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Development and Validation of an LC-MS/MS Method for the Determination of Lamivudine, Tenofovir & Efavirenz in human plasma

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ABSTRACT

Introduction: Therapeutic drug monitoring of Lamivudine, tenofovir and efavirenz, three commonly used antiretroviral drugs, is important to maximize effectiveness while minimizing side effects.

Materials and Methods:

Chromatographic separation was done on Xterra, C18 (2), 150 X 4.6 mm, 5 µm column with a mobile phase composed of 10mM ammonium formate-methanol-acetonitrile in the ratio of 50:25:25 (v/v/v), at a flow rate of 1.0 mL/min. Turbo ion-spray interface (TIS) operated in positive ionization mode was used for the mass spectrometric detection. The MRM transitions monitored were m/z 230.1/112.1

(lamivudine), m/z 288.0/176.2 (tenofovir), m/z 316.2/168.1 (Efavirenz), m/z 248.1/130.1 (Emtricitabine,ISTD) and m/z 287.2/191.2(Abacavir,ISTD). The method was developed and the established calibration ranges are 10-3018 ng/mL, 5-500ng/mL and 20-6021ng/mLfor lamivudine, tenofovir disoproxilfumarateand efavirenz respectively. The slope values are consistent and regression values were found to be more than or equal to 0.99. The accuracy for run size reproducibility batch and dilution integrity (1/2, 1/4) for lamivudine, tenofovir disoproxilfumarateand efavirenz was found to be in the range of 96.9-102.2%. The precision was found to be less than 8.1% for three analytes. Further, the reported method was validated as per the ICH guidelines and found to be well with in the acceptable range.

Results: The method showed good accuracy, low limits of quantification, adequate recovery, minimal matrix effects, and specificity. Analyte stability under multiple storage conditions was demonstrated.

Conclusion: The validated LC-MS/MS method provides a reliable tool for therapeutic drug monitoring and pharmacokinetic studies of anti-HIV regimens. The assay can be applied to large populations, especially in resource-poor settings, to help individualize dosing and improve clinical outcomes while reducing toxicity.

Keywords: *Lamivudine, Tenofovir, Efavirenz, Abacavir and Emtricitabine*

1. INTRODUCTION

Liquid Chromatography-Mass spectrometry (LC-MS) coupled with a Turbo spray interface (TSI) provides a sensitive and powerful technique [1,2]. LC separates mixtures into components, while MS identifies components with high specificity and sensitivity. The coupled technique can analyze biochemical, organic, and inorganic compounds in complex samples [3,4].

Lamivudine, Tenofovir and efavirenz inhibit Human Immuno-deficiency Virus (HIV) reverse transcriptase and lower the amount of HIV in the body. They are administered together as part of highly active antiretroviral therapy (HAART) [5,6]. A bioanalytical method is needed to determine drug concentrations in biological fluids like plasma for purposes of therapeutic drug monitoring and pharmacokinetic studies. LC/MS/MS provides high sensitivity and specificity well-suited for these applications [7]. Method development and validation ensure a reliable technique under given laboratory conditions using available resources. This includes demonstrating that the technique is reproducible and fit for its intended use in analyzing drug levels in biological matrices [8]. The main objective of this study is to develop and validate an efficient LC-MS/MS method for the simultaneous quantification of tenofovir and emtricitabine in human plasma samples to optimize antiretroviral therapy.

2. MATERIAL AND METHODS

Materials

The analytes, Lamivudine , tenofovir and efavirenz, and their internal standards (abacavir and emtricitabine) were procured from Clear synth Ltd, Hyderabad. Methanol and acetonitrile are obtained from Thermo Fischer Scientific, Hyderabad, while ammonium formate, formic acid, and ammonia solution are obtained from Merck Specialities Ltd, Kakinada.

Blank Plasma

K2 Ethylene Diamine Tetra-acetic acid (EDTA) human blank plasma was sourced from Doctor's Pathological Lab in Hyderabad. This plasma was used for method development, validation, and analysis. This technique was utilized to generate calibration standards and quality control samples. The plasma was collected from healthy, non-smoking volunteers who provided informed consent for use for research purposes. Blood samples were collected in K2 EDTA vacutainers from the volunteers [9].

Instrumentation

The study utilized the following instruments for the analysis. An analytical balance model, CP225D from Sartorius, was used. A centrifuge model SW12R from Firlabo, France, was employed. Deep freezers at -86°C and -20°C of the VIP series from Sanyo, USA, were used to store samples. A

High-Performance Liquid Chromatography (HPLC) system from Shimadzu, Japan comprising LC-20 AD pumps, DGU-20 A3 degasser, CTO-ASVP column oven, and SIL HTC autosampler was utilized. A microbalance model CP2P from Sartorius, Germany, was used. Micropipettes of 5-50 µL and 100-1000 µL from Brand, Germany, were employed. An API-4000 mass spectrometer with Turbo ion spray interface in negative ionization mode from MDA, Sciex, Canada, was used. A Turbo Vap LV nitrogen evaporator from Caliper Life Sciences, USA, was used. An Orion star pH meter from Thermo Electron Corporation, USA, was employed. A SpeeDisk-48 solid phase extraction unit from Orochem Technologies, USA, was used. A Powersonic 510 ultrasonic bath from Hwashin Technologies, Korea, and a Spinix vortex from Tarsons, India, were utilized. Purified water was obtained from water purification units, including the Elix 10 and Milli-Q gradient A10 from Millipore, USA. Data acquisition and processing on the LC-MS/MS instrument were performed using Analyst software version 1.5.1.

Chromatographic Conditions

The chromatographic conditions were optimized through a trial and error or scouting approach. Various parameters were varied and tested to determine the optimal conditions for best performance [10]. The mobile phase consisted of 10 mM ammonium formate - methanol -acetonitrile- 50:25:25 (v/v/v). A XterraC18 (2) (150 × 4.6 mm, 5µm) column was used. The flow rate was 1.0 mL/min with an injection

volume of 10 µL. The column oven temperature was 35 ± 1 °C, and the autosampler temperature was 5 ± 1 °C. The retention times were Lamivudine: 1.69 min., tenofovir: 2.04 min., efavirenz: 1.81 min., abacavir: 1.81min internal standard (ISTD), and emtricitabine: 1.7 min (ISTD) and the run time was

3.5 min with a splitness of 40:60.

Mass spectrometric conditions

For mass spectrometric detection, a turbo ion spray interface operated in positive ion mode was used [11]. The multiple reaction monitoring (MRM) transitions monitored were m/z 230.1/112.1 (lamivudine), m/z 288.0/176.2 (tenofovir), m/z 316.2/168.1 (efavirenz), m/z 248.1/130.1 (emtricitabine, ISTD) and m/z 287.2/191.2 (abacavir, ISTD).

The dwell time was set to 200 ms for each transition. Quadrupoles 1 and 3 were operated at unit resolution. The gas/turbo ion spray source parameters were optimized as follows. Nitrogen was used as the ion source gas 1 and was set at 35 psi to aid in analyte spray formation [12]. Nitrogen was also used as ion source gas 2 at 45 psi to stabilize the ion spray. A curtain gas flow of 30 psi of nitrogen was used to improve ion transmission into the mass spectrometer. The collision gas was set at 10 psi of nitrogen for collision-induced dissociation. The ion spray voltage was set to 5000 V to produce maximally charged droplets. The source temperature was maintained at 500°C to assist desolvation of ions and droplets. The interface heater setting was turned on to further aid the desolvation of ions entering the mass spectrometer [13]. The mass spectrometric parameters were optimized to achieve maximum sensitivity. The declustering potential was 70V for lamivudine 41V for tenofovir 31V for efavirenz 55V for emtricitabine and 62V for abacavir. An entrance potential of 10V was applied for all analytes. A collision energy of 38V was used for lamivudine 16V for tenofovir & efavirenz while 31V was used for emtricitabine and 24 for abacavir. The collision cell exit potential was 22V for lamivudine 15V for tenofovir 12V efavirenz 21V for emtricitabine and 15V for abacavir**.**

Stock Solutions and Calibration Standards

Individual stock solutions of 1 mg/mL lamivudne 1 mg/mL tenofovir 1 mg/mL efavirenz 1 mg/mL abacavir and 1 mg/mL emtricitabine were prepared in methanol to quantify the drugs in human plasma using LC-MS/MS [14]. Serial dilutions of these stock solutions were performed to obtain calibration standards and quality control samples. Separately, internal standard stock solutions of 2 mg/mL abacavir and 2 mg/mL emtricitabine were diluted to achieve a 500 µg/mL working concentration of each internal standard [15]. The stock solutions were stored at 2-8^0C and found to be stable for 22 days [16].

Calibration curves ranging from 10-3018 ng/mL, 5-500 ng/mL and 20-6021 ng/mL for lamivudine, tenofovirdisoproxilfumarate and efavirenz respectively were made. Quality control plasma samples were also prepared to standardize the method developed. The lamivudine concentrations used were 10ng/mL, 20 ng/mL, 40 ng/mL, 100 ng/mL, 300 ng/mL, 900 ng/mL, 2415 ng/mL and 3018 ng/mL tenofovir concentrations used were 5 ng/mL, 10 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL ,200 ng/mL ,400 ng/mL and 500 ng/mL, efavirenz concentrations used were 20 ng/mL, 40 ng/mL, 100 ng/mL, 500 ng/mL, 1500 ng/mL ,3000 ng/mL ,5001 ng/mL and 6022 ng/mL representing quality control levels of the lowest limit of quantification quality control (LLOQ), low-quality control (LQC), medium quality control (MQC), high-quality control (HQC) and dilution quality control (DQC) respectively.

Desired drug levels in plasma were achieved by spiking with 1% of the working standard dilutions. Long-term stability quality control samples were prepared in pooled plasma and stored at -70^0C. For

sample preparation, 400 µL of plasma was mixed with 100 µL of the 500 µg/mL internal standard solution. Then 0.4 mL of 0.2% formic acid was added and loaded onto preconditioned Phenomenex Strata-X solid phase extraction cartridges. The cartridges were washed and eluted with 1 mL of 2% ammoniated methanol. The eluate was dried under nitrogen, reconstituted in 400 µL mobile phase, and 10 µL injected into the LC-MS/MS system [17].

3. METHOD VALIDATION

System Suitability and Repeatability Assessment

To assess that the LC-MS/MS system and method were functioning properly, 2 injections of a low-concentration calibration standard and 6 injections of a high-concentration calibration standard were performed for Lamivudine, tenofovir and efavirenz [18]. The low concentration standard was injected to inspect the peak shapes. The coefficient of variation (%CV) of the area ratios from the six high-concentration standard injections should be less than 4% according to guidelines [16].

Carryover and cleaning validation

To evaluate the autosampler performance in terms of carryover, extracted blank samples, high-standard samples, and low-standard samples were injected. The carryover should not be more than 20% and 5% at the retention times of the analytes and internal standards, respectively [19].

Selectivity, sensitivity, and suitability of the assay

The ability of the method to accurately measure analyte concentrations in the presence of endogenous plasma components and exogenous interfering substances was evaluated. Selectivity was assessed by analyzing 8 different lots of drug-free human plasma along with 2 lots each of hemolyzed plasma and lipemic plasma that were processed using the sample preparation method [3]. The effect of co-administered medications was studied by spiking acetaminophen, ibuprofen, ranitidine, and ondansetron into analyte-free plasma at concentrations equivalent to their reported maximum plasma levels found in patients [16]. Any interfering peaks at the retention times of Lamivudine, tenofovir and efavirenz, and their internal standards were monitored to determine the selectivity of the method for the intended purpose in real patient samples.

Evaluation of Matrix Effects

Matrix effects should be evaluated for mass spectrometry-based methods to assess signal suppression or enhancement due to the ionization of analytes in biological matrices. To determine if the method was free from matrix effects, the post-extraction responses of Lamivudine, tenofovir and efavirenz in plasma samples from 10 different donors (including 2 each of hemolyzed and lipemic samples) were compared to the responses in aqueous solutions [20]. Matrix effects were determined at the low and high-quality control levels by calculating the matrix factor for the analyte and corresponding internal standard. Then, the matrix factor of the analyte was normalized to the internal standard matrix factor to obtain the internal standard normalized matrix factor. A value of 1 indicates no matrix effects, while the limits for the normalized matrix factor are 0.85 to 1.15 [21].

Method Linearity

Three calibration curves were analyzed to evaluate linearity. The peak area ratios of the analyte to standard internal response were plotted on the y-axis against concentration on the x-axis [11,22].

Extraction efficiency (Recovery)

Consistent and comparable extraction recovery during sample preparation was necessary for precise and accurate quantitative results. The relative recovery of Lamivudine, tenofovir and efavirenz was evaluated at three concentration levels (low, medium, and high-quality control samples) by comparing the response of post-extraction spiked plasma samples to pre-extraction spiked plasma samples (n = 6) [11]. Absolute recovery was also determined by comparing the response of extracted plasma samples to standard aqueous solutions [23].

Accuracy and precision

The accuracy and precision of the method over a complete run size were evaluated to demonstrate the reproducibility and consistency of the method [24]. A total of 50 replicates each of low, medium, and high-quality control samples were prepared and quantified against freshly prepared calibration curves.

The quality control samples were injected in ascending order of concentration from low to high [25].

Stability evaluation

The stability of Lamivudine, tenofovir and efavirenz was assessed under various conditions: short-term bench top stability at room temperature, freeze-thaw stability after 5 cycles, autosampler stability, dried extract stability, and long-term stability at -70°C [26].

4. RESULTS AND DISCUSSION

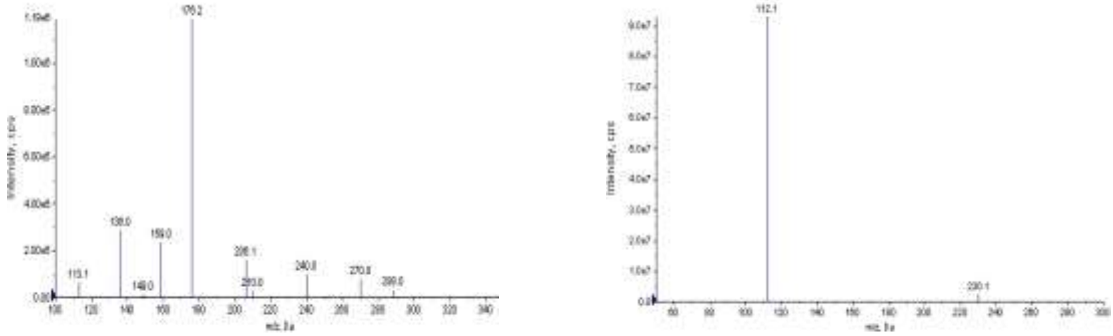
Method Development

To obtain precise and reliable quantification results, it is vital to fine-tune the chromatographic process variables, sample extraction process, and mass spectrometric parameters with equal importance. These different aspects must be tuned and developed in harmony for the best overall performance of the analytical method [27].

Optimization of LC-MS/MS conditions

The analytes (Lamivudine, tenofovir and efavirenz, and their internal standards) were individually infused into the mass spectrometer to optimize the MS conditions for better sensitivity [28]. Initially, a Q1 scan was performed by optimizing the Declustering Potential (DP) and Entrance Potential (EP) to select the parent ion. This was followed by an MS/MS scan to optimize the Collison Energy (CE) and Collison Cell Exit Potential (CXP) to select product ions. In optimizing the mass spectrometric parameters for quantification, the $[M^{+}H]^{+}$ precursor ions observed in the mass spectrum, along with their reproducible fragment ions, were selected for MRM quantification. Unit resolution mode with 300 ms dwell time was employed for every transition. Different stationary phases like C18 and C8 were evaluated. The Xterra column gave a better peak shape, resolution, and required sensitivity. A moderate-strength buffer of 10mM ammonium formate provided a more signal-to-noise ratio. The total run time was 3.5 minutes. The product ion spectra of analytes and internal standards are shown in Figure 1.

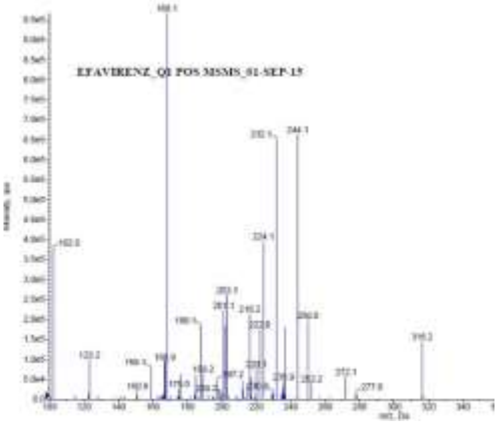
Figure 1. Product ion spectra of a. Lamivudine b. Tenofovir c. Efavirenz d. Emtricitabine and e. Abacavir

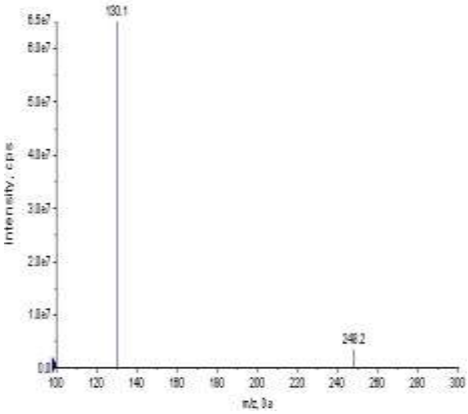


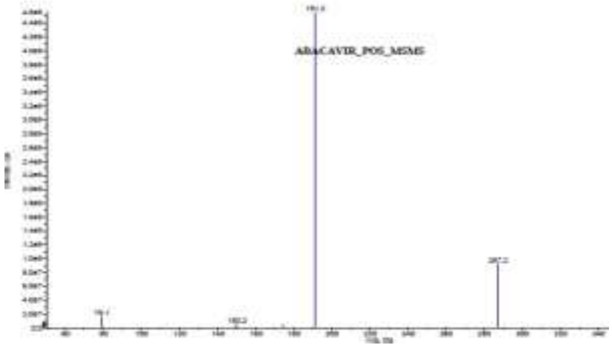
A

B

c.







Sample Preparation Method Development

Both liquid-liquid extraction and solid-phase extraction techniques were evaluated for preparing plasma samples. After considering various methods for solid phase extraction, the Phenomenex

Strata-X cartridges were chosen as the preferred option due to their ability to provide high and uniform extraction recoveries, minimal matrix effect, and clear sample extracts. Although washing the cartridges with 5% methanol initially provided chromatograms with clean peaks, the recovery rates for emtricitabine were observed to be low. Washing the cartridges with 0.1% formic acid solution improved the recovery of emtricitabine. The extraction procedure was carefully optimized and adjusted to obtain similar recoveries for all the analytes (Lamivudine, tenofovir and efavirenz) and their corresponding internal standards (abacavir and emtricitabine). The comparable recoveries for analytes and internal standards, along with acceptable normalized response factors of the internal standards, led to a stable and reproducible quantification method [18].

Method Validation

System Suitability and Repeatability Assessment

The actual % CV values found for the area ratios ranged from 2.5% to 12.6% across the entire method validation, demonstrating that the LC-MS/MS system and method yielded repeatable and reproducible results with good precision [16,29]. This indicated that the system and analytical method were suitable for the intended purpose. The results of the system suitability and repeatability assessment are shown in Table 1.

Table No. 1. Results of system suitability and repeatability assessment

Sl.No	System suitability		
	Parameter	**Acceptance Criteria**	**%CV observed**
01	Retention time	±2.0%	0.61%
02	Capacity factor (k')	1.5 -20	10.23

03	Tailing factor (T)	0.8 -1.5	1.02
Repeatability			
04	Intra-day precision	≤15% at LLOQ, ≤15% at LQC and ≤15% at HQC	0.42% to 2.12%
05	Inter-day precision	≤15% at LLOQ, ≤15% at LQC, and ≤15% at HQC	0.89% to 2.38%

Carryover and cleaning validation

The results in Table 2 show that the carryover is not observed as the peak area in blanks after the Upper Limit of quantification (ULOQ) samples was <20% of LLOQ, indicating good autosampler performance. No interfering peaks were observed in blank water, methanol, or plasma washes, demonstrating the adequacy of the glassware cleaning procedure used [7].

Table No. 2. Results of Carry over, cleaning validation, selectivity and sensitivity

Sl. No.	Parameter	Results
01	Carryover assessment	Peak area of carryover in blank after ULOQ <20% of LLOQ
Glassware cleaning validation		
02	Blank water wash	No peaks >20% of LLOQ
03	Blank methanol wash	No peaks >20% of LLOQ
04	Blank plasma wash	No peaks >20% of LLOQ
05	Selectivity	Interference at LLOQ retention time < 20% of LLOQ response
Sensitivity		
06	Limit of Detection (LOD)	Lamivudine 0.8 ng/mL Tenofovir 0.4 ng/mL Efavirenz 0.2 ng/mL
07	Lower Limit of Quantification (LLOQ)	Lamivudine 2 ng/mL Tenofovir 1 ng/mL Efavirenz 0.5 ng/mL

Selectivity, sensitivity, and suitability of the assay

The interference observed in the blank plasma lots was compared to the mean response of processed samples at LLOQ. The precision and accuracy at the LLOQ level were found to be 6.7% and 96.4% for lamivudine ,5.8% and 94.8% for tenofovir and 8.5% and 96.5% for efavirenz respectively. Assay suitability was also assessed, and no cross-talk was observed between the analytes and internal standards, demonstrating the specificity of the method [25].

Evaluation of matrix effect

Matrix factor values close to 1 indicate little matrix effect. The average matrix factor values for lamivudine, tenofovir and efavirenz were close to 1, ranging from 0.92 to 1.05, 0.91 to 1.09 and 0.92 to

1.09, respectively (results shown in Table 3). This suggests a minimal matrix effect. The %CV (coefficient of variation) values were all below 5%, meeting the typical acceptance criteria for matrix effect evaluation. This indicates good precision and reproducibility in the matrix effect assessment. The matrix factor values were determined for various plasma batches, including hemolytic and lipemic samples. The fact that the matrix factor was consistent across different plasma types indicates the robustness of the method and its suitability for real patient samples. Standard deviation and %CV were slightly higher for HQC samples compared to LQC samples. This is not unexpected as the matrix effect and ion suppression tend to increase at higher analyte concentrations [22].

Table No. 3. Matrix effect data results

ISNMF-Internal standard normalised matrix factor						
Blank Plasma Lots	**Lamivudine**		**Tenofovir**		**Efavirenz**	
	LQC	**HQC**	**LQC**	**HQC**	**LQC**	**HQC**
LOT-1	**0.92**	**0.95**	**0.93**	**1.02**	**0.94**	**0.95**
LOT-2	**0.91**	**0.91**	**0.95**	**1.02**	**1.08**	**0.93**
LOT-3	**1.02**	**0.99**	**0.91**	**0.98**	**0.99**	**0.91**
LOT-4	**1.00**	**1.08**	**0.95**	**0.95**	**0.94**	**0.95**
LOT-5	**1.02**	**0.94**	**0.98**	**1.04**	**0.96**	**1.10**
LOT-6	**1.05**	**0.92**	**0.91**	**1.11**	**0.92**	**1.09**
LOT-7 hemolytic	**0.94**	**0.89**	**0.99**	**0.98**	**1.01**	**0.96**
LOT-8 hemolytic	**0.93**	**0.94**	**1.04**	**1.06**	**1.05**	**0.97**
LOT-9 lipemic	**0.98**	**0.9**	**1.06**	**0.95**	**1.02**	**0.98**
LOT-10	**0.97**	**0.95**	**1.09**	**0.96**	**1.09**	**0.92**
lipemic						
Mean	**0.974**	**0.947**	**0.981**	**1.007**	**1.000**	**0.976**
SD	**0.0481**	**0.0550**	**0.0635**	**0.0527**	**0.0604**	**0.0664**
%CV	**4.9**	**5.8**	**6.5**	**5.2**	**6.0**	**6.8**

Method Linearity

The calibration curves were linear over the range of 10-3018 ng/mL, 5-500 ng/mL and 20-6021 ng/mL for lamivudine, tenofovirdisoproxilfumarate and efavirenz respectively. The slope values were

consistent, and correlation coefficients were ≥ 0.99. The mean measured concentrations of the

calibration standards met the acceptance criteria of accuracy (±15%) and precision (≤15%). Table 4 shows the mean back-calculated concentrations of the calibration standards for tenofovir and emtricitabine.

Table No. 4: Summary of calibration standards

Lamivudine calibration standards

Nominal Conc (ng/ml)	10	20	40	100	300	900	2415	3018
Mean conc found (ng/ml)	908	17.9	35.9	99.4	276.1	950.2	2269.5	3005.2
%Accuracy	98.0	89.5	89.8	99.4	92.0	105.6	94.0	99.6
%Relative error	-2.0	-10.5	-10.3	-0.6	-8.0	5.6	-6.0	-0.4

Tenofovirdisoproxilfumarate calibration standards

Nominal Conc (ng/ml)	5	10	25	50	100	200	400	500
Mean conc found (ng/ml)	4.9	9.5	24.5	49.8	99.7	202.1	401.2	499.5
%Accuracy	98.0	95.0	98.0	99.6	99.7	101.1	100.3	99.9
%Relative error	2.0	5.0	2.0	0.4	0.3	1.1	0.3	0.1

Efavirenz calibration standards

Nominal Conc (ng/ml)	20	40	100	500	1500	3000	5001	6022
Mean conc found (ng/ml)	19.5	38.4	99.5	485.9	1456.7	2857.1	4563.1	5896.4
%Accuracy	97.5	96.0	99.5	97.1	97.1	95.2	91.3	97.9
%Relative error	-2.5	-4.0	-0.5	-2.9	-2.9	-4.8	-8.7	-2.1

Extraction efficiency (Recovery)

The mean recovery for lamivudine at at LQC, MQC and HQC levels was 92%,93% and 96%, respectively, in absolute terms and 96%, 97%, and 95%, in relative terms. For tenofovir at LQC, MQC and HQC levels was 96%, 95% and 96%, in absolute terms and 92%, 94%, and 92%, in relative term. For emtricitabine, the mean recovery at LQC, MQC, and HQC levels was 95%, 96%, and 93% in absolute terms, and 94%, 96%, and 93% in relative terms. The recovery values were within acceptable limits indicating that the extraction method yielded consistent and reproducible recoveries [30].

Accuracy and Precision

The accuracy for the entire run size batch ranged from 96.9-102.2%, while the precision was less than

8.1% for both tenofovir and emtricitabine. For lamivudine, the intra-batch accuracy at LLOQ, LQC, MQC, and HQC levels was 91.8%, 89.5%, 91.7%, and 89.3%, respectively, with %CV ranging from

3.1%- 11.2%. The inter-batch accuracy for lamivudine was 96.6% at LLOQ, 93.5% at LQC, 97.5% at MQC, and 96.5% at HQC level with % CV between 3.6% - 6.8%. For tenofovir, the intra-batch accuracy at LLOQ, LQC, MQC, and HQC levels was 92.5%, 91.5%, 96.8%, and 96.5%, respectively, with %CV ranging from 2.5%- 6.7%. The inter-batch accuracy for tenofovir was 94.8% at LLOQ, 99.7% at LQC, 93.8% at MQC, and 93.4% at HQC level with % CV between 5.8% - 12.6%. For efavirenz, the intra-batch accuracy was 96.4% at LLOQ, 93.5% at LQC, 97.5% at MQC, and 103.5% at HQC level with %CV between 2.9% - 9.6%. The inter-batch accuracy for efavirenz was 96.5% at

LLOQ, 97.8% at LQC, 92.8% at MQC, and 96.4% at HQC level, with %CV ranging from 2.8% - 9.4%. This indicated that the method yielded accurate and precise results over a full run size, establishing its reliability for batch analysis of patient samples [27]. The accuracy and precision results obtained from dilution integrity experiments at 1:2 and 1:4 dilutions were also within acceptable limits. The blank plasma chromatograms are shown in Figure 2, while the chromatograms of the analytes at LLOQC and HQC levels are shown in Figures 3 and 4, respectively.

Figure 2. Blank plasma chromatograms of a. Lamivudine b. Tenofovir c. Efavirenz d. Emtricitabine and e. Abacavir

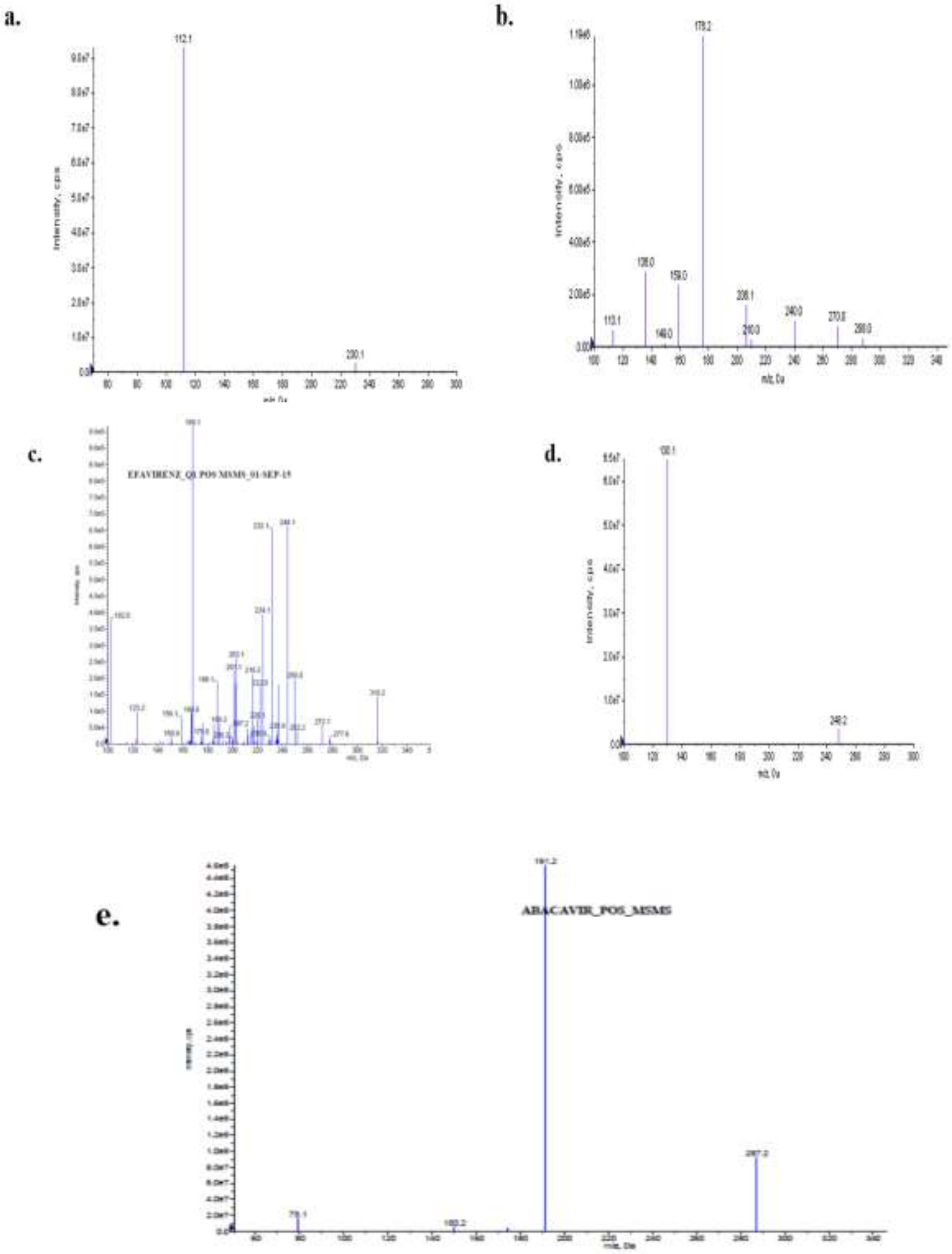


Figure 3. Chromatograms of a. Lamivudine b. Tenofovir c. Efavirenz d. Emtricitabine and e. Abacavir at LLOQC level

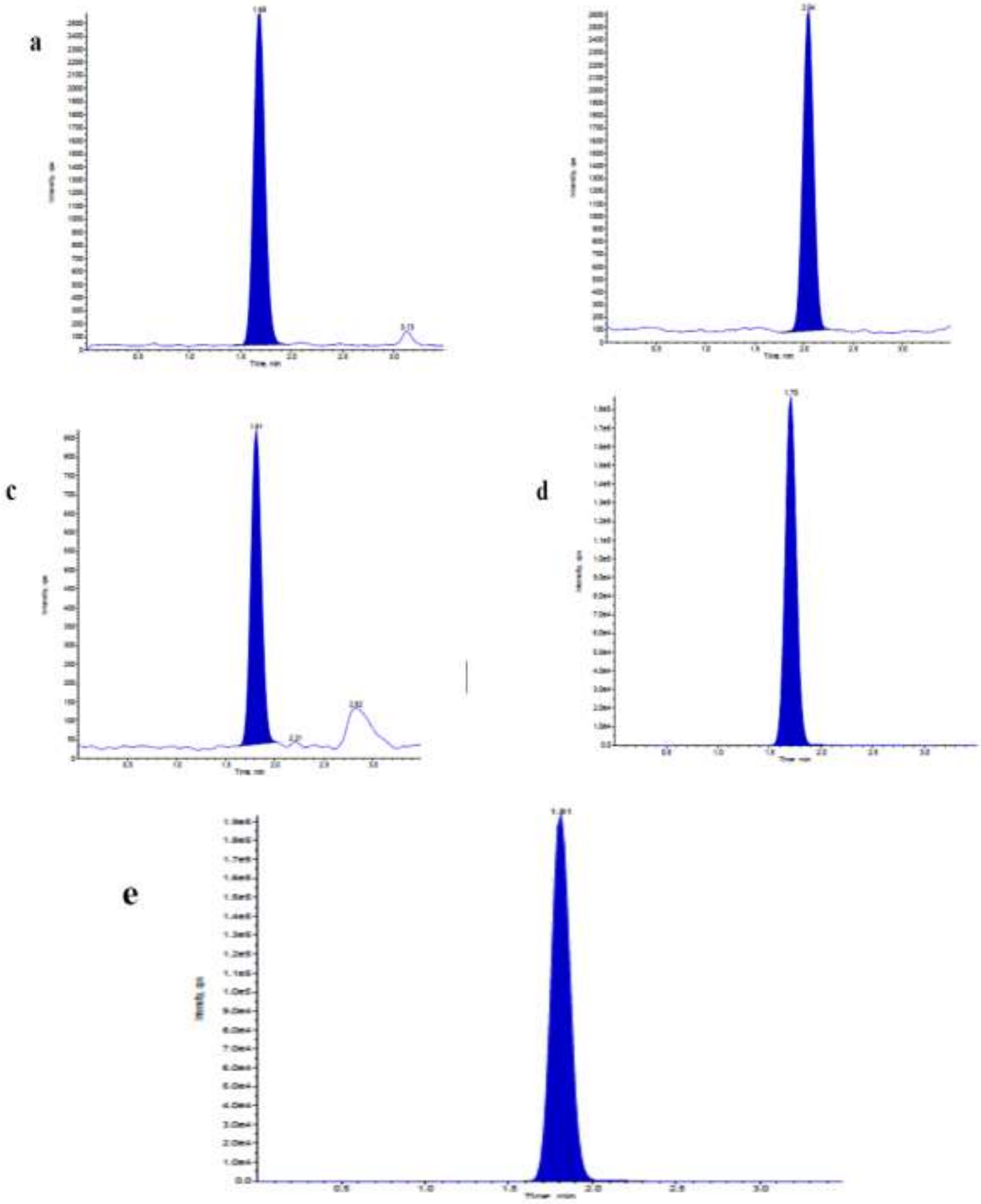
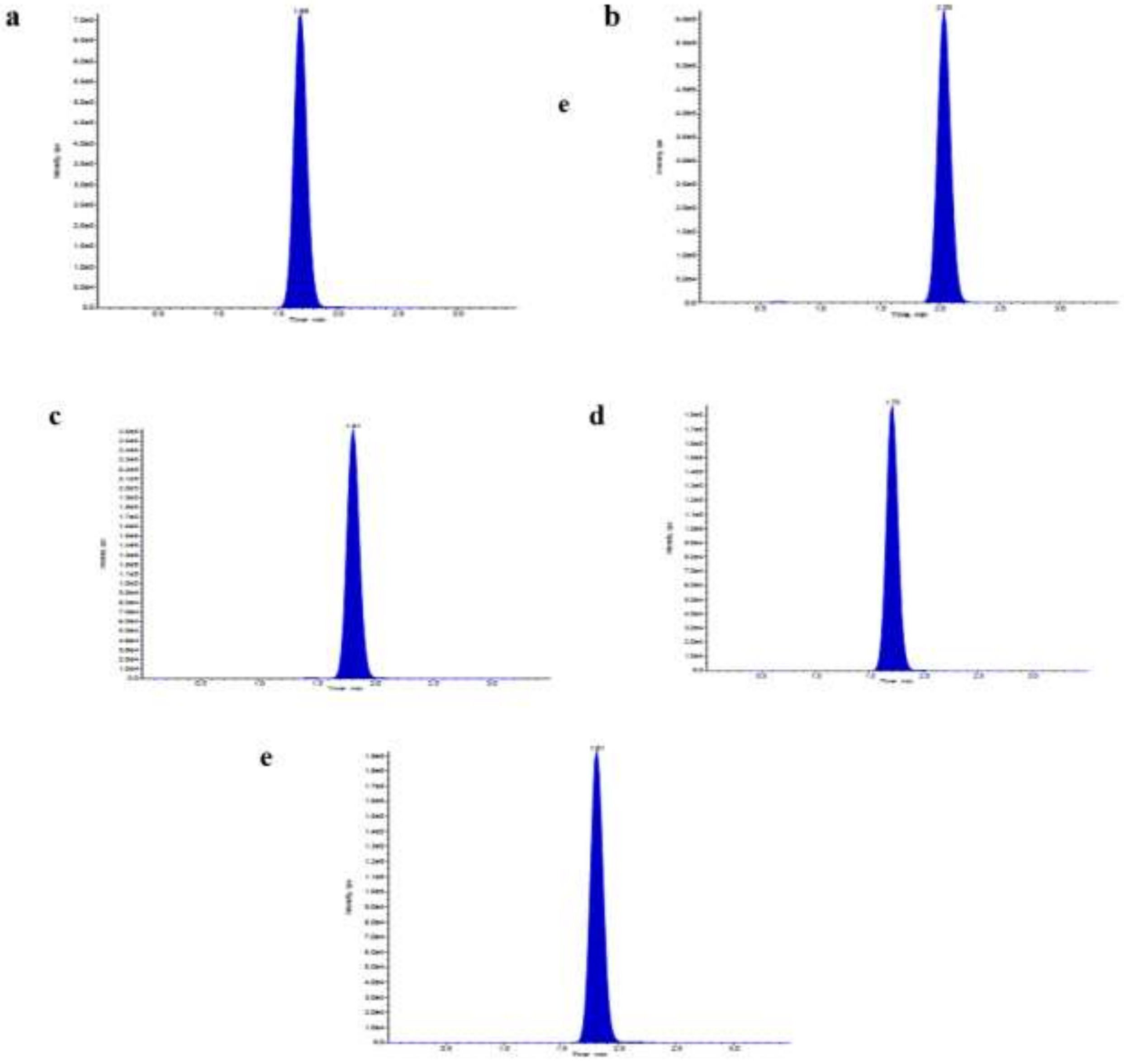


Figure 4. Chromatograms of a. Lamivudine b. Tenofovir c. Efavirenz d. Emtricitabine and e. Abacavir at HQC level



Stability evaluation

As shown in Figure 5, the tenofovir and emtricitabine stock solutions and working dilutions were steady for 31 days when stored at 2-8^{0}C. The % mean stability of the analytes ranged from 88.9% to 109.7%. Stability samples at low and high-quality control concentrations were analyzed using a freshly prepared calibration curve. Lamivudine, Tenofovir and efavirenz were found to be stable in human plasma for approximately 19 hours at room temperature and after undergoing 6 complete freeze-thaw cycles. The established autosampler stability time (after sample preparation but before injection) was 46 hours for lamivudine, tenofovir and efavirenz, while the dried extract stability (after reconstitution of dried extracts) was 52 hours. The analytes were stable in whole blood for 2.5 hours. Long-term stability was evaluated by storing prepared plasma samples at -70 ^{0}C, and lamivudine, tenofovir and efavirenz were found to be stable for at least 39 days under these conditions.

Figure 5: Stability conditions and results

Stability Experiment			Auto sampler stability	Free and thaw stability	Dry extract stability	Room temperature stability	Long term stability	stability in blood
stability condition			46 hrs at 5° C	6 cycles at -70° C ± 15°C	55 hrs at 2-8° C	19 hrs at Room temperature at 25° C ± 5°C	39 days at -70° C ± 15°C	2.5 h Room temperature at 25° C ± 5°C
% Mean stability	**Lamivudine**	**LQC**	91.5	100.2	98.9	99.7	99.7	92.1
		HQC	93.5	100.1	95.5	106.4	97.5	91.2
	Tenofovir	**LQC**	88.9	92.8	109.7	92.8	90.7	102.7
		HQC	93.4	97.7	105.7	93.3	94.2	106.5
	Efavirenz	**LQC**	102.5	96.4	93.4	101.5	108.5	104.2
		HQC	108.7	92.7	99.7	95.4	92.8	99.6

5. CONCLUSION

A faster and more sensitive LC-MS/MS method was developed for quantifying tenofovir and emtricitabine in human plasma using positive electrospray ionization. The method was validated according to Food and Drug Administration (FDA), Brazilian Health Regulatory Agency, and European

Medical Agency guidelines and has a shorter run time of 3.5 minutes. MRM analysis with optimized

MS/MS conditions was used, and solid phase extraction and a Xterra column were used for separation. No significant interference was observed in blank plasma samples. Thus, this method can be used as a reliable and efficient tool for therapeutic drug monitoring and pharmacokinetic studies of antiretroviral therapy.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ABBREVIATIONS:

CXP: Collision Cell Exit Potential; **%CV:** coefficient of variation; **DQC:** dilution quality control; **DP:** Declustering Potential; **EDTA:** Ethylene Diamine Tetra-acetic acid; **EP:** Entrance Potential; **FDA:** Food and Drug Administration; **HAART:** highly active antiretroviral therapy; **HPLC:** High-Performance Liquid Chromatography; **HIV:** Human Immuno-deficiency Virus; **HQC:** high-quality control; **ISTD:** internal standard; **LC-MS:** Liquid Chromatography-Mass spectrometry; **LLOQ:** lowest limit of quantification quality control; **LQC:** low-quality control; **MRM:** multiple reaction monitoring; **MQC:** medium quality control; **TSI:** Turbo spray interface; **ULOQ:** Upper Limit of quantification.

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