

DEVELOPMENT AND VALIDATION OF AN ACCURATE AND PRECISE LC-MS/MS METHOD FOR DETERMINATION OF TENOFOVIR AND EMTRICITABINE IN HUMAN PLASMA

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ABSTRACT

A simple, accurate and precise LC-MS/MS analytical method was developed and validated for the simultaneous assay of tenofovir and emtricitabine which are used for the treatment of HIV-infected patients. Extraction from human plasma was performed on Oasis MCX cartridges. The chromatographic separation was performed with Chromolith Speedrod RP18 column. The total chromatographic run time is 2.8 minutes. Both of the tenofovir and emtricitabine were monitored through positive ESI mode by MRM transitions 288.1>176.2 and 248.1>130.1 respectively. The validated linearity of this method was in the range of 3.030-1512.901ng/mL and 5.050-2521.999ng/mL for tenofovir and emtricitabine respectively. Lower limit of quantitation (LLOQ) for both tenofovir and emtricitabine was achieved up to 3.030ng/mL and 5.050ng/mL respectively with a better signal to noise ratio. The correlation co-efficient (r) value for both the calibration curve was higher than 0.9992 for five precision and accuracy batches.

Keywords: Tenofovir, Emtricitabine, Human plasma, LC-MS/MS, Solid Phase Extraction.

1. INTRODUCTION

Tenofovir disoproxil fumarate (TDF or PMPA¹), ((([[(2R)-1-(6-amino-9H-purin-9-yl) propan-2-yl]oxy] methyl) phosphonic acid) marketed by Gilead Sciences under the trade name Viread, belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (NRTIs), which block reverse transcriptase, a crucial virus enzyme in human immunodeficiency virus 1 (HIV-1) and hepatitis B virus infections².

Tenofovir is indicated in combination with other antiretroviral agents for the treatment of HIV-1 infection in adults and pediatric patients 2 years of age and older². It was found that both tenofovir alone, as well as the tenofovir/emtricitabine combination, significantly decreased the risk of contracting HIV³.

The initial form of tenofovir used in these studies had limited potential for widespread use because it was not absorbed when administered orally. A medicinal chemistry team at Gilead developed a modified version of tenofovir, tenofovir disoproxil fumarate⁴. This version of tenofovir, which is the only version used clinically today, is often referred to simply as "tenofovir". In this version of the drug, the two negative charges of the tenofovir phosphonic acid group are masked, thus enhancing oral absorption.

Since these anti-viral drugs do not interact pharmacokinetically, and have moderate elimination half-lives in plasma, a once-daily combination therapy is possible⁵. Furthermore, the combination of TDF and FTC can result in a greater HIV RNA suppression than either drug alone⁶.

Emtricitabine (FTC) (4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one), with trade name Emtriva (formerly Coviracil), is a nucleoside reverse transcriptase inhibitor (NRTI) for the treatment of HIV infection in adults and children⁷.

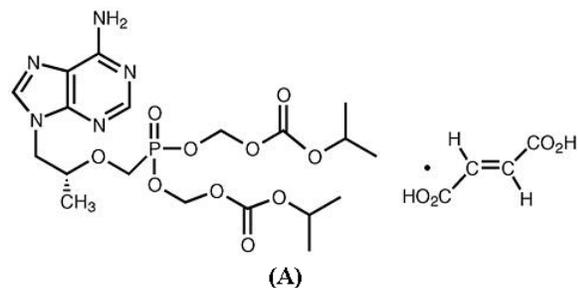


Fig. 1: illustrates chemical structure tenofovir disoproxil fumarate (A) an Emtricitabine (B)

Tenofovir/emtricitabine, trademark Truvada by Gilead Sciences, is a fixed dose combination of two antiretroviral drugs used for the treatment of HIV. It consists of 300 milligrams of tenofovir disoproxil fumarate (of which 245mg tenofovir) and 200 milligrams of emtricitabine. Emtricitabine/tenofovir DF 200 mg/300 mg once daily was an effective backbone for boosted protease inhibitor (PI) based regimens in the initial treatment of HIV-1 infection^{7,8}.

Different methods have been reported in the literature for monitoring plasma levels of tenofovir and emtricitabine individually. Some other techniques used in individual analysis of tenofovir from plasma include HPLC with mass spectrometric^{9, 10}, spectrofluorimetric¹¹, UV and single mass spectrometric¹² and simple UV detection¹³.

Takahashi et al. also report a conventional LC-MS method for quantifying tenofovir individually from human plasma in the concentration range of 0.019–1.567 μ g/ml¹⁴. Emtricitabine has been quantified in presence of other antiretroviral drugs, by Notari et al.¹⁵ and Rebiere et al.¹⁶. Sparidans et al. have quantified emtricitabine individually in human plasma using a validated LC-MS/MS method with a detection limit of up to 5 ng/ml¹⁷.

This paper expresses a marvelous method for the quantification of Tenofovir and Emtricitabine in a human plasma sample, using an optimized solid phase extraction (SPE) procedure using HPLC and triple quad MS/MS detector. This method significantly increases high sample throughput with reproducible and accurate results.

2. EXPERIMENTAL

2.1. Reagents & chemicals

Working standards of Tenofovir (99.82%) and Emtricitabine (99.78%) were obtained from Vivan Life Sciences Pvt. Ltd. (Mumbai, India). Working

standards of clonazepam (99.4%) was obtained from Clearsynth Pvt. Ltd. (Mumbai, India). HPLC grade methanol and formic acid, ammonia were procured from Merck Specialties Pvt. Ltd. (Mumbai, India). Ammonium acetate of purity $\geq 99\%$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetic acid was purchased from Spectrochem (Mumbai, India). Oasis-MCX extraction cartridges were occupied from Waters (Bangalore, India). Water used in the entire analysis was collected from Milli-Q water purification system from Millipore (Bangalore, India). Blank human plasma with K_3EDTA as anticoagulant was obtained from Prathama Blood Centre (Ahmedabad, India) and was stored at $-20^\circ C$ until use.

2.2. Instruments & software

The LC-MS/MS instrument used was a UFLC system (Shimadzu, Japan), with binary prominence pump model LC-20AD with DGU-20A3 degasser, SIL-HTc auto-sampler, CTO-10ASVp column oven and triple quad API 4000 mass detector with ESI interface (Applied Biosystems, MDS Sciex, Canada). Analyst software (version 1.6.2, AB Sciex, Canada) was used for acquiring the chromatographic data.

Peak response area ratio of the analyte to IS was used for construction of the calibration curve. A weighting factor of $1/x^2$ using linear regression was used for curve fitting. The same regression equation was used to back-calculate the quality control samples and results were compared with the theoretical concentration to obtain the accuracy.

2.3. Calibration standard and quality control sample preparation

Stock solution of tenofovir and emtricitabine were prepared by dissolving the precisely individually weighed compounds in water: methanol (75:25v/v) obtaining a final concentration of about 1.0mg/mL. This tenofovir and emtricitabine stock solutions were serially diluted with methanol: water (50:50v/v) to make a series of standard curve working solutions at 75645.033, 33662.040, 16831.020, 7573.959, 3786.979, 1514.792, 302.958, 151.479ng/mL for tenofovir and 126099.968, 56114.486, 28057.243, 12625.759, 6312.880, 2525.152, 505.030, 252.515ng/mL for emtricitabine.

Again tenofovir and emtricitabine stock solutions were serially serially diluted with methanol: water (50:50v/v) to make a series of quality control working solutions at 67240.030, 3765.442,

442.816, 154.986ng/mL for tenofovir and 112088.861, 6276.976, 738.172, 258.360ng/mL for emtricitabine as high, mid, low and LLOQ level respectively.

An eight point standard curve was prepared by spiking the previously screened blank plasma with appropriate amount of respective tenofovir and emtricitabine working standard solutions. The calibration curve ranged from 3.030-1512.901ng/ml and 5.050-2521.999ng/ml for tenofovir and emtricitabine respectively. Aliquots of 400 μ L were stored at $-20^\circ C$ prior to use. All the working standards, stock and working solutions were stored at refrigerator temperature $5^\circ C$.

1mg/mL stock solution of clonazepam internal standard (IS) was prepared in methanol and further diluted with methanol to obtain the working solution of IS of a concentration 100ng/mL.

2.4. Extraction procedure

To 300 μ L aliquoted human plasma, 50 μ L of IS (100ng/mL of Clonazepam in methanol) was added. Then 250 μ L of 10%v/v formic acid was added and mixed properly. Samples were centrifuged at 4000 RPM for 5 minutes at $5^\circ C$. Oasis-MCX, 30mg/1mL extraction cartridges pre-conditioned with 1.0mL methanol followed by 1.0mL of 1%v/v formic acid were used for extraction of analytes and IS. Centrifuged samples were loaded to the pre-conditioned cartridges. Then cartridges were washed with 1.0mL of 1%v/v formic acid followed by 1.0mL of methanol and dried completely by applying full nitrogen pressure. Analytes and IS were eluted with 600 μ L elution solution (10% ammonia (v/v): methanol (15:85)). Eluent was evaporated under nitrogen stream at $45^\circ C$. Dried samples were reconstituted with 400 μ L mobile phase and 10 μ L of the sample was injected to the column.

2.5. Chromatographic conditions and LC-MS/MS parameters

Chromatographic separation was performed at $50^\circ C$ using a column oven, on Chromolith Speedrod RP18 column (50mm \times 4.6mm i.d.) (Merck, India) Chromatographic run was performed with mobile phase A (Acetonitrile) and mobile phase B (20mM ammonium acetate, pH 3.5) with a ratio of (25:75v/v) & 750 μ L/mL flow rate. Throughout the analysis, the samples were maintained in auto-sampler at a $5^\circ C$ temperature. Nitrogen was generated by peak scientific (NM20Z) from compressed air. The analyte(s) and IS were eluted within a run time of 2.8 minutes.

For MS/MS, ESI with positive MRM was selected for ionization. Tenofovir & Emtricitabine were monitored by MRM transitions 288.1>176.2 and 248.1>130.1 respectively. IS was monitored by MRM transition 316.1>270.1. De-clustering Potential (DP) & Entrance Potential (EP) for Tenofovir, Emtricitabine and Clonazepam were 60.0V & 10.0V respectively. Collision Energy (CE) for Tenofovir, Emtricitabine and Clonazepam were 20.0V, 38.0V & 38.0V respectively. Collision Cell Exit Potential (CXP) for Tenofovir, Emtricitabine and Clonazepam were 12.0V, 16.0V & 14.0V respectively. Curtain Gas, Nebulizer Gas, Heater Gas, Capillary voltage, Temperature, Collision Gas for all the MRM transitions were maintained as 40psi, 50psi, 55psi, 5500V, 550°C and 6psi. Dwell time for all the MRM transitions was 200msec.

3. DEVELOPMENT

Preliminary experiments were conducted to achieve the response and better chromatography to precisely quantitate the desired LOQ level. The choice of analytical column and mobile phase composition are playing a vital role in build-up and optimization of chromatography for simultaneous analysis. Various C18 columns with different length and internal diameters with different size of particle size were tried to achieve maximum peak separation from the suppression zone.

Extraction method was finalized after various experiments by changing the extraction buffers, extraction cartridges selection, cartridge conditioning solutions, washing solutions and elution solutions etc. After selection of best mobile phase, MS/MS parameters for both the analytes were tuned along with the mobile phase to optimize the detector response. The injection volume of sample was set so as to maximize the signal to noise ratio of LLOQ sample with adequate chromatography.

4. METHOD VALIDATION

The method validation procedure was based on the recommendations published online by the FDA [18].

4.1. System suitability & carryover

System suitability was performed by six consecutive runs of MQC sample. Carryover of the chromatographic system was evaluated in each experiment throughout the validation by injecting blank sample before and after highest concentration sample.

4.2. Specificity and selectivity

Interference from endogenous compounds was investigated by analysis of ten different blank plasma lots including haemolysed, lipidemic and heparinized plasma. The extracted blank and LLOQ plasma samples from each plasma lot were injected into the chromatographic system. Drug-drug reactivity effect was also examined for the enhancement or suppression in any of analyte.

4.3. Limits of quantification and limit of detection

The lower limit of quantification (LLOQ) was defined as the lowest concentration of calibration curve for which the standard deviation (% CV) and the % bias from the nominal concentration were less than 20%. The upper limit of quantification (ULOQ) was defined as the highest concentration of calibration curve for which both the % CV and the % bias from the nominal concentration were less than 15%. The detection limit was defined as a signal-to-noise ratio of 3:1.

4.4. Linearity, accuracy and precision

Peak area of analyte(s) and IS ratios for the calibration standards were proportional to the concentration of analyte(s) in plasma over the range tested. The calibration curves were fitted by linear regression with weighted least-squares. The linear regression data for the calibration curves (n=5) for the method was determined throughout the validation. Intra-day and inter-day accuracy and precision were determined by assaying extracted samples (n=6) at four different concentrations (QCs) for each drug for 5 analytical batches.

4.5. Dilution integrity

This was especially for the subject samples which are found to contain concentrations exceeding the ULOQ. So, it was ascertained to confirm whether the dilution of these samples before analysis can modify the accuracy of the drug determination.

4.6. Matrix effect

Matrix effect was performed using six different blank plasma lots at LQC and HQC concentration in triplicate by calculating the % accuracy and precision (% CV) which should be in the range of 85-115% and ≤15% respectively.

4.7. Matrix factor

The estimation of the matrix factor was quantified in 6 different plasma lots at low, mid and high

levels of QCs as the peak-area response of analyte(s) added to blank plasma extracts, divided by the peak-area response of standard solution of analyte(s). A value above or below 1 for the matrix factor indicates an ionization enhancement or suppression, respectively. The results were showing that co-eluting plasma matrix components do not appear to affect significantly the ionization of tenofovir and emtricitabine as well as IS.

4.8. Recovery

Extraction efficiency was established from the peak area of the extracted QC samples at low, medium and high levels and expressed as a percentage of peak area of equivalent unextracted samples prepared in aqueous solution.

4.9. Ruggedness

Ruggedness of the method was evaluated using re-injection of one precision and accuracy batch samples using different columns of the same make and also with different analysts.

4.10. Stability

Six replicates of plasma extracted samples at each of the low and high QC concentrations were analyzed for each stability experiment against the freshly prepared calibration standards. All the

stability samples were considered stable if the % accuracy and % CV were found within the range 85-115% and $\leq 15\%$ respectively.

Bench-top stability was assessed by keeping spiked plasma samples for 12 hours in a deep freezer at -20°C and then kept at ambient temperature (20°C) and then extracted and analyzed. Dry extract stability was assessed by keeping the dried samples after extraction in deep freezer at (-20°C) and then reconstituted and analyzed. Freeze and thaw stability was assessed after five freeze-thaw cycles (at -20°C & -70°C) and then plasma samples were extracted and analyzed. Extracted plasma samples were tested for wet extract stability, which included auto-sampler stability and re-injection reproducibility. In auto-sampler stability, samples were analyzed after being stored in the auto-sampler at 5°C . In re-injection reproducibility, the re-injections of previously injected samples were carried out. Long-term stability in plasma was established after the storage of samples in a deep freezer (at -20°C & -70°C).

Stock solutions of tenofovir, emtricitabine and clonazepam and working solutions of tenofovir and emtricitabine were assessed for short term stability at ambient temperature 20°C and for long term stability in a refrigerator at 5°C .

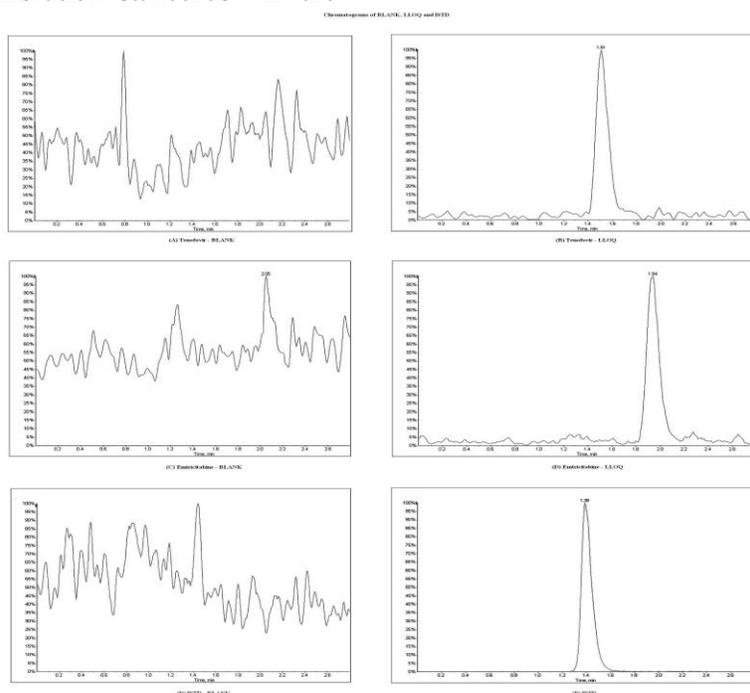


Fig. 2: Chromatograms of extracted Blank of tenofovir (A), emtricitabine (B) and clonazepam (C) and extracted LLOQ of tenofovir (D), emtricitabine (E) and clonazepam (F).

5. RESULTS

5.1. System suitability and carryover

The precision (% CV) of system suitability test was observed within 0.4% for the retention time and 1.9% for the area ratio of analyte(s)/IS. No significant carryover was observed for any of analyte(s) and IS in each experiment.

5.2. Specificity and selectivity

No endogenous substances were found to be interfered at the retention time of analyte(s) or internal standard in blank plasma extracts. It was also determined that there is no drug-drug reaction effect like enhancement or suppression for any analyte.

5.3. Limits of quantification and limit of detection

The % mean accuracy of LLOQ at a concentration 3.030ng/mL and 5.050ng/mL was 99.0% and 100.6% with precision (% CV) of 1.9% and 1.9% for tenofovir and emtricitabine respectively. The mean signal to noise ratio of all LLOQ samples for both tenofovir and emtricitabine were found ≥ 24.0 .

5.4. Linearity, accuracy and precision

The regression coefficient (r) of all five calibration curves was ≥ 0.9992 for both tenofovir and emtricitabine. The method was linear from 3.030-1512.901ng/mL and 5.050-2521.999ng/mL for tenofovir and emtricitabine respectively. Intra-day and inter-day accuracy and precision were determined by assaying six spiked plasma samples at four different concentrations (QCs) for each drug for 5 analytical batches. Accuracy was calculated as the percent deviation from the nominal concentration. Results of accuracy and precision of all 5 batches are shown in table 1 (a & b) and table 2 (a & b).

5.5. Dilution integrity

For dilution integrity, sample was further diluted to five-fold and ten-fold dilutions with drug free blank plasma to bring the concentration within the calibration range.

% accuracy and precision (% CV) of five and ten fold dilutions were 99.1% & 1.3% and 101.4% & 2.2% respectively for tenofovir. % accuracy and precision (% CV) of five and ten fold dilutions were 102.9% & 2.0% and 98.9% & 2.2% respectively for emtricitabine.

5.6. Matrix effect

The % mean accuracy and precision (% CV) of LQC and HQC samples for all 6 different lots were 101.3% & 3.3% and 100.6% & 1.3% respectively for tenofovir and 105.0 % & 2.2 % and 102.7 % & 3.1 % respectively for emtricitabine.

5.7. Matrix factor

A value above or below 1 for the matrix factor indicates an ionization enhancement or suppression, respectively. The mean IS normalized matrix factors for low, mid and high QCs were 1.03, 1.00, 1.01 & 1.01, 1.01, 1.02 for tenofovir and emtricitabine respectively. The results were showing that co-eluting plasma matrix components do not appear to affect significantly the ionization of tenofovir and emtricitabine as well as IS.

5.8. Recovery

The % extraction recoveries at low, medium and high QC levels against the un-extracted aqueous samples were 92.6%, 92.9%, 92.0% and 88.6%, 87.6%, 87.9% respectively for tenofovir and emtricitabine. % mean recovery of tenofovir, emtricitabine and clonazepam was found over 92.5%, 88.0% and 94.6% respectively.

5.9. Ruggedness

The precision (% CV) and % accuracy values for different column were ranging from 2.1% to 5.1% and 99.6% to 107.1% for tenofovir and 3.5% to 4.1% and 99.2% to 106.1% for emtricitabine respectively at all four QC levels. For experiment with different analysts, the results for precision (% CV) and % accuracy were ranging from 1.6% to 4.7% and 100.6% to 106.8% for tenofovir and 1.2% to 5.2% and 101.3% to 104.3% for emtricitabine respectively at all four QC levels.

5.10. Stability

Bench top stability experiment confirmed that tenofovir and emtricitabine were found stable for 6 hours at ambient temperature (20°C). Dry extract stability experiment confirmed that tenofovir and emtricitabine were found stable for 44 hours in deep freezer (-20°C). Freeze and thaw stability experiment confirmed that tenofovir and emtricitabine were found stable for five freeze and thaw cycles in deep freezer (at -20°C & -70°C). Autosampler stability experiment confirmed that tenofovir and emtricitabine were found stable for 54 hours at 5°C. Reinjection reproducibility experiment confirmed that tenofovir and emtricitabine were found stable and reproduce

the same result during reinjection. Both tenofovir and emtricitabine were found stable in plasma for 61 days in deep freezer (at -20°C & -70°C).

Stock solutions of tenofovir, emtricitabine and clonazepam and working solutions of tenofovir and emtricitabine were stable for 6 hours at ambient temperature 20°C and for 11 days in refrigerator at 5°C respectively. The % CV were found $\leq 15\%$ and % bias were found $\pm 10\%$, when the stability samples peak area were compared with the peak area of fresh samples. Table 3 shows the complete summary of stability data for both analytes and IS.

6. CONCLUSION

LC-MS/MS is a powerful analytical tool with superior specificity and sensitivity, which is used in therapeutic drug monitoring. It offers a viable alternative to simple HPLC technology with UV detection and has the advantage of simultaneous measurement of drugs with high throughput. A relatively simple, accurate, precise and reproducible method suitable for use in pharmacokinetic/bioavailability/bioequivalence studies in human subjects to determine tenofovir and emtricitabine in plasma has been developed and validated.

Table 1(a): Summary of calibration curve with back calculated concentrations (Tenofovir)

	Back calculated calibration curve concentrations (ng/mL)								r Value
	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	
Nominal concentration (ng/mL)	3.030	6.059	30.296	75.740	151.479	336.620	673.241	1512.901	
1	3.033	6.076	29.611	76.333	147.201	340.220	686.482	1513.253	0.9998
2	2.999	6.215	29.300	77.016	148.434	342.948	691.893	1525.070	0.9997
3	3.040	6.031	28.544	75.077	144.723	334.415	674.708	1487.226	0.9999
4	3.021	6.198	29.010	76.161	146.732	338.943	683.755	1507.064	0.9996
5	2.901	5.904	28.512	75.239	145.177	335.662	677.378	1493.296	0.9993
Mean concentration (ng/mL)	2.999	6.085	28.995	75.965	146.453	338.438	682.843	1505.182	
SD	0.0568	0.1279	0.4768	0.8053	1.5152	3.4519	6.9286	15.2308	
% CV	1.9	2.1	1.6	1.1	1.0	1.0	1.0	1.0	
% Mean Accuracy	99.0	100.4	95.7	100.3	96.7	100.5	101.4	99.5	

SD, standard deviation; CV, coefficient of variance

Table 1(b): Summary of calibration curve with back calculated concentrations (Emtricitabine)

	Back calculated calibration curve concentrations (ng/mL)								r Value
	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	
Nominal concentration (ng/mL)	5.050	10.101	50.503	126.258	252.515	561.145	1122.290	2521.999	
1	5.104	9.853	50.889	128.934	255.394	554.509	1147.478	2490.788	0.9997
2	4.965	10.440	50.357	127.732	253.106	549.652	1137.527	2469.301	0.9998
3	5.224	9.380	50.404	128.428	254.853	553.886	1146.693	2489.636	0.9992
4	5.077	9.810	50.705	128.480	254.505	552.590	1143.516	2482.199	0.9998
5	5.033	9.826	51.239	130.000	257.621	559.483	1157.896	2513.542	0.9996
Mean concentration (ng/mL)	5.081	9.862	50.719	128.715	255.096	554.024	1146.622	2489.093	
SD	0.0959	0.3777	0.3639	0.8371	1.6457	3.5786	7.4197	16.1258	
% CV	1.9	3.8	0.7	0.7	0.6	0.6	0.6	0.6	
% Mean Accuracy	100.6	97.6	100.4	101.9	101.0	98.7	102.2	98.7	

SD, standard deviation; CV, coefficient of variance

Table 2(a): Summary of intra-batch and inter-batch precision and accuracy (Tenofovir)

Level	Nominal Concentration (ng/mL)	Intra-batch				Inter-batch			
		n	Mean back calculated concentration (ng/mL) ^a	%RE	%CV	n	Mean back calculated concentration (ng/mL) ^b	%RE	%CV
HQC	1344.801	6	1347.889	0.2	1.0	30	1356.979	0.9	1.1
MQC	75.309	6	75.233	-0.1	4.8	30	76.968	2.2	4.8
LQC	8.856	6	8.885	0.3	2.0	30	8.921	0.7	2.1
LLOQ QC	3.100	6	3.226	4.1	5.2	30	3.228	4.1	4.8

RE, relative error; CV, coefficient of variance; n, total number of observations.

^a Mean of 6 replicates at each concentration.^b Mean of 6 replicates for five precision and accuracy batches.**Table 2(b): Summary of intra-batch and inter-batch precision and accuracy (Emtricitabine)**

Level	Nominal Concentration (ng/mL)	Intra-batch				Inter-batch			
		n	Mean back calculated concentration (ng/mL) ^a	%RE	%CV	n	Mean back calculated concentration (ng/mL) ^b	%RE	%CV
HQC	1344.801	6	2309.799	3.0	4.0	30	2303.437	2.8	2.4
MQC	75.309	6	123.688	-1.5	5.5	30	125.304	-0.2	5.0
LQC	8.856	6	15.319	3.8	2.0	30	15.391	4.3	2.2
LLOQ QC	3.100	6	5.233	1.3	3.3	30	5.269	2.0	4.4

RE, relative error; CV, coefficient of variance; n, total number of observations.

^a Mean of 6 replicates at each concentration.^b Mean of 6 replicates for five precision and accuracy batches.**Table 3(a): Stability of Tenofovir under different conditions (n = 6)**

Stability	Storage condition	Level	Nominal concentration (ng/mL)	Mean back calculated concentration (ng/mL)	%CV	%RE
Bench Top	At 20±5°C ambient temperature for 6 hours	HQC	1344.801	1352.092	1.7	0.5
		LQC	8.856	8.937	7.0	0.9
Auto-sampler	At 5±1°C in autosampler for 54 hours	HQC	1344.801	1362.725	1.0	1.3
		LQC	8.856	9.053	2.1	2.2
Freeze and Thaw	For 5 freeze and thaw cycle at -20°C	HQC	1344.801	1322.797	2.7	-1.6
		LQC	8.856	8.744	2.4	-1.3
Freeze and Thaw	For 5 freeze and thaw cycle at -70°C	HQC	1344.801	1335.841	1.4	-0.7
		LQC	8.856	8.767	2.9	-1.0
Dry Extract	At -20±5°C in deep freezer for 44 hours	HQC	1344.801	1367.879	2.1	1.7
		LQC	8.856	8.951	5.1	1.1
Long Term Plasma Stability	At -20±5°C in deep freezer for 61 days	HQC	1344.801	1307.503	1.5	-2.8
		LQC	8.856	8.670	3.7	-2.1
Long Term Plasma Stability	At -70±10°C in deep freezer for 61 days	HQC	1344.801	1318.841	2.2	-1.9
		LQC	8.856	8.700	2.6	-1.8
Tenofovir Stock Solution	At 5±3°C in refrigerator for 11 days	ULOQ	-	-	2.5	2.6
Tenofovir Stock Solution	At 20±5°C ambient temperature for 6 hours	ULOQ	-	-	2.8	0.7
Tenofovir Working Solution	At 5±3°C in refrigerator for 11 days	ULOQ	-	-	3.4	2.9
		LLOQ	-	-	2.8	0.6
Tenofovir Working Solution	At 20±5°C ambient temperature for 6 hours	ULOQ	-	-	2.4	1.5
		LLOQ	-	-	2.5	-1.4

CV, coefficient of variance; RE, relative error; n, total number of observations.

Table 3(b): Stability of Emtricitabine under different conditions (n = 6)

Stability	Storage condition	Level	Nominal concentration (ng/mL)	Mean back calculated concentration (ng/mL)	%CV	%RE
Bench Top	At 20±5°C ambient temperature for 6 hours	HQC	2241.777	2306.956	3.6	2.9
		LQC	14.763	14.969	3.0	1.4
Auto-sampler	At 5±1°C in autosampler for 54 hours	HQC	2241.777	2279.445	3.4	1.7
		LQC	14.763	15.480	1.9	4.9
Freeze and Thaw	For 5 freeze and thaw cycle at -20°C	HQC	2241.777	2202.626	2.3	-1.7
		LQC	14.763	14.577	3.2	-1.3
Freeze and Thaw	For 5 freeze and thaw cycle at -70°C	HQC	2241.777	2213.214	3.8	0.2
		LQC	14.763	14.785	3.9	-1.3
Dry Extract	At -20±5°C in deep freezer for 44 hours	HQC	2241.777	2230.929	3.1	-0.5
		LQC	14.763	14.945	1.9	1.2
Long Term Plasma Stability	At -20±5°C in deep freezer for 61 days	HQC	2241.777	2198.304	2.7	-1.9
		LQC	14.763	14.576	3.4	-1.3
Long Term Plasma Stability	At -70±10°C in deep freezer for 61 days	HQC	2241.777	2207.527	3.2	-1.5
		LQC	14.763	14.625	3.1	-0.9
Emtricitabine Stock Solution	At 5±3°C in refrigerator for 11 days	ULOQ	-	-	1.0	1.1
Emtricitabine Stock Solution	At 20±5°C ambient temperature for 6 hours	ULOQ	-	-	2.1	3.7
Emtricitabine Working Solution	At 5±3°C in refrigerator for 11 days	ULOQ	-	-	1.7	0.6
		LLOQ	-	-	2.0	-0.2
Emtricitabine Working Solution	At 20±5°C ambient temperature for 6 hours	ULOQ	-	-	1.5	2.4
		LLOQ	-	-	1.2	1.1

CV, coefficient of variance; RE, relative error; n, total number of observations.

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