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Simultaneous quantification of emtricitabine and tenofovir in human plasma using high-performance liquid chromatography after solid phase extraction

Naser L. Rezk *, Rustin D. Crutchley, Angela D.M. Kashuba

Clinical Pharmacology/Analytical Chemistry Core, Center for AIDS Research, 3320 Kerr Hall, CB# 7360, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

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Abstract

An accurate, sensitive and simple reverse-phase (RP) high-performance liquid chromatography (HPLC) assay for the simultaneous quantitative determination of emtricitabine and tenofovir in human blood plasma is described. Using 200 μL of plasma and BOND ELUT-C18 Varian columns, the solid phase extraction (SPE) method results in a clean baseline and high extraction efficiencies (100% for emtricitabine and 98.6% for tenofovir). An Atlantis™ dC-18 analytical column is used along with an 18 min linear gradient elution of phosphate buffer (pH 5.7) and methanol to provide sharp peaks for emtricitabine at 280 nm, tenofovir at 259 nm, and the internal standard 2′,3′didoxyuridine (DDU) at 262 nm. The method was validated over the range of 10–10,000 ng/mL for both analytes, and is accurate (average accuracies of three different concentrations ranged from 98 to 105% for emtricitabine and 97 to 103% for tenofovir) and precise (within- and between-day precision ranged from 1.7 to 3.7% and 3.7 to 5.2%, respectively). This method is suitable for use in clinical pharmacokinetic studies and is nimble enough for therapeutic drug monitoring.

Keywords: HIV; Antiretroviral; NRTI; NtRTI; HPLC; Chromatography

1. Introduction

Emtricitabine (FTC; 5-fluro-1-(2*R*, 5*S*)-[2-9hydroxymethyl]-1,3-oxathiolan-5-yl) is a (−) entantiomer of a thio analogue of cytidine (Fig. 1). FTC is classified as a dideoxycytidine nucleoside analogue reverse transcriptase inhibitor, and is potent and selective against HIV types I and II and hepatitis B virus [1,2]. FTC is phosphorylated by cellular enzymes to form emtricitabine 5′-triphosphate, which competes with deoxycytidine 5′-triphosphate and terminates the amino acid chain of newly forming viral DNA. Tenofovir diproxil fumarate (TDF; 9-[(*R*)-2-[[bis[[(isopropoxycarbonyl) oxy] methoxy] phosphinyl] methoxy] propyl), formerly known as PMPA, is a nucleotide reverse transcriptase inhibitor belonging to the class of acyclic nucleoside phosphonates, and is active against a variety of drug resistant HIV-I strains in vitro [6,7]. TDF is found as tenofovir (TNF; 9-[(*R*)-2-(phosphonomthoxy)-propyl] adenine) in blood plasma (Fig. 1) and is diphosphorylated intracellularly to its active moiety. Both FTC and TDF have favorable side effect profiles [5] and long elimination half-lives, which allow for once daily therapy [3,4].

Recently, the combination of FTC and TDF has demonstrated significantly greater HIV RNA suppression compared to the combination of zidovudine and lamivudine [8,9]. FTC and TDF are currently co-formulated in one convenient fixed dose tablet called Truvada™ (Gilead Sciences, Foster City, CA, USA). Therefore, an analytical method capable of simultaneously analyzing these two drugs in blood plasma is needed. Of great concern with any analytical method for antiretroviral agents is sample volume: an assay to measure

* Corresponding author. Tel.: +1 919 843 0596; fax: +1 919 962 0644.
E-mail address: naser2@med.unc.edu (N.L. Rezk).

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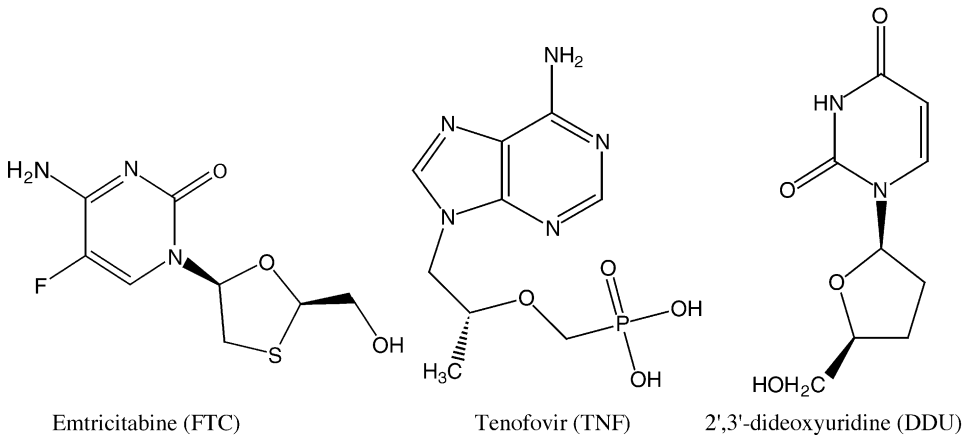

Fig. 1. Chemical structure of FTC, TNF and DDU.

drug concentrations in sample volumes of 200 μL or less is most preferable, in order for the assay to be utilized in multiple settings on fresh and frozen samples.

This paper describes a novel method for the quantification of FTC and TNF in a 200 μL plasma sample, using an optimized solid phase extraction (SPE) procedure combined with a rapid reverse-phase (RP) high-performance liquid chromatography (HPLC) method and photodiode array detection (DAD).

2. Experimental

2.1. Chemicals

All reagents were obtained from standard suppliers; reagent grade (>94% purity). Analytical grade 2′,3′didoxyuridine (DDU; 100% purity) was purchased from Sigma Chemical Company (St. Louis, MO, USA). TNF and FTC were obtained from the NIH AIDS Research & Reference Reagent Program (McKesson HBOC BioServices, Rockville, MD, USA). HPLC grade chemicals were purchased from Fisher Scientific (Norcross, GA, USA). Purified compressed nitrogen gas was obtained from National Welders Supply (Charlotte, NC, USA). Drug-free pooled plasma was obtained from whole blood anticoagulated with sodium EDTA (Biological Specialty Corporation, Colmar, PA, USA).

2.2. Equipment

A high-performance liquid chromatography system consisting of an Agilent Technologies (Wilmington, DE, USA) Model HP1100 binary pump, an HP1100 degasser, an HP1100 auto sampler, an HP1100 UV-DAD-detector, and HP ChemStation software (Version A.08.03) run on a Compaq Evo computer (operated with Windows 2000 Professional), was used for this method.

2.3. Preparation of standards

2.3.1. Preparing the standard master stock solution

Individual clear stock solutions of TNF and FTC were prepared as 1 mg/mL concentrations. For tenofovir, 5.3 mg of tenofovir monohydrate (molecular weight 305.23 g) powder was accurately weighed and dissolved in 50 μL of 5.0 M sodium hydroxide solution and the volume adjusted using HPLC-grade water in a 5 mL volumetric flask. FTC was prepared in a 5.0 mL volumetric flask by dissolving 5.0 mg of powder in HPLC-grade water. The master stock solution was prepared as a composite of both analytes (1.0 mL each), adjusted to a final concentration of 100 μg/mL in a 10 mL volumetric flask using 8.0 mL of HPLC-grade water. This 100 μg/mL master stock solution was used to prepare eight working solutions (10, 25, 50, 100, 500, 1000, 5000 and 10,000 ng/mL) in drug-free pooled plasma. Plasma quality control (QC) samples at 75, 750 and 7500 ng/mL were prepared from another 100 μg/mL of master stock solution.

Solutions of potential drugs of interference (primarily antiretroviral agents) were prepared from pure standard or clear filtered extracts of the pharmaceutical formulation. These solutions were prepared in 100% methanol to a final concentration of 1.0 mg/mL and diluted with mobile phase before injection onto the HPLC system at concentrations of 10 μg/mL.

2.4. Internal standard (IS) preparation

An internal standard stock solution was prepared by accurately weighing 10 mg of 2′,3′didoxyuridine and dissolving this in a volumetric flask with HPLC-grade water to achieve a final concentration of 1.0 mg/mL. The internal standard working solution was prepared by diluting an aliquot from the stock solution in 150 mM ammonium acetate buffer (pH adjusted to 5.0 using 33% acetic acid) to a final concentration of 2.0 μg/mL.

2.5. Sample pre-treatment

Patient blood samples were collected in potassium EDTA tubes and centrifuged (2800 rpm for 15 min at 4 °C) within 15 min of collection. Plasma was carefully transferred to sterile cryovials and stored at −70 °C until analysis. Prior to extraction, all plasma samples were heated for 30 min at 58 °C to inactivate HIV virus.

On the day of analysis, the internal standard working solution, calibrators, QC samples, and patient samples were brought to room temperature. A 200 μL aliquot of each patient sample, calibrators, and QC samples, along with a 200 μL of internal standard working solution was directly transferred to a conditioned and labeled SPE column according to the extraction method below.

2.6. SPE extraction method

Solid phase extraction columns (1.0 mL, 200 mg BOND ELUT-C18 Varian, Harbor City, CA, USA) were placed on a vacuum elution manifold (20-SPE System, Waters, Milford, MA, USA). The cartridges were rinsed with 0.5 mL of methanol, and then conditioned with 0.5 mL of 150 mM ammonium acetate (pH 5.0). Two hundred microlitres of the working internal standard solution was transferred to the conditioned column followed by 200 μL of blank plasma, calibrators, QC samples, or patient samples. Both internal standard and plasma samples were allowed to pass through the column bed with minimal suction (1–3 mmHg). The column was further washed with 900 μL of 100 mM ammonium acetate buffer (pH 7.0), and then suctioned to dryness for 2 min using 10–15 mmHg of vaccum. The retained drugs were then eluted with 500 μL of methanol. The eluent solution was evaporated to dryness under a gentle nitrogen stream at 45 °C, and the residue was reconstituted with 100 μL of mobile phase (19:1, A/B; see below for composition). The resulting solutions were sonicated for 30 s, carefully vortexed for 30 s, then centrifuged at 12 000 rpm for 5 min. The supernatants were transferred to 200 μL HPLC autosampler vials (Microvials, Agilent Technologies) and 80 μL was injected onto the column.

2.7. High-performance liquid chromatographic conditions

An Atlantis™ dC-18 analytical column (150 mm × 3.9 mm, 5.0 μm particle size, Waters Corp., Milford, MA, USA), with an Atlantis™ dC-18 guard column (20 mm × 3.9 mm, 5.0 μm particle size, Waters Corp.) were used for analyte separation and quantitation. During elution, the absorbance was monitored by three different wavelengths; 262 nm for DDU, 280 nm for FTC and 259 nm for TNF.

Mobile phase A was composed of 20 mM phosphate monobasic and 4 mM of tetrabutylammonium hydrogen sulfate (pH 5.7). This buffer solution was filtered through a 0.45 μm membrane filter (Millipore, Milford, MA, USA) before use. Mobile phase B was 100% methanol. The chromatographic separation of analytes was performed with a gradient elution according to the schedule in Table 1, with an extra 2 min after the run time. The analysis was performed at 25 °C, with a mobile phase flow rate of 1.0 mL/min.

Table 1
The elution gradient delivery system of mobile phases A and B over 18 min of run time

Time (min)	A (%)	B (%)
0.00	95	5
2	95	5
12	50	50
14	15	85
16	15	85
18	95	5

2.8. Specificity and selectivity

Interference from endogenous plasma constituents was investigated by analysis of six separate male and female blank plasma samples, in addition to the blank plasma samples injected during each analytical run. Interference from 22 commonly used medications/medication classes was also investigated. These included atorvastatin, propranolol, gemfibrazil, chlorhexadine, nystatin, acyclovir, acetylsalicylic acid, and the nucleoside reverse transcriptase inhibitors; zalciatbine, lamivudine, didanosine, stavudine, zidovudine and abacavir. Also includes the protease inhibitors indinavir, amprenavir, nelfinavire, saqunavir, atazanavir, ritonavir, lopinavir and non-nucleosides reverse transcriptase inhibitors, nevirapine and efavirenz.

2.9. Limits of quantification and limit of detection

The lower limit of quantification (LLQ) was defined as the concentration for which both the relative standard deviation (R.S.D.) and the percent deviation from the nominal concentration were less than 20%. The upper limit of quantification (ULQ) was defined as the concentration for which both the relative standard deviation and the percent deviation from the nominal concentration were less than 15% [10]. The detection limit was defined as a signal-to-nose ratio of 3:1.

2.10. Stability

HIV-infected patient samples are routinely heated at 58 °C to inactivate the virus prior to handling. Heat inactivation studies were performed to verify the stability of all the drugs in plasma under these conditions. An additional stability test was performed to verify the stability of the drugs in the autosampler tubes while waiting for HPLC analysis. The samples were left at room temperature for 24 h prior to analysis. The stability during sample handling was also verified by subjecting samples to three freeze-thaw cycles. Quality con-

Table 2
Summary of analyte retention times, extraction efficiency (%), linearity range, slope, r^2, and intercept values

Analyte	Retention time (min)	Range (ng)	Extraction efficiency (%)	r^2	Slope ± S.D.	Intercept ± S.D.
IS	7.6		97.2			
FTC	8.4	10–10,000	100.0	0.999	0.44 ± 0.01	4.54 ± 0.56
TNF	10.6	10–10,000	98.6	0.999	0.31 ± 0.01	14.51 ± 4.34

trol samples at three concentrations 75, 750 and 7500 ng/mL were utilized in these stability tests.

2.11. Extraction recovery

Recovery of the two analytes (FTC, and TNF) after solid phase extraction was determined by comparing observed peak area in extracted plasma for each compound, to those of non-processed standard solutions.

3. Results

3.1. Linearity

The peak area ratios of drug to IS for the calibration standards were proportional to the concentration of each drug in plasma over the range tested. The linear regression data for the calibration curves ($n = 3$) are shown in Table 2. The mean ± S.D. of three standard curve slopes and intercepts, and the linear range for each analyte is also shown in Table 2. The 10–10 000 ng/mL range was linear for both analytes (Table 3). The regression coefficient (r^2) of all calibration curves was ≥0.999.

3.2. Selectivity

A representative chromatogram of a blank plasma sample containing internal standard is shown in Fig. 2. The approximate retention times for the two analytes and internal standard are listed in Table 2. No endogenous substances interfered with any of the analytes in blank plasma extracts. The retention times for potential drugs of interference were either different from the compounds of interest ($n = 10$), or out of the run time range ($n = 12$). The following are the compounds and their respective retention times eluted within the run time:

Table 3
Summary of back-calculated values for the standard curves, and relative standard deviation and accuracy (%) of all eight levels of calibration for tenofovir and emtricitabine

Theoretical concentration (ng/mL)	FTC			TNF		
	Observed concentration (ng/mL)	R.S.D. (%)	Accuracy (%)	Observed concentration (ng/mL)	R.S.D. (%)	Accuracy (%)
10	9.3	8.1	92.8	10.5	14.3	105.0
25	22.5	6.6	90.2	25.5	5.5	101.8
50	46.1	7.6	92.3	46.8	4.9	93.7
100	96.0	6.6	96.0	98.3	0.6	98.3
500	516.2	4.5	103.2	516.2	4.5	103.2
1000	1063.3	4.8	106.3	1025.0	2.8	102.5
5000	5076.6	0.4	101.5	4958.4	0.6	99.2
10000	10651.2	3.7	106.5	10557.8	3.6	105.6

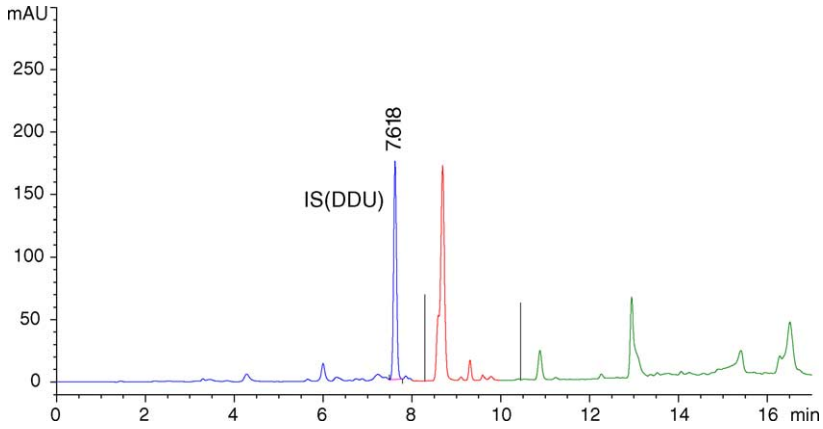

Fig. 2. Chromatogram of the assay for blank plasma and the internal standard (IS) DDU. Vertical lines indicate elution time for FTC and TNF.

acyclovir 6.1 min, acetylsalisalic acid 14.8 min, zidovudine 11.6 min, stavudine 8.7 min, abacavir 14.3 min, lamivudine 6.8 min, didanosine 8.1 min, zalcitabine 6.7 min, nevirapine 15.0 min and indinavir 14.4 min.

3.3. The limit of quantification

The low limit of quantification for both analytes was 10 ng/mL. The upper limit of quantification was 10,000 ng/

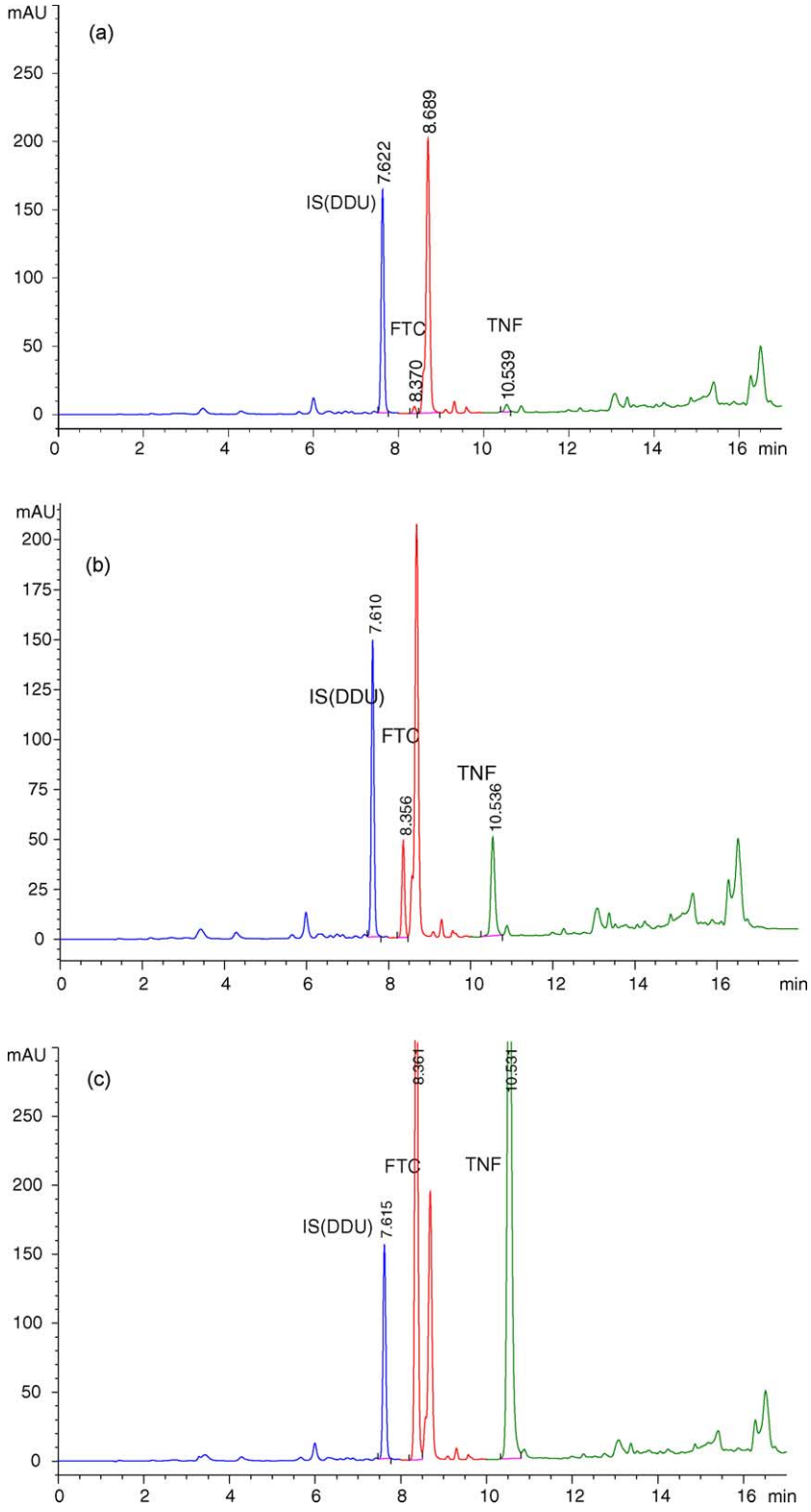

Fig. 3. (a) Chromatogram of FTC and TNF at: (a) 75 ng/mL; (b) 750 ng/mL; (c) 7500 ng/mL and the internal standard (IS) DDU.

Table 4
Summary of accuracy and precision (%) during method validation at low, medium and high concentrations

Analyte	Concentration (ng/mL)	Accuracy (%)	Precision (%)	
			Within-day C.V. (%), $N = 3$	Between-day C.V. (%), $N = 9$
FTC	75	98	1.7	1.9
	750	106	2.2	1.1
	7500	105	3.7	2.8
TNF	75	102	3.2	5.2
	750	97	2.7	1.6
	7500	103	3.7	0.9

mL. Chromatograms for the QC samples 75, 750 and 7500 ng/mL are shown in Fig. 3a–c, respectively.

3.4. Accuracy, precision

Results from the validation of this method in human plasma were within acceptable ranges. The accuracy of all analytes ranged from 97 to 106% with a mean of 102%. Precision and accuracy throughout the concentration range of the control samples for the three levels of validation are presented in Table 4. The mean intra-day precision was <3.7%. The mean inter-day precision for both analytes was 5.2%, with mean coefficients of variation ranging from 0.9 to 5.2%.

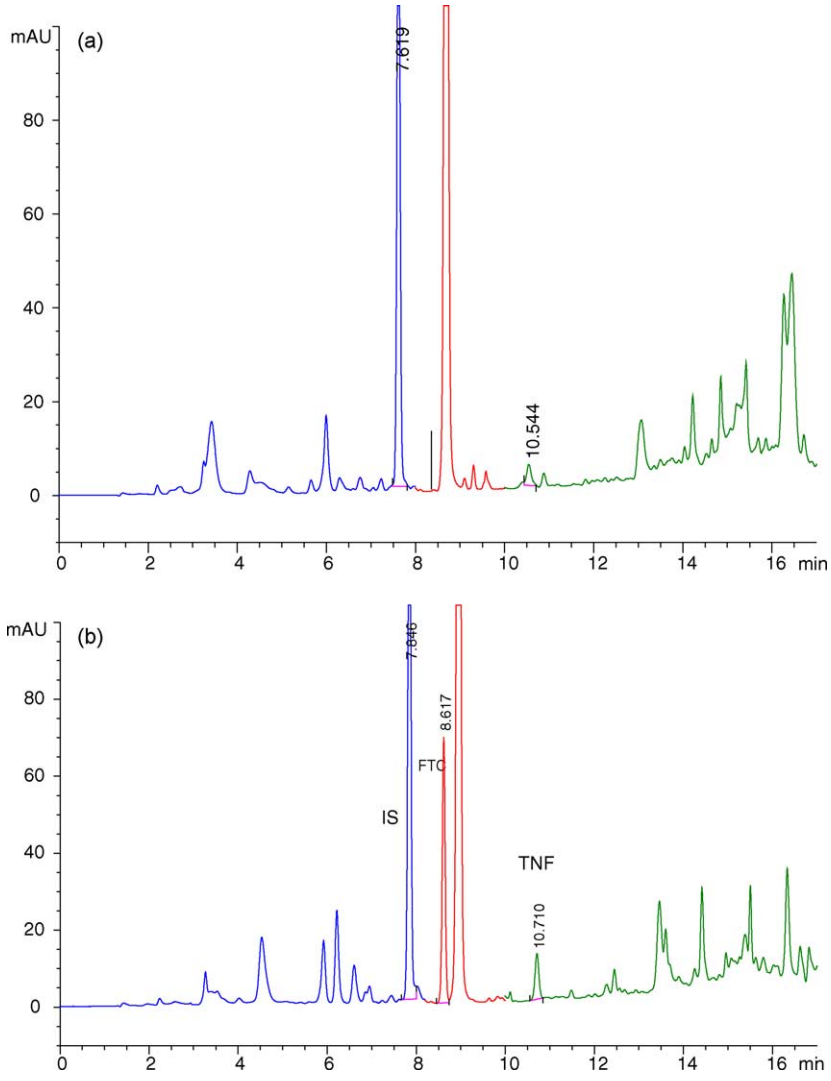

Fig. 4. (a) Chromatogram of a patient sample taken 12 h after administration of 300 mg dose of TDF. The sample contained 77.2 ng/mL of TNF. (b) Chromatogram of a patient sample taken 4 h after administration of 200 mg dose of FTC and 300 mg dose of TDF. The sample contained 1053.6 ng/mL of FTC and 165.0 ng/mL of TNF.

3.5. Extraction recovery

The extraction efficiencies for FTC and TNF using solid phase extraction columns were calculated using the ratio of the analyte concentration in blood plasma to the identical concentrations of the analytes prepared in mobile phase without extraction. The analyte recoveries were 98.6% for TNF and 100% for FTC.

3.6. Stability

FTC, TNF, and DDU were stable under all conditions tested. All assayed concentrations were within the acceptance criteria of ±15% deviation from the nominal concentration.

3.7. Analysis of patient samples

The applicability of described method examined by analyzing patient plasma samples collected from HIV-infected patients. Patients were using tenofovir once a day or tenofovir plus emtricitabine once a day. For patient samples and QC calculations, calibration curves were obtained by weighted ($1/\text{concentration}^2$) least squares linear regression analysis. Fig. 4a illustrates a chromatogram from one patient sample taken 12 h after the tenofovir Viread® dose and Fig. 4b one patient sample taken 4 h after the Truvada™ dose.

4. Discussion

The combination of TNF and FTC is commonly used as part of an antiretroviral regimen in both treatment-naïve and treatment experienced patients due to its potency, limited pill burden, and once daily dosing. This paper describes the development and validation of a bioanalytical method for the simultaneous quantitative determination of these two compounds. To date, no analytical method that simultaneously measures these two compounds has been developed. The challenges in creating this methodology were extracting TNF with an acceptable level of recovery (extraction efficiency >90%), and eliminating any plasma interference for the analytes of interest.

Due to the high polarity of TNF, liquid-liquid extraction was not a useful technique. Recently, in a method published by Sentence et al. [11], TNF was extracted from 1.0 mL of blood plasma on a 500 mg C-18 solid phase matrix. In order to achieve a TNF extraction efficiency of 63.7%, Sentence et al. used 1.0 mL of 0.6% trifluoroacetic acid (TFA) to lower the pH to ∼1.5. However, when we tried this method, the SPE columns repeatedly clogged, and the low extraction efficiency did not make the method easily reproducible. Additionally, this method is too cumbersome to be suitable for high throughput, involving several chloroform clean-up steps.

In our extraction procedure, we achieve a high level of extraction efficiency not only for TNF, but also for FTC. This makes the assay highly reproducible and allows us to lower the limit of quantification. Furthermore, this extraction procedure uses less solvent, which decreases both the length of time to perform the assay and the overall cost of the assay. We found the parameter most responsible for our high TNF recovery was the ionic strength of the buffer: as the buffer concentration increased, recovery increased.

The chromatographic conditions of this method were optimized for a short 20 min run time, which includes 2 min for re-equilibration. The gradient conditions are simple and applicable to any binary pump system. This gradient is excellent in cleaning the column of all endogenous plasma components from each injection.

The analytical column chosen for this method was based on its power to retain the two analytes of interest. The Atlantis column provided sharper peaks than any of the other five columns testes, which increased the assay sensitivity. Furthermore, we achieved good resolution of our three analyte peaks from all other endogenous plasma interferences and possible concomitant antiretroviral drugs.

Detecting FTC at 280 nm increased this method's sensitivity approximately 30% compared to using 259 nm. The 280 nm wavelength was also recently used by Cass et al. [12] in determining the enantiomer purity of FTC. DDU was monitored at 262 nm, a more specific wavelength for DDU which reduced the potential for matrix interference.

5. Conclusion

Our validated method is suitable for the simultaneous quantification of FTC and TNF. The rugged, efficient solid phase extraction method provides exceptional sample clean up and high recoveries using 200 μL of plasma. Due to the popularity of the combination of FTC and TNF in treating HIV-infected patients, this assay will be most useful. The high extraction efficiency, low limit of quantification, and wide linear dynamic range make this a suitable method for use in clinical trials and therapeutic drug monitoring of HIV-infected patients.

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