Rapid and Specific Liquid Chromatographic Tandem Mass Spectrometric Determination of Tenofovir in Human Plasma and its Fragmentation Study

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Abstract

A simple, specific, and high throughput liquid chromatography tandem mass spectrometry method is developed for the determination of tenofovir, a nucleotide reverse transcriptase inhibitor, in human plasma using adefovir as internal standard. Plasma samples are prepared by solid-phase extraction of the analyte and internal standard using Waters Oasis MCX cartridges (1 cc, 30 mg). The chromatographic separation is achieved on a reversed-phase Chromolith, C18 analytical column (100 mm × 4.6 mm, 5 µm) under isocratic conditions. The mobile phase consists of 0.5% formic acid in water and acetonitrile (90:10, v/v) to give a run time of 1.8 min. The protonated precursor → product ion transitions for tenofovir and IS are monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring and positive ion mode. The fragmentation pathways for tenofovir are studied by varying the collision energy (5–55 V) using nitrogen as CAD gas. A linear dynamic range of 3.1–1002.0 ng/mL is established using 0.2 mL plasma sample. The method is fully validated for its sensitivity, selectivity, accuracy and precision, matrix effect, recovery, stability, and dilution integrity. It is applied to a bioequivalence study in 43 human subjects after oral administration of 300 mg tablet formulation under fasting conditions.

Introduction

Tenofovir {9-[(*R*)-2-(phosphonomethoxy)-propyl]adenine, TFV} belongs to a unique class of nucleotide analogues in which a phosphonate group is bonded to the alkyl side chain of various purines and pyridimines (1). This is a class of potent nucleotide antiviral agents with demonstrated activity against a broad range of viruses such as hepatitis B virus (HBV), herpes viruses like cytomegalovirus, and retroviruses such as HIV (2). Because TFV is not sufficiently bioavailable by oral route, it is administered orally as a prodrug in the form of tenofovir disoproxyl fumarate (3). The prodrug readily undergoes esterase hydrolysis to give TFV, which is phosphorylated by nucleoside diphosphate kinase into the active diphosphate form. Unlike other nucleoside analogues, TFV does not require the initial phosphorylation reaction, which is often a rate-limiting step. This reduction in the phosphorylations requirement allows a more rapid and complete conversion of the drug to its active metabolite (4). TFV is currently prescribed to HIV-infected patients once daily as a part of combination therapy with other antiretroviral drugs. As TFV has an intracellular half-life greater than 60 h and a long serum half-life (17 h), it allows less frequent dosing as compared to many other nucleoside analogs (6). Moreover, TFV retains its activity and shows more favorable resistance profile against a variety of drug-resistance HIV-1 strains in vitro (6).

Due to extensive use of TFV in anti-HIV therapy, it has become essential to develop a sensitive, specific, and high-throughput bioanalytical assay for its routine measurement in subject samples. Several analytical methods are developed and validated to determine TFV concentration alone (7–13) or in combination with other antiretroviral drugs (14–16) in human plasma. Gehrig et al. (14) have determined twelve antiretroviral drugs including TFV in plasma using triple quadrupole mass spectrometry with electrospray ionization (ESI-MS). Similarly, Bezy and co-workers (15) have employed high-performance liquid chromatography (HPLC)–UV to simultaneously analyze eight nucleoside reverse transcriptase inhibitors approved under HIV-1 therapy in rat plasma. A recent combination of TFV and emtricitabine (Emtriva), which has significantly greater HIV RNA suppression compared to zidovudine and lamivudine, was quantified using HPLC after solid-phase extraction (SPE) from human plasma (16). The sensitivity achieved was 10 ng/mL and the separation was possible in a run time of 18 min under gradient conditions. Very recently during preparation of the manuscript, Saux et al. (17) have quantitated

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seven nucleoside/nucleotide reverse transcriptase inhibitors in human plasma by LC–MS. Plasma samples were precipitated by acetonitrile using 100 µL plasma volume with the limit of quantitation for TFV at 5 ng/mL. The separation of all seven drugs was achieved in a run time of 14 min. An anion exchange isolation of tenofovir-diphosphate from human peripheral blood mononuclear cells, coupled with dephosphorylation, desaltation, and detection by LC–MS has been proposed by King et al. (18). Other procedures reported for the determination of TFV alone in human plasma are described using HPLC–UV (8), HPLC–fluorescence (9,10), and LC–tandem MS (11–13) methodology. The salient features of these methods, along with figures of merit, are compared and summarized in Table I.

Thus, in the present study, the aim was to develop and validate a specific, sensitive, and high-throughput method for routine determination of TFV in subject samples. The proposed validated LC–MS–MS method exhibited excellent performance in terms of selectivity, ruggedness, and efficiency (1.8 min per sample). Though the method is less sensitive than one used in a previous study (13), the overall analysis time is significantly less compared to all other reported procedures. The method ensures the estimation of TFV up to 48 h with desired accuracy and precision for elimination phase concentration in human volunteers for bioequivalence study. Also reported are the experiments done to study the fragmentation behavior of TFV by varying collision energy (5–55 V) using nitrogen as the collision activation dissociation (CAD) gas.

Experimental

Chemicals and materials

Reference standards of TFV (99.09%) and adefovir (internal standard, IS) (99.6%) were procured from Matrix Labs Ltd. and Samex Overseas (Ahmedabad, India), respectively. HPLC-grade acetonitrile, methanol, formic acid, hydrochloric acid, and ammonia solution were obtained from Merck Specialties Pvt. Ltd. (Mumbai, India). Water used in the entire analysis was prepared from Milli-Q water purification system procured from Millipore (Bangalore, India). Waters Oasis MCX extraction cartridges (1 cc, 30 mg) were procured from Milford, MA. Blank human plasma was obtained from Supratech Micropath Laboratory & Research Institute (Ahmedabad, India) and was stored at –20°C until use.

Table I. Salient Features of Bioanalytical Methods Developed for TFV in Human Plasma*

Sr. no.	Extraction procedure (plasma volume)	Chromatography column [length (mm) × i.d. (mm), particle size (µ)]	Elution; mobile phase; injection volume; on-column loading†	Analytical run time	Detection technique	LLOQ (ng/mL)	Ref. no.
1	SPE with Supel-clean TM LC-18 cartridge (1.0 mL)	Symmetry shield RP18 (250 × 4.6, 5)	Gradient; ACN + pH 6 buffer containing 15mM Na_2HPO_4 and 10mMg TBAHS; 150 µL; 2400 ng	12.0 min	HPLC-UV	10.0	8
2	PP with MeOH (0.2 mL)	Cluzeau C8 plus satisfaction (250 × 3, 4)	Isocratic; Phosphate buffer with 5mM TBAC–ACN, pH 6.0 (85:15, v/v); 40/80 µL; 80 ng	> 11.0 min	HPLC-fluorescence	5.0	9
3	PP with TCA (0.2 mL)	Chromspher C8 (150 × 4.6, 5)	Gradient; 10mM SP and 5mM TBAHS buffer pH 7.0 + (ACN + 7.0 pH buffer, 50;50, v/v); 50 µL; 50 ng	20.0 min	Ion-pair HPLC-fluorescence	20.0	10
4	PP with TFA (0.25 mL)	Synergi Polar RP (150 × 2.0, 4)	Isocratic; 3% ACN–1%AA in DI H_2O; 10 µL; 7 ng	7.0 min	LC–MS–MS	10.0	11
5	PP with ACN (0.5 mL)	Sun Fire C1 (50 × 2.1, 3.5)	Gradient; 0.3% TFA + ACN + 100mM AmA; 5 µL; 39 ng	12.0 min	LC–MS	19.0	12
6	SPE with Waters Oasis MCX cartridge (0.2 mL)	Waters Atlantis dC18 (100 × 2.1, 3)	Isocratic; HA/AA buffer, pH 6.75-MeOH (93:7, v/v); 25 µL; 75 ng	10.0 min	LC–MS–MS	1.0	13
7	SPE with Waters Oasis MCX cartridge (0.2 mL)	Phenomenex, Chromolith C18 (100 × 4.6, 5)	Isocratic; 0.5% FA in ACN–H_2O (10:90, v/v); 10 µL; 20 ng	1.8 min	LC–MS–MS	3.1	Present method

* PP: protein precipitation; SPE: solid-phase extraction; TCA: trichloroacetic acid; TFA: trifluoroacetic acid; ACN: acetonitrile; TBAC: tetrabutylammonium chloride; TBAHS: tetrabutylammonium hydrogen sulphate; AA: acetic acid; DI: deionized water; AmA: ammonium acetate; HA: hydroxylamine; FA: formic acid.
† At ULOQ.

Liquid chromatographic conditions

A Shimadzu LC-VP HPLC system (Kyoto, Japan) consisting of LC-10AD prominence pump, SIL-HTc autosampler, CTO 10 ASvp column oven, and a DGU-14A degasser was used for setting the reverse-phase LC conditions. The separation of TFV and adefovir was performed on a Phenomenex analytical column, type Chromolith C18, 100 mm × 4.6 mm (length × inner diameter), with 5 µm particle size, and was maintained at 40°C in column oven. The mobile phase consisted of 10% acetonitrile and 90% water containing 0.5% formic acid (v/v). For isocratic elution, the flow rate of the mobile phase was kept at 1.5 mL/min. The total chromatographic run time was 1.8 min. The auto sampler temperature was maintained at 5ºC and the pressure of the system was 500 psi. The total eluant from the column was split in 75:25 ratio; flow directed to the ISP interface was equivalent to 375 µL/min.

Mass spectrometric conditions

Ionization and detection of analyte and IS were carried out on a triple quadrupole mass spectrometer, MDS SCIEX API 4000 (Toronto, Canada), equipped with ESI (TIS interface of the API 4000) and operating in positive ion mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor parent → product ion (*m/z*) transitions for tenofovir 288.1 → 176.2 and 274.4 → 256.0 for IS (Figures 1A and 1B).

The source-dependent parameters maintained for both tenofovir and adefovir were Gas 1 (Nebuliser gas): 40 psig; Gas 2 (heater gas flow): 50 psig; ion spray voltage: 5500 V; turbo heater temperature: 450°C; interface heater: ON; entrance potential: 10 V; CAD: 10 psig; and curtain gas, nitrogen: 30 psig. The optimum values for compound-dependent parameters like declustering potential, collision energy, and cell exit potential set were 68, 35, and 15 V for TFV and 45, 28, and 10 V for IS, respectively. Quadrupoles 1 and 3 were maintained at unit mass resolution and the dwell time was set at 200 ms. Analyst software version 1.4.1 was used to control all parameters of LC and MS.

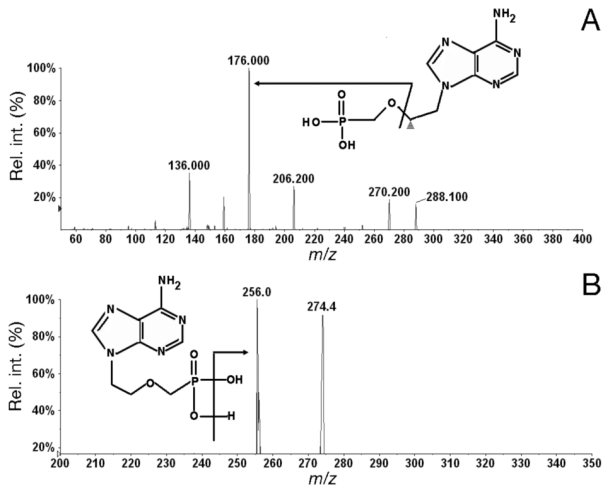

Figure 1. Product ion mass spectra of TFV (A) and IS adefovir (B) in positive ionization mode.

Standard stock, calibration standards, and quality control sample preparation

The standard stock solution of 10 mg/mL of TFV was prepared by dissolving the requisite amount in methanol. Calibration standards and quality control (QC) samples were prepared by spiking (2%) blank plasma with stock solution. Calibration curve standards were made at 3.1, 6.1, 17.5, 50.1, 125.3, 250.5, 501.0, and 1002.0 ng/mL concentrations while quality control samples were prepared at four levels; viz., 875.5 ng/mL (high quality control, HQC), 105.1 ng/mL (middle quality control, MQC), 9.1 ng/mL (low quality control, LQC), and 3.2 ng/mL [lower limit of quantitation (LLOQ) QC]. Stock solution (1000 ng/mL) of the IS was prepared by dissolving 25.0 mg of adefovir in 25.0 mL of methanol. An aliquot of 0.75 mL of 0.200 mg/mL of this solution was further diluted to 50.0 mL in the same diluent to obtain a solution of 3 µg/mL. All solutions (standard stock, calibration standards, and quality control samples) were stored at 2–8°C until use.

Protocol for sample preparation

Prior to analysis, all frozen subject samples, calibration standards, and quality control samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 0.200 mL of spiked plasma sample, 50 µL IS was added and vortexed for 10 s. Next, 50 µL of 0.1 N hydrochloric acid was added and vortex-mixed for 10 s. Centrifugation of the samples was done at 3200 × g, at 10°C for 2 min. The samples were loaded on Oasis MCX (1 cc, 30 mg) extraction cartridges which were preconditioned with 1 mL of methanol followed by 1 mL of water. The cartridges were washed with 1 mL of 0.1 N hydrochloric acid followed by 1 mL methanol. Drying of cartridges was done for 2 min by applying 25 psi pressure at 2.4 L/min flow rate. Elution of TFV and IS from the cartridges was carried out with 1 mL of 5% ammonia solution into pre-labeled tubes. The eluate was evaporated to dryness in a thermostatically controlled water-bath maintained at 40°C under the stream of nitrogen for 3 min. After drying, the residue was reconstituted in 100 µL of mobile phase and 10 µL was used for injection in the chromatographic system.

Bio-analytical method validation

A through and complete method validation of TFV in human plasma was done following the USFDA guidelines (19).

The carryover effect of the autosampler was evaluated by sequentially injecting solutions of aqueous standard (tenofovir), reconstitution solution (0.5% formic acid in water–acetonitrile, 90:10 v/v), standard blank, and extracted standard (TFV), equivalent to highest standard in the calibration range.

The linearity of the method was determined by analysis of standard plots associated with an eight point standard calibration curve. Five linearity curves containing eight non-zero concentrations were analyzed. Peak area ratios of analyte/IS obtained from MRM were utilized for the construction of calibration curves, using weighted ($1/x^2$) linear least squares regression of the plasma concentrations and the measured peak area ratios. Back-calculations were made from these curves to determine the concentration of TFV in each calibra-

tion standard. A correlation coefficient (r^2) greater than 0.99 was desirable for all the calibration curves. The lowest standard on the calibration curve was to be accepted as the LLOQ, if the analyte response was at least five times more than that of drug free (blank) extracted plasma. In addition, the analyte peak of LLOQ sample should be identifiable, discrete, and reproducible with a precision (%CV) not greater than 20.0 and accuracy within 80.0–120.0%. The deviation of standards other than LLOQ from the nominal concentration should not be more than ± 15.0%.

The selectivity/specificity of the method towards endogenous plasma matrix components, metabolites, and concomitant medications was assessed in twelve batches (6 normal of K3 EDTA, 2 haemolyzed, 2 lipemic, and 2 heparinized) of blank human plasma. They were processed using the proposed extraction protocol and the set chromatographic conditions of TFV at the LLOQ level. Cross talk of MRM channels for analyte and IS were checked using the highest concentration from the linear calibration curve and the working solution of IS. The effect of potential concomitant antiretroviral drugs (nucleoside reverse transcriptase inhibitors—zidovudine, didanosine, stavudine, abacavir; non-nucleoside reverse transcriptase inhibitors—efavirenz; protease inhibitors—ritonavir, lopinavir; herpes virus inhibitor—acyclovir and valacyclovir) was studied under the same conditions and their possible interference at the elution time of analyte and IS was observed. A check for interference due to commonly used medications in human volunteers was done for paracetamol, chlorpheniramine maleate, caffeine, acetylsalicylic acid, and ibuprofen. Their stock solutions (1000 µg/mL) were prepared by dissolving the requisite amount in methanol–water (50:50, v/v). Also, working solutions (1.0 µg/mL) were prepared in the mobile phase and 5 µL was injected to check any possible interference at the elution time of TFV and IS.

The extraction efficiency (recovery) of TFV was performed at LQC, MQC, and HQC levels. It was evaluated by comparing the mean area response of six replicates of extracted samples (spiked before extraction) to that of unextracted samples (spiked after extraction) at each QC level. The recovery of IS was similarly estimated. As per the acceptance criteria, the recovery of the analyte need not be 100.0%, but should be consistent, precise, and reproducible.

For determining the intra-day accuracy and precision, replicate analysis of plasma samples of TFV was performed on the same day. The run consisted of a calibration curve and six replicates of LLOQ, LQC, MQC, and HQC samples. The inter-day accuracy and precision were assessed by analysis of five precision and accuracy batches on three consecutive validation days. The precision of the method was determined by calculating the percent coefficient of variation (%CV) for each level. The deviation at each concentration level from the nominal concentration was expected to be within ± 15.0% except LLOQ, for which it should not be more than 20.0%. Similarly, the mean accuracy should not deviate by ± 15.0% except for the LLOQ where it can be ± 20.0% of the nominal concentration.

To study the effect of matrix on analyte quantitation with respect to consistency in signal enhancement/suppression, it was checked in six different batches. Six samples each at LQC and HQC levels were prepared from six different batches of plasma (total 12 samples) and checked for the % accuracy and precision (%CV) in both the QC samples. This was assessed by comparing the back calculated value from the QC's nominal concentration. The deviation of the standards should not be more than ± 15.0% and at least 90% of the lots at each QC level should be within the aforementioned criteria.

Stability experiments were carried out to examine the analyte stability in stock solutions and in plasma samples under different conditions. Short term and long term stock solution stability at room temperature was assessed by comparing the area response of stability sample of analyte and IS with the area response of sample prepared from fresh stock solutions. The solutions were considered stable if the deviation from nominal value was within ± 10.0%. Autosampler stability, bench top stability, dry extract stability, and freeze-thaw stability were performed at LQC and HQC using six replicates at each level. The samples were considered stable if the deviation from the mean calculated concentration of freshly thawed quality control samples was within ± 15.0.

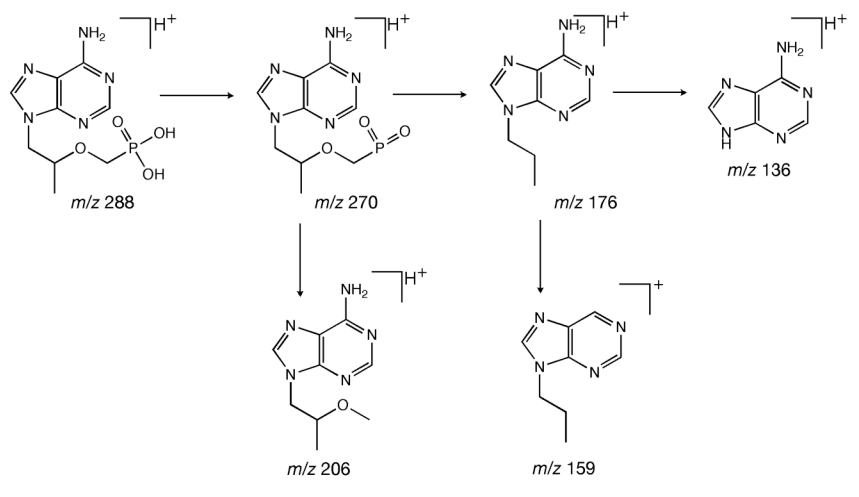

Figure 2. Observed mass fragmentation pathway for protonated precursor ion (*m/z* 288) of TFV.

To authenticate the ruggedness of the proposed method, it was done on two precision and accuracy batches. The first batch was analyzed by different analysts while the second batch was analyzed on two different columns.

Dilution integrity experiment was evaluated by diluting the stock solution prepared as spiked standard at concentration of 2500 ng/mL in the screened plasma. The precision and accuracy for dilution integrity standards at 1/5th (500 ng/mL) and 1/10th (250 ng/mL) dilution were determined by analyzing the samples against calibration curve standards.

Bioequivalence study

The design of the study comprised of "A randomized, open label, two treatment,

two period, two sequences, single dose, crossover, bioequivalence study of tenofovir disoproxil fumarate 300 mg tablets (test and reference—IREAD from Gilead Sciences Inc., Foster City, CA) in healthy human adult male subjects, under fasting conditions". Each volunteer was judged to be in good health through medical history, physical examination, and routine laboratory tests. Written consent was taken from all the volunteers after informing them about the objectives and possible risks involved in the study. The work was approved and subject to review by the Institutional Ethics Committee, an independent body comprised of five members including a lawyer, a medical doctor, a social worker, a pharmacologist, and an academician. The procedures followed while dealing with human subjects were based on International Conference on Harmonization, E6 Good Clinical Practice guidelines (20). A single oral dose of 300 mg drug was given to the volunteers with 240 mL of water. Blood samples were collected at 0.0 (predose), 0.17, 0.33, 0.50, 0.67, 0.83, 1.00, 1.25, 1.50, 1.75, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 6.00, 8.00, 10.00, 12.00, 24.00, 36.00, 48.00, and 72.00 h after oral administration of the dose for test and reference formulation in labeled K3 EDTA-vacuettes. Plasma was separated by centrifugation and kept frozen at –20°C until analysis. During study, volunteers had a standard diet while water intake was free. The samples were processed based on the proposed extraction protocol for quantification of TFV.

Results and Discussion

Bioanalytical method development

The method development was initiated to achieve adequate sensitivity, minimum overall analysis time (plasma processing and chromatographic run), and the use of a small plasma volume for processing, which is crucial for antiretroviral drugs, especially for variable combination therapy. To develop a rapid and sensitive method, it was equally necessary to optimize the chromatographic and mass spectrometric conditions, as well as to have an efficient extraction procedure for TFV. The present study was conducted using ESI as the ionization source as it gave high intensity for the analyte and IS and a good linearity in regression curves. The ESI mass spectrum for TFV and adefovir ion the positive mode was dominant with protonated $(M+H)^+$ ions, as both can be easily protonated. Addition of acid helped in further enhancing the intensity of these ions to obtain protonated precursor ion peaks at *m/z* 288.1 and 274.4 for TFV and IS, respectively. There are five nitrogen atoms which could be protonated, but possibly due to resonance stabilization after the first protonation, the basicity of the other nitrogen atoms is considerably reduced. The mass fragmentation pattern of TFV revealed several peaks of significant intensity by varying collision energy from 5–55 V with nitrogen as CAD gas. The protonated precursor ion of TFV was stable up to 30 V collision energy with very little fragmentation, which may be attributed to its high stability and possibly due to low molecular mass of nitrogen as CAD gas compared to argon (21). Further increase in collision energy up to 35 V gave two fragments at *m/z* 176 and 270 with relative intensity of approximately 21%. The low abundance ions at *m/z* 270 from protonated precursor of tenofovir presumably result via loss of water molecule, followed by elimination of phosphono methoxy group to give an ion at *m/z* 176. The dominant fragment observed in the range of 35–45 V energy was at *m/z* 176 (relative intensity, 100%), hence was selected as the product ion for quantitation. The ion at *m/z* 176 is further fragmented between collision energy 50–55 V by the loss of propyl group to give an intense signal for adenine—the parent moiety of tenofovir at *m/z* 136. Though the fragments at *m/z* 159 (81.3%) and 136 (100%) had higher relative intensities above 50 V collision energy, but their absolute intensity was too low to be used for further study. The observed fragmentation pathway for the protonated precursor ion of tenofovir, *m/z* 288, is shown in Figure 2. The MRM state file parameters like nebulizer gas, CAD gas, ion spray voltage, and temperature were suitably optimized to obtain a consistent and adequate response for the analyte. A dwell time of 200 ms for TFV and IS was adequate and no cross talk was observed between their MRMs.

Chromatographic analysis of TFV and IS was initiated under isocratic conditions to obtain adequate response, sharp peak shape, and a short run time. The use of volatile buffers like hydroxylamine–acetic acid, ammonium formate, and ammonium phosphate in combination of methanol–acetonitrile for the separation of TFV and other antiretroviral drugs has been documented in earlier reports (13,15). It was also observed that the pH of the mobile phase and selection of the right column are important criteria. Thus, separation was tried using various combinations of methanol–acetonitrile, acidic buffers and additives like formic acid on different reversed-phase columns with 5 µm particle size [viz., Chromolith RP-18 (100 × 4.6 mm), Kromasil (50 and 100 × 4.6 mm), ACE Cyano (150 and 250 × 4.6mm), and Gemini C-18 (50 × 4.6mm)] to find the optimal mobile phase that produced the best sensitivity, efficiency, and peak shape. The analytes showed nonlinear behavior on the Gemini C-18 column and thus it was not considered for further study. The Chromolith RP-18 column offered superior peak shape, efficient separation, and reproducibility for TFV and IS from endogenous plasma matrix. The mobile phase consisting of 0.5% formic acid in water and acetonitrile (90:10 v/v) ratio with pH approximately 3.0–3.5 was found most suitable for eluting the analyte and IS in a run time of 1.8 min.

Initially, the extraction of TFV was carried out via protein precipitation with common solvents like acetonitrile, methanol, and acetone, but the recovery was poor (< 10%) in all solvents with frequent clogging of the column. Liquid–liquid extraction technique was also tested to isolate the drugs from plasma using diethyl ether, dichloromethane, methyl tert butyl ether, and isopropyl alcohol (alone and in combination) as extracting solvents. However, the recovery was inconsistent with some ion suppression (greater than 15% CV). Finally, optimization of the SPE process was done on Waters Oasis HLB, Waters Oasis MCX, and Phenomenex Strata cartridges. Addition of strong acid like HCl during sample preparation helped in breaking the drug-protein binding and maintaining the analyte in the ionized form. Thus, better

Table II. Summary of Calibration Curves with Back-Calculated Concentration for TFV*

	STD-1	STD-2	STD-3	STD-4	STD-5	STD-6	STD-7	STD-8			
	Nominal concentration (ng/mL)								Regression parameters		
ID no.	3.1	6.1	17.5	50.1	125.3	250.5	501.0	1002.0	Slope	Intercept	r^2
1	3.1	6.1	17.1	48.0	124.3	247.9	561.6	1010.5	0.0024	0.0001	0.9984
2	3.0	6.5	17.4	47.2	132.4	237.2	500.1	1072.6	0.0027	0.0004	0.9982
3	3.1	6.1	17.3	49.3	123.5	238.8	576.0	999.5	0.0025	0.0013	0.9977
4	3.0	6.7	17.3	48.4	119.6	247.3	531.8	1035.9	0.0026	0.0010	0.9983
5	3.0	6.5	17.2	48.6	121.2	233.8	562.1	999.0	0.0026	0.0001	0.9975
Mean	3.1	6.4	17.3	48.3	124.2	241.0	546.3	1021.9	0.0026	0.0006	0.9980
S.D.	0.07	0.29	0.12	0.78	4.96	6.31	30.45	32.93			
% CV	2.4	4.6	0.7	1.6	4.0	2.6	5.6	3.2			
% Accuracy	99.9	103.8	98.4	96.4	99.1	96.2	109.0	102.0			

* CV = coefficient of variance; S.D. = standard deviation; r = correlation coefficient.

retention was provided on the Waters Oasis MCX as compared to other cartridges. Also, use of acid and methanol during washing step gave consistent recovery, especially at the LLOQ level with minimum matrix interference.

According to the U.S. FDA guideline, the IS should ideally mirror the analyte in as many ways as possible. It should preferably belong to the same class, with the same physicochemical and spectral properties, to improve the method ruggedness. Adefovir, a nucleotide analog, was selected as an IS due to its structural similarity to TFV. Also, both drugs are used concurrently in HBV/HIV-coinfected patients with no indications of drug-drug interactions (22). Moreover, there was no significant effect of IS on analyte recovery, sensitivity, or ion suppression.

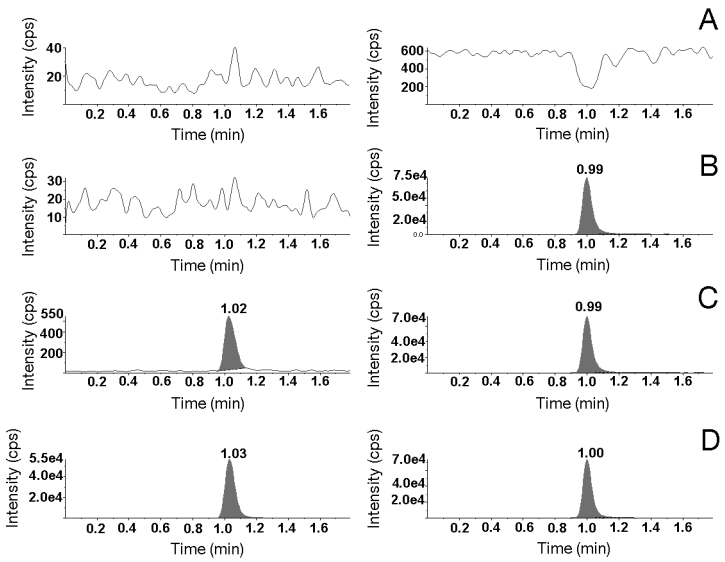

Figure 3. MRM ion-chromatograms of TFV (288.1 → 176.2) in double blank plasma (A), blank plasma with IS (B), LLOQ (C), and real subject sample at 1.0 h (C_{max} 326.6 ng/mL) after administration of 300 mg dose (D).

Auto sampler carry-over, linearity, and LLOQ

Carry-over evaluation was performed in each analytical run so as to ensure that it does not affect the accuracy and the precision of the proposed method. There was no carry-over observed during autosampler carry-over experiment. Also, no enhancement in the response was observed in double blank after subsequent injection of highest calibration standard (aqueous and extracted) at the retention times of tenofovir and IS, respectively. Moreover, no ghost peaks appeared during the analysis of blank samples.

All five calibration curves analyzed during the course of validation were linear for the standards ranging from 3.1 to 1002.0 ng/mL. A straight-line fit was made through the data points by least square regression analysis and a constant proportionality was observed. The mean value for slope, intercept, and correlation coefficient (r^2) observed were 0.0026, 0.0006, and 0.9980, respectively. The accuracy and precision (%CV) observed for the calibration curve standards ranged from 96.2 to 109.0% and 0.7 to 5.6, respectively. The LLOQ achieved was 3.1 ng/mL for TFV. Table II summarizes the back-calculated concentration with accuracy and precision data for five linearties.

Selectivity, recovery and accuracy, and precision

The selectivity of the method towards endogenous plasma matrix was ascertained in twelve batches of human plasma by analyzing blank and spiked plasma samples at LLOQ concentration. No endogenous peaks were observed at the retention time of the analyte for any of the batches. Figures 3A–3D demonstrate the selectivity experiments with the chro-

Table III. Intra-Batch and Inter-Batch Precision and Accuracy for TFV*

		Intra-batch				Inter-batch			
QC ID	Nominal conc. (ng/mL)	*n*	Mean conc. observed (ng/mL)†	% CV	% Accuracy	*n*	Mean conc. observed (ng/mL)‡	% CV	% Accuracy
HQC	875.5	6	874.3	2.6	99.9	30	888.9	3.0	101.5
MQC	105.1	6	104.9	2.8	99.9	30	104.2	3.5	99.2
LQC	9.1	6	9.1	3.3	100.0	30	8.9	4.4	97.5
LLOQ QC	3.2	6	3.0	3.2	94.7	30	2.9	7.4	90.6

* CV, coefficient of variance; *n*, total number of observations.
† Mean of 6 replicates at each concentration.
‡ Mean of 6 replicates for five precision and accuracy batches.

matograms of extracted blank plasma, the peak response of TFV at LLOQ and IS, and real patient sample at 1.0 h after oral administration of 300 mg tablet formulation. The retention times for TFV and IS were 1.01 and 0.98 min, respectively. None of the concomitant medications studied showed interfering signals at the retention times of tenofovir or adefovir (IS). This demonstrates that the method is highly specific and free from interference due to matrix components and other prescribed medications.

Six replicates at LQC, MQC, and HQC levels were prepared for recovery determination. The % mean recovery for tenofovir at HQC, MQC, and LQC levels was 70.1, 68.8, and 75.4%, respectively, while the recovery of IS was 79.3%.

The intra- and inter-batch accuracy and precision were determined in five batches at LLOQ QC, HQC, MQC, and LQC levels with six replicates for each batch. Precision (%CV) for intra- and inter-batch ranged from 2.6 to 7.4. The intra- and inter-batch accuracy results were within 90.6 to 101.5% at the QC levels. The detailed results are presented in Table III.

Table IV. Matrix Effect Data of TFV at HQC and LQC Levels in Six Different Lots of Human Plasma

	HQC		LQC	
	Nominal concentration (ng/mL)			
	875.5		9.1	
Replicate no.	Calculated concentration (ng/mL)	% Accuracy	Calculated concentration (ng/mL)	% Accuracy
1	875.9	100.0	9.1	100.0
2	839.4	95.9	8.3	91.0
3	888.2	101.4	8.9	97.6
4	856.7	97.9	8.9	96.9
5	886.5	101.2	8.5	93.2
6	888.4	101.5	8.4	92.1
Mean		99.7		95.1
SD		2.31		3.57
% CV		2.3		3.7

Matrix effect and stability results

Matrix effects may arise due to co-elution of some unintended components present in biological samples or which are added as part of analysis. These components may not give a signal in MRM of target analyte but can certainly decrease or increase the analyte response dramatically to affect the sensitivity, accuracy, and precision of the method. Thus, assessment of matrix effect constitutes an important and integral part of validation for quantitative LC–MS–MS method for supporting pharmacokinetics studies. The matrix effect for the intended method was assessed by using chromatographically screened human plasma. Precision (% CV) for LQC and HQC samples observed was 3.7% and 2.3%, while the accuracy found at these levels was 95.1% and 99.7%, respectively (Table IV). There was no significant signal enhancement or suppression due to endogenous plasma matrix at the retention times of tenofovir and IS. Thus, the method was rugged and gave accurate and consistent results when applied to subject sample analysis.

Stock solutions for short term and long-term stability of the analyte and IS were stable at room temperature for a minimum period of 6 h and between 2–8°C for 7 days, respectively. TFV in control human plasma (bench top) at room temperature was stable at least for 6 h at 25°C and for a minimum of three freeze and thaw cycles. Spiked plasma samples stored at –20°C and –70°C for long-term stability experiments were stable for a minimum 93 days. Dry extract stability of the spiked quality control samples stored at –20°C was determined up to 24 h. Autosampler stability of the spiked quality control samples maintained at 5°C was determined up to 21.5 h. Different stability experiments in plasma and the values for the precision and percent change are shown in Table V.

Method ruggedness and dilution integrity

Ruggedness was performed by using two precision and accu-

racy batches. The first batch was analyzed by two different analysts, while the second batch was analyzed on two different columns. Precision and accuracy for calibration curve standards and LLOQ were between 0.02 to 10.48% and 95.8 to 108.2%, respectively, which is within the acceptance criteria. For both the experiments the precision and accuracy for LLOQ, low, middle, and high quality control samples ranged from 2.1 to 7.9% and 89.9 to 104.5%, respectively, which are within the acceptance limit of 15% in precision and 85.0 to 115.0% in mean accuracy.

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentration above the upper limit of quantitation (ULOQ), which may be encountered during real subject sample analysis. The precision for dilution integrity of 1/5th and 1/10th dilution were 4.4 and 3.3%, while the accuracy results were 98.2 and 94.5%, respectively, which is within the acceptance limit of 15% for precision (CV) and 85.0 to 115.0% for accuracy.

Application of the method in human subjects

The proposed validated method was successfully applied for the assay of tenofovir in healthy male volunteers in the age group of 18 to 45 years. Figure 4 shows the mean plasma concentration-time profile following 300 mg dosing of tenofovir to 43 human subjects under fasting conditions up to 72 h. In all, approximately 3000 samples, including the calibration and QC samples with volunteer samples, were run and analyzed during a period of 10 days and the precision and accuracy for calibration and QC samples were well within the acceptable limits. The pharmacokinetic parameters (viz., plasma concentration maximum C_{max}, area under the plasma concentration-time curve from zero hour to the last measurable concentration $AUC_{0–t}$, area under the plasma concentration-time curve from zero hour to infinity $AUC_{0–inf}$, time point of plasma concentration maximum T_{max}, elimination rate constant K_{el}, and half life of drug elimination during the terminal phase $t_{1/2}$) were calculated for tenofovir in test and reference formulations. The mean pharmacokinetic parameters obtained for the test and reference formulation are presented in Table VI. The 90% confidence interval of individual ratio geometric mean for test/reference was within 80–125% for $AUC_{0–t}$, $AUC_{0–inf}$, and C_{max}. It has been observed that the value for C_{max} depends on dose strength. In the present study, the values for C_{max}, T_{max}, and $t_{1/2}$ are comparable with those reported earlier (22). These observations confirm the bioequivalence of the

Table V. Stability of TFV Under Different Conditions (*n* = 6)

Storage conditions	Mean comparison Mean comparison samples	Calculated conc. (ng/mL) Mean stability samples	% Change*
Bench top stability			
HQC	874.3 ± 22.5	894.4 ± 29.3	–2.3
LQC	9.1 ± 0.3	9.2 ± 0.1	–0.6
Wet extract stability			
HQC	879.3 ± 15.3	869.7 ± 22.5	1.1
LQC	9.1 ± 0.4	9.4 ± 0.3	–4.2
Dry extract stability			
HQC	908.1 ± 17.0	934.5 ± 20.5	–2.9
LQC	8.6 ± 0.5	9.6 ± 0.4	–12.0
Freeze and thaw stability			
HQC	874.3 ± 22.5	919.2 ± 84.7	–5.1
LQC	9.1 ± 0.3	9.0 ± 0.4	1.7
Long term stability (in matrix)			
HQC	848.1 ± 22.6	783.0 ± 23.3	8.1
LQC	8.5 ± 0.2	8.0 ± 0.5	4.8

$$\text{* \% Change} = \frac{\text{Mean stability samples} - \text{Mean comparison samples}}{\text{Mean comparison samples}} \times 100$$

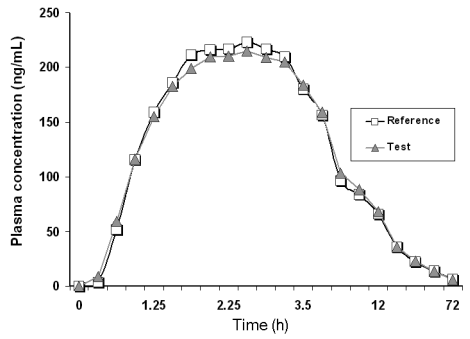

Figure 4. Mean plasma concentration-time profile of TFV after oral administration of 300 mg (test and reference) tablet formulation to 43 healthy volunteers.

Table VI. Mean Pharmacokinetic Parameters Following Oral Administration of 300 mg Tablet Formulation (Test and Reference) of TFV in 43 Healthy Human Subjects

Parameter	Test reference Mean ± %RSD	Mean ± %RSD
C_{max} (ng/mL)	313.3 ± 85.3	358.0 ± 113.5
T_{max} (h)	1.1 ± 0.4	1.0 ± 0.4
$t_{1/2}$ (h)	18.6 ± 2.2	18.5 ± 1.8
$AUC_{0-72\,h}$ (h.µg/mL)	2375.7 ± 451.3	2504.8 ± 648.5
AUC_{0-inf} (h.µg/mL)	2529.6 ± 486.5	2657.8 ± 690.1

test sample with the reference product in terms of rate and extent of absorption. Furthermore, there was no adverse event during the course of the study.

Conclusion

The bioanalytical methodology for tenofovir described in this manuscript is highly specific and rugged for therapeutic drug monitoring, both for analysis of routine samples of single dose or multiple dose pharmacokinetics and also for clinical trial samples with desired sensitivity, precision, accuracy, and high throughput. The method involved a simple and specific sample preparation by SPE followed by isocratic chromatographic separation in 1.8 min. The overall analysis time is promising compared to other reported procedures for tenofovir. The established LLOQ is sufficiently low to conduct a pharmacokinetic study with 300 mg test formulation of tenofovir (alone or in combination with other drugs) in healthy human volunteers. Also, the study on fragmentation of tenofovir indicates the stability of the protonated precursor ions, possibly due to resonance stabilization.

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