Original Article

Bio-analytical Method Development and Validation for Simultaneous Determination of Bictegravir, Emtricitabine, and Tenofovir Alafenamide Fumarate in Human Plasma by LC-MS/MS

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ABSTRACT

Aim: The current method was developed as a novel and reliable quantitative liquid chromatography-mass (tandem) spectrometry (LC-MS/MS) method for the estimation of analytes like Bictegravir (BIC) Tenofovir Alafenamide Fumarate (TNF), and Emtricitabine (EMT) in plasma of human simultaneously. **Materials:** Naproxen (NPX) is used as the internal standard for the current study. The 'Precipitation Extraction technique' is used for the present study. The Zorbax XDB C_{18} analytical column (2.1 X 50 and particle size of 5μm) is used for the chromatographic separation with isocratic natured mobile phase, which consists of Acetonitrile: Formic acid (0.1%) in water (70:30, v/v), at a flow rate of 0.15 mL/minute. **Methods:** The parent→production conversions were observed at m/z 450.1 → 289.1 (BIC), m/z 248.3 → 130.03 (EMT), m/z 477.3 → 270.04 (TNF), and m/z 231.12 → 184.82 (NPX) on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring (MRM) positive ion mode. The three compounds were found to possess primary groups hence positive manner was selected for the LC-MS/MS study. **Results:** The subject method validation was performed for the concentration of range 2-500 ng for Bictegravir (BIC), Emtricitabine (EMT), and Tenofovir Alafenamide Fumarate (TNF). The obtained mean recoveries for the three-drug moieties from samples of spiked plasma were found reproducible. **Conclusion:** Hence, based on the above, the method was proved to be rugged and rapid, with a least total run time of 3.0 min. The current method was successfully validated according to the FDA, EMA, and ICH guidelines, and the stability studies were evaluated accordingly.

Keywords: Bictegravir, Emtricitabine, Tenofovir Alafenamide Fumarate, Liquid Chromatography-Mass Spectrometry, Extraction, Stability.

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INTRODUCTION

Human Immunodeficiency Virus (HIV) is a deadly viral infectious disease that affects the immune system, increasing the other risks and illnesses. On progression, the disease may develop into an advanced illness stage known as Acquired Immuno Deficiency Virus (AIDS). Many anti-retroviral medicines that are commercially accessible were used to treat, mitigate or control HIV infection. 'BIKTARVY' is marketed with the trade name for the oral tablet of anti-retroviral medication that includes three drugs (Bictegravir+ Emtricitabine+ Tenofovir AF). Of the above three medicines, 'Bictegravir' acts as an integrase strand transfer inhibitor for HIV-1, and it bounds to albumin and α1-acid glycoprotein extensively.[1] Emtricitabine and Tenofovir Alafenamide Fumarate is an HIV-1 nucleoside analogue reverse transcriptase inhibitor.

As a result, 'BIKTARVY' may be regarded as an absolute regimen for individuals infected with HIV-1 (Type-1).[2-4] The Integrase strand transfer inhibitors (INSTIs) combine two nucleoside reverse transcriptase inhibitors and other anti-retroviral treatment components. These INSTIs are available

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as singlet formulations and/or regimens, convenient to administer, easy to take, and have potential observance advantages.[5] INSTIs have better therapeutic efficacy and tolerability than other anti-retrovirals, and they are given to both treatment-naive and treatment-experienced HIV patients in clinical practice.[6] In comparison to other medicines in the class, BIC seems to be a highly promising INSTI.[7]

Bictegravir is an 'Integrase strand transfer inhibitor' with a strong *in vitro* barrier, highly resistible to significant interactions of the drug, acts selectively against infection of HIV. The drug, 'Bictegravir,' compared to other INSTIs, has the best *in-vitro* resistance profile of the widely marketed antiretrovirals.[8-9] Emtricitabine and Tenofovir Alafenamide Fumarate inhibit HIV reverse transcriptase from synthesizing DNA, causing viral DNA chain termination and stopping HIV replication.[10-11]

Therapeutic Drug Monitoring (TDM) has been proven helpful for optimizing posology and identifying instances with poor adherence, ultimately leading to greater efficacy and reduced toxicity.[12]

Based on the US Food and Drug Administration (FDA), it is proven that the 'Bictegravir' has been authorized as a daily-once fixed-dose regimen to treat HIV-1 infection.[13-14] Figure 1, Figure 2, and Figure 3 depict the chemical structures of three active medicinal components.

The Following is the Dosing Schedule

Bictegravir (50mg) + Emtricitabine (20 mg) + Tenofovir Alafenamide Fumarate (25mg).

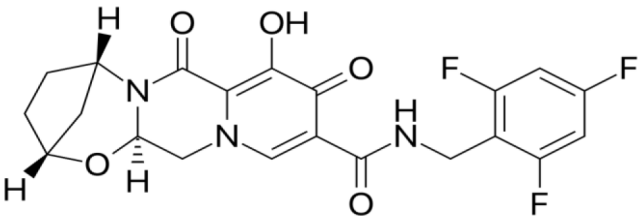

Figure 1: Chemical Structure of Bictegravir.

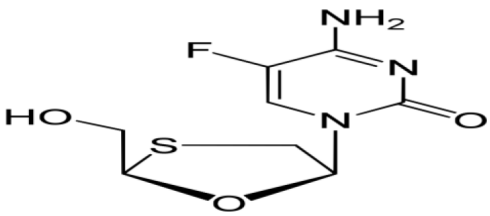

Figure 2: Chemical Structure of Emtricitabine.

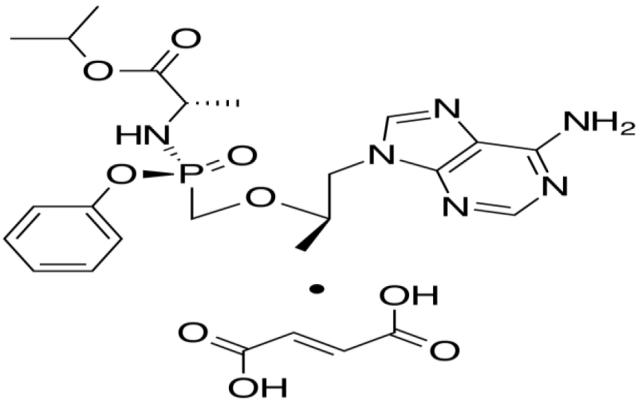

Figure 3: Chemical Structure of Tenofovir Alafenamide fumarate.

Table 1: Comparison of parameters between the existing and current methods.

Sl. No.	Parameter	Existing Method (Raju *et al.*)[21]	Current Method
1	Column	Zorbax C_{18} Column (150 X 4.6 mm, 5µm)	Zorbax XDB C_{18} Column (2.1 X 50 mm, 5µm)
2	Mobile Phase	Methanol: 0.1% Formic acid in Water (85: 15 % v/v)	Acetonitrile: 0.1% Formic acid in Water (70: 30 % v/v)
3	Flow Rate	1.0 mL/min	0.15 mL/min
4	Run Time	4 min	3 min
5	Retention Time of peaks	Comparatively more	Comparatively less
6	Internal Standard	Three Internal Standards were used for three individual drugs	Single Internal Standard used for all three drugs

Based on the studies of 'Department of Health and Human Services,' the present mix of regimens is designed to deal with HIV-1 patients.[15-17] Biktarvy is not advisable to be used with other antiretrovirals.[18] A literature search revealed that there are few stability-indicating RP-HPLC isocratic elution techniques for estimating the medicines of interest. However, there are few techniques available for Emtricitabine, Bictegravir, and Tenofovir AF from pharmaceutical preparations.[19] The proposed approach was shown to be more sensitive and reliable. Table 1 shows the facts. As a result, we established a new, reliable, and efficient technique for quantifying the subject medicines in this study. Because drug stability affects drug moieties' chemical, pharmacological, and toxicological properties, stability studies are conducted according to FDA, EMA, and ICH standards,[20] and the findings are published.

BIC is proved to possess a rich resistance barrier and has an enhanced resistance when compared to remaining INSTIs (such as Dolutegravir (DTG), Raltegravir (RAL), and Elvitegravir (EVG)) available in the market. Bictegravir (BIC) is a recently accepted formulation, co-formulated with Tenofovir Alafenamide (TAF) and Emtricitabine (EMT), for the treatment of HIV infection in patients receiving anti-retrovirals.

Based on the executed Literature review, it is noticed that one method was available for the "Development and Validation of a bio-analytical method for simultaneous determination of Bictegravir, Emtricitabine, and Tenofovir Alafenamide Fumarate in human plasma by LC-MS/MS." However, the current experimental study was proved to be more economical and with lesser analytical time. The details are as tabulated as follows in Table 1.

MATERIALS AND METHODS

Reference and Working Standards

The Reference Standards of Bictegravir, Emtricitabine and Tenofovir Alafenamide Fumarate with the purity of 99% w/w were acquired from 'Hetero Labs', Hyderabad. Naproxen with purity 99% w/w was procured from 'Hetero Labs', Hyderabad. The same was employed for the current study.

Chemicals and Reagents

Acetonitrile – Merck, Methanol - HPLC grade (Rankem), Milli-Q Water HPLC grade (Merck), K2 EDTA Human Plasma.

Instruments used for Method Development and Validation

The Instruments used for the current research work were elaborated for the Make and Model Table 2.

Equipment and LC-MS/MS Assay Conditions

Acquity UPLC (WATERS) system, complete with solvent supply pumps (LC-20AD), degasser (DGU-20 A3), Column oven (CTO-AS), and a high-performance autosampler (SIL HTC) were utilized. Mass spectrometric detection was carried out using multi-power reaction monitoring (MRM) mode on a three-piece instrument (Mass Analyzer – Waters – Quattro Premier XE). The positive ionization interface has been utilized, and Mass Lynx, version 4.1, has been used for data processing.

Mass Spectrometry Conditions

The fundamental concept of MS is the generation and detection of ions separated by their mass-to-charge (m/z) ratios. The method is developed by scanning the parent and its corresponding fragment ions in the mass spectrometer. To this aim, 500ng/mL Analyte Solutions and Internal Standard were prepared with the blend of Acetonitrile: 0.1% v/v Formic acid in 70:30 percent and infused with an Injection volume of 10µL. The parent and product weights were scanned in full scan mode to solve each analyte/ISTD. Each analyte and the respective internal standard were monitored in the range of 100 to 600 atomic mass units and reported. After identifying the parent ion, scanned further using MS/MS mode to attain the product ions. The collision gas is the Nitrogen gas, and the sheath gas is the zero air, and the resolution was measured in unit mass. For Multiple Reaction Monitoring (MRM), the fragment ion has a greater intensity was selected.

Table 2: Instruments used for Method Development and Validation.

Sl. No	Instrument	Make and Model
1	Mass Spectrophotometer	WATERS, Quattro Premier XE, Software: Mass Lynx, Ver 4.1
2	UPLC	WATERS, Acquity UPLC
3	Analytical Balance	Scale-Tec, Axis
4	Refrigerator	Blue Star, CHFSD100DHSW
5	Centrifuge	REMI, Medico Plus
6	Vortex	REMI, CM101
7	Micro-Pipettes	Thermo-scientific

Tuning of Mass Spectroscopy

The Tuning was performed for the three analytes based on their Molecular weight and found that the respective Parent ions and daughter ions were successfully identified. Hence it is evident that the three analytes are 'ionizable' and 'polar.' Therefore, Electron Spray Ionization (ESI) mode was selected for the study.

After choosing the parent, optimizing composite and the gas parameters with 0.15 mL/min flow rate was ensured in injection flow analysis to achieve acceptable values. The syringe pump was connected to a detector and a "T" connector to the LC pump. For the detection of ions through Gas Chromatography, the 'Positive ionization mode' was employed.

Optimized Conditions for Mass-Spectrophotometric Method

A summary of Chromatographic and mass spectrometric conditions is as follows under Table 3.

The Mass Spectra of the three analytes (Bictegravir, Emtricitabine and Tenofovir Alafenamide Fumarate)

Table 3: The Optimized Conditions for Mass-Spectrophotometric Method.	
UPLC	**WATERS ACQUITY UPLC SYSTEM**
Mass	QUATTRO PREMIER XE
Polarity	Positive ion mode
Ion Source	Electron ion Spray
Detection ions:	
Bictegravir	450.1 amu (Parent), 289.0 amu (Daughter)
Tenofovir Alafenamide Fumarate	477.3 amu (Parent), 270.04 amu (Daughter)
Emtricitabine	248.3 amu (Parent), 130.03 amu (Daughter)
Naproxen (Internal Standard)	231.12 amu (Parent), 184.82 amu (Daughter)
Column	Agilent Zorbax XDB C_{18} 2.1X50 mm, 5µ
Target oven temperature of Column	30°C
Target Peltier temperature	5°C
Mobile Phase for experiment	Acetonitrile: 0.1% Formic acid (70:30 v/v)
Flow Rate	0.15 mL/min
Volume of injection	10µL
Sample loop option	Partial loop with needle overfills
Curve Type	6
Retention Time	Tenofovir AF – 0.88 min Emtricitabine – 0.85 min Bictegravir – 1.05 min Naproxen (Internal Standard) – 1.20 min
Run Time	3.0 min

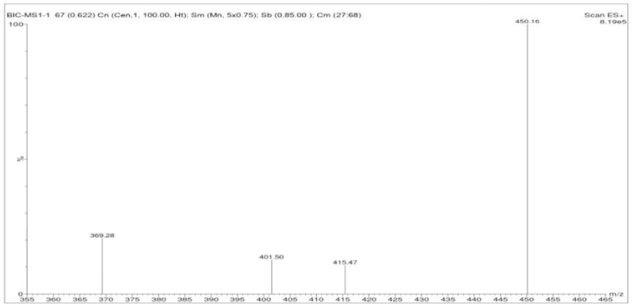

Figure 4: Bictegravir Parent ion identification.

and the Internal Standard (Naproxen) were detailed in following Figures 4-11;

The Mass Reaction Monitoring (MRM) conditions were detailed under Table 4.

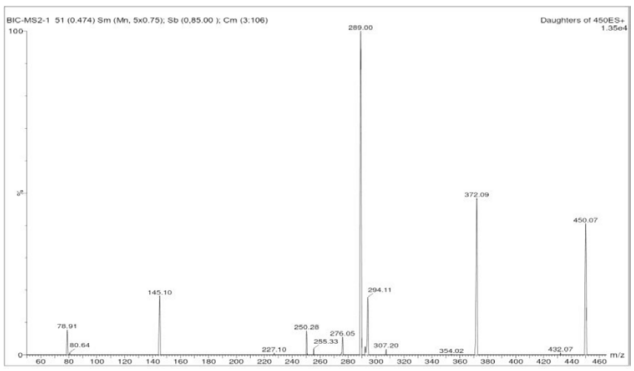

Figure 5: Bictegravir Daughter ion identification.

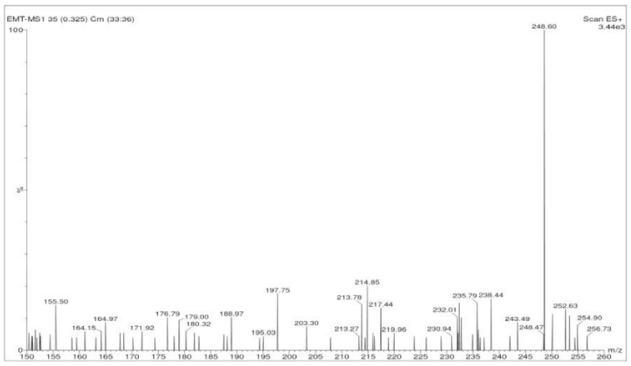

Figure 6: Emtricitabine Parent ion identification.

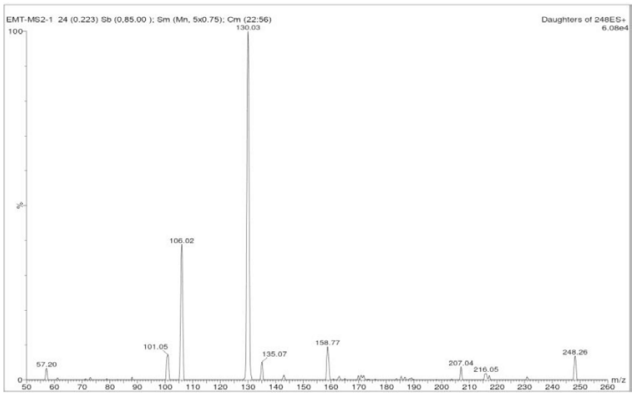

Figure 7: Emtricitabine Daughter ion identification.

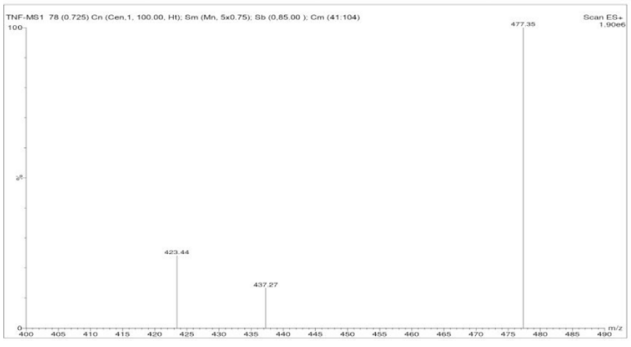

Figure 8: Tenofovir Alafenamide Fumarate Parent ion identification.

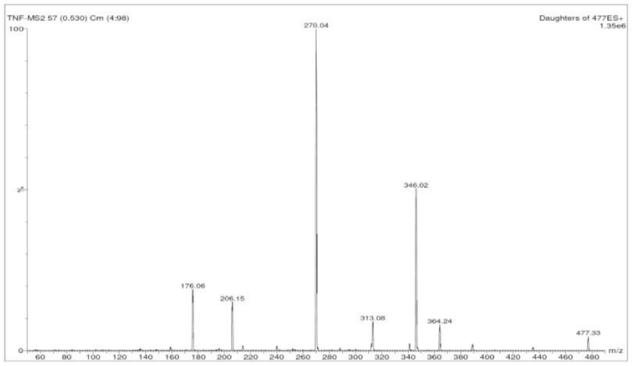

Figure 9: Tenofovir Alafenamide Fumarate Daughter ion identification.

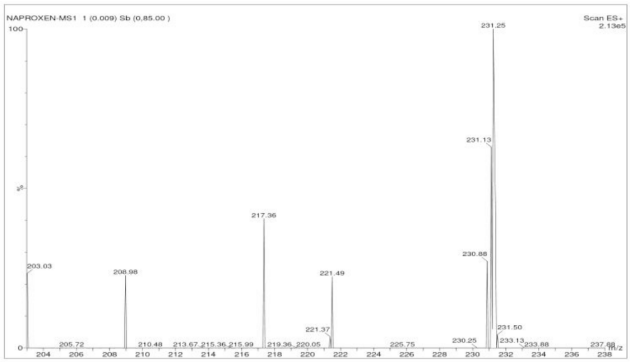

Figure 10: Naproxen Parent ion identification.

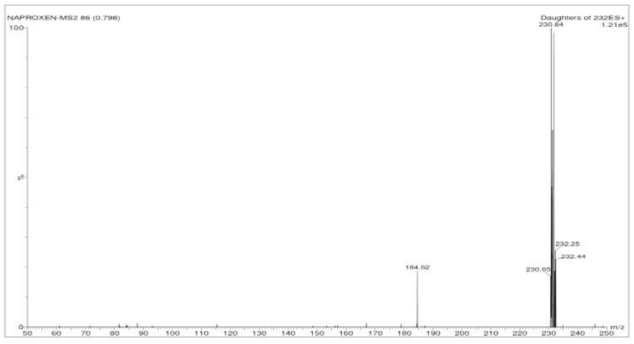

Figure 11: Naproxen Daughter ion identification.

The remaining parameters set for the current method are tabulated below in Table 5.

Optimization of extraction procedure

'Protein Precipitation' can be described as a way of reducing the interference of the matrix with the analyte. Trichloroacetic acid and perchloric acid were used as the precipitant agents. Several organic solvents, such as methanol, ethanol, Acetonitrile, and acetone, are broadly used to remove the plasma proteins and enhance the compatibility with the current method of HPLC technique. The matrix of the sample is diluted using the precipitating agent and vortexed. The filtration technique is used to remove the bulk proteins after this centrifugation. The resulting supernatant liquid or filtrate is examined immediately for the interested analyst. The analyte obtained from the 'protein precipitation technique' should be readily soluble in the reconstituted solvent.

Table 4: Mass Reaction Monitoring (MRM) conditions.

MRM Conditions	Value
Source Temperature	120°C
Desolvation Temperature	400°C
Capillary voltage (KV)	3.00
Desolvation Gas (L/hr)	650
Cone gas (L/hr)	100
RF Lens	0
Extractor	3
Gas cell pirami pressure	0.05
Cone (V)	30
Collision Energy	Bictegravir – 25, Tenofovir AF – 30, Emtricitabine – 15, Naproxen – 20
Multiplier	550

Table 5: Parameters for Mass-Spectroscopic method.

Parameters	Value
Low Resolution Mass (LM1)	15
High Resolution Mass (HM1)	15
Ion Energy (IE1)	1
Low Resolution Mass (LM2)	15
High Resolution Mass (HM2)	15
Ion Energy (IE2)	1

Sample Extraction Procedure

The stored plasma samples have been collected from the freezer maintained at room temperature, and thereby exposed to the following process.

Each sample of 200 µL aliquot was taken into a 5 mL tube (made of polypropylene). To this, 50 µL of ISTD solution (containing 1 µg/mL Naproxen) and 1 mL of Acetonitrile was added as precipitating agent. The contents were vortexed for mixing and were precipitated. The supernatant liquid of 0.8 mL was collected from the resulting solution, filled into the sample vials, and injected onto the LC-MS/MS instrument.

Optimization of Procedure

The standard solutions of three analytes were analyzed to attain the definite optimized chromatographic

conditions. The details of the numerous trials performed are tabulated in Table 6.

Therefore, based on the above Chromatographic trials, the Trial-V was found to be optimized; hence the method was developed using the same experimental conditions.

Table 6: The details of the Trials performed prior to the optimization of method parameters.

Trial No.	Conditions	Remarks
Trial-I	Flow Rate-0.25 mL/min, Column-ACQUITY UPLC BEH C_{18} 2.1X50mm, 1.7µm, ACN:0.1% Formic acid (70:30), Diluent- 50% Methanol, Blank- Diluent	A deformed peak shape was observed.
Trial-II	Flow Rate-0.15 mL/min, Column-Agilent Zorbax XDB C_{18} 2.1×50 mm, 5µ, ACN:0.1% Formic acid (80:20), Diluent- 50% Methanol, Blank- Diluent	Tailing of peaks was observed.
Trial-III	Flow Rate-0.20 mL/min, Column-Agilent Zorbax XDB C_{18} 2.1×50 mm, 5µ, ACN:0.1% Formic acid (80:20), Diluent- 50% Methanol, Blank- Diluent	The resolution of peaks was not attained
Trial-IV	Flow Rate-0.20 mL/min, Column-Agilent Zorbax XDB C_{18} 2.1×50 mm, 5µ, ACN:0.1% Formic acid (70:30), Diluent- 50% Methanol, Blank- Diluent	Low resolution observed
Trial-V (Optimized)	Flow Rate-0.15 mL/min, Column-Agilent Zorbax XDB C_{18} 2.1X50 mm, 5µ, ACN:0.1% Formic acid (70:30), Diluent- 50% Methanol, Blank- Diluent	The obtained peaks were definite with good peak shape, free from tailing, and with high resolution

PREPARATION OF MOBILE PHASE

Preparation of Formic acid (0.1%)

Accurately pipette out 1.0 ml Formic acid and dissolve in 1000 ml of HPLC grade water. Finally, the solution was filtered using a 0.45 Micron membrane filter and sonicated for 10 min.

Preparation of Mobile Phase

Accurately measured 300 ml (30%) of 0.1% Formic acid and 700 ml (70%) of Acetonitrile were mixed and filtered through a 0.45 µ filter under vacuum filtration.

Diluent Preparation

500 ml Methanol and 500 ml Water (50 percent Methanol) were measured to get a 50% solution and filtered using a 0,45 µ vacuum filtering filter.

Needle Wash and Seal Wash Solutions

Diluent (50% Methanol) is used as Needle wash and Seal wash solutions.

Preparation of Internal Standard Solution

The Naproxen, Internal Standard Concentration of 1µg/mL, was prepared using Methanol and Water combination (50:50 v/v).

Preparation of the Standard Solutions of Emtricitabine, Tenofovir AF, and Bictegravir

Preparation of Standard Stock Solutions

A 99.9% pure Bictegravir (BIC), Emtricitabine (EMT), and Tenofovir Alafenamide Fumarate (TNF), obtained from M/S. Hetero Laboratories, Hyderabad, were utilized as standard/ reference to develop the stock solutions for study. Stock solutions of BIC, TNF, EMT, and ISTD (Internal Standard - Naproxen) were prepared by weighing each analyte working standards equal to 10.0 mg into individual 10.0 mL volumetric flasks, dissolved with 5.0 mL of methanol, and diluted to the mark with methanol. The stock solutions are stored in the refrigerator at 1-10°C.

Mixed Standard Solution Preparation

Weighed accurately and transferred one mg of each analyte into a 1 ml diluent and vortexed to dissolve 1000 µg/mL each of Emtricitabine, Tenofovir AF, and Bictegravir (Mixed Stock solution).

Preparation of Working Solutions

Pipette 20 µL of each above stock solution into a 2 ml diluent containing 10 µg/ mL of each analyte. Pipette 100 µL of each above solutions into a 2 ml diluent containing 500 ng/ mL of each analyte. The resulting solutions were used for Tuning the Mass Spectrophotometric, Multiple Reaction Monitoring parameters. 20 µL of solution each from respective 10 µg/mL Solutions were taken, and 2 mL of diluent was added to get 100 ng/mL concentration solutions. The prepared working solutions were stored at room temperature, and daily fresh dilutions were made during analysis. All the volumetric measurements were made using calibrated micropipettes.

The preparation procedure of Calibration Standards spiked with plasma and Quality Control Samples

The working standard solutions (freshly prepared) were used by spiking the blank plasma to produce the 'Calibration standards' and 'Quality control (QC) samples. The Blank plasma lots were collected from screened, healthy individuals and combined before usage.

Calibration standards for concentrations of 2, 10, 50, 250 and 500 ng/mL for Bictegravir, Tenofovir Alafenamide Fumarate, and Emtricitabine were prepared. The Quality control samples at 3.7 -LQC ng/mL, 250 -MQC ng/mL and 400 -HQC ng/mL for BIC and TNF; and 2.2 -LQC ng/mL, 250 -MQC ng/mL, and 400 -HQC ng/mL for EMT were prepared.

Optimization of Sample Extraction Procedure

In optimizing the extraction procedure, 'Protein precipitation' was selected to get consistent and reproducible results with low matrix effects for the intended mass spectrophotometric analysis. A 950 µL of human plasma was added to each analyte sample and vortexed. Added 1 mL of ACN to the solution, vortexed for 5min and centrifuged for 10 min. 0.8 mL of the supernatant solution has been collected from the resultant solution, filled into the sample vials, and injected into the LC-MS/ MS system.

Mobile Phase and System Suitability Criteria

The mobile phase composition was established during the Method Development. A large mobile phase volume was prepared by adding 700 mL of Acetonitrile to 300 mL of 0.1% formic acid to provide consistent results throughout the validation and research analyses. A mobile phase produced a system suitability solution at LLOQ concentration (i.e., 2 ng/mL). A System Suitability Test (SST) was conducted during the validation studies to ensure that the system performance is acceptable for study. As part of the SST test, six system suitability solutions were prepared in duplicate and injected. The instrument performance was found acceptable if the response rate's Coefficient of Variance (%CV) was ~15.0%. Therefore, the instrument performance was ensured before performing the analysis.

Method Development

Methodological development begins with scanning parent and fragment ion analyte solutions utilizing 200 ng/mL BIC, TNF, EMT as analytes, and Naproxen (NPX) as an Internal standard. The analyte solution was injected, and the parent weight of the analyte was scanned. In addition, the parent ion was examined for the MS/MS mode product ions. The protons have been adjusted into positive mode utilizing electro-sprinkler technology based on analytes and ISTD's capacity to receive them. Scanning in the range between 100 and 600 amu has been carried out. Their polarity and the presence in their structures of highly ionized functional groups like the amine or carboxylic acid have great sensitivity to antiviral products in mass ionization analyses compared to non-polar and ionizable organics. All parent peaks and daughter ion peaks were eluted appropriately. In optimizing compound parameters for all three analytes, Tenofovir AF used comparatively high collision energy to get an appropriate response and the respective Mass chromatograms were detailed in following Figures 12-14.

Method Validation

The development and validation of bioanalytical method(s) are of paramount importance concerning drug discovery and development. A 'Bio-analytical method' is the quantitative measurement for the concentration of a drug and/or its metabolite in biological fluids, which may include blood, urine, plasma, serum, and saliva or in extracts of tissue. 'Bio-analytical Method Validation' is the process of demonstrating that the developed analytical method provides accurate results that ensure the quantitative evaluation of medication for the intended use.

They comprise all criteria determining data quality. There are several parameters that are included in 'Method Validation.' The Stability study was also conducted and evaluated concerning the drugs of interest. The current study includes the following.

1. Specificity/ Sensitivity
2. Selectivity
3. Calibration Curve and Linearity Range

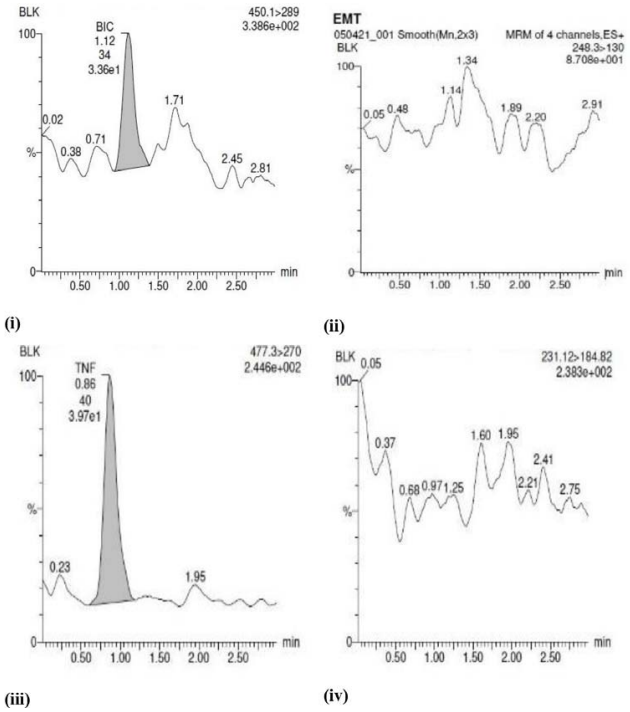

Figure 12: Chromatograms of (i) BIC, (ii) EMT (iii) TNF (iv) Naproxen in Blank Plasma.

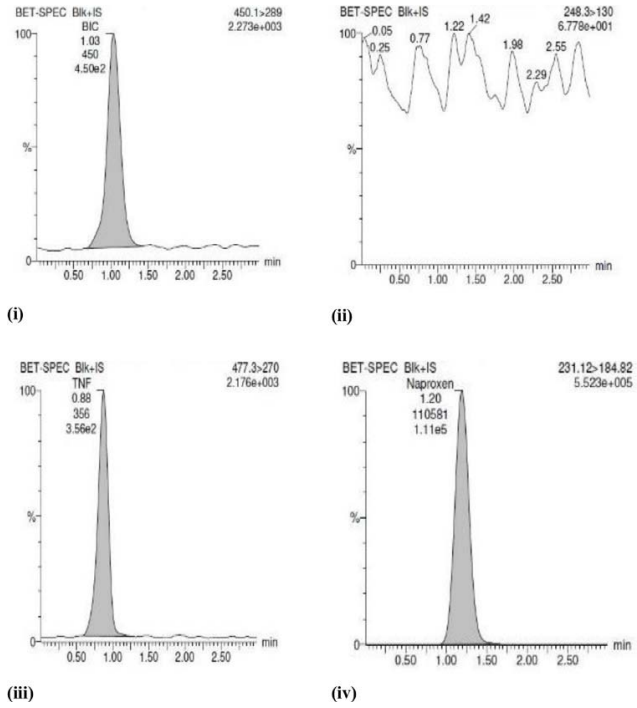

Figure 13: Chromatograms of (i) BIC, (ii) EMT, (iii) TNF, (iv) Naproxen in Blank Plasma with Internal Standard.

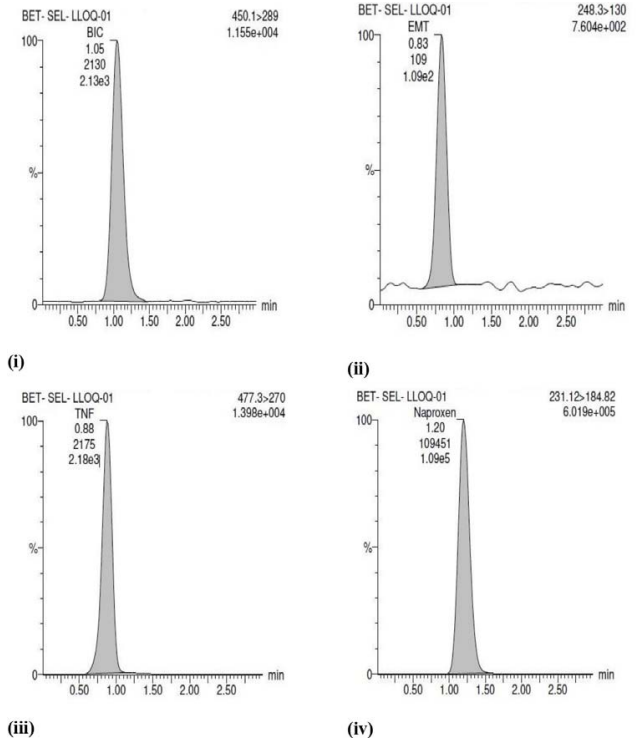

Figure 14: Chromatograms of (i) BIC, (ii) EMT (iii) TNF (iv) Naproxen in LLOQ Samples.

4. Quantification Range of Lower Limit of Quantification 'LLOQ' and Upper Limit of Quantification 'ULOQ'
5. Fresh Quality Control (QC) Samples
6. Accuracy
7. Precision
8. Recovery
9. Matrix Factor
10. Stability Study

Specificity/ Sensitivity

The ability to quantify and differentiate the analyte signal in the presence of components/excipients that are intended to be present in the subject sample.

Procedure

One Blank of 200 µL of human plasma at LLOQ concentration of 2 ng/mL with six replicates were analyzed.

Selectivity

Selectivity is defined as the capacity to measure the analysis technique and determine the presence of endogenous components in the matrix, which might include metabolites, impurities, matrix components, decomposition products, etc. The 'selectivity' of the procedure was analyzed and assessed using Blank plasma, LLOQ sample, and blank plasma along with internal standard (ISTD).

Procedure

One Blank, one Blank + ISTD, one Bictegravir, one Tenofovir AF, and one Emtricitabine sample vials were prepared. From aqueous solutions of 1 mg/ mL, 10µL was taken into 2mL of diluent to get 5 µg/mL concentration. From this solution of each, 50 µL was taken, and 950 µL plasma was added. From the resulting solution, 200µL was taken, added Internal Standard (ISTD), and vortexed. Added 1 mL of ACN, vortexed for 5 min, centrifuged for 10 min, and 0.8 mL of the supernatant solution was transferred to the respective Auto-sampler vials.

Calibration Curve

The selection of proper quantitation range based on expected concentration range determines the 'Calibration curve.'

A calibration curve should be constructed by spiking the matrix with known analyte concentrations and by evaluating sample solutions in the same biological matrix as planned for the research study. During method development, the calibration standards' concentrations are chosen based on the concentration range selected for the current study.[22] Based on the concentration range, the concentrations for the individual standards should be selected.

The calibration curve should consist of a blank sample (without internal standard), one zero (matrix sample with internal standard), and 5-8 non-zero standards, including the LLOQ concentration samples.

Linearity

The 'Linearity' parameter is defined as the capacity of the bioanalytical process to provide analytical results that are directly proportional to the sample concentration within the range of the standard curve.

Therefore, the linearity can be defined according to the relevant calibration curve(s).

Procedure

The concentration levels of a minimum of five to eight are selected for the determination of linear range. The linearity excludes the blank and zero samples. The linearity of the individual calibration curve was determined by plotting the peak area ratio 'y-axis' of analytes against the nominal concentration 'x-axis' of analytes. The details for the preparation of spiked plasma are as follows in Table 7.

The calibration curves were found linear with a correlation coefficient (R^2) of more than 0.99 between 2 and 500 ng/mL for BIC, TNF, and EMT.

Quantification Range

The 'Quantification Range' is the concentration range that includes the LLOQ (Lower Quantification Limit) and ULOQ (Upper Quantitative Limit) of the 'Linearity Range,' depending on the concentration-response.

The Lower quantification limit (LLOQ) is defined as the lowest quantity of an analyte. The upper quantification limit (ULOQ) is defined as the highest quantity of analyte in BIC, TNF, and EMT samples measured with accuracy and Precision less than 20%.

Therefore, based on the obtained Linearity Range, the Lower Limit of Quantification (LLOQ) was recognized as '2 ng/mL' and the Upper Limit of Quantification (ULOQ) as '500 ng/mL'.

Table 7: Details for the preparation of spiked plasma.

Preparations of spiked plasma					
Conc. of Stock (µg/mL)	Stock Volume mL	Plasma Volume	Final Vol. (mL)	Final Conc. (ng/mL)	Details
10	0.050	0.950	1.000	500	CC5
5	0.050	0.950	1.000	250	CC4
1	0.050	0.950	1.000	50	CC3
0.100	0.050	0.950	1.000	10	CC2
0.040	0.050	0.950	1.000	2	CC1

Fresh QC Samples

The fresh quality control samples (QCs) should be used to determine the accuracy and stability of the analyte molecules. QCs assist to evaluate the method performance and analysis stability. The performance QCs are provided to determine the correctness and accuracy of the technique.

Procedure

The Quality Control 'QC' samples in duplicate (that includes a minimum of 3 concentrations- 3 times of the LLOQ, midrange, and high end) should be selected for the study to evaluate the accuracy and stability of the developed method. The sample preparations for the spiked plasma samples of three subject analytes (BIC, TNF and EMT) was detailed as follows in Tables 8 and 9.

Accuracy

The 'exactitude' of a bioanalytical technique may be defined by reference to the test results against the analyte's nominal concentration. The accurateness of samples having known analyte concentrations is evaluated with the parameter 'Accuracy.' It is a measure of closeness of test results obtained by that method to the true value.

Accuracy should be assessed on samples spiked with known amounts of the analyte, the quality control samples, and should be evaluated by at least 3 levels and by 5 concentration measurements. The accuracy of the developed method is assessed using the percent variance coefficient (percent CV).

Table 8: The three levels of QC sample preparation details for EMT.
Fresh QC Samples

Preparations of spiked plasma – EMT					
Conc. of Stock (µg/mL)	Stock Volume mL	Plasma Volume	Final Vol. (mL)	Final Conc. (ng/mL)	Details
8.8	0.05	0.95	1	440	HQC
5	0.05	0.95	1	250	MQC
0.044	0.05	0.95	1	2.2	LQC

Table 9: The three levels of QC sample preparation details for TNF and BIC.

Preparations of spiked plasma – TNF and BIC					
Conc. of Stock (µg/mL)	Stock Volume mL	Plasma Volume	Final Vol. (mL)	Final Conc. (ng/mL)	Details
8	0.05	0.95	1	400	HQC
5	0.05	0.95	1	250	MQC
0.074	0.05	0.95	1	3.7	LQC

Precision

The 'precision' for a developed method can be defined as the closeness between the individually obtained results of pre-defined experimental conditions. The Precision is conducted with QC samples (which includes the LLOQC, LQC, MQC, and HQC in 6 replicates individually) and estimated against a set of standards of the calibration curve.

Intermediate Precision assesses time accuracy and may include several analyzers, appliances, reagents, and labs.

Recovery

The 'Recovery' of the analyte is the measure of detector response received from a quantity of analyte extracted from the plasma (biological matrix) compared against the response obtained from pure standard nominal concentration.

The analyte's regeneration does not need to be 100%, but it should be constant, accurate, and repeatable for an analyte and the internal standard.

Procedure

The extraction recoveries for BIC, TNF, and EMT were determined by comparing the responses from samples of plasma spiked before extraction against the samples of plasma extracted and spiked after the extraction. Mean recoveries of BIC, EMT and TNF were found to be 81.23%, 82.67%, and 87.76%, respectively, with % CV for the three levels ranging between 8.22 to 10.44%.

Matrix Factor

The combined effect of a matrix that includes all components of the sample solutions other than the respective analytes during the measurement of the sample quantities is termed the 'Matrix Effect.' The degree of 'Matrix Effect' is measured with the term 'Matrix Factor.'

Co-eluting components from the matrix may affect and alter the ionization process. However, the Accuracy and Precision of the technique may not be impacted by any observable response in matrix blanks owing to MS selectivity detection.

The 'Matrix Effect' may also be used to assess the quantitative measurement of matrix effects due to the removal or improvement of ionization from a mass spectrometric detector.

The 'Matrix Effect' can be evaluated and represented as 'Matrix Factor,' and this 'Matrix Factor' shall be calculated concerning each analyte of the study.

Procedure

Blank plasma was extracted from six different sources (that includes one hemolytic and one lipemic lot) to study the matrix effect. After extraction, the residue was reformed using a mobile phase containing a defined amount of analyte (LQC level and ISTD; post extracted samples) and evaluated in parallel compared to the aqueous samples.

After extraction, a mobile phase with a known analyte level (LQC level and ISTD; samples extracted after extraction) was reconstructed and analyzed against the aqueous samples. The Matrix Factor for Analyte /ISTD has been compared against the peak response in the absence of matrix ions against the peak response in the presence of matrix ions.

Each response ratio of the post extracted matrix lot and mean of aqueous samples is compared.

Stability

'Stability' may be assessed under certain circumstances for chemical or physical compatibility in a particular matrix at specific intervals. The stability of each analyte is thus determined in the biological matrix at desired stock temperatures.

In addition, the length of sample preparation, sample handling, and analytical run time must be examined throughout an ambient temperature period.

All stability assessments must utilize the samples produced in a suitable blank, a biological matrix that is interference-free using a newly developed stock solution from the analyte.

Procedure

The respective samples were tested for different stability conditions such as Long-Term, Short-Term, and Bench Top Stability, and the results obtained for the %Stability were calculated. Hence the stability of each analyte can be evaluated based on the conducted stability study and preparations details tabulated in Table 10, 11 and 12.

Statistical analysis

The data was processed using the 'Mass Lynx software (Version - 4.1),' and the results were calculated in terms of 'Mean' with 'Standard deviation,' %RSD, and 'Coefficient of regression' for the Accuracy, Precision, and linearity parameters respectively.

RESULTS AND DISCUSSION

System Suitability

The 'specificities' are regarded as a measure based on the analysis to ensure the optimal functioning of the system used for the study. The analyte peaks were found eluted specifically with the best resolution; hence the developed method was '*Specific and Sensitive.*'

Table 10: The stability sample preparation details for BIC and TNF (Bench-Top and Long-Term).

Preparations of spiked plasma – BIC and TNF Bench-Top and Long-Term Stability Study					
Conc. of Stock in µg/mL	**Stock Volume in mL**	**Plasma Volume in mL**	**Final Volume in mL**	**Final Concentration in ng/mL**	**Details**
0.074 (old stock)	0.05	0.95	1	3.7	LQC (Old)
0.074 (Fresh stock)	0.05	0.95	1	3.7	LQC (Fresh)
8 (old stock)	0.05	0.95	1	400	HQC (Old)
8 (Fresh stock)	0.05	0.95	1	400	HQC (Fresh)

Table 11: The stability sample preparation details for EMT (Bench-Top and Long-Term).

Preparations of spiked plasma – EMT Bench-Top and Long-Term Stability Study					
Conc. of Stock in µg/mL	**Stock Volume in mL**	**Plasma Volume in mL**	**Final Volume in mL**	**Final Concentration in ng/mL**	**Details**
0.044 (old stock)	0.05	0.95	1	2.2	LQC (Old)
0.044 (Fresh stock)	0.05	0.95	1	2.2	LQC (Fresh)
8.8 (old stock)	0.05	0.95	1	440	HQC (Old)
8.8 (Fresh stock)	0.05	0.95	1	440	HQC (Fresh)

Table 12: The stability sample preparation details for BIC, EMT and TNF (Short-Term).

Preparations – BIC, EMT, TNF Short Term Stability Study					
Conc. of Stock (µg/mL)	**Stock Volume (mL)**	**Diluent Volume (mL)**	**Final Volume (mL)**	**Final Concentration (ng/mL)**	**Details**
5 (old stock)	0.05	0.95	1	250	MQC (Old)
5 (Fresh stock)	0.05	0.95	1	250	MQC (Fresh)

Selectivity

The analyte peaks of interest were eluted accordingly with no interference of excipients that tend to be present; hence the method is said to be *'Selective.'*

Calibration Curve and Linearity Range

A matrix-based calibration curve was prepared and utilized in the unknown samples tested to calculate the analyte concentration. For the three subject analytes. The Calibrating Curves were found linear with each correlation coefficient (R^2) of more than 0.9926 between 2 and 500 ng/mL for BIC, TNF, and EMT. The results are detailed in Table 13.

Quality Control Samples

Fresh quality control (QC) samples were integrated from different concentration levels (one in 3 X LLOQ, one in the middle, and one at the upper end of the range). The accuracy and stability of the aqueous samples were evaluated.

Table 13: Results of Calibration Curve.

Analyte	Nominal Concentration mg/ml	Mean Found Concentration mg/ml	% CV
BIC	2	2.0	0%
	10	10.4	4%
	50	52.3	4.6%
	200	213.6	6.8%
	500	468.9	-6.2%
EMT	2	1.9	-5.0%
	5	5.5	10%
	30	28.9	-3.66%
	200	222.5	11.25%
	500	504.7	0.94%
TNF	2	2.0	0%
	10	10.0	0%
	50	52.5	5%
	200	218.9	9.45%
	500	479.7	-4.06%

Accuracy and Precision

The Upper and Lower Quantification Limit should be established with appropriate accuracy and Precision, both of which must be defined as LOQ. The results of Precision are tabulated in Table 14.

Extraction Recovery

Recovery is the response of the detector acquired from the analyte quantity added and removed from the matrix, compared to the response achieved for the pure, authentic standard concentration. BIC, TNF, and EMT (at the low, medium, and high concentrations) have been retrieved by comparing the response in pre-extraction plasm samples (n=6) with those in extraction plasma samples. The mean recovery of BIC, EMT, and TNF was shown to range between 81.23%, 94.95%, and 87.76%, with a CV of between 1.2 and 13.9%, respectively. Results are tabulated in Table 15.

Matrix effect

The components from the Co-eluting matrix can restrain or improve ionization but may not have any effect on the obtained result. Matrix Effect is evaluated from six

Table 14: Results of Precision.

Analyte	Level of QC	Nominal concentration in ng/mL	Intra Batch		Inter Batch	
			Mean found concentration in ng/mL	%CV	Mean found concentration in ng/mL	%CV
BIC	LLOQ QC	2	2.1	5	2.1	5
	LQC	3.7	3.9	5.40	3.8	2.70
	MQC	250	268.4	7.36	265.3	6.12
	HQC	400	416.9	4.25	414.3	3.58
EMT	LLOQ QC	2	1.9	-5	2.0	0
	LQC	2.2	2.1	-4.5	2.1	-4.5
	MQC	250	260.7	4.28	265.8	6.32
	HQC	440	451.0	2.5	455.2	3.45
TNF	LLOQ QC	2	2.0	0	1.9	-5
	LQC	3.7	3.5	5.41	3.7	0
	MQC	250	259.0	3.60	253.9	1.56
	HQC	400	418.3	4.58	411.6	2.90

Table 15: Results of Extraction Recovery.

Analyte	QC Level	Extracted Samples	Post Extracted spiked samples	%Recovery	%Mean Recovery	Standard deviation	%CV
BIC	LQC	3813	4742	80.40	81.23	6.68	8.22
	MQC	246011	278663	88.28			
	HQC	379179	505531	75.00			
EMT	LQC	156	202	77.22	82.67	8.63	10.44
	MQC	21369	23069	92.63			
	HQC	64070	81957	78.17			
TNF	LQC	3912	4219	92.72	87.76	7.92	9.02
	MQC	232482	252873	91.93			
	HQC	377035	479513	78.62			
Extraction Recovery at MQC level for Internal Standard							
Internal Standard		**Extracted Samples**	**Post Extracted spiked samples**	**%Recovery**			
Naproxen		121145	149963	80.78			

different lots. The comparison of each post-extracted matrix lot response ratio against the respective aqueous samples. Matrix Factor for analyte or for the ISTD was assessed by comparing the peak response of matrix ions presence to that of absence.[22] Results are tabulated in Table 16, 17 and 18.

Table 16: Results of Bictegravir Matrix Effect.

Analyte	Bictegravir		
	Analyte MF	ISTD MF	IS Normalized Factor
Lot I	0.7436	0.6502	1.143
Lot II	0.7123	0.6496	1.096
Lot III	0.6952	0.6745	1.031
Lot IV	0.6596	0.6211	1.061
Lot V	0.7621	0.6513	1.170
Lot VI	0.7820	0.6425	1.217
Mean	-	-	1.119
% Std Dev	-	-	0.069
% CV	-	-	6.2

Table 17: Results of Emtricitabine Matrix Effect.

Analyte	Emtricitabine		
	Analyte MF	ISTD MF	IS Normalized Factor
Lot I	0.7100	0.6502	1.101
Lot II	0.7352	0.6311	1.165
Lot III	0.7196	0.6576	1.094
Lot IV	0.6985	0.6623	1.055
Lot V	0.6598	0.6104	1.081
Lot VI	0.7215	0.6533	1.104
Mean	-	-	1.100
%Std Dev	-	-	0.037
%CV	-	-	3.4

Table 18: Results of Tenofovir Alafenamide Fumarate Matrix Effect.

Analyte	Tenofovir Alafenamide Fumarate		
	Analyte MF	ISTD MF	IS Normalized Factor
Lot I	0.7830	0.6502	1.204
Lot II	0.8123	0.6129	1.204
Lot III	0.7651	0.6753	1.133
Lot IV	0.7448	0.6549	1.137
Lot V	0.8213	0.6312	1.301
Lot VI	0.7622	0.6458	1.180
Mean	-	-	1.193
% Std Dev	-	-	0.061
% CV	-	-	5.1

Stability

The stability of the three analytes was assessed under specified stability conditions and based on the respective experimental conditions, and the % Stability was evaluated accordingly.

Benchtop Stability

Stability Benchtop (BT) was assessed to ensure that degradation of analytes does not happen during the analysis or extraction of experimental samples. The six quality control samples were collected from the freezer and positioned at room temperature (~25°C). Within 6 hr, representatives of stability standards and sample quality control were processed and evaluated from comparison samples. To estimate the percentage stability, stability concentration and comparison samples were assessed and determined.

Long-term Stability

The storage stability has been evaluated to validate the potency of the analyte that remains unaffected in the matrix throughout the study. The samples were stored at -20 ± 5°C. After 6 hr of duration, the calibration standards and the QC samples from comparison samples were analyzed. By comparison of the average stability sample concentration with the average sample concentration, the stability samples, and comparison samples were evaluated, and the percentage stability was determined.

Short-term stability

To validate the analyte stability inside the test system matrix, shorter storage stability from collecting samples to sample analysis was evaluated. Six duplicates of each piece were processed and evaluated with newly raised calibration standards and quality control samples after storage of the models under room temperature under lab conditions (aqueous comparison samples). The percentage stability was determined by comparing the average stability sample concentration with the average sample concentration.

All the results obtained for stability studies of Bictegravir, Emtricitabine and Tenofovir Alafenamide Fumarate were detailed in Table 19.

CONCLUSION

The newly developed Bio-analytical method determines reasonable Specificity and is found to be selective concerning the three subject drugs (Emtricitabine, Bictegravir, and Tenofovir Alafenamide Fumarate). The current Bio-analytical method development, validation, and establishment of stability study for the existing

Table 19: Results 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 Hr @ 25°C)	BIC	LQC	4024	3813	105.53
		HQC	380541	379179	100.36
	EMT	LQC	162	156	103.84
		HQC	60182	64070	93.93
	TNF	LQC	4110	3912	105.06
		HQC	356853	377035	94.65
Long-Term (6 Hr @ -20°C)	BIC	LQC	3998	3813	104.85
		HQC	367067	379179	96.81
	EMT	LQC	152	156	97.43
		HQC	57515	64070	89.77
	TNF	LQC	4061	3912	103.80
		HQC	349075	377035	92.58
Short-Term (25°C @ Room Temperature)	BIC	MQC	325761	334802	97.30
	EMT	MQC	46559	45405	102.54
	TNF	MQC	300126	294555	101.89

method were performed as per the FDA, EMA and ICH guidelines and found in line with the authorized standards. Economic solvents are employed for analysis, and the results obtained were found reproducible. No interferences or any existence of impurities were reported. The subject drugs are effectively analyzed and attained with higher resolution with lesser retention times compared to the pre-existing methods. The results obtained for the validation parameters 'Accuracy', 'Precision' and '% Recovery' were within the respective specification over the respective 'Linearity' range.

The current method was also proved to be having less Matrix effect. The Stability studies were also found satisfactory with obtained % Stability determined.

Therefore, this proposed Bio-analytical method is recommended for regular analytical purposes in view of providing reliable and reproducible results for the simultaneous estimation of chosen anti-retroviral fixed-dose regimen (Emtricitabine, Bictegravir, and Tenofovir Alafenamide Fumarate) quantitatively through LC-MS/MS technique.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

LC-MS/MS: Liquid Chromatography-Mass Spectrometry; **BIC:** Bictegravir; **TNF:** Tenofovir Alafenamide Fumarate; **EMT:** Emtricitabine; **NPX:** Naproxen; **MRM:** Multiple Reaction Monitoring; **FDA:** Food and Drug Administration; **EMA:** European Medicines Agency; **ICH:** International Conference for Harmonisation; **HIV:** Human immunodeficiency virus; **AIDS:** Acquired Immuno Deficiency Virus; **INSTIs:** Integrase strand transfer inhibitors; **DNA:** Deoxyribonucleic acid; **TDM:** Therapeutic Drug Monitoring; **RP-HPLC:** Reverse Phase - High Performance Liquid Chromatography; **DTG:** Dolutegravir; **RAL:** Raltegravir; **EVG:** Elvitegravir; **UPLC:** Ultra-performance Liquid Chromatography; **ISTD:** Internal Standard; **ESI:** Electron Spray Ionization; **LQC:** Lower Quality Control Concentration; **MQC:** Middle Quality Control Concentration; **HQC:** Higher Quality Control Concentration; **ACN:** Acetonitrile; **SST:** System Suitability Test; **LLOQ:** Lower Limit of Quantification; **ULOQ:** Upper Limit of Quantification; **QC:** Quality Control.

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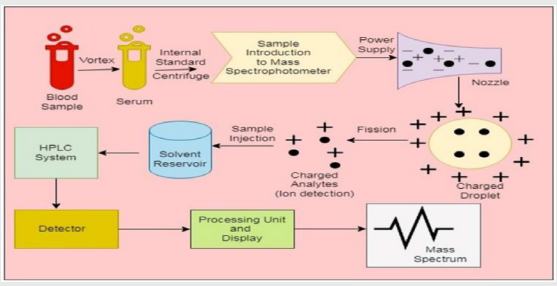
PICTORIAL ABSTRACT



SUMMARY

- The least retention times were achieved for the current method.
- The use of economic solvents enhanced the application of the subject method.
- The lower Matrix effect evaluated aids in assessing samples using human plasma.
- The lower calibration range is appropriate for all three drug moieties.

About Authors

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