International Journal of
Medicinal Chemistry & Analysis
www.ijmca.com
e ISSN 2249 - 7587
Print ISSN 2249 - 7595

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF EFAVIRENZ AND EMTRICITABINE IN HUMAN PLASMA BY LC-MS/MS

Balammal G[1*] and Saravana Kumar A[2]

[1]Research Scholar, Pacific Academy of Higher Education and Research University, Pacific Hills, Udaipur – 313024, Rajasthan, India.
[2]Sri Venkateswara College of Pharmacy, Chittoor, Andhra Pradesh – 517 127, India.

ABSTRACT

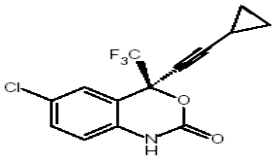
A sensitive, robust and selective liquid chromatographic – tandem mass spectrometric method (LC-MS/MS) was developed & validated for Efavirenz and Emtricitabine quantification in human EDTA plasma. Sample preparation was based on solid phase extraction using mixture of methanol/ammonium acetate 5mM (80/20v/v) to extract the drug and internal standard from plasma. Chromatography was performed on c18 analytical column, retention time were for Efavirenz, Emtricitabine and IS was around 2.34min, 1.52min and 1.76min respectively. The ionization was optimized using ESI(+) selectivity was achieved by tandem mass spectrometric analysis using MRM functions for Efavirenz, Emtricitabine & Atenolol. Analytical curve ranged from 25 - 5000 ng/mL for Efavirenz and 20 - 4000 ng/mL for Emtricitabine. Inter day precision and accuracy of the quality control samples were <15 % relative standard deviation (RSD), analyte stability during sampling processing and storage were established. The developed and validated bioanalytical method for Efavirenz and Emtricitabine drugs shows satisfactory linearity, precision, accuracy and stability. The practical extraction procedure based on solid phase extraction technique for the specific drug used in the study provides a high and precise recovery from plasma. The method can be particular useful for pharmacokinetics and pharmacodynamics.

Keywords: Efavirenz, Emtricitabine, Validation, LC-MS/MS.

INTRODUCTION

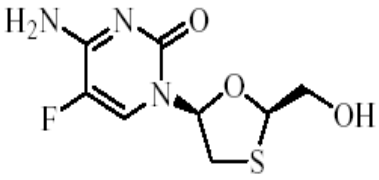
Efavirenz is a human immunodeficiency virus type 1 (HIV-1) specific, non-nucleoside, reverse transcriptase inhibitor (NNRTI) [1]. Efavirenz is used to treat HIV infection. It is never used alone and is always given in combination with other drugs. The decision on when to start treatment should take into account CD4 count, HIV viral load, treatment history, resistance profiles and patient preference [2].

Figure 1. Structure of Efavirenz



Emtricitabine, a synthetic nucleoside analog with activity against human immunodeficiency virus type 1 (HIV-1) reverse transcriptase. Emtricitabine is an analogue of cytidine. The drug works by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA [3].

Figure 2. Structure of Emtricitabine

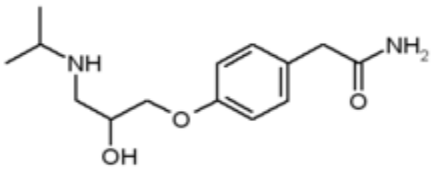


Corresponding Author: - **Balammal G** Email: bala.hospira@gmail.com

By interfering with this process, which is central to the replication of HIV, Emtricitabine can help to lower the amount of HIV, or "viral load", in a patient's body and can indirectly increase the number of immune system cells (called T cells or CD4+ T-cells) [4].

Atenolol, a synthetic, beta1-selective (cardioselective) adrenoreceptor blocking agent. Atenolol can be used to treat cardiovascular diseases such as hypertension, coronary heart disease, arrhythmias, and treatment of myocardial infarction after the acute event. Patients with compensated congestive heart failure may be treated with Atenolol as a co medication (usually together with an ACE inhibitor, a diuretic and a digitalis-glycoside, if indicated) [5].

Figure 2. Structure of Atenolol



MATERIALS AND METHODS

Efavirenz, Emtricitabine as working standard and Atenolol as internal standard. HPLC grade Methanol, Acetonitrile was procured from J.T.Baker, Milli-Q Water from Merck. Other reagents and equipment listed in Table 1 and 2.

Preparation of Stock Solution

Preparation of Emtricitabine and Efavirenz Stock solution

Weigh accurately 10 mg of Emtricitabine and Efavirenz working Standards and transfer into a 10 ml volumetric flask. Dissolve the Emtricitabine in water followed by Efavirenz in Methanol and make up the volume with the same to produce a solution of 1 mg/ml strength of Emtricitabine and Efavirenz.

IS Stock solution

0.01008gm IS was weighed and dissolved in 5 ml of HPLC Grade Methanol in a 10 ml clean volumetric flask.The solution was then sonicated for 5 min in ultrasonicator. The volume was made up to the mark with the same. Stock concentration was calculated by using given below formula to get concentration of 1005.984 µg/ml.

$$\text{Stock conc.} = \frac{\text{Weight taken(g) x mol. weight x assay percent x 1000X1000}}{\text{Total volume, (ml) x mol. weight}} \times 100$$

IS Stock Dilution

Dilute the IS stock solution with diluent solution to acquire ~40µg/mL of IS (Working solution).

Preparation of Calibration Curve Samples

Spiking Efavirenz in plasma for calibration curves

Linearity standards for Efavirenz in plasma was prepared by taking respective volume of aqueous dilutions mentioned in Table No 3 and then diluted in serial dilution manner in 2 percent of total plasma volume. These linearity standards were vortexed for 2-3 min to get uniform concentration.

Spiking Emtricitabine in plasma for calibration curves

Linearity standards for Emtricitabine in plasma was prepared by taking respective volume of aqueous dilutions mentioned in Table No 4 and then diluted in serial dilution manner in 2 percent of total plasma volume. These linearity standards were vortexed for 2-3 min to get uniform concentration.

About 1.20 ml of calibration curve samples was aliquated in polypropylene tubes and stored in deep freezer at-50˚c.

Preparation of Quality Control Samples (QC)

Spiking plasma for quality control samples of Efavirenz

Quality control samples at three concentrations level for Drug in plasma was prepared by taking respective volume of aqueous dilutions mentioned in Table No 5 and then diluted in serial dilution manner in 5 percent of total plasma volume. These Quality control samples then vortexed for 2-3 min to get uniform concentration (Table 5).

Spiking plasma for quality control samples of Emtricitabine

Quality control samples at three concentrations level for Drug in plasma was prepared by taking respective volume of aqueous dilutions mentioned in Table No 6 and then diluted in serial dilution manner in 5 percent of total plasma volume. These Quality control samples then vortexed for 2-3 min to get uniform concentration.

About 1.20ml of quality control samples were aliquated in polypropylene tubes and stored in deep freezer below -50˚c (Table 6).

Evaluation

The standard curves were calculated from the peak area ratio (P.A.R.) of Drug / IS using linear regression $y = ax + b$ with $1/x^2$ weighing. Drug concentrations (ng/mL) for QCs in a batch calculated by interpolating the peak area ratios from the corresponding standard curve. The measured peak area ratios of the QC samples were converted into concentration using the following equation,

$$\text{Drug concentration} = \frac{\text{P.A.R. (Drug / IS)} - b}{a}$$

Where a = slope of the corresponding standard curve,
b = intercept of the corresponding standard curve.

The concentrations were reported in nanogram per milliliter plasma.

Tuning of Drug and Internal standard

For Drug and IS tuning was done by manually using of solution made in diluent MeOH: water (50:50) using syringe needle. First scanning was performed for Q1 to get mass of parent ion. Then this ion goes to Q2 where fragmentation takes place by collision energy leads to the formation of daughter ions. This daughter ion scanned in Q3. Formation of ions and traveling from one quadrapole to another all activities performed by setting different potential .C.E, C.X.P, F.P and DP.

Pretreatment of biological sample

In bioanalysis the method development step additionally require the extraction trial in order to recover the analyte and internal standard from highly complicated biological matrix one should have knowledge about the nature of the Drug, its molecular weight, polarity, pKa, ionic character and the solubility parameter. Selection of internal standard should be on the basis of structural similarity, physicochemical properties related to the analyte to be quantified. In initial stages of method development our focus is on achieving our LOQ level with precision and accuracy and checking for interference at the retention time of analyte and internal standard. Method development trials involve a lot of exercises based on chemistry of molecule and efficiency of our extraction procedure in order to achieve good results. Optimization of mobile phase, buffer, and column are essential part of method development. The peak shape, retention time of analyte and internal standard, column, flow rate are optimized by making aqueous solution of Drug and internal standard in a set of different mobile phase compositions.

Extraction Technique

Sample preparation

Retrieve one set of CC and minimum two set of QC's along with all samples of one or more periods of one or more subject from the deep freezer and allow them to thaw at room temperature.

Precipitation Technique

Vortex the thawed sample to ensure complete mixing of contents. Aliquot 0.1mL of sample in poly propylene tubes and 25µL of IS dilution Vortex the content for 5 seconds. Add 2mL of acetonitrile and vortex thoroughly for 5 seconds. Centrifuge the content at 3000rpm at 4˚C for 5 minutes. Transfer the clear aqueous layer into the HPLC vials for injection.

Chromatographic Condition

Extraction Method: Solid phase extraction
Quantification parameter: Peak area ratio
Linearity range: 54.6 to 5248.5ng /ml
Column: Hypurity Advance C-18, 50×4.6mm, 5µ
Mobile phase: Buffer: methanol (20:80v/v)
Flow rate: 0.8ml/min
Detection: Positive MRM
Injection volume: 5 µl
Column oven temperature: 35˚c
Sample cooler temperature: 10˚c
Retention time: For Drug around 1.90 Min and
for IS around 1.85Min
Run time: 3.0 min

Preparation of reagents

Mobile phase buffer (pH3.5 ±0.05)-5Mm Ammonium acetate buffer

385.4 mg of ammonium acetate buffer was weighed accurately. It was then transferred into1000 ml reagent bottle. To this 900 ml Milli-Q/ HPLC water was added and mixed well to dissolve. This content was transferred to 1000ml volumetric flask and the volume was made with Milli-Q/HPLC water. The pH of solution was exactly set to 3.5 ± 0.05 using Acetic Acid. It was then Filter & sonicated.

Diluent

Milli-Q/HPLC water: methanol (50:50) was mixed. Then it was sonicated.

Injection needle wash solution (v/v)

330 ml of milli-Q /HPLC water, 330 ml of HPLC grade methanol and 340 ml of HPLC grade acetonitrile were mixed. It was then sonicated.

METHOD VALIDATION

System suitability test

This test is performed in order to check suitability of system with optimized final condition and to maintain performance of system reproducibility in changing environment. The percent coefficient of variation for peak area ratio of analyte to internal standard and for retention time were under the acceptance criteria i.e. % C.V was less than 5 % for LC-MS/MS based procedures. Retention time for Drug and IS was around 1.90 min and 1.85min respectively [6].

Performance Data

Inter-run Precision and Accuracy

The inter-run precision and accuracy data were obtained by measuring 4 different standard curves including a blank, standard zero + IS (standard Curve 1-4). Six sets of quality control samples were assayed with each of the standard curves. The statistical data on the accuracy (expressed percent deviation of the back-calculated versus the nominal values) and precision (expressed as

coefficient of variation of the back- calculated values) of these standards and quality control samples are shown in Table No 7 and 8 respectively and the summary of the standard curve parameters is shown in Table No 9 and 10.

Selectivity

The selectivity is generally defined as the lack of interfering peaks at the retention time of the assayed Drug and the internal standard in the chromatograms. This was confirmed by the injection of the extracts of blank plasma samples, and samples spiked with internal standard and the working solution resulting in the LOQ concentration. In addition, the influence of Hemolysed, Lipemic plasma, plasma collected in EDTA Vacutainers and stored in polypropylene and glass tubes for interference at Drug and IS RT were also assayed (Table No 11 and 12) [7].

Matrix Effect

Matrix effect was checked by processing six lots of plasma samples. LQC and HQC stock solutions were spiked post extraction in duplicate (100% recovery). Similarly, internal standard was also spiked post extraction. The samples were acquired using the LC-MS/MS method for Drug. % CV Drug area in LQC and HQC samples was compared across the six lots to access matrix effect on ionization if any (Table No 13 and 14).

Extraction Recovery

The Recovery [8] of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery was calculated by preparing separately pure aqueous solutions of analyte and IS representing 100.0% extraction of QC samples at LQC, MQC and HQC concentrations. Concurrently, six replicates of LQC, MQC and HQC were extracted as per the method SOP and recovery of analyte and IS was evaluated by injecting six replicates of the pure "non-extracted" solutions and six replicates of the extracted QC samples at each LQC, MQC and HQC concentrations (Table No 15, 16 and 17).

Dilution Integrity

Dilution integrity for Drug was evaluated by preparing QC samples with concentration of 1.7 times the concentration of the highest standard, a stock solution of 17.0100ng/mL in plasma was prepared and this was diluted to 2 fold and 4 fold of the original concentration and analysed against a fresh calibration curve (Table No 18 and 19).

STABILITY TESTINGS [9]

Bench Top Stability in Plasma

The stability of Drug in plasma at room temperature was examined by spiking and keeping the six sets of HQC and LQC at room temperature for 6.0 hours without protection from light. The test samples were calculated using a fresh calibration standard (Table No 20 and 21).

Stability after Repeated Freezing / Thawing Cycles

Freeze thaw stability in plasma was assayed using freshly prepared calibration curve standards against six replicates of LQC and HQC samples previously frozen and thawed over three cycles. The freeze and thaw stability was evaluated at the end of first, second and third cycle. These samples were analyzed and calculated with a freshly prepared standard curve (Table No 22 and 23).

Long Term Plasma Stability

Long-term plasma stability of Drug in plasma sample was assured by analyzing in replicate quality control samples. Back calculation concentration were arrived at by using freshly prepared calibration curve [10] (Table No 24 and 25).

Short Term Stock Solution Stability

The stability of Drug in the stock solution was examined at room temperature for a period of 6.0 hours Table No 26 and 27).

Table 1. Chemicals and reagents used

Reagents	Manufacturer
Acetonitrile(HPLC Grade)	J.T.Baker
Ammonium Acetate	Sigma Aldrich
Formic acid	Sigma Aldrich
Methanol (HPLC Grade)	J.T.Baker
Milli-Q /HPLC water	Merck
Human plasma (Sodium citrate)	Prathama blood bank
Oasis HLB 30 mg SPE cartridges	Waters
Sodium hydroxide Pellets (AR Grade)	Merck
Acetic acid	Fluka

Table 2. Instrument used

Name	Specification (Model / Brand)	Company
LC-MS/MS	API 2000	MDS-SCIEX
Detection	MRM	Applied Biosystems
Data acquisition system	Analyst 1.4.1	Applied Biosystems

HPLC	Autosampler SIL-HTc, Pump LC-10ADVP Column Oven CTO-10AVP	Shimadzu
Centrifuge	Multifuge 3SR	Heraeus
Sample Extractor	Multi pulse Vortexer	Glas-Col
Analytical Balance	XS205 DU	Mettler Toledo
Cyclo Mixer	CM 101	Remi Equipment Pvt.Ltd.
Solid Phase Extractor	Waters manifold	Waters
Nitrogen Evaporator	Turbo Vap® LV	Zymark
Column	Hypurity Advance C-18, 50 x 4.6mm,5µm	Thermoelectron
	Hypersil,BDS C-8 ,150 x 4.6, 5micron	Thermoelectron
	Hypersil,hypurity 50 x 4.0,5 micron	Thermoelectron
	Inertsil,cyano , 150 x 4.6 , 5micron	Thermoelectron

Table 3. Linearity standards for Efavirenz in plasma

Solution ID	Working stock solution con. (ng/mL)	Volume of stock (ml)	Final volume (ml)	Final con. (ng/mL)	Spiking solution ID
AQ-STD-8	598795.20	0.1	10	5987.9520	**STD-8**
AQ-STD-7	425144.59	0.1	10	4251.4459	**STD-7**
AQ-STD-6	225326.63	0.1	10	2253.2663	**STD-6**
AQ-STD-5	78864.32	0.1	10	788.6432	**STD-5**
AQ-STD-4	21293.37	0.1	10	212.9337	**STD-4**
AQ-STD-3	10646.68	0.1	10	106.4668	**STD-3**
AQ-STD-2	5323.34	0.1	10	53.2334	**STD-2**
AQ-STD-1	2661.67	0.1	10	26.6167	**STD-1**

Table 4. Linearity standards for Emtricitabine in plasma

Spiking solution ID	Spiking conc.(ng/ml)	Spiking volume (µL)	Final volume (ml)	Final conc (ng/ml)	Sample ID
AQ-STD-8	396780.80	0.20	10	3967.8080	**STD-8**
AQ-STD-7	238068.48	0.20	10	2380.6848	**STD-7**
AQ-STD-6	95227.39	0.20	10	952.2739	**STD-6**
AQ-STD-5	52375.07	0.20	10	523.7507	**STD-5**
AQ-STD-4	20950.03	0.20	10	209.5003	**STD-4**
AQ-STD-3	8380.01	0.20	10	83.8001	**STD-3**
AQ-STD-2	4190.01	0.20	10	41.9001	**STD-2**
AQ-STD-1	2095.00	0.20	10	20.9500	**STD-1**

Table 5. Spiking plasma for quality control samples of Efavirenz

Solution ID	Working stock solution con. (ng/mL)	Volume of stock (ml)	Final volume (ml)	Final con. (ng/mL)	Spiking solution ID
AQ-DR-02	419156.64	0.1	10	4191.5664	**AQHQC**
AQM1QC	251493.98	0.1	10	2514.9398	**AQMQC**
AQMQC	7544.82	0.1	10	75.4482	**AQLQC**
AQMQC	2670.87	0.1	10	26.7087	**AQLLOQ**

Table 6. Spiking plasma for quality control samples of Emtricitabine

Spiking solution ID	Spiking conc. (ng/ml)	Spiking volume (µL)	Final volume (ml)	Final conc. (ng/ml)	Sample ID
AQHQC	3471.8320	0.50	25	3471.8320	**HQC**
AQMQC	1735.9160	0.50	25	1735.9160	**MQC**
AQLQC	60.7571	0.50	25	60.7571	**LQC**
AQLOQQC	21.2650	0.50	25	21.2650	**LLOQ**

Table 7. Calculated Quality Control Data of Efavirenz (Inter-run)

	LLOQ	LQC	MQC	HQC
	Nominal Concentration (g/mL)			
	26.7087	**75.4482**	**2514.9398**	**4191.5664**
P & A I	26.0782	68.9809	2338.6197	3793.3725
	21.1872	72.0952	2228.3806	3720.9628
	29.4644	80.7063	2236.6233	3853.8221
	30.1041	69.3984	2027.6141	3620.2903
	22.5068	80.3493	2064.7417	3797.7271
	28.1496	83.7486	2139.5253	3815.8695
Mean	**26.2484**	**75.8798**	**2172.5841**	**3767.0074**
S.D ±	**3.7005**	**6.4671**	**117.0652**	**83.9156**
%CV	**14.10**	**8.52**	**5.39**	**2.23**
% Nominal	**98.28**	**100.57**	**86.39**	**89.87**
P & A II	21.5174	67.4809	2106.7689	4120.1447
	26.8086	60.9881	2241.8129	4365.9271
	21.9202	75.4570	2284.6288	4271.4478
	24.7918	67.3800	2279.9454	NA
	17.5193	66.3251	2499.3312	4623.6035
	17.5931	70.282	2282.2337	4229.1909
Mean	**21.6917**	**67.9855**	**2282.4535**	**4322.0628**
S.D ±	**3.7439**	**4.7632**	**126.1457**	**190.2688**
%CV	**17.26**	**7.01**	**5.53**	**4.40**
% Nominal	**81.22**	**90.11**	**90.76**	**103.11**
P & A III	28.6277	71.4077	2376.3019	4479.0441
	22.6187	74.6476	2122.7069	4269.1660
	31.1443	79.6843	2130.0891	4089.1503
	31.4509	87.7085	2332.7097	4262.4016
	21.2899	79.9298	2204.2620	3925.4659
	29.1828	90.5494	2428.7167	4422.4918
Mean	**27.3857**	**80.6546**	**2265.7977**	**4241.2866**
S.D ±	**4.3660**	**7.3583**	**131.0797**	**206.6278**
%CV	**15.94**	**9.12**	**5.79**	**4.87**
% Nominal	**102.53**	**106.90**	**90.09**	**101.19**

Table 8. Calculated Quality Control Data of Emtricitabine (Inter-run)

	LLOQ	LQC	MQC	HQC
	Nominal Concentration (g/mL)			
	21.2650	**60.7571**	**1735.9160**	**3471.8320**
P & A I	23.3637	56.0425	1845.7614	3083.8646
	24.7084	49.0440	1683.6629	3137.8346
	26.4248	58.2804	1757.2744	3273.3689
	19.9350	63.8379	1499.3617	3014.4214
	24.2980	56.3445	1620.1201	3281.4718
	15.0897	57.4163	1551.0511	3181.2181
Mean	**22.3033**	**56.8276**	**1659.5386**	**3162.0299**
S.D ±	**4.1357**	**4.7547**	**129.4552**	**105.3702**
%CV	**18.54**	**8.37**	**7.80**	**3.33**
% Nominal	**104.88**	**93.53**	**95.60**	**91.08**
	18.1800	63.2900	1533.8884	3286.9706
P & A II	18.0024	54.1824	1661.9222	3384.6202
	18.4694	58.5996	1778.1554	3173.4531
	21.3004	50.2116	1703.2709	NA
	20.8254	54.0228	1810.9452	3636.4794

	19.5781	69.1870	1630.4771	3365.5932
Mean	**19.3926**	**58.2489**	**1686.4432**	**3369.4233**
S.D ±	**1.4130**	**6.9882**	**101.1944**	**170.8562**
%CV	**7.29**	**12.00**	**6.00**	**5.07**
% Nominal	**91.19**	**95.87**	**97.15**	**97.05**
P & A III	22.4912	62.5795	1976.1014	3581.9104
	19.5757	67.7895	1729.6047	3538.2354
	22.0773	65.1150	1729.9176	3350.4461
	30.2901	70.5655	1907.0431	3632.9664
	23.1595	66.9745	1646.5386	3359.5164
	26.7197	69.1464	2003.4425	3644.1136
Mean	**24.0523**	**67.0284**	**1832.1080**	**3517.8647**
S.D ±	**3.8262**	**2.8647**	**149.0575**	**131.7628**
%CV	**15.91**	**4.27**	**8.14**	**3.75**
% Nominal	**113.11**	**110.32**	**105.54**	**101.33**

Table 9. Back-calculated Concentrations of Calibration Curve Standards for Efavirenz in Human plasma

Std ID	Nominal Concentration (ng/mL)	Back calculated concentration (ng/mL)			Mean	S.D	%CV	% Nominal
		P & A I	P & A II	P & A III				
STD 1	26.6167	25.3329	27.0214	26.2410	26.19843	0.845054	3.23	98.43
STD 2	53.2334	57.2879	57.2879	58.1767	57.73230	0.513149	0.89	108.17
STD 3	106.4668	111.6147	98.6529	102.9080	104.39187	6.607077	6.33	98.05
STD 4	212.9337	209.5273	218.0115	204.6453	210.72803	6.763516	3.21	98.96
STD 5	788.6432	814.1116	782.9899	758.1285	785.07667	28.049827	3.57	99.55
STD 6	2253.2663	2038.1192	2277.3079	2172.6818	2162.70297	119.906177	5.54	95.98
STD 7	4251.4459	4460.8272	4583.6558	4911.6687	4652.05057	233.072730	5.01	109.42
STD 8	5987.9520	5710.4411	5704.7222	5919.1425	5778.10193	122.178179	2.11	96.50
	r^2	0.9952	0.9972	0.9930				
	Slope	0.0004	0.0003	0.0002				
	Intercept	0.0004	0.0016	0.0018				

Table 10. Back-calculated Concentrations of Calibration Curve Standards for Emtricitabine in Human plasma

Std ID	Nominal Concentration (ng/mL)	Back calculated concentration (ng/mL)			Mean	S.D	%CV	% Nominal
		P & A I	P & A II	P & A III				
STD 1	20.9500	21.1794	20.8806	21.2194	21.0931	0.1851	0.88	100.68
STD 2	41.9001	38.0036	41.4684	40.8427	40.1049	1.8465	4.60	95.72
STD 3	83.8001	91.6642	84.1292	78.6051	84.7995	6.5553	7.73	101.19
STD 4	209.5003	235.9766	223.4339	237.5867	232.3324	7.7483	3.33	110.90
STD 5	523.7507	560.3357	567.2414	561.6959	563.0910	3.6581	0.65	107.51
STD 6	952.2739	830.8053	859.2393	931.4318	873.8255	51.8748	5.94	91.76
STD 7	2380.6848	2303.3710	2302.3483	2391.4812	2332.4002	51.1682	2.19	97.97
STD 8	3967.8080	3777.3657	3931.0969	3512.3357	3740.2661	211.8314	5.66	94.27
	r^2	0.9890	0.9958	0.9974				
	Slope	0.0013	0.0009	0.0003				
	Intercept	0.0844	0.0561	0.0036				

Table 11. Selectivity-Efavirenz

Plasma Lot ID	Specificity (Blank)		Selectivity (Spiked)		% Interference		Area ratio	S/N Ratio (≥5)	
	Analyte	IS peak	Analyte	IS peak	Analyte (<20%)	IS(<5%)	Analyte/IS (<20%)	Analyte	IS
LOT 1	0	135	3941	190024	0.0000	0.071044	0.0207	14	5744
LOT 2	0	149	3108	142095	0.0000	0.104859	0.0219	10	4884
LOT 3	0	227	2816	146830	0.0000	0.154601	0.0192	11	3562
MEAN	0.00000	170.33333	3288	159650	0.0000	0.11017	0.02060	11.6667	4730.0000
SD	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	0.001353	N/AP	N/AP
%CV	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	6.57	N/AP	N/AP

N/AP - Not Applicable; % of Lots passing = **100%**

Table 12. Selectivity-Emtricitabine

Plasma Lot ID	Specificity (Blank)		Selectivity (Spiked)		% Interference		Area ratio	S/N Ratio (≥5)	
	Analyte	IS peak	Analyte	IS peak	Analyte (<20%)	IS(<5%)	Analyte/IS (<20%)	Analyte	IS
LOT 1	0	135	14477	190024	0.0000	0.071044	0.0762	237	5744
LOT 2	0	149	11588	142095	0.0000	0.104859	0.0816	206	4884
LOT 3	0	227	11356	146830	0.0000	0.154601	0.0773	228	3562
MEAN	0.0000	170.3333	12474	159650	0.0000	0.11017	0.07836	223.66667	4730.0000
SD	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	0.002824	N/AP	N/AP
%CV	N/AP	N/AP	N/AP	N/AP	N/AP	N/AP	3.60	N/AP	N/AP

N/AP - Not Applicable; % of Lots passing = **100%**

Table 13. Matrix effect-Efavirenz

S. No	Analyte		Internal Standard			Matrix Factor	
	Aqueous Area	Spiked Area	Aqueous Area	Spiked Area		Analyte	Internal Standard
1	12827	11475	194730	209895		0.9797	1.0674
2	10825	11967	189344	204918		1.0217	1.0421
3	11476	12055	208685	200578		1.0292	1.0201
4	11723	12070	193781	199048		1.0305	1.0123
Mean	11712.75	11891.75	196635.00	203609.75	**MEAN**	1.01528	1.03547
SD	833.828270	281.523682	8369.30	4872.27	**SD**	0.024036	0.024778
CV	7.12	2.37	4.26	2.39	**%CV**	**2.37**	**2.39**
					% Matrix Effect	**1.02**	**1.04**

Table 14. Matrix effect-Emtricitabine

S.No	Analyte		Internal Standard			Matrix Factor	
	Aqueous Area	Spiked Area	Aqueous Area	Spiked Area		Analyte	Internal Standard
1	52326	49280	194730	209895		1.0037	1.0674
2	47032	49698	189344	204918		1.0122	1.0421
3	49789	51845	208685	200578		1.0560	1.0201
4	47243	49145	193781	199048		1.0010	1.0123
Mean	49097.5	49992	196635.00	203609.75	**MEAN**	1.01822	1.03547
SD	2490.438047	1257.563517	8369.30	4872.27	**SD**	0.025614	0.024778
% CV	5.07	2.52	4.26	2.39	**%CV**	**2.52**	**2.39**
					% Matrix Effect	**101.82**	**103.55**

Table 15. Extraction Recovery – IS

IS Stock (ng/mL)				
S. No		**IS AREA**		**IS AREA**
1	**Unextracted (Aqueous)**	90275	**Extracted (Spiked)**	85369
2		81929		89801
3		91880		84770
4		84662		84804
5		88312		85434
6		86124		92413
	Mean	87197.00	**Mean**	87098.50
	SD	3687.20	**SD**	3224.77
	%CV	**4.23**	**%CV**	**3.70**
% Recovery=99.89%				

Table 16. Extraction Recovery – Efavirenz

S. No	**AQS HQC**	**EXTD HQC**
1	235693	247835
2	252501	255067
3	234964	240352
4	247688	269759
5	235292	247849
6	249530	250113
Mean	242611.33	251829.17
SD ±	8140.81	9984.27
%CV	**3.36**	**3.96**
S. No	**AQS MQC**	**EXTD MQC**
1	160544	170748
2	158021	153222
3	163963	165748
4	162140	157960
5	153665	160938
6	162316	162213
Mean	160108.17	161804.83
SD ±	3739.60	6081.03
%CV	**2.34**	**3.76**
S. No	**AQS LQC**	**EXTD LQC**
1	4504	5699
2	5163	5376
3	5669	4957
4	4513	4844
5	4502	4738
6	4659	4902
Mean	4835.00	5086.00
SD ±	481.30	371.49
CV %	**9.95**	**7.30**

Table 17. Extraction Recovery – Emtricitabine

S. No	**AQS HQC**	**EXTD HQC**
1	806968	716830
2	821108	752908
3	813786	692824
4	834142	792394
5	790676	689106
6	838184	729802

Mean	817477.33	728977.33
SD ±	17675.08	39088.04
%CV	**2.16**	**5.36**
S. No	**AQS MQC**	**EXTD MQC**
1	448676	402785
2	416267	377634
3	459548	399554
4	438802	373125
5	423531	386207
6	437084	373745
Mean	437318.00	385508.33
SD ±	15866.06	13038.25
%CV	**3.63**	**3.38**
S. No	**AQS LQC**	**EXTD LQC**
1	13811	13105
2	14372	14098
3	14899	13036
4	14321	13350
5	15917	12952
6	15466	13408
Mean	14797.67	13324.83
SD ±	786.35	418.59
CV %	**5.31**	**3.14**

Table 18. Results –Efavirenz QCs – Diluted by factor 2 times and 4 times

Run No.	**Dilution factor 2 4191.5664 ng/mL**	**Dilution factor 4 2095.7832 ng/mL**
	Actual Concentration (ng/mL)	
1	3636.0335	1860.7154
2	3735.0498	1875.1300
3	3616.0525	1783.7452
4	3669.5981	1824.0682
5	3834.2419	1778.7439
6	3721.8048	1851.3020
Mean	3702.1301	1828.9508
SD ±	79.7004	40.5690
%CV	**2.15**	**2.22**
% Nominal	**88.32**	**87.27**

Table 19. Results –Emtricitabine QCs – Diluted by factor 2 times and 4 times

Run No.	**Dilution factor 2 3471.8320 ng/mL**	**Dilution factor 4 1735.9160 ng/mL**
	Actual Concentration (ng/mL)	
1	2980.5531	1583.2118
2	3175.2189	1530.5225
3	3191.5027	1482.5042
4	3176.1745	1530.2014
5	3096.7912	1465.0000
6	3170.3993	1608.7634
Mean	3131.7733	1533.3672
SD ±	81.2525	55.5847
%CV	**2.59**	**3.63**
% Nominal	**90.21**	**88.33**

Table 20. Stability in plasma at room temperature and light for 6.0 hours.-Efavirenz

	LQC - 0.00 HRS	HQC - 0.00 HRS	LQC - 6.00 HRS	HQC - 6.00 HRS
Acutal ng/ml	**75.4482**	**4191.5664**	**75.4482**	**4191.5664**
	78.4498	4817.2678	73.8271	3908.3647
	84.3904	3775.7251	61.0126	4854.0385
	67.1407	4554.4731	76.2088	3810.6640
	68.2128	3831.1124	66.8090	4001.8492
	56.9685	4106.0135	71.3236	3894.1189
	74.9743	3734.7538	60.7702	3869.8350
Mean	71.6894	4136.5576	68.3252	4056.4784
SD	9.6674	452.5253	6.5462	395.6375
%CV	**13.49**	**10.94**	**9.58**	**9.75**
%Nominal	**95.02**	**98.69**	**90.56**	**96.78**

Table 21. Stability in plasma at room temperature and light for 6.0 hours.-Emtricitabine

	LQC - 0.00 HRS	HQC - 0.00 HRS	LQC - 6.00 HRS	HQC - 6.00 HRS
Acutal ng/ml	**60.7571**	**3471.8320**	**60.7571**	**3471.8320**
	61.9408	3275.5882	53.2181	3143.0744
	62.1693	3225.1530	51.6944	3366.5439
	60.7450	3196.5213	53.5936	2972.1188
	56.0099	2878.1979	52.8436	2874.9142
	53.6592	3032.2217	51.3855	3087.4321
	55.5077	2953.6786	61.6545	3157.3422
Mean	58.3387	3093.5601	54.0650	3100.2376
SD	3.7088	161.7171	3.8166	169.3305
%CV	**6.36**	**5.23**	**7.06**	**5.46**
%Nominal	**96.02**	**89.10**	**88.99**	**89.30**

Table 22. Stability of samples after repeated freeze /cycle - Efavirenz

	LQC-FT4	HQC-FT4
Acutal ng/ml	**75.4482**	**4191.5664**
	89.3544	3507.1099
	80.6390	3574.3799
	79.1770	3472.3994
	75.9955	3572.7265
	70.5798	4125.0156
	80.5013	3885.3290
Mean	79.3745	3689.4934
SD	6.1850	258.9634
%CV	**7.79**	**7.02**
%Nominal	**105.20**	**88.02**

Table 23. Stability of samples after repeated freeze /cycle - Emtricitabine

	LQC	HQC
Acutal ng/ml	**60.7571**	**3471.8320**
	57.3868	3363.9966
	60.0248	3175.3977
	57.7337	3248.4978
	56.6143	3268.7895
	51.8528	3573.7658
	52.1114	3150.5474
Mean	55.9540	3296.8325

SD	3.2806	155.2234
%CV	**5.86**	**4.71**
%Nominal	**92.09**	**94.96**

Table 24. Long term plasma stability of Efavirenz

For Efavirenz							
0 DAY				**8th DAY**			
S.No	**Analyte Area**	**ISTD Area**	**Area Ratio**	**S.No**	**Analyte Area**	**ISTD Area**	**Area Ratio**
1	514590	224824	2.2889	1	441901	199349	2.2167
2	530830	228948	2.3186	2	423235	212545	1.9913
3	491958	196287	2.5063	3	419833	193511	2.1696
4	521929	216112	2.4151	4	395832	194538	2.0347
5	494271	212280	2.3284	5	406337	187201	2.1706
6	499578	229165	2.1800	6	384385	176963	2.1721
		Mean	2.33953			Mean	2.12583
Stability of the Stock =90.87%							
For IS							
0 DAY				**8th DAY**			
S.No	**Analyte Area**	**ISTD Area**	**Area Ratio**	**S.No**	**Analyte Area**	**ISTD Area**	**Area Ratio**
1	374911	165346	0.4410	1	391101	167825	0.4291
2	380989	169557	0.4450	2	380918	158536	0.4162
3	410335	150331	0.3664	3	386583	172142	0.4453
4	375960	167589	0.4458	4	400655	158529	0.3957
5	399070	175045	0.4386	5	371109	170275	0.4588
6	365224	136938	0.3749	6	377832	156379	0.4139
		Mean	0.41863			Mean	0.42650
Stability of the Stock =101.88%							

Table 25. Long term stability of Emtricitabine

For Analyte							
0 DAY				**8th DAY**			
S.No	**Analyte Area**	**ISTD Area**	**Area Ratio**	**S.No**	**Analyte Area**	**ISTD Area**	**Area Ratio**
1	1674558	224824	7.4483	1	1356729	199349	6.8058
2	1518974	228948	6.6346	2	1426057	212545	6.7094
3	1465727	196287	7.4673	3	1257088	193511	6.4962
4	1510505	216112	6.9895	4	1250675	194538	6.4289
5	1403031	212280	6.6093	5	1163664	187201	6.2161
6	1490741	229165	6.5051	6	1082307	176963	6.1160
		Mean	6.94234			Mean	6.46209
Stability of the Stock = 93.08%							
For IS							
0 DAY				**8th DAY**			
S.No	**Analyte Area**	**ISTD Area**	**Area Ratio**	**S.No**	**Analyte Area**	**ISTD Area**	**Area Ratio**
1	1143299	165346	0.1446	1	1100032	167825	0.1526
2	1154668	169557	0.1468	2	1012843	158536	0.1565
3	953863	150331	0.1576	3	1101761	172142	0.1562
4	1070927	167589	0.1565	4	1025367	158529	0.1546
5	1094918	175045	0.1599	5	1043559	170275	0.1632
6	871143	136938	0.1572	6	1024126	156379	0.1527
		Mean	0.15377			Mean	0.15597
Stability of the Stock = 101.43%							

Table 26. Short term stability of Efavirenz

FOR ANALYTE							
0 HOURS				6 HOURS			
S.No	Analyte Area	ISTD Area	Area Ratio	S.No	Analyte Area	ISTD Area	Area Ratio
1	555372	260142	2.1349	1	553753	240441	2.3031
2	557296	225385	2.4726	2	581204	240230	2.4194
3	555422	232803	2.3858	3	520667	243067	2.1421
4	560141	236078	2.3727	4	545384	260154	2.0964
5	555151	257473	2.1562	5	527658	225165	2.3434
6	568720	249055	2.2835	6	548087	251719	2.1774
		Mean	2.30095			Mean	2.24695
Stability of the Stock = 97.65%							
FOR IS							
0 HOURS				6 HOURS			
S.No	Analyte Area	ISTD Area	Area Ratio	S.No	Analyte Area	ISTD Area	Area Ratio
1	576738	250018	0.4335	1	349673	142933	0.4088
2	549528	237477	0.4321	2	554677	241067	0.4346
3	575975	191838	0.3331	3	351039	142909	0.4071
4	570598	241367	0.4230	4	550764	207095	0.3760
5	572244	200426	0.3502	5	564061	218917	0.3881
6	553029	254837	0.4608	6	555102	231273	0.4166
		Mean	0.40546			Mean	0.40520
Stability of the Stock = 99.94%							

Table 27. Short term stability of Emtricitabine

FOR ANALYTE							
0 HOURS				6 HOURS			
S.No	Analyte Area	ISTD Area	Area Ratio	S.No	Analyte Area	ISTD Area	Area Ratio
1	1698834	260142	6.5304	1	1804516	240441	7.5050
2	1743609	225385	7.7361	2	1757453	240230	7.3157
3	1766967	232803	7.5900	3	1724181	243067	7.0934
4	1747755	236078	7.4033	4	1728731	260154	6.6450
5	1829299	257473	7.1048	5	1532927	225165	6.8080
6	1740584	249055	6.9888	6	1826388	251719	7.2557
		Mean	7.22556			Mean	7.10381
Stability of the Stock = 98.32%							
FOR IS							
0 HOURS				6 HOURS			
S.No	Analyte Area	ISTD Area	Area Ratio	S.No	Analyte Area	ISTD Area	Area Ratio
1	1687205	250018	0.1482	1	1117530	142933	0.1279
2	1589926	237477	0.1494	2	1741599	241067	0.1384
3	1266716	191838	0.1514	3	1105289	142909	0.1293
4	1683139	241367	0.1434	4	1451418	207095	0.1427
5	1349772	200426	0.1485	5	1442588	218917	0.1518
6	1689377	254837	0.1508	6	1593764	231273	0.1451
		Mean	0.14862			Mean	0.13919
Stability of the Stock = 93.66%							

Figure 3. Efavirenz-LLOQ

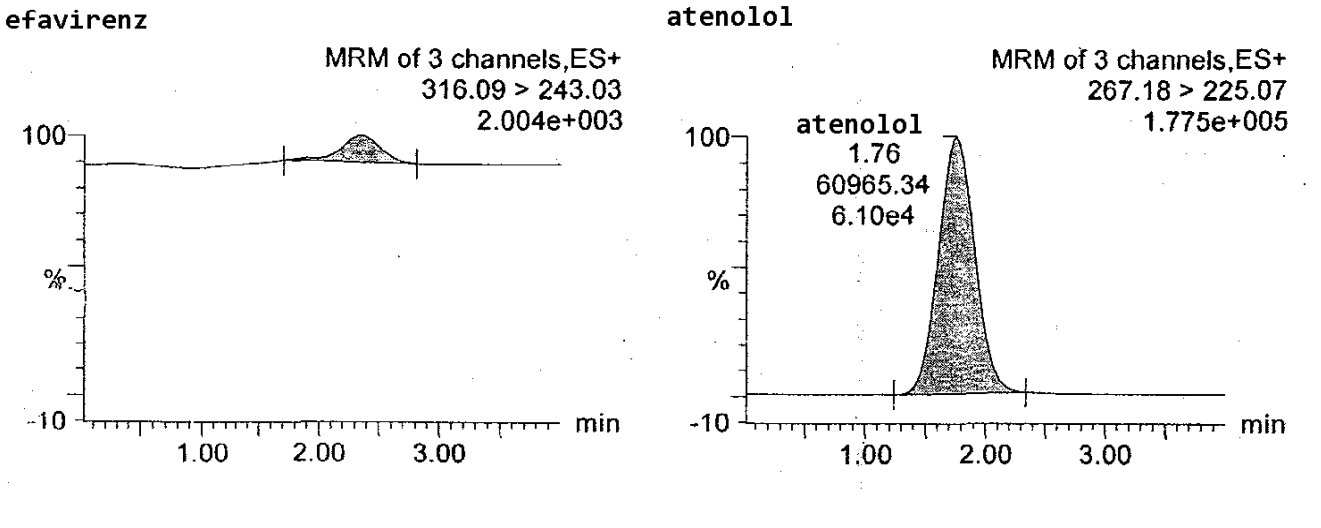
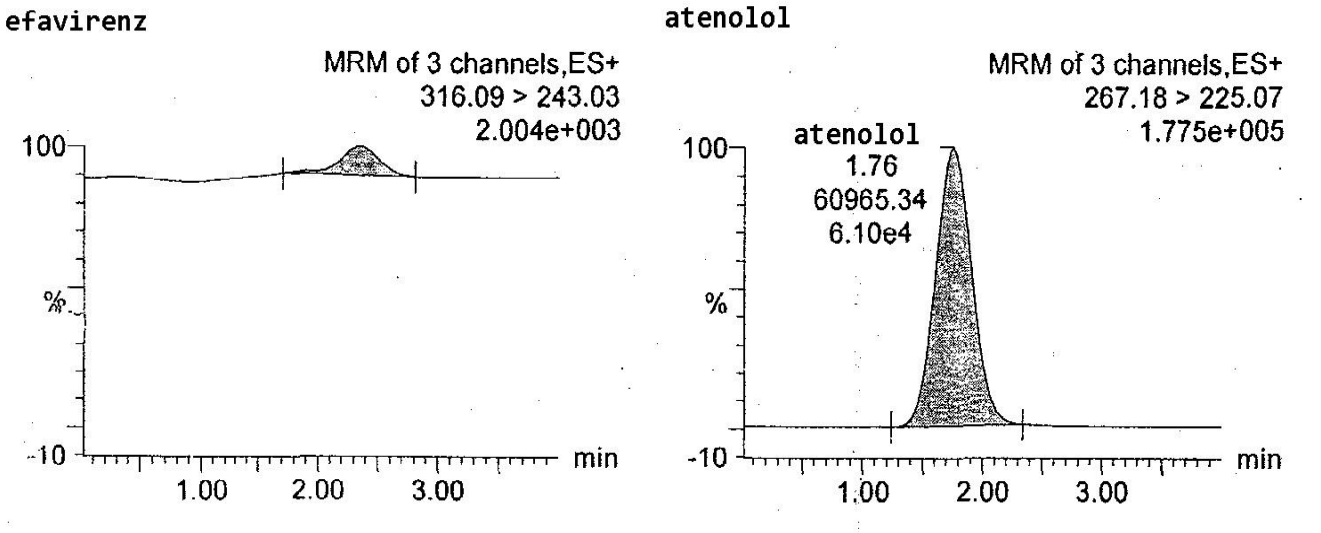


Figure 4. Emtricitabine-HQC



RESULTS AND DISUSSION

LC-MS/MS method which was developed and validated according to currently accepted FDA guidelines of bioanalytical method validation. The following parameters were tested;

Linearity

Efavirenz linearity was established in the range of 26.6167/ml to 5987.9520 ng/ml with coefficient of correlation 0.99 which meets the acceptance criteria (Table 3). Emtricitabine linearity was established in the range of 20.9500/ml to 3967.8080ng/ml with coefficient of correlation 0.99 which meets the acceptance criteria (Table 4).

Acceptance criteria: The coefficient of correlation should be greater than or equal to 0.99

Sensitivity

Sensitivity can be expressed as the slope of the linear regression in the calibration curve and it is measured at the same time in the linearity test.

The % C.V and in the Precision and accuracy of Efavirenz LLOQ was found to be **14.10**%, **17.26**%, and **15.94**%and the %nominal in the precision and accuracy for LLOQ was found to be **98.28**%, **81.22**%, and **102.53**% which where within acceptance criteria (Table 7). The % C.V and in the Precision and accuracy of Emtricitabine LLOQ was found be **18.54**%, **7.29**%, and **15.91**%and the %nominal in the precision and accuracy for LLOQ was found to be **104.88**%, **91.19**%, and **113.11**% which where within acceptance criteria (Table 8).

Acceptance criteria: The lowest standard in the calibration curve should be considered as lower limit of quantification of the method and the precision and

accuracy for LLOQ between batch and with in batch should be less than or equal to 20% respectively.

Selectivity

This was evaluated by injecting extracted blank plasma samples and comparing any interference with the response of the extracted LOQ samples processed with IS using proposed precipitation technique and chromatographic / mass spectrometric conditions. The results obtained were in acceptance criteria and interference peak at retention time of drug was found to be 0% and for internal standard it was found to be 0 % (Table 11, 12).

Acceptance criteria: The response of interference peak at the RT of analyte should be less than are equal to 20% of the mean area response of LLOQ standards.

For IS response of interference peak at the retention time of IS should be less than or equal to 5% of mean IS area response.

Accuracy

Accuracy is expressed in terms of %nominal and the values of % nominal obtained in precision and accuracy batch were with the acceptance criteria.

Acceptance criteria: The within batch and between batch accuracy for LQC, MQC and for HQC should be with in 85%to 115% and for LOQQC it should be within 80%to 120 % (Table 7-10).

Precision

Precision is expressed in terms of %C.V and the values obtained in precision and accuracy batch were with in acceptance criteria.

Acceptance criteria: The within batch and between batch precision for LQC, MQC and for HQC should be less than or equal to 15% and for LOQQC it should be within 20% (Table 7-10).

Matrix Effect

The results were obtained within acceptance criteria, % CV for Efavirenz was found to be 2.37% and % CV for Emtricitabine was found to be 2.52%. Similarly % CV for IS was found to be 2.39%. (Table 13,14).

Acceptance criteria: the matrix effect is nullified if the % C.V is less than or equal to 15%. At least 80% of all batches should meet the acceptance criteria.

Extraction Recovery

The mean recovery of Efavirenz for the low, medium and high QCs were 105.19%, 101.06% and 103.80% respectively. The mean recovery of Emtricitabine for the low, medium and high QCs were 90.05%, 88.15% and 89.17% respectively. The internal standard peak area of extracted quality control samples were compared to the internal standard peak area of aqueous quality control standards. Mean recovery for IS was 99.89%and the% CV was found to be 1.4% and the method meets the acceptance criteria (Table 15-17).

Acceptance criteria: The recovery for the analyte is acceptable if the % C.V for the extracted and unextracted standards at LQC, MQC, M1QC and at HQC is less than or equal to 15% and the % C.V between LQC, MQC, M1QC and HQC should be less than or equal to 20%.The recovery of IS is acceptable if the % C.V is less than or equal to 15% from extracted and un extracted samples.

Dilution Integrity

The accuracy of the samples Efavirenz for 2 fold and 4 fold dilutions of Drug were **88.32**% and **87.27**%respectively, Precision of the Drug for both the dilutions 2 fold and 4 fold was **2.15**% and **2.22**% respectively, which is within the acceptance criteria of ≤ 15%. The accuracy of the samples Emtricitabine for 2 fold and 4 fold dilutions of Drug were **90.21**% and **88.33**%respectively, Precision of the Drug for both the dilutions 2 fold and 4 fold was **2.59**% and **3.63**% respectively, which is within the acceptance criteria of ≤ 15%. The recalculated concentrations of these diluted samples agreed with their original nominal concentration, which proves the ability to dilute specimen in a linear fashion (Table 18,19).

Acceptance criteria: The precision and accuracy of the dilution integrity of QC samples should be less than or equal to 15% and 85% to 115% respectively.

Stability Evaluation

Stability of Efavirenz in plasma-Bench top stability

The stability of drug in plasma in bench top stability study is expressed in % change and %nominal and the nominal concentration of LQC was **75.4482**ng/ml and the mean concentration of stability samples was found to be 71.6894ng/ml. so the %nominal at LQC was found to be **95.02**% and the mean concentration of stability samples at HQC was found to be **4191.5664**ng/ml. so the %nominal at HQC was found to be **98.69**%, were within acceptance criteria (Table 20).

Stability of Emtricitabine in plasma-Bench top stability

The stability of drug in plasma in bench top stability study is expressed in % change and %nominal and the nominal concentration of LQC was **60.7571**ng/ml and the mean concentration of stability samples was found to be 58.3387ng/ml. so the %nominal at LQC was found to be **96.02**% and the mean concentration of stability samples at HQC was found to be **3471.8320**ng/ml. so the %nominal at HQC was found to be **89.10**%, were within acceptance criteria (Table 21).

Stability after Repeated Freezing / Thawing Cycles

The stability of Efavirenz in plasma in after repeated freezing and thawing stability study is expressed in % change and %nominal and the nominal concentration

of LQC was 75.4482 ng/ml and the mean concentration of stability samples was found to be after FT-4 was found to be 79.3745ng/ml. so the % nominal at LQC at FT-4 was found to be 105.20%. The nominal concentration of HQC was 4191.5664 ng/ml and the mean concentration of stability samples after FT-4 was found to be 3689.4934 ng/ml. So the %nominal at HQC was found to be 88.02%, which were within acceptance criteria (Table 22). The stability of Emtricitabine in plasma in after repeated freezing and thawing stability study is expressed in % change and %nominal and the nominal concentration of LQC was 60.7571ng/ml and the mean concentration of stability samples was found to be after FT-4 was found to be 55.9540ng/ml. so the % nominal at LQC at FT-4 was found to be 92.09%. The nominal concentration of HQC was 3471.8320ng/ml and the mean concentration of stability samples after FT-4 was found to be 3296.8325ng/ml. So the %nominal at HQC was found to be 94.96%, which were within acceptance criteria (Table 23).

Long Term Stock Solution Stability at 2-8 °C

The stability of Efavirenz and IS in the stock solution were examined at refrigerated condition for a period of 10 days .The % Stability of drug and IS after 10 days found to be 90.87% and 101.88 % respectively (Table 24).

The stability of Emtricitabine and IS in the stock solution were examined at refrigerated condition for a period of 10 days .The % Stability of drug and IS after 10 days found to be 93.08% and 101.43% respectively (Table 25).

Acceptance criteria: The mean response of stability samples of analyte and IS versus comparison samples should be within range of 90 to 110%.

Short Term Stock Solution Stability at Room Temperature

The stability of Emtricitabine, Efavirenz and IS in the stock solution examined at room temperature for a period of 6.0 Hrs. The % Stability of Efavirenz and IS after 6.0Hrs found to be 97.65% and 99.94% respectively which were within the acceptance criteria (Table 26). The % Stability of Emtricitabine and IS after 6.0Hrs found to be 98.32% and 93.66% respectively which were within the acceptance criteria (Table 27).

Acceptance criteria: The mean response of stability samples of analyte and IS versus comparison samples should be within range of 90 to 110%.

CONCLUSION

The developed and validated bioanalytical method for Efavirenz and Emtricitabine drugs shows satisfactory linearity, precision, accuracy and stability. The practical extraction procedure based on solid phase extraction technique for the specific drug used in the study provides a high and precise recovery from plasma .The method can be particular useful for pharmacokinetics and pharmacodynamics.

REFERENCES

1. MY Liu, SN Meng, HZ Wu, S Wang, MJ Wei. Pharmacokinetics of single-dose and multiple-dose memantine in healthy chinese volunteers using an analytic method of liquid chromatography-tandem mass spectrometry. *Clinical Therapeutics*, 30(4), 2008, 641–653.
2. Almeida AA, DR Campos, G Bernasconi et al. Determination of memantine in human plasma by liquid chromatography-electrospray tandem mass spectrometry: application to a bioequivalence study. *Journal of Chromatography B*, 848(2), 2007, 311-316.
3. Koeberle MJ, PM Hughes, CG Wilson and GG Skellern. Development of a liquid chromatography-mass spectrometric method for measuring the binding of memantine to different melanins. *Journal of Chromatography B*, 787(2), 2003, 313-322.
4. Jaiswal YS, Grampurohit ND, Bari SB. Recent trends in validation of Bio-anlatyical methods. *Analytical letters*, 40(13), 2007, 2497-2505.
5. Xu X, Lan J, Korfmacher W. Rapid bio-analytical method development using LC- MS/MS in drug discovery. *Analytical Chemistry*, 77, 2005, 389A-394A.
6. Ardrey RE, Ardrey Robert. Liquid chromatography-mass spectrometry: an introduction. London: J. Wiley. ISBN 0-471-49801-7: 2003.
7. Korfmacher WA. Principles and application of LC/MS in drug discovery. *Drug Discovery Today*, 10(20), 2005, 1357-1367.
8. Hsieh Y and Korfmacher WA. Increasing speed and throughput when using HPLC-MS/MS Systems for Drug Metabolism and Pharmacokinetic Screening. *Current Drug Metabolism*, 7, 2006, 479-489.
9. Schwartz Jae C, Michael W Senko, John EP Syka. A two-dimensional quadrupole ion trap mass spectrometer. *Journal of the American Society for Mass Spectrometry*, 13, 2002, 659-669.
10. Espinosa Boscha M, Ruiz Sanchez AJ, Sanchez Rojasc F, Bosch Ojedac B. Analytical methodologies for the determination of omeprazole: An overview. *Journal of Pharmaceutical and Biomedical Analysis*, 44, 2007, 831-844.