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High Throughput Lc-Ms/Ms Method for the Quantitation of Emtricitabine in Human Plasma by Solid Phase Extraction Using 96 Well Plate Format

Tangudu Nagabhusana Rao[1*], Guntuku Girija Sankar[1] and Lade Jyothi Rani[2]

[1]College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, Andhra Pradesh, 530003, India.
[2]Mallareddy Institute of Pharmaceutical Sciences, Dhulapally, Secunderabad-14, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author TNR was done the method development and completed the validation under the supervision of author GGS. Author TNR wrote the first draft of the manuscript. Author LJR managed the literature searches and contributed in manuscript writing. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To develop and validate sensitive, high-throughput Liquid chromatography–mass spectrometry method for the quantification of Emtricitabine in human plasma using Lamivudine as an internal standard (ISTD) using 96 well plate format solid phase extraction (SPE) technique.
Methodology: The samples were extracted from plasma using solid phase extraction (SPE) in 96 well plate format. After extraction the analyte and internal standard were analyzed on Inertsil ODS column with dimensions 4.6 X 100 mm, 5 µm using a mobile phase consisting of Methanol: 0.2% formic acid in water 85:15, v/v. The precursor and product ions of the analytes were monitored on a triple quadrupole instrument operated in the positive ionization mode.
Results: The method was validated with concentration range of 5.024 to 5023.725 ng/mL. The relative recoveries were ranging from 60.7 to 65.9%. Three validation batches were performed with five QC levels (LLOQ, LQC, MQC II, MQC and HQC). Across three validation runs the inter batch

**Corresponding author: E-mail: tangudur@gmail.com;*

precision (%CV) was ≤ 9.2% and the accuracy was between 98.9–106.0%. All the stability experiments found satisfactory.
Conclusion: According to the method validation results, the current method was found to be specific, accurate, sensitive, precise and high throughput method. This method can be used for the estimation of Emtricitabine in human plasma during routine analysis.

Keywords: Emtricitabine; lamivudine; solid phase extraction; 96 well plates.

1. INTRODUCTION

Emtricitabine (FTC) is a nucleoside reverse transcriptase inhibitor (NRTI) which is used in the treatment of human immunodeficiency virus (HIV) in adults and children. It is usually prescribed as 200 mg capsules and as a fixed dose combination with other antiretroviral agents like tenofovir, efavirenz etc. FTC is included in current World Health Organization (WHO) model List of Essential Medicines (EML) and various international guidelines for the treatment of HIV infection. Lamivudine (3TC) and FTC are structurally similar and are considered clinically equivalent. However FTC has a longer half-life over 3TC, which is a potential advantage of its use in antiretroviral treatment [1-3].

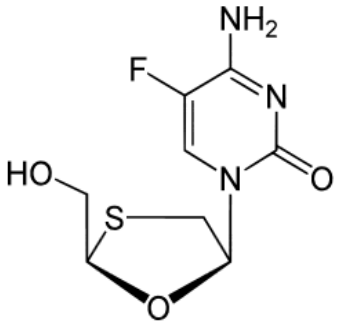

Fig. 1. Structure of Emtricitabine

Some methods [4-16] were reported for the quantitation of FTC in formulations individually or in other combinations. However very few LC-MS/MS methods were available for determination of only Emtricitabine in human plasma. Peepliwal et al. [11] developed a HPLC-UV method with a LOQ of 100 ng/mL. Droste et al. [12] developed a gradient HPLC-fluorescence method in plasma with a LOQ of 10 ng/mL, using 500 µL sample volume and run time of 15 min. Supriya et al. [15] developed a LC-MS/MS method in plasma with a LOQ of 50 ng/mL, which is insufficient to appropriately monitor elimination period in bioequivalence studies. Yadav et al. [16] developed a LC-MS/MS method in plasma with a LOQ of 29 ng/mL using liquid-liquid extraction, which is prone to matrix effects and less selective over solid phase extraction.

The aim of the current study was to develop and validate a sensitive and high throughput LC-MS/MS method using solid phase extraction technique in 96 well plate format as per FDA and EMA guidelines [17-19].

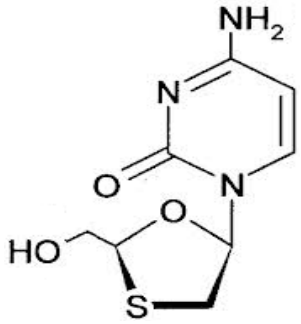

Fig. 2. Structure of Lamivudine

2. EXPERIMENTAL DETAILS

2.1 Chemicals and Reagents

Working standards of Emtricitabine (Purity ~98%) and Lamivudine (purity ~98%) were obtained from sigma Aldrich. LC–MS grade methanol was purchased from Thermo Fisher Scientific India Private limited (Mumbai, India). Formic acid (FA) and Ortho Phosphoric Acid (OPA) were purchased from Merck. HPLC grade water was obtained from Milli-Q water purification system (Millipore). Human plasma with K2 EDTA anticoagulant was obtained from Doctor's pathological lab (Hyderabad, India). Waters Oasis HLB 96 well plates 30 µm (10 mg) were purchased from Waters Corporation (Milford, MA, USA).

2.2 Instrumentation

Agilent 1200 Series HPLC with a binary pump was used for the analysis. API-4000 triple quadrupole mass spectrometer (MDS SCIEX, Toronto, Canada) equipped with turbo ion spray inter-face was used for mass spectrometric detection. Multiple Reaction Monitoring (MRM) modes were used for quantitation. The hardware and data capturing were controlled by analyst software.

2.3 Chromatographic Conditions

Inertsil ODS, 4.6 X 100 mm, 5 µm analytical column was used to perform the chromatographic separation. Isocratic mobile phase consisting of 15:85 v/v 0.2% formic acid: Methanol at a flow rate of 1 mL/min was used. The auto sampler temperature was set at 4°C±2°C and injection volume was 5 µL. The column oven temperature was set at 35.0±2.0°C. Retention Time of Emtricitabine was 1.2 min and Lamivudine was 1.0 min with total chromatographic run time of 3.0 min.

2.4 Mass Spectrometric Conditions

2.4.1 Ionization mode

Positive ionization.

2.4.2 Resolution

Q1 Unit; Q3 Unit.

2.4.3 MRM conditions

Refer Table 1 for optimized MRM conditions.

2.4.4 Source/Gas parameters

Refer Table 2 for optimized Source/Gas parameters.

2.5 Preparation of Calibration Standards and Quality Control Samples

Stock solutions of Emtricitabine and internal standard (Lamivudine) were prepared by dissolving accurately weighed amounts in methanol to give a final concentration of 1 mg/mL. Individual working solutions of analyte were prepared by appropriate dilution of their stock solutions in 50% Methanol in water. All the solutions were stored in refrigerator at below 10°C and were brought to room temperature before use. Working solution of internal standard (Lamivudine, 3 µg/mL) was prepared daily in 50% methanol in water and was stored at room temperature.

Calibration standards and quality control (QC) samples were prepared by spiking blank K2 EDTA human plasma with the working solutions (5%) prepared from independent stock weightings. K2 EDTA anticoagulant blank plasma collected from healthy volunteers was screened individually and pooled before use. Calibration standards were prepared at concentrations of 5.024, 10.047, 26.315, 105.259, 248.794, 526.295, 1196.125, 2727.165, 4210.360 and 5023.725 ng/mL. Quality control samples were prepared at 5.024 ng/mL (LLOQ QC), 14.354 ng/mL (LQC), 440.174 ng/mL (MQC II), 2440.095 ng/mL (MQC) and 4018.980 ng/mL (HQC).

2.6 Sample Preparation

Calibration standards and QC's were processed by 96 well plate solid phase extraction using Ezypress Positive Pressure SPE Manifold. 5 µL of each working solutions of Emtricitabine were spiked into 190 µL of human plasma to prepare CC and QC in human plasma. 100 µL of spiked plasma was aliquoted for CC's and QC's processing. 10 µL of internal standard was added to each tube except for blank plasma samples and 10 µL of 50:50v/v MeoH: Water was added to blank plasma sample. All the samples were vortexed. To these samples 150 µL of 5% OPA solution was added and vortexed. The Waters Oasis HLB 96 well plate 30 µm (10 mg) cartridges conditioned with 500 µL of methanol followed by 500 µL of Milli Q water. All the samples were loaded onto the cartridge. The cartridges were washed with 600 µL (two aliquots of 300 µL each) of Milli Q Water. Finally the samples were eluted with 800 µL (two aliquots of 400 µL each) of methanol into 96 well collection plate. The eluent was evaporated under a gentle stream of nitrogen using a TurboVap 96, at a temperature of approximately 50°C. The dried samples were reconstituted with

Table 1. MRM conditions

Parameters	Q1 (amu)	Q3 (amu)	Dwell time (msec)	DP (volts)	CE (volts)	CXP (volts)	EP (volts)
Emtricitabine	248.2	130.1	200	22	32	12	12
Lamivudine	230.3	112.2	200	25	32	15	12

Table 2. Source/Gas parameters

Parameters	CUR (psi)	GS1 (psi)	GS2 (psi)	IS (Volts)	CAD (psi)	TEMP (°C)
Source/Gas	40	45	40	5000	6	400

100 µL of mobile phase and vortexed to mix. 5 µL of the of this sample was injected onto the LC-MS/MS system.

2.7 Methods Validation

The EMEA and USFDA guidelines were followed to complete the method validation of Emtricitabine in human plasma. Different experiments were performed on different days to evaluate selectivity, sensitivity, linearity, precision, accuracy, recovery, matrix effect, dilution integrity and different stabilities. Each run was organized with a set of spiked standard samples, blank (with ISTD and without ISTD) and quality control samples specific to validation parameters. Standard samples were analyzed at the beginning of the run and quality control samples were distributed consistently throughout the validation runs.

Selectivity and specificity towards endogenous and exogenous components of plasma was evaluated using six different individual lots of human plasma. The blank plasma lots were extracted (without addition of ISTD), and injected on LC–MS/MS. Later selectivity in each lot was evaluated by comparing the blank peak responses against the mean peak response observed in plasma spiked LLOQ sample (n = 6). Linearity of the method was assessed using three calibration curves analyzed on three different days. Each plot was associated with a ten point non-zero concentrations spread over the dynamic range. A quadratic regression with weighing factor (1/X2) was performed on peak area ratios versus analyte concentrations. Peak area ratios for plasma spiked calibration standards were proportional to the concentration of analytes over the established range.

Precision and accuracy batches of Intra (within day) and inter (between day) batches were evaluated at five distinct concentrations (LLOQ, LQC, MQC II MQC and HQC). Each concentration level was evaluated in terms of %CV and relative error respectively. The extraction recovery of Emtricitabine was determined at different QC levels of LQC, MQC II, MQC and HQC. The relative recoveries were evaluated by comparing the peak areas of extracted samples (spiked before extraction) with that of un-extracted samples (blank extracts spiked after extraction).

The matrix effect of the plasma was checked at low QC and high QC level using six different blank plasma lots (including one hemolytic and one lipemic lot). Matrix factor for analyte and internal standard was calculated in each lot by comparing the peak responses of post extraction samples (blank extracts spiked after extraction) against the peak responses of equivalent aqueous samples prepared in mobile phase. Internal standard (ISTD) normalized matrix factor in each lot was later evaluated by comparing the matrix factor of analyte and internal standard.

Matrix stability and aqueous solutions stability of analytes were evaluated after subjecting to different conditions and temperatures that could mimic the regular analysis. Matrix stability was evaluated in terms of freeze–thaw stability, bench top stability, long-term stability, and extracted sample stability. Freeze–thaw stability was evaluated after seven freeze (at -70°C) thaw (at room temperature) cycles. Bench top stability was assessed at room temperature and the long-term stability was evaluated at both -20°C and -70°C. Stability of extracted samples was determined after reconstitution (in-injector stability at 4°C). Stability in whole blood was evaluated at room temperature. All the stability assessments were made at LQC and HQC level by comparing the stability samples against freshly prepared samples.

Analyte stability in stock and working solutions were assessed at room temperature (short-term stability) and at 1-10°C (long-term stability). All stability samples were compared against freshly prepared stock solutions or working solutions. Before each analytical run, system suitability was evaluated by injecting six replicates of MQC sample to check the system precision and chromatography. System suitability was considered acceptable when the coefficient of variation for response ratios was less than 4.0%.

3. RESULTS AND DISCUSSION

3.1 Method Development

The optimization of extraction procedure along with chromatographic and mass spectrometric conditions was necessary to get consistent and

reliable estimation of analytes. Analyte and Internal standard were tuned in positive polarity mode using electrospray ionization technique. The Q1 and the product ion scans were made in infusion mode. The compound and gas parameters were optimized in flow injection analysis. The [M+H] peaks were observed at m/z of 248.2 and 230.3 for Emtricitabine and Lamivudine, respectively. Most intense product ions were found at m/z of 130.1 and 112.2 for both Emtricitabine and Lamivudine (Fig. 3) by applying sufficient collision activated dissociation gas and collision energy. Increase in source temperature beyond 400°C augmented the intensity. A 5% change in ionspray voltage and gas parameters did not affect the signal intensity.

Isocratic mode mobile phase was selected In the optimization of chromatographic conditions as no cross talk was observed between analytes and ISTD. Usage of methanol over acetonitrile in the mobile phase showed significant improvement in the signal intensities. Addition of 0.2% formic acid in mobile phase gave good chromatographic peak shapes and further increase in the buffer concentration was resulted in loss of response. A flow rate of 1 mL/min was used to lower the run time.

Protein precipitation and liquid–liquid extraction techniques were deliberately avoided as sample extraction methods to reduce base line noise and to get clean sample. Solid phase extraction initiated with individual HLB cartridges. Later on the method was shifted and optimized using 96 well plate format. Impact of different solutions and their concentration on recovery of analytes was monitored and the final optimized conditions are depicted in Section 2.6. During the optimization of chromatographic conditions and extraction procedure, more emphasis was given to improve the sensitivity and recovery. No significant matrix effects were observed with the proposed chromatographic and extraction conditions.

The method uses low plasma sample volume of only 100 µL. Positive electrospray ionization with a mobile phase containing methanol: 0.2% formic acid in water (85:15, v/v) was used to detect the drugs. During method development more emphasis was given to optimize the chromatographic conditions and to minimize the high baseline noise that is observed after more number of sample injections. No cross interference was observed between the analyte and ISTD. The method utilizes an injection volume of 5 µL and with chromatographic run time of only 3.0 min.

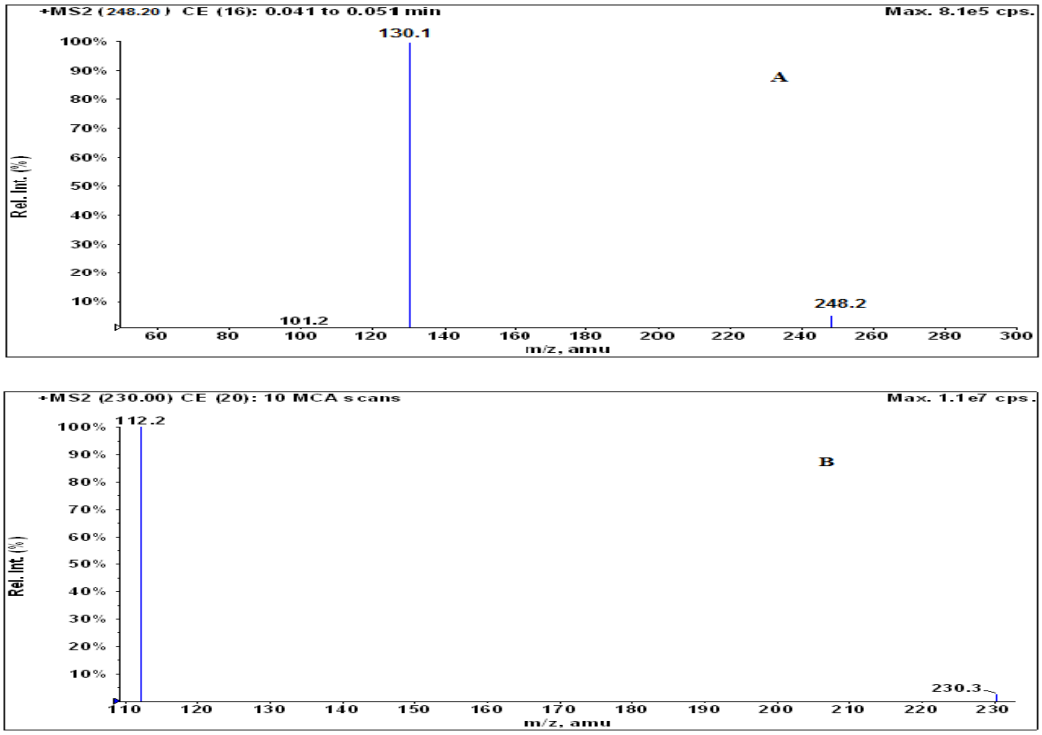

Fig. 3. Product ion spectra of Emtricitabine (A) and Lamivudine (B, ISTD)

3.2 Selectivity

Blank plasma samples from six different individual lots along with one lipemic and one hemolytic lot were processed and analyzed without addition of analyte and internal standard. In addition, each lot was spiked with LLOQ concentration and internal standard). All samples were processed and analyzed. Significant matrix interference (>20% of the analyte peak area of the LLOQ sample, >5% of the internal standard peak area of the blank matrix) was not observed in any of the six blank samples at the retention time of analyte or the internal standard. Representative chromatograms of a blank plasma, Zero standard and LLOQ samples are provided in Figs. 4-6.

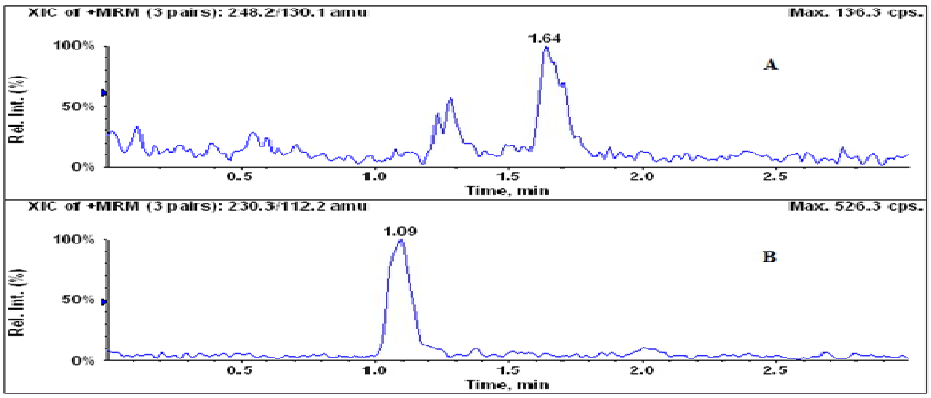

Fig. 4. Representative chromatogram of blank plasma

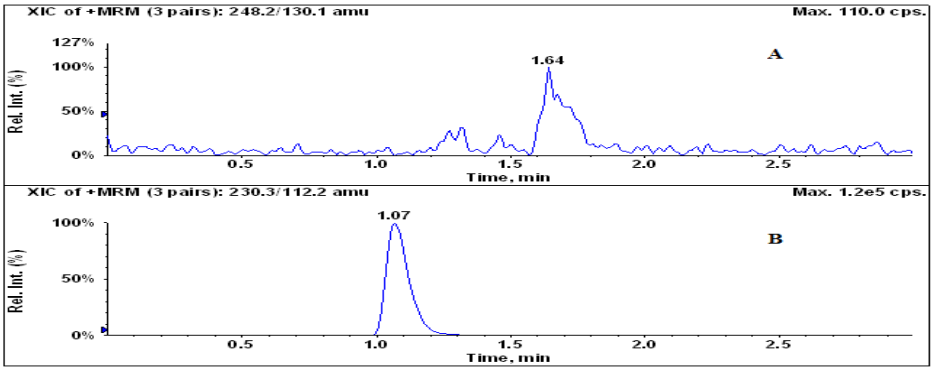

Fig. 5. Representative chromatogram of zero standard

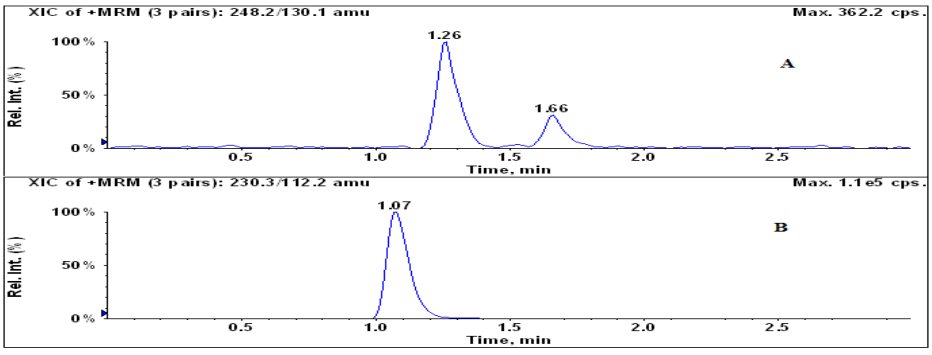

Fig. 6. Representative chromatogram of LLOQ

3.3 Linearity and Sensitivity

Calibration curves with eight points (CS1 to CS8) were spiked and used for the data sets. The calibration curve range was from 5.024 to 5023.725 ng/mL. The quadratic regression of the curves for peak area ratios versus concentration was weighted by 1/x2 (reciprocal of the square of the analyte concentration). The assay produced quadratic calibration curves over the concentration range. The r values, slopes and intercepts were calculated from three intra and inter day calibration curves. The r values for the calibration curve were ≥0.9995. The observed mean back calculated concentrations with accuracy (% Nominal) and precision (%CV) are presented in Table 3. The lower limit of quantitation (LLOQ) for determination of analytes was found to be 5.024 ng/mL. At LLOQ (n = 6) accuracy (% Nominal) was 97.0% with a %CV of 5.3%.

3.4 Precision and Accuracy

Intra-assay precision and accuracy were evaluated using replicates (N=6) from each of the five QC concentrations (LLOQ, LQC, MQC-II, MQC and HQC) of first PA batch. The intra batch precision was between 3.0 to 5.3% with % Nominal between 97.0 to 104.0.

The LLOQ, LQC, MQC-II, MQC and HQC samples from the three PA validation batches were used to assess the inter-assay precision and accuracy. The inter batch precision was between 2.7 to 10.4% with % Nominal between 98.9 to 106.0 Results of precision and accuracy are presented in Table 4.

3.5 Matrix Effect

Co-eluting components of matrix can enhance or suppress the ion- ization. This might not result in a detectable response in matrix blanks due to selectivity of the MS detection. However they can affect the precision and accuracy of the assay. The potential for variable matrix related ion suppression was evaluated in six independent sources (containing one hemolytic and one lipemic lot) of human plasma, by calculating the ISTD normalized matrix factor. The mean ISTD normalized matrix factor was ranged between 0.9846 and 1.0070 with a %CV of 1.4 to 3.2 as shown in Table 5.

Table 3. Summary of calibration standards of Emtricitabine in human plasma

Analyte	Nominal (ng/mL)	Mean (ng/mL)	%CV	% nominal
	5.024	5.028	2.2	100.1
	10.047	9.991	4.6	99.4
	26.315	26.677	0.8	101.4
	105.259	103.054	1.3	97.9
Emtricitabine	248.794	252.437	0.7	101.5
	526.295	534.924	1.0	101.6
	1196.125	1157.301	1.9	96.8
	2727.165	2750.305	2.1	100.8

%CV, percent coefficient of variation;
a Mean of 3 replicates at each concentration

Table 4. Intra batch and inter batch precision and accuracy of Emtricitabine in human plasma

QC level	Nominal conc. (ng/mL)	Intra batch[a]			Inter batch[b]		
		Mean conc found (ng/mL)	% CV	% Nominal	Mean conc found (ng/mL)	% CV	% Nominal
LLOQQC	5.024	4.873	5.3	97.0	5.327	10.4	106.0
LQC	14.354	13.987	4.7	97.4	14.511	6.5	101.1
MQC II	440.174	457.822	3.5	104.0	455.894	2.9	103.6
MQC	2440.095	2427.825	3.0	99.5	2413.064	2.7	98.9
HQC	4018.980	4002.863	4.1	99.6	4008.464	3.9	99.7

%CV, percent coefficient of variation. Conc., Concentration
a 6 replicates at each concentration; b 18 replicates at each concentration

Table 5. Matrix effect of Emtricitabine in human plasma

Lot #	LQC			HQC		
	MF of analyte	MF of ISTD	ISTD normalized factor	MF of analyte	MF of ISTD	ISTD normalized factor
1	0.982	0.991	0.991	1.009	1.032	0.978
2	0.996	0.964	1.034	1.004	1.007	0.997
3	0.993	1.008	0.985	0.961	1.001	0.960
4	1.017	1.038	0.980	1.023	1.026	0.997
5	1.079	1.019	1.059	1.009	1.023	0.986
6	1.042	1.048	0.994	1.050	1.061	0.989
Mean	-		1.0070	-		0.9846
SD			0.03175			0.01415
% CV			3.2			1.4
N			6			6

MF: Matrix factor

3.6 Extraction Recovery and Dilution Integrity

Six samples each of LQC, MQC-II, MQC and HQC which were spiked in human plasma were processed and analyzed along with unextracted samples. Unextracted samples were prepared by spiking the LQC, MQC-II, MQC and HQC working solutions in extracted blank samples followed by reconstitution solution. Percent recovery LQC, MQC-II, MQC and HQC samples were 60.7%, 63.2%, 65.9% and 63.7%, respectively. Mean % recovery was 63.38% with %CV of 3.4%, as shown in Table 6. Percent recovery of internal standard was 76.4. Dilution integrity experiment was carried out at 3 times the ULOQ concentration. After 1/5, 1/10 and 1/50 dilution the mean back calculated concentration for dilution QC samples was within 85–115% of nominal value with a %CV of ≤2.5 as shown in Table 7.

3.7 Stability

Stability experiments were performed in both plasma and aqueous samples. The stock solution of analyte was found to be stable for a period of 6 h at room temperature and upto 17 days at 1-10°C. Stock dilution of analyte in 50% methanol was found to be stable up to 6 h at room temperature. All the stability evaluations in plasma were performed against freshly spiked calibration standards using freshly prepared quality control samples (comparison samples). The analyte was found to be stable up to 23 h 50 min on bench top at room temperature. The analyte was found to be stable over 8 freeze-thaw cycles. The processed samples were found to be stable up to 76 h in autosampler at 4°C. Reinjection reproducibility was done for 60 h. The analyte was found to be stable in plasma at both -20°C and -50°C over a period of 42 days. No significant degradation of analytes was

Table 6. Recovery of Emtricitabine in human plasma

Analyte		A	B	% recovery	Mean recovery	% CV
	LQC	8768.8	14436.3	60.7	63.38	3.4
Emtricitabine	MQC II	276442.2	437689.5	63.2		
	MQC	1530833.8	2322282.8	65.9		
	HQC	2487426.7	3903743.5	63.7		
Lamivudine		39782.7	52151.6	76.3	-	-

B: Mean peak response of Un extracted samples
A: Mean peak response of extracted samples

Table 7. Dilution integrity of Emtricitabine in human plasma

Dilution factor[a]	% nominal	% CV
1/5	100.1	1.8
1/10	99.2	2.5
1/50	98.1	1.7

a: Six replicates at each dilution factor

Table 8. Stability data of Emtricitabine in human Plasma

Stability	QC Level	A	%CV	B	%CV	% change
Bench top stability at room temperature (23 hrs 50 min)	LQC	14.4153	2.6	16.1793	3.6	-10.9
	HQC	3938.9387	1.0	4036.9967	3.7	-2.4
Freeze-thaw (after 8 cycle)	LQC	14.2110	3.7	14.6487	4.8	-3.0
	HQC	3986.0332	1.1	4099.2860	1.9	-2.8
Auto sampler stability (76 hrs)	LQC	13.1727	3.7	15.3233	10.2	-14.0
	HQC	3535.3845	2.6	3990.8285	13.9	4.7
Long term stability for 42 days (below -20°C)	LQC	13.2022	3.1	14.6487	4.8	-9.9
	HQC	3981.2535	1.5	4099.2860	1.9	-2.9
Long term stability for 42 days (below -50°C)	LQC	12.8728	2.5	14.6487	4.8	-12.1
	HQC	3930.4442	1.6	4099.2860	1.9	-4.1

observed over the stability duration and conditions. The stability results presented in Table 8 were within 85-115%.

4. CONCLUSION

A sensitive, rapid, high throughput and accurate liquid chromatography with electrospray ionization tandem mass spectrometry using 96 well plate solid phase extraction method was developed for determination of Emtricitabine in human plasma chromatographic run time of 3.0 min. This method offers a high selectivity with a LLOQ of 5 ng/mL. The sample extraction utilizes a low plasma volume of 100 µL and given consistent and reproducible recoveries for analyte and ISTD with minimum plasma interference and matrix effect. Compared to reported methods [10-16] this is high sensitive method with low processing volume using high throughput solid phase extraction in 96 well plate format. This validated method can be successfully implemented in clinical and tox studies. The 96 well plate high throughput method format can reduces the overall processing time, which allowing to process and analyze more than 180 samples in single time.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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