Turk J Pharm Sci 2025;22(3):191-206
DOI: 10.4274/tjps.galenos.2025.70718

ORIGINAL ARTICLE

Simultaneous Quantification of Doravirine, Lamivudine, and Tenofovir Disoproxil Fumarate in Human Plasma by UPLC-MS/MS: Method Development and Validation

Narasimha KANJARLA, Balaraju KATTA*

Chaitanya (Deemed to be University), Department of Pharmacy, Hyderabad, Telangana, India

ABSTRACT

Objectives: A novel, high-throughput liquid chromatography tandem mass spectrometry (UPLC–MS/MS) technique has been developed that uses Etravirine (ETR) as the internal standard (IS) to simultaneously quantify Doravirine (DOR), Lamivudine (LAM), and Tenofovir Disoproxil Fumarate (TDF) in human plasma. The procedure employs a precipitation extraction technique to analyze analytes from human plasma. This study aims to develop and validate a novel and reliable stability-indicating UPLC–MS/MS method for the simultaneous determination of DOR, LAM, and TDF in human plasma, using ETR as the IS.

Materials and Methods: ETR, based on its stable-isotopic nature and structural and physicochemical similarity to the analytes of interest, was used as an IS. Precipitation extraction was the technique used to prepare samples. An agilent zorbax XDB C18 analytical column (2.1 × 50 mm, 3.5 μm) was used for chromatographic separation, and its isocratic mobile phase consists of acetonitrile and buffer (5 mM of ammonium formate with 0.1 % formic acid) in the ratio 80:20, *v/v*, at a flow rate of 0.120 mL/min.

Results: The parent-to-product ion transitions for the drugs were as follows : LAM: m/z 231.08 amu → 112.00 amu, TDF: m/z 288.33 amu → 176.17 amu, DOR: m/z 426.38 amu → 112.02 amu, and IS ETR: m/z 437.36 amu → 164.97 amu. These transitions were observed using a triple quadrupole mass spectrometer in the multiple reaction monitoring (MRM) positive ion mode. The compound's basic group content determined which positive mode to choose. For DOR, LAM and TDF, the method was validated throughout concentration ranges of 2.5–1000 ng/mL with correlation coefficients (r^2) values obtained were found to be 0.99. From spiked plasma samples, the mean recovery outcomes were observed and found to be DOR, LAM, and TDF was 83.39%, 87.33%, and 85.56%. With a 3.0-minute total run time, the approach was shown to be reliable and quick.

Conclusion: A triple quadrupole mass spectrometer running in the MRM positive ion mode was used to track these transitions. The compounds' functional group content served as the basis for choosing the positive mode. The mean recovery values were obtained for three APIs from spiked plasma samples. The run times were found to be both reliable and quick. The method was proven to produce precise and specific results for the determination of selected drugs through the current study. The method is stable when exposed to various stress conditions, demonstrating minimal degradation. The current method was validated as per the ICH M10 guidelines and was found to meet the desired acceptance criteria. The developed bioanalytical method, validated in accordance with ICH M10 guidelines, demonstrated high accuracy, precision, and reproducibility for the simultaneous quantification of DOR, LAM, and TDF. Its streamlined design and reliable performance make it a valuable tool for routine analysis.

Keywords: Doravirine, Tenofovir Disoproxil Fumarate, lamivudine, UPLC-MS/MS, quantification

---

***Correspondence:** balrajkatta@gmail.com, **ORCID-ID:** orcid.org/0009-0002-8297-0934

Received: 19.05.2024, **Accepted:** 02.06.2025 **Publication Date:** 01.08.2025

Cite this article as: KANJARLA N, KATTA B. Simultaneous quantification of Doravirine, Lamivudine, and Tenofovir Disoproxil Fumarate in human plasma BY UPLC-MS/MS: method development and validation. Turk J Pharm Sci. 2025;22(3):191-206

INTRODUCTION

In the domain of pharmaceutical research, the creation of precise and efficient analytical methods for quantifying drug compounds in biological matrices is crucial for ensuring the effectiveness and safety of therapeutic interventions. Human immunodeficiency virus (HIV) represents one of the most pressing global health issues of our time, impacting millions worldwide. HIV belongs to the lentivirus family, a type of retrovirus known for its ability to target and debilitate the immune system, particularly CD4 cells (T-helper cells), which are vital for coordinating the body's immune response against infections.[1,2]

The US Food and Drug Administration approved the tablet [containing Doravirine (DOR), Lamivudine (LAM), and Tenofovir Disoproxil Fumarate (TDF): 100 mg/300 mg/300 mg] on August 30, 2018 to treat HIV-1 infection in adults who have never received antiretroviral therapy. Alternatively, they were be used to replace an existing antiretroviral regimen in people who were virologically suppressed for at least six months on a stable antiretroviral regimen, had no history of treatment failure, and had no known substitutions linked to resistance to these tablets.[2]

Data from clinical trials showing the effectiveness of combining DOR, LAM, and TDF in lowering HIV virus type 1 (HIV-1) viral load and raising cluster of differentiation four positive ($CD4^+$) cell counts served as the foundation for the approval. With this approval, HIV treatment has advanced significantly, and patients now have a powerful, well-tolerated choice for treating their condition.[3-6] The fixed-dose combination pill was meant to be taken orally once a day, with or without meals. It functions by inhibiting the replication of HIV, thereby decreasing the viral load in the body and delaying the advancement of the disease.

This research aimed to develop and validate a novel and reliable stability-indicating high-throughput liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method for the simultaneous determination of DOR, LAM, and TDF (Figure 1-3) in human plasma. Developing and validating such a method is pivotal for facilitating accurate pharmacokinetic studies, monitoring therapeutic drug levels, and ensuring compliance with regulatory standards.

It is observed that no methods are available for the "Simultaneous Quantification of DOR, LAM, and Tenofovir Disoproxil Fumarate in Human Plasma by UPLC-MS/MS". Different publications were available for the simultaneous estimation of these drugs using HPLC and HPTLC methods in tablet formulation.[13-20] While HPLC methods are indeed well-established, UPLC-MS/MS offers superior sensitivity, specificity, and the ability to detect and quantify analytes at lower concentrations, which is critical for the simultaneous estimation of DOR, LAM, and TDF in a biological matrix. These advantages make UPLC-MS/MS a more suitable choice for the objectives of our work. Hence, the current experimental study will be beneficial for the simultaneous estimation of the aforementioned drugs in a biological matrix.

MATERIALS AND METHODS

Resources and techniques

Working standards and references

The samples of LAM, TDF, and DOR (with 99% *w/w* purity) were obtained from Hetero Labs Limited, Hyderabad. The same supplier provided the 99% *w/w* pure ETR, which was used as an internal standard (IS).

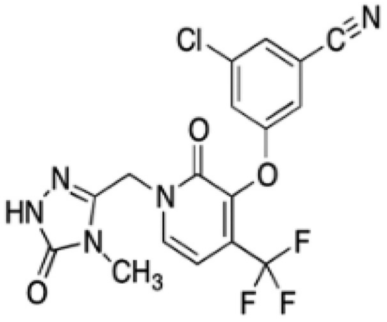


Figure 1. Structure of Doravirine

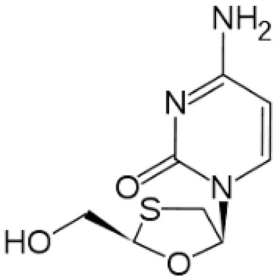


Figure 2. Structure of Lamivudine

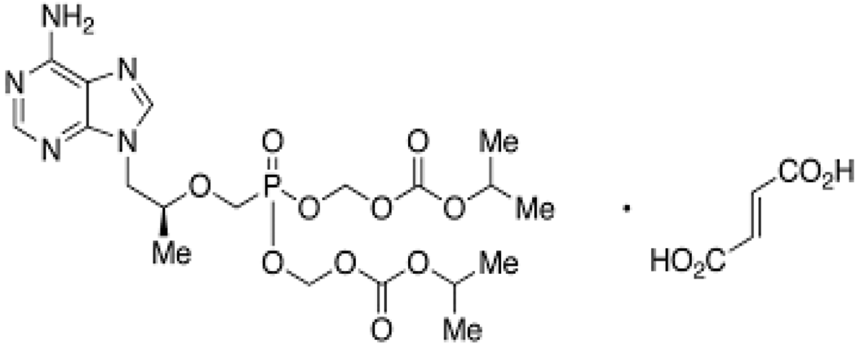


Figure 3. Structure of Tenofovir Disoproxil Fumarate

Chemicals-reagents

The research employed the following reagents and chemicals: HPLC-grade methanol (from Merck), K2EDTA human plasma, Milli-Q water (from Merck), and acetonitrile (from Merck).

Instruments utilized

Waters Acquity UPLC and Waters Quattro Premier XE Mass Spectrometer systems were utilized for method development and validation of the simultaneous quantification of DOR, LAM, and TDF. The UPLC system comprised a tunable UV detector, a quaternary pump, and an auto-injector. The MS/MS system, featuring a heated electrospray ionization (ESI) probe, delivers high sensitivity and ultra-fast detection. Designed to minimize contamination, the mass spectrometer incorporates a high-temperature heating block, heated ESI probe, drying gas, and heated desolvation line. Data analysis and interpretation were performed using MassLynx software (Version 4.1). Collectively, this instrumentation provides a robust platform for accurate and efficient quantification of the target compounds.

Equipment and UPLC-MS/MS assay conditions

In this study, a SIL HTC, a high-performance autosampler, a column oven (CTO-AS), a degasser DGU-20A3, and an Acquity UPLC system from Waters were used. The Quattro Premier XE type mass analyzer from Waters was the apparatus used for mass spectrometric detection in multiple reaction monitoring (MRM) mode. The data processing was performed using MassLynx, version 4.1, and the analysis was performed using a positive ionization interface.

Conditions of MS

The fundamental principle of MS revolves around the generation and detection of ions separated based on their mass-to-charge (m/z) ratios. In the method developed for this study, 500 ng/mL solutions of analytes and IS were prepared using a mixture of acetonitrile and buffer in an 80:20 ratio. These solutions were then infused into the mass spectrometer with an injection volume of 10 µL. Initially, full scan mode was employed to scan the parent and corresponding fragment ions for each analyte and IS pair. Subsequently, following the parent ion's identification, scanning was performed through MS/MS to obtain the resultant ions. Nitrogen gas was utilized as the collision gas, while zero air served as the sheath gas. Unit mass was used to measure the resolution. The fragment ion with the highest intensity, which was used for multiple reaction monitoring, was chosen for quantification.

Tuning of MS

The process confirmed the successful identification of parent ions and daughter ions for all analytes based on their molecular weights. Therefore, the analytes have been proved to be polar and ionizable. Consequently, for the investigation, the ESI method was chosen.

Optimized conditions

Instrument Setup: The analysis was performed using the Waters ACQUITY UPLC System coupled with a QUATTRO PREMIER XE mass spectrometer.

Ionization Conditions: Positive ion mode was selected as the polarity, with Electron Ion Spray (EIS) used as the ion source.

Detected Ions:

DOR: Parent ion at 426.38 amu; Daughter ion at 112.02 amu

TDF: Parent ion at 288.33 amu; Daughter ion at 176.17 amu

LAM: Parent ion at 231.08 amu; Daughter ion at 112.00 amu

Etravirine (ETR) (used as IS): Parent ion at 437.36 amu; Daughter ion at 164.97 amu

Chromatographic Conditions: Separation was achieved using an Agilent Zorbax XDB C18 column (2.1 × 50 mm, 3.5 µm particle size). The column oven temperature was maintained at 30 °C, and the Peltier temperature was set to 10 °C.

Mobile Phase & Flow Parameters: The mobile phase consisted of acetonitrile and buffer in an 80:20 *(v/v)* ratio, delivered at a flow rate of 0.120 mL/min. A sample volume of 5.0 µL was injected using a partial loop with needle overfill technique.

Retention Times & Run Duration: Tenofovir DF: 1.11 min, LAM: 1.00 min, DOR: 1.20 min and ETR (IS): 1.72 min. Total run time was 3.0 minutes. The mass spectra are illustrated in Figures 4-11.

Extraction procedure

Protein precipitation serves as a method to mitigate matrix interference in the analyte. Precipitating agents like trichloroacetic acid and perchloric acid are employed for this purpose. The precipitating agent is diluted with the sample matrix before it is vortexed. Subsequent centrifugation and filtration were employed to eliminate high-molecular-weight proteins. The obtained filtrate was analyzed. It's essential that the reconstituted solvent easily dissolve any protein precipitated during the precipitation process.

Procedure for extracting samples

Each sample was placed into a 5 mL polypropylene tube and weighed at approximately 200 milligrams. To this, the sample was mixed with 1 mL of acetonitrile (a precipitating agent) and 50 µL of Internal Standard (ISTD) solution (1 µg/mL ETR). Vortexing thoroughly mixed the materials to aid in precipitation. Eight-tenths milliliters of the liquid supernatant were collected after precipitation, placed in vials, and then injected into the UPLC-MS/MS for examination.

Procedure optimization

The ideal chromatographic conditions were established by analyzing the standard solutions of the three analytes.

Preparation of mobile phase

0.1% formic acid solution

Precisely dispense 1.0 mL of formic acid. Dissolve the dispensed formic acid in 1000 mL of water (HPLC grade). Filter and sonicate the solution for 10 minutes to ensure complete mixing and dissolution.

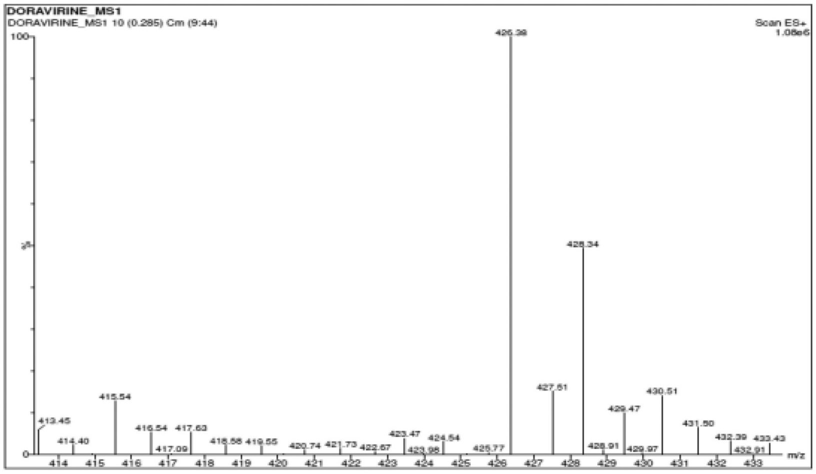


Figure 4. Parent ion of Doravirine

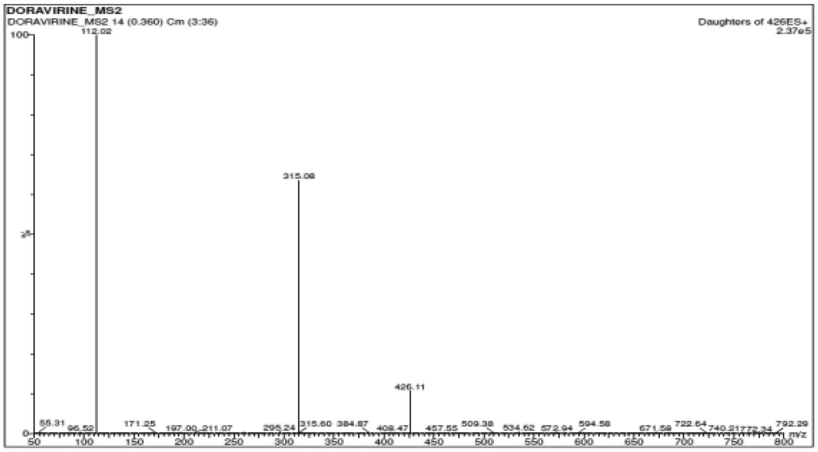


Figure 5. Daughter ion of Doravirine

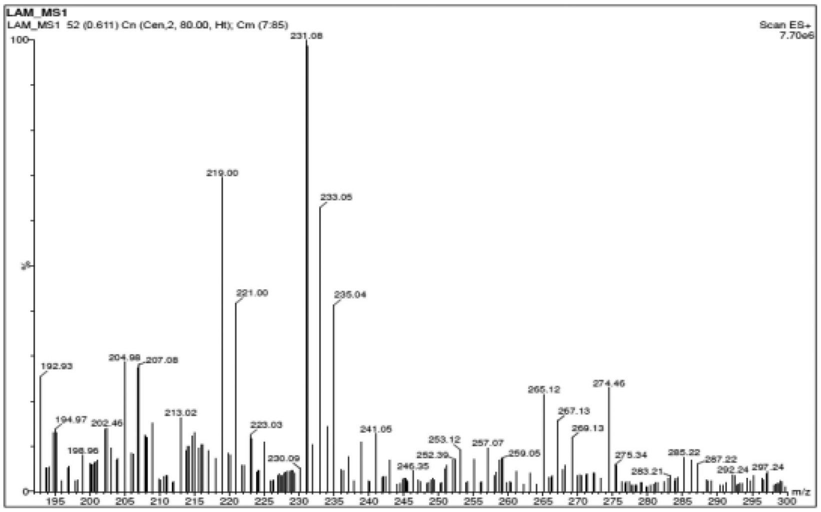


Figure 6. Parent ion of Lamivudine

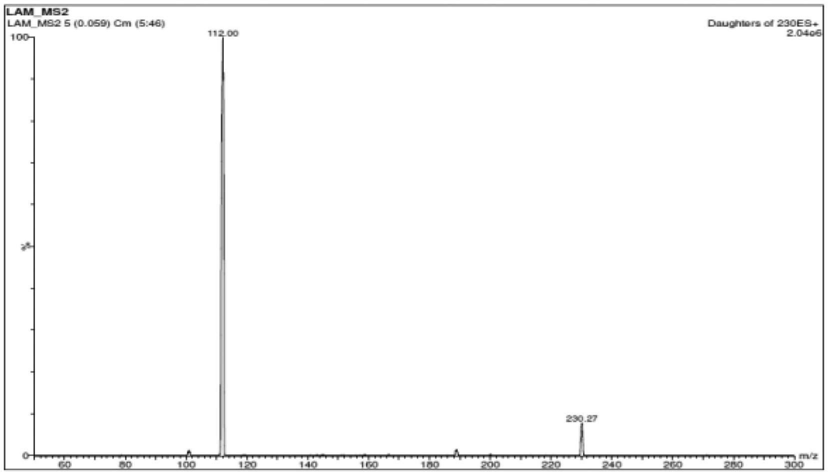


Figure 7. Daughter ion of Lamivudine

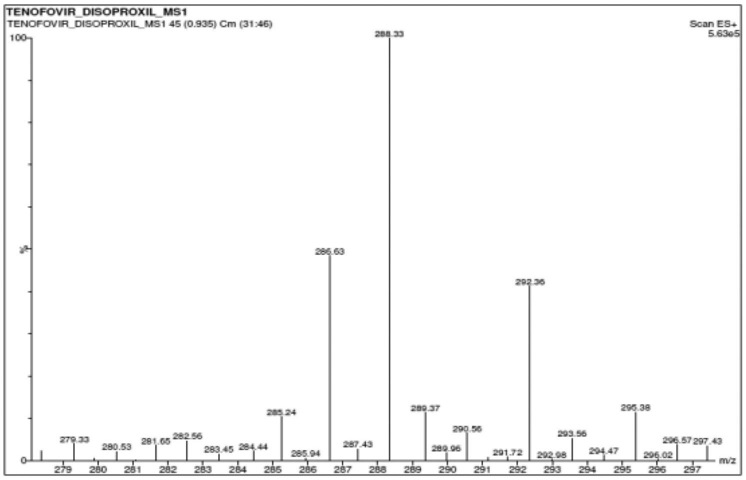


Figure 8. Parent ion of Tenofovir Disoproxil Fumarate

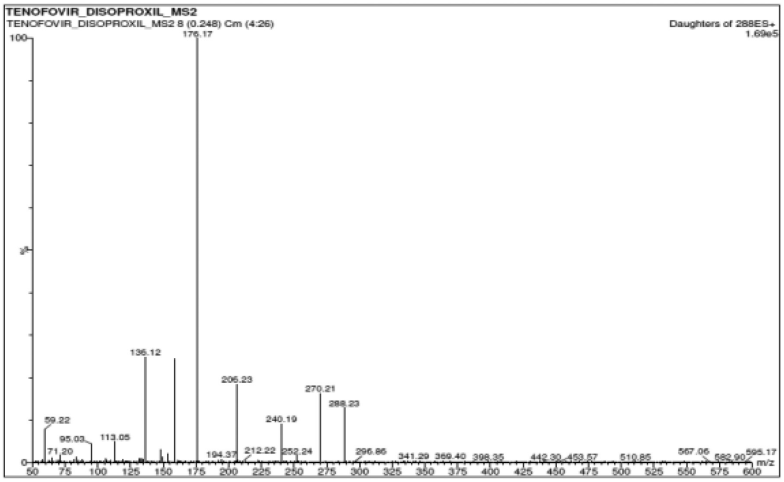


Figure 9. Daughter ion of Tenofovir Disoproxil Fumarate

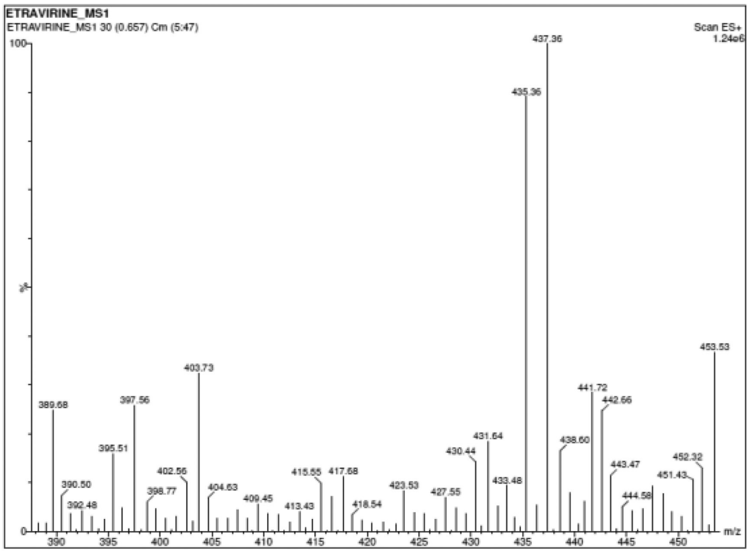


Figure 10. Parent ion of

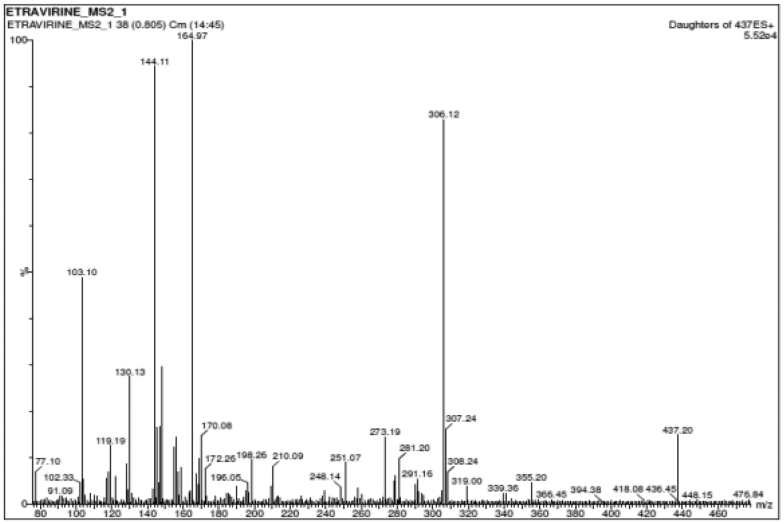


Figure 11. Daughter ion of Etravirine

Preparation of buffer

Combine 5 mM of ammonium formate with 0.1% formic acid.

Preparation of mobile phase

Accurately measure 800 mL (80%) of acetonitrile and 200 mL (20%) of buffer. Mix the components thoroughly and filter using the vacuum.

Diluent preparation

Measure 800 mL of acetonitrile and 200 mL of water to obtain an 80% acetonitrile solution. Filter the mixture using a vacuum (0.45 μm).

Needle wash & seal wash solutions

Diluent solution (80% acetonitrile).

Internal standard solution

Mix ETR to achieve a concentration of 1 μg/mL using a combination of methanol: water in a 50:50 volume ratio (*v/v*).

Preparation of standard solutions of DOR, LAM, & TDF

Typical stock resolutions

Weigh 1.0 mg of each analyte (DOR, LAM, TDF) and ISTD (ETR) in 10.0 mL flasks. Add 5.0 mL methanol, and then dilute each solution to the mark with methanol/water in the ratio 50:50 (*v/v*).

Mixed standard solution

Accurately weigh 1 mg of each analyte (DOR, LAM, TDF) into a 1 mL diluent. Vortex the solution thoroughly to dissolve. This results in a concentration of 1000 μg/mL for each analyte. This solution constitutes the mixed stock solution containing DOR, LAM, and TDF at a concentration of 1000 μg/mL each.

Working solutions

Pipette 20 μL of each solution into a 2 mL diluent, achieving a concentration of 10 μg/mL or 10000 ng/mL. Pipette 200

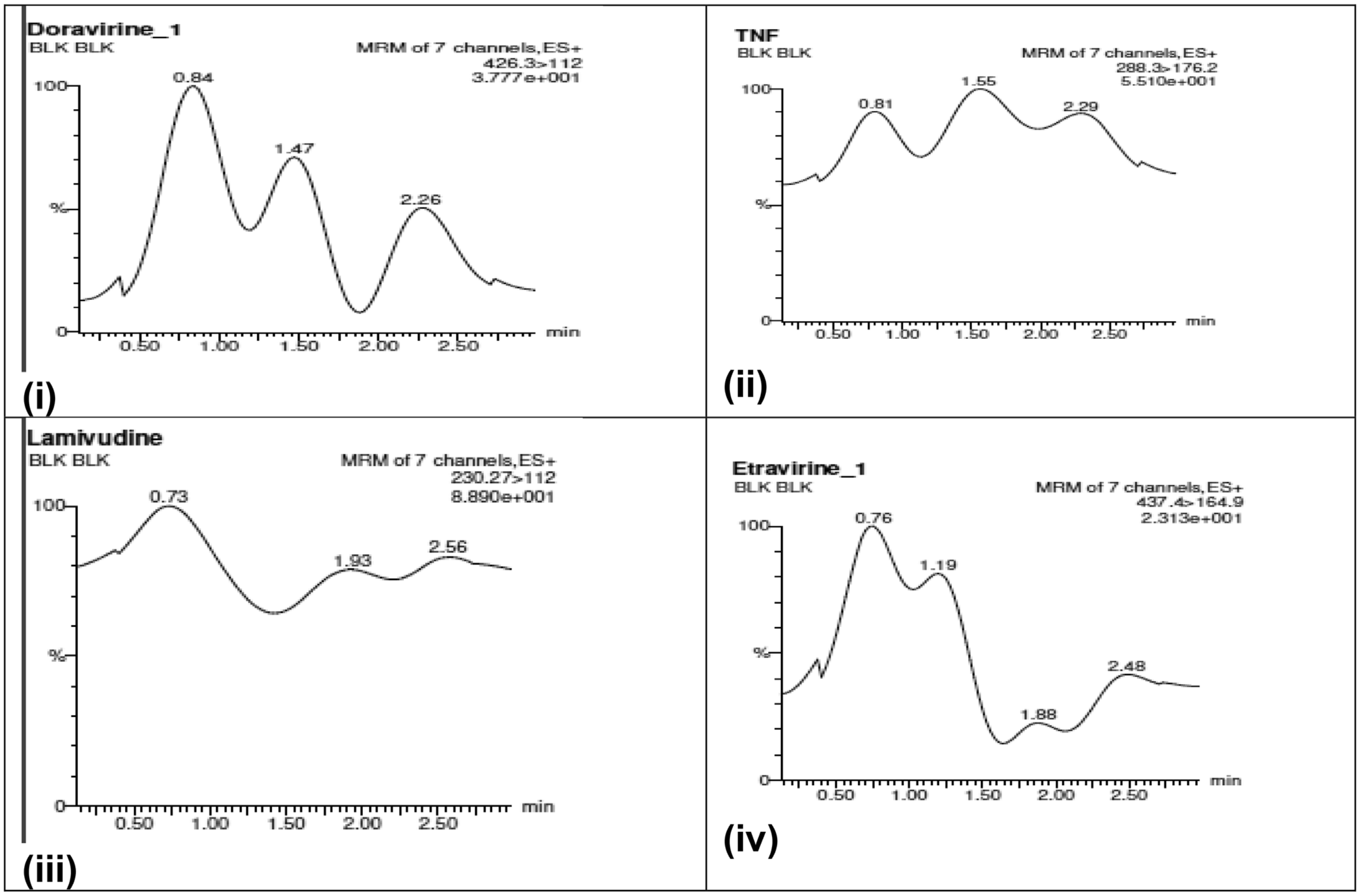


Figure 12. Blank Chromatograms of (i) DOR, (ii) TDF, (iii) LAM & (iv) ETR
DOR: Doravirine, TDF: Tenofovir disoproxil fumarate, LAM: Lamivudine, ETR: Etravirine

µL of each resulting solution into a 2 mL diluent to obtain a concentration of 1000 ng/mL using a methanol:water mixture in the ratio 50:50 (*v/v*).

Preparing quality control samples and plasma-spiked calibration standards

Calibration standards were prepared at 2.5, 10, 50, 250, 500, and 1000 ng/mL concentrations for the combination of DOR, LAM, and TDF. Quality control (QC) samples were prepared at concentrations of 7.4 ng/mL for Low-Quality Control (LQC), 480 ng/mL for Middle-Quality Control (MQC), and 900 ng/mL for High-Quality Control (HQC) for DOR, LAM & TDF.

Sample extraction

To guarantee uniformity, 950 µL of human plasma was used in each analyte sample. After adding 1 mL of acetonitrile (ACN) to the mixture, the centrifugation was run for 10 min, followed by vortexing for 5 min. After centrifugation, 0.8 mL of the solution's supernatant was carefully taken from the supernatant, put into vials, and then injected into the UPLC-MS/MS apparatus for examination.

Criteria for system suitability and mobile phase

Each composition was determined, resulting in the preparation of a large volume of mobile phase combining 800 mL of acetonitrile with 200 mL of buffer. This was done to ensure comparable outcomes throughout the study and validation. For System Suitability Testing (SST), a mobile phase was prepared to achieve the Lower LLOQ concentration, which was 2.5 ng/mL in this case. The System Suitability Test (SST) should be assessed.

Method development

After injecting the analyte solution, each analyte's parent ion weight was scanned. Furthermore, the parent ions were examined to determine the product ions in MS/MS mode. Scanning was conducted in the range of 100 to 600 atomic mass units. All ion peaks were eluted accurately. During the optimization of compound parameters for all three analytes, TDF required relatively high collision energy to achieve an appropriate response, and the respective mass chromatograms are detailed in Figures 12 and 13 below.

Method validation

The concentration of a medication, and possibly its metabolites, in biological samples, including blood, urine, plasma, serum, and tissue extracts, can be quantitatively measured using a bioanalytical technique. The process of method validation guarantees that the analytical approach yields precise results for the quantitative evaluation of drugs.

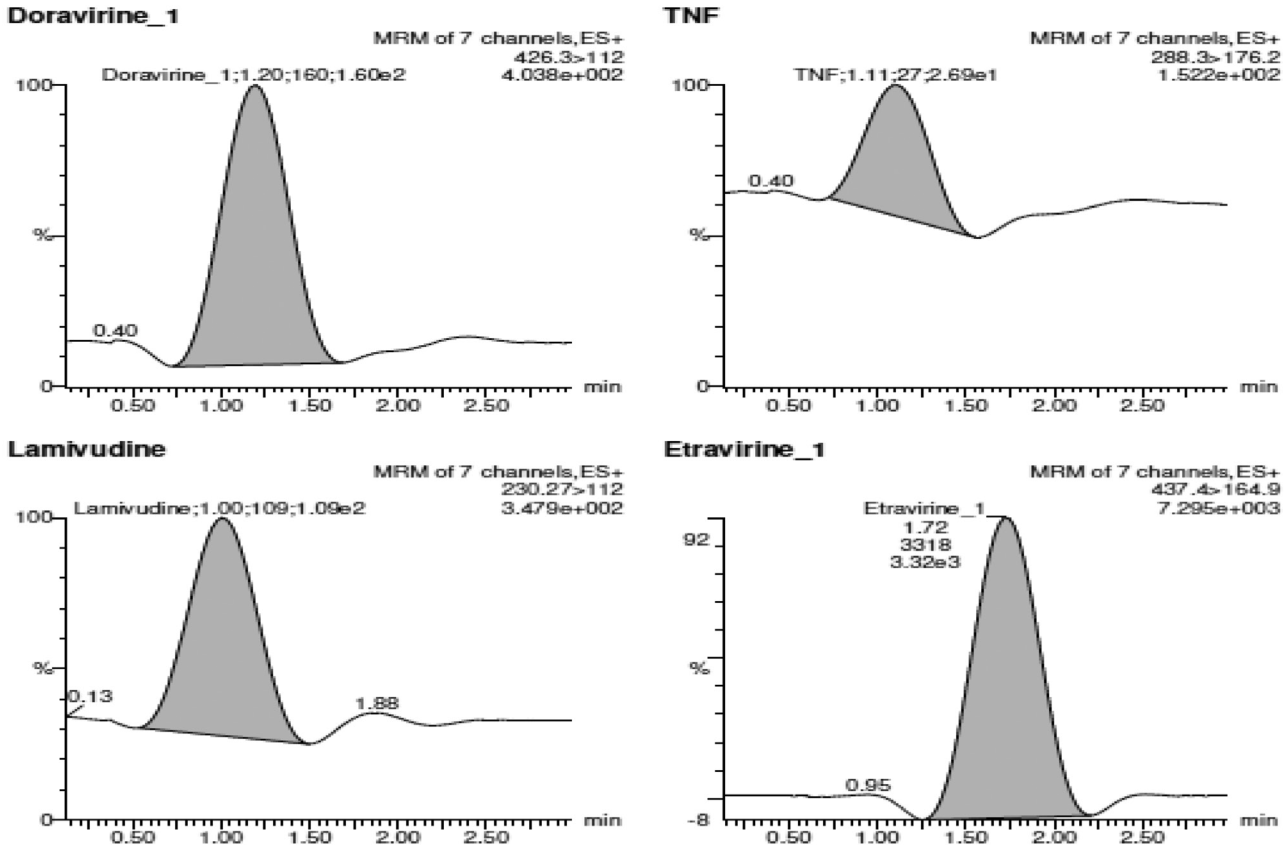


Figure 13. Sample Chromatograms of (i) DOR, (ii) TDF, (iii) LAM & (iv) ETR
DOR: Doravirine, TDF: Tenofovir disoproxil fumarate, LAM: Lamivudine, ETR: Etravirine

Method validation encompasses various parameters, including:

Specificity-sensitivity

The capacity to measure and distinguish the analyte signal when the sample contains the expected constituents or excipients. The capacity to measure and distinguish the analyte signal when the sample contains the expected constituents or excipients. *Procedure:* Six replicates were used to analyze a blank sample of 200 μL of human plasma at the LLOQ value of 2.5 ng/mL.

Selectivity

The capacity of the analysis technique to detect the presence of endogenous components in the matrix, such as metabolites, impurities, or decomposition products.

Procedure: Analysis was performed on plasma, LLOQ sample, and blank human plasma using an IS.

Calibration curve

Constructed by testing within the same biological matrix sample solutions that were intended, and spiking the matrix with known analyte concentrations.

Linearity

The ability of the bioanalytical procedure to yield outcomes that are exactly proportionate to the concentration of the sample within the standard curve's range.

Procedure: To find the linear range, concentration levels containing at least five to eight standards are used. The details for the preparation of spiked plasma are given in Table 1. The calibration curves for DOR, LAM, and TDF were determined to be linear, with correlation coefficients (r^2) exceeding 0.99 across the concentration range of 2.5 to 1000 ng/mL.

Quantification range

In this study, 2.5 ng/mL was found to be the LLOQ based on the established linearity range, and the Upper Limit of Quantification (ULOQ) was determined to be 1000 ng/mL for DOR, LAM, and TDF samples.

Fresh samples

Fresh sample QCs are essential for assessing the accuracy and stability of analyte molecules. They help evaluate method performance and analysis stability, ensuring the correctness and accuracy of the technique. These performance QCs play a crucial role in validating the reliability and precision of the analytical method.

Procedure: The study's QC samples are chosen to assess the precision and stability of an established method. They are prepared in duplicate and cover a minimum of three concentrations, including the LLOQ, the mid-range, and the high end. The methods used to prepare spiking plasma samples of the three subject analytes (DOR, LAM, TDF) are detailed in Table 2 as follows.

Table 1. Information on how to prepare spiked plasma

Plasma spiking preparations

Concentration of stock (μg/mL)	Stock volume (mL)	Plasma volume (mL)	Final volume (mL)	Final concentration (ng/mL)	Details
20.00	0.1	0.90	1.000	1000	CC6
10.00	0.1	0.90	1.000	500	CC5
5.00	0.1	0.90	1.000	250	CC4
1.00	0.1	0.90	1.000	50	CC3
0.10	0.1	0.90	1.000	10	CC2
0.04	0.1	0.90	1.000	2.5	CC1

Table 2. The specifics of the three QC sample preparation stages for Doravirine, Lamivudine, and Tenofovir Disoproxil Fumarate

Plasma spiking preparations – Doravirine, Lamivudine, and Tenofovir Disoproxil Fumarate

Concentration of stock (μg/mL)	Stock volume (mL)	Plasma volume (mL)	Final volume (mL)	Final concentration (ng/mL)	Details
18.00	0.1	0.9	1	900	HQC
9.50	0.1	0.9	1	480	MQC
0.08	0.1	0.9	1	7.4	LQC

HQC: High-Quality Control, MQC: Middle-Quality Control, LQC: Low-Quality Control

Accuracy

Samples spiked with known concentrations of the analyte, such as QC samples, should be used to assess accuracy. Five concentration measurements and at least three levels should be included in this evaluation. The accuracy of the developed method is assessed using its percentage coefficient of variation.

Precision

The precision of a developed method refers to the agreement between individually obtained results under predefined experimental conditions. Precision is evaluated using QC samples, including the Lower Limit of Quantification QC (LLOQC), Lower-Quality Control (LQC), Medium Quality Control (MQC), and High-Quality Control (HQC), each analyzed in six replicates.

Recovery

This paper describes the contrast between the detector response derived from a pure standard nominal concentration and the response derived from an analyte extraction quantity from plasma (biological matrix). It is sufficient that the analyte recovery be consistent, precise, and reproducible for both the analyte and the IS; 100% recovery is not necessary.

Procedure: Extraction recoveries for DOR, LAM, and TDF were assessed by comparing the responses obtained from plasma samples spiked before extraction to those spiked after extraction. The mean recoveries for DOR, LAM, and TDF were determined to be within the specified range (80-120%).

Matrix factor

The "matrix effect" refers to the combined influence of all components present in the sample solution, except for the analytes being measured during the sample quantification process. It is assessed by determining the "matrix factor," which measures the degree of this effect. It is calculated for each analyte in the study to assess the extent of matrix interference.

Procedure: Blank plasma was collected from six sources, including one hemolytic and one lipemic lot, in order to study the matrix effect. To construct post-extracted samples, the residue was reconstituted using a mobile phase that contained an ISTD and a set amount of analyte (LQC level). These samples, in addition to aqueous samples, were then assessed. The matrix factor for each analyte/ISTD was determined by comparing the peak response in the presence of matrix ions to the peak response in their absence. Each response ratio of the post-extracted matrix samples was compared to the mean response of the aqueous samples to assess the matrix effect.

Stability

Stability is evaluated to determine the chemical or physical compatibility of analytes under specific conditions and intervals, ensuring their reliability in a particular matrix. Furthermore, the stability analysis encompasses evaluating the impact of sample preparation, handling procedures, and analytical runtime over a specified period.

Procedure:

BT, short-term stability, and long-term stability are among the situations that are tested for stability. To evaluate the stability of each analyte, the data obtained on the percentage stability (% Stability) was determined. Details of the stability study are presented in Tables 3 and 4.

RESULTS

System suitability

The specificity serves as an indicator of the system's optimal performance during analysis, ensuring accurate results. The analyte peaks demonstrated specific elution with optimal resolution, affirming the method's specificity and sensitivity.

Selectivity

The method exhibited selectivity, as the analyte peaks were eluted without interference from other components present, as shown in Figure 14, ensuring accurate detection and quantification.

Table 3. Details of the production of stability samples for Doravirine, Lamivudine, and Tenofovir Disoproxil Fumarate (Benchtop and Long-Term)

Preparations of spiked plasma – Doravirine and Tenofovir Disoproxil Fumarate Benchtop and Long-Term Stability study

Concentration of stock in µg/mL	Stock volume (mL)	Plasma volume (mL)	Final volume (mL)	Final concentration (ng/mL)	Details
0.08	0.1	0.9	1	7.4	LQC (Old)
0.08	0.1	0.9	1	7.4	LQC (Fresh)
18.00	0.1	0.9	1	900	HQC (Old)
18.00	0.1	0.9	1	900	HQC (Fresh)

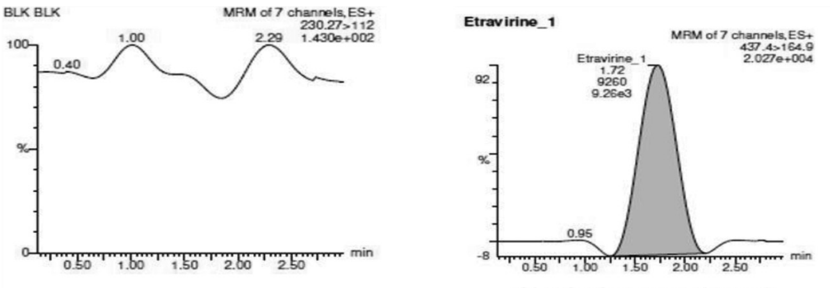
HQC: High-Quality Control, LQC: Low-Quality Control

Table 4. The stability sample preparation details for Doravirine, Lamivudine, and Tenofovir Disoproxil Fumarate (Short-Term)

Preparations – Doravirine, Lamivudine, and Tenofovir Disoproxil Fumarate Short Term Stability Study

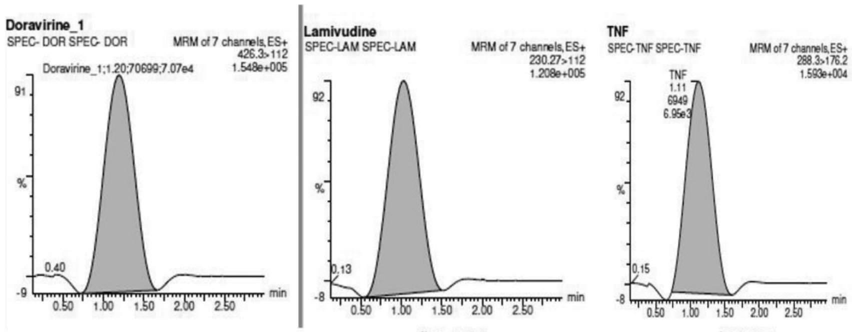
Concentration of stock (µg/mL)	Stock volume (mL)	Diluent volume (mL)	Final volume (mL)	Final concentration (ng/mL)	Details
Old stock (9.5)	0.1	0.9	1	480	MQC - Old
Fresh stock (9.5)	0.1	0.9	1	480	MQC - Fresh

MQC: Middle-Quality Control



a) Blank

b) Blank + ETR (ISTD)



c) DOR

d) LAM

e) TDF

Figure 14. Chromatograms of a) Blank, b) Blank + ETR (ISTD), c) DOR, d) LAM & e) TDF

DOR: Doravirine, TDF: Tenofovir disoproxil fumarate, LAM: Lamivudine, ETR: Etravirine

Linearity range and calibration curve

A calibration curve with a matrix basis was constructed and applied to determine the analyte concentrations in unknown samples. The calibration curves for DOR, LAM, and TDF were found to be linear, with r^2 0.99 within the concentration range of 2.5 to 1000 ng/mL. Detailed results are provided in Table 5, and calibration curves are displayed in Figure 15.

QCs samples:

Three concentration levels of fresh QC samples were prepared: one in the center, one at the top end of the range, and one at three times the Lower Limit of Quantification (LLOQ). The precision and stability of these watery samples were evaluated.

Precision and accuracy:

The Upper and Lower Quantification Limits, which together constitute the range for the Limit of Quantification (LOQ), must each exhibit appropriate precision and accuracy. Table 6 displays the precision findings.

Extraction recovery

In contrast to the response derived from a pure, genuine standard concentration, recovery describes the detector response obtained from the amount of analyte supplied, which is then extracted from the matrix. The recovery of DOR, TDF, and LAM at low, medium, and high concentrations was ascertained by comparing the pre-extraction reaction plasma samples (n=6) with the post-extraction plasma samples. With a coefficient of variation (CV) ranging from 1.2 to 13.9 %, the mean recovery for DOR, LAM, and TDF was 83.39%, 87.33%, and 85.56%, respectively. Table 7 displays the results.

Matrix effect

Co-eluting matrix components may have a positive or negative effect on ionization, but they may not impact the outcome. By comparing each post-extracted matrix lot's response ratio to the corresponding aqueous samples, the matrix effect was evaluated using samples from six distinct lots. By contrasting the peak response in the presence and absence of matrix ions, the matrix factor for each analyte or the ISTD was determined. Results are presented in Tables 8, 9, and 10.

Benchtop stability

Benchtop stability (BT) was evaluated to make sure the analytes do not degrade during sample analysis or extraction. Six QC samples were removed from the freezer and allowed to come to room temperature, or around 25 °C. In fewer than six hours, stability standards and QC samples were produced, and the outcomes were compared to control samples. By contrasting the concentrations of the stability and control samples, the percentage stability of the sample concentrations was computed.

Long-term stability

Storage stability was evaluated to ensure that the analytes stay stable in the matrix throughout the study. After six hours at −20

Table 5. Calibration curve outcomes

Analyte	Nominal concentration (ng/mL)	Mean found concentration (ng/mL)	CV%	Parameters
DOR	2.5	2.1	00%	• Slope: 0.00875556 • Y-intercept: 0.015462 • r^2: 0.99929 • Regression equation: y = 0.00875556x + 0.015462 • Linearity range: 2.5 - 1000 ng/mL
	10	11.1	11%	
	50	49.8	6.8%	
	100	107.3	7.3%	
	500	476.3	-4.74%	
	1000	992.2	-0.78%	
TDF	2.5	2.2	-12.0%	• Slope: 0.00321555 • Y-intercept: 0.00158833 • r^2: 0.993768 • Regression equation: y = 0.00321555x + 0.00158833 • Linearity range: 2.5 - 1000 ng/mL
	10	9.2	-8.0%	
	50	51.1	4.2%	
	100	111.5	11.5%	
	500	481.6	-3.68%	
	1000	1007.1	0.71%	
LAM	2.5	2.6	4%	• Slope: 0.000851902 • Y-intercept: 0.00409457 • r^2: 0.998670 • Regression equation: y = 0.000851902x + 0.00409457 • Linearity range: 2.5 - 1000 ng/mL
	10	8.7	-13%	
	50	50.4	4.4%	
	100	109.4	9.4%	
	500	445.9	-10.82%	
	1000	1040.9	4.09%	

DOR: Doravirine, LAM: Lamivudine, TDF: Tenofovir Disoproxil Fumarate, CV: Coefficient of variation

± 5 °C, samples were examined using calibration standards and QC samples. Percentage stability was determined by comparing the average concentration of the stability sample with that of the control samples.

Short-term stability

A shorter storage stability study was carried out from sample collection to sample analysis in order to verify the stability of the analytes within the test system matrix. After keeping the samples at room temperature in a laboratory setting, six replicates of each sample were processed and assessed using freshly made calibration standards and QC samples (aqueous comparison samples). The percent stability was ascertained by contrasting the average concentration of the stability samples with that of the comparison samples.

All the outcomes obtained from the stability studies of DOR, LAM, and TDF are presented in Table 11. MRM chromatograms of DOR, TDF, LAM and ETR are shown in Figure 16.

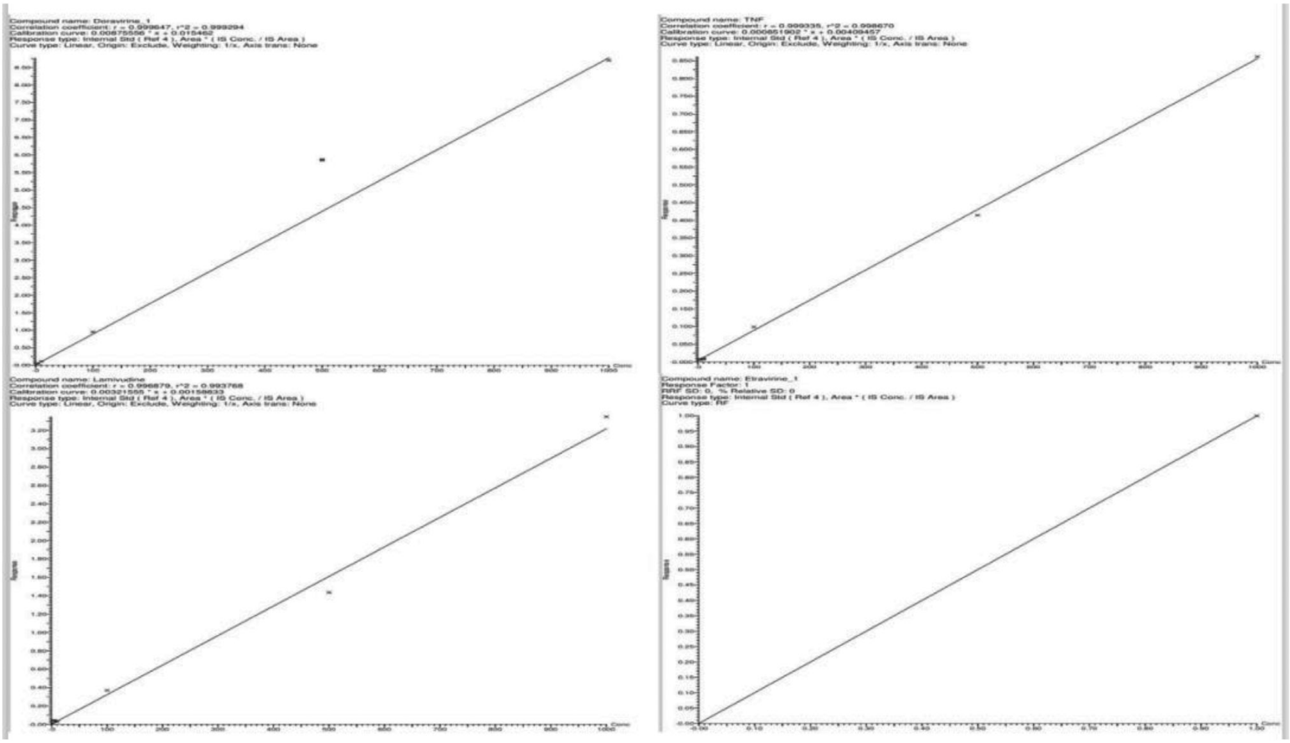


Figure 15. Calibration curves of DOR, TDF, LAM & ETR

DOR: Doravirine, TDF: Tenofovir disoproxil fumarate, LAM: Lamivudine, ETR: Etravirine

Table 6. Outcomes of precision

Analyte	Level of QC	Nominal concentration (ng/mL)	Intra batch		Inter batch	
			Mean found concentration (ng/mL)	CV%	Mean found concentration (ng/mL)	CV%
DOR	**LQC**	7.4	6.8	-1.08	6.5	-1.18
	MQC	480	439.9	-4.23	423.4	-5.31
	HQC	900	991.3	5.06	1070.2	7.01
LAM	**LQC**	7.4	8.5	5.23	8.8	6.54
	MQC	480	501.9	8.8	521.4	5.6
	HQC	900	986.7	5.23	1026.1	7.1
TDF	**LQC**	7.4	7.9	2.1	8.5	2.6
	MQC	480	501.4	4.2	522.8	6.2
	HQC	900	912.1	2.2	1005.6	5.9

DOR: Doravirine, LAM: Lamivudine, TDF: Tenofovir Disoproxil Fumarate, CV: Coefficient of variation, HQC: High-Quality Control, MQC: Middle-Quality Control, LQC: Low-Quality Control

Table 7. Results of extraction recovery

Analyte	QC level	Extracted samples	Extracted spiked samples (post)	% of recovery	% of mean recovery	Standard deviation	CV%
DOR	**LQC**	978	1169	83.66	83.39	1.84	2.21
	MQC	17327	20365	85.08			
	HQC	32625	40063	81.43			
LAM	**LQC**	538	623	86.36	87.33	2.30	2.64
	MQC	7704	8564	89.96			
	HQC	12380	14451	85.67			
TDF	**LQC**	149	174	85.63	85.56	1.92	2.25
	MQC	1893	2165	87.44			
	HQC	3231	3865	83.60			
Internal standard		**Extracted samples**	**Extracted spiked samples (post)**	**% of recovery**		**% of mean recovery**	
Etravirine **MQC** **HQC**	**LQC**	3871	4618	83.82		81.75	
	3587	4423	81.10				
	4168	5189	80.32				

DOR: Doravirine, LAM: Lamivudine, TDF: Tenofovir Disoproxil Fumarate, CV: Coefficient of variation, HQC: High-Quality Control, MQC: Middle-Quality Control, LQC: Low-Quality Control

Table 8. Outcomes of Doravirine Matrix Effect

Analyte	Doravirine		
	Analyte MF	ISTD MF	IS normalized factor
Lot-I	0.791	0.720	1.099
Lot-II	0.747	0.606	1.233
Lot-III	0.637	0.620	1.027
Lot-IV	0.762	0.712	1.070
Lot-V	0.711	0.696	1.022
Lot-VI	0.667	0.709	0.941
Mean	-	-	**1.065**
SD%	-	-	0.098
CV%	-	-	9.204

MF: Matrix factor, ISTD: Internal standard, CV: Coefficient of variation, SD: Standard deviation

Table 9. Outcomes of Lamivudine matrix effect

Analyte	Lamivudine		
	Analyte MF	ISTD MF	IS normalized factor
Lot-I	0.410	0.720	0.569
Lot-II	0.574	0.794	0.723
Lot-III	0.488	0.750	0.651
Lot-IV	0.533	0.774	0.689
Lot-V	0.441	0.852	0.518
Lot-VI	0.497	0.821	0.605
Mean	-	-	**0.626**
SD%	-	-	0.077
CV%	-	-	12.24

MF: Matrix factor, ISTD: Internal standard, CV: Coefficient of variation, SD: Standard deviation

Table 10. Outcomes of Tenofovir Disoproxil Fumarate matrix effect

Analyte	Tenofovir Disoproxil Fumarate		
	Analyte MF	ISTD MF	IS normalized factor
Lot-I	0.627	0.720	0.871
Lot-II	0.722	0.690	1.046
Lot-III	0.768	0.680	1.129
Lot-IV	0.762	0.678	1.124
Lot-V	0.653	0.714	0.916
Lot-VI	0.691	0.691	1.000
Mean	-	-	**1.014**
SD%	-	-	0.106
CV%	-	-	10.49

MF: Matrix factor, ISTD: Internal standard, CV: Coefficient of variation, SD: Standard deviation

Table 11. Outcomes of stability studies of all three analytes

Stability	Analyte	QC level	Mean concentration of fresh stability sample	Mean concentration of old comparison sample	Stability %
Bench top (6 hrs @ 25 °C)	**DOR**	**LQC**	978	1107	88.35
		HQC	32625	34706	94.00
	LAM	**LQC**	538	600	89.67
		HQC	12380	12339	100.33
	TDF	**LQC**	149	178	83.71
		HQC	3231	3533	91.45
Long-term (6 hrs @ -20 °C)	**DOR**	**LQC**	971	1198	81.05
		HQC	29103	32400	89.82
	LAM	**LQC**	856	976	87.70
		HQC	13988	13561	103.15
	TDF	**LQC**	172	163	105.52
		HQC	3654	3403	107.38
Short-term (25°C @ room temperature)	**DOR**	**MQC**	3330	3261	102.12
	LAM	**MQC**	17675	18421	95.95
	TDF	**MQC**	25498	24958	102.16

DOR: Doravirine, LAM: Lamivudine, TDF: Tenofovir Disoproxil Fumarate, HQC: High-Quality Control, MQC: Middle-Quality Control, LQC: Low-Quality Control

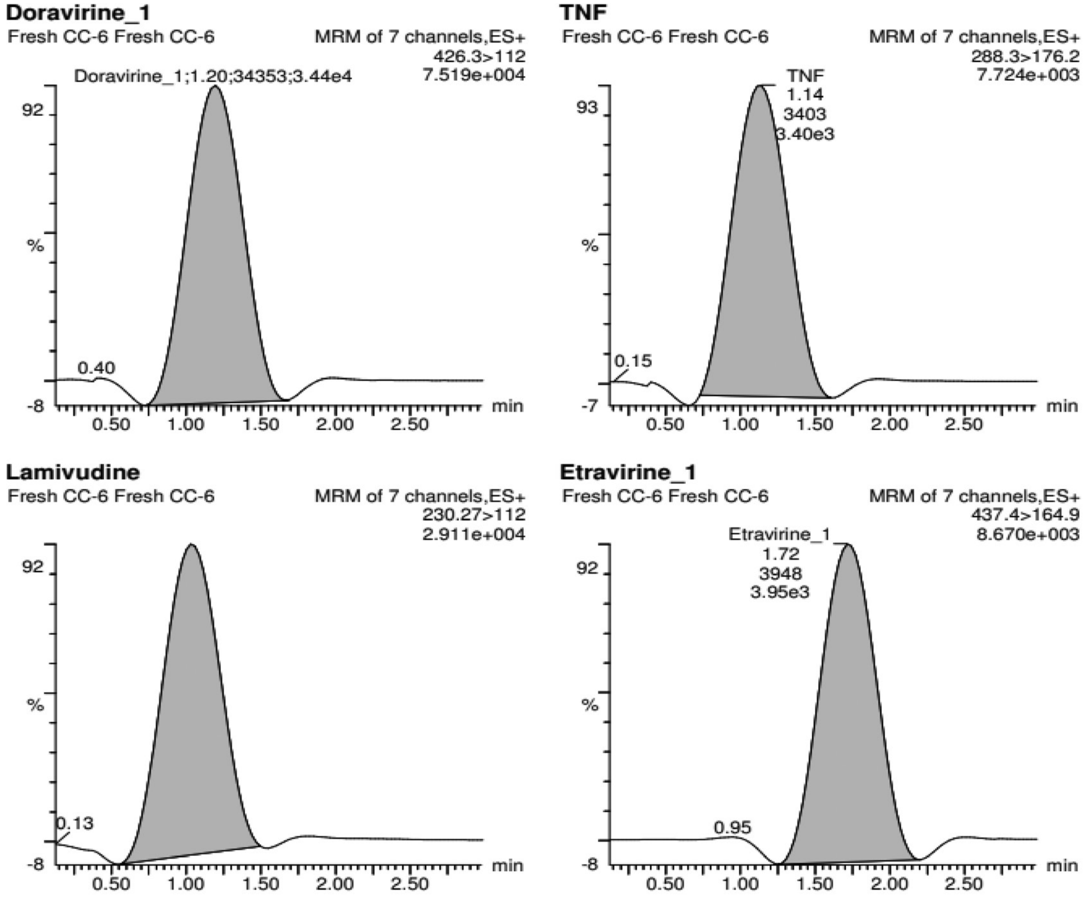


Figure 16. MRM Chromatograms of (i) DOR, (ii) TDF, (iii) LAM & (iv) ETR

DOR: Doravirine, TDF: Tenofovir disoproxil fumarate, LAM: Lamivudine, ETR: Etravirine

DISCUSSION

The bioanalytical method developed in this study demonstrates robust specificity and selectivity for the quantification of DOR, LAM, and TDF in human plasma. Method development, validation, and stability assessments adhered to regulatory standards outlined by the FDA, EMA, and ICH M10, thereby ensuring global applicability and reliability of the results. The strategic use of cost-effective solvents not only enhanced the method's economic viability but also contributed to reproducible outcomes with no observable interferences or impurities.

Compared to conventional analytical methods, particularly traditional HPLC, the newly optimized approach via UPLC-MS/MS offers superior sensitivity and specificity. The inclusion of tandem mass spectrometry enables the detection of analytes at lower concentration levels, a critical aspect of therapeutic drug monitoring. Validation parameters such as accuracy, precision, and percent recovery consistently fell within acceptable limits across the defined linearity range, substantiating the method's robustness.

Additionally, the method exhibited a minimal matrix effect, and stability studies revealed that the analytes remained stable under various conditions, affirming the method's suitability for routine clinical application. Notably, the use of columns with smaller particle sizes and high-pressure tolerance allowed for significantly shorter retention times and enhanced resolution. This translated into improved compound separation and reduced risk of co-elution, which is particularly beneficial when analyzing complex biological matrices. The increased throughput of the method presents a valuable advantage for clinical laboratories requiring rapid sample processing without compromising analytical performance.

CONCLUSION

We developed and validated a highly sensitive, reproducible, and robust bioanalytical method using the UPLC-MS/MS technique. The method demonstrated excellent performance across all parameters and complied fully with ICH guidelines. Given its reliability and consistency, this approach is well-suited for routine quantification of LAM, DOR, and TDF in analytical applications.

Ethics

Ethics Committee Approval: Not required.

Informed Consent: Not required.

Acknowledgments

The authors would like to express their profound thanks to M/S KSHETRA ANALYTICALS, Hyderabad, for providing crucial research facilities and direction during the progression and completion of this study. We would also like to thank "Hetero Labs Ltd.", Hyderabad, for kindly providing gift samples of the drug compounds used in this investigation.

Footnotes

Authorship Contributions

Concept: N.K., B.K., Design: N.K., B.K., Data Collection or Processing: N.K., B.K., Analysis or Interpretation: N.K., B.K., Literature Search: N.K., B.K., Writing: N.K., B.K.,

Conflict of Interest: The authors declare no conflicts of interest.

Financial Disclosure: The authors declared that this study received no financial support.

REFERENCES

1. Bekker LG, Beyrer C, Mgodi N, Lewin SR, Delany-Moretlwe S, Taiwo B, Masters MC, Lazarus JV. HIV infection. Nat Rev Dis Primers. 2023;9:42.
2. Full Prescribing Information (PIL) of Delstrigo Tablets, US FDA.
3. Colombier MA, Molina JM. Doravirine: a review. Curr Opin HIV AIDS. 2018;13:308-314.
4. Boyle A, Moss CE, Marzolini C, Khoo S. Clinical pharmacodynamics, pharmacokinetics, and drug interaction profile of Doravirine. Clin Pharmacokinet. 2019;58:1553-1565.
5. Blevins SR, Hester EK, Chastain DB, Cluck DB. Doravirine: a return of the NNRTI class? Ann Pharmacother. 2020;54:64-74.
6. Johnson MA, Moore KH, Yuen GJ, Bye A, Pakes GE. Clinical pharmacokinetics of Lamivudine. Clin Pharmacokinet. 1999;36:41-66.
7. Taylor K, Fritz K, Pellegrini MV, Parmar M. Lamivudine. 2024 Feb 28. In: StatPearls [Internet].
8. Kumar PN, Patel P. Lamivudine for the treatment of HIV. Expert Opin Drug Metab Toxicol. 2010;6:105-114.
9. Gallant JE, Deresinski S. Tenofovir Disoproxil Fumarate. Clin Infect Dis. 2003;37:944-950.
10. Miller MD. K65R, TAMs and tenofovir. AIDS Rev. 2004;6:22-33.
11. Nastri BM, Pagliano P, Zannella C, Folliero V, Masullo A, Rinaldi L, Galdiero M, Franci G. HIV and drug-resistant subtypes. Microorganisms. 2023;11:221.
12. Johnson M, Kumar P, Molina JM, Rizzardini G, Cahn P, Bickel M, Mallolas J, Zhou Y, Morais C, Kumar S, Sklar P, Hanna GJ, Hwang C, Greaves W; DRIVE-SHIFT Study Group. Switching to Doravirine/Lamivudine/Tenofovir Disoproxil Fumarate (DOR/3TC/TDF) maintains HIV-1 virologic suppression through 48 weeks: results of the DRIVE-SHIFT trial. J Acquir Immune Defic Syndr. 2019;81:463-472.
13. Kokkirala TK, Kancherla P, Alegete P, Suryakala D. A simple alternative and improved HPLC method for the estimation of Doravirine, Lamivudine, and Tenofovir Disoproxil Fumarate in solid oral dosage form. Biomed Chromatogr. 2021;35:e5121.
14. Attaluri T, Seru G, Varanasi SNM. Development and validation of a stability-indicating RP-HPLC method for the simultaneous estimation of Bictegravir, Emtricitabine, and Tenofovir Alafenamide Fumarate. Turk J Pharm Sci. 2021;18:410-419.
15. Tanuja A, Ganapaty S. Bio-analytical method development and validation for simultaneous determination of bictegravir, emtricitabine, and tenofovir alafenamide fumarate in human plasma by LC-MS/MS. Indian Journal of Pharmaceutical Education and Research. 2022;56:1190-1205.
16. Schauer AP, Sykes C, Cottrell ML, Imaz A, Podzamczer D, Kashuba AD. Validation of an LC-MS/MS assay for the simultaneous determination of bictegravir, Doravirine, and raltegravir in human plasma. J Pharm Biomed Anal. 2022;220:115010.
17. Marakatham S, Shanmugapandiyan P. Bioanalytical method development and validation of Doravirine, Lamivudine and Tenofovir Disoproxil Fumarate using HPLC in human plasma. Research Journal of Pharmacy and Technology. 2021;14:4087-4091.
18. Godela R, Gummadi S. A simple stability indicating RP-HPLC-DAD method for concurrent analysis of Tenofovir Disoproxil Fumarate, Doravirine and Lamivudine in pure blend and their combined film coated tablets. Ann Pharm Fr. 2021;79:640-651.
19. Rathod SM, Patel NC, Patel PU. Simultaneous determination of emtricitabine, tenofovir alafenamide fumarate and dolutegravir sodium by validated stability-indicating RP-HPLC-DAD method. Ann Pharm Fr. 2023;81:94-106.
20. Anderson MS, Gilmartin J, Fan L, Yee KL, Kraft WK, Triantafyllou I, Reitmann C, Guo Y, Liu R, Iwamoto M. No meaningful drug interactions with Doravirine, Lamivudine and Tenofovir Disoproxil Fumarate coadministration. Antivir Ther. 2019;24:443-450.