The Pharmaceutical and Chemical Journal, 2016, 3(4):157-168

Available online www.tpcj.org

Research Article

ISSN: 2349-7092
CODEN(USA): PCJHBA

Pharmacokinetic and bioequivalence study of Tenofovir Disoproxil Fumarate under fasting conditions on Argentine healthy-volunteers. Optimization and validation of SPE-LC-MS/MS for determination of Emtricitabine, Lamivudine and Tenofovir in human plasma

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Abstract A pharmacokinetic study based on single oral administration of Tenofovir disoproxil fumarate 300 mg (tablets) to Argentinean male volunteers under fasting conditions is presented. The obtained values for test and reference products were 238±62 and 261±73 ng mL^{-1} for C_{max}; 1658±466 and 1801±529 ng mL^{-1} h for AUC_{0-48h}; 1845±579 and 2015±639 ng mL^{-1} h for $AUC_{0-\infty}$, respectively. The 90% confidence intervals obtained by analysis of variance were 85.0-97.2% for ln-C_{max}, 87.2-97.9% for ln-AUC_{0-48h} and 85.5-98.1% for ln-$AUC_{0-\infty}$, which are within the acceptance range of 80-125%. Both products were bioequivalent in terms of rate and extent of drug absorption and therefore interchangeable. The analytical methodology was optimized and validated for determination of Emtricitabine (FTC), Lamivudine (3TC) and Tenofovir (TFV) in human plasma samples; which is the pharmaceutical combination most commonly used for treating HIV-infected patients. The analytical methodology was based on solid phase extraction (SPE) using Oasis® MCX mixed-mode cartridges coupled to Liquid Chromatography and electrospray tandem mass spectrometry (LC-ESI-MS/MS). The analytical methodology was validated according to the US Food and Drug Administration guidelines. Under optimized conditions, the analytical methodology lead to work within a clinical of 11-5434, 11-5452 and 13-397 ng mL^{-1} for FTC, 3TC and TFV, respectively; resulting wider than others previously reported. The intra and inter batch precision (%RSD) across three validation runs was less than 14%. The accuracy determined at four QC levels (LOQ, Low, Middle and High) was within 13%, in terms of percent relative error.

Keywords Pharmacokinetic; Bioequivalence; LC-ESI-MS/MS; Emtricitabine; Lamivudine; Tenofovir; HIV

Introduction

Since the identification of the human immunodeficiency virus (HIV) in 1983 [1], which causes acquired immunodeficiency syndrome (AIDS), numerous research efforts have been invested worldwide in an attempt to combat this pandemic disease. Among the main antiretrovirals (ARV) developed to treat AIDS, combinations

containing Tenofovir Disoproxil Fumarate (TDF), Emtricitabine (FTC) and Lamivudine (3TC) are particularly recommended for HIV treatment [2]. TDF is an acyclic nucleoside phosphonate diester analog of adenosine monophosphate. TDF is rapidly absorbed and converted to Tenofovir (TFV) after oral doses, with peak plasma concentrations occurring after 1 to 2 hours. Its bioavailability is about 25%, however, it can be enhanced when TDF is taken with a high fat meal. TFV is widely distributed into body tissues, particularly in kidney and liver [3]. Binding of TFV to plasma and serum proteins is less than 1% and 7%, respectively. The terminal elimination half-life of TFV is 12 to 18 hours [3]. TDF requires initial diester hydrolysis for its conversion to TFV and subsequent phosphorylations by cellular enzymes to form tenofovir diphosphate. Tenofovir diphosphate inhibits the activity of HIV-1 Reverse Transcriptase (RT)after its incorporation into a DNA chain termination by competing with the natural substrate deoxyadenosine 5'-triphosphate. Tenofovir diphosphate is a weak inhibitor of mammalian DNA polymerases alpha and beta, and mitochondrial DNA polymerase gamma, thus conferring this drug a high specificity for reverse transcriptases of viral origins [4].

The fixed-dose combination of FTC and TDF drugs is a simple, highly efficacious combination, with a superior safety profile compared with thymidine analogues [5]. FTC is a synthetic nucleoside analogue with activity against HIV RT [6]. After oral doses it is rapidly and extensively absorbed from the gastrointestinal tract, with a peak plasma concentrations occurring after 1 to 2 hours. Bioavailability is reported to be 93% for the capsules. Binding to plasma proteins it is reported to be less than 4%. The plasma elimination half-life is about 10 hours [3]. FTC is dosed once daily, without regard to food intake and after its absorption is readily anabolized by cellular enzymes to form its monophosphate, diphosphate and finally, triphosphate chemical forms. The triphosphate form is the active intracellular moiety that inhibits RT [7].

Another commonly employed RT inhibitor is 3TC, a nucleoside analog with a long history of use in human HIV treatments [8]. The 3TC is rapidly absorbed after oral doses and peak plasma concentrations are achieved in about 1 hour. Its absorption is delayed, but not reduced, when ingested with food. The bioavailability is between 80 and 87%. Binding to plasma protein is reported to be up to 36%. An elimination half-life of 5 to 7 hours was reported after a single dose [3]. The 3TC is phosphorylated to its putative active metabolite, lamivudine-5'-triphosphate, initially, by deoxycytidine kinase and then, by other human cellular kinase enzymes. The main way of action of 3TC-triphosphate (3TC-TP) is the inhibition of RT via DNA chain termination after incorporation of the nucleotide analogue [9]. Treatment of HIV infections with an antiviral regiment including 3TC is highly desirable because it shows lower toxicity compared to other nucleoside derivatives [10].

Comparative head-to-head pharmacokinetic studies among pharmaceutical products are required to demonstrate bioequivalence of formulations commercially available. As it is well known, pharmacokinetic parameters not only depend on the commercial formulation of the administered dose, but also on the ethnicity of the bioequivalence study volunteers and conditions of medicine intake, among others parameters [10–13]. To the best knowledge of the authors, there are no reports of pharmacokinetic studies of tenofovir disoproxil fumarate 300 mg (tablets) carried out on Argentinean male volunteers under fasting conditions. A key step in conducting a bioequivalence study is the development of analytical methodologies for a quantitative determination of the pharmaceutical ingredient in the selected biological samples with sufficient sensitivity and specificity [14–16]. Development of an appropriate analytical methodology thus plays an important role for assurance of confident results in bioequivalence studies of ARV formulations. In this sense, the figures of merits of the analytical methodology are decisive, and must contemplate the situation under study. Recently, different analytical methods were reported in the literature for monitoring plasma levels of FTC [17–20], TFV [10,21] and 3TC [22–24]. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), is the most promising instrumental technique for determining ARV since highly selectivity and sensitivity. However, the major challenge when determining plasma concentration of TFV using LC-MS/MS, is to circumvent ion suppression effects that occur in the ion source due to the high polarity of this analyte [17]. Among the reported sample preparation techniques for determination of TFV by LC-MS/MS, SPE was the suggested technique since it leads to efficient extraction and concentration of TFV and eliminates matrix effects [21, 25-26]. Yadav *et al.* reported the use of Oasis® MCX mixed-mode cartridges for the analysis of FTC, 3TC and TFV in human plasma by LC-MS for a bioequivalence study of TDF, FTC and 3TC in healthy Indian

subjects [10]. However, the figures of merits of this analytical methodology (detection limits and working linear range) were non-successful for following up bioequivalence studies carried out in this work.

The aim of this work was to optimize and validate an analytical methodology for determining concentration of TFV, FTC and 3TC in human plasma according to the US Food and Drug Administration (FDA) guidelines [2,14], and based on previous reports [10, 18–20, 27]. This methodology was the employed to evaluate and compare the rate and extent of absorption of test and reference formulations of TDF 30 mg administered to Argentinean healthy-volunteers under fasting conditions.

Experimental Work

Chemicals and Reagents

Emtricitabine (USP, LOT F0J163, 99.8% w/w), Lamivudine (USP, LOT H1L282, 99.7% w/w), Tenofovir (USP, LOT F0J005, 99.8% w/w), and Tenofovir-d_7 (TLC PHARMACHEM, LOT 1299-001A3, 98.3% w/w) standard were supplied by various pharmaceutical industry sponsors. Stock solutions (600 µg mL^{-1} each ARV) were prepared by dissolving an appropriate amount of each compound in metanol (MeOH). Working solutions were prepared daily by dilutions of the stock solutions with MeOH. HPLC grade water, acetonitrile (ACN) and MeOH were purchased from Carlo Erba, France, and Ammonium acetate (NH_4Ac) was purchased from Sigma-Aldrich, Germany. All reagents were analytic grade or above.

LC-MS/MS Instrumentation

The assay was carried out by using Ultra-Fast Liquid Chromatography (UFLC) coupled to a triple quadrupole instrument, described as follows: UFLC Shimadzu (LC 20AD), automatic injector Shimadzu (SIL-20A XR), coupled to an API 3200 (Applied Biosystems, Ontario, Canada). Liquid chromatographic analysis was carried out on a Hypersil Gold C_{18} column (100 mm × 2.1mm i.d., 3 µm particle, Thermo Fisher Scientific, USA). Data were acquired and processed using Analyst software (version 1.5.1; ABSciex, Toronto, Canada).

Sampling and Sample Conditioning

Samples were obtained from volunteers previously enrolled at the Biopharmaceutical Analysis Center Dominguez Lab S.R.L.. The informed-consent form was approved by the Ethic Committee prior obtaining the consent from the subjects. The nature and purpose of the informed-consent was explained to all volunteers, and was performed according to the principles of the Declaration of Helsinki [28]. Sampling was carried out using a catheter system (BD Saf-T-Intima™, BD Vacutainer®) and syringes of 5 mL. The blood sample was collected into heparinized polypropylene 4 mL tubes (NAHEP PLH 13X75 4.0 PLBL GN, BD Vacutainer®, Broken Bow NE 68822 US) and centrifuged at 3000 × g for plasma separation. Aliquots of 1 mL were preserved in polypropylene 2 mL cryovials and frozen at -20 °C ± 5 °C until analysis.

Sample preparation using SPE

Subject samples, calibration standards, and quality control (QC) samples were thawed and allowed to equilibrate at room temperature, prior analysis. An aliquot of 700 µL of plasma was spiked with 100 µL of internal standard (Tenofovir-d_7 4.8 µg mL^{-1}) and vortex-mixed for 10 s. Further, 1.5 mL HCl 0.2 N was added and vortex-mixed for 10 s. Centrifugation of the samples was carried out at 10°C, 3000 × g for 5 min. Each prepared sample was loaded on Oasis® MCX (3 cc, 60 mg) extraction cartridge, which was preconditioned as follows: cartridge activation was carried out flushing it with 3 mL of MeOH followed by 3 mL of water. Then, the cartridge was pH conditioned by rinsing it with 1 mL HCl 0.1 N followed by 1 mL MeOH. Finally, the cartridge was dried with a gentle N_2 stream (20 psi, 1 min). Elution of analytes and internal Standard (IS) from the cartridges was carried out with 1 mL NH_4Ac 5% methanolic solution into prelabeled tubes. The eluate was evaporated to dryness at 40°C under a gentle N_2 stream. After drying, the residue was reconstituted in 500 µL of ACN: H_2O 50:50 and 25 µL were used for injection in the chromatographic system.

Preparation of Calibration Standards and Quality Control Samples

Calibration curve consisted of blank samples (matrix sample processed without internal standard), zero samples (matrix sample processed with internal standard) and non-zero plasma standards, covering the expected range of

concentrations to be quantified. Calibration standards were prepared by spiking control human plasma with working solutions containing the analytes to be quantified.

Quality Control (QC) samples have the purpose to monitor the precision and accuracy of the quantification method during the assays. These samples were prepared by spiking control human plasma with working solutions prepared for quality controls containing the analytes to be quantified. QC samples were prepared at four levels: lower limit of quantification quality control - LLOQ QC - (11, 11 and 13 ng mL^{-1} for FTC, 3TC and TFV, respectively); low quality control -LQC- (28 ng mL^{-1} for all ARVs); middle quality control – MQC - (2377, 2370 and 208 ng mL^{-1} for FTC, 3TC and TFV, respectively) and high quality control-HQC- (4755, 4741 and 312 ng mL^{-1} for FTC, 3TC and TFV, respectively).

Bioanalytical Method Validation

The selectivity of the method towards endogenous plasma matrix components was assessed in three different batches (4 normal, 2 hemolyzed, 2 lipemic) of blank plasma.

The linearity of the method was determined by analysis of three linearity curves containing eight non-zero concentrations. The ratio of area response for FTC, 3TC, TFV and to TFV–d7 obtained from MRM was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted (1/x) linear regression. A correlation coefficient (r) value > 0.99 was desirable for all the calibration curves. The lowest standard on the calibration curve was accepted as the LLOQ, if the analyte response was at least five times more than that of drug-free (blank) extracted plasma. For determining the intra-assay accuracy and precision, replicate analysis of plasma samples of FTC, 3TC, TFV was performed on the same day. The run consisted of a calibration curve and five replicates of LLOQ QC, LQC, MQC, and HQC samples. The inter-assay accuracy and precision were assessed by analyzing three precision and accuracy batches on three consecutive validation days. The deviation at each concentration level from the nominal concentration was expected to be within ±15%, except for LLOQ which it should be within ±20%. Similarly, the mean accuracy should not deviate by ±15%, except for LLOQ where it can be ±20% of the nominal concentration.

As a part of method validation, stability was evaluated. Analytes were considered stable if the percent relative error (%RE) of the mean test responses were within 15% of appropriate controls (LQC, MQC, and HQC). The stability of spiked human plasma stored at room temperature (bench-top stability) was evaluated for 6 h. Extracted samples were tested for stability after being stored in the auto sampler pending analysis at ambient temperature (22±3 °C) for 12 h. Freeze–thaw stability was assessed by QC samples that had been frozen at −20 °C and thawed three times. The long term stability in human plasma when stored at −20 °C was tested after 159 days.

Design of the Bioequivalence Study

The conducted bioequivalence study was a single-dose, randomized-sequence, open label and 2-way crossover. The volunteers were confined into the research center 12 h before drug administration and for 24 h after administration. The volunteers were continuously monitored by medical supervision throughout the confinement period of the study. After a 10 h overnight fast, the volunteers received a single 300 mg dose of TDF of the test, or reference formulation, with 240 mL of water in random order, with 2 study periods separated by 7 days of washout period. The washout period was determined based on 5 to 7 times the $T_{1/2}$ of TFV (12-15 h). Approximately 4 mL of blood for TFV assay was drawn into heparinized tubes through an indwelling cannula before (0 h) and at 0.25 h, 0.5 h, 0.75 h, 1 h, 1.25 h, 1.50 h, 2 h, 2.5 h, 3 h, 4 h, 6 h, 8 h, 10 h, 24 h and 48 hours after drug administration. After washout period, the study was repeated in the same manner to complete the cross-overdesign.

Pharmacokinetics and Statistical Analysis

The plasma concentration-time data after oral administration of a single dose(s) of test and reference treatment were analyzed using a noncompartmental method (WinNonlin v.6.02, Pharsight Corporation, St. Louis, MO, USA). The maximal concentration (C_{max}) and the time for maximal concentration (T_{max}) were obtained directly for the plasma concentration–time curves of TFV. The area under the plasma concentration–time curve ($AUC_{0–last}$), was calculated using the linear trapezoidal rule. The area under the concentration–time curve extrapolated to infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0–last}$, $+C_t/Ke$, where C_t is the last measured concentration, and Ke is the slope of the linear regression of the natural log (ln)-transformed plasma concentration–time in the terminal phase. The plasma

elimination half-life ($T_{1/2}$) was calculated as 0.693/Ke [20]. The bioequivalence between the two formulations was evaluated based on the 90% CI transformed back for the geometric mean ratios of $AUC_{0-\infty}$, AUC_{0-48h} and C_{max}, which were within acceptance range of 80-125% according to the local and international guidelines [29,30].

Results & Discussion

Method Optimization

For consistent and reliable determination of the target analytes and the IS, optimization of the sample preparation procedure, as well as the chromatographic method optimization along with spectrometric mass detection parameteres were carried out. Analytes and internal standard (ISTD) were tuned in both positive and negative polarity modes using electrospray ionization technique. Declustering potential (DP), entrance potential (EP), collision energy (CE) and collision exit potential (CXP) were all optimized to allow the highest transduction signal with low background noise. Signal optimization was performed by constant infusion of 100 ng mL^{-1} drug solutions in H_2O:ACN (50:50) at a rate of 50 ng mL^{-1}. The mass spectrometer was used in the multiple reaction monitoring (MRM) mode and the *m/z* transition for quantification collected in positive mode were 248.0 → 130.0, 230.0 → 112.1, 288.1 → 159.1 and 295.3 → 183.5 for FTC, 3TC, TFV and TFV–d_7, respectively. The remaining operative conditions were as follows: 250 ms dwell time, 5500 V ion spray voltage, 50 psi nebulizer gas (GS1:N_2), 30 psi curtain gas, 7 psi collision gas (N_2), 50 psi drying gas (GS2:N_2), 500 °C drying gas temperature and 100 °C source temperature.

The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes for the analytes and internal standards, as well as a short running time. The chromatographic separation was carried out using a three-component mobile phase of MeOH:ACN:10mM NH_4Ac (33:22:45). The optimum flow rate gradient was set at 0.21 mL min^{-1} for 0.50 min increasing to 0.42 mL min^{-1} at 0.55 min and maintained for 1.50 min, and returning to 0.21 mL at 1.60 mL min^{-1} to the end of the run. With this gradient flow the best chromatographic peak and the best signal was obtained to reach the limits of quantification required. The column temperature was kept at 40°C along the run. The injection volume was 25 µL and the total running time was 4 min. The retention time for the target analytes were 1.30, 1.30, 1.2 and 1.2 min for FTC, 3TC, TFV and TFV–d_7, respectively.

In the selection of sample preparation method, the recovery of the analytes after solid phase extraction (SPE) was determined by comparing observed peak area in extracted plasma for each compound, to those of blank samples processed and spiked with working solutions containing the analytes to be quantified. SPE is a widely used procedure for cleanup of complex environmental samples prior to LC–MS/MS [31,32]. SPE and instrumental efficiency are conditioned by sample matrix characteristic and physicochemical properties of analytes. The Oasis® HLB (3 cc, 60 mg) cartridges was developed for the extraction of a wide range of acidic, basic, and neutral compounds from various matrices using a simple and generic protocol. Under these conditions, concomitants of the plasma sample are also retained and may be co-eluted together with the target compounds in the elution step. The Oasis® MCX cartridges contain a novel mixed-mode polymeric sorbent that has been designed to achieve higher selectivity for extracting basic compounds through its cation-exchange groups. Therefore, we incorporated into our method a passage through a mixed-mode Oasis® MCX cartridge for cleanup of the sample extracts. To effectively retain the analytes on the MCX sorbent, it was necessary to acidify the sample extracts before loading for retaining the analytes in their cataionc form. Sample concomitants were removed during the washing step with 1 mL of methanol and methanolic washing also lead to shorter drying steps. Analysis of the eluate from the washing steps showed no loss of the analytes during this step. After the methanolic washing step, the analytes were eluted with an ammonium hydroxide–methanol solution. Figure 1 shows a comparison of extraction recoveries of FTC, 3TC, TFV and TFV-d_7 for two different SPE cartridge, Oasis® HLB and Oasis® MCX, and their reconmended eluant solvent or solution (MeOH for Oasis® HLB and NH_4Ac 5% in MeOH for Oasis® MCX). The recovery of the all ARVs increased as the eluant volume increased. For TFV y TFV-d_7, the highest analytical response was achieved for the Oasis® MCX cartridge. The low analytical response for these two analytes, TFV and TFV-d_7, might be due to their high affinity for the sorbent phase of the HLB SPE cartdrige, which was strongly conditionated for the pK_a of these

analytes (10.2). The cartridge Oasis® MCX was found to meet the criteria of clean injection extracts, sensibility and consistent recovery. In order to reduce the drying time, 1 mL elution volume was chosen for further assays.

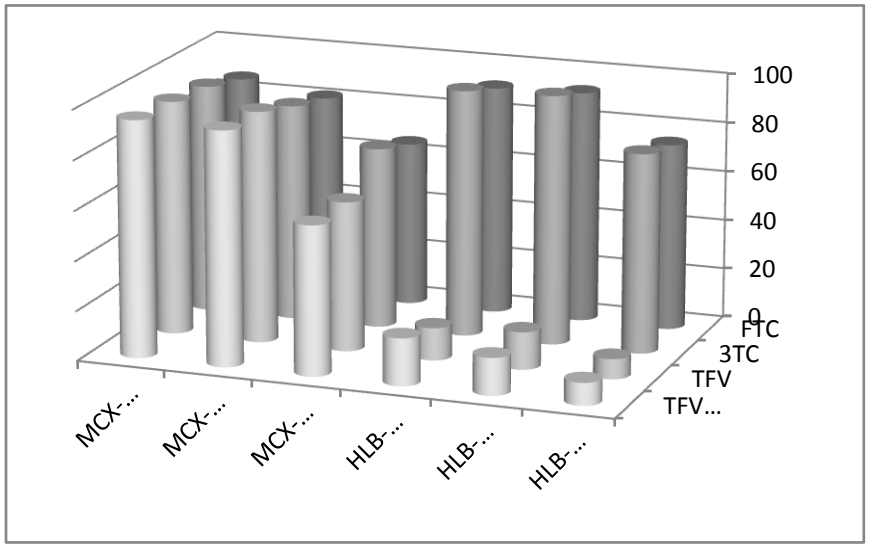


Figure 1: Comparison of extraction recoveries of FTC, 3TC, TFV and TFV-d7 for two different SPE cartridge, Oasis® HLB and Oasis® MCX, and their recommended eluant solvent or solution (MeOH for Oasis® HLB and NH_4Ac 5% in MeOH for Oasis® MC).

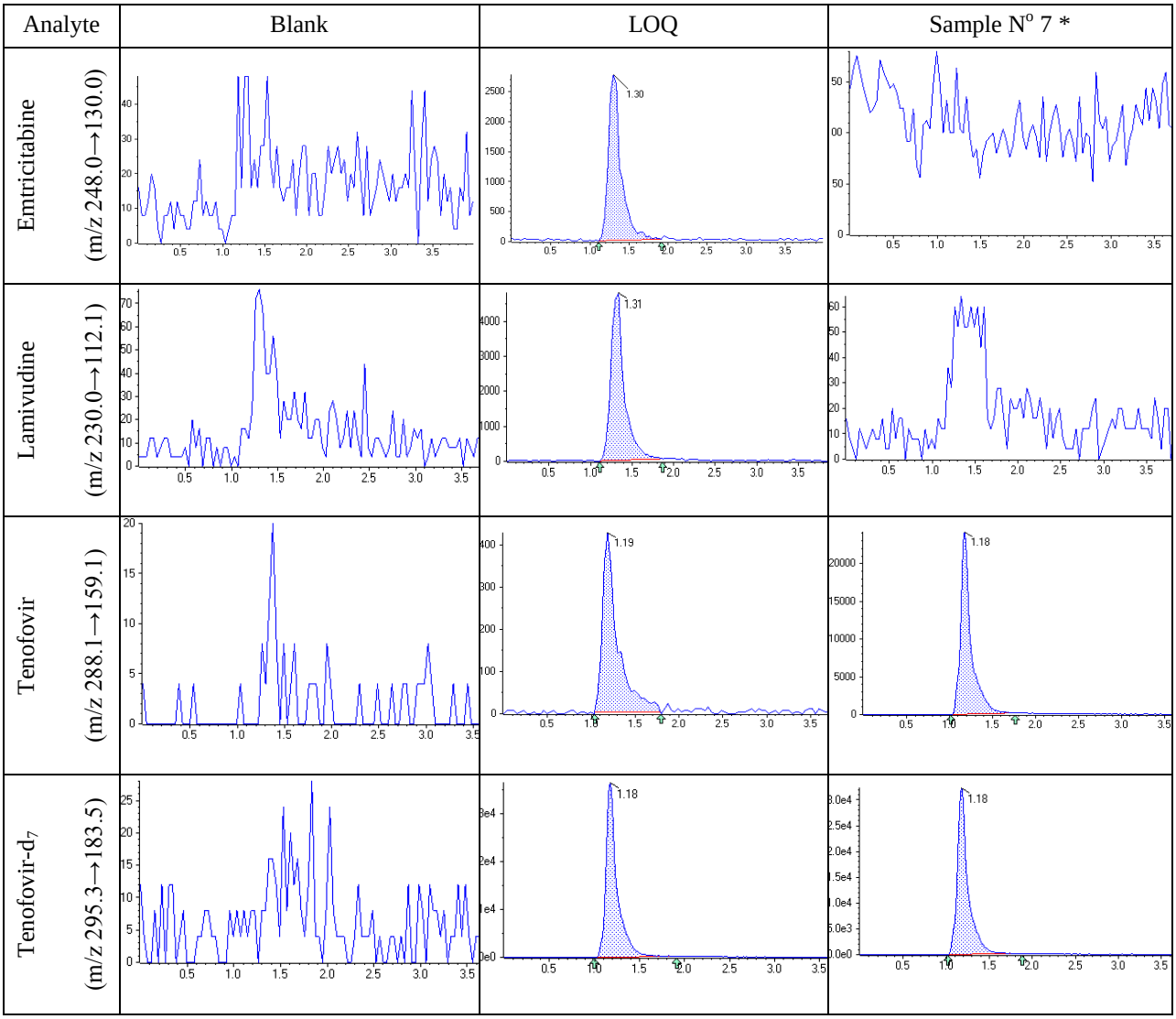
Analytical Performance and Method Validation

The proposed analytical methodology was validated according to bioanalitycal methods validation guide [14–16]. Multi-component method was evaluated in terms of selectivity, linearity, accuracy and precision intra-day and inter-day; LLOQ, recovery and stability.

Selectivity was evaluated by comparing chromatograms of six blank plasma samples from six different sources to make sure that there were no significant interfering peaks neither at retention time nor at LLOQ of the analytes and IS (Figure 1).

A carryover study was performed to estimate a possible effect either on the accuracy or the precision of the proposed analytical methodology. A blank sample was analyzed after injection of the highest concentration level of the calibration standard with the internal standard. No analytical response was observed at the retention time of neither the target ARV nor the internal standard. The calibration curves showed a satisfactory linearity within the following concentration range: 11-5434 ng mL^{-1}, 11-5452 ng mL^{-1}, and 13-397 ng mL^{-1} for FTC, 3TC and TFV, respectively. These concentrations were chosen based on the expected pharmacokinetic profiles of these drugs in a bioequivalence study. This wide linear dynamic range ensures the estimation of all three antiretrovirals with desired accuracy and precision at the elimination phase concentration expected to be found in human subjects, thus making our method suitable to be implemented in the bioanalytical step of a bioequivalence study [27,33]. The working linear range of the methodology achieved under optimized conditions resulted wider than those previously reported [10,13,18–20,27,34-35]. Calibration data was fitted by using a 1/x curve resulting coefficients of correlation (r) > 0.99 for all analytes. Blank plasma was spiked with ARVs standards achieving different concentration levels. No significant chromatographic signals of endogenous ARVs were observed for any of the plasma batches at the target analytes retention times. Inter- and intra-assay precision and accuracy were determined by analyzing the LOQ and three QC levels as described above. For both inter- and intra-assay data, the %RE for either analyte at any QC concentration level was < 13%, while the percent relative standard deviation (%RSD) was < 10%. The precision and accuracy for all of the four tested QC levels and the LLOQ were within the acceptable range of 15%. The recoveries of FTC, 3TC, and TFV were 90% (% RSD 12), 102% (% RSD 7) and 82% (% RSD 9), respectively. The analytical figures of merits were summarized in Table 1. The stability of ARVs in human plasma after three freeze-thaw

cycles, short-term room stability (6 h), post-processing stability (12 h remain at the auto-sampler temperature) and long-term stability (159 days at -20°C) were evaluated. The analyzed ARVs were considered to be stable in human plasma or extracts when accuracy was within 15% (Table 2). The proposed methodology meets standards required by international guidelines for pharmacokinetic studies of drugs, as well as for therapeutic monitoring of HIV patients.



*: Bioequivalence Study, Sample 7, Time 1.50 h, Volunteer 1, Period 1, Test Formulation. Axis "y": Intensity (cps); Axis "x": Time (minutes).

Figure 2: MRM chromatograms for emtricitabine, lamivudine, tenofovir and tenofovir-d_7 resulting from analysis of blank, LOQ plasma samples and volunteer sample

Pharmacokinetic parameters and statistical analysis

A total of 24 volunteers were screened and enrolled (mean weight, 74.9 ± 10.0 Kg; mean age 22.9 ± 3.8 and mean height 1.76 ± 0.07 m) in the study for administration of the 300 mg TDF formulation. All subjects completed the study. Each batch contained a calibration curve, the unknown samples to be quantified and quality control samples (LQC, MQC and HQC), interleaved at each volunteers. TFV appeared in the plasma of all volunteers within 0.5 h after administration and achieved maximal concentrations from 0.5 to 2 h. The mean values of the pharmacokinetic parameters (AUC_{0-48h} and $AUC_{0-\infty}$,C_{max}, T_{max}, $T_{1/2}$ and K_e) for the 24 subjects for the test (T) and reference

(Viread®) products are presented in Table 3. The mean concentration-time curves for both formulations of TFV are shown in Figure 3. No adverse effects of TDF were detected in any of the study periods. The geometric least square means, ratio (A/B)% of geometric least square mean and 90% confidence interval (90% CI) for the ratio (A/B)% based on root mean square error obtained from ANOVA for the pharmacokinetic parameters C_{max}, AUC_{0-48h} and $AUC_{0-\infty}$ were summarized in Table 4. Period and formulation effects for ln-transformed pharmacokinetic parameter C_{max} AUC_{0-48h} and $AUC_{0-\infty}$ and sequence effects of C_{max} were no statistically significant ($p > 0.05$). The ratio of geometric least square means for the (A/B) of C_{max}, AUC_{0-48h} and $AUC_{0-\infty}$ were 91.9%, 92.3% and 91.6%, respectively. The 90% confidence interval for the (A/B) of C_{max} AUC_{0-48h} and $AUC_{0-\infty}$ were 85.1 – 97.2 %, 87.2 – 97.7% and 85.5 – 98.18%, respectively. The intra-subject variability for ln-transformed data of the C_{max}, AUC_{0-48h} and $AUC_{0-\infty}$ were 13.6%, 11.5% and 13.9%, respectively. The power of the test for the ln-transformed pharmacokinetic parameters C_{max}, AUC_{0-48h} and $AUC_{0-\infty}$ were 99.97%, 100.00% and 99.96%, respectively.

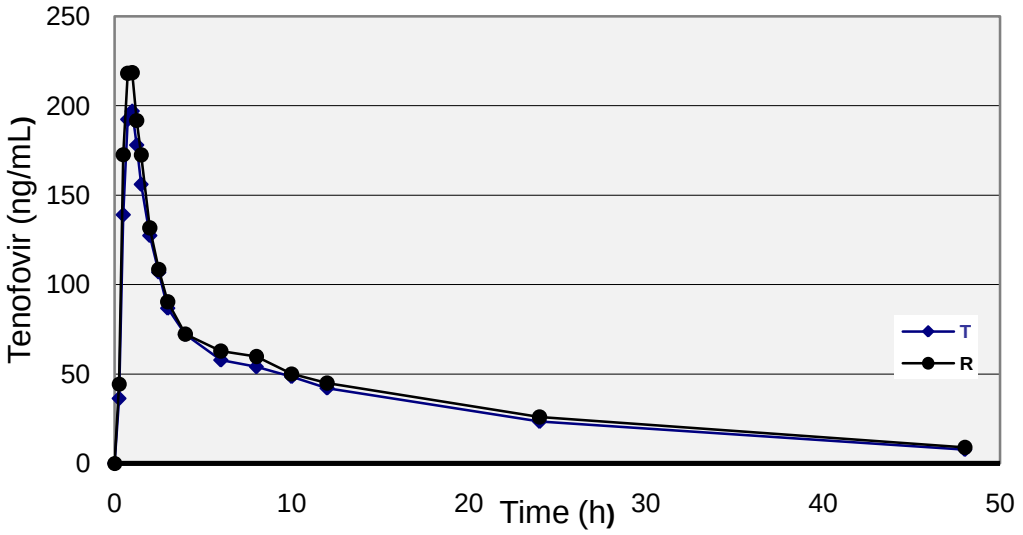


Figure 3: Mean concentration–time profiles in plasma following the oral administration of TDF 300 mg (tablets) of Viread® (R) or generic TDF (T) formulation to healthy volunteers.

Table 1: Analytical figures of merit of SPE LC-MS/MS for analysis of ARVs in human plasma

Analyte	**FTC**		**3TC**		**TFV**	
LOQ (%RSD)	11 (13)		11 (1)		13 (7)	
Linear Range (ng mL^{-1})	11 – 5434		11 – 5452		13 – 397	
Correlation coefficient	r = 0.99		r = 0.99		r = 0.99	
Accuracy Intra-day	**Concentration (ng mL^{-1})**	**%RE**	**Concentration (ng mL^{-1})**	**%RE**	**Concentration (ng mL^{-1})**	**%RE**
	31	8	27	-6	30	7
	2626	11	2369	0	217	4
	5359	13	4979	5	324	4
Accuracy Inter-day						
	27.9	-2	27	-7	27	-3
	2372	0	2183	-8	208	0
	4822	1	4559	-4	307	-2

Table 2: Stability of the ARVs in plasma samples at different conditions

Stability		Room temperature (6.0 h)			Autosampler (12 h)			Freeze and thaw (cycle 3)			Long-term (159 days)		
		Conc. (ng mL^{-1})	%RE	%RSD	Conc. (ng mL^{-1})	%RE	%RSD	Conc. (ng mL^{-1})	%RE	%RSD	Conc. (ng mL^{-1})	%RE	%RSD
TFV	LQC	29	3	9	26	-6	10	27	-3	6	32	15	12
	MQC	212	2	2	186	-10	2	217	4	2	208	0	8
	HQC	306	-2	2	276	-12	2	324	4	3	299	-4	7
3TC	LQC	26	10	14	22	-13	7	26	-8	5	28	-3	2
	MQC	2178	8	4	2180	-9	4	2167	-9	6	2335	-2	4
	HQC	4474	6	1	4183	-12	4	4484	-5	6	4435	-6	3
FTC	LQC	32	12	7	25	-12	6	26	-9	7	28	-4	7
	MQC	2673	12	8	2132	-11	6	2303	-4	2	2463	3	2
	HQC	5383	13	4	4367	-9	3	4776	0	3	5047	5	4

Table 3: Pharmacokinetic properties of 2 oral formulations of single-dose TDF 300 mg (tablets) in healthy subjects (N = 24).* Values are mean (SD).

Property	Test †	Viread ® ‡
C_{max}, (ng mL^{-1})	237.6 (61.8)	261.3 (73.1)
T_{max}, (h)	0.98 (0.42)	0.85 (0.31)
$T_{1/2}$, (h)	14.64 (3.62)	15.22 (4.18)
Ke, (h^{-1})	0.051 (0.015)	0.049 (0.015)
$AUC_{0–48h}$, (ng mL^{-1} h)	1658 (466)	1801 (529)
$AUC_{0–\infty}$, (ng mL^{-1} h)	1845 (579)	2015 (639)

$AUC_{0–48h}$ = AUC from time 0 (baseline) to time 48 h; $AUC_{0–\infty}$ = AUC from baseline to infinity.

*No significant between-treatment differences were found.

†Trademark of Argentine (test formulation).

‡Trademark of Gador S.A., Darwin 429, Buenos Aires, Argentina (reference formulation).

Table 4: Comparison of 90% CIs of ln C_{max}, ln $AUC_{0–48h}$, and ln $AUC_{0–\infty}$, the probability of exceeding the limits of acceptance and power test in TDF 300 mg (tablets).

Parameter	Mean Ratio %	90% CI	Probability of Exceeding Limits of Acceptance		
			<80%	>125%	Power
In C_{max} % ratio	90.9	85.0-97.2	0.00168	< 0.00000	0.9997
In $AUC_{0–48h}$ % ratio	92.3	87.2-97.9	0.00014	< 0.00000	1.0000
In $AUC_{0–\infty}$ % ratio	91.6	85.5-98.1	0.00130	< 0.00000	0.9996

$AUC_{0–48h}$ = AUC from time 0 (baseline) to time 48 h; $AUC_{0–\infty}$ = AUC from baseline to infinity

Conclusion

A pharmacokinetic study based on single oral administration of TDF 300 mg tablets to Argentinean male volunteers is presented. The results showed that the estimated point and the 90% CIs for the corresponding ratios of C_{max}, $AUC_{0–48h}$, and $AUC_{0–\infty}$ were within the acceptable range and met the predetermined criteria for bioequivalence ranges of 80-125% ($p < 0.05$) as suggested by the US-FDA bioequivalence guideline. The study demonstrates

bioequivalence between two pharmaceutical products commercially available. The analytical methodology was optimized and validated for determination of FTC, 3TC and TFV in human plasma samples at a concentration range suitable for pharmacokinetic analysis in humans. Due to the popularity of the combination of FTC, 3TC and TDF pharmaceutical in treating HIV-infected patients, this analytical methodology could result useful for monitoring therapeutic treatments employing pharmacological formulation of these three drugs. The analytical methodology was optimized and validated according to the US-FDA guidelines. The working linear range of the methodology achieved under optimized conditions resulted wider than those previously reported. The optimized analytical methodology was sensitive enough as to detect trace concentrations of TFV in human plasma of volunteers subjected to a pharmacokinetic study. The method exhibited excellent performance in terms of selectivity, precision, accuracy and short run time of analysis. These features of the method allowed its application in a head-to-head bioequivalence study of test and reference TFV formulations.

Acknowledgements

GAH and MNB are doctoral fellows of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) co-funded by DominguezLab S.R.L. GJH, SRH and JA are research investigators of CONICET. The authors thank DominguezLab S.R.L. for the equipment facilitated for carrying out analytical assays. Financial support was provided by 06/M062 (Res. Nº4540/2013-R) SeCTyP-UNCuyo project.

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