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A Validated LC-MS/MS Method for the Simultaneous Quantification of Dolutegravir, Lamivudine and Tenofovir disoproxil fumarate in Human Plasma

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ABSTRACT

Objectives: *This study aimed to develop and validate a novel, sensitive and high-throughput liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the simultaneous quantification of Dolutegravir (DTG), lamivudine (3TC) and tenofovir disoproxil fumarate (TDF) in human plasma using abacavir (ABC) as the internal standard (IS). A simple protein precipitation technique was employed for plasma sample extraction. The developed method was evaluated for its analytical performance, reliability and stability study to ensure its suitability for routine bioanalytical applications*

Materials and Methods: *Abacavir shows physicochemical similarity to the analytes of interest, was used as an IS. Plasma samples were prepared using a protein precipitation extraction technique. Chromatographic separation was achieved using an analytical column Thermo Hypersil C18 (4.6 X100 mm,5.0µm particle size). An isocratic mobile phase comprising acetonitrile and an aqueous buffer containing 5 mM ammonium formate and 0.1% formic acid, in a ratio of 80:20 (v/v), was used at a flow rate of 0.6 mL/min.*

Results: *The method demonstrated linearity over a concentration range of 8-8000 ng/mL for DTG, 4-4000 ng/mL for 3TC,1-800 ng/mL for TDF respectively, with regression coefficient of approximately 0.99. The mean extraction recoveries from spiked human plasma samples were 90% for DTG, 89.33% for 3TC, and 84.56% for TDF. With a total chromatographic run time of 4.5 minutes, the method offered rapid analysis and showed potential for high-throughput bioanalytical applications.*

Conclusion: *A rapid and reliable LC–MS/MS method was developed for the simultaneous quantification of Dolutegravir (DTG), lamivudine (3TC) and tenofovir disoproxil fumarate (TDF) in human plasma, using abacavir (ABC) as the internal standard (IS). The method demonstrated excellent extraction recoveries and a short chromatographic run time of 4.5 minutes, making it suitable for high-throughput bioanalysis. Following validation in accordance with ICH M10 guidelines, the method was assessed for accuracy, precision, selectivity, sensitivity, linearity and other relevant validation parameters. Its analytical performance supports its potential application in routine bioanalytical investigations and pharmacokinetic studies.*

Keywords: *HIV, antiretroviral therapy, lamivudine, dolutegravir, tenofovir, LC-MS/MS, method validation.*

INTRODUCTION

In pharmaceutical research, the development of accurate, sensitive and reliable analytical methods for quantifying drugs in biological matrices is essential for evaluating drug exposure, therapeutic efficacy and safety. Human immunodeficiency virus (HIV) remains a major global public health concern and

primarily targets CD4-positive T lymphocytes, which play a crucial role in coordinating immune responses [1,2]. Progressive depletion of these cells weakens the immune system and increases susceptibility to opportunistic infections. Antiretroviral therapy (ART) suppresses viral replication, preserves immune function and improves clinical outcomes [1,3]. Therefore, reliable bioanalytical methods for quantifying antiretroviral agents in human plasma are important for pharmacokinetic investigations, therapeutic drug monitoring, and pharmaceutical research [4].

The fixed-dose combination of dolutegravir (50 mg), lamivudine (300 mg), and tenofovir disoproxil fumarate (300 mg) is administered orally once daily and is indicated as a complete regimen for the treatment of HIV-1 infection in eligible adults and pediatric patients, according to the applicable product prescribing information [4,5].

Clinical evidence has supported the use of dolutegravir (DTG), lamivudine (3TC), and tenofovir disoproxil fumarate (TDF) in Fixed dose Combination (FDC)as an antiretroviral regimen for HIV-1 infection. This combination targets viral replication through complementary mechanisms, helping achieve and maintain virological suppression and supporting recovery of CD4+ T-cell counts. The World Health Organization included the TDF + 3TC + DTG regimen among its preferred first-line treatment options. When administered as a fixed-dose combination (FDC), the regimen can simplify treatment by reducing pill burden and supporting adherence. The dosing instructions, including whether administration with food is necessary, should be confirmed against the prescribing information for the specific product [6-8].

The present study aimed to develop and validate a sensitive, precise, accurate, reproducible and reliable liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the simultaneous quantification of dolutegravir (DTG), lamivudine (3TC), and tenofovir disoproxil fumarate (TDF) using abacavir (ABC) as an internal standard (IS). Figure 1,2,3 shows the structures of analytes and figure 4 shows the structure of Abacavir, which is used as internal standard. The method was intended to provide efficient chromatographic separation, adequate selectivity and reproducible quantitative performance for the simultaneous analysis of all three antiretroviral drugs. The development and validation of this analytical method are essential for ensuring reliable pharmacokinetic investigations, enabling therapeutic drug monitoring, and meeting established regulatory requirements for analytical method performance. Available literature suggests a potential research gap in the simultaneous quantification of dolutegravir (DTG), lamivudine (3TC), and tenofovir disoproxil fumarate (TDF) in human plasma using abacavir (ABC) as an internal standard (IS) by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Different publications were available for the estimation of these drugs [9-17].

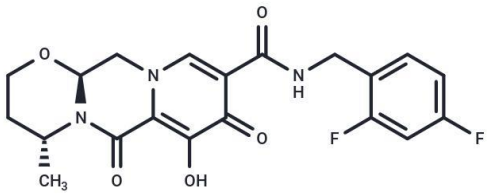


Figure 1: Structure of Dolutegravir (DTG)

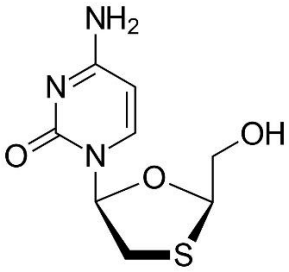


Figure 2: Structure of Lamivudine (3TC)

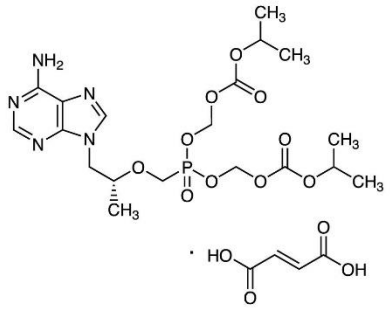


Figure 3: Structure of tenofovir disoproxil fumarate (TDF)

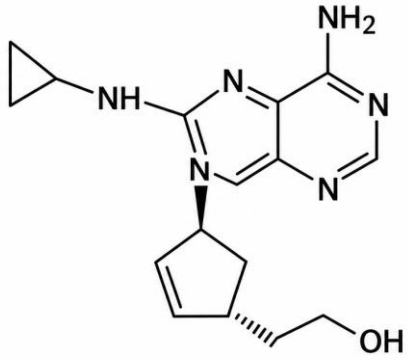


Figure 4: Structure of Abacavir (ABC)

MATERIALS AND METHODS

Chemicals and Reagents

Analytical grade API of DTG,3TC, TDF and ABC(IS) were obtained from Med Chem Express (MCE). Methanol and tert-butyl methyl ether (TBME), Ethyl Acetate, Diethyl ether, Formic acid, Ammonium formate 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 3000 LC-MS/MS mass spectrometer equipped with an electrospray ionization (ESI) source operating in multiple reaction mode. System In-build Analyst Software was used for data interpretation.

Chromatographic Conditions

Chromatographic separation was achieved using isocratic elution throughout the analysis, with mobile phase comprising acetonitrile and an aqueous buffer containing 5mM ammonium formate with 0.1% formic acid, in a ratio of 80:20 (v/v), was used at a flow rate of 0.6 mL/min. Thermo Hypersil C18 (4.6 X100 mm, 5.0µm particle size) column was used and autosampler temperature was set at 10^0C. Injection volume kept 20 µL with a total run time of 4.5 min.

PREPARATION OF STANDARD SOLUTIONS

Preparation of Stock Solution and Spiking Solution

The master stock solutions of DTG,3TC and TDF 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 Abacavir 1 µg/mL Abacavir solution was prepared and used as the internal standard for sample analysis.

Primary stock solutions of DTG,3TC and TDF 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:

Liquid-liquid extraction (LLE) and Protein precipitation technique (PPT) both were employed. LLE Screened with Tert-Butyl-Methyl-Ether (TBME), Ethyl Acetate and Diethyl ether. 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 among all the extracting and precipitating agents.

250 µL 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 10^0C and 250 µL 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 ≤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 ≤ 20% of the LLOQ response for the analyte and ≤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 ≤20% of the mean analyte response at LLOQ QC and ≤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 ± 15% of respective nominal concentration. Precision for QC's (Percent Coefficient of Variation (%CV)) ≤ 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 ±15% of the nominal concentration (±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 atleast 67% of total QC samples should be within ± 15% & for LLOQ ± 20% Atleast 50% of QC samples at each level should be within ± 15% & for LLOQ QC ± 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 ± 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 ± 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 ± 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 ± 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 ± 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 DTG,3TC, TDF and ABC(IS) was found < 5% of mean area of Analyte and IS respectively. Hence system suitability test (SST) is found within acceptance criteria for all the drugs and IS.

Selectivity and Specificity

The analytes peaks were found eluted specifically with best resolution with no interference; showing specificity and selectivity of method. PPT provided a clean baseline with acceptable signal response, provided efficient elution with well-defined peak shapes, facilitating retention times of analytes 1.52min, 2.77 min, 2.09 min, 1.52 min for DTG,3TC, TDF and ABC(IS) respectively. The analysis time was short, which makes it an attractive method for routine analysis. Representative chromatograms are shown in fig. 5 and fig.6.

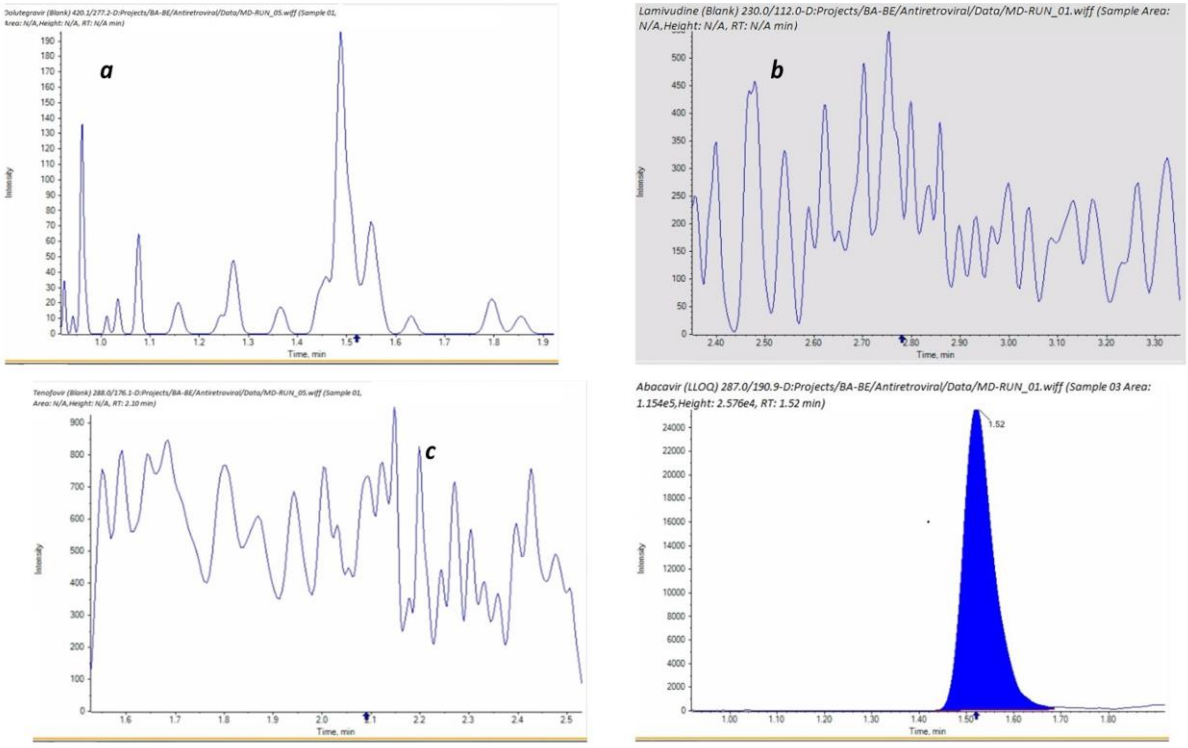


Figure 5: LC-MS/MS chromatogram for DTG (a), 3TC (b), TDF (c), and ABC as IS (d) blank sample extract from PPT with acetonitrile.

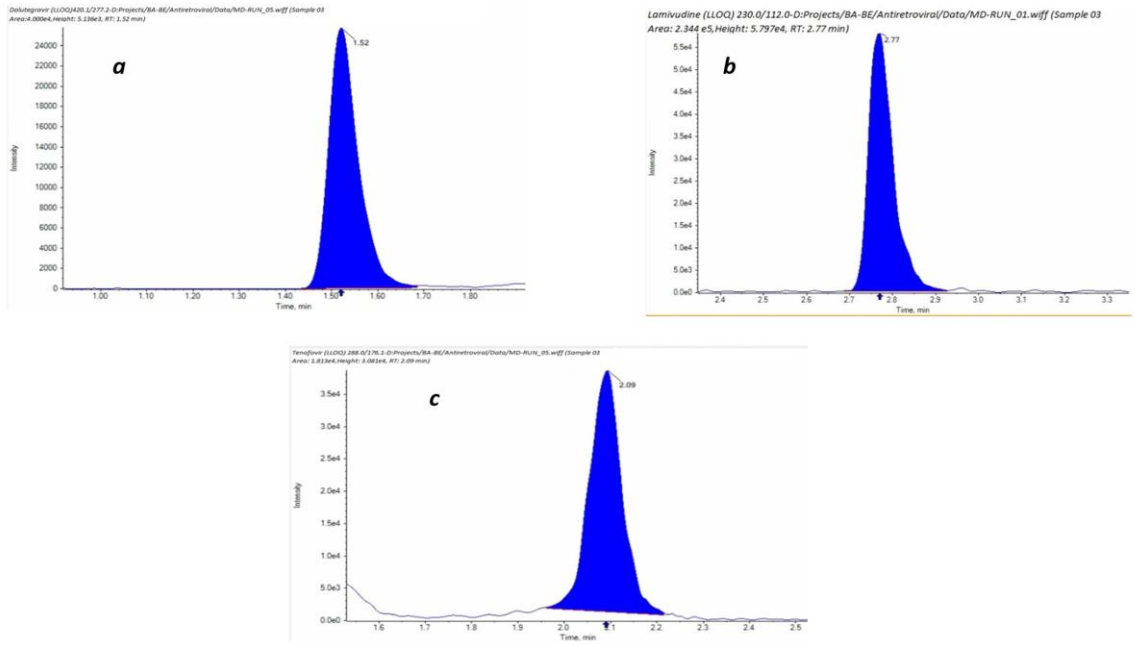


Figure 6: LC-MS/MS chromatogram for plasma spiked with DTG (a), 3TC(b), TDF (c) at the lower limit of quantification (LLOQ) Concentration 2.5 ng/mL by PPT.

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.7).

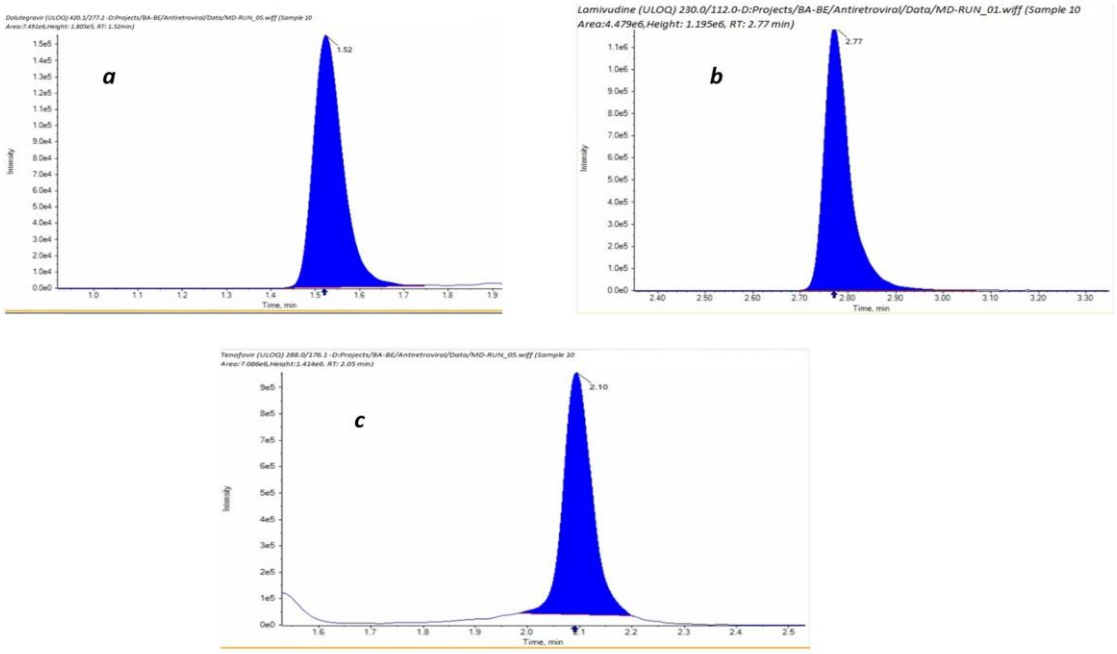


Figure 7: LC-MS/MS chromatogram for with DTG (a), 3TC (b), TDF(c) Upper limit of quantification (ULOQ) concentration 2500 ng/mL by PPT.

Linearity

A calibration curve was constructed with matrix basis and applied to determine the analyte concentration in unknown samples. The calibration curves for DTG,3TC & TDF were found to be linear. Linearity response was assessed in the concentration ranges 8-8000 ng/mL for DTG, 4-4000 ng/mL for 3TC and 1-800 ng/mL for TDF. The calibration curve lines were drawn to give the linear regression equations Y = m X + C. The correlation coefficient square (r2) was 0.99364, 0.99708 and 0.99176 for DTG,3TC & TDF respectively. Detailed calibration curves are displayed in figure 8,9 & 10.

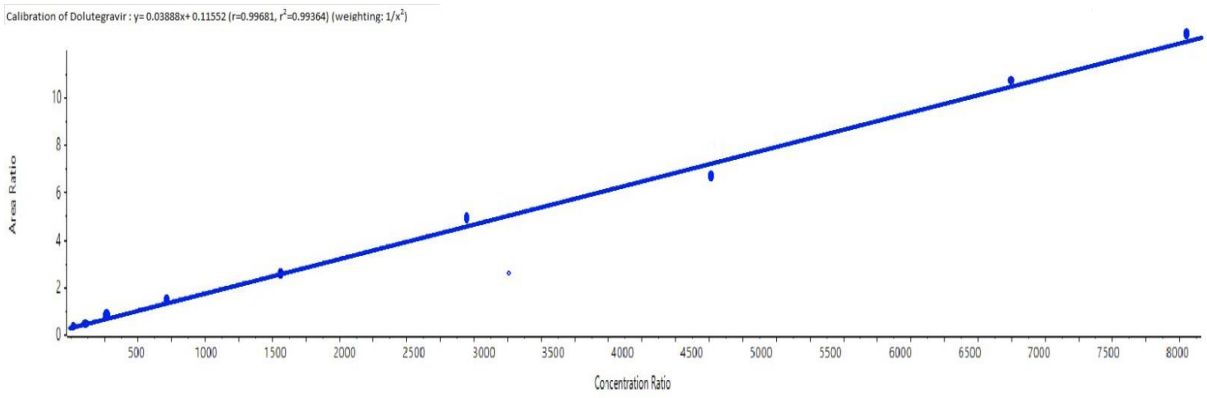


Figure 8: Calibration curve of DTG

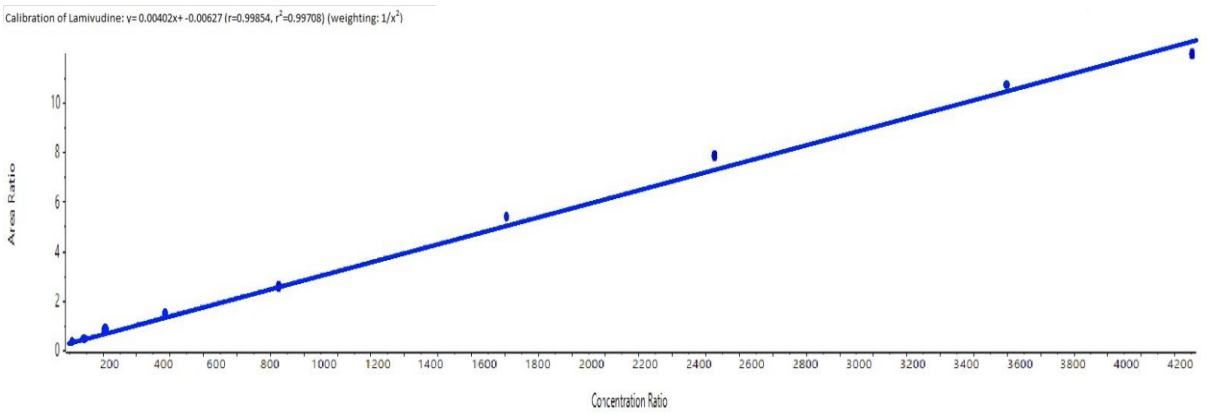


Figure 9: Calibration curve of 3TC

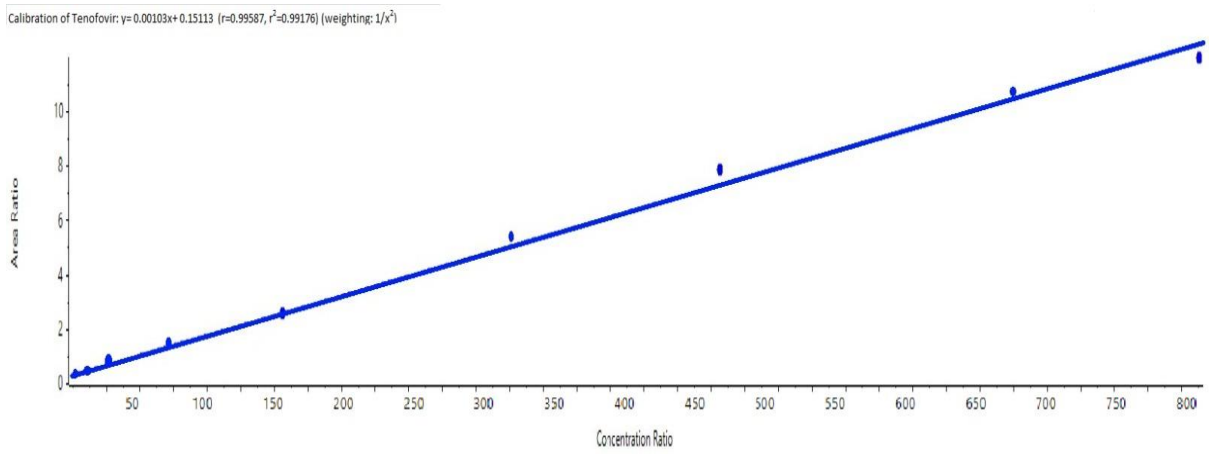


Figure 10: Calibration curve of TDF

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. Corelation coefficient (r^2) for all the calibration curve was found > 0.99. Quality control (QC) samples were within acceptance criteria (QC Samples within ± 15% limit and for LLOQ QC ± 20% limit). Precision & Accuracy batch found within acceptance criteria for DTG,3TC & TDF

Reinjection reproducibility

All standard CC point was passing through acceptable limit. Corelation coefficient (r^2) for calibration curve was found 0.99589 for DTG, 0.99402 for 3TC and 0.99723 for TDF respectively. Quality control (QC) samples were within acceptance criteria (QC Samples within ± 15% limit and for LLOQ QC within ± 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 ± 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 11 hours and found within acceptance criteria for DTG,3TC & TDF. Processed sample stability was performed after 23 hours and meet the required guideline for all three drugs. Freeze thaw stability for DTG,3TC & TDF was performed at 3 and 5 cycles passing through acceptance limit. Autosampler stability studies were investigated after 64 hours for DTG,3TC & TDF found within a limit.

The developed LC–MS/MS method demonstrated efficient chromatographic resolution, yielding well-defined and distinct peaks for DTG, 3TC, TDF, and ABC (IS) within a short analytical run time of 4.5 min. The absence of significant endogenous interference in blank plasma samples confirmed the excellent selectivity of the method. Protein precipitation with acetonitrile yielded consistent and reproducible extraction recoveries for all analytes and the internal standard. The method exhibited good linearity across the selected concentration ranges, with correlation coefficients greater than 0.99. The accuracy and precision results were within the prescribed acceptance criteria. Furthermore, analyte recovery remained consistent and reproducible across all quality control (QC) levels. Matrix effect assessment revealed negligible ion suppression or enhancement, indicating minimal interference from plasma constituents and supporting the reliability of the developed method.

Stability assessments demonstrated that DTG, 3TC and TDF retained their stability under various storage and sample-processing conditions. The incorporation of ABC as an internal standard enhanced analytical reproducibility and reduced variability, thereby improving the overall reliability and consistency of the developed method.

CONCLUSIONS

A rapid, sensitive and precise LC–MS/MS method was successfully developed and validated for the simultaneous quantification of dolutegravir (DTG), lamivudine (3TC) and tenofovir (TDF) in human plasma, using abacavir (ABC) as the internal standard. The developed method complied with bioanalytical validation requirements, exhibiting acceptable accuracy, precision, extraction recovery, matrix effects and stability under the evaluated conditions. The proposed analytical technique is simple, reliable, cost-effective and time-efficient, enabling the simultaneous determination of all three antiretroviral drugs. Owing to its satisfactory validation performance and short analytical run time, the method is suitable for routine bioanalytical applications, pharmacokinetic studies and therapeutic drug monitoring of antiretroviral agents.

Conflicts of Interest

The authors declare no conflict of interest.

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