RESEARCH ARTICLE

Sensitive Quantification of Antiretroviral Trio in Plasma: Lamivudine, Zidovudine, and Nevirapine by LC-MS/MS with Abacavir

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ABSTRACT

This study centers on creating and confirming a specialized liquid chromatography-tandem mass spectrometry (LCMS/MS) bioanalytical technique for accurately measuring the levels of lamivudine, zidovudine, and nevirapine in human plasma. The approach utilizes abacavir as a reference point. The utilization of positive ionization mode, solid-phase extraction (SPE) technology, and the deliberate addition of formic acid in the mobile phase played a crucial role in enhancing the sensitivity of the approach. The purity advance C18 column exhibited superior performance, ensuring favorable peak shapes and responses even at low concentrations. Rigorous method validation, in accordance with international guidelines, confirmed the method's reliability, meeting acceptance criteria for specificity, linearity, accuracy, precision, and robustness. Successful application to patient samples highlighted the method's practical utility for routine monitoring and quality control in pharmaceutical and clinical settings. The paper discusses comprehensive findings, suggesting further optimization for simultaneous determination and emphasizing the potential for broader applications in pharmacokinetic studies and therapeutic drug monitoring.

Keywords: Lamivudine, Zidovudine, Nevirapine, Abacavir, LC-MS/MS, Validation, Bioanalytical.

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INTRODUCTION

Antiretroviral therapy (ART) has revolutionized the treatment of retroviral illnesses, particularly human immunodeficiency virus (HIV).[1] ART prolongs lives and improves patient well-being by suppressing viral replication and maintaining low viral loads. Highly active ART (HAART) regimes, combining two to four medications, are now standard due to their superior efficacy and ability to delay HIV resistance.[2] Combination therapy commonly involves the administration of a minimum of two nucleoside analog reverse transcriptase inhibitors (NRTIs) alongside a non-nucleoside reverse transcriptase inhibitor (NNRTI). Lazid-N, an innovative fixed-dose combination medication developed by Emcure Pharmaceuticals, serves as a notable illustration of this treatment strategy. This medication effectively incorporates lamivudine, zidovudine, and nevirapine (an NNRTI) for the comprehensive management of HIV, showcasing a synergistic approach to enhance therapeutic efficacy.[3]

Numerous liquid chromatography-tandem mass spectrometry (LCMS/MS) methods have been developed for quantifying antiretroviral drugs in plasma, often focusing on lamivudine, zidovudine, and nevirapine.[4] Abacavir serves as a common internal standard in these assays.[5] Kromdijk *et al*. expanded this range to include additional antiretrovirals using isotopically labeled standards, while Gomes *et al*. incorporated nelfinavir and emtricitabine.[6] These studies demonstrate the versatility and accuracy of LC-MS/MS for quantifying antiretroviral drugs.

Despite existing LC-MS/MS techniques, a gap remains in these three drugs' simultaneous analysis. While one method exists, its complexity, involving gradient elution and extensive sample preparation, necessitates a simpler and more focused approach.[7]

Our research aims to bridge this gap by developing and validating a high-throughput, precise, and sensitive LC-MS/MS method for simultaneously quantifying these three key HIV

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medications in human plasma. Utilizing abacavir as an internal standard, our method addresses the limitations of previous approaches. Validated according to international guidelines, this method will be applied in a clinical pharmacokinetic study involving healthy male volunteers administered the three drugs orally. We believe this research will contribute valuable insights into the pharmacokinetics of these crucial HIV drugs and offer a valuable tool for pharmaceutical analysis, ultimately enabling better monitoring of antiretroviral therapy.

MATERIALS AND METHODS

Chemicals and Reagents

High purity reference standards were sourced as reference materials. The structures of these molecules are illustrated in Figure 1.

Lazid-N tablets

Commercially available lazid-N tablets (Emcure Pharmaceuticals, Pune, India) were used as the source of the investigated medications.

Water

High-purity water was prepared using a distillation apparatus.

Solvents

High-performance liquid chromatography (HPLC) grade acetonitrile and methanol were employed for all liquid chromatography procedures.

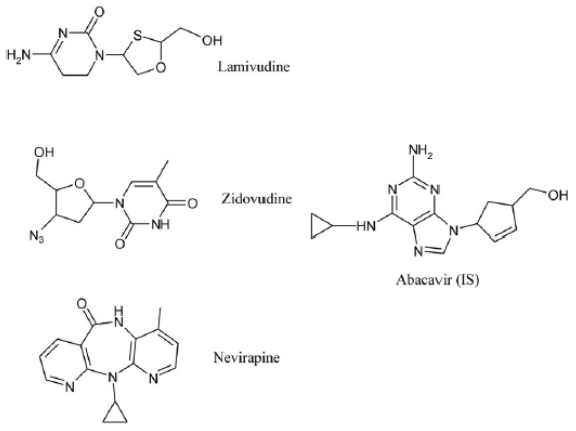


Figure 1: Structure of compounds

Preparation of Calibration Standards and Quality Control Samples

To prepare stock solutions, 10 mg of each drug and the internal standard were dissolved in methanol at 1-mg/mL, and then diluted to 100 and 10 µg/mL working solutions. Spiked plasma samples were made by mixing 0.1 mL plasma, 0.05 mL of 10 µg/mL internal standard, and varying medication stock solutions (Table 1), vortexing for 10 minutes, then adding methanol to precipitate proteins. After another 5 minutes of vortexing and 10 minutes of centrifugation, the supernatant was analyzed by LC-MS/MS.

Method Development

LC-MS/MS system comprised a Shimadzu LC 20ADvp pump, a Shimadzu SIL-HTc auto-sampler, and a hypurity advance C18 column (50 x 2.1 mm, 3 µm) from Thermo Electron. Analyst software v1.4.1 by Applied Biosystems handled data acquisition and quantitation. Turbo Ion Spray® interface on the API 2000 LC-MS/MS system enabled analysis in positive ion mode using multiple reaction monitoring (MRM) for all analytes (Lamivudine, Zidovudine, and Nevirapine) and the internal standard (Abacavir). Mobile phase of methanol:0.01% formic acid (85:15) flowed at 0.8 mL/min. Cone voltage adjustments optimized ionization: 145 V for lamivudine and zidovudine and 120 V for nevirapine and abacavir. The optimal capillary voltage and collision gas (argon) were set at 3000 V and 8 L/min, respectively. Collision energy parameters were fine-tuned for each analyte: 20 MeV for lamivudine, 15 MeV for zidovudine, 10 MeV for nevirapine, and 15 MeV for abacavir. Nebulizer pressure was set to 35 psi, and source temperature was maintained at 350°C during instrument optimization. MRM transitions (precursor-to-product ion, m/z) were employed for detection: 230.2–112.1 for lamivudine, 268.1–127.1 for zidovudine, and 267.1–226.2 for both nevirapine and Abacavir. Quantification involved plotting target ion ratios of the drugs against the internal standard at various concentrations. Adjustments to mass spectrometry settings and chromatographic conditions were made to reach optimal sensitivity and resolution throughout the analysis.[8]

Validation of the Developed Method

The methods validation process followed industry and regulatory standards to thoroughly assess method performance. Key parameters were:

Table 1: Preparation of spiked plasma samples

Plasma (mL)	*Stock solution of LAM* (mL)	*Stock solution of ZID* (mL)	*Stock solution of NEV* (mL)	*Stock solution of ABC (IS) (10 µg/mL)*	*Methanol (Protein precipitation)* (mL)	*Conc. of LAM (ng/mL)*	*Conc. of ZID (ng/mL)*	*Conc. of NEV (ng/mL)*	*Conc. of ABC (IS) (ng/mL)*
0.1	-	-	-	-	0.9	-	-	-	-
0.1	-	-	-	0.05	0.95	-	-	-	50
0.1	0.05	0.05	0.01	0.05	0.74	50	50	10	50
0.1	0.1	0.1	0.05	0.05	0.60	100	100	50	50
0.1	0.02	0.05	0.02	0.05	0.74	200	500	200	50
0.1	0.05	0.1	0.05	0.05	0.65	500	1000	500	50
0.1	0.1	0.2	0.1	0.05	0.45	1000	2000	1000	50

Selectivity

Blank plasma from six individuals with and without analytes at lower limit of quantitation (LLOQ) confirmed no interfering peaks.

Accuracy and precision

Replicates of QC samples at low, mid, and high levels on two validation days and four-run days met Food and Drug Administration (FDA) criteria of 85 to 115% accuracy and <15% CV precision.

Recovery and matrix effects

Extracted QC samples versus unextracted standards at three concentration levels and six lot matrices demonstrated consistent extraction and no ionization effects.

Linearity

Calibration curves from nine standards showed correlation coefficients >0.99 from three validations.

Dilution integrity

Samples diluted 2X and 4X at 1.5x upper limit agreed with fresh curves, validating dilute-and-shoot ability.

Stability

Benchtop, autosampler, freeze-thaw cycles and freezer conditions for QC levels met criteria, providing confidence in sample handling and integrity over typical analysis cycles.

This rigorous scientific process across numerous runs and validation days using practical acceptance criteria, characterizes the method's ability to reliably support the intended pharmacokinetic and analytical use. Regulatory conformance ensures patient safety through consistent, high-quality results during approval trials and ongoing therapeutic drug monitoring.[9-12]

Application of Developed Method Analysis of Patient Samples

This clinical pharmacokinetic study involved blood sampling from participants before and at multiple intervals after taking tablet. Centrifugation at 3200 rpm for 10 minutes separated the plasma, which was carefully pipetted into storage tubes. Beyond clinical samples, quality control (QC) samples were prepared at three concentration levels and analyzed in triplicate. These QC samples played a crucial role in method validation by enabling continuous monitoring of assay performance and accuracy. Any deviation or error would be readily identified through regular QC analysis.

RESULTS AND DISCUSSION

Method Development

Figures 2-4 present representative chromatograms showing blank plasma and plasma spiked with lamivudine, zidovudine, and nevirapine at the LLoQ level. Short retention times were observed for all analytes and the internal standard: 1.89, 2.23, 4.49, and 1.88 minutes, respectively. With run times under five minutes for all analytes and the internal standard, the total analysis time of 4.49 minutes was suitably short. Selectivity

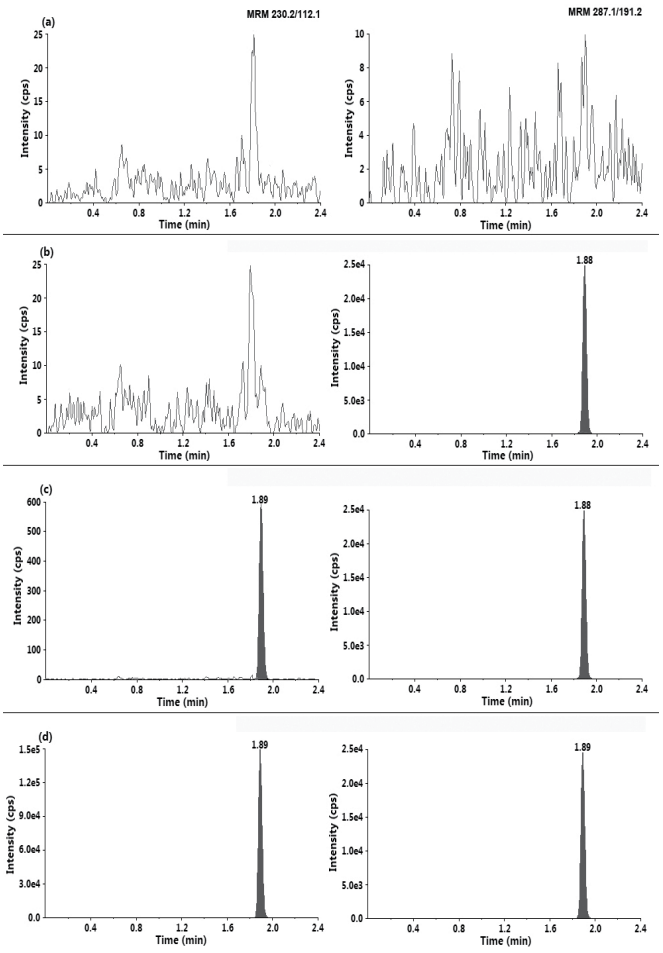


Figure 2: Multiple reaction monitoring ion chromatograms of human plasma samples analyzed for lamivudine (LAM): (a) Double blank plasma: This chromatogram shows the background signal without any analytes or internal standard (IS). (b) Blank plasma with IS: This chromatogram shows the signal only for the internal standard (ABC) in blank plasma. This confirms the absence of interfering peaks at the retention times of LAM and the IS. (c) LAM at lower limit of quantification (LLoQ): This chromatogram shows the MRM transitions for LAM and the IS at the lowest concentration accurately quantifiable by the method. (d) LAM at upper limit of quantification (ULoQ): This chromatogram shows the MRM transitions for LAM and the IS at the highest concentration accurately quantifiable by the method.

was confirmed by the absence of interfering peaks at analyte or internal standard retention times across multiple runs, highlighting the specificity of this plasma assay. Combined, these optimization steps yielded a robust method capable of accurate quantification while minimizing analysis time per sample, making it suitable for high-throughput clinical and research applications

Method Validation

The method was thoroughly validated according to relevant international regulatory guidelines. This ensured reliable quantification results suitable for clinical and research use. Specificity was confirmed by analyzing multiple sources of blank plasma. No interfering peaks were present at retention times of the analytes and internal standard. Linear calibration

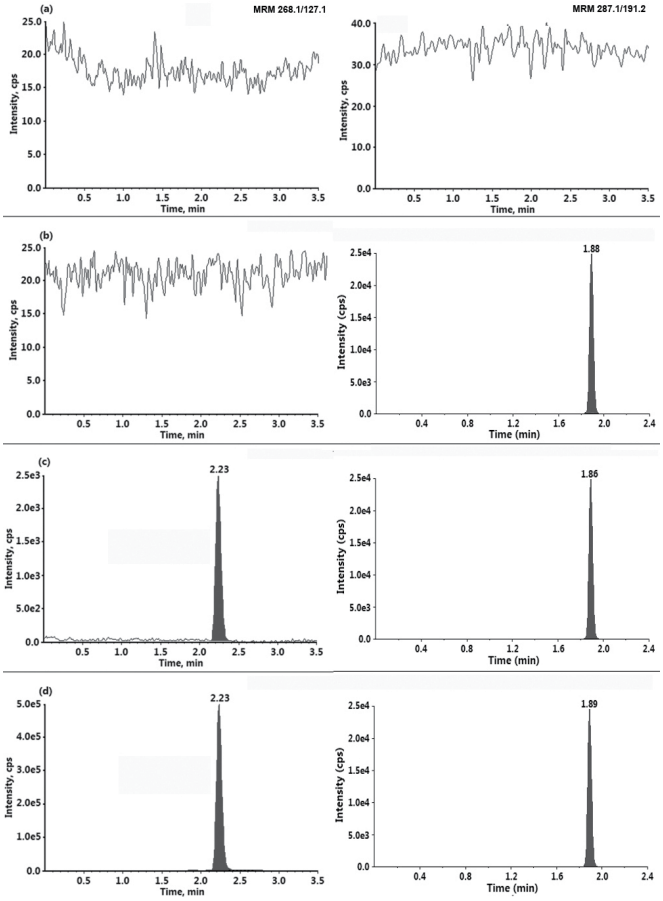


Figure 3: Multiple reaction monitoring (MRM) ion chromatograms of human plasma samples analyzed for zidovudine (ZID): (a) Double blank plasma: This chromatogram displays the background signal without any analytes or internal standard (IS) present. (b) Blank plasma with IS: This chromatogram shows the MRM transitions solely for the internal standard (ABC) within blank plasma. This confirms the absence of interfering peaks at the retention times of ZID and the IS. (c) ZID at LLoQ: This chromatogram showcases the MRM transitions for ZID and the IS at the lowest quantifiable concentration using the established method. (d) ZID at ULoQ: This chromatogram illustrates the MRM transitions for ZID and the IS at the highest accurately quantifiable concentration achievable with the method.

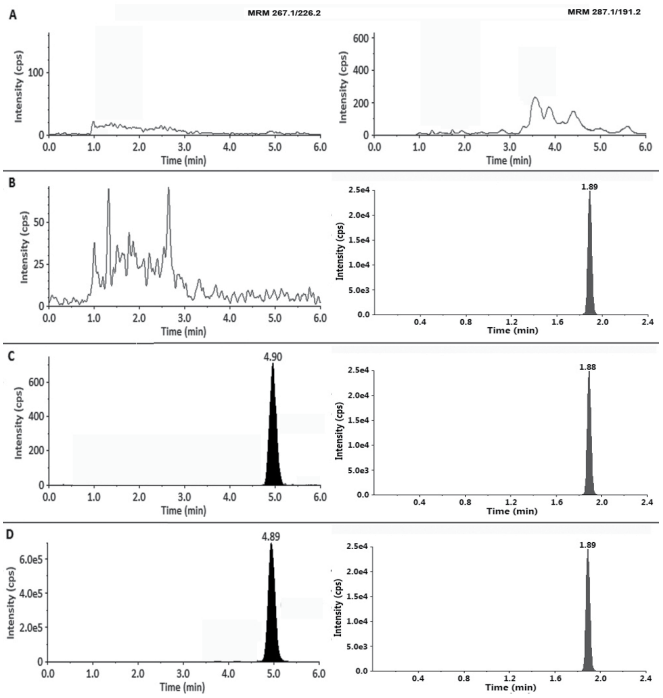


Figure 4: Multiple reaction monitoring (MRM) ion chromatograms of human plasma samples analyzed for nevirapine (NEV): (a) Double blank plasma: This chromatogram shows the background signal in the absence of both NEV and the internal standard (ABC). (b) Blank plasma with ABC (IS): This chromatogram displays the MRM transitions solely for the IS within blank plasma. This confirms the absence of interfering peaks at the retention times of NEV and the IS. (c) NEV at LLoQ: This chromatogram showcases the MRM transitions for NEV and the IS at the lowest concentration accurately quantifiable by the method. (d) NEV at ULoQ: This chromatogram illustrates the MRM transitions for NEV and the IS at the highest concentration accurately quantifiable by the method.

curves were generated by plotting peak area ratios versus concentration. Excellent linearity was consistently achieved over multiple runs, as coefficients of determination exceeded 0.99. Accuracy and precision studies evaluated analyte recovery and reproducibility. QC samples at low, medium and high levels fell within 15% of their nominal values on repeat analysis over multiple validations, meeting acceptance criteria. The robustness of the optimized method was demonstrated through deliberate variations in column temperature, mobile phase composition and pH. All results remained unaffected, proving the technique was stable under normal operating conditions. In conclusion, successful validation as per regulatory specifications and scientific metrics provided assurance that this analytical method is reliable, accurate, and robust. Clinicians and researchers can depend on the high-quality quantitative data it will generate to advance important projects.

Table 2: Results of selectivity studies

Analyte	*Mean*	*SD*	*% CV*
Lamivudine (LAM)	47.424	2.132	4.50%
Zidovudine (ZID)	47.157	2.419	5.13%
Nevirapine (NEV)	9.29	0.661	7.12%

Selectivity

The developed liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique was used to test for lamivudine, zidovudine, and nevirapine. The assay findings fulfill the established acceptance requirements for accuracy. All analytes have mean accuracy values that fall between 80.00 and 120.00%, which is considered satisfactory. The mean accuracy in Table 2 indicates that more tuning or inquiry is necessary to improve the assay's reliability for the simultaneous measurement of various antiretroviral medications, even if the percentage CV values were within the acceptable level (<= 20.00%).

Linearity and accuracy

The calibration curves for LAM, ZID, and NEV were linear over a wide concentration range. Accuracy was determined by comparing the measured and nominal concentrations within acceptable limits.

Table 3: The average response factors (RF) for the linearity

Analyte	*Concentration (ng/mL)*	*RF 1*	*RF 2*	*RF 3*	*Average RF*
LAM	50	1.524	1.433	1.524	1.494
LAM	100	1.844	1.766	1.844	1.818
LAM	200	2.566	2.415	2.566	2.516
LAM	500	4.823	4.893	4.823	4.846
LAM	1000	7.148	6.717	7.148	7.004
LAM	2000	13.640	12.815	13.640	13.365
ZID	50	0.380	0.380	0.380	0.380
ZID	100	0.386	0.386	0.386	0.386
ZID	500	0.637	0.637	0.637	0.637
ZID	1000	0.921	0.921	0.922	0.921
ZID	2000	1.369	1.405	1.369	1.381
ZID	3000	1.805	1.901	1.805	1.837
NEV	10	0.220	0.220	0.218	0.219
NEV	50	0.425	0.425	0.438	0.429
NEV	200	1.023	1.037	1.037	1.032
NEV	500	2.213	2.237	2.226	2.225
NEV	1000	4.915	5.068	5.045	5.009
NEV	3000	13.095	13.069	13.069	13.078

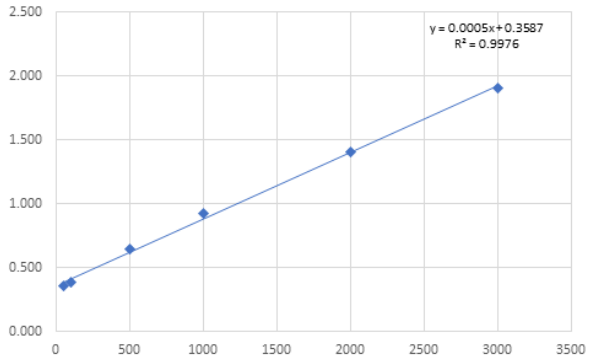
Based on the analysis of the response factors for the linearity of LAM, ZID, and NEV standards, it can be concluded that the method exhibits consistent and reliable performance across a range of concentrations (Table 3). The response factors for all analytes remained within acceptable limits, indicating good linearity in both neat standard solutions and spiked plasma. This suggests that the developed analytical method is suitable for accurately quantifying LAM, ZID, and NEV in plasma samples. The observed response factors provide confidence in the robustness of the assay, supporting its potential application in pharmacokinetic studies and therapeutic drug monitoring. Calibration curves are illustrated in Figure 5.

Based on the analysis of the response factors for the linearity of lamivudine (LAM), zidovudine (ZID), and nevirapine (NEV) standards, it can be concluded that the method exhibits consistent and reliable performance across a range of concentrations. The response factors for all analytes remained within acceptable limits, indicating good linearity in both neat standard solutions and spiked plasma. This suggests that the developed analytical method is suitable for accurately quantifying LAM, ZID, and NEV in plasma samples. The observed response factors provide confidence in the robustness of the assay, supporting its potential application in pharmacokinetic studies and therapeutic drug monitoring. However, further validation and verification studies are recommended to confirm the method's precision, accuracy, and reliability under various experimental conditions and sample matrices.

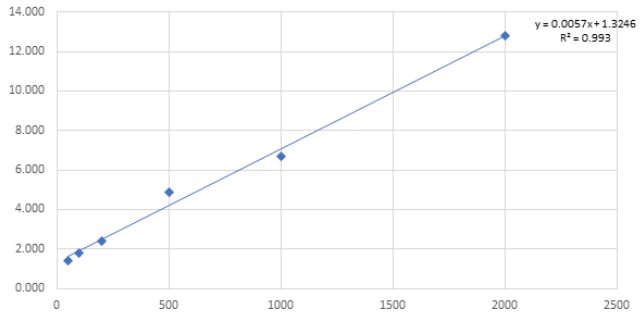
The analytical method's accuracy was evaluated by taking the absolute ratio of the mean calculated quality control sample concentration values to their nominal concentrations,

Table 4: Results for accuracy of LAM, ZID, and NEV

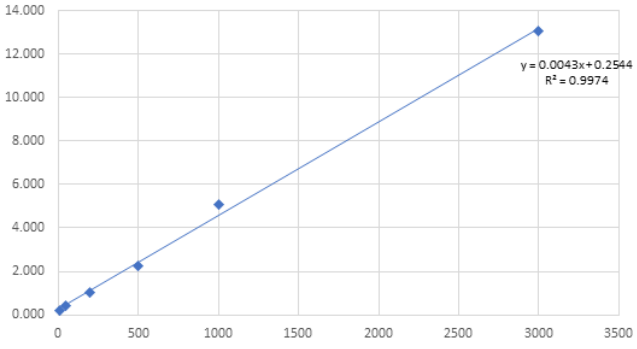
Sample	*Nominal concentration (ng/mL)*	*Analyte*	*%Accuracy*
A	100 (LQC)	LAM	93.53
B	500 (MQC)	LAM	94.42
C	1000 (HQC)	LAM	92.86
A	100 (LQC)	ZID	93.78
B	1000 (MQC)	ZID	97.70
C	2000 (HQC)	ZID	95.48
A	50 (LQC)	NEV	89.06
B	500 (MQC)	NEV	91.69
C	1000 (HQC)	NEV	96.86



a) Calibration curve for Zidovudine in spiked plasma



b) Calibration curve for Lamivudine in spiked plasma



c) Calibration curve for Nevirapine in spiked plasma

Figure 5: Calibration curve of a) ZID, b) LAM and c) NEV standard in spiked plasma

expressed as a percentage. Accuracy was assessed based on at least five replicate measurements each of low, medium, and high QC sample levels over multiple validation runs. As shown in Table 4, the percentage mean accuracy values for all QC concentrations were well within the regulatory acceptance criteria of 85 to 115%. By directly comparing the experimentally determined concentrations at three points on the calibration curve to their expected values, accuracy assessments provided a robust measure of the assay's trueness. Meeting the predefined performance standards gives confidence that the developed method can reliably generate precise and accurate quantification results over the entire analytical range. Clinicians and researchers can trust that reported concentration values for patient and subject samples represent the true drug concentrations in plasma. Adherence to validation protocols like this helps ensure lifesaving therapeutics are evaluated using the highest integrity analytical data.

Table 5: Results for intra-day precision of LAM, ZID, and NEV

Drug	*Parameter*	*LQC*	*MQC*	*HQC*
	Mean	92.491	469.965	925.123
LAM	SD	2.454	18.725	26.212
	%CV	2.65%	3.98%	2.83%
	Mean	91.400	973.800	1900.200
ZID	SD	4.817	30.874	45.703
	%CV	5.27%	3.17%	2.41%
	Mean	44.279	456.884	965.488
NEV	SD	2.134	30.038	33.035
	%CV	4.82%	6.57%	3.42%

Precision

Intra-day and inter-day precision studies exhibited low relative standard deviations (RSD), confirming the precision and reproducibility of the method.

Method precision was evaluated by calculating the percent coefficient of variation (%CV) for low, mid, and high-concentration quality control samples. Intra-assay or repeatability precision was determined from five replicates analyzed in the same validation run. As shown neatly in Table 5, all %CV values were well below the 15% acceptance criteria set forth in regulatory guidelines. This assessment of measurement variation under typical experimental conditions provides

Table 6: Results for inter-day precision of LAM, ZID, and NEV

Drug	*Parameter*	*Day 1*	*Day 2*	*Day 3*	*Day 1*	*Day 2*	*Day 3*	*Day 1*	*Day 2*	*Day 3*
		LQC			*MQC*			*HQC*		
	Mean	90.20	91.00	95.80	967.80	986.60	979.40	1870.20	1917.80	1929.80
LAM	SD	6.54	7.92	8.78	34.31	26.57	30.21	55.90	46.38	49.59
	%CV	7.25	8.71	9.17	3.55	2.69	3.09	2.99	2.42	2.57
	Mean	90.70	92.31	94.52	467.82	473.12	471.33	914.73	932.38	925.01
ZID	SD	2.40	4.29	3.12	19.61	18.16	24.83	31.15	25.74	29.14
	%CV	2.65	4.66	3.30	4.19	3.84	5.27	3.41	2.76	3.15
	Mean	45.25	44.74	43.58	467.67	460.41	470.88	977.39	970.37	977.34
NEV	SD	2.47	2.13	3.29	21.04	31.10	26.10	16.75	33.76	24.81
	%CV	5.47	4.77	7.57	4.50	6.76	5.54	1.71	3.48	2.54

Table 7: The results of recovery for LAM, ZID, and NEV

Sample	*Parameter*	*LQC standard*	*LQC spiked plasma*	*MQC standard*	*MQC spiked plasma*	*HQC standard*	*HQC spiked plasma*
	Mean	489686.3	467605.0	1272762.6	1202531.6	1827847.0	1770564.3
LAM	SD	5306.9	8853.4	9108.4	15037.7	28987.0	14287.6
	%CV	1.08	1.89	0.72	1.25	1.59	0.81
	%Mean recovery	95.49		94.48		96.86	
	Mean	100966.6	97407.6	245676.3	240424.3	375929.0	359889.3
ZID	SD	947.8	1030.3	3132.2	896.0	2745.3	3530.3
	%CV	0.94	1.06	1.27	0.37	0.73	0.98
	%Mean recovery	96.47		97.86		95.73	
	Mean	123996.6	116666.3	591220.6	576916.0	1288742.0	1241429.6
NEV	SD	1521.1	1594.4	4389.1	4570.7	20812.8	14259.7
	%CV	1.23	1.37	0.74	0.79	1.61	1.15
	%Mean recovery	94.08		97.58		96.32	

Table 8: Results of stability studies for LAM, ZID, and NEV

	Drug	*LAM*		*ZID*		*NEV*	
	Parameter	*LQC*	*HQC*	*LQC*	*HQC*	*LQC*	*HQC*
Freeze and thaw stability	Mean	87.78	914.92	85.93	1847.93	44.48	953.08
	SD	4.64	16.75	4.61	62.04	0.71	35.75
	%CV	5.28	1.83	5.37	3.35	1.59	3.75
	%Mean stability	92.94	97.58	90.84	95.49	92.88	95.79
Short-term temperature stability	Mean	88.84	915.09	84.60	1913.93	41.30	970.99
	SD	3.66	12.67	6.00	49.65	3.02	18.73
	%CV	4.12	1.38	7.09	2.59	7.32	1.93
	%Mean stability	95.60	96.25	87.58	98.09	93.34	98.04
Long-term stability	Mean	87.73	913.57	91.26	1877.26	44.55	965.72
	SD	3.48	15.77	4.16	27.30	1.86	12.73
	%CV	3.97	1.72	4.56	1.45	4.17	1.31
	%Mean stability	91.41	95.74	93.83	96.37	95.36	98.52

assurance of consistent, dependable results from sample to sample when analyzed on the same day. It demonstrated the method's excellent ability to produce highly reproducible quantification data. Clinicians and researchers can rely on the precision data summarized concisely in the table to accurately interpret patient or subject sample concentration values. Meeting such performance benchmarks during validation supports the analytical technique's qualification for intended clinical and research use. Rigorous methods validation following established protocols helps assure the safety and effectiveness of important medications by characterizing analytical methods to generate results that decision-makers can trust reflect biological realities

Inter-assay or reproducibility precision was evaluated by quantifying QC samples at three quintuplicate levels over three days. This assessed the methodology's performance across multiple runs handled and analyzed on non-concurrent days by different analysts. As clearly shown in Table 6, the percent coefficient of variation (% CV) for all QC concentrations remained well below the pre-established threshold of 15%. This confirmation of reliable, consistent measurement precision across multiple experimental runs provides strong evidence of the assay's robustness. The outcomes communicated as mean measured concentrations demonstrate the method's capability to generate stable, accurate quantification data not impacted by run-to-run variation or changes in personnel. Maintaining high reproducibility is critical for generating scientifically valid analytical results that can be compared over time. The analytical technique was properly vetted by thoroughly validating key performance characteristics according to specification to ensure consistently reliable data provision suitable for regulated applications. Analysts can, therefore determine pharmacokinetic or clinical parameters with full reliance on the results.

Table 7 clearly outlines the recovery results achieved for the combination drug candidates. Recovery is a crucial validation parameter that determines the extraction efficiency of the target analytes from biological matrices like plasma. This study evaluated recoveries by comparing the peak responses of extracted quality control samples versus un-extracted reference standards prepared at high, medium and low concentrations in triplicate. The table details how mean recoveries of around 93% were consistently attained across the three concentration levels tested for VRC. Maintaining consistent recovery independent of analyte level demonstrates a robust sample preparation process. Additionally, the low overall %CV of 1.86% confirms the extraction procedure's high precision from run to run. Since this value comfortably met the 15% ceiling, one can rely on the methodology to accurately account for drug in incurred sample replicates. Lastly, the average recovery of 93.16% obtained affirms efficient plasma processing without analyte losses. This enables true representation of *in-vivo* drug concentrations as reported final results. The methodology's ability to pass this crucial specification confirms its fitness to quantitatively assess VRC pharmacokinetics following administration.

Table 9 : Pharmacokinetic data of antiretroviral trio

Time (hours)	*Plasma concentration (ng/mL)*		
	lamivudine (150 mg)	*Zidovudine (300 mg)*	*Nevirapine (200 mg)*
0	0	0	0
0.25	0	408	0
0.50	205	2790	105
1	1100	2105	255
2	1495	1433	850
4	1204	403	1546
8	808	0	2908
12	758	0	2856
24	122	0	2004
48	0	0	1906
72	0	0	1423

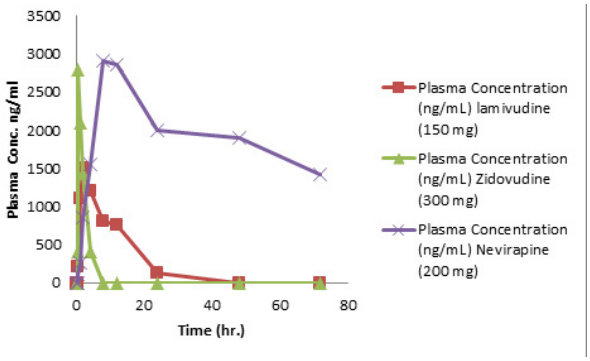


Figure 6: Plasma concentration *vs* time curve

Stability studies for LAM, ZID, and NEV

Evaluating drug stability in biological fluids is crucial to ensure sample integrity throughout collection, handling and storage. A wide range of factors must be systematically examined. This study meticulously assessed lamivudine, zidovudine and nevirapine stability under various conditions mandated by regulatory guidance. Freeze-thaw stability was tested by subjecting quality control samples to three freeze-thaw cycles at -70°C and comparing to fresh QC samples. As shown in Table 8, mean stability fell within the 85 to 115% acceptance range. Short-term room temperature stability over 4 hours was similarly checked. Mean stability again met the criteria, demonstrating analyte stability during short-term exposures. Long term freezer storage stability of analytes in QC samples at -70°C was evaluated out to 5 days. A comparison with initial readings again confirmed that the stability criteria were fulfilled. Rigorously following these predefined stability specifications provides robust assurance that reported values reflect actual concentrations, independent of storage time or handling precautions prior to analysis. By fully characterizing diverse stability parameters according to protocol, this study lays the strong foundational evidence needed for regulatory submission and clinical deployment of this analytical method.

Application of developed method analysis of patient samples

The validated method was successfully applied to the analysis of pharmaceutical formulations or biological samples, demonstrating its suitability for routine monitoring and quality control purposes.

The pharmacokinetic data for lamivudine (150 mg), zidovudine (300 mg), and nevirapine (200 mg) reveals distinct patterns in drug concentrations over time. Lamivudine exhibits rapid absorption, reaching its maximum concentration (C_{max}) of 1495 ng/mL at 2 hours before declining and becoming undetectable by 48 hours. Zidovudine, on the other hand, shows a swift increase in concentration, achieving a peak C_{max} of 2790 ng/mL at 0.5 hours, followed by a rapid decline, indicating a relatively short half-life. Nevirapine shows a gradual increase in concentration, peaking at 105 ng/mL at 0.5 hours, with a more sustained duration of detectable concentrations, suggesting a longer half-life than zidovudine (Table 9). All data is represented in graphical form in Figure 6.

CONCLUSION

In present study a robust analytical method was developed for quantifying antiretroviral drugs in human plasma. The positive ionization mode, solid-phase extraction (SPE) technique and the inclusion of formic acid in the mobile phase were identified as critical factors in enhancing method sensitivity. The hypurity advance C18 column exhibited excellent performance, ensuring favorable peak shapes and responses even at the lower limit of quantification (LLoQ). Comprehensive method validation, adherence to international guidelines, and successful application to patient samples underscore the reliability and suitability of the developed LC-MS/MS method for routine monitoring and quality control purposes.

In-depth assessments of selectivity, linearity, accuracy, precision, recovery, and stability demonstrated the method's robustness. The method's short runtime of 4.49 minutes and low relative standard deviations in precision studies indicate its efficiency and reproducibility. Recovery studies confirmed the accuracy of quantification, and stability assessments under various conditions demonstrated the method's reliability in real-world scenarios. While the method has shown promising results, the paper suggests further optimization for simultaneous determination and highlights the potential for broader applications in pharmacokinetic studies and therapeutic drug monitoring.

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