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Research Article

Quantitative Bio-Analysis of Tenofovir Disoproxil Fumarate, Lamivudine and Efavirenz Simultaneously in Human Plasma Using Reverse-Phase Liquid Chromatography

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Abstract

A simple, sensitive, specific and accurate reverse-phase liquid chromatography method was developed and validated for the simultaneous quantitative determination of Tenofovir disoproxil fumarate (TDF), Lamivudine (3TC) and Efavirenz (EFV) with UV detection at 254 nm in human plasma. The method involves single step extraction of drugs from human plasma with Methanol. The compounds were separated with gradient elution using mobile phase A: Water with 0.1% Tetrahydrofuran (THF); mobile phase B: Acetonitrile with 0.1% THF on C18 (2) (250 × 4.6 mm, 5µ) column. The method was validated according to International Conference on Harmonisation (ICH) guidelines over the range of 1 - 6 µg/ml for TDF and 3TC and 2 - 12 µg/ml for EFV. The method was shown intra and inter - day accuracy and precision %RSD values less than 2 hence the method has proved to be accurate and precise. The percentage recoveries of three drugs from plasma were greater than 90%. The method was proved to be specific to the three drugs and the retention times were 7.32, 9.6 and 11.79 minutes for 3TC, TDF and EFV respectively. The LOD/LOQ for TDF, 3TC and EFV were 0.019/0.0585, 0.0061/0.0184 and 0.0126/0.038 µg/ml respectively. This validated method was applied to determine the drug concentrations in patents living with human immunodeficiency virus (PLHIV) infection who were receiving antiretroviral treatment with TLE containing regimen. Owing to its improved sensitivity, this method could be useful in pharmacokinetic and therapeutic drug monitoring studies in PLHIV infection receiving tenofovir based regimen.

Keywords: Tenofovir Disoproxil Fumarate; Lamivudine; Efavirenz; Human Immunodeficiency Virus

Abbreviations

HAART: Highly Active Anti-Retroviral Therapy; PLHIV: Patients Living with Human Immunodeficiency Virus; NRTI: Nucleotide Reverse Transcriptase Inhibitor; NNRTI: Non-Nucleoside Reverse Transcriptase Inhibitor; PI: Protease Inhibitor; TDF: Tenofovir Disoproxil Fumarate; 3TC: Lamivudine; EFV: Efavirenz; ICH: International Conference on Harmonisation; RP-UFLC: Reverse Phase Ultrafast Liquid Chromatogram; APIs: Active Pharmaceutical Ingredients

Introduction

Highly Active Anti-Retroviral Therapy (HAART) has been proved to reduce the morbidity and mortality in patients living with human immunodeficiency virus (PLHIV) infection [1]. HAART treatment consists of a three drug regimen with two - nucleoside or nucleotide reverse transcriptase inhibitor (NRTI) backbone in combination with a non - nucleoside reverse transcriptase inhibitor (NNRTI) or protease inhibitor (PI) [2]. Tenofovir based regimen is the next preferred first line treatment option for patients who are susceptible to Zidovudine based regimen treatment [3]. In Tenofovir based regimen Tenofovir disoproxil fumarate (TDF; Figure 1a) {9-[(R)-2-[[bis[[isopropoxy- carbonyl) oxy] methyl] phosphinyl] methoxy] propyl] adenine fumarate} and Lamivudine (3TC; Figure 1b) are the NRTIs and Efavirenz (EFV; Figure 1c) {2H-3,1-benzoxazin-2-one,6-chloro-4-(cyclo-propylethynyl)-1,4-dihydro-4-(trifluoromethyl)} is a NNRTI [4]. This drug is administered as a single, once daily dose (TLE regimen) offered good patient compliance and viral suppression.

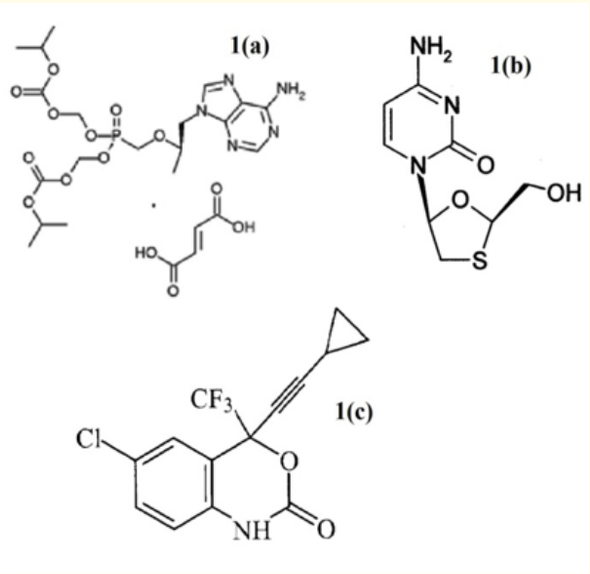

Figure 1: (a) Tenofovir disoproxil fumarate (b) Lamivudine and (c) Efavirenz.

Various analytical methods like ultra violet (UV) spectrophotometric analysis of TDF [5], 3TC [6] and EFV [7], High performance thin layer chromatography (HPTLC) of TDF [8], 3TC [9] and EFV [10] has been reported in bulk and dosage forms and liquid chromatography/tandem mass spectrometry (LC/MS) of TDF [11], 3TC with Zidovudine [12] and EFV [13] in plasma were reported. Several High Performance Liquid Chromatography (HPLC) methods with UV detection for simultaneous estimation of TDF, 3TC and EFV [4,14,15] in pharmaceutical dosage forms have been reported. HPLC method for individual estimation of TDF [16,17], 3TC [18,19] and EFV [20-24] in plasma were described. Also HPLC methods for estimation of TDF [25,26], 3TC [27,28] and EFV [29] with other antiretroviral drugs in dosage forms as well as in plasma were reported. To the best of the author's knowledge, literature survey revealed no HPLC analytical methods were reported for simultaneous quantitation of TDF, 3TC and EFV in plasma. The purpose of this study is to develop and validate according to International Conference on Harmonisation (ICH) [30] a simple, rapid, precise and accurate reverse phase ultrafast liquid chromatogram (RP-UFLC) method for the simultaneous estimation of TDF, 3TC and EFV in human plasma which could be applied for pharmacokinetic and therapeutic drug monitoring studies in PLHIV using this single dose regimen.

Experimental

Chemicals

Active Pharmaceutical Ingredients (APIs) of Tenofovir disoproxil fumarate (TDF) and Efavirenz (EFV) were kindly supplied by Hetero Labs (Hyderabad, Telangana, India) and Lamivudine (3TC) was a kind gift from Aurobindo Pharma (Hyderabad, India). Deionized Water used for analysis was obtained from Milli-Q water purification system (Millipore®, USA). Acetonitrile and Methanol of HPLC grade and Tetrahydrofuran (THF) were obtained from Merck (India). Pooled human blood was obtained from healthy human volunteer with given consent for method validation in blank drug free plasma.

Instrumentation

Chromatographic analysis was performed by the Ultra-Fast Liquid Chromatography (UFLC; Shimadzu, Kyoto, Japan) system with gradient capability binary pump (LC-20AD). Chromatography was equipped with UV/Visible dual wavelength absorbance detector (SPD-A20) and a Rheodyne injector port with 20 µl loop volume. Data acquisition was done by use of Lab solutions software. The mobile phase degassing was done on Ultrasonic cleaner D150H sonicator. Weighing of materials was done with 0.1 mg precision balance. Processing of plasma samples involved vortex mixing and centrifugation, and were performed using CM 101 cyclo mixer and centrifuge respectively.

Chromatographic conditions

The RP-UFLC analysis was performed at ambient temperature with gradient elution on analytical column C18 (2), 250 × 4.6 mm; 5µ particle size (Luna 5µ, Phenomenex) using Water with 0.1% THF as mobile phase A, Acetonitrile with 0.1% THF as mobile phase B and the flow rate was maintained at 1 ml/min. The detection wavelength was set to 254 nm. Gradient program was executed as depicted in the table 1. Mobile phases were freshly prepared and filtered through 0.22 µm membrane filter and degassed for 30 minutes on sonicator prior to analysis.

Preparation of standards

Standard stock solution was prepared by transferring accurately weighed 10 mg of TDF, 3TC and 20 mg of EFV together in to a 10 ml of volumetric flask and volume was made up to the mark with Methanol to get 1000 µg/ml of TDF, 3TC, and 2000 µg/ml of EFV final concentrations. Serial dilutions from stock solution were done using Methanol as diluents.

Chromatographic Conditions			
Stationary Phase			Phenomenex Luna C18 (2) column, (250 × 4.6 mm; 5**µ particle size)**
Mobile phase			A: Water with 0.1% THF; B: Acetonitrile with 0.1% THF
Flow rate			1.0 ml/min
Detection wave length			254nm
Injection volume			20 **µl**
Gradient Time Program			
Time (min)	**Mobile phase**		**Program**
	A %	**B%**	
0.03	90	10	Isocratic
3	75	25	Linear gradient
5	15	85	Linear gradient
9	10	90	Linear gradient
12	90	10	Linear gradient
15	90	10	Isocratic

Table 1: Chromatographic conditions and gradient time program of validated method.

Sample preparation

After collection of whole blood in potassium - EDTA tubes, immediately centrifuged at 4000 rpm for 20 minutes, then clear supernatant plasma was separated and stored at -20°C till process. Calibration standards in plasma were prepared as described by Yin., *et al.* 2014 [20] with few modifications, in brief 100 µl of blank drug free plasma was transferred in to 2 ml centrifuge tubes and 200 µl of combine solution (100 µl of appropriate concentration of different diluted standard aliquots and 100 µl of Methanol for protein precipitation) was added. To achieve liquid-liquid extraction above mixture was vortexed for 60 seconds and was laid aside for 10 minutes at room temperature followed by centrifuged at 5000 rpm for 15 minutes. Supernatant was separated and 20 µl was injected into UFLC system for analysis.

Limit of detection (LOD) and Limit of quantification (LOQ)

Sensitivity of method for three analytes were determined by calculating limit of detection (LOD) and limit of quantification (LOQ) from concentration that gives a signal to noise ratio of 3 and the lowest concentration with a signal to noise ratio of 10 respectively [30].

Linearity and Range

To establish the linearity of the method six calibration standards in triplicate were prepared by adding aliquots of methanolic drug solution to the plasma followed by the same extraction procedure as of sample preparation. The resulted solutions give the final concentration ranges in plasma of TDF and 3TC 1 - 6 µg/ml (1, 2, 3, 4, 5 and 6 µg/ml) and of EFV 2 - 12 µg/ml (2, 4, 6, 8, 10 and 12 µg/ml). Individual calibration curves using linear regression analysis were plotted to observed peak area of analyte against concentration of the analyte and from this data slope, intercept and coefficient of variation were obtained.

Accuracy and Precision of method

Accuracy of the method in plasma was evaluated using spiked plasma quality control (QC) samples of 50, 100 and 150% of analytes concentrations of 2.5, 5 and 7.5 µg/ml for TDF and 3TC and 5, 10 and 15 µg/ml for EFV. To evaluate the intra and inter day precision QC samples of low, middle and high (1, 3, 6 µg/ml) for TDF and 3TC and (2, 6, 12 µg/ml) for EFV were prepared. All the QC samples were prepared in triplicate following the same procedure as for sample preparation, using a separate set of stock solutions. Freshly prepared calibration and QC samples were used for analysis at different days and values are reported in mean ± SD (%RSD).

Recovery of analytes from plasma

Recovery of each drug from plasma was calculated by the method of comparing the peak area of drug that underwent extraction with plasma to that of peak area of identical concentrations of methanolic solution.

Specificity

Specificity of the method was determined by observing any interfering substances at elution of the analytes of interest [30]. It was done by comparing the blank plasma chromatogram with analyte spiked plasma chromatogram.

Robustness and Ruggedness

To determine the robustness and ruggedness of the method higher concentration range (i.e. 6 µg/ml of TDF and 3TC and 12 µg/ml of EFV) in triplicate was used for analysis. Robustness was checked by changing the mobile phase flow rate to 0.8 and 1.2 ml/min, peak modifiers to 0.1% formic acid (FA) and 0.1% diethyl amine (DEA) and the varied mobile phase composition respect to time in gradient system. The changes in retention times were reported as %RSD.

Ruggedness of the method was assessed by comparing the analysis performed by the two analysts in the same laboratory and the P value less than 0.05 is considered as significant change in the validated method.

Samples

According to the protocol previously approved by the Ethics Committee of Kakatiya Medical College (No: KIEC/MGM/KMC/NCT/2016/P02; KIEC, Warangal) and with the informed consent of the patient a twelve adult HIV patients who were attending the outpatient clinic of the ART centre, MGM Hospital, Warangal, and clinically stable i.e. CD4 count > 500 cells/mm^3 took part in the study. Patients with co-morbid conditions were exempted from the study and plasma samples were collected in potassium - EDTA tubes followed by processing of patients plasma for estimation as described under sample preparation procedure. Estimations of plasma TDF, 3TC and EFV in all the samples were undertaken within 24 - 48h of blood collection.

Results and Discussion

Method optimization

As TDF, 3TC and EFV drugs exhibit different physicochemical properties it was difficult to separate them simultaneously under isocratic conditions. As the instrument is binary pump system, various trials were tried by dividing mobile phase in to an aqueous and organic phase systems. In set of first trials, Millipore Water was taken as an aqueous phase system and Methanol and Acetonitrile individually was taken as an organic phase system and in an another set of trials aqueous phase was replaced with various buffers at different pH. In each set every time two phase and three phase systems at different compositions were attempted, Mobile phases for isocratic two phase system of Methanol: Water; Acetonitrile: Water; different buffers of Sodium, Potassium and Ammonium at different pH with Acetonitrile and Methanol and three phase system of Water: Methanol: Acetonitrile; at different compositions were attempted but none of them gave satisfactory chromatographic separations. In isocratic elution, consistently 3TC and TDF were eluted near the solvent front and EFV was not eluted within 25 minutes. Thus a gradient method was considered, and a good chromatographic separation of TDF, 3TC and EFV was obtained with mobile phase A: Water with 0.1% THF and mobile phase B: Acetonitrile with 0.1% THF.

Method Validation

Limit of detection (LOD) and Limit of quantification (LOQ)

Though the published methods reported low LOD/LOQ (in µg/ml) for simultaneous estimation of these three drugs using different RP-HPLC methods by various authors i.e. Bhavsar., *et al.* 2012 [4] (TDF: 0.01/0.1, 3TC: 0.018/0.05 and EFV: 0.02/0.07); Srinatha., *et al.* 2014 [14] (TDF: 0.08/0.27, 3TC: 0.04/0.16, EFV: 0.25/0.84); and Vanaja., *et al*. 2013 [15] (TDF: 0.03/0.102, 3TC: 0.02/0.09, EFV: 0.03/0.111), all of them were in dosage forms.

Actual concentration for TDF and 3TC (µg/ml)	**Found concentration (µg/ml) [n=3] Mean ± SD (%RSD)**		**Actual concentration for EFV (µg/ml)**	**Found concentration (µg/ml) [n=3] for EFV Mean ± SD (%RSD)**
	TDF	**3TC**		
1	1.15 ± 0.015 (1.321)	1.13 ± 0.015 (1.344)	2	2.21 ± 0.0001 (0.004)
2	1.82 ± 0.015 (0.838)	1.93 ± 0.005 (0.299)	4	4.09 ± 0.0057 (0.141)
3	2.99 ± 0.015 (0.511)	2.62 ± 0.173 (6.621)	6	5.73 ± 0.006 (0.1007)
4	4.08 ± 0.021 (0.509)	4.09 ± 0.01 (0.245)	8	7.85 ± 0.0001 (0.001)
5	4.84 ± 0.01 (0.206)	5.15 ± 0.015 (0.296)	10	9.72 ± 0.006 (0.059)
6	6.11 ± 0.006 (0.094)	5.95 ± 0.01 (0.168)	12	12.35 ± 0.066 (0.539)
Correlation Coefficient	0.994 ± 0.001 (0.058)	0.991 ± 0.001 (0.101)		0.994 ± 0.001 (0.101)
Slope	4018.7 ± 7.57 (0.188)	8208.3 ± 6.658 (0.081)		8674 ± 1 (0.0115)
y intercept	- 614.7 ± 23.514 (3.82)	1341.67 ± 15.14 (1.12)		- 984.8 ± 33.23 (3.37)
LOD (µg/ml)	0.019	0.0061		0.0126
LOQ (µg/ml)	0.0585	0.0184		0.038

Table 2: Calibration data and LOD and LOQ of three drugs.

None of the earlier methods have not been described the LOD/LOQ values for simultaneous estimation of these three drugs in human plasma. Except the LOD for TDF by Bhavsar., *et al.* in dosage form, for the first time present method achieved even low LOD/LOQ in human plasma for simultaneous estimation of TDF, 3TC and EFV i.e. 0.019/0.0585, 0.0061/0.0184 and 0.0126/0.038 µg/ml respectively (Table 2). This reveals that the method is highly sensitive and using this method small drug concentration can be estimated in patient's blood plasma.

Linearity and Range

Areas of the calibration standards were proportional to the concentrations of analytes in plasma. Calibration data for three drugs were shown in table 2 and were fitted by linear least-square regression. The coefficient of determination was found to be greater than 0.99 for three drugs and was represented as mean ± SD (%RSD) [TDF: 0.994 ± 0.001 (0.058); 3TC: 0.991 ± 0.001 (0.101) and EFV: 0.994 ± 0.001 (0.101)]. The %RSD was found to be < 2% according to ICH guidelines. Thus the method is linear over the range of 1 - 6 µg/ml for TDF and 3TC and 2 - 12 µg/ml for EFV.

Accuracy and Precision and Recovery

The results from the validation of the method in human plasma for accuracy are listed in table 3. Average accuracy for intra-day (%RSD) ranged from 0.38 to 0.69, 0.40 to 0.93 and 0.19 to 0.38 for TDF, 3TC and EFV respectively. The average accuracy for inter-day (%RSD) was ranged from 0.36 to 0.99 for TDF, 0.11 to 0.64 for 3TC and 0.19 to 0.57 for EFV.

Parameters	Actual Concentration (µg/ml)	Found Concentration (µg/ml) Mean ± SD (%RSD)		Mean % of Recovery ± SD (%RSD)	
		Intra Day	Inter Day	Intra Day	Inter Day
TDF					
50%	2.5	2.46 ± 0.02 (0.69)	2.48 ± 0.025 (0.99)	99.16 ± 0.43 (0.43)	99.42 ± 0.69 (0.7)
100%	5.0	4.89 ± 0.03 (0.66)	4.93 ± 0.033 (0.68)	99.08 ± 0.56 (0.56	99.28 ± 0.08 (0.08)
150%	7.5	7.45 ± 0.03 (0.38)	7.45 ± 0.027 (0.36)	99.63 ± 0.18 (0.18)	99.63 ± 0.18 (0.18)
3TC					
50%	2.5	2.45 ± 0.023 (0.93)	2.46 ± 0.02 (0.64)	99.07 ± 0.06 (0.06)	99.26 ± 0.19 (0.19)
100%	5.0	4.93 ± 0.027 (0.55)	5.03 ± 0.005 (0.11)	99.31 ± 0.46 (0.46)	100.14 ± 0.42 (0.42)
150%	7.5	7.40 ± 0.03 (0.4)	7.43 ± 0.011 (0.15)	99.49 ± 0.35 (0.35)	99.69 ± 0.46 (0.46)
EFV					
50%	5.0	4.94 ± 0.013 (0.26)	4.92 ± 0.024 (0.48)	99.49 ± 0.43 (0.43)	99.35 ± 0.32 (0.32)
100%	10	9.85 ± 0.019 (0.19)	9.82 ± 0.056 (0.57)	99.51 ± 0.64 (0.64)	99.26 ± 0.54 (0.54)
150%	15	14.9 ± 0.056 (0.38)	15.012 ± 0.028 (0.19)	99.63 ± 0.22 (0.22)	100.08 ± 0.31 (0.31)

Table 3: Data of accuracy.

The precision was determined for low, middle and high concentrations of the three drugs and were tabulated in table 4. The method was precise, intra-day precision (%RSD) ranged from 0.44 to 0.7 for TDF, 0.43 to 0.74 for 3TC and 0.22 to 0.51 for EFV. The inter-day precision (%RSD) ranged from 0.72 to 0.78, 0.35 to 0.58 and 0.56 to 0.68 for TDF, 3TC and EFV respectively. As the results of accuracy (< 5%) and precision (< 10%) was found to be within the limits of ICH guidelines, the method was proved to be accurate and precise.

Present method shown 96% plasma recovery for 3TC which was similar to that of Zheng., *et al.* (> 95%) [19]. Percentage recovery for EFV in several liquid-liquid extraction procedures reported by earlier authors (75.6 - 80.3%) Yin., *et al.* 2014 [20]; (> 78%) Sailaja., *et al.* 2007 [22]; (92.7 - 94.1%) Mogatle., *et al.* 2009 [24] and solid phase extraction (SPE) procedure described by (> 83%) Sarasa-Nacenta., *et al.* 2001 [21] were lower than reported herein i.e. 98%. Current method showed 91% plasma recovery for TDF and this is high when comparing with expensive SPE for TDF

from previous reports i.e., 66.7% [17] and 80.6% [26]. Hence the protein precipitation with Methanol in liquid-liquid extraction method offers high plasma recoveries of TDF, 3TC and EFV and is more economic too.

Parameters	Actual Concentration (µg/ml)	Found Concentration (µg/ml) Mean ± SD (%RSD)		Mean % of Recovery ± SD (%RSD)	
		Intra Day	Inter Day	Intra Day	Inter Day
TDF					
Low	1	0.98 ± 0.006 (0.6)	0.99 ± 0.007 (0.72)	99.18 ± 0.45 (0.45)	99.58 ± 0.53 (0.53)
Middle	3	2.95 ± 0.02 (0.7)	2.95 ± 0.023 (0.78)	99.28 ± 0.16 (0.16)	99.22 ± 0.13 (0.13
High	6	5.96 ± 0.026 (0.44)	5.96 ± 0.043 (0.73)	99.65 ± 0.22 (0.22)	99.54 ± 0.5 (0.5)
3TC					
Low	1	0.99 ± 0.007 (0.74)	0.99 ± 0.004 (0.41)	99.34 ± 0.36 (0.36)	99.39 ± 0.36 (0.37)
Middle	3	2.95 ± 0.017 (0.57)	2.97 ± 0.017 (0.58)	99.32 ± 0.27 (0.27)	99.49 ± 0.16 (0.17)
High	6	5.95 ± 0.026 (0.43)	6.00 ± 0.021 (0.35)	99.59 ± 0.13 (0.13)	11.11 ± 0.57 (0.57)
EFV					
Low	2	1.99 ± 0.05 (0.23)	1.97 ± 0.012 (0.63)	99.67 ± 0.17 (0.17)	99.28 ± 0.30 (0.30)
Middle	6	5.93 ± 0.03 (0.51)	5.92 ± 0.04 (0.68)	99.44 ± 0.17 (0.17)	99.31 ± 0.18 (0.18)
High	12	11.95 ± 0.027 (0.22)	11.87 ± 0.067 (0.56)	99.95 ± 0.47 (0.47)	99.43 ± 0.04 (0.04)

Table 4: Data of precision.

Specificity and System suitability

As visualized in figure 2a and 2b, the chromatogram of three drugs showed good separation and they were not interfered with endogenous plasma components with retention times of 7.32, 9.6 and 11.79 minutes for 3TC, TDF and EFV respectively. For this reason the validated method has proven to be specific towards three drugs.

The detailed data for system suitability of validated method is listed in table 5. The theoretical plates (N) number greater than 2000 (according to ICH guidelines) for TDF, 3TC and EFV was signify the column efficiency in simultaneous separation of these three drugs. Tailing factor (T) for TDF, 3TC and EFV was found to be less than 2 the limit specified in ICH guidelines reveal that the peaks were well resolved with good sharpness and with good symmetry. Retention factor (K′) gives a measure of the location of the peak of interest with respect to the peak of non-retained components and the range should be 1 - 10. The K′ values of present drugs were within the range and it imparts that the effective elution of three drugs from the non-retained components (obtained at 2.45 minutes). Separation factor (α) and resolution factor (Rs) are often used to measure the how well two peaks are separated and are essential for quantitation. In this method the α and R_s for 3TC to TDF and TDF to EFV were met the criteria of ICH guidelines i.e. $\alpha > 1$ and $R_s > 2$. Hence the validated method proved to offer good resolution of three peaks with well separation.

S. NO	Parameters	Values obtained			Limits
		TDF	3TC	EFV	
1	USP Theoretical plates (N)	12764.16	7421.16	19252.07	T > 2000
2	USP Tailing factor (T)	1	0.75	0.98	T < 2
3	USP Retention factor (K′)	2.92	1.98	3.81	1-10
4	USP Separation factor (α)	1.47	-	1.31	$\alpha > 1$
5	USP Resolution factor (R_s)	5.61	6.51	-	R > 2

Table 5: System suitability parameters for TDF, 3TC and EFV.

Robustness and Ruggedness

Results of robustness and ruggedness are tabulated in table 6. ICH defines robustness as a measure of the method's capability to remain unaffected by small, but deliberate variations in method parameters and %RSD should be less than 2. In this method change in R_t of three analytes with small changes in flow rate, peak modifiers and mobile phase composition were observed while the other analytical parameters kept constant. The %RSD of R_t values for TDF, 3TC and EFV was found to be < 2 and the low value of RSD indicates the method was robust.

Robustness [Mean R_t ± SD (%RSD)]				
Condition	**Modification**	**TDF**	**3TC**	**EFV**
Flow rate (ml/min)	0.8	9.90 ± 0.007 (0.071)	7.62 ± 0.004 (0.053)	12.14 ± 0.006 (0.047)
	1 (Optimized)	9.60 ± 0.005 (0.052)	7.32 ± 0.004 (0.057)	11.79 ± 0.003 (0.025)
	1.2	9.46 ± 0.006 (0.065)	7.56 ± 0.295 (3.903)	11.51 ± 0.009 (0.082)
Peak modifiers	0.1% FA	9.63 ± 0.008 (0.082)	7.33 ± 0.006 (0.082)	11.81 ± 0.005 (0.043)
	0.1% DEA	9.62 ± 0.005 (0.055)	7.33 ± 0.003 (0.036)	11.81 ± 0.005 (0.04)
	0.1% THF (Optimized)	9.60 ± 0.005 (0.052)	7.32 ± 0.004 (0.057)	
Mobile phase composition	T/%B: 0.03/15;3/20;5/80;9/90;12/15;15/15	9.42 ± 0.006 (0.068)	7.27 ± 0.009 (0.132)	11.75 ± 0.008 (0.068)
	T/%B: 0.03/10;3/25;5/85;9/90;12/10;15/10 (Optimized)	9.60 ± 0.005 (0.052)	7.32 ± 0.004 (0.057)	11.79 ± 0.003 (0.025)
	T/%B: 0.03/10;3/15;5/85;9/90;12/10;15/10	9.73 ± 0.012 (0.128)	7.46 ± 0.01 (0.149)	11.86 ± 0.018 (0.152)

Ruggedness							
Sample (n=3)	**Concentration (µg/ml)**	**Found concentration (µg/ml) Mean ± SD (%RSD)**			**Retention time (min) Mean ± SD (%RSD)**		
		Analyst I	**Analyst II**	**P value**	**Analyst I**	**Analyst II**	**P value**
TDF	6	5.95 ± 0.074 (1.239)	5.96 ± 0.106 (1.77)	0.802	9.602 ± 0.05 (0.052)	9.61 ± 0.005 (0.053)	0.322
3TC	6	5.86 ± 0.093 (1.586)	5.797 ± 0.056 (0.981)	0.394	7.32 ± 0.056 (0.109)	7.32 ± 0.004 (0.056)	0.558
EFV	12	11.87 ± 0.235 (1.986)	11.783 ± 0.179 (1.523)	0.627	11.80 ± 0.012 (0.104)	11.8 ± 0.012 (0.103)	0.851

Table 6: Robustness and Ruggedness data of validated method.

*T: Time; FA: Formic Acid; DEA: Diethyl Amine; THF: Tetrahydrofuran; P < 0.05 is considered as significant difference.

Ruggedness is a measure of the reproducibility of a test result under normal, expected operating condition from instrument to instrument and from analyst to analyst. Present validated method shows the P values greater than 0.05 indicates that there is no significant variation of results from analyst to analyst hence the method has good reproducibility.

Demographic data and Analysis of patient samples

Table 7 shows the demographic data of 12 patients (6 male and 6 female) with mean age of 56 ± 9.16 years with mean weight of 54.83 ± 14.14 and the mean duration of the disease was 7.21 ± 4.39. Figure 2(c) and 3 shows a typical chromatogram and drug

concentrations in patient blood plasma respectively. Minimum/ Maximum (in mg/dL) concentration of TDF, 3TC and EFV from the patient's plasma samples were found to be 0.014/0.47, 0.005/0.05 and 0.22/1.01 respectively. This validated method demonstrated detection of minute quantities in plasma samples of PLHIV taking TLE regimen, thus this analytical method has proven its applicability in pharmacokinetic, toxicological and therapeutic drug monitoring studies.

S. NO	Gender	Age in years (Mean ± SD 56 ± 9.16)	Weight in Kgs (Mean ± SD 54.83 ± 14.14)	Duration of the disease in years (Mean ± SD 7.21 ± 4.39)	CD_4 count cells/mm^3
1	F	46	48	10	513
2	F	51	85	9	1248
3	M	65	43	1	955
4	F	65	60	11	804
5	M	43	62	2	699
6	M	45	76	11	660
7	M	64	58	1.6	630
8	F	63	39	8	593
9	F	55	45	10	569
10	M	55	53	11	607
11	F	50	46	11	506
12	M	70	43	1	550

Table 7: Demographic data of the patients.

F: Female; M: Male

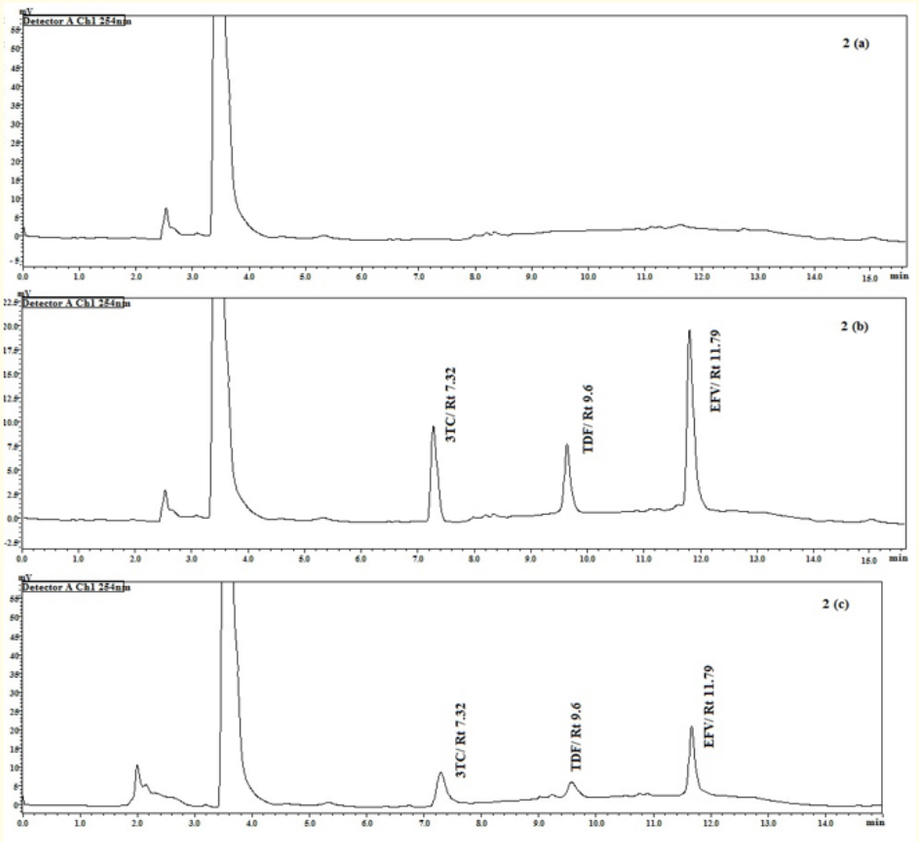

Figure 2: (a) Chromatogram of blank plasma (b) Chromatogram of spiked plasma with 6 µg/ml of 3TC, TDF and 12 µg/ml of EFV (c) Chromatogram of extracted plasma samples of PLHIV.

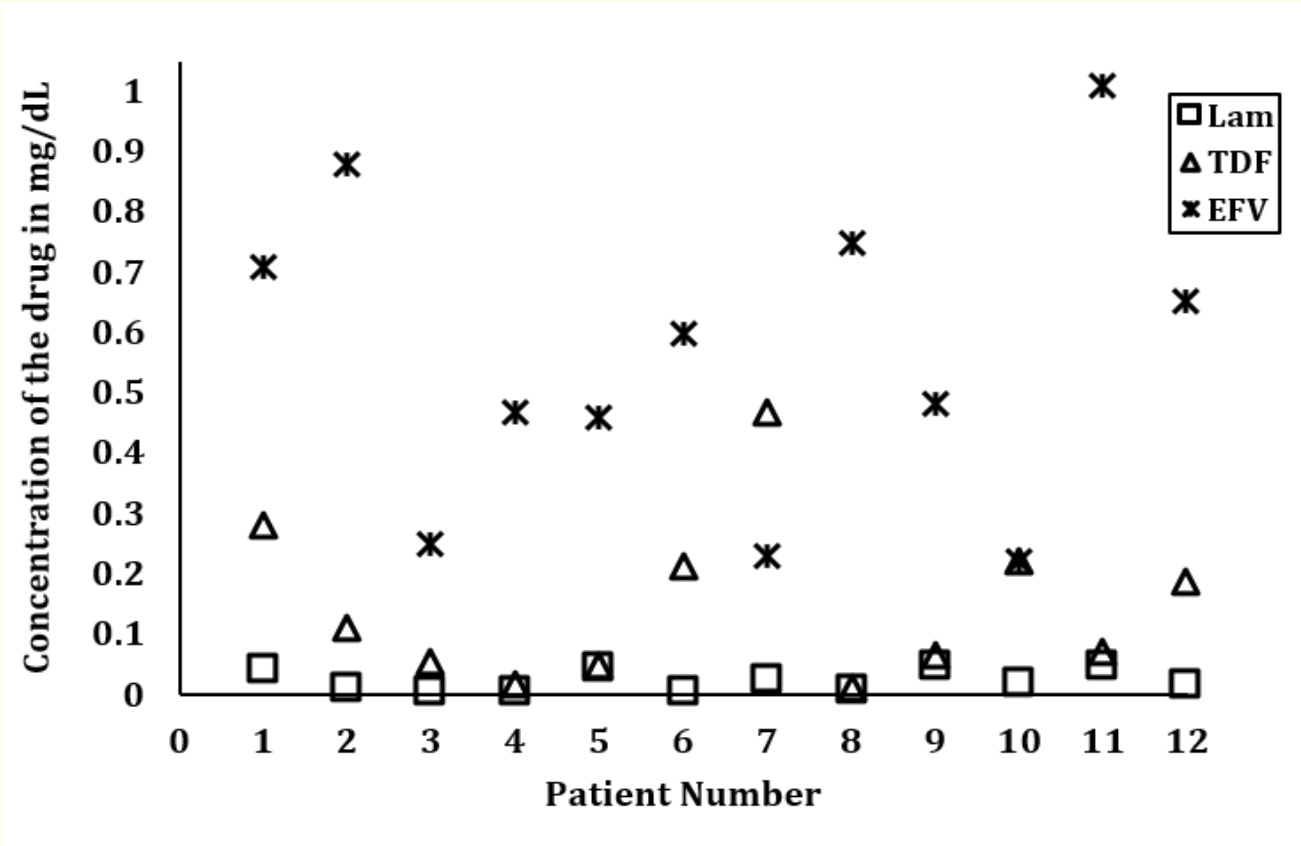

Figure 3: Concentrations of 3TC, TDF and EFV in mg/dL in patient's plasma samples.

Conclusion

A simple, sensitive, specific and validated reverse phase UFLC method for the simultaneous quantitation of tenofovir disoproxil fumarate, lamivudine and efavirenz in plasma with gradient elution is achieved. The method is rapid and involved simple extraction procedure. The chromatogram yields well resolved peaks for TDF, 3TC and EFV with good intra- and inter-day precisions. The easy sample preparation and small sample volume makes this method highly suitable for pharmacokinetic, toxicological and therapeutic drug monitoring studies in PLHIV.

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