

Development and Validation of High Performance Liquid Chromatography Tandem Mass Spectrometry (HPLC-MS/MS) Method for Determination of Tenofovir in Small Volumes of Human Plasma

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Abstract

Determination of plasma tenofovir (TFV) concentrations in small plasma volumes and in a short time period is most desirable in a clinical setting. The HPLC-MS/MS method was developed and validated for the determination of TFV. Plasma sample volumes of 10 μ L were extracted by protein precipitation. Cimetidine, used as internal standard (ISTD) and TFV were separated on a C18 (Phenomenex Kinetex™ 30 mm \times 2.1 mm, 2.6 μ m) reversed phase column with a pre-column. The gradient mobile phase consisted of 10 mmol/L ammonium acetate in water and acetonitrile/methanol (50:50, v/v). TFV and ISTD retention times were 0.27 and 0.90 minutes, respectively, with a run time of 2 minutes. Transition of the parent to the product ion of TFV (m/z 288.072 \rightarrow 176.038) and ISTD (m/z 253.13 \rightarrow 158.987) were monitored in positive ionization mode. Calibration curves were linear (average r^2 , 0.9958) over a TFV concentration range of 12.5-600 ng/mL. The mean recovery was 96.9%. Accuracy, inter and intra-assay of three quality controls and lower limit of quantification (12.5 ng/mL) were within the acceptable limit of <15% relative standard error. TFV was stable at studied conditions and neither matrix effect nor significant carry-over was observed. A distinct, reliable and robust method for determination of TFV in a small volume (10 μ L) of human plasma was developed, validated and incorporated to determine the plasma TFV levels in 30 HIV-infected women on antiretroviral treatment.

Keywords: Tenofovir plasma levels; Small plasma volumes; Clinical application; Antiretroviral treatment

Introduction

Tenofovir disoproxil fumarate (TDF) is a prodrug of tenofovir diphosphate (TFV), a nucleotide analogue of adenosine monophosphate with potent activity against human immunodeficiency virus (HIV-1 and HIV-2) [1,2]. The absorption of TDF following an oral dose of 300 mg/day is described by first-order kinetics with an absorption rate constant of 1.03 h⁻¹. Its oral bioavailability is approximately 25% in fasting state and is enhanced by a high-fat meal [3,4]. The volume of distribution of TFV in adults is 0.813 L/kg and is best described by a two-compartment model. The concentrations of TFV in the cerebrospinal fluid are only 5% of plasma concentrations [3,5]. It is minimally bound to human plasma or serum proteins *in vitro*. The drug is not a substrate of the cytochrome P-450 enzyme system but is mainly excreted unchanged in urine. After intracellular uptake, TFV inhibits the activity of HIV-1 reverse transcriptase by competing with the natural substrate deoxyadenosine 5'-triphosphate for incorporation into DNA [1,2]. A dose of 300 mg/day TDF is used in combination with other antiretroviral drugs to manage HIV-1 infection in adults with proven improved efficacy [6]. The maximum plasma TFV concentration ranges from 195.6-306.3 ng/mL and the trough concentrations from 33.5-62.1 ng/mL [7]. Elevated plasma trough TFV concentration (>90 ng/mL) is associated with renal impairment and bone mineral loss [8,9] and this suggests the need of monitoring plasma TFV as a way of managing adverse effects experienced by patients. The monitoring of TFV in plasma, not only does it confirm patients' compliance but also ensure acceptable plasma drug exposure.

To this effect, several LC-MS/MS analytical methods to determine TFV as a single drug or in combination with other antiretroviral drugs in plasma have been published [10-17,20,21,24-26]. The analytical methods differ with the steps involved in sample preparation, the cost of the method and the duration or run time of the assay.

The aim of the present study was to develop and validate HPLC-MS/MS method for the determination of TFV in human plasma.

Sample preparation was improved by using smaller volumes of plasma and run time was reduced while maintaining the desired sensitivity and stability for clinical application.

Materials and Methods

Chemicals and reagents

Tenofovir monohydrate USP (C₉H₁₄N₅O₄P.H₂O) reference standard (Figure 1) was obtained from Industrial Analytical (Pty) Ltd, South Africa (CAS Number 206184-49-8). Cimetidine (C₁₀H₁₆N₆S) as internal standard (ISTD) (Figure 1) was sourced from Sigma-Aldrich, USA (CAS Number 51481-61-9). HPLC water was obtained from a Milli-Q water purification system (Millipore SAS 67120 Moisheim, France). Ammonium acetate, LC/MS/MS hyper grade acetonitrile and methanol were sourced from Merck (Pty) Ltd, South Africa. Drug-free plasma containing sodium citrate anticoagulant was kindly donated from healthy volunteers and stored at -23°C.

Instrumentation

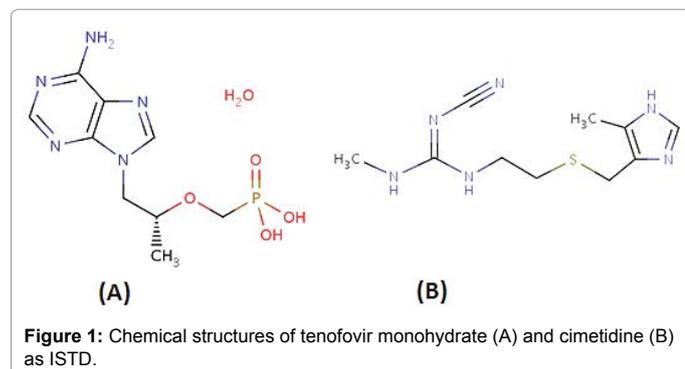
An Agilent 1290 Infinity HPLC System consisting of binary pump with two identical high pressure (1200 bar) pumps; a two-channel solvent degasser and four-channel inlet solvent selection valve and

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CTC PAL HTx-xt auto sampler with a 20 μL sample loop were used for analysis. Mass spectrometric detection was performed on an AB SCIEX 4000 QTRAP[®] MS/MS system. Quantitation was performed in multiple reactions monitoring mode (MRM) and Analyst[®] 1.6 software was used to manage and execute analysis. MultiQuant 3.0 was used to quantify results and create reports.

Chromatographic conditions

Chromatographic separation was carried out on C18 (Phenomenex Kinetex[™] 30 mm \times 2.1 mm, 2.6 μm) reversed phase column with a pre-column (UHPLC C18, 2.1 mm ID). Column and auto sampler tray temperatures were maintained at 20°C and 19°C, respectively. A gradient was used to elute TFV and ISTD as shown in Table 1. The total chromatographic run time was 2 minutes. Mobile phase A consisted of 10 mmol/L ammonium acetate in water and phase B consisted of methanol: acetonitrile (50:50, v/v).

Mass spectrometric conditions

Optimization of the signal was performed by constant injection of high concentration of TFV and ISTD. The transition of the parent to product ion was studied with the use of a turbo spray ionization source, operating in the positive ionization mode. The transition of parent \rightarrow production (m/z) for TFV was 288.072 \rightarrow 176.038. Dwell time (msec), declustering potential (DP), collision energy (CE) and collision exit potential (CXP) were optimized at 200 msec, 106, 35 and 8 volts, respectively. The parent \rightarrow product ion (m/z) for ISTD was 253.13 \rightarrow 158.987. The dwell time was 200 msec. DP, CE and CXP was 61, 21 and 12 volts, respectively.

Preparation of stock and internal standard solutions

The stock solution was prepared by dissolving 8.5 mg of TFV reference standard in 50 mL of Milli-Q water to a final concentration of 170 $\mu\text{g/mL}$. Stock quality control (QC) solution was prepared by dissolving 9.1 mg of TFV reference standard in 50 mL of Milli-Q water to a concentration of 182 $\mu\text{g/mL}$. This solution was then further diluted with Milli-Q water to a final QC concentration of 170 $\mu\text{g/mL}$. 10.08 mg of ISTD was dissolved in 25 mL of methanol: water (40:60, v/v) to a final concentration of 403.2 $\mu\text{g/mL}$. This solution was diluted to 10 ng/ml with Milli-Q water on the day of analysis. All solutions were clearly labeled and stored in Eppendorf tubes at -23°C.

Preparation of calibration standards and quality control samples

Calibration standards were prepared by spiking 4235 μL of drug-free plasma with 15 μL of TFV stock solution which was then diluted with drug-free plasma. The nine calibration standard concentrations were 12.5, 25, 50, 100, 200, 300, 400, 500 and 600 ng/mL.

QC samples were prepared at 30, 150 and 450 ng/mL for low quality control (LQC), medium quality control (MQC) and high quality control (HQC), respectively by spiking 4235 μL of drug-free plasma with 15 μL of stock QC solution and then diluting appropriately with drug-free plasma. Calibration standards and quality control samples were stored in Eppendorf tubes at -23°C in 100 μL aliquots. Prior to analysis, all frozen samples from participants, calibration standards and quality control were left to thaw unassisted until equilibrated with room temperature.

Sample pre-treatment

Nine calibration standards and QC plasma samples in Eppendorf tubes were heated in a water bath at 57°C for 30 minutes and were left to equilibrate to room temperature. 25 μL (10 ng/mL) of ISTD stock solution was added to 10 μL of the plasma sample. To precipitate plasma proteins, 100 μL of methanol: acetonitrile (50:50 v/v) was added to the sample. The sample was then vortex mixed for 10 seconds, left in a freezer at -23°C for 10 minutes, vortex mixed for a second time and then centrifuged for 4 minutes at 6000 rpm. 50 μL of the supernatant was transferred into a clean auto sampler vial, 120 μL of Milli-Q water was added and vortex mixed. 20 μL of this solution was injected onto the column for analysis.

The same pre-treatment procedure was performed for all human plasma samples. Heating of plasma samples in a water bath ensured inactivation of HIV.

Method validation

The analytical method was validated for linearity, sensitivity, accuracy, precision, selectivity, carry-over, recovery, matrix effect and stability according to the European Medicines Agency and US Food and Drug Administration guidelines for bio analytical method validation [18,19]. Each analytical validation run comprised nine spiked standards, QC samples, zero blank (with ISTD, without TFV) and blank (without ISTD or TFV) samples.

Clinical application

This method was developed to determine plasma TFV concentrations of 30 HIV-1-infected women in a pilot cross-sectional sub-study within the Prospective Urban and Rural Epidemiology-South Africa study (PURE-SA). Women were all on the first line antiretroviral regimen, taking a TDF dose of 300 mg *nocte*. This investigation was approved by Human Research Ethics Committee (HREC) of North-West University in Potchefstroom (NWU-00016-10-A1 on 12/04/2013). Participants signed the informed consent to have their blood drawn for analysis. Blood was collected in tubes containing sodium citrate anticoagulant between 11-14 hours post TDF dose and plasma was stored at -80°C until analyzed.

Results

Method development

TFV and ISTD were successfully separated on C18 (Phenomenex Kinetex[™] 30 mm \times 2.1 mm, 2.6 μm) reversed phase column with a

Run time (min)	Flow rate ($\mu\text{L}/\text{min}$)	A (%)	B (%)
0.00	250	95.0	5.0
0.10	250	95.0	5.0
0.60	250	5.0	95.0
1.30	250	5.0	95.0
1.40	250	90.0	10.0
2.00	250	90.0	10.0

Table 1: Chromatography gradient elution.

pre-column. Retention times for TFV and ISTD were 0.27 and 0.90 minutes, respectively. Representative chromatographic profiles of the upper limit of quantification (600 ng/mL) and zero blank samples are presented in Figure 2.

In order to achieve optimal separation of TFV and ISTD, sharp peak shape and shortest possible run-time was used as criteria and we performed chromatographic analysis via gradient elution. Ultra C18 column, 20% carbon load (100 Å pores, 100 mm × 2.1 mm, 5 µm, Restek, Bellefonte) and C18 (Phenomenex Kinetex™ 30 mm × 2.1 mm, 2.6 µm) columns were employed in order to obtain the best separation efficiency. The C18 (Phenomenex Kinetex™ 30 mm × 2.1 mm, 2.6 µm) column provided better peak shape and shorter run-time. The mobile phases tested to provide the best resolution were: acetonitrile/ ammonium (150 mM) formate in water and ammonium acetate (10 mmol/L) in water/ methanol: acetonitrile (50:50, v/v). The latter mobile phase was found more suitable for eluting TFV and ISTD with better resolution at a flow rate of 250 µL/min.

Linearity and sensitivity

Linearity and sensitivity were determined by a nine-point TFV calibration standard curve on three separate days. The curve was constructed (Y-axis) using peak area ratios of chromatograms (TFV peak area/ISTD peak area) versus (X-axis) nominal concentration of TFV over the concentration range of 12.5-600 ng/mL. A linear regression weighting factor of 1/x best described the linearity of the calibration curve with regression coefficient (r^2) ranging from 0.9942-0.9970 with an average of 0.9958. The lower limit of quantification (LLOQ) was 12.5 ng/mL and the signal was more than 5 times the signal of the blank sample (Figure 3) using the FDA/ EMA guidelines [18,19]. Back calculated mean concentrations with percentage relative standard deviation and accuracy are presented in Table 2.

Accuracy and precision

Accuracy and precision for the analyte were determined by analyzing QCs including the lower limit of quantification samples (HQC, MQC, LQC and LLOQ) with the standard calibration curve. Sets of samples were analyzed within a single analytical run (within-run precision) and between different runs (between-run precision) on different days. Table 3 summarizes the accuracy and precision results.

Selectivity and carry-over

Selectivity was demonstrated by analyzing six drug-free plasma samples obtained from six random individual donors in sodium citrate tubes spiked at LLOQ. Negligible interference in one donor's plasma was observed at the TFV retention time (0.27 minutes); blank and TFV peak area counts were 96.909 and 988.236 (9.8%), respectively [18,19]. Chromatograms of the blank and spiked samples from five donors showed no endogenous peaks at TFV retention time or internal standard retention time (0.90 minutes). Hence, the method was selective and unaffected by interference from endogenous components in the matrix.

Carry-over was assessed by injecting blank samples after the upper limit of quantification (600 ng/mL) of the calibration standard in each analytical run. TFV carry-over for three runs was negligible (0.00, 0.00 and 0.286 ng/mL) with average of 0.095 ng/mL (0.76% of LLOQ). This was within the acceptable range of not greater than 20% of the LLOQ. There was no observed carry-over in the ISTD.

Recovery

Extraction recovery of TFV from sodium citrate plasma was determined at 4 concentrations (HQC, MQC, LQC and LLOQ) in triplicate by comparing analyte-internal standard area ratio of samples spiked in plasma with those of spiked in Milli-Q water. The mean recovery of TFV at HQC, MQC, LQC and LLOQ was 99.4 ± 11.0 ,

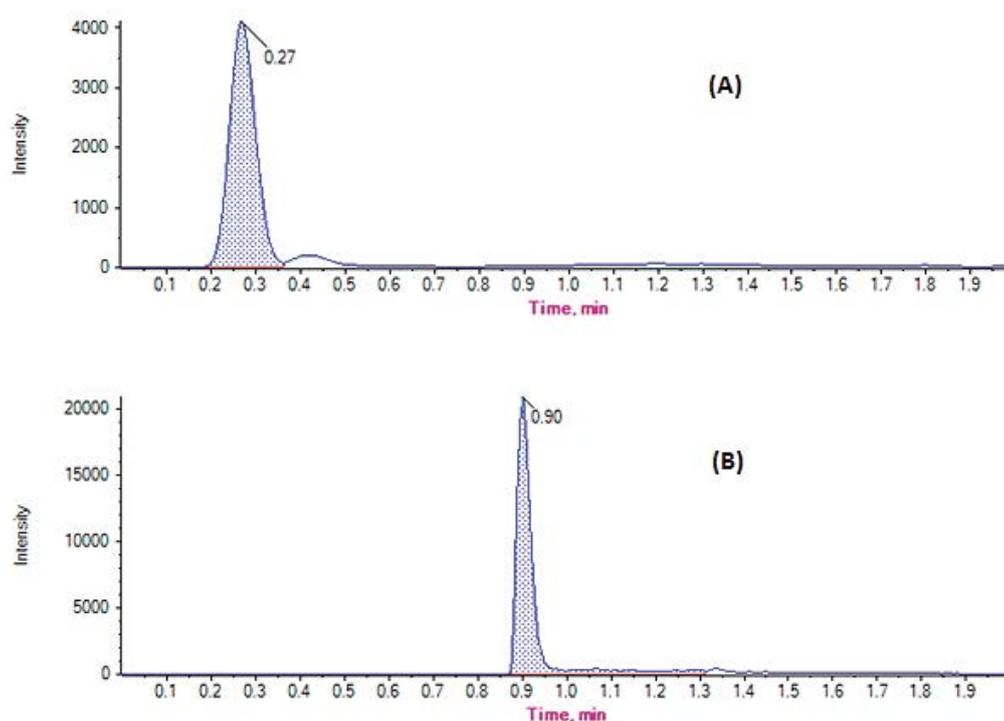


Figure 2: Representative chromatograms of TFV (A) 600 ng/mL sample and ISTD (B) sample.

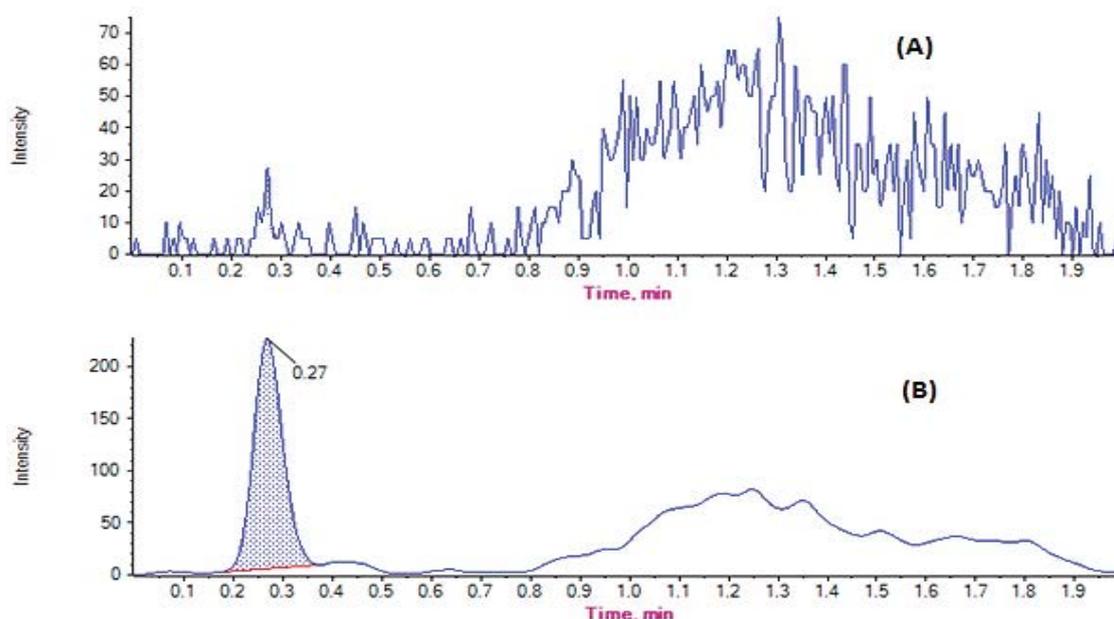


Figure 3: Representative chromatograms of blank sample (A) and TFV (B) spiked at 12.5 ng/mL (LLOQ).

Nominal Conc. (ng/mL)	^a Calculated Mean Conc. (ng/mL)	^b %RSD	Accuracy (%)
12.5	13.2	2.0	105.5
25	25.8	4.7	103.4
50	47.4	6.1	94.8
100	94.9	5.8	94.9
200	199.7	13.3	99.9
300	308.0	3.6	102.7
400	388.5	2.9	97.1
500	503.1	6.2	100.6
600	606.9	6.2	101.1

^aMean of 3 injections on three separate days; ^bPercent relative standard deviation.

Table 2: Summary of standard calibration curve concentrations.

Nominal conc. (ng/mL)	Calculated conc. (ng/mL)	Accuracy (%)	Precision (%RSD)
Within-run assay			
12.5 ^a	12.2	98.2	3.6
30 ^b	30.2	100.6	5.9
150 ^b	146.4	97.6	7.9
450 ^b	453.1	100.7	11.2
Between-run assay			
12.5 ^c	11.5	92.0	3.6
30 ^d	28.0	93.2	8.8
150 ^d	139.0	92.5	14.9
450 ^d	399.2	88.7	5.9

^an=5, ^bn=6, ^cn=3, ^dn=4.

Table 3: Within-run (intra) and between-run (inter) assay performance for TFV in plasma.

100.1 ± 2.3, 92.8 ± 3.7 and 95.3 ± 2.0%, respectively. The overall mean extraction recovery (96.9%) was high and acceptable.

Matrix effect

Co-elution of some endogenous compounds from the matrix can affect sensitivity, precision and accuracy of the bio-assay. The matrix

effect was thus determined by assessing variability in instrument response from six lots of plasma of individual donors. Each plasma lot was spiked with TFV at 30, 150 and 450 ng/mL and analyzed in 6 runs. The overall coefficient of variation (CV) for the calculated concentrations at three levels was less than 15% as presented in Table 4. No significant differences were observed in peak areas of the ISTD.

Stability

Stability of TFV was investigated in plasma spiked at LQC, MQC and HQC. Samples were heated in a water bath at 57°C for 30 minutes and analyses were performed in quadruplicate. The coefficient of variation ranged between 2.6-12.8% and accuracy was between 93.3-99.1%. After a second freeze-thaw cycle at -23°C of the same QC plasma samples, analyses were performed in triplicate and accuracy ranged between 90.3-93% with the coefficient of variation between 5.1-10.0%. The mean concentration of freeze-thaw samples compared to heated samples decreased by 8.0, 2.6 and 5.9% for LQC, MQC and HQC, respectively. Hence, TFV was stable after heating in a water bath and after two freeze-thaw cycles.

Clinical application

This validated assay was successfully applied in measuring TFV in plasma samples of 30 HIV-infected women and investigated the association of TFV plasma levels and its toxicity on renal function and bone metabolism. TFV could not be quantified in 5 participant's plasma samples as these levels were below the LLOQ. The mean concentration in 25 participants was found to be 114.0 ± 78.9 ng/mL. The lowest and highest concentration was 17.2 and 434.2 ng/mL, respectively corresponding to mid-dose concentration [9]. The concentrations were all in the range of 12.5-600 ng/mL. The typical chromatograms of high and low TFV plasma concentrations from two participants are presented in Figure 4.

Discussion

In several published analytical methods where TFV was analyzed in the presence of other antiretroviral drugs [11,12,14-16,20-22,23,24] or alone [10,13,17,25,26], fairly large plasma volumes were used:

	LQC (30 ng/mL)		MQC (150 ng/mL)		HQC (450 ng/mL)	
	Calculated conc. (ng/mL)	Accuracy (%)	Calculated conc. (ng/mL)	Accuracy (%)	Calculated conc. (ng/mL)	Accuracy (%)
1	26.64	88.8	149.31	99.5	466.43	103.7
2	33.16	110.5	133	88.7	405	90.0
3	28.9	96.3	130.52	87.0	403	89.6
4	24.1	80.3	117.73	78.5	331	73.6
5	29.65	98.8	146.4	97.6	444.02	98.7
6	28.42	94.7	121.8	81.2	362.2	80.5
Mean		94.9		88.7		89.3
%CV		10.7		9.6		12.5

Table 4: Matrix effect at LQC, MQC and HQC in six lots of plasma.

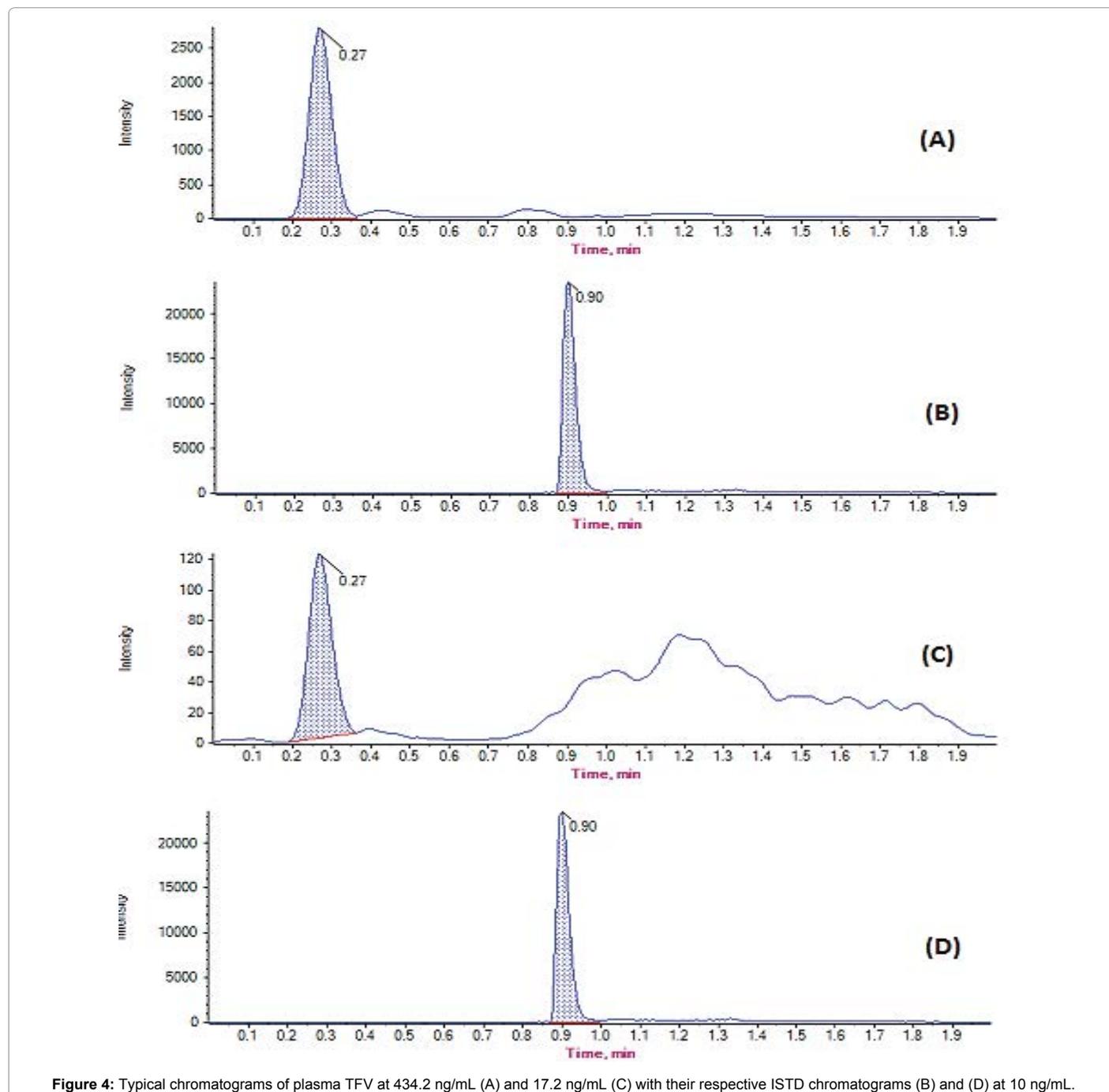


Figure 4: Typical chromatograms of plasma TFV at 434.2 ng/mL (A) and 17.2 ng/mL (C) with their respective ISTD chromatograms (B) and (D) at 10 ng/mL.

volumes between 100-200 μ L and 200-1000 μ L, respectively for protein precipitation or solid phase extraction were documented. This is the first method, to our knowledge, where only 10 μ L of plasma was used for extraction of TFV by protein precipitation, making it preferable and suitable for clinical application. Furthermore, this method is convenient for the emerging interest in therapeutic drug monitoring of TDF in HIV-infected patients [3,8]. A method [13] with a similar run time (1.8 minutes) to this method (2 minutes) was reported in the literature. However, the former required a solid phase extraction procedure which is expensive and more time consuming in sample pre-treatment than the current method.

The method developed is robust, fast, accurate, and sensitive with high extraction recovery and it covered the whole range of expected plasma concentration [9]. A mixture of acetonitrile and methanol (50:50, v/v) made a better precipitating solvent than acetonitrile or methanol alone. Protein precipitation was further enhanced by refrigeration of samples in addition to vortex mixing. Dilution of supernatant with water at neutral pH produced a higher TFV intensity chromatogram compared to acidic or basic water. Similarly, the intensity of TFV chromatograms decreased with decreasing auto sampler temperatures but was optimal at 19°C.

TFV was stable after at least 2 freeze-thaw cycles, which was in agreement with previously published methods [23,24]. Additionally, this method showed that plasma TFV was stable when exposed to a temperature of 57°C in a water bath for 30 minutes.

Conclusion

A simple, fast, reliable and robust HPLC-MS/MS method has been developed and validated for determination of TFV in human plasma. This developed method is suitable for clinical application and was applied for analysis of small volumes (10 μ L) of plasma TFV in HIV-1-infected women with desired sensitivity and accuracy.

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Conflict of Interest

The analytical part of this study was funded by Pharmacen and the PCDDP, North-West University, Potchefstroom Campus, South Africa. The authors declare no conflict of interest.

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