Research Article

Development and Validation of LC-MS/MS Method for Simultaneous Estimation of Emtricitabine, Tenofovir Alafenamide and Dolutegravir in Human Plasma

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ABSTRACT

A sensitive and rapid liquid chromatography tandem mass spectrometry (LC-MS/MS) method has been developed and validated for simultaneous quantification of Emtricitabine (EMT), Tenofovir alafenamide (TAF) and Dolutegravir (DTG) in human plasma. The analytes were extracted with 0.1% v/v formic acid in water using solid phase extraction technique. The chromatographic separation was developed using Kinetex biphenyl 100 A column C18 (100 mm × 4.6 mm, 5µm) with mobile phase of 0.1% v/v formic acid in water and 0.1% v/v formic acid in acetonitrile in gradient elution. The chromatographic run time for EMT, TAF and DTG was obtained at 0.991, 1.287, 1.854 min, respectively. The mass transitions have been monitored in electron spray ionization (Positive mode and triple quadrupole as m/z 248.05→130.15 for EMT, m/z 477.10→270.10 for TAF, m/z 420.15→276.95 for DTG. The proposed method has been validated with a linear range of 20 to 5000 ng/ml for EMT, 2 to 500 ng/ml for TAF and 30 to7500 ng/ml for DTG. The intrarun and interrun precision are 3.64% for EMT, 4.36% for TAF, 4.5% for DTG at their respective LOQ levels. The method was validated for linearity, accuracy, precision, specificity and stability. The developed LC-MS/MS method has been successfully applied to simultaneous quantification of Emtricitabine, Tenofovir alafenamide and Dolutegravir in human plasma without any interference.

Keywords: Emtricitabine; Tenofovir alafenamide; Dolutegravir; LC-ESI/MS/MS.

INTRODUCTION

AIDS (acquired immune deficiency syndrome) is a set of symptoms and illnesses that develop as a result of advanced HIV infection, which has destroyed the immune system. Antiretroviral combination therapy is useful in AIDS.[1] Emtricitabine is nucleoside reverse transcriptase inhibitors (NRTI) used in HIV infection. Emtricitabine works by inhibiting reverse transcriptase, preventing transcription of HIV RNA to DNA. Emtricitabine is a synthetic nucleoside analogue of cytidine.[2] Tenofovir alafenamide is NRTI and it is first approved pro-drug, which block reverse transcriptase, a crucial virus enzyme in human immunodeficiency virus 1 (HIV-1) and hepatitis B virus infections. Tenofovir alafenamide is more stable than TDF (Tenofovir diphosphate).[3-4] Dolutegravir inhibit HIV integrase by binding to the site and blocking the strand transfer of retroviral DNA integration in the host cell. Dolutegravir is antiretroviral drug acts by HIV integrase inhibition. Dolutegravir, emtricitabine and tenofovir alafenamide is a three-drug combination of dolutegravir (integrase strand transfer inhibitor [INSTI]), emtricitabine (FTC) and tenofovir alafenamide (TAF), both HIV nucleoside analogue reverse transcriptase inhibitors (NRTIs) and is indicated for use alone as a complete regimen for the treatment of HIV-1 infection in adults and paediatric patients.[5]

The literature review revealed a high-performance liquid chromatography (HPLC)[6-9] and liquid chromatography mass spectrometry (LC-MS)[10-17] are reported for monitoring of plasma level of Emtricitabine, Tenofovir alafenamide and Dolutegravir individually or in combination with others drugs. Some HPLC techniques are reported for the analysis of drug in pharmaceutical dosage forms.[18-23] This paper proposed the method for simultaneous quantification of Emtricitabine, Tenofovir alafenamide and Dolutegravir in human plasma using optimized solid phase extraction (SPE) techniques for the sample preparation and LC-MS/MS for the method development with shorter retention time, superior specificity and sensitivity, which is used in therapeutic drug monitoring.

MATERIALS AND METHODS

Chemicals and reagents

The reference standards of Emtricitabine (99.69%), 15ND2 Emtricitabine (94.17%), Tenofovir alafenamide (98.10%), D5 Tenofovir alafenamide (97.03%) were procured from Simson Pharma Ltd. (Mumbai, India), Clearsynth Labs ltd (Mumbai, India), Daicel Chiral Tech (Hyderabad, India) respectively. Dolutegravir (99.54%) and D6 Dolutegravir (99.66) were procured from Vivan Life Science (Thane, India). HPLC-grade acetonitrile and methanol were procured from Mallinckrodt Baker (Phillipsburg, NJ). GR-grade formic acid was obtained from Merck Specialties Pvt. Ltd. (Mumbai, India). Water was prepared from Milli-Q water purification system from Millipore (Bangalore, India). Strata-X extraction cartridges (30 mg/ml) were procured from Phenomenex (California, US). Blank Human plasma was obtained from Veeda Clinical Research (Ahmedabad, India) and was stored at -20˚C until use.

Instruments

The LC–MS/MS instrument equipped with UFLC system (Shimadzu, Japan), having dual prominence pump model LC-20AD with DGU-20A3 degasser, CTO-10ASVp column oven, SIL-HTc auto-sampler and triple quad API 4000 mass detector with ESI interface (Applied Biosystems, MDS Sciex, Canada) was used for chromatographic separation and quantification of analytes.

Calibration standards and quality control samples

The stock solution of EMT, TAF, 15ND2 EMT (ISTD), D5 TAF (ISTD) were prepared in water-methanol (50:50, v/v) to get concentration of 2000 µg/ml, 1000 µg/ml, 1000 µg/ml, 1000 µg/ml respectively. The stock solution of DTG and D6 Dolutegravir (ISTD) were prepared in dimethyl sulphoxide to get concentration of 2000 µg/ml and 1000 µg/ml respectively. Calibration standards solutions were prepared at eight points by spiking the previously screened blank plasma with suitable aliquot of EMT, TAF, DTG to get concentration of 20 to 5000 ng/ml for EMT; 2 to 500 ng/ml for TAF; 30 to 7500 ng/ml for DTG. Emtricitabine, Tenofovir and Dolutegravir were serially diluted with methanol: water (50:50 v/v) to make a series of quality control working solutions at 3750, 2250, 450, 60 and 20 ng/ml for EMT, 375, 225, 45, 6 and 2 ng/ml for TAF and 5625, 3375, 675, 90 and 30 ng/ml for DTG at high, middle 1, middle 2, low and LLOQ level respectively. The stock solutions, Quality control solutions, Calibration standards were stored at 2-8˚C until use.

Sample preparation

A 200 µl of human plasma transfers into pre-labelled tube and 50 µl of mixed internal standard solutions (2 µg/mL of EMT 15 ND2, 0.5 µg/mL of TAF D5 and 1 µg/mL of DTG D6) was added. 0.4 ml of 0.1% v/v formic acid in water was added and mix properly. Samples were centrifuged at 4000 RPM for 5 minutes at 5°C. Strata-X extraction cartridges pre-conditioned with 1.0 mL methanol followed by 1.0 mL of 1%v/v formic acid were used for extraction of analytes and internal standards. Centrifuged samples were loaded to the pre-conditioned cartridges. Then cartridges were washed with 1.0mL of 1%v/v formic acid followed by 1.0mL of methanol and dried completely by applying full nitrogen pressure. Analytes and Internal standards were eluted with 1 ml of acetonitrile. Eluent was evaporated under nitrogen stream at 45°C. Dried samples were reconstituted with 300µL mobile phase and 10µL of the sample was injected to the column.

Liquid Chromatography and mass spectrometric condition

LC instrument measured the separation of three analytes and internal standards response on a Kinetex 5µ Biphenyl C18 100A column (100×4.6mm i.d.). The mobile phase consists of 0.1%v/v formic acid in water (mobile phase A) and 0.1%v/v formic acid in acetonitrile (mobile phase B) with flow rate of 1.0ml/min. The gradient elution was performed by adjusting mobile phase A and B (50:50, v/v) for 1.6 min, then increase the mobile phase B to 80 % for 1.6 min to 2.2 min, then to 50% mobile phase B for 2.2 to 3 min. The total run time was 3.5 min. The auto sampler temperature was maintained at 5 ± 0.3 ˚C. Column oven temperature was maintained at 40 ± 5 ˚C.

Detection and ionization of analytes and internal standards were carried out on a triple quadrupole mass spectrometer Shimadzu 8040 (Kyoto, Japan) equipped with ESI operation in positive ion mode with multiple reaction monitoring (MRM) transition. The mass transition was monitored at m/z 248.05→130.15 for EMT, m/z 477.10→270.10 for TAF, m/z 420.15→276.95 for DTG. Compound specific values of mass spectrometer are listed in Table 1. The data acquisition software, lab solution version 5.72 was used to control all the parameter of LC and MS.

Table 1: Compound specific values of mass spectrometer

Parameter	EMT	EMT IS	TAF	TAF IS	DTG	DTG IS
Mass transition (m/z)	248.05→ 130.15	251.05→ 131.05	477.10→ 270.10	482.25→ 269.95	420.15→ 276.95	426.20→ 282.09
Q1 pre-bias (V)	-13	-13	-15	-15	-22	-22
Q3 pre-bias (V)	-23	-23	-27	-27	-27	-27
Collision energy (CE)	-13	-13	-32	-32	-29	-29
Dwell time (ms)	50	50	50	50	50	50

METHOD VALIDATION

The method was validated for selectivity, precision and accuracy, recovery, stability as per FDA guidelines.[24]

System suitability and carryover

System suitability experiment was performed by injecting six consecutives of MCQ samples. Carryover of the chromatographic system was evaluated in each experiment throughout the validation by injecting blank sample before and after highest concentration sample.

Specificity

Interference from endogenous compounds was investigated by analysis of ten different blank plasma lots including haemolysed, lipidemic and heparinized plasma. The extracted blank and LLOQ plasma samples from each plasma lot were injected into the chromatographic system. The specificity will be evaluated by comparing the

responses of interfering peak at the retention time of drug and internal standards in the standard blank against the response of the respective extracted LLOQ.

Linearity

Linearity of the method was performed at eight different concentrations. The peak area ratio of individual standard to that internal standard, EMT/EMT-IS, TAF/TAF-IS, and DTG/DTG-IS were used for regression analysis. Each calibration curve was analysed individually by linear regression with weighted least-squares. A correlation coefficient (r^2) value of greater than 0.99 was desirable for all curves, the lowest standard of linearity was considering as a LLOQ, if analyte peak response is 20 times more than a blank plasma. Deviation of standards other than LLOQ from nominal concentration should not be more than ±15%.

Accuracy and precision

For developing intra batch accuracy and precision, replicate analysis of plasma samples was determined on same day at five concentration level (LLOQ, QC, LQC, MQC 1, MQC 2 and HQC) and inter batch accuracy and precision were assessed on three consecutive days. The precision (%CV) at each concentration level from nominal concentration should not be greater than 15%. Similarly, the mean accuracy should be within 85 to 115%, except LLOQ QC where it should be 80 to 120% of nominal concentration.

Dilution integrity

This was especially for the subject samples which are found to contain concentrations exceeding the ULOQ. So, it was ascertained to confirm whether the dilution of these samples before analysis can modify the accuracy of the drug determination. Dilution integrity was conducted by using six replicates of DQC (1/10) samples. Process and analyze the DQC (1/10) samples along with two replicates of CC standards and two replicates of batch qualifying QC samples.

Recovery

Extraction efficiency was established from the peak area of the extracted QC samples at low, medium and high levels and expressed as a percentage of peak area of equivalent un-extracted samples prepared in aqueous solution. Recovery was calculated by comparing the mean peak area response at extracted sample (spiked before extraction) with that of un-extracted sample (spiked after extraction) at HQC, MQC and LQC level.

Matrix effect

Matrix effect was performed using six different blank plasma lots at LQC and HQC concentration in triplicate by calculating the % accuracy and precision (% CV) which should be in the range of 85-115% and ≤15% respectively.

Matrix factor

The estimation of the matrix factor was quantified in 6 different plasma lots at low, mid and high levels of QCs as the peak-area response of analytes added to blank plasma extracts, divided by the peak-area response of standard solution of analytes. A value above or below 1 for the matrix factor indicates an ionization enhancement or suppression, respectively. The results were showing that co-eluting plasma matrix components do not appear to affect significantly the ionization of analytes as well as internal standard.

Stability

Stability was evaluated by comparing stability samples against freshly prepared standard solution with identical concentration. Freeze and thaw stability was assessed after five freeze thaw cycle stored at -20±5°C and -70± 8°C and then plasma samples were extracted and analyzed. Bench-top stability was assessed at the HQC and LQC level by keeping spiked plasma samples for 12 hours in a deep freezer at -20°C and then kept at ambient temperature (20°C) and then extracted and analyzed. Dry extract stability was assessed by keeping the dried samples after extraction in deep freezer at (-20°C) and then reconstituted and analyzed. All the stability samples were considered stable if the % accuracy and % CV were found within the range 85-115% and ≤15% respectively.

RESULTS

Method development

The objective of this work was to develop and validate a simple, rapid and sensitive LC-MS/MS method for the simultaneous quantification of EMT, TAF and DTG in human plasma. Various mobile phase optimizations, sample extraction procedures and chromatographic conditions were optimized to get desired sensitivity, selectivity and linearity. The samples were extracted by solid phase extraction technique using starata-x cartridges. The EMT, TAF, and DTG exist in unionized form in the basic PH, the plasma samples containing analytes and internal standards were treated with 0.1%v/v formic acid in water after acidifying with formic acid to strong binding of analytes to copolymer of SPE cartridges. The analytes were eluted with acetonitrile. It was found that best signal was achieved with positive ion electron spray ionization (ESI) mode. Further optimization of LC condition, mobile phase 0.1%v/v formic acid in water (mobile phase A), 0.1%v/v formic acid in acetonitrile (mobile phase B) were shown good peak shapes.

METHOD VALIDATION

System suitability and carryover

The precision (% CV) of system suitability test was observed within 0.4% for the retention time and 1.84 % for the area ratio of analytes/internal standard. No significant carryover was observed for any of analytes and internal standards in each experiment.

Specificity

Specificity was performed at blank plasma and plasma spiked with analytes and internal standards at LLOQ level. The Chromatogram of extracted blank plasma and

extracted Emtricitabin, Tenofovir alafenamide and Dolutegravir and their Internal standards at LLOQ are shown in Fig. 1 and 2, respectively. No interfering peak was observed at the retention time of analytes or the internal standard in blank plasma extract containing K3EDTA as anticoagulant.

Linearity

All the calibration curves were linear over the concentration range of 20 to 5000 ng/ml, 2 to 500 ng/ml and 2 to 7500 ng/ml for EMT, TAF, DTG respectively. The correlation coefficient (R^2) value was 0.9959, 0.9968 and 0.9989 for EMT, TAF, DTG respectively. Results of calibration are shown in Table 2.

Precision and accuracy

The precision and accuracy were found within ± 20% for LLOQ and ± 15% for other quality control samples for all three analytes. The results of intra-run and inter-run precision and accuracy are shown in Table 3.

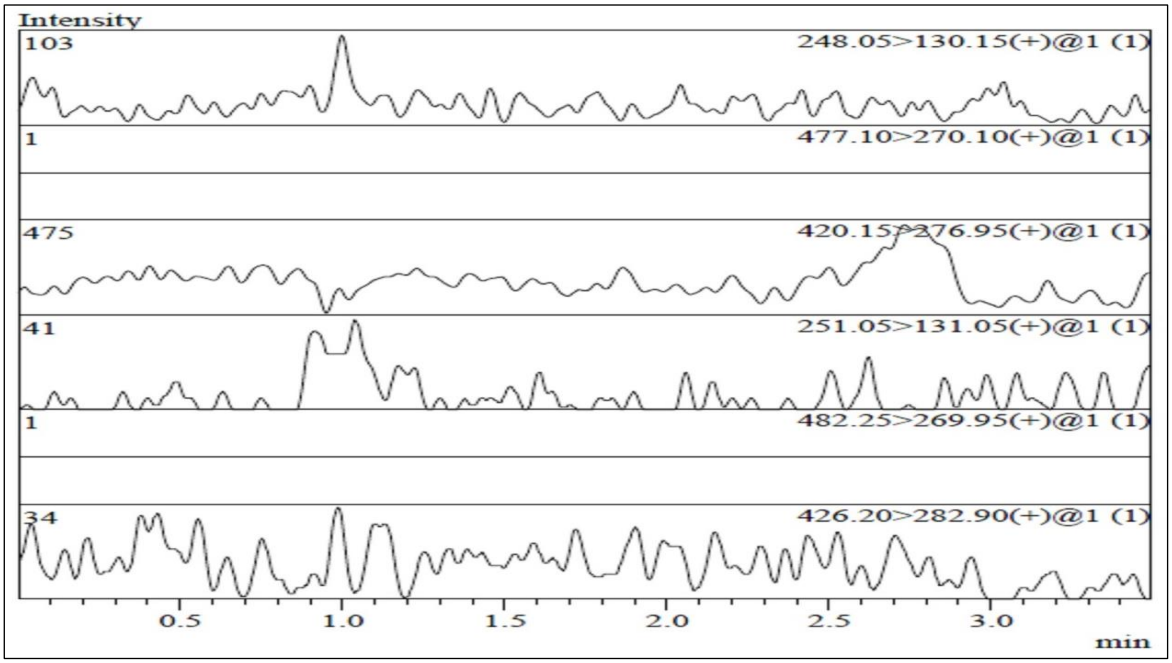

Figure 1: Chromatograms of extracted Blank of Emtricitabin, Tenofovir alafenamide and Dolutegravir and their Internal standards

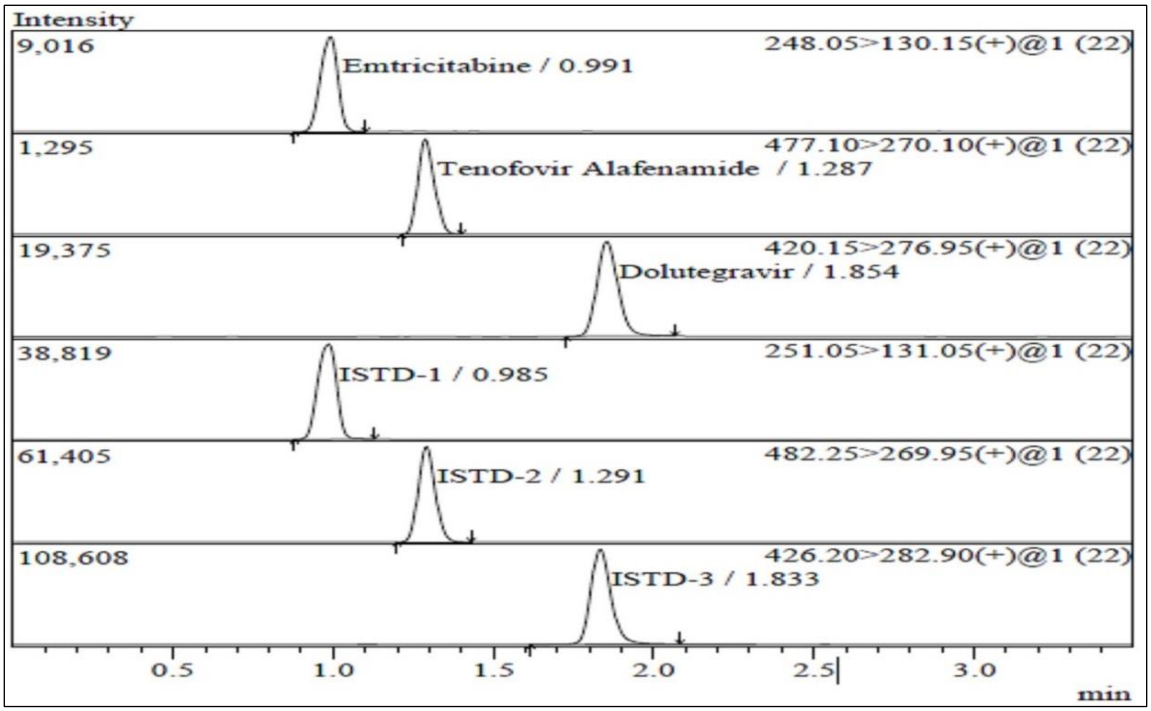

Figure 2: Chromatogram of extracted Emtricitabin, Tenofovir alafenamide and Dolutegravir and their Internal standards at LLOQ

Table 2 Calibration curve with back calculated concentrations (EMT, TAF and DTG)

Drug	STD ID	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8
EMT	**Nominal conc. (ng/ml)**	**5000**	**4000**	**2000**	**1000**	**500**	**250**	**40**	**20**
	Mean	4598.71	3756.8	2028.1	1077.8	531.9	252.9	40.9	19.6
	SD	222.241	17.154	69.795	61.705	7.495	14.549	0.68	0.070
	%CV	4.83	0.46	3.44	5.72	1.46	5.75	1.67	0.36
	% Mean accuracy	91.97	93.92	101.41	107.79	102.78	101.19	102.49	98.45
	R^2	0.9959							
TAF	**Nominal conc. (ng/ml)**	**500**	**400**	**200**	**100**	**50**	**25**	**4**	**2**
	Mean	495.460	391.25	201.87	105.41	49.37	24.16	4.09	1.98
	SD	33.61	7.44	10.34	9.37	0.17	0.69	0.39	0.04
	%CV	6.78	1.90	5.12	8.89	0.33	2.84	9.43	1.97
	% Mean accuracy	99.09	97.81	100.94	105.41	98.47	96.64	102.30	99.10
	R^2	0.9968							
DTG	**Nominal conc. (ng/ml)**	**7500**	**6000**	**3000**	**1500**	**750**	**375**	**60**	**30**
	Mean	7303.94	5849.06	3047.60	1551.98	763.96	363.59	61.38	29.68
	SD	287.96	79.86	113.87	30.48	5.60	4.33	0.629	0.372
	%CV	3.94	1.37	3.74	1.96	0.73	1.19	1.02	1.25
	% Mean accuracy	97.39	97.48	101.59	103.47	101.86	96.96	102.30	98.96
	R^2	0.9989							

Table 3(a) Results of Intra-run and Inter-run precision and accuracy of EMT

Level	Spiked conc. (ng/ml)	Intra-run			Inter-run		
		Mean conc. found (ng/ml)	% CV	% Accuracy	Mean conc. found (ng/ml)	% CV	% Accuracy
LLOQ	20	20.5	2.7	102.5	19.2	2.9	96.3
LQC	60	61.6	3.0	102.8	60.3	4.1	100.6
MQC 2	450	444	0.8	98.6	446.9	3.4	99.1
MQC 1	2250	2126.1	1.5	94.5	2126.1	2.2	94.4
HQC	3750	3625.2	3.6	96.6	3548.7	4	94.6

Table 3(b): Results of Intra-run and Inter-run precision and accuracy of TAF

Level	Spiked conc. (ng/ml)	Intra-run			Inter-run		
		Mean conc. found (ng/ml)	% CV	% Accuracy	Mean conc. found (ng/ml)	% CV	% Accuracy
LLOQ	2	2.1	3.8	106.2	2	5.3	101.4
LQC	6	6	2.0	100.9	59	3.7	98.6
MQC 2	45	43.2	1.4	96.1	43.5	3.2	96.7
MQC 1	225	213.7	2.0	95.0	218	3.3	97.3
HQC	375	392.2	4.3	104.6	374.8	4.5	99.9

Table 3(c): Results of Intra-run and Inter-run precision and accuracy of DTG

Level	Spiked conc. (ng/ml)	Intra-run			Inter-run		
		Mean conc. found (ng/ml)	% CV	% Accuracy	Mean conc. found (ng/ml)	% CV	% Accuracy
LLOQ	30	30.9	3.7	103.3	29.3	2.9	97.7
LQC	90	91.6	3.7	101.7	86.6	2.7	96.2
MQC 2	675	957.2	1.7	97.3	654.5	3.5	96.9
MQC 1	3375	3179	2.2	94.2	3192.8	1.9	94.6
HQC	5625	5638.8	4.5	100.2	5486	5.5	97.5

Dilution integrity

For dilution integrity, sample was further diluted to ten-fold dilutions with drug free blank plasma to bring the concentration within the calibration range. % accuracy and precision (% CV) were 94.33% & 1.59 % and 94.20 % & 3.32 % and 90.82 % & 2.01 % for EMT, TAF and DTG, respectively.

Matrix effect

Matrix effect was carried to check whether any suppression or enhancement in response is observed or not due to matrix. Matrix effect was performed at HQC and LQC level. The result of matrix factor is shown in Table 4.

Table 4 Matrix effect of EMT, TAF and DTG at LQC level

Level	EMT	TAF	DTG
Nominal concentration (ng/ml)			
	60	6	90
LQC 1	0.98	0.24	1.03
LQC 2	1.01	0.24	1.02
LQC 3	0.98	0.24	1.01
LQC 4	0.99	0.24	1
LQC 5	0.99	0.24	1.03
LQC 6	0.98	0.25	1.03
Mean	0.98	0.24	1.03
% CV	1.20	2.87	2

Matrix factor

Matrix factor was performed to check matrix suppression or enhancement effect. A value above or below 1 for the matrix factor indicates an ionization enhancement or suppression, respectively. The mean IS normalized matrix factors were of 0.985, 0.244, 1.032 at low QCs and 1.008, 0.244, 1.012 at high QCs for EMT, TAF and DTG, respectively.

Recovery

The recovery results are presented in Table 5. The recovery was found by comparison between extracted and un-extracted QC sample. The mean recovery of EMT, TAF and DTG were 71.22%, 94.32%, 94.09%, respectively and mean recovery of EMT IS, TAF IS and DTG IS were found to 66.08%, 92.32%, 91.87% respectively.

Table 5 Recovery of EMT, TAF and DTG at HQC, MQC and LQC level

Level	EMT	TAF	DTG
HQC	69.96%	96.43%	97.75%
MQC 1	68.49%	91.96%	91.91%
LQC	75.21%	94.57%	92.62%
% Overall recovery	71.22%	94.32%	94.09%

Stability

Bench top stability, Freeze and thaw stability, dry extract stability studies were found that EMT, TAF and DTG are stable within the acceptance criteria. Stock and working solutions of EMT, TAF and DTG were stable for 12 hours at ambient temperature 20°C and for long term stability in refrigerator at 5°C. The % CV were found ≤15% and % bias were found ±10%, when the stability samples peak area was compared with the peak area of fresh samples. The results of stability studies are shown in Table 6.

Table 6 Stability of EMT, TAF and DTG under various conditions (n = 3)

	EMT		TAF		DTG	
Stability	**Mean stability ± SD**	**% Accuracy**	**Mean stability ± SD**	**% Accuracy**	**Mean stability ± SD**	**% Accuracy**
Bench top stability, ambient,12 h						
LQC	57.3 ± 3	95.55	5.3 ± 0.2	89.03	86.1 ± 5	95.71
HQC	3730.2 ±52.4	99.47	355.4 ± 4.3	94.78	5717.9 ± 87.1	101.65
Freeze thaw stability, -20 ± 5˚C, 5 cycles						
LQC	55.3 ± 0.8	92.25	6.1 ± 0.1	103.20	85.5 ± 1	95.07
HQC	3438.7 ± 51	91.70	345.4 ± 4	92.13	5319.3 ± 124.1	94.57
Freeze thaw stability, -70 ± 8˚C, 5 cycles						
LQC	55.6 ± 3.2	92.77	5.3 ± 0.3	89.02	84.3 ± 5.9	93.67
HQC	3502.8 ± 1.7	93.41	354.7 ± 6.5	94.59	5359.6 ± 58.1	95.28
Dry extract stability, -20±5°C in deep freezer for 44 hours						
LQC	57.1 ± 1.8	95.3	5.6 ± 0.1	93.6	85.9 ± 2.8	95.4
HQC	3404 ± 142.9	90.7	368.5 ± 13.9	98.2	5184.8 ± 158.4	92.1

DISCUSSION

The method was capable of separating and detecting EMT, TAF and DTG, significantly within shortest retention time of 0.991, 1.287, 1.854 min, respectively. The % accuracy and precision were found within ± 20% for LLOQ and ± 15% for other quality control samples for EMT, TAF and DTG. The matrix factor results were showing that co-eluting plasma matrix components appear to affect significantly the ionization of TAF. There was no interfering peak was observed at the retention time of analytes and internal

standards in different blank plasma lots including hemolyzed, lipidemic, and heparinized plasma extract containing K3EDTA as anticoagulant. The low level of LOD and LOQ indicated the higher sensitivity of the developed methods for the identification and quantification of EMT, TAF and DTG. Recovery results indicating good extraction efficiency of analytes from plasma using solid phase extraction techniques. Stability studies was shown that the stock and working solutions of EMT, TAF and DTG were stable in bench top, freeze thaw and dry extract.

CONCLUSION

The proposed method is accurate, precise and reproducible for simultaneous quantification of EMT, TAF and DTG in human plasma with superior specificity and sensitivity, which is used in therapeutic drug monitoring. The method was validated with consideration of USFDA, ANVISA and EMA guidelines and suitable for BA-BE studies.

ABBREVIATIONS

EMT: Emtricitabine; TAF: Tenofovir alafenamide; DTG: Dolutegravir

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AUTHORS' CONTRIBUTIONS

DM was developed and validated the method. RP and ND were supervised the method. RP reviewed the data and supported for writing the manuscript. The authors have read and approved the final manuscript.

CONFLICT OF INTERESTS

The authors declare that they have no Conflict of interests.

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