

New Validated Liquid Chromatography Coupled with Tandem Mass Spectrometry Method Development for Quantification of Tenofovir in Human Plasma Co-administered with Dolutegravir and Lamivudine

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Abstract

Aim: A selective and reproducible liquid chromatography coupled with tandem mass spectrometry method was developed for the quantification of Tenofovir in human plasma, in the presence of Dolutegravir and Lamivudine.

Methodology: The method employed solid-phase extraction using strong cation exchange (SCX) cartridges and chromatographic separation on a ZORBAX SB C8 column with mobile phase 0.1% formic acid in water: acetonitrile (90:10, v/v). Detection was achieved via electrospray ionization in positive mode, monitoring multiple reaction

monitoring transitions of Tenofovir and its deuterated internal standard. **Results and Discussion:** The retention time for Tenofovir was approximately 1.70 min, with a total run time of 3.0 min/sample. The calibration curve was linear over the range of 3.0–1055.7 ng/mL, with correlation coefficients consistently above 0.9988 and an lower limit of quantification (LLOQ) of 3.052 ng/mL, demonstrating excellent sensitivity. Mean recovery for Tenofovir ranged from 59.88% to 62.11%, with a percent coefficient of variation below 1%, confirming the reproducibility of the extraction process. **Conclusion:** The method was validated as per regulatory guidelines, demonstrating acceptable precision, accuracy, recovery, and stability across a wide concentration range. This assay is suitable for pharmacokinetic, bioequivalence, and therapeutic drug monitoring applications in HIV patients receiving combination therapy.

Key words: Accuracy, dolutegravir, lamivudine, Liquid chromatography coupled with tandem mass spectrometry, LLOQ, recovery, tenofovir

INTRODUCTION

Tenofovir, Dolutegravir, and Lamivudine form the cornerstone of modern antiretroviral therapy, routinely prescribed in fixed-dose combinations for effective HIV management [Figure 1]. Precise quantification of these drugs in human plasma is fundamental for pharmacokinetic characterization, therapeutic drug monitoring, and bioequivalence assessment, each playing a critical role in optimizing individual patient outcomes as well as in clinical trial settings. Despite notable progress in bioanalytical

sciences, reliably measuring these agents simultaneously within complex biological matrices remains technically demanding due to potential interferences and the need for high specificity,

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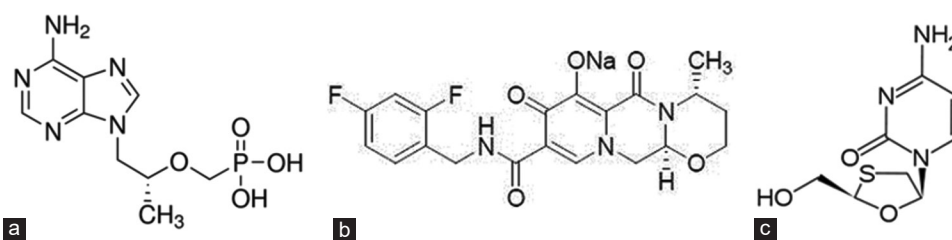


Figure 1: Chemical structure of (a) Tenofovir, (b) Dolutegravir, and (c) Lamivudine

Table 1: Calibration curve standards for analyte

Batch ids	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	STD9	STD10
Nominal concentration, ng/mL	3.000	6.001	10.557	21.115	52.787	105.575	263.937	527.874	844.598	1055.748
P&A01	3.058	5.839	10.443	20.65	53.09	104.396	269.978	526.635	841.203	1086.71
P&A02	2.999	6.144	10.273	20.433	53.481	103.058	273.493	524.062	834.556	1086.505
P&A03	3.087	5.562	10.948	20.413	54.762	103.562	267.376	523.897	836.031	1083.884
Mean	3.048	5.8483	10.5547	20.4987	53.7777	103.672	270.2823	524.8647	837.2633	1085.7
Standard deviation	0.04484	0.2911	0.35108	0.13114	0.87459	0.67575	3.06983	1.53537	3.49065	1.57575
% Coefficient of variation	1.47	4.98	3.33	0.64	1.63	0.65	1.14	0.29	0.42	0.15
Accuracy	101.6	97.46	99.98	97.08	101.88	98.2	102.4	99.43	99.13	102.84

values represent the mean back-calculated concentration ($n=3$) across multiple analytical runs.

sensitivity, and robustness. This thesis addresses these analytical challenges by presenting the development and regulatory-compliant validation of a selective liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) method tailored for Tenofovir measurement when co-administered with Dolutegravir and Lamivudine. Leveraging solid-phase extraction and advanced chromatographic separation, this method aims to provide robust data for pharmacokinetic, bioequivalence, and therapeutic studies, bridging current gaps in reliable multi-drug quantification for HIV combination therapy.^[1-3]

Simultaneous quantification of Tenofovir alongside Dolutegravir and Lamivudine presents analytical challenges, including matrix complexity, ion suppression, and potential interference from concomitant medications. LC-MS/MS has emerged as a preferred technique for such applications, offering high sensitivity, specificity, and throughput. This study describes the development and validation of a robust LC-MS/MS method for Tenofovir quantification in human plasma,^[4-9] employing solid-phase extraction and optimized chromatographic conditions. The method adheres to international regulatory guidelines and is suitable for clinical and research applications involving HIV combination therapy.^[10-13]

MATERIALS AND METHODS

Chemicals and reagents

Tenofovir and Tenofovir D6 (internal standard) were obtained from Simson Pharma (Mumbai, India). LC-grade acetonitrile and

methanol, GR-grade formic acid, and ammonium acetate were procured from Merck. Human plasma (K2EDTA) was sourced from a certified biobank. Milli-Q water was used throughout.

Instrumentation

Chromatographic separation was performed using a Shimadzu UFLC XR system coupled to an AB SCIEX API 4500 triple quadrupole mass spectrometer. Analyst 1.5.1 software was used for data acquisition and processing.

Optimized conditions

- Column: ZORBAX SB C8 (4.6 × 150 mm, 5 μm)
- Mobile phase: 0.1% formic acid in water: acetonitrile (90:10, v/v)
- Flow rate: 1.0 mL/min (70% split)
- Injection volume: 10 μL
- Run time: 3.0 min
- Retention time: ~1.70 min

Mass spectrometric conditions

- Ionization mode: Positive ESI
- Scan type: Multiple reaction monitoring
- Transitions: Tenofovir (m/z 288.2 → 176.2); Tenofovir D6 (m/z 294.2 → 182.0)
- Source parameters: CUR 35, GS1 40, GS2 45, IS 5500, TEM 600°C
- Compound parameters: DP 80, EP 10, CE 35, CXP 12, Dwell Time 200 ms.

Sample preparation

After thawing the required number of plasma samples at room temperature and thoroughly mixing them, a specific amount of internal standard working solution is added to

all tubes except the standard blank, which instead receives diluent; following this, a measured volume of plasma is transferred to each prepared tube and mixed briefly. Extraction buffer is then introduced to each tube, and the mixture is vortexed again to ensure even distribution. For extraction, Orochem SCX 1CC (30 mg) cartridges are conditioned with methanol and high-purity water, the prepared samples are loaded carefully onto the cartridges, and the cartridges are washed with extraction buffer before being dried under vacuum. The analytes are eluted using an elution solution into labeled vials, after which the eluates are evaporated to dryness under nitrogen at a controlled temperature. The residues are then reconstituted with an appropriate reconstitution solution, mixed until

Table 2: Calibration curve parameters of calibration curve (CC) standard for analyte

Batch ids	Slope	Intercept	r	r ²
P&A01	0.00918	−0.00288	0.9998	0.9996
P&A02	0.00919	−0.00258	0.9996	0.9992
P&A03	0.00925	−0.00499	0.9992	0.9984

Table 3: Inter-batch validation data

QC ID	Actual Conc. (ng/mL)	Mean values		MEAN	Standard deviation	% Coefficient of variation	% Accuracy
		P&A-I	P&A-II				
HQC	802.569	765.6488	773.26	769.454	5.382	0.7	95.87
MQC1	497.593	483.8863	480.236	482.061	2.581	0.5	96.88
MQC2	73.644	74.8442	75.5095	75.177	0.470	0.6	102.08
LQC	8.035	8.1158	8.2122	8.164	0.068	0.8	101.61
LLOQ QC	3.000	3.03213	3.0338	3.033	0.001	0.0	101.10

Values represent the mean of six determination samples ($n = 18$) for each Quality Control (QC) level.

Table 4: Intra-batch validation data

QC ID	Actual Conc. (ng/mL)	Mean values		MEAN	Standard deviation	% coefficient of variation	% Accuracy
		P&A-I	P&A-II				
HQC	802.569	765.6488	773.3575	769.503	5.451	0.7	95.88
MQC1	497.593	483.8863	479.3007	481.594	3.243	0.7	96.78
MQC2	73.644	74.8442	74.805	74.825	0.028	0.0	101.60
LQC	8.035	8.1158	8.2805	8.198	0.116	1.4	102.03
LLOQ QC	3	3.03213	3.1045	3.068	0.051	1.7	102.28

Table 5: Summarized stability parameters of both analyte and its active metabolite

Stability	Storage condition and duration	Level	Nominal concentration (ng/mL)	Mean Stability sample (mean±Standard deviation, ng/mL)	% coefficient of variation	Accuracy
Bench top	Room temperature and 7 h	HQC	802.569	812.6672±5.71597	0.70	100.25
		LQC	8.035	8.2555±0.20068	2.43	101.15
Processed sample stability	Refrigerated (2–8°C) and 3 days	HQC	802.569	818.2610±11.29905	1.38	100.94
		LQC	8.035	8.1575±0.15809	1.94	99.95
Processed sample stability	Room temperature (24±4°C) and 26 h	HQC	802.569	806.3943±5.31703	0.66	99.48
		LQC	8.035	8.1422±0.19696	2.42	99.76
Freeze/thaw (on water bath)	−70±10°C and 5 cycles	HQC	802.569	809.1202±5.24113	0.65	99.81
		LQC	8.035	8.0565±0.07498	0.93	98.71
Auto sampler	5±3°C	HQC	802.569	811.4442±6.588129	0.81	100.1
		LQC	8.035	8.0677±0.19046	2.36	98.85

dissolved, and a designated aliquot from each is placed into autosampler vials for LC-MS/MS analysis.^[14,15]

RESULTS AND DISCUSSION

Our new LC-MS/MS approach offers excellent sensitivity, selectivity, and reliability for measuring Tenofovir levels in human plasma – right alongside Dolutegravir and Lamivudine, with no issues. All validation metrics align with key standards from the FDA, proving it's sturdy enough for real-world bioanalysis. The lowest detectable level (LLOQ-3.000 ng/mL) easily picks up the plasma concentrations that matter clinically. We tackled matrix effects head-on with a streamlined prep process, delivering precise results even in tricky biological samples. Plus, testing with co-administered drugs showed clean separation of peaks, making it ideal for pharmacokinetic work and checking drug interactions.

System suitability

Six replicate injections confirmed a percent coefficient of variation (%CV) <2% for retention time and <5% for peak area ratio.

Specificity and selectivity

No interference was observed in 11 plasma lots, including hemolytic, lipemic, and heparinized samples. Concomitant drugs (paracetamol, caffeine, diclofenac, etc.) did not interfere.

Matrix effect

Matrix factor values ranged from 0.98 to 1.00. ISTD-normalized matrix factor %CV was <2%.

Linearity and sensitivity

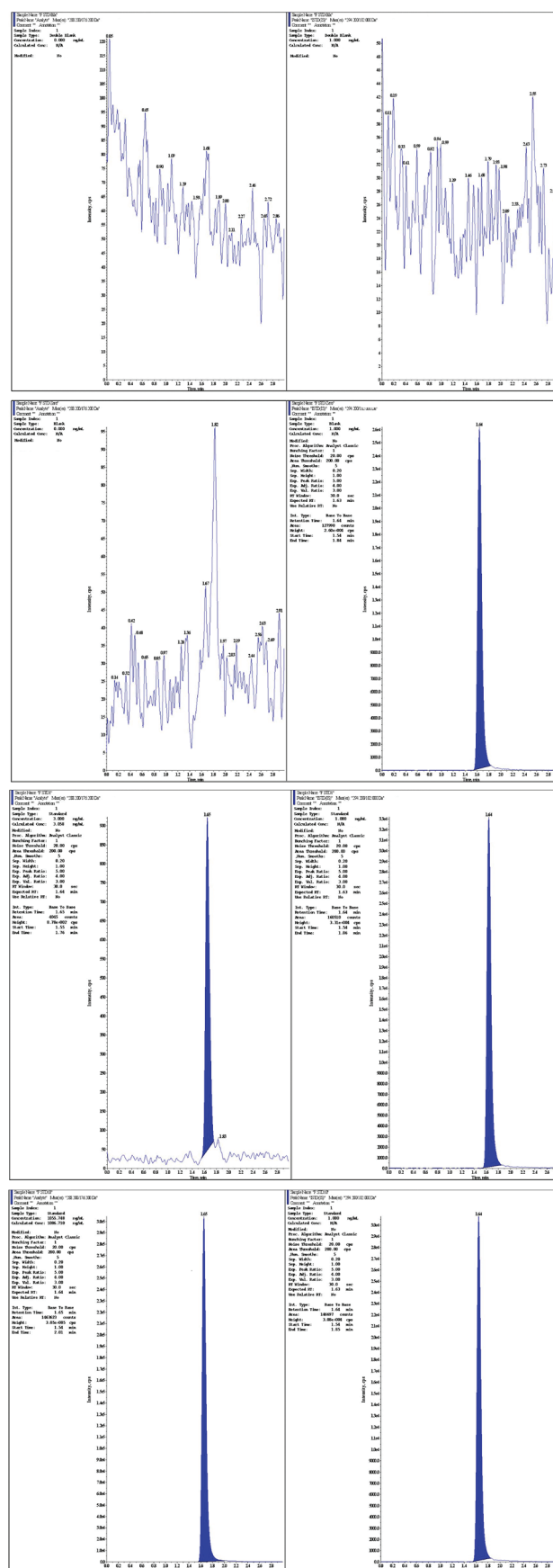
Calibration curves were linear over 3.0–1055.7 ng/mL ($r \geq 0.9988$). LLOQ was 3.052 ng/mL with %CV 4.12% and accuracy 101.77% [Tables 1 and 2].^[16,17]

Precision and accuracy

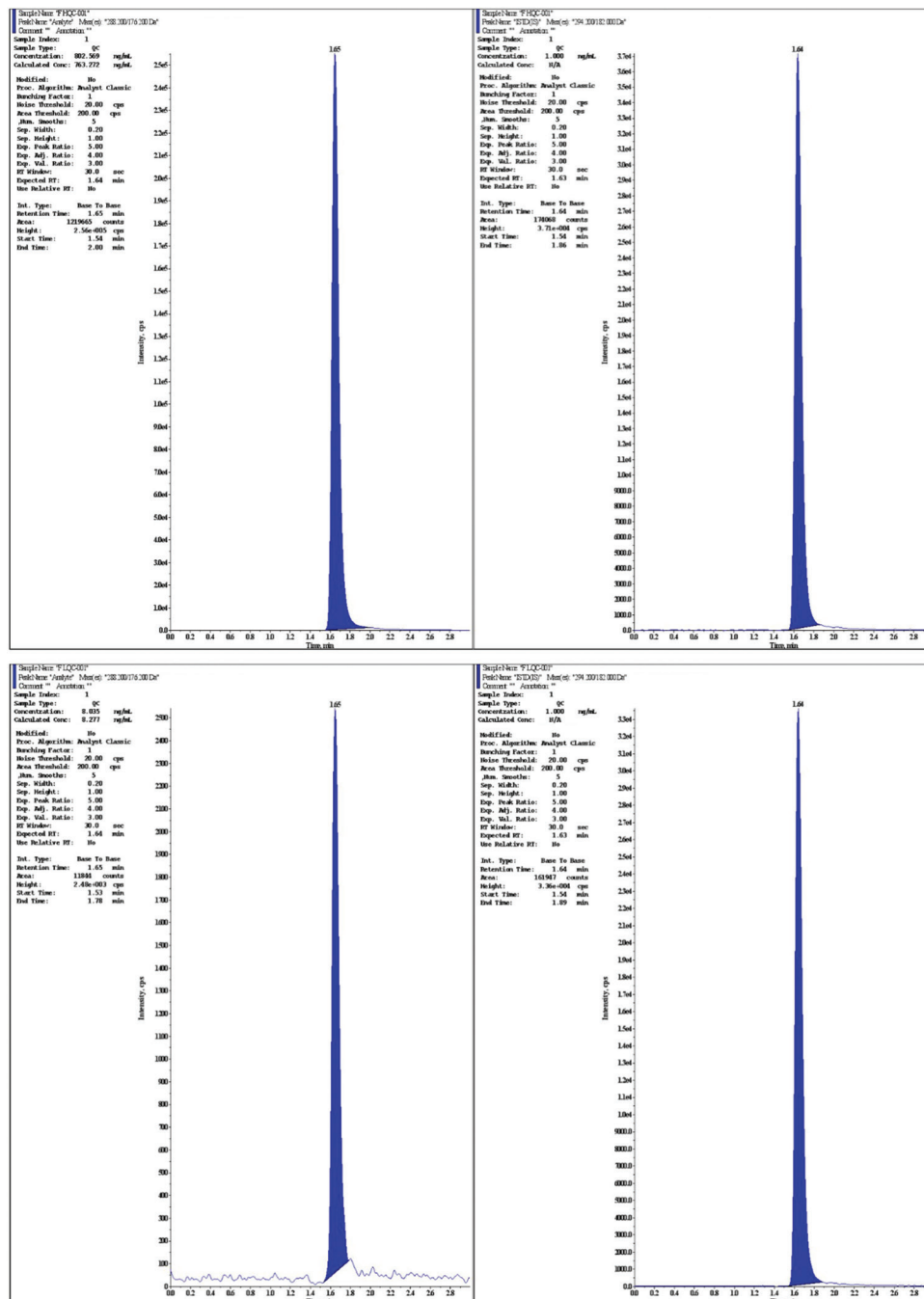
- Intra-batch: %CV 0.89–3.34; accuracy 95.40–101.63%
- Inter-batch: %CV 0.94–4.32; accuracy 96.04–102.09% [Tables 3 and 4].

Recovery

Mean recovery for Tenofovir was ~60–62% with %CV <1%. ISTD recovery was ~60.6% with %CV <1%.



QC level (nominal concentration, ng/mL)	Mean area response (n=6)		Recovery (B/A%)		Global % recovery	
	A (Post extracted area)	B (Extracted area)	Analyte	IS	Analyte	IS
HQC (802.569)	2867492.8	1746271.3	60.90	60.71	60.97	60.64
MQC1 (497.593)	1779440.0	1065577.3	59.88	60.25		
LQC (8.035)	28041.0	17416.7	62.11	60.97		



Stability studies

Tenofovir was stable under freeze-thaw, bench-top, autosampler, wet/dry extract conditions, with %CV <5% and accuracy within 85–115% [Table 5].

Compared to earlier HPLC-UV or LC-MS/MS methods reported for Tenofovir, the present method achieved shorter run times, improved sensitivity, and enhanced reproducibility. Unlike prior approaches that struggled with co-elution issues, this method successfully separated Tenofovir from

Dolutegravir and Lamivudine, demonstrating superior selectivity.^[18]

Limitations

The study was limited to Tenofovir quantification in plasma; extension to other biological matrices (e.g., intracellular compartments, urine) requires further validation. In addition, while Dolutegravir and Lamivudine were included, other antiretroviral co-medications were not assessed.

CONCLUSION

A robust LC-MS/MS method was successfully developed and validated for the quantification of Tenofovir in human plasma samples co-administered with Dolutegravir and Lamivudine. The analytical procedure, based on solid-phase extraction and chromatographic separation using a ZORBAX SB C8 column, demonstrated consistent retention time (~1.70 min) and a short run time of 3.0 min, supporting efficient sample throughput. The method exhibited high specificity, with no interference from endogenous plasma components or commonly co-administered medications. Calibration curves were linear across the range of 3.0–1055.7 ng/mL, with correlation coefficients exceeding 0.9988 and LLOQ accuracy above 101%, confirming excellent sensitivity and reliability. Intra- and inter-batch precision and accuracy values were within acceptable limits (%CV <5%, accuracy 95–102%), and recovery for Tenofovir and its internal standard averaged approximately 61%, with minimal variability (%CV <1%). Matrix effects were negligible, and stability assessments under various conditions, including freeze-thaw cycles, bench-top exposure, autosampler storage, and processed sample holding, confirmed the analyte's integrity. Collectively, these findings affirm the method's suitability for pharmacokinetic profiling, therapeutic drug monitoring, and bioequivalence studies in HIV patients receiving combination antiretroviral therapy.^[19]

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