Journal of Pharmaceutical and Biomedical Analysis 48 (2008) 918–926

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for simultaneous determination of tenofovir and emtricitabine in human plasma and its application to a bioequivalence study

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ARTICLE INFO

Article history:
Received 27 May 2008
Received in revised form 23 July 2008
Accepted 23 July 2008
Available online 5 August 2008

Keywords:
Tenofovir
Emtricitabine
Lamivudine
LC–MS/MS
Human plasma

ABSTRACT

A rapid and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method has been developed and validated for simultaneous quantification of Tenofovir (TEN) and Emtricitabine (EMT) in human plasma using Chromolith Speed Rod RP18. The mass transition ion-pair has been followed as *m/z* 288.10 → 176.10 for TEN, *m/z* 248.20 → 130.20 for EMT and *m/z* 230.10 → 112.10 for Lamivudine (LAM). The method involves solid phase extraction from plasma, simple isocratic chromatographic conditions and mass spectrometric detection using an API 5000 instrument that enables detection at nanogram levels. Lamivudine was used as the internal standard. The proposed method has been validated with a linear range of 10–600 ng/ml for TEN and 25–2500 ng/ml for EMT. The intrarun and interrun precision values are within 12.0% for TEN and 15.6% for EMT at their respective LOQ levels. The overall recoveries for TEN and EMT were 84.3% and 68.5%, respectively. Total elution time was as low as 2 min.

1. Introduction

Tenofovir (TEN; 1-(6-aminopurin-9-yl) propan-2-yloxymethyl-phosphonic acid) belongs to the nucleotide analogue reverse transcriptase inhibitors (NtRTIs) class of antiretroviral drugs. Tenofovir disoproxil fumarate is an acyclic nucleoside phosphonate diester analogue of adenosine monophosphate. It is the first nucleotide reverse transcriptase inhibitor approved for use in combination with other antiretroviral agents in the treatment of HIV-1 infection in the United States. Unlike the nucleoside reverse transcriptase inhibitors, which must undergo three intracellular phosphorylation steps for activation, nucleotide analogues such as tenofovir require only two such steps. This reduction in the phosphorylation requirement has the potential to produce more rapid and complete conversion of the drug to its pharmacologically active metabolite [1].

Emtricitabine (EMT; 4-amino-5-fluoro-1-[2-(hydrxoymethyl)-1,3-oxathiolan-5-yl]-pyrimidin-2-one) is a nucleoside reverse transcriptase inhibitor (NRTI) with activity against Human Immunodeficiency Virus Type 1 (HIV-1). Its action is such that it blocks HIV reverse transcriptase. It is often used in conjunction with other anti-HIV medicines to treat people with HIV infection.

When compared with stavudine, EMT is found to be a more effective virological agent when taken as a first-line treatment drug with didanosine and efavirenz by people with HIV-1 [2].

TEN and EMT are NRTIs that are approved for use in combination with other antiretrovirals in the treatment of HIV-infected adults. Chi et al. have concluded that a single dose of TEN and EMT at delivery, reduced resistance to non-nucleoside reverse transcriptase inhibitors at 6 weeks after delivery by half and hence this treatment should be considered as an adjuvant to intrapartum nevirapine [3].

Different methods have been reported in the literature for monitoring plasma levels of TEN and EMT individually. Rezk et al. have reported a simultaneous method for the estimation of TEN and EMT in human plasma using a validated HPLC method [4] with a rather long run time. Some other techniques used in individual analysis of TEN from plasma include HPLC with

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doi:10.1016/j.jpba.2008.07.022

mass spectrometric [5,6], spectrofluorimetric [7], UV and single mass spectrometric [8] and simple UV detection [9]. Bezy et al. have also reported a method involving the simultaneous analysis of several antiretroviral nucleosides in rat-plasma by HPLC with UV detection using acetic acid/hydroxylamine buffer [10]. King et al. quantified TEN alone from human peripheral blood mononuclear cells (hPBMCs) using a validated LC–MS/MS method [11] with an assay range of 50–10,000 fmol per sample and a lower limit of quantification of 10 fmol per million cells with 5 million hPBMCs used. Pruvost et al. reported a specificity enhancement method using LC-positive ESI-MS/MS for the measurement of nucleotides supported by its application in determining carbovir triphosphate, lamivudine triphosphate and tenofovir diphosphate simultaneously in human peripheral blood mononuclear cells [12]. Takahashi et al. also report a conventional LC–MS method for quantifying TEN individually from human plasma in the concentration range of 0.019–1.567 μg/ml [13]. EMT has been quantified in presence of other antiretroviral drugs, by Notari et al. [14] and Rebiere et al. [15]. Sparidans et al. have quantified EMT individually in human plasma using a validated LC–MS/MS method with a detection limit of up to 5 ng/ml [16].

Since there is no specific method available in the literature for the simultaneous estimation of EMT and TEN in human plasma using LC–MS/MS as the detection system with a sufficiently short run time, the aim of this study was the development and validation of a simple, specific, rapid and sensitive LC–MS/MS method for the determination of TEN and EMT in human plasma.

2. Experimental

2.1. Chemicals and reagents

The reference standards of TEN was obtained from Cipla (Mumbai, India), LAM was obtained from Cadila Healthcare Ltd. (Ahmedabad, India) and EMT was obtained from Matrix Labs. (Bangalore, India). High purity water was prepared in-house using a Milli-Q water purification system obtained from Millipore (Bangalore, India). HPLC grade methanol and acetonitrile were purchased from Baker (Mumbai, India). Extra pure ammonium acetate and formic acid were purchased from Fluka (Steinheim, Germany). Analytical reagent grade ammonia solution was purchased from Merck (Darmstadt, Germany). Drug free (blank) heparinised human plasma was obtained from healthy volunteers that had previously signed a consent form and was stored at −20 °C prior to use. Oasis MCX 30 mg/1cc SPE cartridges containing a mixed-mode polymeric sorbent with both reverse phase and cation exchange functionalities were employed. These cartridges were obtained from Waters (Massachusetts, U.S.A.). The subject samples obtained post-study conduction were also kept stored at −20 °C.

2.2. Calibration curves and quality control samples

Two separate stock solutions each of EMT and TEN were prepared for bulk spiking of calibration curve and quality control samples for the method validation exercise as well as the subject sample analysis. For bulk spiking, screened blank plasma from six different lots with least interference at the retention time of the analytes and the internal standards was pooled together and used.

The stock solutions of TEN were prepared in water:methanol (80:20, v/v) at free base concentration of 1000 μg/ml. The stock solutions of EMT and internal standard, LAM were prepared in methanol at free base concentration of 1000 μg/ml. Aliquots of these stock solutions were kept stored under refrigeration at 4 °C for determination of stock solution stability.

Primary dilutions were prepared from stock solutions by water:methanol (40:60, v/v). The secondary dilutions and subsequent working solutions were prepared as and when required using the same diluent as those for the primary dilutions. These working standard solutions thus prepared were used to prepare the calibration curve and quality control samples.

An eight point standard curve was prepared by spiking the previously screened blank plasma with appropriate amount of both TEN and EMT. The calibration curve ranged from 10 to 600 ng/ml and 25 to 2500 ng/ml for TEN and EMT, respectively. Quality control samples were prepared at three concentration levels of 30, 250 and 450 ng/ml for TEN and 75, 1200 and 1800 ng/ml for EMT in a manner similar to the preparation of calibration curve samples from the stock solution.

2.3. Sample preparation

A 0.2 ml aliquot of human plasma sample was mixed with 50 μl of internal standard working solution (0.5 μg/ml of LAM) and 0.2 ml of 0.1 M hydrochloric acid and loaded onto an Oasis MCX cartridge that was pre-conditioned with 1 ml methanol followed by 1 ml water. The extraction cartridge was washed with 1 ml of 0.1 M hydrochloric acid followed by 1 ml methanol. TEN, EMT and LAM were eluted with 1 ml of 5% ammonia in methanol. The samples were evaporated to dryness under a stream of nitrogen at 40 °C and reconstituted using 750 μl of 1% acetic acid in 10 mM ammonium acetate buffer; 3 μl of the eluant was injected into the LC–MS/MS system.

2.4. High performance liquid chromatography and mass spectrometric condition

Chromatographic separation was carried out on a Shimadzu HPLC with a Chromolith Speed Rod RP18 column (50 mm × 4.6 mm) purchased from Merck (Mumbai, India). A mobile phase consisting of acetonitrile and ammonium acetate (pH 3.0, 40 mM) (20:80, v/v) was delivered with a flow rate of 0.7 ml/min. The total run time for each sample analysis was 2.0 min. Mass spectra were obtained using an API 5000 (Applied Biosystems, Canada) equipped with electrospray ionization source. The mass spectrometer was operated in the multiple reaction-monitoring (MRM) mode. Sample introduction and ionization was electrospray ionization in the positive ion mode. The spray voltage and source temperature were 5500 V and 600 °C, respectively. Nitrogen gas was used as collision gas. The Declustering Potential (DP), Collision Energy (CE), Cell Exit Potential (CXP) were optimized during tuning as 61, 91, 61; 19, 37, 25; 18, 26, 16 eV for EMT, TEN and LAM, respectively. The Entrance Potential (EP) was kept constant as 10 eV for both the analytes and the internal standard. The mass transition ion-pair was selected as m/z 288.10 → 176.10 for TEN, m/z 248.20 → 130.20 for EMT and m/z 230.10 → 112.10 for LAM. The parent and product ion spectra for EMT, TEN and LAM are represented in Figs. 1–6, respectively. The data acquisition software used was Analyst version 1.4.2. For quantification, the peak area ratios of the target ions of the drugs to those of the internal standard were compared with weighted $(1/x \times x)$ least squares calibration curves in which the peak area ratios of the calibration standards were plotted versus their concentrations.

2.5. Validation

The method has been validated for selectivity, linearity, precision, accuracy, recovery and stability. Selectivity was performed by

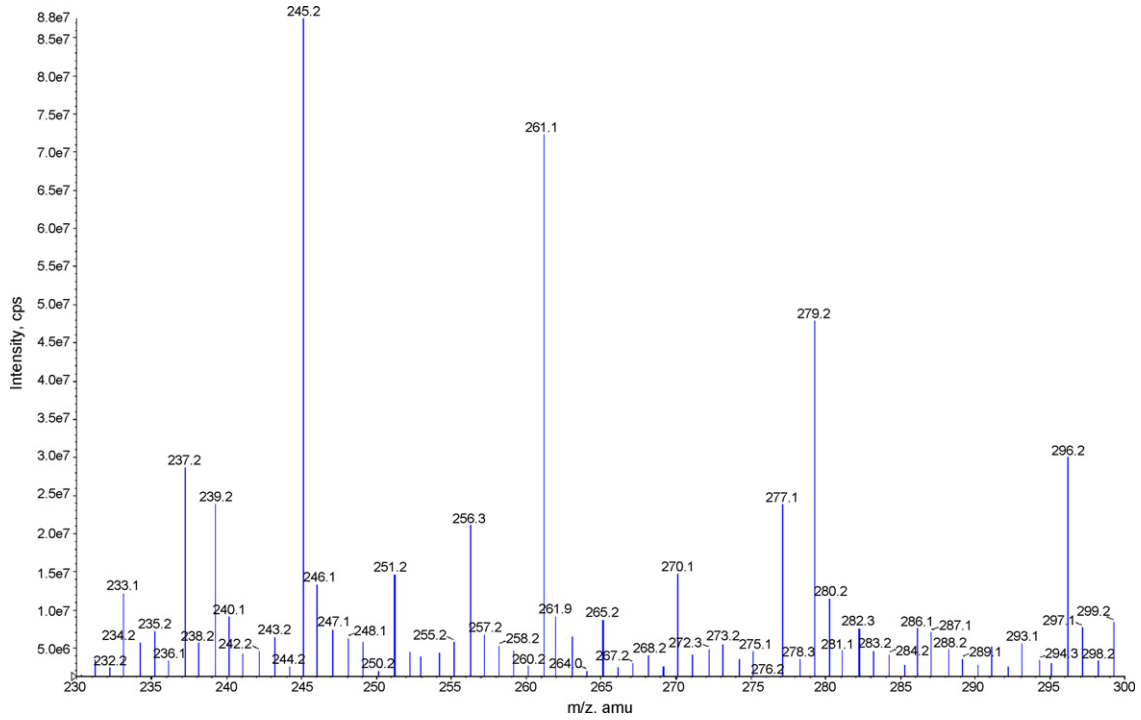

Fig. 1. Representative spectra of parent ion of Emtricitabine.

analyzing the blank plasma samples from 12 different sources (or donors) to test for interference at the retention time of TEN, EMT and internal standard LAM. These 12 sources consisted of six normal controlled plasma lots and three controlled plasma lots each of haemolysed and lipemic plasma containing heparin as the anticoagulant.

The intrarun and interrun accuracy were determined by replicate analysis of the three quality control levels along with the LOQ (Lower Limit of Quantitation) and ULOQ (Upper Limit of Quantitation) levels that were extracted from the sample batch. In each of the precision and accuracy batches, six replicates at each quality control level inclusive of the LOQ and ULOQ levels were analysed.

Accuracy is defined as the percent relative error (%RE) and was calculated using the formula $\%RE = (E-T)\ (100/T)$ where E is the experimentally determined concentration and T is theoretical concentration. Assay precision was calculated by using the formula % R.S.D. = (S.D./M) (100) where M is the mean of the experimentally determined concentrations and S.D. is the standard deviation of M.

The extraction efficiencies of TEN, EMT and LAM were determined by analysis of six replicates at low, medium and high quality

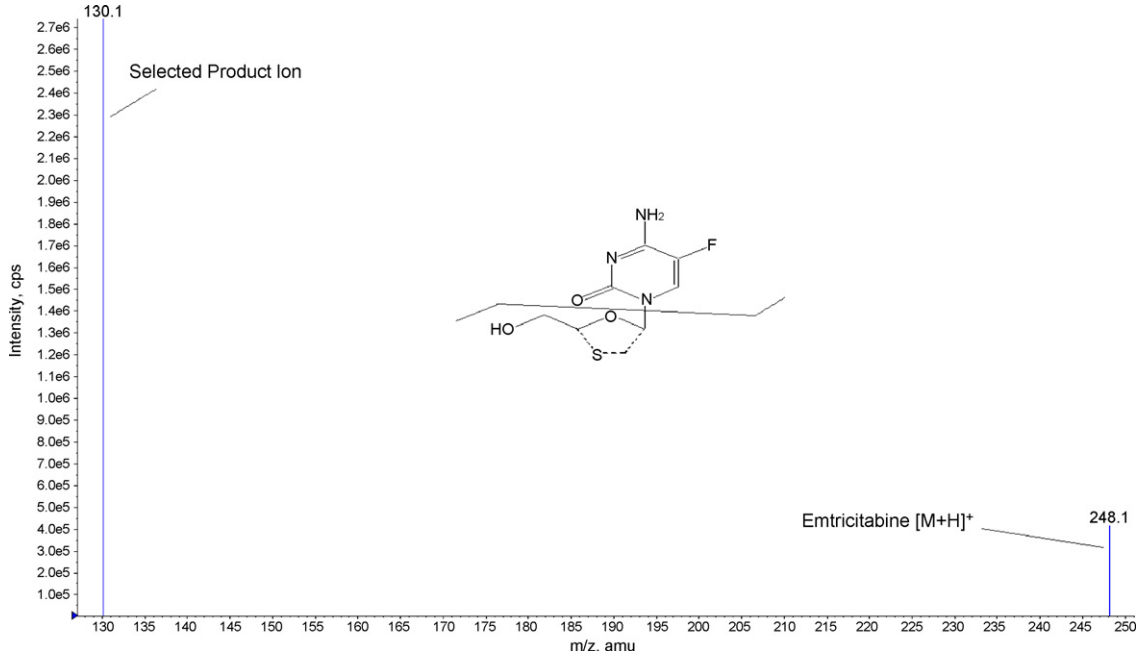

Fig. 2. Representative spectra of product ion of Emtricitabine. The transition monitored is m/z 248.20 → 130.20.

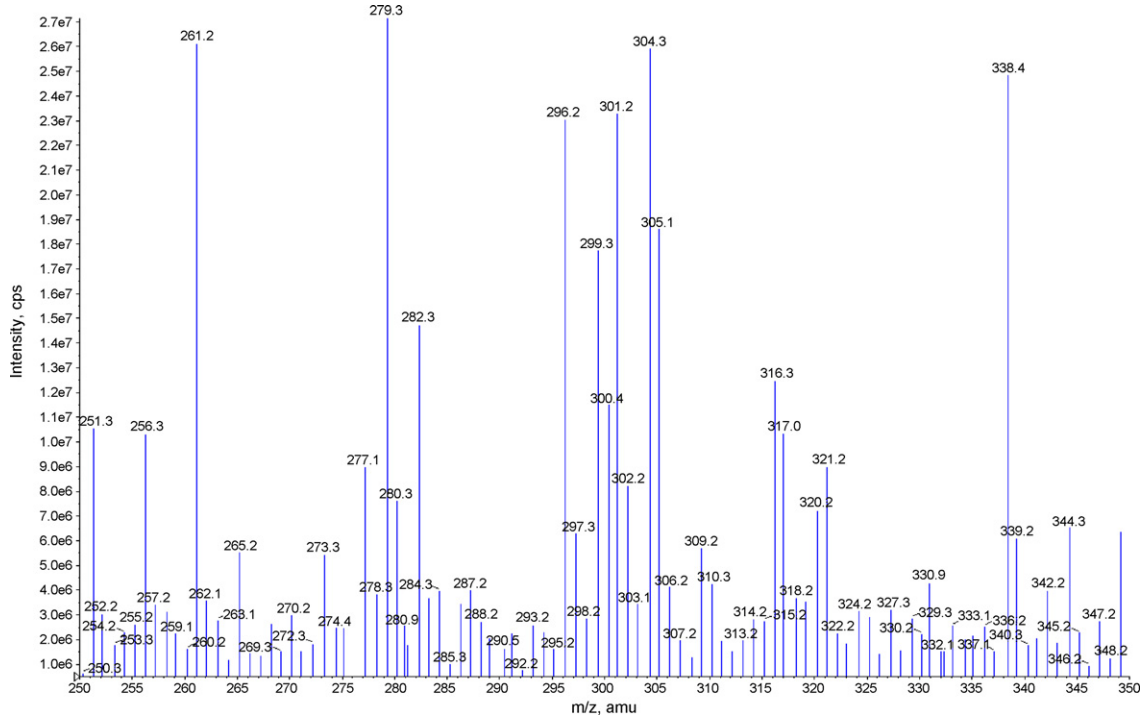

Fig. 3. Representative spectra of parent ion of Tenofovir.

control concentrations for TEN, EMT and at one concentration for the internal standard, LAM. The percent recovery was evaluated by comparing the peak areas of extracted analytes to the peak areas of non-extracted standards.

The assessment of matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionization) constitutes an important and integral part of validation for quantitative LC–MS/MS method for supporting pharmacokinetics studies. It was performed by processing six lots of different normal controlled plasma samples in quadruplet ($n = 4$). LQC and HQC working solutions were spiked post extraction in duplicate for each lot. The R.S.D. for six values at each level was calculated by taking the mean

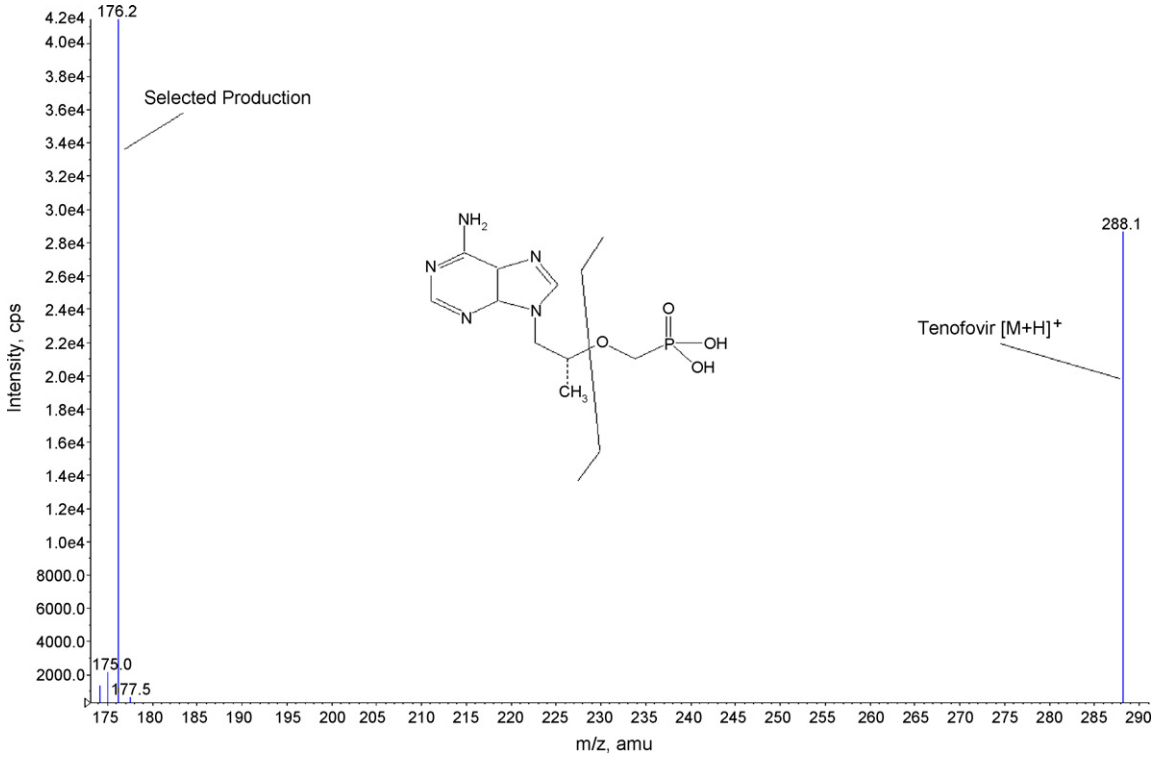

Fig. 4. Representative spectra of product ion of Tenofovir. The transition monitored is m/z 288.10 → 176.10.

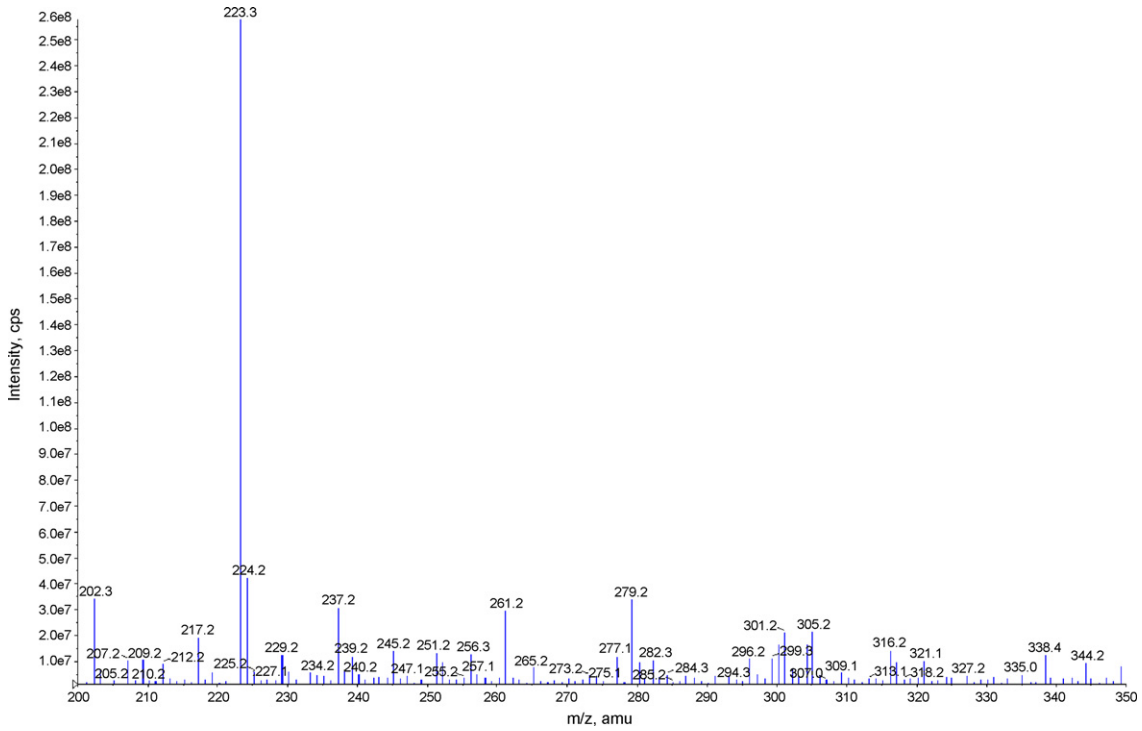

Fig. 5. Representative spectra of parent ion of Lamivudine.

value obtained by injecting the post extracted samples prepared in duplicate from each plasma lot. The R.S.D. of the area ratios of post spiked recovery samples at LQC and HQC levels were within 5% and 4%, respectively for TEN and within 5% and 7%, respectively for EMT. For the internal standard, LAM, the R.S.D. of the area ratios over both LQC and HQC levels was less than 9%. These results found were well within the acceptable limit set i.e. the R.S.D. of area ratio to be within ±15% at each level tested for the two analytes and within ±20% over both the levels tested for the internal standard. Moreover, the minor suppression of analyte signal due to endogenous matrix interferences does not affect the quantification of analytes and IS peak which was confirmed by the post-column infusion technique.

As a part of the method validation, stability was evaluated. Analytes were considered stable if the recovery of the mean test responses were within 15% of appropriate controls. Analytes were tested using the quality control samples whenever appropriate. The stability of spiked human plasma kept at

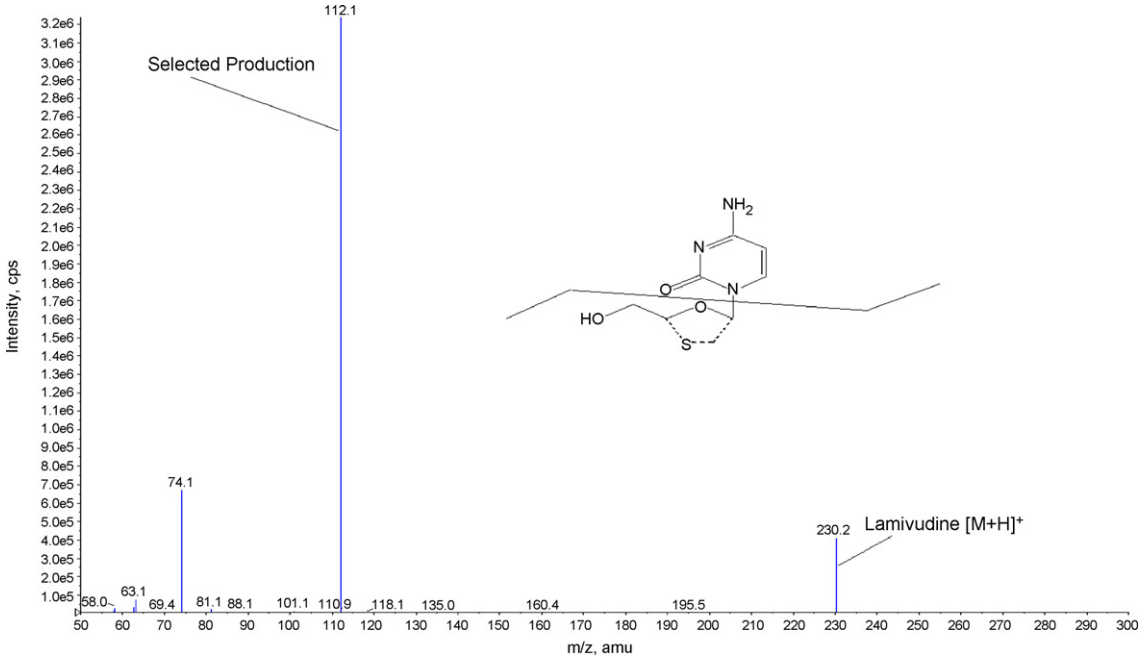

Fig. 6. Representative spectra of product ion of Lamivudine. The transition monitored is *m/z* 230.10 → 112.10.

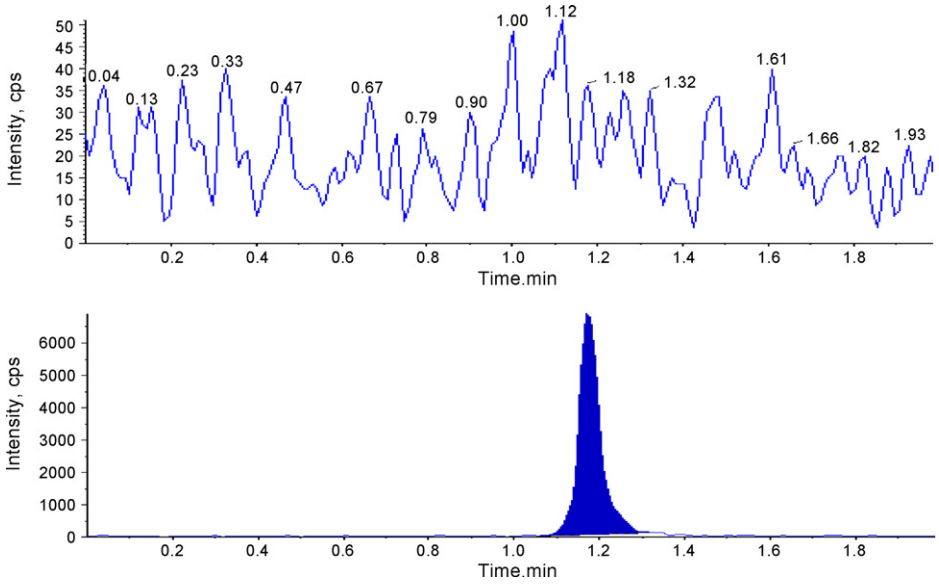

Fig. 7. Representative chromatogram of plasma blank and plasma spiked with Emtricitabine at the lower limit of quantification (25 ng/ml).

room temperature of about 25 °C (bench top stability) was evaluated for 25 h. The processed sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0), with the samples that were re-injected after keeping in the auto sampler at 10 °C for 30 h and at 52 h. The freeze–thaw stability was conducted by comparing the stability samples that had been frozen and thawed three times, with freshly spiked quality control samples. Six aliquots of each low and high concentration were used for the freeze–thaw stability evaluation. The dry extract stability was conducted by comparing the stability samples that had been evaporated and stored at 4 °C. These dry residue samples were then reconstituted after 20 h and compared versus freshly spiked quality control samples at low and high concentrations of the quality control levels.

2.6. Application of method

The method has been successfully used to quantitate TEN and EMT in forty-three human volunteers under fasting conditions after administration of a single combination tablet containing 300 mg Tenofovir Disoproxil Fumarate and 200 mg Emtricitabine tablets as an oral dose. The study design was a randomized, two-period, two-sequence, two-treatment single dose, open label, bioequivalence study using TRUVUDA manufactured by Gilead Sciences, Inc., U.S.A., as the reference formulation. The study was conducted according to current GCP guidelines and after signed consent of the volunteers. The study was also approved by an authorised Ethics committee before being conducted.

There were totally twenty six time points per period after the blood collection was done post dosing inclusive of the predose

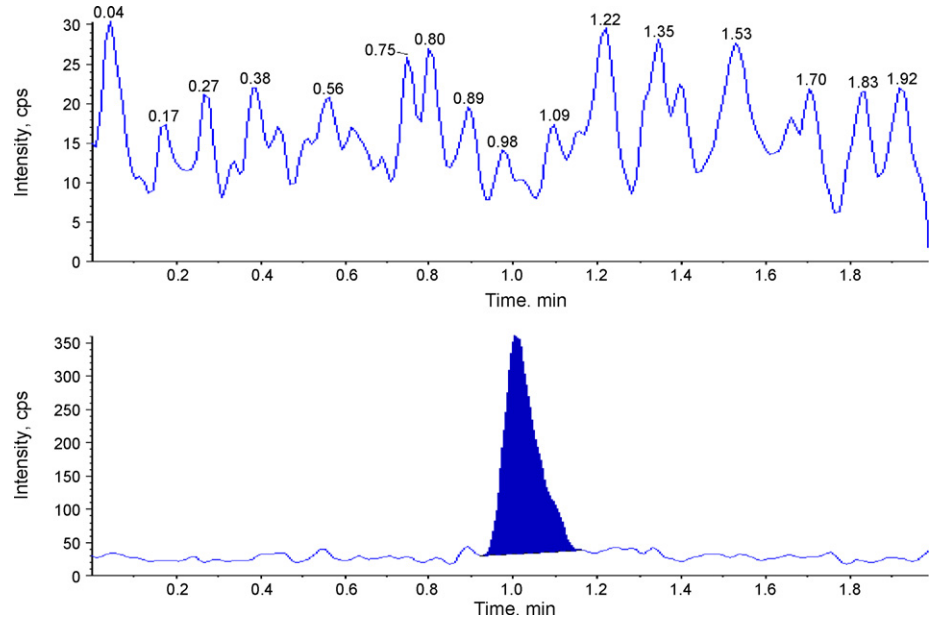

Fig. 8. Representative chromatogram of plasma blank and plasma spiked with Tenofovir at the lower limit of quantification (10 ng/ml).

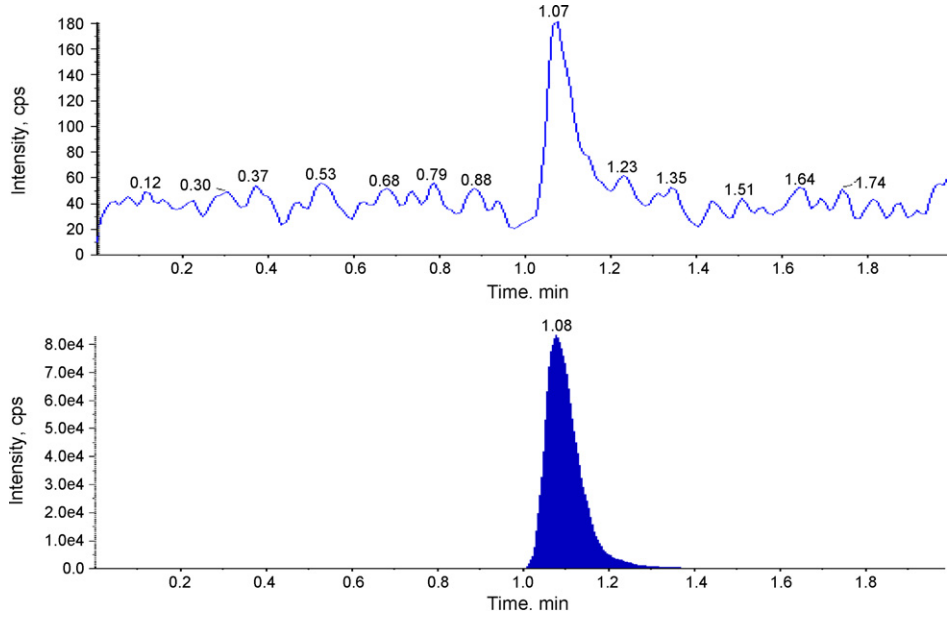

Fig. 9. Representative chromatogram of plasma blank and plasma spiked with Lamivudine (internal standard).

sample. The blood samples were collected in separate vacutainers containing heparin as anti-coagulant. The plasma from these samples was separated by centrifugation at 4000 rpm kept within the range of 2–8 °C. The plasma samples thus obtained were stored at −20 °C till analysis. Post analysis the pharmacokinetic parameters were computed using SAS 9.1.3 software. The statistical data which were evaluated were C_{max} (maximum observed drug concentration during the study), AUC_{0-t} (area under the plasma concentration–time curve measured to the last quantifiable concentration, using the trapezoidal rule), AUC_{0-inf} (AUC_{0-t} plus additional area extrapolated to infinity, calculated using the formula $AUC_{0-t} + C_t/K_{el}$, where C_t is the last measurable drug concentration), T_{max} (time to observe maximum drug concentration), K_{el} (apparent first-order terminal rate constant calculated from a semi-log plot of the plasma concentration versus time curve, using the method of least square regression) and $T_{1/2}$ (terminal half-life as determined by quotient $0.693/K_{el}$).

3. Results and discussion

3.1. Method development

The goal of this work was to develop and validate a simple, rapid and sensitive assay method for the simultaneous extraction and quantification of TEN and EMT, suitable for pharmacokinetic studies. To achieve the goal, during method development different options was evaluated to optimize sample extraction, detection parameters and chromatography. Since TEN and EMT exist in unionized form in the basic pH, the plasma samples were treated with 0.1 M hydrochloric acid and loaded onto the Waters Oasis MCX SPE cartridges. Oasis MCX cartridges were selected as they were found to be the most reproducible and gave less batch to batch variation when compared with other cartridges. After acidification with hydrochloric acid the strong binding of analytes to the copolymer of SPE cartridge enables sufficient clean up. It was found that that the best signal was achieved with positive ion electrospray ionization (ESI) mode. Further optimization of chromatographic conditions increased the signal of analytes. A mobile phase containing 40 mM ammonium acetate (pH 3.0) buffer in combination with acetonitrile resulted in improved signal. Use of short Chromolith Speed Rod RP18 (50 mm × 4.6 mm i.d.) column resulted in reduced flow rate and reduced run time as low a 2.0 min. The column oven temperature was kept at ambient temperature (about 25 °C).

3.2. Selectivity

Representative chromatogram obtained from blank plasma and plasma spiked with LOQ standard for EMT and TEN is presented in Figs. 7 and 8, respectively. Similarly a representative chromatogram obtained from blank plasma and at its nominal concentration for

Table 1
Intrarun precision and accuracy ($n = 6$) of TEN and EMT in human plasma

Analyte	Spiked concentration (ng/ml)	Mean calculated concentration (ng/ml)	%R.S.D.	%RE
TEN	10.1	9.4	12.0	−6.2
	30.0	30.5	7.3	1.4
	250.3	244.9	7.3	−2.1
	450.5	469.9	3.4	4.3
	600.2	639.1	3.1	6.5
EMT	25.5	23.5	15.6	−7.8
	75.1	73.8	4.4	−1.8
	1201.7	1221.5	4.6	1.6
	1802.5	1838.6	3.8	2.0
	2501.5	2507.9	5.7	0.3

Table 2
Interrun precision and accuracy ($n = 6$) of TEN and EMT in human plasma

Analyte	Spiked concentration (ng/ml)	Mean calculated concentration (ng/ml)	%R.S.D.	%RE
TEN	10.1	9.7	9.4	−3.1
	30.0	30.4	6.6	1.1
	250.3	244.0	5.1	−2.5
	450.5	451.8	5.0	0.3
	600.2	618.0	5.6	3.0
EMT	25.5	24.0	11.4	−6.1
	75.1	74.8	4.5	−0.4
	1201.7	1196.8	4.6	−0.4
	1802.5	1778.2	5.4	−1.3
	2501.5	2339.8	7.6	−6.5

Table 3
Summary of statistics for target parameters, test versus reference following a single dose of Tenofovir Disoproxil Fumarate (300 mg) and Emtricitabine (200 mg) to forty-three volunteers under fasting conditions

Analyte	Treatment	C_{max} (ng/ml)	T_{max} (h)	AUC_{0-inf} (ng h/ml)	$T_{1/2}$(h)
EMT	Test	1736.9	1.45	9239.6	4.1
	Reference	1717.7	1.51	9052.3	4.6
TEN	Test	310.7	0.96	2486.7	18.5
	Reference	299.8	0.98	2454.1	19.2

LAM (Internal Standard) is shown in Fig. 9. No interfering peak of endogenous compounds was observed at the retention time of analytes or the internal standard in blank human plasma containing Heparin as anti-coagulant from six different lots of normal controlled plasma and three each of lipemic and heamolysed plasma.

The possibility of interference of other anti-HIV drugs mentioned in section 1 was excluded on the basis of their mass spectra.

3.3. Linearity

The peak area ratios of calibration standards were proportional to the concentration of analytes in each assay over the nominal concentration range of 10–600 ng/ml for TEN and 25–2500 ng/ml for EMT. The calibration curves appeared linear and were well described by least squares lines. A weighing factor of $1/concentration^2$ was chosen to achieve homogeneity of variance. The correlation coefficients were ≥0.9934 for TEN and ≥0.9922 for EMT. Across the eight points taken as calibration standards, the R.S.D. obtained over five batches was ≤11.1 and ≤13.8 for EMT and TEN, respectively. The recovery in terms of %RE obtained was within the range of −5.2% to 9.8% and −10.7% to 3.7% for EMT and TEN, respectively.

3.4. Lower limits of quantitation and of detection

Although the LOQ for TEN and EMT is 10 ng/ml and 25 ng/ml, it was possible to detect concentrations from real samples up to 0.5 ng/ml and 1 ng/ml for TEN and EMT, respectively. However these values obtained from subject sample analysis were not considered during statistical analysis as they were below the LOQ validated.

3.5. Precision and accuracy

The intrarun precision was ≤7.3% for TEN and ≤5.8% for EMT whereas at the LOQ levels it was ≤12.0% for TEN and ≤15.6% for EMT, respectively. The intrarun accuracy in terms of %RE was within the range of −6.2% to 6.5% and −7.8% to 2.0% for TEN and EMT, respectively across all the five levels tested (Table 1). The interrun precision and accuracy were determined by pooling all individual assay results of replicate ($n = 6$) quality control samples over the five separate batch runs. The interrun precision was ≤6.6% for TEN and ≤7.6% for EMT whereas at the LOQ levels it was ≤9.4% for TEN and ≤11.4% for EMT, respectively. The interrun accuracy was within the range of −3.1% to 3.0% for TEN and −6.5% to −0.4% for EMT, respectively (Table 2).

3.6. Recovery

Six replicates at low, medium and high quality control concentration for TEN and EMT were prepared for recovery determination. The mean recovery for TEN and EMT were 84.3% and 68.5% with R.S.D. values of 19.7% and 17.6%, respectively. The mean recovery for LAM was 93.3% with an R.S.D. of 19.1%.

3.7. Stability

Bench top, dry extract and process stabilities for TEN and EMT were investigated at LQC and HQC levels. The results revealed that TEN and EMT were stable in plasma for at least 25 h at room temperature of about 25 °C and 52 h in the auto sampler at 10 °C. It was confirmed that repeated freeze and thawing (three cycles) of plasma samples spiked with TEN and EMT at LQC and HQC level did not affect the stability of TEN and EMT. Dry extract stability was also evaluated for a period of 20 h at LQC and HQC levels. The long term stability results also indicated that both the analytes,

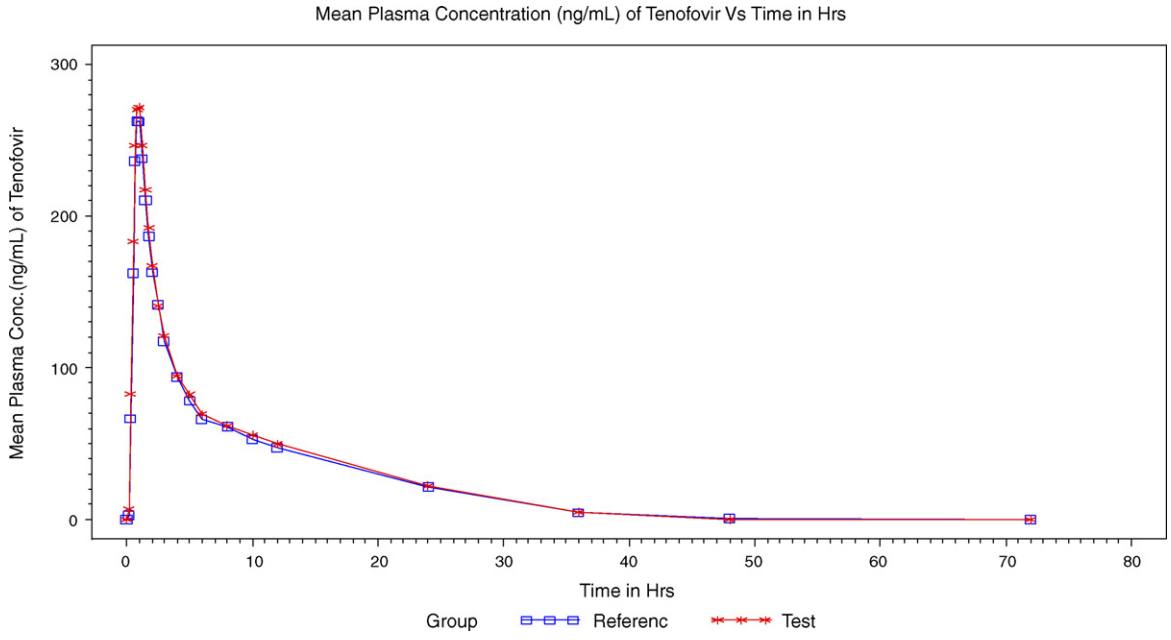

Fig. 10. Concentration versus time profile over 72 h of Tenofovir in human plasma from 43 subjects receiving a single oral dose of 300 mg Tenofovir Disoproxil Fumarate and 200 mg Emtricitabine tablet.

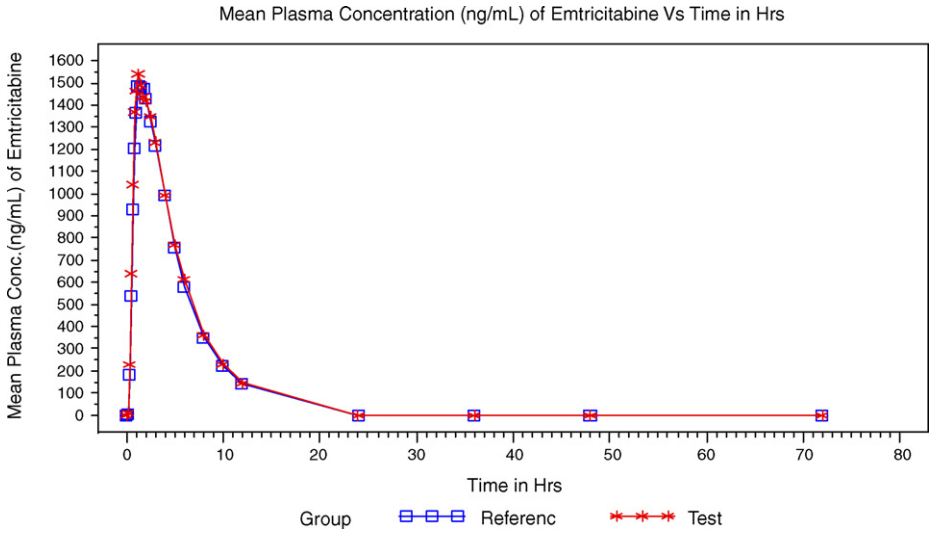

Fig. 11. Concentration versus time profile over 72 h of Emtricitabine in human plasma from 43 subjects receiving a single oral dose of 300 mg Tenofovir Disoproxil Fumarate and 200 mg Emtricitabine tablet.

namely TEN and EMT, were stable in matrix for up to 34 days at a storage temperature of −20 °C. This long term stability data generated was sufficient enough as it covered the entire study period right from the collection of blood samples to the final date of analysis. The stability of the main stock solutions of EMT, TEN and LAM was also proved by comparing their stored aliquots at refrigerated temperature of 4 °C versus freshly prepared stocks. This was done by comparing the areas obtained from aqueous samples prepared at the MQC level from both the stability and the freshly prepared stock solutions. It was found that the stock solutions of EMT, TEN and LAM were stable for at least 24 h at room temperature (about 25 °C) and under refrigeration at 4 °C for up to 12 days.

3.8. Application

The results obtained after application of this method to the bioequivalence study discussed above proved the present method to be suitable enough for its intended purpose. All the statistical parameters computed post-study are tabulated in Table 3. The concentration versus time profile for both the drugs is presented in Figs. 10 and 11 for TEN and EMT, respectively.

4. Conclusion

A simple, specific, rapid and sensitive analytical method for the determination of TEN and EMT in human plasma has been developed. Most of the analytical methods reported, for quantitation of EMT and TEN individually or simultaneously from human plasma, require laborious extraction procedure like liquid–liquid extraction, long run time and high quantification limit. The presented method provided excellent specificity and linearity with a limit of quantification of 10 ng/ml for TEN and 25 ng/ml of EMT. Although it was possible to go down much further in the LOQ determination of both drugs, the results obtained post subject analysis show that the LOQ values selected are sufficient enough to give data for calculation of the required pharmacokinetic data and establish bioequivalence. The other major advantage of this method over all those referenced is the short run time of 2 min which allows the quantitation of over 200 plasma samples per day.

Although the recoveries and accuracies in the method of Rezk et al. [4] seem to be better than those in the present article, the advantage of the presented method is the above mentioned short run time. The somewhat lower recovery for EMT does not have a major impact as it is consistent over the three levels checked during the recovery study.

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