

Quantitative Assessment of Lamivudine, Lopinavir and Ritonavir in Human Plasma Using a Validated LC–MS/MS Method

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ABSTRACT:

The simultaneous determination of antiretroviral drugs with diverse physicochemical properties in biological matrices is analytically challenging. Lamivudine is a hydrophilic nucleoside reverse transcriptase inhibitor, whereas Lopinavir and Ritonavir are relatively lipophilic protease inhibitors. The present study was designed to develop and validate a simple and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method to quantify Lamivudine, Lopinavir and Ritonavir in human plasma using Tenofovir as internal standard. The method exhibited highly sensitive range for 6.129–3000 ng/mL for Lamivudine, 50.433–20173.143 ng/mL for Lopinavir and 50.358–20143.302 for Ritonavir. The analytes were extracted from plasma using protein precipitation technique. Lamivudine, Lopinavir and Ritonavir were separated using Thermo Hypersil C18 column with a gradient mobile phase consisting 0.1% Formic acid in Water: Acetonitrile (10:90) v/v at 0.5 mL/min with 4 min run time. The validated parameters were within the acceptance criteria as per the regulatory guidelines and the validated calibration curve ranges for all the analytes have correlation coefficient (r^2) value > 0.99 with above 90% recovery. The developed method was found to be suitable for pharmacokinetic and therapeutic drug monitoring studies involving antiretroviral drugs.

KEYWORDS

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INTRODUCTION

Human immunodeficiency virus (HIV) infection continues to be a significant global public health issue, with the widespread use of combination antiretroviral therapy (ART) greatly enhancing HIV management [1–3]. Generally, antiretroviral therapy includes the use of multiple drugs with various mechanisms to inhibit viral replication, minimize drug resistance, and enhance clinical results [4,5]. Most HAART treatments include three or four anti-HIV drugs targeted to different viral proteins [6]. Antiretroviral therapy combines drugs from different classes, including NRTIs, NNRTIs and Protease inhibitors (PIs), targeting different stages of HIV replication. Combination therapy is essential for maintaining viral suppression, particularly in drug-resistant HIV and helps reduce the emergence of further drug resistance [7–9]. Nucleoside reverse transcriptase inhibitors (NRTIs) are commonly combined with one or more protease inhibitors (PIs) to target multiple stages of the HIV replication cycle. Among the PIs used in HAART, Lopinavir is co-formulated with a low dose of Ritonavir in “Kaletra”. Ritonavir inhibits CYP3A-mediated metabolism of Lopinavir, thereby enhancing and prolonging its systemic exposure. In addition, administration of alone Lopinavir (LPV) has insufficient bioavailability, Ritonavir (RTV) can inhibit P-glycoprotein-mediated drug efflux, further contributing to the pharmacokinetic boosting of Lopinavir [10–12]. Lamivudine (3TC) is a nucleoside reverse transcriptase inhibitor (NRTIs),

for their effective and complementary ability to counteract the human immunodeficiency virus (HIV), the pathogen responsible for Acquired Immuno Deficiency Syndrome (AIDS) [13,14]. The determination of antiretroviral drug concentrations in biological matrices is important for pharmacokinetic studies, therapeutic drug monitoring, bioequivalence assessment, and evaluation of inter-individual variability in drug exposure [15,16].

Simultaneous estimation of 3TC, LPV and RTV is challenging due to their different physicochemical properties, extraction efficiencies, chromatographic retention, and MS responses. Endogenous plasma components may also cause ion suppression or enhancement, affecting LC–MS/MS accuracy and reproducibility [17]. Lamivudine is relatively polar and hydrophilic, whereas Lopinavir and Ritonavir are highly lipophilic, resulting in differences in extraction efficiency, chromatographic retention, recovery and mass spectrometric response. Additionally, endogenous plasma components may cause ion suppression or enhancement, potentially affecting LC–MS/MS accuracy, precision and reproducibility [18].

Cahn and *et al* formulated dual therapy with Lopinavir and Ritonavir plus Lamivudine versus triple therapy with Lopinavir and Ritonavir plus two NRTIs in antiretroviral therapy-naïve adults with HIV-1 infection at 19 centres in six countries. At 48 weeks, viral suppression (<50 copies/mL) was achieved in 88.3% of patients receiving dual therapy

compared with 83.7% receiving triple therapy. The dual regimen also showed fewer drug-related adverse events and lower treatment discontinuation, suggesting that Lopinavir/Ritonavir plus Lamivudine may be an effective and better-tolerated alternative to conventional triple therapy [19].

Arribas *et al* demonstrated in the OLE randomized, open-label, non-inferiority trial that dual therapy with Lamivudine (3TC) and Ritonavir-boosted Lopinavir (LPV/r) was clinically non-inferior to conventional triple therapy for maintaining HIV-1 viral suppression in virologically suppressed patients. At 48 weeks, treatment response was observed in 87.8% of patients receiving LPV/r plus 3TC compared with 86.6% receiving triple therapy, with similar tolerability. These findings support the clinical relevance of the 3TC, LPV and RTV combination and emphasize the need for reliable simultaneous bioanalytical methods for its quantification in human plasma [20]. Human plasma is a complex matrix containing proteins, phospholipids and endogenous metabolites that may interfere with analysis. Therefore, sensitive and selective methods are essential for reliable antiretroviral drug quantification. LC–MS/MS is widely used for simultaneous quantification in complex biological matrices [21,22].

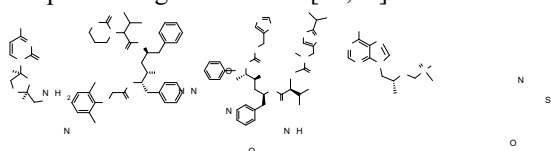


Figure.1: Chemical structures of Lamivudine (3TC), Lopinavir (LPV), Ritonavir (RTV), Tenofovir (TFV)

This study aimed to develop a simple, sensitive, reproducible method using protein precipitation for plasma quantification, supporting pharmacokinetic and therapeutic monitoring studies. The clinical relevance of the 3TC, LPV and RTV combination is supported by clinical studies demonstrating that therapy with Lamivudine and Ritonavir-boosted Lopinavir effectively maintains HIV-1 viral suppression, emphasizing the importance of reliable simultaneous bioanalytical quantification of these drugs in human plasma.

The novelty of the present study is to highly active antiretroviral therapy (HAART) uses combinations of antiretroviral drugs to improve the management of HIV infection, making reliable measurement of drug concentrations important for pharmacokinetic and therapeutic investigations. The proposed method addresses the analytical challenges associated with the distinct physicochemical properties of these drugs, particularly the hydrophilic nature of lamivudine compared with the more lipophilic lopinavir and ritonavir.

The practical importance of the method is its ability to provide selective, sensitive, accurate and reproducible quantification of all three drugs from a single plasma sample. The optimized sample-preparation procedure minimizes endogenous

interference and provides suitable extraction performance and compatibility with LC–MS/MS analysis. The validated method therefore be useful for pharmacokinetic, bioavailability, bioequivalence and therapeutic drug-monitoring studies.

MATERIALS AND METHODS

Chemicals and Reagents

Analytical grade API of 3TC, LPV, RTV and TFV(IS) were obtained from Med Chem Express (MCE). Methanol and tert-butyl methyl ether (TBME), Formic acid, Acetonitrile purchased from Loba Chemie Pvt Ltd. Ultrapure water was prepared in-house by use of a Milli-Q water-purification system. Blank plasma was collected within in-house from healthy, disease-free human subjects by collecting their blood samples through vein puncture and collected plasma stored under refrigerated conditions until analysis.

Instrumentation

The LC–MS/MS system consisted of a high-performance liquid chromatography system coupled with a triple quadrupole SCIEX API 4000 LC-MS/MS mass spectrometer equipped with an electrospray ionization (ESI) source operating in multiple reaction mode.

Chromatographic Conditions

Chromatographic separation was achieved using gradient elution throughout the analysis, with the mobile-phase composition programmed according to the optimized gradient conditions.

Thermo Hypersil C18 (4.6 X100 mm, 5.0µm particle size) column, temperature was set at 30°C and autosampler temperature was set at 10°C. The flow rate settled at 0.5 mL/min, with a gradient elution of mobile phase combination 0.1% Formic acid: Acetonitrile (10:90) v/v. Injection volume kept 20 µL with a total run time of 4 min.

PREPARATION OF STANDARD SOLUTIONS

Preparation of Stock Solution and Spiking Solution

The master stock solutions of 3TC, LPV and RTV having concentration 5 mg/mL are prepared in 50% methanol (diluent). Master stock solutions of each analyte were prepared at a concentration of 500 µg/mL. From these, secondary stock solutions of 50 µg/mL were prepared using the appropriate diluent. Subsequently, working stock solutions of 1 µg/mL were prepared by further dilution of the 50 µg/mL solutions. A 950 µL aliquot of blank plasma was spiked with 50 µL of stock solution (50 µg/mL of each analyte), vortexed for 30s and used as the ULOQ plasma sample. A 950 µL aliquot of blank plasma was spiked with 50 µL of stock solution (1 µg/mL of each analyte), vortexed for 30s and used as the LLOQ plasma sample. From 10 µg/mL solution of Tenofovir 1 µg/mL Tenofovir solution was prepared and used as the internal standard for sample analysis.

Primary stock solutions of 3TC, LPV, RTV and TFV were prepared separately in methanol at a concentration of 1 mg/mL. Working standard

solutions were prepared by serial dilution with mobile phase. Calibration curve (CC) standards and quality control (QC) samples were prepared by spiking blank human plasma with appropriate working solutions.

Optimization of extraction procedure

Protein precipitation technique (PPT) is routine sample pretreatment technique. During the initial stages of method development, PPT was carried out using methanol and acetonitrile as precipitating agent. As a result, acetonitrile was selected to obtain a clean extract and lower background noise with high sensitivity.

250 μ L of spiked plasma and internal standard were taken in Ria vial with 1 mL precipitating agent/denaturing agent acetonitrile. Vortex the Ria vial for 30 second on vortexer and centrifuge the vial at 12000 RPM for 10 minutes at 10°C and 250 μ L of supernatant collected into labelled autosampler vial for analysis.

METHOD DEVELOPMENT AND VALIDATION

For method development and its validation, serial dilutions were done to prepare various concentration stock, working standard of various concentrations was prepared by taking aliquots of standard solution and diluted to get required concentration for calibration plot; further they processed and analysed as per method validation protocol.

The developed method was validated according to ICH bioanalytical method validation guidelines [23]. The validation parameters Selectivity, Specificity, Carryover effect, Sensitivity, Linearity, Precision and Accuracy, Matrix effect, Recovery, Reinjection reproducibility, Hemolysis effect and Plasma Stability were evaluated under the validation section.

VALIDATION PARAMETERS

System suitability test (SST)

The System Suitability Test (SST) is performed before sample analysis to verify instrument performance, including signal-to-noise ratio, peak shape, retention time, response reproducibility, and carryover. It typically involves 6 replicate injections of a spiked sample of MQC level. Acceptance requires $\leq 5\%$ CV for analyte/IS area ratios and individual peak areas.

Selectivity and Specificity

Selectivity and Specificity assess a bioanalytical method's ability to accurately measure an analyte in the presence of endogenous matrix components and other interferences. Six different blank plasma lots are analyzed. The method is acceptable if interference is $\leq 20\%$ of the LLOQ response for the analyte and $\leq 5\%$ of the IS response.

Carry Over Effect

Carry-over was assessed by sequentially injecting a blank matrix sample, duplicate LLOQ QC samples, duplicate ULOQ QC samples and duplicate blank matrix samples following the ULOQ injections. Responses at the analyte and internal standard (IS) retention times in the blank samples were compared

with the mean responses of the processed LLOQ QC samples.

Acceptance Criteria: The response of interfering peaks in blank samples should be $\leq 20\%$ of the mean analyte response at LLOQ QC and $\leq 5\%$ of the mean IS response. Blank samples injected after the ULOQ should also meet these limits.

Matrix effect

Matrix effect refers to changes in the analyte response caused by co-eluting endogenous components from the biological matrix. It is evaluated by comparing post-extracted plasma samples with neat standard solutions. Matrix effects may cause ion suppression or enhancement, reduced sensitivity, baseline noise, retention time shifts and distorted peak shapes. During validation, at least six different normal matrix lots, including hemolyzed and lipemic plasma, should be assessed. For each lot, at least two replicates of LQC and HQC are analyzed and compared with six replicates of aqueous LQC and HQC samples.

Acceptance Criteria: Accuracy of at least 67 % (2/3) of LQC & HQC samples at each level from each lot should be $\pm 15\%$ of respective nominal concentration. Precision for QC's (Percent Coefficient of Variation (%CV)) $\leq 15\%$.

Linearity

The calibration curve consisted of one blank plasma sample, one zero sample containing only the internal standard (IS), and eight plasma samples spiked with analyte standards. It was constructed by plotting the analyte-to-IS peak area ratio against analyte concentration. At least three calibration curves were evaluated during method validation. The curve was described by the linear equation $y = ax + b$, where y represents the analyte-to-IS peak area ratio and x represents the analyte concentration (ng/mL). The regression coefficient, slope and y-intercept were determined using least-squares weighted ($1/x^2$) regression.

Precision and Accuracy

Accuracy and precision were assessed through within-run (intra-day) and between-run (inter-day) evaluations. Intra-day performance was determined by analyzing six replicates at each QC level in a single analytical run, whereas inter-day performance was assessed across at least three analytical runs performed over a minimum of two days.

Acceptance Criteria: Accuracy and precision are considered acceptable when at least 67% of all QC samples are within $\pm 15\%$ of the nominal concentration ($\pm 20\%$ for LLOQ QC), with at least 50% of QC samples at each concentration level meeting these limits. The calibration curve must satisfy the acceptance criteria. Overall precision and accuracy are calculated using all valid analytical batches, excluding batches with failed calibration curves. A minimum of three acceptable precision and accuracy batches is required and used for statistical data.

Sensitivity

The LLOQ designates the sensitivity of the method. Sensitivity was evaluated as part of the precision and

accuracy assessment by processing and analysing six replicates of LLOQ QC samples at the LLOQ concentration in at least three precision and accuracy (P&A) batches.

Recovery

Six replicates of quality control (QC) samples at the LQC, MQC and HQC levels were prepared and processed according to the extraction procedure. Blank matrix extracts were externally spiked with the analyte and internal standard (IS) to prepare unextracted samples at each QC level. Six replicate injections of the unextracted samples were analyzed. The mean recovery, standard deviation (SD) and percent coefficient of variation (%CV) for the analyte and IS were calculated at each QC level.

$$\% \text{ Recovery of Analyte} = \frac{\text{Area of analyte in extracted Sample}}{\text{Mean Area of analyte in Unextracted Sample}}$$

Extraction recovery was determined by comparing peak areas obtained from extracted samples with those of unextracted samples at corresponding concentrations.

Reinjection reproducibility

Reinjection reproducibility is performed by using samples of one of the Precision and Accuracy (PA) batch which is already acquired and accepted. The Entire PA Batch is reinjected to prove Reinjection reproducibility. Concentration of QCs were back calculated by using respective CC.

Acceptance Criteria: Accuracy of at least 67% of total QC samples should be within $\pm 15\%$ & for LLOQ $\pm 20\%$ At least 50% of QC samples at each level should be within $\pm 15\%$ & for LLOQ QC $\pm 20\%$ Calibration Curve should pass as per criteria.

Hemolysis Effect

Hemolyzed plasma whose matrix contained 2-5%(v/v) lysed whole blood. Preparation of Hemolyzed Quality Control (QC) samples: LQC & HQC prepared in Hemolyzed plasma. These Hemolyzed QCs (LQC & HQC) were processed along with non-hemolyzed CC and QCs using identified extraction procedure. Above samples were analysed and results were compared.

Acceptance Criteria: LQC and HQC should be within $\pm 15\%$

Stability Studies

1. Bench top Stability

Bench-top stability was evaluated at the LQC and HQC levels using six replicates each. Stability samples stored in the deep freezer were thawed to room temperature, processed and analyzed alongside freshly prepared calibration curve (CC) standards and QC samples. The concentrations were back-calculated using the corresponding CCs and stability was assessed based on the elapsed time between sample removal from the freezer and sample processing.

Acceptance Criteria: LQC and HQC should be within $\pm 15\%$

2. Processed Sample Stability

Processed sample stability was evaluated at the LQC and HQC levels. Six replicates of each QC level were processed according to the extraction procedure and kept on the bench top immediately after processing. Freshly prepared calibration curve (CC) standards and QC samples were processed and analyzed concurrently with the stability samples. The concentrations of the fresh and stability QC samples were back-calculated using the respective CCs. Stability duration was calculated from the time the processed samples were placed on the bench top until their transfer to the autosampler.

Acceptance Criteria: LQC and HQC should be within $\pm 15\%$

3. Freeze thaw (FT) stability

Freeze–thaw (FT) stability was evaluated at LQC and HQC levels using six replicates after at least three freeze–thaw cycles. QC samples were stored at -70°C , thawed unassisted at room temperature, and refrozen for each cycle. After completion of the required cycles, FT stability samples were processed and analyzed along with freshly prepared calibration curve (CC) standards and QC samples. The concentrations of FT stability samples were back-calculated using the respective calibration curve and compared with freshly prepared QC samples to assess stability.

Acceptance Criteria: LQC and HQC should be within $\pm 15\%$

4. Autosampler Stability

The stability was performed at LQC and HQC level. Fresh stock solution was used for spiking of CC Standards and QC samples for stability study evaluation. Six replicates of LQC and HQC were processed as per extraction procedure. Immediately after processing, these samples were placed in Autosampler at specified temperature. The time of placing autosampler stability samples in autosampler is recorded. Concentration of fresh QC and autosampler stability QC samples were back calculated using respective CC. The actual time of stability were calculated from time of placing the stability samples in the autosampler to the first stability QC sample injection.

Acceptance Criteria: LQC and HQC should be within $\pm 15\%$

RESULTS AND DISCUSSION

System suitability Test (SST)

To perform the SST 6 injections at extracted MQC level is injected and analysed. System suitability test is performed daily before start of analysis. It is observed that % CV of area ratio for 3TC, LPV, RTV and IS was found $< 5\%$ of mean area of Analyte and IS respectively. Hence system suitability test (SST) is found within acceptance criteria for both the drugs and IS.

Selectivity and Specificity

The analytes peaks were found eluted specifically with best resolution with no interference of excipients showing specificity and selectivity of

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method. PPT provided a clean baseline with acceptable signal response, provided efficient elution with well-defined peak shapes, facilitating retention times of analytes 2.77min, 2.09 min, 2.19 min, 1.46 min for 3TC, LPV, RTV and IS (TFV) respectively. The analysis time was short, which makes it an attractive method for routine analysis. Representative chromatograms are shown in fig. 2 and fig.3.

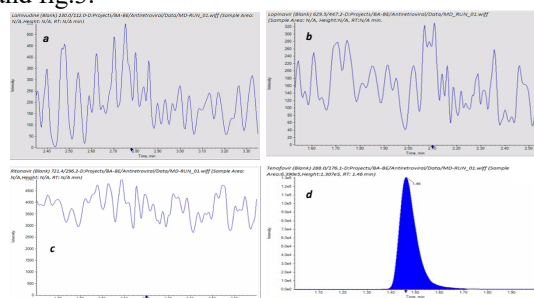


Figure 2: LC-MS/MS chromatogram for 3TC (a), LPV (b), RTV (c) and TFV (d) blank sample extract from PPT with acetonitrile.

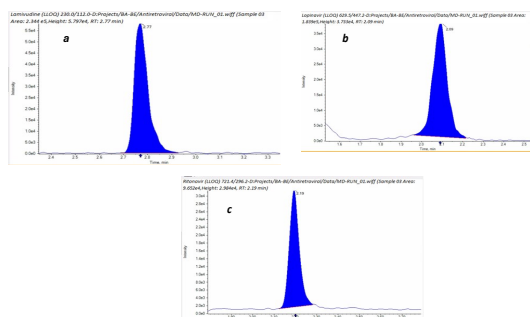


Figure 3: LC-MS/MS chromatogram for plasma spiked with 3TC (a), LPV (b), RTV (c) at the lower limit of quantification (LLOQ) Concentration 50 ng/mL by PPT.

Carryover effect

Carry over evaluation was carried out to ensure that precision and accuracy of the procedure are not affected. The carry over for the analytes and IS was within the acceptance limit. No interfering peaks co-eluted with analytes and IS after subsequent injection of ULOQ (fig.4).

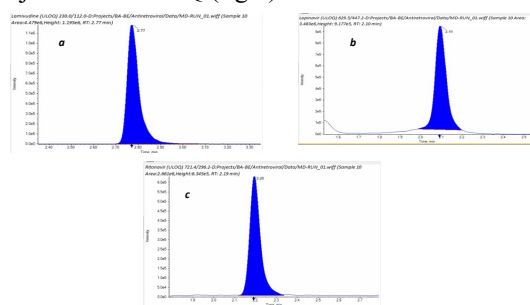


Figure 4: LC-MS/MS chromatogram for with 3TC (a), LPV (b), RTV (c) Upper limit of quantification (ULOQ) concentration 2500 ng/mL by PPT.

Linearity

A calibration curve was constructed with matrix basis and applied to determine the analyte concentration in unknown samples. The calibration curves for 3TC, LPV and RTV were found to be linear. Linearity response was assessed in the

concentration ranges 6.129-3000 ng/mL for 3TC and 50.433-20173.143 ng/mL for LPV and 50.358-20143.302 ng/mL for RTV. The calibration curve lines were drawn to give the linear regression equations $Y = mX + C$. The correlation coefficient square (r^2) was 0.99068, 0.9940 and 0.99645 for 3TC, LPV and RTV respectively. Detailed results obtained are shown in table 1 and calibration curves are displayed in figure 5,6,7.

Analyte	Nominal concentration (ng/mL)	Back calculated concentration (ng/mL)	% Accuracy	Parameters
3TC	6.129	5.600	91.369	r^2: 0.99068 - Slope: 0.021393 - Intercept: 0.000179 - Regression equation: $y = 0.0026x + 0.04442$ - Linearity range: 6.129-3000 ng/mL - LLOQ: 6.139 ng/mL
	12.259	12.100	98.703	
	153.232	155.210	101.291	
	306.465	291.550	95.133	
	612.930	590.950	96.414	
	1225.859	1120.330	91.391	
	1838.789	1730.850	94.130	
	2604.951	2388.620	91.695	
	3064.648	2752.550	89.816	
LPV	50.433	49.255	97.664	r^2: 0.99700 - Slope: 0.021393 - Intercept: 0.000179 - Regression equation: $y = 0.00244x + 2.41953$ - Linearity range: 50.433-20173.143 ng/mL - LLOQ: 50.433 ng/mL
	100.866	93.544	92.741	
	1008.657	991.120	98.261	
	2017.314	1980.552	98.178	
	4034.629	3654.251	90.572	
	8069.257	7685.983	95.250	
	12103.886	11789.562	97.403	
	17147.172	16586.362	96.729	
		19152.395	94.940	
	20173.143			
RTV	50.358	47.365	94.057	r^2: 0.99822 - Slope: 0.021393 - Intercept: 0.000179 - Regression equation: $y = 0.00362x + 0.35735$ - Linearity range: 50.358-20143.302 ng/mL
	100.717	92.782	92.121	
	1007.165	978.350	97.139	
	2014.330	1899.652	94.307	
	4028.660	3892.889	96.630	
	8057.321	7896.452	98.003	
	12085.981	11789.623	97.548	
	17121.807	16489.220	96.305	
	20143.302	18796.636	93.315	

3TC: Lamivudine. LPV: Lopinavir, RTV: Ritonavir

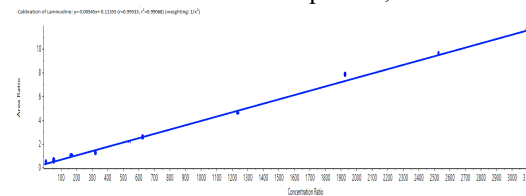


Figure 5: Calibration curve of 3TC

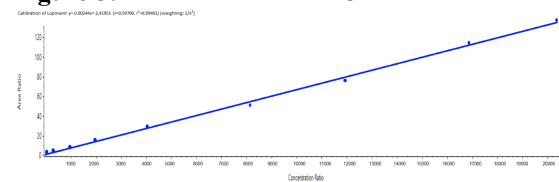


Figure 6: Calibration curve of LPV

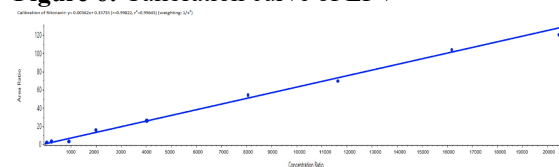


Figure 7: Calibration curve of RTV

Precision and Accuracy

Three P & A batches were analysed including intraday and interday batches. Six samples of each quality control (QC) were analysed against a single standard calibration curve (CC). A standard CC for all the batches was meet the acceptance criteria. All standard CC point was found within acceptable limit. Correlation coefficient (r^2) for all the calibration curve was found > 0.99 . Quality control (QC) samples were within acceptance criteria (QC Samples within $\pm 15\%$ limit and for LLOQ QC $\pm$

20% limit). Precision & Accuracy batch found within acceptance criteria for 3TC, LPV and RTV. Table 2 displays the outcomes of precision.

Table 2. Outcomes of precision

An alyt es	Le vel of QC	Nom inal conc entr ation (ng/ mL)	Inter batac h		Intra batac h	
			Mea n foun d conc entr ation (ng/ mL)	% CV	Mea n foun d conc entr ation (ng/ mL)	% CV
3T C	LL		6.159	12.	5.883	1
	QC			53		2
		6.073				.
						5
						8
	LQ		15.99	8.7	15.66	9
	C	16.39	3	3	6	.
		7				1
						8
	M		1115.	6.4	1122.	5
LP V	QC	1062.	345	4	000	.
		774				7
						4
	H		2378.	7.2	2423.	3
	QC	2353.	144	2	049	.
		285				9
						8
	LL		47.98	7.4	49.34	1
	QC	50.41	1	5	6	1
		6				.
						5
						1
	LQ		137.1	7.0	138.5	6
	C	138.1	77	1	04	.
		39				5
						9
	M		7140.	4.7	6965.	5
	QC	7058.	950	4	285	.
		196				1
						8

H QC		1571	6.0	1531	4
		1562	8.185	1	4.937
		8.862			7
					0
RT V	LL QC	50.2 27	46.12 3	8.77 37	49.5 10.0 0
	LQ C	137. 622	139.3 73	6.40 148.	10.7 6
					8.71
	M QC	703 1.75	7044. 576	6.10 692	6.56
		6		2	
					5.07
	HQ C	155 70.3	1523 7.892	5.03 12.4	155
		17		48	

3TC: Lamivudine. LPV: Lopinavir, RTV: Ritonavir, CV: Coefficient of variation, LLOQC: Lower Limit Quality Control, LQC: Low-Quality Control, MQC: Middle-Quality Control, HQC: High-Quality Control

Reinjection reproducibility

All standard CC point was passing through acceptable limit. Corelation coefficient (r^2) for calibration curve was found 0.9996 for 3TC, 0.99401 for LPV and 0.99645 for RTV respectively. Quality control (QC) samples were within acceptance criteria (QC Samples within $\pm 15\%$ limit and for LLOQ QC within $\pm 20\%$ limit).CC and QC meets the Acceptance Criteria hence reinjection reproducibility batch passed the validation parameter.

Hemolysis Effect

Concentration of hemolyzed LQC and HQC was back calculated against the standard CC. concentration of hemolyzed LQC and HQC was found within acceptance criteria of quality control samples (QC Samples within $\pm 15\%$ limit). Hemolysis effect passes the validation criteria.

Plasma Stability Studies

Stability studies were performed at LQC and HQC level. Concentration of fresh QC and Stability QC samples were back calculated using respective CC. Bench top stability was performed at 9 hours and found within acceptance criteria for 3TC, LPV and RTV. Processed sample stability was performed after 20 hours and meet the required guideline for all three drugs. Freeze thaw stability for 3TC, LPV and RTV was performed at 3 and 5 cycles passing through acceptance limit. Autosampler stability studies were investigated after 56 hours for 3TC, LPV and RTV found within a limit.

Table 3. Summary of Validation Results

Parameter		Q	3TC	LPV	RTV
		C			
Selectivity	Analyte (%CV at LLOQ)	-	5.23	3.69	3.99
	Internal standard (% CV at IS)	-	0.77	0.77	0.77
Specificity		-	Spec	Spec	Spec
Carryover	Analyte (%Interference LLOQ)	-	1.68	0.55	0.54
	IS (% Interference)	-	0.10	0.10	0.10
Linearity	Linearity range (ng/mL)		6.12	50.43	50.35
			9-3064	3-2017	8-2014
			.648	3.143	3.302
	r ²	-	0.99	0.997	0.998
	Regression equation		y = 0.00545x + 0.11355	y = 0.00244x + 0.53	y = 0.00262x + 0.357
Matrix effect	Precision, CV (%)	L	1.83	1.89	2.94
		Q			
		C			
Recovery		H	0.36	0.04	0.04
		Q			
		C			
	Mean (%)	L	92.8	91.91	91.76
		Q	4		
		C			
		M	90.9	91.91	92.28
		Q	8		
		C			
		H	91.3	94.71	94.38
		Q	0		
		C			

The developed LC–MS/MS method provided efficient chromatographic separation with well-resolved peaks for 3TC, LPV, RTV and TFV within a short runtime of 4 min. No significant endogenous interference was observed in blank plasma samples, demonstrating excellent selectivity. Protein precipitation using acetonitrile produced consistent and reproducible recoveries for all three analytes along with internal standard. The method exhibited

good linearity over the selected concentration ranges with correlation coefficients exceeding 0.99. Accuracy and precision values were within acceptable limits. The recovery of analytes was consistent and reproducible across all QC levels. Matrix effect studies indicated negligible ion suppression or enhancement.

Stability studies confirmed that 3TC, LPV and RTV remained stable under various storage and processing conditions. The use of TFV as an internal standard improved method reproducibility and minimized analytical variability.

CONCLUSIONS

A rapid, selective and sensitive LC–MS/MS method was successfully developed and validated for simultaneous estimation of Lamivudine, Lopinavir and Ritonavir in human plasma using Tenofovir as internal standard. The method demonstrated acceptable accuracy, precision, recovery, matrix effect and stability according to bioanalytical validation guidelines. The short runtime and simple protein precipitation extraction make the method suitable for high-throughput pharmacokinetic and bioequivalence studies. The proposed method for simultaneous estimation of 3TC, LPV and RTV were found to be simple, accurate, economical and rapid. It is suitable for routine bioanalysis, pharmacokinetic investigations and therapeutic drug monitoring studies involving antiretroviral drugs.

Conflicts of Interest

The authors declare no conflict of interest.

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