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Selective Determination of Antiretroviral Agents Tenofovir, Emtricitabine, and Lamivudine in Human Plasma by a LC–MS–MS Method for a Bioequivalence Study in Healthy Indian Subjects

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Abstract

A selective, sensitive, rugged, and high throughput liquid chromatography tandem mass spectrometry method is developed for the determination of one nucleotide tenofovir (TFV) and two nucleosides emtricitabine (FTC) and lamivudine (3TC) reverse transcriptase inhibitors in human plasma. Plasma samples were prepared by solid-phase extraction of the analytes and acyclovir (ACV) as internal standard using Waters Oasis MCX cartridges. The chromatographic separation is achieved in a run-time of 3.0 min on an ACE 5 CN column (150 mm × 4.6 mm, 5 µm) under isocratic conditions. The mobile phase consisted of 0.5% formic acid in water and acetonitrile (55:45, v/v). The protonated precursor → product ion transitions for TFV, FTC, 3TC, and internal standard were monitored on a triple quadrupole mass spectrometer operating in the multiple reaction monitoring (MRM) and positive ion mode. A linear dynamic range of 4.0–802 ng/mL, 15.0–3006 ng/mL, and 20.1–4023 ng/mL is established for TFV, FTC, and 3TC, respectively, using 0.2 mL plasma sample. The method is fully validated for its sensitivity, selectivity, accuracy and precision, ion suppression, matrix effect, recovery, stability, and dilution integrity. It is successfully applied to a bioequivalence study of [300(TFV) + 200(FTC) + 300(3TC)] mg tablet formulation in 43 healthy human subjects under fasting conditions.

Introduction

Nucleoside/nucleotide reverse transcriptase inhibitors (N(t)RTIs) play a vital role in combination therapy to manage acquired immunodeficiency syndrome (AIDS) (1). The need for such a therapy has arisen due to the development of resistance by the causative human immunodeficiency virus (HIV) to single anti-HIV drugs and to minimize potential dose-dependent side effects (2). The U.S. Department of Health and Human Services has recommended highly effective combination regiments of antiretroviral drugs to combat mortality and morbidity in humans (3). The current regiment for treating HIV infection is to use a combination of at least three drugs, a practice sometimes called HAART (highly active antiretroviral therapy) (4). This potent combination generally includes two nucleoside reverse transcriptase inhibitors (NRTI) and a non-nucleoside reverse transcriptase (NNRTI) or protease inhibitor. Nucleoside reverse transcriptase inhibitors are intracellularly phosphorylated to their corresponding triphosphorylated derivatives, which compete with the corresponding natural nucleotide for binding to HIV reverse transcriptase. Although non-nucleoside reverse transcriptase inhibitors act as non competitive inhibitors of the HIV reverse transcriptase. The protease inhibitors prevent T-cells that are infected from producing new copies of the virus and prevent maturation of the HIV virus (5–8). This complimentary/synergistic action of different classes of anti-HIV drugs in combination therapy has increased survival rate and improved quality of life for HIV-positive patients (4). Although these combinations show significant clinical efficacy in the treatment of HIV, they can result in drug-drug interactions with large inter-individual pharmacokinetic variability (9). Also, the complexity of the dosing regiment and recurrent adverse effects can lead to drug resistance and virological failure (10). Thus, simultaneous determination of these drugs in biological matrices, along with their pharmacokinetic study, can assist in checking their effectiveness and treatment compliance to prevent adverse events and to formulate optimum dosages.

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 (11). It is active against a broad range of viruses such as hepatitis B virus (HBV), herpes viruses like cytomegalovirus (CMV), and retroviruses such as HIV (12). Because TFV is not sufficiently bioavailable by oral route, it is administered orally as a pro-drug in the form of

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tenofovir disoproxyl fumarate (TDF) (13). The pro-drug 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 phosphorylation requirement allows more rapid and complete conversion of the drug to its active metabolite (14). TFV is currently prescribed to HIV-infected patients as VIREAD and in fixed dose combination therapy with emtricitabine as TRUVADA and in addition with efavirenz under the brand name ATRIPLA.

Emtricitabine {FTC, 5-flouro-1-(2*R*, 5S)-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine} is a deoxycytidine analog nucleoside reverse transcriptase inhibitor (NRTI) that has demonstrated potent and selective inhibitory activities against human immunodeficiency (HIV type I and II) and hepatitis B virus (HBV) (15,16). It is readily anabolized in a stepwise manner to form its monophosphate, diphosphate, and triphosphate forms. The triphosphate form is the active intracellular species that completely inhibits HBV DNA polymerase (17). FTC is rapidly and extensively absorbed following oral administration and reaches peak plasma levels in about 3 h with maximum plasma levels of 1.8 µg/mL for single 200 mg oral dose (18). The major advantage of FTC over other NRTIs is its favorable pharmacokinetic profile that permits once daily dosing; it has a long mean plasma elimination half-life of 8–10 h and the intracellular half-life of emtricitabine triphosphate of 39 h after multiple doses of 200 mg daily (19). Apart from combination formulation, emtricitabine alone is also available under the brand name of EMTRIVA.

Lamivudine (3TC), a nucleoside reverse transcriptase inhibitor, is a synthetic dideoxynucleoside derivative with potent activity against human immunodeficiency virus (HIV) and hepatitis B virus (HBV) (20). Treatment of HIV infections with an antiviral regiment that includes 3TC is highly desirable because it shows lower toxicity compared to other nucleoside derivatives (21). It is administered orally and is rapidly absorbed with a bioavailability of more than 80% in humans (22). Like the other two drugs, 3TC is also available as EPIVIR and in combination with other antiretrovirals like zidovudine as COMBIVIR, with abacavir as EPZICOM, and a triple combination with abacavir and zidovudine as TRIZIVIR.

Due to extensive use of these drugs in combination therapy, it has become essential to develop competent bioanalytical assays for their routine measurement in subject samples. Several analytical methods are developed and validated to determine TFV (23–30), FTC (31–34), and 3TC (35–39) individually in different biological matrices. EMTRIVA a potent combination of TFV and FTC, which has significantly greater HIV RNA suppression compared to zidovudine and 3TC, was quantified using gradient high-performance liquid chromatography (HPLC) after solid-phase extraction from human plasma (40). The sensitivity achieved was 10 ng/mL, and the separation was possible in a run-time of 18 min under gradient conditions. A binary combination of TFV and adefovir has been studied by liquid chromatography–tandem mass spectrometry (LC–MS–MS) in human plasma (41). Both the analytes were eluted in a run-time of 4.5 min, and the lower limits of quantitation achieved were 1.0 ng/mL. Recently, the analytical behavior of FTC in human serum along with atazanavir and indinavir has been investigated using electrospray ionization (ESI) ion trap mass spectrometry, LC, gas chromatography, and polarography (42). Simultaneous determination of 3TC and zidovudine has been extensively studied in rat plasma and amniotic fluids (43), rat tissues (43,44), human seminal plasma (45), and serum (46). In ternary combinations, several LC–MS–MS methods have been reported for 3TC along with zidovudine and abacavir (47), stavudine and nevirapine (48,49), stavudine and efavirenz (50), zidovudine and nevirapine by ion-pair HPLC (51), and lopinavir and ritonavir by MALDI-TOF/TOF (52). Other methods present simultaneous determination of these drugs in combination with more than five antiretrovirals in serum (53), rat plasma (54), intracellular medium (55), and human plasma (55–61). Bezy and co-workers (54) have employed HPLC–UV to simultaneously analyze TFV and 3TC along with six nucleoside reverse transcriptase inhibitors approved under HIV-1 therapy in rat plasma. Another HPLC procedure (59) for the simultaneous determination of FTC and 3TC along with 14 other drugs (six HIV protease, six nucleoside reverse transcriptase, and two non-nucleoside reverse transcriptase inhibitors) has been described. The sensitivity achieved by this method was 100 ng/mL and 5 ng/mL, respectively, employing 600 µL human plasma for solid-phase extraction. Gehrig et al. (60) have characterized eighteen antiretroviral drugs including TFV, FTC, and 3TC in plasma using triple quadrupole mass spectrometry with ESI. Saux et al. (61) have quantified seven nucleoside/nucleotide reverse transcriptase inhibitors in human plasma by LC–MS. Plasma samples were precipitated by acetonitrile with the limit of quantitation of 5 ng/mL for TFV and 10 ng/mL for FTC and 3TC, respectively. The separation of all seven drugs was achieved in a run-time of 14 min.

Thus, in the present study the aim was to develop and validate a selective, sensitive, rugged, and high throughput method for routine determination of TFV, FTC, and 3TC in subject samples. Also, a systematic matrix effect evaluation was conducted for selective determination of all three drugs in presence of matrix components and other antiretrovirals. The validated LC–ESI-MS–MS method exhibited excellent performance in terms of selectivity, ruggedness, and efficiency (3.0 min per sample). The wide linear dynamic range ensures the estimation of all three antiretrovirals up to 72 h with desired accuracy and precision for elimination phase concentration in human volunteers for bioequivalence study.

Experimental

Chemicals and materials

Reference standards of tenofovir (98.0%) and lamivudine (99.6%) were procured from Samex Overseas (Surat, India), while emtricitabine (100.0%) and acyclovir (internal standard, IS) (99.82%) were obtained from Matrix Laboratories Limited (Secunderabad, India) and Arochem Industries (Thane, India), respectively. HPLC-grade methanol and acetonitrile were

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purchased from Mallinckrodt Baker (Phillipsburg, NJ). GR-grade ammonia solution, hydrochloric acid, and formic acid were obtained from Merck Specialties Pvt. Ltd. (Mumbai, India), while ammonium formate was from Sigma Aldrich (St. Louis, MO). Water used in the entire analysis was prepared from Milli-Q water purification system procured from Millipore (Bangalore, India). Oasis MCX extraction cartridges (1 cc, 30 mg) were procured from Waters (Milford, MA). Blank human plasma was obtained from Supratech Micropath (Ahmedabad, India) and was stored at –20°C until use.

LC 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 reversed-phase LC conditions. The separation of analytes and acyclovir (IS) was performed on ACE 5 CN analytical column (Advanced Chromatography Technologies, Aberdeen, Scotland) (150 × 4.6 mm, 5-µm particle size) and was maintained at 40°C in the column oven. The mobile phase consisted of 0.5% formic acid in water and acetonitrile (55:45, v/v). For isocratic elution, the flow rate of the mobile phase was kept at 1.0 mL/min. The total chromatographic run-time was 3.0 min. The autosampler temperature was maintained at 5°C, and the pressure of the system was 850 psi. The total eluant from the column was split in 75:25 (v/v) ratio; flow directed to the ISP interface was equivalent to 250 µL/min.

Mass spectrometric conditions

Ionization and detection of analytes and IS was carried out on a triple quadrupole mass spectrometer, MDS SCIEX API 4000 (Toronto, Canada), equipped with ESI (TIS interface of the API 4000) operating in positive ion mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor protonated precursor → product ion (*m/z*) transitions for TFV (288.3→176.1), FTC (248.0 →130.2), 3TC (230.1→112.1), and ACV (226.2 → 152.2), as shown in Figure 1.

The source dependent parameters maintained for TFV, FTC, 3TC, and ACV (IS) were Gas 1 (Nebulizer gas): 40 psig; Gas 2 (heater gas flow): 60 psig; ion spray voltage: 5500 V, turbo heater temperature: 450°C; interface heater: ON; entrance potential: 10 V; collisional activation dissociation (CAD): 6 psig; and curtain gas (CUR), nitrogen: 10 psig. The optimum values for compound dependent parameters like declustering potential, collision energy, and cell exit potential set were 75, 35, and 11.5 V; 40, 6, and 8 V; 45, 6, and 7 V; 50, 25, and 10 V for TFV, FTC, 3TC, and ACV, respectively. Quadrupole 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–MS.

Standard stock, calibration standards, and quality control sample preparation

The standard stock solutions of TFV (0.5 mg/mL), FTC (1.0 mg/mL), and 3TC (2.0 mg/mL) were prepared by dissolving their requisite amount in methanol. Mixed intermediate stock solution (10 mL) for plasma spiking was prepared by mixing 1.8 mL of TFV, 3.4 mL of FTC, 2.3 mL of 3TC, and 2.5 mL of methanol–water (50:50, v/v). Calibration standards and quality control (QC) samples were prepared by spiking (2% of total plasma volume) blank plasma with stock solution. Calibration curve standards were made at 4.0, 8.0, 16.0, 40.1, 80.2, 160.3, 401, and 802 ng/mL for TFV; 15.0, 30.1, 60.1, 150, 301, 601, 1503, and 3006 ng/mL for FTC; 20.1, 40.2, 80.5, 201, 402, 805, 2012, and 4023 ng/mL for 3TC. QC samples were prepared at four levels, viz. 720, 2504, 3630 ng/mL (high quality control, HQC); 54.7, 205, and 276 ng/mL (middle quality control, MWC); 11.5, 43.2, and 57.9 ng/mL (low quality control, LQC); and 4.04, 15.2, and 20.4 ng/mL (lower limit of quantification quality control, LLOQ QC) for TFV, FTC, and 3TC, respectively. Stock solution (0.5 mg/mL) of the internal standard was prepared by dissolving 5 mg of ACV in 10.0 mL of

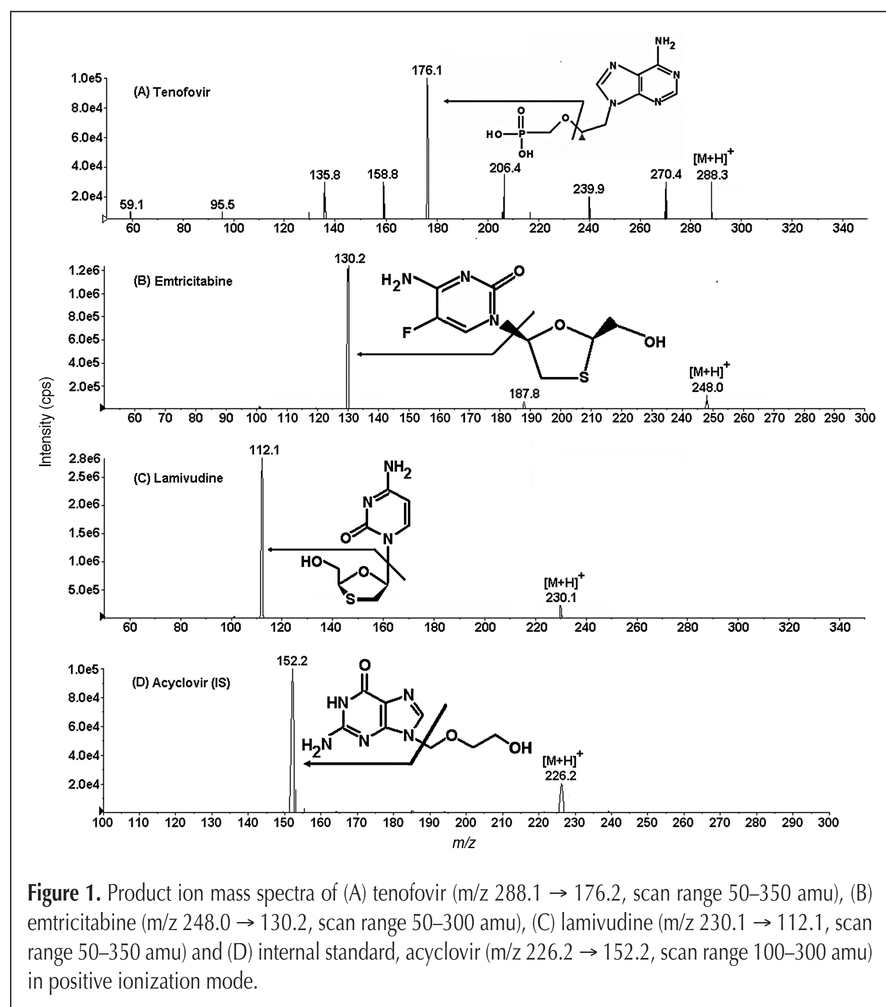

Figure 1. Product ion mass spectra of (A) tenofovir (m/z 288.1 → 176.2, scan range 50–350 amu), (B) emtricitabine (m/z 248.0 → 130.2, scan range 50–300 amu), (C) lamivudine (m/z 230.1 → 112.1, scan range 50–350 amu) and (D) internal standard, acyclovir (m/z 226.2 → 152.2, scan range 100–300 amu) in positive ionization mode.

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methanol–water (50:50, v/v). An aliquot of 0.1 mL of 0.5 mg/mL of this solution was further diluted to 50 mL in the same diluent to obtain a solution of 1 µg/mL. All the solutions (standard stock, calibration standards, and QC samples) were stored at 2–8°C until use.

Protocol for sample preparation

Prior to analysis, all frozen subject samples, calibration standards, and QC samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 0.200 mL of spiked plasma sample, 50 µL internal standard was added and vortex-mixed for 10 s. Further, 0.200 µL of 0.1 N HCl was added and vortex-mixed for 10 s. Centrifugation of the samples was done at 3200 × g for 2 min at 10°C. 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, 0.1 N HCl 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 of nitrogen. Elution of analytes and IS from the cartridges were carried out with 1 mL of 5% ammonia solution in methanol into prelabeled tubes. The eluate was evaporated to dryness in a thermostatically controlled water-bath maintained at 40°C under a stream of nitrogen. After drying, the residue was reconstituted in 150 µL of mobile phase, and 5 µL was used for injection in the chromatographic system.

Bioanalytical method validation

A thorough and complete method validation in human plasma was done following the U.S. FDA guidelines (62).

The carryover effect of the autosampler was evaluated by sequentially injecting solutions of analytes (aqueous standard), reconstitution solution (0.5% formic acid in water–acetonitrile, 55:45 v/v), standard blank, and extracted standards of analytes, 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 nonzero concentrations were analyzed. Peak-area ratios of analyte/IS obtained from multiple reaction monitoring 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, FTC, and 3TC in each calibration standard. The correlation coefficient (r^2) > 0.99 was desirable for all the calibration curves. The lowest standard on the calibration curve was accepted as the lower limit of quantitation (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 (percent coefficient variation, %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. Cross-talk of MRM channels for analytes 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) was studied under the same conditions, and their possible interference at the elution time of analyte and IS was observed. Checking for interference due to commonly used medications in human volunteers was done for acetaminophen, chlorpheniramine maleate, caffeine, acetylsalicylic acid, and ibuprofen. Their stock solutions (1000 µg/mL) were prepared by dissolving requisite amounts in methanol–water (50:50, v/v). Further, 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 analytes and IS.

The extraction efficiency (recovery) of TFV, FTC, and 3TC was performed at LQC, MQC, and HQC levels. It was evaluated by comparing the mean area response of five 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 analytes 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 was performed on the same day. The run consisted of a calibration curve and five 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 %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.

Matrix ion suppression effects on the multiple reaction monitoring LC–MS–MS sensitivity were evaluated by the post-column analytes infusion experiment. A standard solution containing all the analytes (at MQC level in methanol) was infused post column via a "T" connector into the mobile phase at 10 µL/min employing an in-built infusion pump. Aliquots of 5 µL of extracted control plasma were then injected into the column by the autosampler, and multiple reaction monitoring LC–MS–MS chromatograms were acquired for TFV, FTC, and 3TC, respectively. Any dip in the baseline upon injection of double blank plasma (without IS) would indicate ion suppression, while a peak at the retention time of analytes indicates ion enhancement. To study the effect of matrix on analyte quantification with respect to consistency in signal enhancement/suppression, it was checked in five different batches and checked for % accuracy and precision (%CV) at the LLOQ level. This was assessed by comparing the back-

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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 three 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.

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 concentrations of 1780, 6680, and 8940 ng/mL in the screened plasma for TFV, FTC, and 3TC, respectively. The precision and accuracy for dilution integrity standards at 1/5th (356, 1336, and 1788 ng/mL) and 1/10th (178, 668, and 894 ng/mL) for TFV, FTC, and 3TC, respectively, were determined by analyzing the samples against calibration curve standards.

Bioequivalence study design

The design of the study comprised of a randomized, open-label, two-treatment, two-period, two-sequences, single-dose, crossover bioequivalence study of 300 mg tenofovir disoproxil fumarate (test and reference VIREAD from Gilead Sciences Inc., Foster City, CA) + 200 mg emtricitabine (test and reference EMTRIVA from Gilead Sciences Inc.) and 300 mg lamivudine (test and reference EPIVIR from GlaxoSmithKline, Waltha, MA) tablet formulation 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 comprising five members including a lawyer, medical doctor, social worker, pharmacologist, and an academician. The procedures followed while dealing with human subjects were based on International Conference on Harmonization, E6 Good Clinical Practice (ICH, E6 GCP) guidelines (63). Oral dose of test and reference formulation was given to the volunteers with 240 mL of water. Blood samples were collected at 0.0 (pre-dose), 0.17, 0.33, 0.50, 0.67, 0.83, 1.0, 1.25, 1.5, 1.75, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 8.0, 10.0, 12.0, 24.0, 36.0, 48.0, and 72.0 h 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.

Results and Discussion

Bioanalytical method development

The method development was initiated to achieve adequate selectivity, sensitivity, and minimum overall analysis time (plasma processing and chromatographic run) using small plasma volume for processing, which is crucial for antiretroviral drugs, especially for variable combination therapy. Optimum mass acquisition parameters were obtained by direct infusion of 500 ng/mL solution for TFV, FTC, and 3TC at a flow rate of 10 µL/mL. This was done by maintaining declustering potential and ion spray voltage at 50 V and 5500 V, respectively. The present study was conducted using positive ESI as it gave higher response under MRM with a signal-to-noise ratio ≥ 110 and a good linearity in regression curves compared to the negative mode. The ESI Q1 mass spectrum of TFV, FTC, 3TC, and IS was dominant with protonated precursor $(M+H)^+$ ions as all the drugs can be easily protonated. The most stable and consistent product ions for TFV, FTC, 3TC, and IS were observed at *m/z* 176, 130, 112, and 152, respectively. The ion at *m/z* 176 resulted from the loss of a water molecule and phosphono methoxy group from TFV. Both the protonated precursor ion of FTC and 3TC showed a selective loss of a sugar group during CAD to give an ion at *m/z* 130 and 112, respectively. For ACV, the protonated guanine species (*m/z* 152.2) was the most abundant ion seen in the product ion mass spectra. The MRM state file parameters like nebuliser gas, CAD gas, ion spray voltage, and temperature were suitably optimized to obtain a consistent and adequate response for the analytes. To have better ionization efficiency for tenofovir, the column effluent was split, and only 25% was directed to the ISP interface. A dwell time of 200 ms for all the three analytes and IS was adequate, and no cross-talk was observed between their MRMs.

Chromatographic analysis of the analytes and IS was initiated under isocratic conditions to obtain adequate response, sharp peak shape, and a short run-time. It was also observed that the pH of the mobile phase and selection of the right column was crucial for efficient separation. It has been reported earlier (54), the importance of volatile buffers like hydroxylamine-acetic acid, ammonium formate, and ammonium phosphate in combination of methanol–acetonitrile for the separation of TFV, 3TC, and other antiretroviral nucleosides. Rezk et al. (40) have used phosphate buffer (pH 5.7) for the separation of TFV and FTC. Thus, separation was tried using various combinations of methanol–acetonitrile with acidic buffers and additives like formic acid on different reversed-phase columns. ACE 5 CN analytical column (150 mm × 4.6 mm) with 5-µm particle size gave the best results with regard to sensitivity, efficiency, and peak shapes compared to Chromolith RP_{18} (100 × 4.6 mm), Kromasil (50 and 100 × 4.6 mm), and Gemini C_{18} (50 × 4.6mm) columns. A mobile phase consisting of 0.5% formic acid in water–acetonitrile (55:45, v/v) ratio and having pH ~3.5 was found most suitable for eluting the analytes and IS in a run-time of 3.0 min.

Solid-phase extraction of the analytes and IS was tried on Waters Oasis HLB, Waters Oasis MCX, and Phenomenex Strata cartridges for quantitative recoveries. Addition of strong acid

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like HCl during sample preparation helped in maintaining the analytes in the ionized with better retention on Waters Oasis MCX as compared to other cartridges. Quantitative and precise recoveries were obtained at all the QC levels with minimum matrix interference.

Acyclovir, a nucleoside analog, was used as an internal standard in the present study. It was competent to minimize any analytical variation due to solvent evaporation, integrity of the column, and ionization efficiency. Moreover, there was no drug-drug interaction with the other three antiretrovirals. It had similar chromatographic behavior, and all four drugs were quantitatively extracted via solid-phase extraction.

Autosampler carryover and linearity

Carryover evaluation was performed in each analytical run so as to ensure that it does not affect the accuracy and precision of the proposed method. There was no carryover observed during autosampler carryover experiment. Also, no enhancement in the response was observed in the double blank after subsequent injection of highest calibration standard (aqueous and extracted) at the retention time of TFV, FTC, 3TC, and ACV, respectively. Moreover, no ghost peaks appeared during the analysis of blank samples.

All three calibration curves analyzed during the course of validation were linear for the standards ranging from 4.0 to 802 ng/mL; 15.0 to 3006 ng/mL; and 20.1 to 4023 ng/mL for TFV, FTC, and 3TC, respectively. A straight-line fit was made through the data points by least square regression analysis to give the mean linear equation $y = 0.0013x - 0.0002$ for TFV; $y = 0.0019x + 0.0013$ for FTC; and $y = 0.0031x + 0.0012$ for 3TC, where y is the peak-area ratio of the analyte to the IS and x the concentration of the analyte. The standard deviation values for slope, intercept, and correlation coefficient (r) observed during the course of validation were 0.0008, 0.0006,

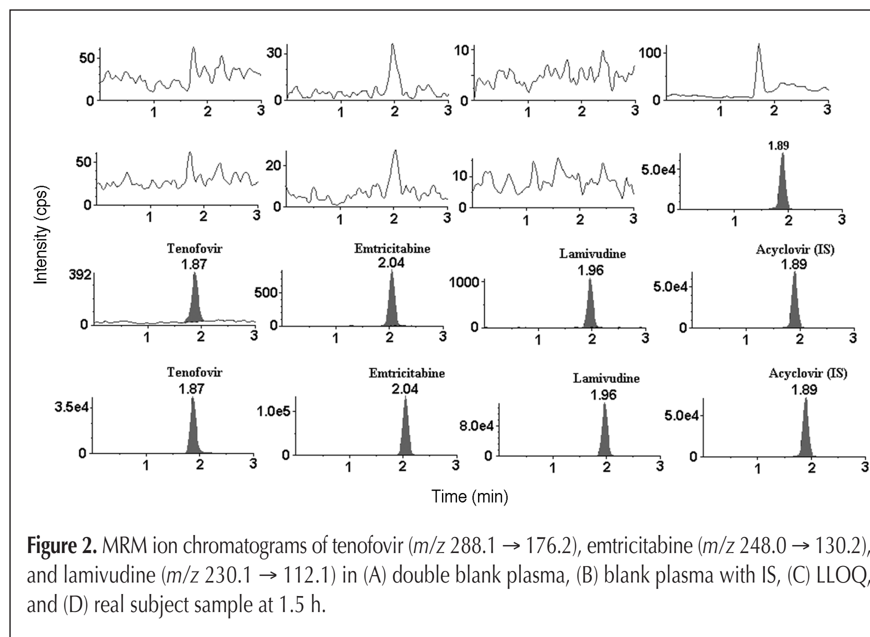

Figure 2. MRM ion chromatograms of tenofovir (m/z 288.1 → 176.2), emtricitabine (m/z 248.0 → 130.2), and lamivudine (m/z 230.1 → 112.1) in (A) double blank plasma, (B) blank plasma with IS, (C) LLOQ, and (D) real subject sample at 1.5 h.

Table I. Comparison of Intra-batch and Inter-batch Precision and Accuracy for Tenofovir, Emtricitabine, and Lamivudine

	Intra-batch					Inter-batch			
QC ID	Nominal concentration (ng/mL)	n	Mean found concentration (ng/mL)*	% CV	% Accuracy	n	Mean found concentration (ng/mL)†	% CV	% Accuracy
Tenofovir									
HQC	720	5	728	3.3	101.1	25	730	3.3	101.5
MQC	54.7	5	53.7	1.9	98.2	25	52.5	3.6	96.0
LQC	11.5	5	11.7	4.4	101.7	25	11.1	5.9	96.8
LLOQ	4.04	5	4.22	2.9	104.4	25	3.90	8.7	96.4
Emtricitabine									
HQC	2504	5	2574	1.8	95.2	25	2571	4.3	95.1
MQC	205	5	199	2.9	96.6	25	206	3.2	100.4
LQC	43.2	5	42.1	1.5	97.5	25	43.4	4.0	100.5
LLOQ	15.2	5	15.2	3.0	100.2	25	14.7	4.7	96.6
Lamivudine									
HQC	3630	5	3516	0.9	96.9	25	3449	2.1	95.0
MQC	276	5	273	1.3	98.9	25	271	2.6	98.2
LQC	57.9	5	56.6	1.0	97.7	25	56.8	2.7	98.0
LLOQ	20.4	5	19.7	4.0	96.6	25	19.2	4.9	94.0

* Mean of 5 replicates at each concentration.
† Mean of 25 replicates at each concentration.

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and 0.0003 for TFV; 0.0027, 0.0010, and 0.0009 for FTC; and 0.0032, 0.0006, and 0.0007 for 3TC, respectively.

Selectivity of the method

The selectivity of the method towards endogenous plasma matrix was ascertained in twelve batches of human plasma by analyzing blanks and spiked plasma samples at LLOQ concentration. No endogenous peaks were observed at the retention time of the analytes for any of the batches. Figure 2 demonstrates the selectivity experiments with the chromatograms of extracted blank plasma, blank plasma with IS, peak response of TFV, FTC, and 3TC at LLOQ and a real sample at 1.5 h after oral administration of [300(TFV) + 200(FTC) + 300(3TC)] mg tablet formulation. The retention time for TFV, FTC, 3TC, and IS were at 1.87, 2.04, 1.96, and 1.89 min, respectively. None of the concomitant medications considered showed interfering signals at the retention time of TFV, FTC, 3TC, or IS. This demonstrates that the method is highly selective and free from interference due to matrix components and other prescribed medications.

Recovery, accuracy, and precision

Five replicates at LQC, MQC, and HQC levels were prepared for recovery determination. The % mean recovery at HQC, MQC, and LQC levels for TFV, FTC, and 3TC were 78.3, 76.7, and 79.4%; 86.0, 83.8, and 86.7%; and 85.3, 79.8, and 84.0%, respectively. The recovery of internal standard was 83.4%.

The intra- and inter-batch accuracy and precision was determined in five batches at LLOQ QC, HQC, MQC, and LQC levels with five replicates for each batch. Precision (%CV) for intra-batch and inter-batch ranged from 1.9 to 8.7 for TFV, 1.8 to 4.7 for FTC, and 0.9 to 4.9 for 3TC, respectively. The accuracy results for intra-batch and inter-batch were within 94.0–104.4% for TFV, FTC, and 3TC, respectively, at all QC levels. The detailed results are presented in Table I.

Ion suppression, 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 result in ion suppression/enhancement, decrease/increase in sensitivity of analytes over a period of time, increase baseline, imprecision of data, drift in retention time, and distortion or tailing of a chromatographic peak (64). Thus assessment of matrix effect constitutes an important and integral part of validation for quantitative LC–MS–MS method for supporting pharmacokinetics studies. Post-column infusion experiment results indicated no ion suppression or enhancement at the retention time of TFV, FTC, and 3TC. Fur-

Table II. Matrix effect assessment of Tenofovir, Emtricitabine, and Lamivudine at the LLOQ Level in Six Different Lots of Human Plasma (*n* = 5)

Plasma Lot ID	Tenofovir	Emtricitabine	Lamivudine
LLOQ, nominal concentration (ng/mL)			
	4.0	15.0	20.1
Calculated concentration (ng/mL)			
ME LLOQ1	3.9	16.4	21.0
ME LLOQ2	4.3	15.8	22.3
ME LLOQ3	4.3	16.1	20.9
ME LLOQ4	4.1	17.0	23.2
ME LLOQ5	4.2	16.9	22.9
Mean	4.2	22.1	
SD	0.1	0.5	1.1
% CV	3.6	3.1	4.8
% Accuracy	104.0	109.4	109.7

Table III. Stability of Tenofovir, Emtricitabine, and Lamivudine Under Various Conditions (*n* = 3)

	Tenofovir		Emtricitabine		Lamivudine	
	Calculated concentration (ng/mL)		Calculated concentration (ng/mL)		Calculated concentration (ng/mL)	
Stability, temperature, duration	Mean stability + SD	%Change*	Mean stability + SD	%Change*	Mean stability + SD	%Change*
Bench top stability, ambient, 7 h						
HQC	723 ± 10.7	0.4	2560 ± 30.6	–5.3	3365 ± 73.3	–7.3
LQC	10.6 ± 0.5	–7.6	40.3 ± 0.2	–6.5	54.0 ± 0.2	–6.8
Wet extract stability, 5°C, 39 h						
HQC	804 ± 17.9	7.8	2666 ± 65.9	4.3	3410 ± 29.9	2.1
LQC	10.6 ± 0.5	8.0	44.8 ± 1.4	1.1	55.4 ± 0.9	–4.5
Dry extract stability, –20°C, 27 h						
HQC	712. ± 6.7	–1.0	2668 ± 17.4	–1.3	3420 ± 88.2	–5.8
LQC	11.0 ± 0.1	–3.7	43.6 ± 2.0	1.0	57.2 ± 0.9	–1.2
Freeze-thaw stability, –20°C, 3 cycles						
HQC	716 ± 12.6	–0.5	2545 ± 21.5	–5.8	3503 ± 47.9	–3.5
LQC	10.7 ± 0.2	–6.3	41.3 ± 2.2	–4.2	55.6 ± 1.6	–4.0
Long-term plasma stability, –20°C, 45 days						
HQC	679 ± 9.5	–5.7	2455 ± 43.7	–5.3	3350 ± 36.7	–7.7
LQC	11.3 ± 0.5	–1.3	40.3 ± 1.0	–6.5	52.8 ± 1.7	–8.9
Long-term plasma stability, –70°C, 45 days						
HQC	704 ± 19.1	–2.9	2441 ± 31.0	–9.7	3296 ± 30.1	–9.2
LQC	11.4 ± 0.7	–0.6	40.4 ± 1.2	–6.3	53.8 ± 1.9	–7.1

* %Change = [(Mean stability samples – Mean comparison samples)/Mean comparison samples] × 100.

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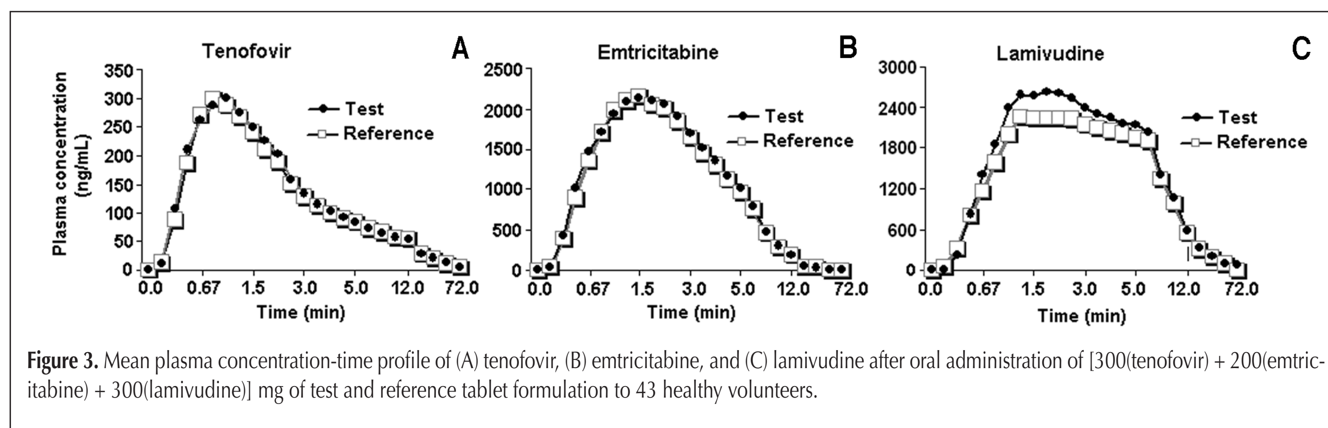

Figure 3. Mean plasma concentration-time profile of (A) tenofovir, (B) emtricitabine, and (C) lamivudine after oral administration of [300(tenofovir) + 200(emtricitabine) + 300(lamivudine)] mg of test and reference tablet formulation to 43 healthy volunteers.

Table IV. Pharmacokinetic Parameters of Tenofovir, Emtricitabine, and Lamivudine after Oral Administration of Tablet Formulation* to 43 Healthy Human Subjects

Parameter	Tenofovir Mean + SD		Emtricitabine Mean + SD		Lamivudine Mean + SD	
	Test	Reference	Test	Reference	Test	Reference
C_{max} (ng/mL)	313.7 ± 85.8	358.0 ± 113.5	2412.6 ± 537.3	2410.1 ± 463.2	2750.9 ± 692.5	2789.9 ± 638.3
T_{max} (h)	1.1 ± 0.84	1.0 ± 0.4	1.6 ± 0.7	1.6 ± 0.5	1.5 ± 0.7	1.5 ± 0.7
$t_{1/2}$ (h)	18.6 ± 2.2	18.5 ± 1.8	5.4 ± 1.1	5.5 ± 0.8	5.0 ± 1.9	5.1 ± 1.7
$AUC_{0–72 h}$ (h.ng/mL)	2376.8 ± 452.2	2505.2 ± 648.7	12477.5 ± 2037.3	12192.1 ± 2267.2	11811.3 ± 2694.4	11876.4 ± 2719.8
$AUC_{0–inf}$ (h.ng/mL)	2530.6 ± 487.2	2658.1 ± 690.3	12832.5 ± 2044.3	12576.0 ± 2282.1	12087.9 ± 2654.5	12145.9 ± 2720.5

* [300(TFV) + 200(FTC) + 300(3TC)] mg.

ther, the matrix effect for the intended method was assessed by using chromatographically screened human plasma. The precision (%CV) values for LLQC samples observed were 3.6, 3.1, and 4.8%, while the accuracy found at this level was 104.0, 109.4, and 109.7% for TFV, FTC, and 3TC, respectively (Table II). 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 analytes and IS were stable at room temperature for minimum period of 7 h and between 2–8°C for 7 days, respectively. TFV, FTC, and 3TC in control human plasma (bench top) at room temperature were stable at least for 7 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 experiment were stable for a minimum of 45 days. Dry extract stability of the spiked quality control samples stored at –20°C was determined up to 27 h. Autosampler stability of the spiked quality control samples maintained at 5°C was determined up to 39 h. Different stability experiments in plasma and the values for the precision and percent change are shown in Table III.

Method ruggedness and dilution integrity

For ruggedness study, the precision and accuracy for calibration curve standards and LLOQ were between 0.3–5.6% and 96.2–104.4% for all analytes, which is within the acceptance criteria. For both the experiments the precision and accuracy for LLOQ, all QC samples ranged from 0.5 to 6.6% and 92.3 to 100.7% for TFV, FTC, and 3TC, 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 quantification (ULOQ), which may be encountered during real subject sample analysis. The precision for dilution integrity of $1/5^{th}$ and $1/10^{th}$ dilution were 1.6 and 4.3%; 1.8 and 3.9%; and 1.6 and 5.1% for TFV, FTC, and 3TC, respectively, while the accuracy results were 106.7, 106.5%; 101.1, 105.3%; and 101.9, 105.2% for TFV, FTC, and 3TC, respectively, which are 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 TFV, FTC, and 3TC in healthy male volunteers in the age group of 18 to 45 years. Figure 3 shows the mean plasma concentration-time profile following [300(TFV) + 200(FTC) + 300(3TC)] mg dosing 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 over a period of 10 days,

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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 TFV in test and reference formulations. The mean pharmacokinetic parameters obtained for the test and reference formulation are presented in Table IV. 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. These observations confirm the bioequivalence of the test sample with the reference product in terms of rate and extent of absorption. Further, there was no adverse event during the course of the study.

Conclusion

The bioanalytical methodology presented for simultaneous estimation of TFV, FTC, and 3TC in human plasma is highly selective and rugged for routine measurement of these drugs. The LLOQ and wide linear dynamic range makes it suitable for use in clinical studies. The method involved an efficient and specific sample preparation by solid phase extraction followed by isocratic chromatographic separation in 3.0 min. The small plasma requirement for processing is beneficial, especially for children infected with HIV. The overall analysis time is promising compared to other reported procedures for simultaneous determination of these three antiretrovirals.

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