High Throughput LC-MS/MSMethod for the Quantitation of Efavirenz in Human Plasma by Solid Phase Extraction using 96 Well Plate Format

Lade Jyothi Rani[*1], Sangeetha.J ,R[2].V.ValliKumariTangudu[3] Nagabhusana Rao[4]

[*1,2,3] Mallareddy Institute of Pharmaceutical Sciences, Dhulapally, Secunderabad,India.

[4] India College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, Andhra Pradesh, India

E-Mail : ladejyothirani@gmail.com

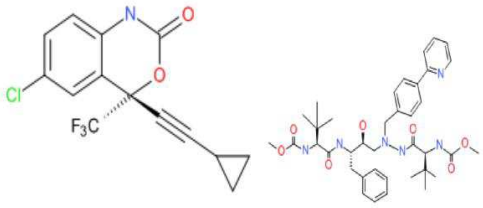
Abstract-

HighthroughputLiquidchromatographymassspectrometrymethodhasbeendevelopedandvalidatedforthequantificationofEfavirenzinhumanplasmausing Atazanavirasaninternalst andard (ISTD). Following solid phase extraction (SPE) in 96 well plate format, the analyte and ISTD were run on ACE C18 4.6 x75 mm (5.0 µm) column using an isocratic mobile phase consisting of Methanol:0.1% formic acid in water (90:10, v/v).Theprecursorandproductionsofthedrugswere monitoredonatriplequadrupoleinstrumentoperated inthepositiveionizationmode.Themethodwasvalidatedovera concentration range of4.999 to 5095.155ng/mLwithrelativerecoveriesrangingfrom 72.5to76.4%. The inter batch precision (%CV) across three validation runs was ≤ 5.8%. The Inter batch percent nominaldetermined at five QC levels (LLOQ, LQC, MQC II, MQC and HQC) was between98.1 to 101.0%. According to the validated results, the proposed method was found to be specific, accurate, precise and high throughput method and could be used for the estimation of Efavirenz in human plasma and can be applied for the routine analysis.

Key Words: Efavirenz, Atazanavir, Solid Phase Extraction and 96 well plates

INTRODUCTION

Efavirenz (EFV) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) which is used in the treatment of human immunodeficiency virus type 1 (HIV-1). It is generally used in combination with other drugs as a part of highly active antiretroviral therapy (HAART). EFV acts by inhibiting reverse transcriptase enzyme, which is essential for viral RNA to transcribe into DNA [1-2]. Several methods were available for its determination in biological matrices [3-7] using HPLC. However very few methods were reported for the determination of Efavirenz in plasma using Mass Spectrometry [8-12]. The aim of the current study was to develop a selective and sensitive LC-MS/MS method with relatively low run time for its application to a clinical study. As part of it developed an isocratic LC-MS/MS method with a LOQ of 5 ng/mL using atazanavir (ATZ) as internal standard. The method uses a highly selective solid phase extraction technique in 96 well plate format for sample preparation. Positive electrospray ionization with a mobile phase containing methanol: 0.1% formic acid in water (90:10, v/v) was used to detect the drugs. During method development more emphasis was given to establish a simple and fast processing method with minimal matrix effects. No cross interference was observed between the analyte and ISTD. The method utilizes a processing volume of 25 µL and injection volume of 5 µL. The chromatographic run time was 3.2 min.[1]



Structure of EfavirenzStructure of Atazanavir

MATERIALS AND METHODS

2.1 Chemicals and reagents

Working standards of Efavirenz andAtazanavirwere obtained from USP. LC– MS grade methanol was obtained from Thermo Fisher Scientific India Pvt. Ltd. (Mumbai, India). Formic acid was pro-cured from Merck Specialties Pvt. Ltd. (Mumbai, India). HPLC water was obtained from Milli-Q water purification system (Millipore). Human plasma containing K_2 EDTA anticoagulant was obtained from Doctor's pathological lab (Hyderabad, India). Strata-X 30 mg 96 well plates were obtained from Phenominex (USA).

2.2 Instrumentation

Agilent 1200 Series equipped with a binary pump for solvent delivery was used for the analysis. Mass spectrometric detection was performed on API-4000 triple quadrupole mass spectrometer (MDS SCIEX, Toronto, Canada) equipped with turbo ion spray inter-face. Quantitation was performed in multiple reaction monitoring (MRM) mode and Analyst software (SCIEX) was used for controlling the hardware and data handling.

2.3 Chromatographic conditions

Chromatographic separation was performed on ACE C18 4.6 x75 mm (5.0 µm), analytical column. Isocratic mobile phase consisting of Methanol : 0.1% formic acid in water (90:10, v/v) was delivered at a flow rate of 0.7 mL/min. The auto sampler was set at 4□C±2□C and the injection volume was 5 µL. The column oven temperature was set at35.0 ± 2.0°C. Retention Time of Efavirenz was 2.2 min and Atazanavirwas 2.2. The total chromatographic run time was 3.2 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

Parameters	Q1 (amu)	Q3 (amu)	Dwell	DP	CE	CXP	EP
Efavirenz	316.6	244.3	200	42	24	16	10
Atazanavir	705.6	335.3	200	51	33	14	10

2.4.4 Source/ Gas parameters

Parameters	CUR (psi)	GS1 (psi)	GS2 (psi)	IS (Volts)	CAD (psi)	TEMP (°C)
Source/Gas	9	45	44	5200	6	450

2.5 Preparation of stock solutions and working solutions

EFV and ISTD stock solutions were prepared at 1-10 °C.The working solutions of EFV were prepared in 50% methanol using the stock solution. Internal standard working solution (containing 500 ng/mL ATZ) was also prepared in 50% methanol and used in the assay. The prepared working solutions were stored at 1-10 °C. All the volumetric measurements were made using calibrated micropipettes.

2.6 Preparation of calibration standards and quality control samples

Calibration standards and quality control (QC) samples were prepared by spiking blank plasma (5%) with working solutions.

plasma volume to avoid unintended changes in sample matrix. Blank plasma lots obtained from healthy volunteers were individually screened and pooled before use. Calibration standards for EFVwere made at concentrations of 4.999, 9.998, 24.995, 52.874, 105.749, 254.758, 504.709, 1057.485, 2018.835, 4085.738 and 5095.155 ng/mL. Quality control samples were prepared at 4.999 ng/mL (LLOQ QC), 14.420 ng/mL (LQC), 403.767 ng/mL (MQC

II), 2499.510 ng/mL (MQC) and 3845.400 ng/mL (HQC). LLOQ QC samples were prepared only during validation batch runs.[2-3]

Sample extraction p rocedure

Calibration standa rds, QC's were processed using Ezypress Positive Pressure SPE Manifold by using 2 5 µL of Plasma Volume

For CC and QC spike 5 µL of each working solutions of Didanosine into 95 µL of human p lasma. Aliquot 25 µL of spiked plas ma for CC's and QC's preparation.

Add 10 µL of intern al standard (1000 ng/mL ATZ) to each tube except for blank plasma samples.

Add 10 µL of 5 0:50v/v MeoH: Water to blank plas ma samples

Add 25 µL of 2% formic acid solution to each tub e and vortex.

Condition the Stra ta-X 30 mg 96 well plate (Phenomenex, USA) with 1000µL of metha nol followed by 1000 µL of Milli Q water.

Load the samples on to the cartridge.

Wash the cartridge with 1000 µL of Milli Q water

Elute the sample with 200 µL of mobile phase into 9 6 well collection plate.

Inject 5 ∝L of the sample onto the LC-MS/MSsyst em.[4-5]

RESULTS AND DISCUSSION

3.1 Method develop ment

Selection of internal standard is highly important in the development of reliable and precise analytical met hod.ATZ has shown consistent recoveries along with analyte and more importantly, its chromatographic behavior was almost similar with analyte (with same RT of 2.2 min.) and resulted in minimal matrix suppressions.

Method development was initiated with scanning of the compo unds for parent and product ions to perfor m multiple reaction monitoring. 250 ng/m L solutions of EFV and ATZ were separately prepared in 50% methanol and are infu sed using a syringe pump at a rate of 10 µ L/min. Based on their ability to accept the protons, analyte and ISTD was tuned in positive mode using electrospray ionization technique. The [M+H]+ peaks were observed at m/z of 316.4 for EFV and at 705.6 for ATZ. The abundant product ions were found at m/z of 244.3 for EFV and at 335.3 for ATZ (Figure 4.1), by applying a ppropriate collision energy[6-7]

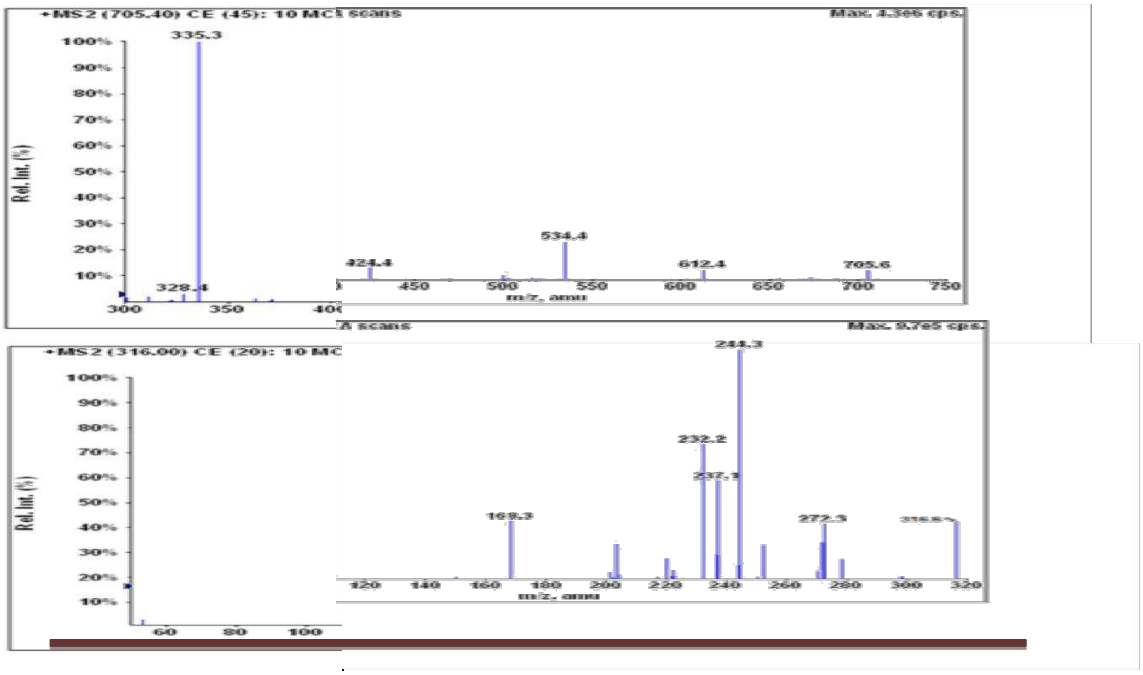


Figure 1: Product ion spectra of EFV (A) and ATZ (B, ISTD)

LC conditions for analyte and ISTD were set under isocratic mode. Optimization of mobile phase was initiated with methanol and milli-Q water. Replacement of milli-Q water with 0.1% formic acid in water has augmented the analyte response by 200%, however no significant increase was identified in ATZ response. Incorporation of buffer even at 1 mM concentration has shown a negative impact on signal intensities. Use of methanol in mobile phase has provided comparatively high and consistent response ratios for analyte and ISTD. During the optimization of stationary phase Zodiac C18 (100 X 3.0, 3 µm), Oyster ODS3 (100 X 4.6, 5 µm) and ACE C18 4.6 x75 mm (5.0 µm) columns were verified. ACE C18 4.6 x75 mm (5.0 µm) has given a more selective chromatography with symmetrical peaks. C8 columns have produced as highly tailed chromatographic peaks with retention time at 2.0 min. A flow rate of 0.7mL/min was used to minimize the run time.

Inspite of high partition coefficient values for analyte and ISTD, liquid-liquid extraction and precipitation techniques were avoided to eliminate unintended matrix related variations during study analysis. A solid-phase extraction procedure was employed out using Strata-X 30 mg 96 well plates. Strata-X is a polymeric based sorbent with both hydrophilic and lipophilic sites and which can provide consistent results for polar as well as non-polar compounds. Extraction procedure was initiated with a default protocol using 1 mL methanol and milli-Q water in conditioning step and 1 mL of water in washing step. The final elution of compounds was made using mobile phase.

The elution was initially performed with acetonitrile followed by evaporation and reconstitution with mobile phase, however it is later replaced with direct elution to minimise the processing time. Use of 2 X 1 mL milli-Q water wash and 1 min. drying of the cartridges before elution has given clean sample extracts and direct elution does not reduced the column performance.[8-10]

Method validation

Validation runs were conducted on separate days.Each precision and accuracy validation run was executed with a set of spiked calibration standard samples distributed at eleven concentration levels over the dynamic range, a blank (without ISTD), a zero sample (blank with ISTD) and QC samples (n=6 at each of four concentration levels; LLOQ, low, medium and high).

Standard samples were analyzed at the beginning of each validation run and other samples were distributed randomly throughout the run. Results of the QC samples from three intra and inter day runs were used to evaluate the accuracy and precision of the method. Sensitivity (at lower limit of quantification), dilution integrity (two, ten and fifty) and ruggedness of the method were also determined. System suitability test was performed before each validation or study analytical run to verify the system performance. As part of it, six replicate injections of system suitability standard (containing analyte at ULOQ with ISTD) were made and results are considered acceptable if the %CV for response ratios was ≤ 4.0.[11]

Selectivity

Method selectivity was evaluated by analyzing human K_2 EDTA plasma blank matrix from ten different individual matrix lots along with one lipemic and one hemolytic plasma lot. Peak responses in blank lots were compared against the mean response of spiked LLOQ samples (n = 6) and negligible interferences were observed in all the screened lots, at the retention time of analyte and ISTD. Figure(s) 2, 3 and 4 illustrate the selectivity of the method with the chromatograms of blank plasma, blank plasma with internal standard and LLOQ sample respectively.

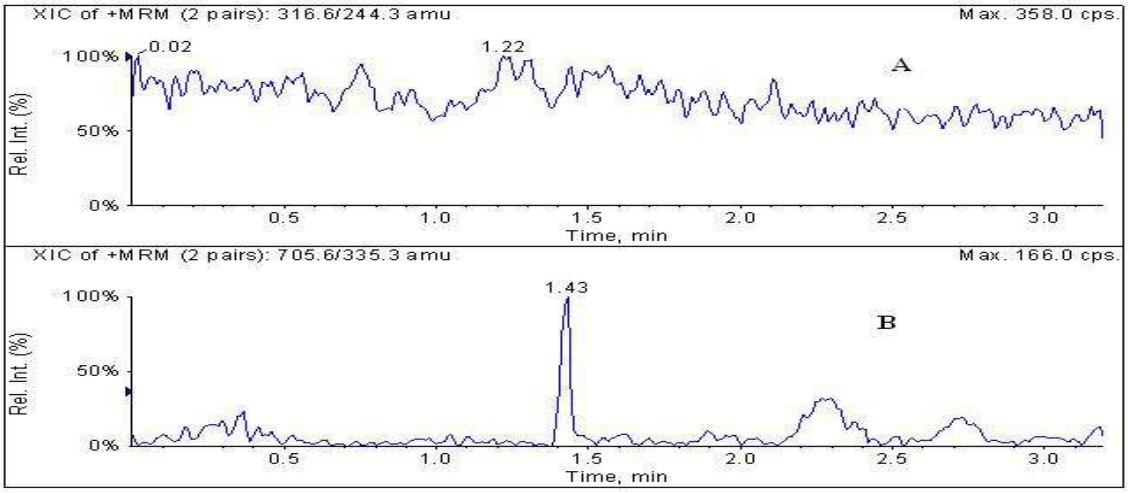


Figure 2: Representative chromatogram of EFV (A) and ATZ (B) in blank plasma

Sensitivity

The lower limit of quantification (LLOQ) is defined as the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. This was demonstrated by injecting six replicates of extracted LLOQ samples against a calibration curve. The lower limit of quantification (LLOQ) was found to be 4.999 ng/mL. At LLOQ accuracy (% Nominal) for EFV was ranged from 95.4 to 101.8%, with a %CV of ≤ 6.6.

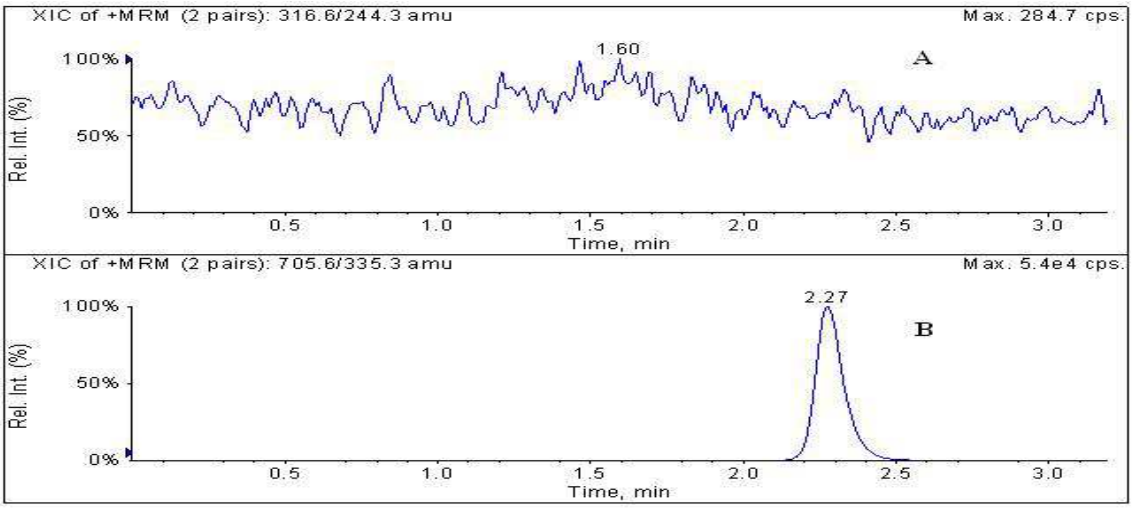


Figure 3: Representative chromatogram of EFV (A) and ATZ (B) in blank plasma with internal standard

Linearity

Calibration curves were linear from 4.999 to 5095.155 ng/mL with correlation coefficient (r) values more than 0.9996. The r values, slopes and intercepts were calculated using weighted ($1/X^2$) linear regression analysis with three intraday and inter day calibration curves. The observed mean back calculated concentrations with accuracy and precision (%CV) from 3 linearity curves were given in Table 1.

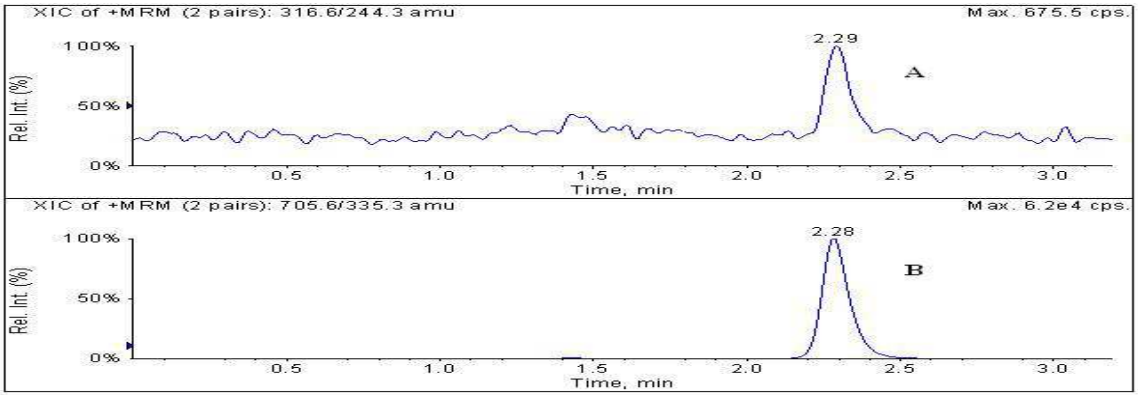


Figure 4: Representative chromatogram of EFV (A) and ATZ in LLOQ sample

Table 1: Summary of calibration standards

	Nominal conc. (ng/mL)	Mean found conc.a	%CV	%Nom
Efavirenz	4.999	5.0570	1.2	101.2
	9.998	9.7257	3.1	97.3
	24.995	24.9703	2.4	99.9
	52.874	52.874	1.3	101.7
	105.749	105.749	1.3	101.9
	254.758	254.758	2.2	98.5
	504.709	504.709	0.8	101.2
	1057.485	1057.485	2.4	99.5
	2018.835	2018.835	2.1	97.4
	4085.738	4085.738	0.9	100.3
	5095.155	5095.155	3.0	102.1
%Nom - Percent Nominal				
%CV - Percent coefficient of variation				
a - Mean of 3 replicates at each concentration				

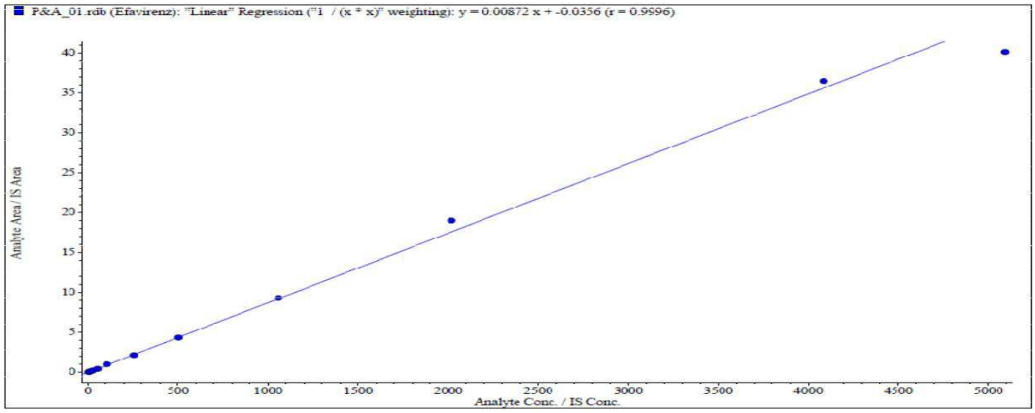


Figure 5: Calibration Curve of Efavirenz

Precision and accuracy

The precision of a method is defined as the closeness of replicate determinations of an analyte by an assay and accuracy is the closeness of determined value to the true value. Each precision and accuracy run consisting of QC samples (6 replicates each of the LLOQC, LQC, MQC II, MQC and HQC) were analyzed and back calculated against a set of calibration curve standards. Intra and inter batch precision and accuracy evaluation was done using four different batches analyzed on different days. Results of intra and inter batch accuracy and precision were shown in Table 2.

Table 2: Intra batch and inter batch precision and accuracy

Analyte	QC Level	Nominal Conc. (ng/mL)	Intra batch			Inter batch		
			Mean found conc .a (ng/mL)	% Nom	% CV	Mean found conc. b (ng/mL)	% Nom	% CV
Efavirenz	LLOQ	4.999	5.0902	101.8	6.6	4.9044	98.1	5.8
	LQC	14.420	14.4948	100.5	4.6	14.5635	101.0	4.3
	MQC II	403.767	405.7673	100.5	1.8	402.5061	99.7	2.1
	MQC	2499.51	2485.6662	99.4	2.2	2449.0624	98.0	3.3
	HQC	3845.40	3732.6320	97.1	2.5	3780.6173	98.3	2.3
a - Mean of 6 replicates at each				b - Mean of 18 replicates at each concentration				
%Nom – Percent Nominal				%CV - Percent coefficient of variation				

Matrix effect

The matrix effect was investigated by extracting blank plasma from six different sources, including one hemolytic and one lipemic lot. After extraction, residue from each blank lot was reconstituted with mobile phase having known amount of analyte (at LQC and HQC level along with ISTD; post extracted samples) and analyzed along with equivalent aqueous samples. Response ratio obtained from each of the post extracted matrix lot was compared against the mean response ratio of aqueous samples. Further matrix factor for analyte / ISTD was calculated by comparing the peak response in presence of matrix ions to that of peak

response in absence of matrix ions. The IS normalized matrix factor for EFV at both low and high QC level was ranged between 0.9772 to 1.0111 with a % CV of ≤ 3.4, as shown in Table 3.

Table 3: Matrix effect results

LQC level			
	MF for analyte	MF for ISTD	IS normalized MF
Lot 1	1.011	1.005	1.006
Lot 2	0.998	0.980	1.019
Lot 3	0.952	0.998	0.955
Lot 4	1.004	0.964	1.042
Lot 5	1.023	0.974	1.051
Lot 6	0.959	0.963	0.995
Mean			1.0111
%CV			3.4
HQC level			
	MF for analyte	MF for ISTD	IS normalized MF
Lot 1	0.984	1.018	0.967
Lot 2	0.994	1.017	0.978
Lot 3	0.983	1.008	0.975
Lot 4	0.994	1.015	0.980
Lot 5	1.006	1.017	0.989
Lot 6	0.996	1.023	0.974
Mean			0.9772
%CV			0.8
MF - Matrix factor		%CV - Percent coefficient of variation	

Extraction recovery

Recovery 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 of the analyte need not to be 100%, but the extent of recovery of analyte and internal standard should be consistent, precise and reproducible. The extraction recovery of EFV was determined (at low, medium II, medium and high concentration) by comparing the responses from plasma samples spiked before extraction (n=6) with those from plasma samples extracted and spiked after extraction. The mean recovery of EFV was found to be 74.4% with %CV of 2.4 across the four levels, as given in Table 4.

Table 4: Extraction recovery for Efavirenz

Analyte	QC Level	A	B	% Recovery	%Mean	% CV
Efavirenz	LQC	13854	18381	75.4	74.44	2.4
	MQC II	359941	496566	72.5		
	MQC	1923154	251713	76.4		
	HQC	2815642	382973	73.5		
Extraction recovery for internal standards						
Analyte		A	B	%Recovery		
Atazanavir		2343672	2812662	83.3		
A - Mean response in extraction samples						
B - Mean response in post extraction spiked samples						

Dilution integrity

Dilution integrity of the method was evaluated after 1/2 and 1/5 dilution. The mean back calculated concentrations for 1/2 and 1/5 dilution samples were within 85-115% of their nominal concentration with a %CV of ≤3.4. The results of dilution integrity were depicted in Table 5.

Table 5: Results of dilution integrity

Analyte	Dilution Factor	Nominal conc.	Mean found conc.[a] (ng/mL)	%Nom	%CV
Efavirenz	2	15352.020	15604.3722	100.6	3.4
	5		17242.2928	112.3	2.7

a – Mean of 6 replicates at each dilution factor / concentration ; %CV – Percent coefficient of variation; %Nom – Percent Nominal

Ruggedness

Six samples each of LLOQ, LQC, MQC II, MQC and HQC in human plasma were analyzed along with CC samples. The ruggedness was assessed by changing analyst, solvent lot and different column of same make. Results are summarized in Table 6. The data obtained were within acceptance criteria.

Table 6: Ruggedness

Analyte	QC Level	% Nominal	% CV
Efavirenz	LLOQ QC	95.2	8.8
	LQC	100.8	3.3
	MQC II	99.0	2.5
	MQC	97.1	1.8
	HQC	99.0	2.0

N = 6 at each level

Extended Precision and Accuracy Batch

The Extended Precision and Accuracy batch was assessed by processing and analyzing CC samples along with 38 samples each of LQC, MQC II, MQC and HQC in human plasma. Calibration standards were used to determine the accuracy of quality control samples. The batch of 152 samples analysed was found to acceptable for the maximum no of sample that can be processed during routine sample analysis. Results are summarized in Table 7. The data obtained were within acceptance criteria.[12]

Table 7: Extended PA Batch

Analyte	QC Level	% Nominal	% CV
Efavirenz	LQC	101.8	13.1
	MQC II	93.7	5.0
	MQC	91.1	3.0
	HQC	94.0	6.7

N = 38 at each level

Reinjection reproducibility was performed by injecting the previously passed precision and accuracy batch after a period of 70 hr 33 min. The reinjected quality control samples concentrations were back calculated against initially injected CC curve. The % CV of back calculated concentrations for all quality control samples of LQC, MQC II, MQC and HQC concentration levels ranged from 2.2 to 4.6, which are within the acceptance limit of 15.00%. The % mean accuracy of back calculated concentrations for all quality control samples at LQC, MQC II, MQC and HQC concentration levels were ranged from 98.3 to 106.4 which is within acceptance limit 85.00–115.00%.

Stability

Stability of analyte in both solutions and in human plasma was evaluated after subjecting to different conditions and temperatures that could encounter during regular analysis. All the stability assessments were made at LQC and HQC level (n=6) by comparing the stability samples against freshly prepared samples (comparison samples).

Stability in aqueous solutions

Stability of analyte and ISTD in stock solutions and in working solutions was assessed at room temperature (short-term stability) and at 1-10 °C (long-term stability). The stock solutions were prepared in methanol and the working solutions were prepared in 50% methanol and all comparisons were made against freshly prepared stock solutions or working solutions. The stock and working solutions were stable up to 6.0 hrs at room temperature and up to 25 days at 1-10 °C

Stability in biological matrix

Demonstration of analytestability in matrix should mimic the conditionsunder which the study samples were collected, stored, processed and analyzed. These establishments include bench-top stability, freeze-thaw stability, long-term stability to assess the processing and storage conditions. In addition the stability in processed samples was also demonstrated using in-injector stability evaluations. All matrix stability evaluations were performed using freshly prepared calibration standards and quality control samples (comparison samples).

Freeze and thaw stability

From a practical standpoint, it is often necessary to expose room temperature. The first freeze cycle was

made for 24 hrs followed by 12 hrs in the subsequent cycles. After subjecting to 8 freeze-thaw cycles, six replicates each of low and high quality control stability samples were processed and analyzed along with freshly spiked calibration standards and quality control samples (comparison

and the %stability was computed by comparing the mean concentration of stability samples against the mean
concentration of comparison samples

Bench-top stability

Bench-top stability was evaluated to confirm that analyte After a period of 26 hrs 25 min the stability

samples were processed and analyzed along with freshly spiked calibration standards and quality control samples (comparison samples). The concentration of stability and comparison samples were back calculated and the %stability was computed by comparing the mean concentration of stability samples against the mean concentration of comparison samples.

In-injector stability

Stability of processed samples in the instrument need to b quality control samples (comparison

samples). The concentration of stability and comparison samples were back calculated and the %stability was computed by comparing the mean concentration of stability samples against the mean concentration of comparison samples.

Long-term stability

Long-term storage stability was assessed to confirm analy standards and quality control samples (comparison samples). The concentration ofstability and comparison samples
were back

calculated and the % stability was assessed by comparing the mean concentration of stability samples against the mean concentration of comparison samples.

Whole Blood Stability

The whole blood stability was evaluated by comparing the response ratios of stability samples (n=6 at LQC and HQC) against the freshly prepared samples. After spiking in whole blood the samples were placed at room temperature over a period and plasma was separated by centrifugation at 3000 rpm (along with freshly spiked samples, comparison samples) and then processed and analyzed as per established conditions. EFV was stable at room temperature up to 2 hrs and the %change at low and high QC levels was found to be 1.5 and 3.1 respectively.

The stability evaluations in matrix have comfortably met t [13-15]

Table 8: Stability results for Efavirenz

Stability	QC Level	A	% CV	B	% CV	% Change
Bench-top (26 hrs 25 min at ~25 °C)	LQC	15.5953	3.6	14.9760	5.9	4.1
	HQC	4048.8998	2.9	4083.9373	5.2	-0.9
Freeze-thaw (8th cycle)	LQC	12.4192	4.8	13.4005	4.9	-7.3
	HQC	3614.3335	1.1	3808.5518	2.5	-5.1
In-injector(at 4 °C for 102 hrs 35 min)	LQC	14.3140	3.0	14.4232	1.9	-0.8
	HQC	3745.4280	2.0	3817.2032	1.6	-1.9
Long-term stability(at -20 °C for 90 days)	LQC	14.7145	5.5	15.0823	3.0	-2.4
	HQC	3833.8193	1.9	3783.1157	2.8	1.3
Long-term stability(at -70 °C for 90 days)	LQC	14.3983	4.8	15.0823	3.0	-4.5
	HQC	3864.6050	2.5	3783.1157	2.8	2.2
A - Mean concentration (ng/mL) of stability samples						
B - Mean of concentration (ng/mL) comparison samples						

CONCLUSION

A simple, rapid and rugged LC–MS/MS method was developed and validated for the determination EFV in human K_2 EDTA plasma. The method validation was performed as per USFDA and EMA guidelines and has comfortably met the acceptance criteria. The current method was a highly selective and high throughput solid phase extraction technique for sample processing and isocratic mobile phase for chromatography. The method has added advantage of low sample processing volume, low processing time and uses direct sample injection without evaporation after solid phase extraction. The assay can be used to support multiple clinical studies and regulatory approvals

.

REFERENCES

[1]. Vrouenraets SM, Wit FW, Van Tongue J and Lange JM (2007). Efavirenz: a review. Expert Opin Pharmacother. 8(6):851-71.

[2]. Joy YF., John KL., Florence M., Derrick G., Kirsten LW., Evguenia SS., Katyna BE. and Michael DM. (2009). The triple combination of tenofovir, emtricitabine and efavirenz shows synergistic anti-HIV-1 activity in vitro: a mechanism of action study. Retrovirology. 6:44.

[3]. Lakshmi SA., Kishore KK., Ravi KDVR., Mohan KC., Yugandhar NM. and Srinubabu G. (2007). Development and validation of a liquid chromatographic method for determination of efavirenz in human plasma. Chromatographia. 65(5): 359-361.

[4]. Matthews CZ., Woolf EJ., Mazenko RS., Haddix-Wiener H., Chavez-Eng CM., Constanzer ML., Doss GA. and Matuszewski BK. (2002). Determination of efavirenz, a selective non-nucleoside reverse transcriptase inhibitor, in human plasma using HPLC with post-column photochemical derivatization and fluorescence detection. J Pharm Biomed Anal. 28(5): 925-934.

[5]. Geetha Ramachandran, A.K. Hemanth Kumara, Soumya Swaminathan, P. Venkatesan, V. Kumaraswami and David J. Greenblat (2006). Simple and rapid liquid chromatography method for determination of efavirenz in plasma. Journal of Chromatography B. 835: 131–135.

[6]. Marıa Sarasa-Nacenta, Yolanda Lopez-Pu, Luis F. Lopez-Corte, Josep Mallolas,, Jose M. Gatell and Xavier Carne (2001). Determination of efavirenz in human plasma by high-performance liquid chromatography with ultraviolet detection. Journal of Chromatography B. 763:53–59.

[7]. Benjamin U. Ebeshi, Oluseye O. Bolaji, Margaret Oluka, Vaikosen N. Edebi, Julius O. Soyinka and Anastasia Guantai (2014). Simple Reversed-Phase High Performance Liquid Chromatographic Estimation of the Antiretroviral Agent Efavirenz from Human Plasma. British Journal of Pharmaceutical Research. 4(1): 145-157.

[8]. Supriya NH., Ashish AM. and Meena C.
   a. (2011). Tandem mass spectrometric estimation of Efavirenz from human plasma and comparison of various statistical approaches for evaluation of incurred sample reanalysis. Annals of Biological Research. 2(4): 126-135.

[9]. Theron A., Cromarty D., Rheeders M. and Viljoen MJ. (2010). Determination of salivary efavirenz by liquid chromatography coupled

[10]. with tandem mass spectrometry. Chromatogr B Analyt Technol Biomed Life Sci. 878(28): 2886-2890.

[11]. Danilo Cesar Galindo Bedor, Jose Homero de Souza Filho, Virna Ligiane Souza Ramos, Talita Mota Gonçalves et al. (2011). A sensitive and robust LC-MS/MS method with monolithic column and electrospray ionization for the quantitation of efavirenz in human plasma: Application to a bioequivalence study. Quim Nova. 34(6): 950-955.

[12]. Kwon-Bok K., Hyunmi K., Fen J., Chang-Woo Y., Soo KB., Zeruesenay D., Jae-Gook S. and Kwang-HL. (2011). Rapid and simultaneous determination of efavirenz, 8-Hydroxyefavirenz, and 8,14-Dihydroxyefavirenz using LC–MS–MS in human plasma and application to pharmacokinetics in healthy volunteers. Chromatographia. 73(3): 263-271.

[13]. Praveen Srivastava, Ganesh S. Moorthy, Robert Gross, Jeffrey S. Barrett. (2013). A Sensitive and Selective Liquid Chromatography/Tandem Mass Spectrometry Method for Quantitative Analysis of Efavirenz in Human Plasma. Plos One. 8(6):1-9.

[14]. Guidance for Industry – Bioanalytical Method Validation (CDER, FDA), 2001.

[15]. EMEA Guideline on Bioanalytical Method Validation, 2011.