IJBPAS, September, Special Issue, 2021, 10(9): 748-760

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

AN LC-MS/MS METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF FOSAMPRENAVIR IN BIOLOGICAL SAMPLES

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Received 25th June 2021; Revised 28th July 2021; Accepted 29th Aug. 2021; Available online 25th Sept. 2021

https://doi.org/10.31032/IJBPAS/2021/10.9.1057

ABSTRACT

The main aim of the present work was to develop a sensitive liquid chromatography-electrospray ionization tandem mass spectrometric technique for the quantitation of fosamprenavir in biological matrices. Chromatographic elution was attained thru a Phenomenex C_{18} stationary column having the dimensions of 50 × 4.6 mm and particle size of 5 µm. Isocratic elution was processed with 0.1%V/V formic acid and methanol in the ratio of 25:75 V/V as mobile phase with flow rate of 0.70 ml/min. Liquid-liquid extraction was performed for drug and internal standard isolation with an ethyl acetate and methanol (4:1) solvent system. Parent and product ions were monitored at m/z 586.19→57.07 for fosamprenavir and 420.1→136.0 for dolutegravir internal standard on multiple reaction monitoring. Linearity graph of drug was rectilinear in concentration over 1.0. to 2100.0 ng/ml having r^2(correlation coefficient) value more than 0.999. Percentage of RSD findings were ≤3.081% for inter and intraday accuracy and precision. This procedure has good recoveries and %recovery findings of lower quality control (LQC), median quality control (MQC) and higher quality control(HQC) samples were 97.35%, 98.61% and 102.65% respectively. Fosamprenavir has more stability for longer time when subjected for different stability environments and the technique was effectively relevant to routine analysis of fosamprenavir in biological matrix.

Keywords: HIV, Fosamprenavir , LC-MS/MS, FDA guidelines, Accuracy, Precision

INTRODUCTION

Fosamprenavir is an antiretroviral agent used for the treatment and postexposure prophylaxis of human immunodeficiency virus (HIV-1) infection. Fosamprenavir is a prodrug that is rapidly hydrolyzed to amprenavir by cellular phosphatases in the gut epithelium as it is absorbed **[1-3]**. Amprenavir is an inhibitor of HIV-1 protease. During HIV replication, HIV protease cleaves viral polypeptide products of the Gag and Gag-Pol genes to form structural proteins of the virion core and essential viral enzymes. Amprenavir interferes with this process by binding to the active site of HIV-1 protease, thereby preventing the processing of viral Gag and Gag-Pol polyprotein precursors, resulting in the formation of immature non-infectious viral particles **[4-6]**. Fosamprenavir is hydrolyzed by cellular phosphatases to the antiretroviral protease inhibitor amprenavir. This hydrolysis allows for the slow release of amprenavir, reducing the number of pills a patient must take. In the gut epithelium during absorption, fosamprenavir is rapidly and almost completely hydrolyzed to amprenavir and inorganic phosphate prior to reaching the systemic circulation. Amprenavir is metabolized in the liver by the cytochrome P450 3A4 (CYP3A4) enzyme system. Excretion of unchanged amprenavir in urine and feces is minimal. Chemically fosamprenavir designated as {[(2*R*,3*S*)-1-[*N*-(2-methylpropyl)(4-aminobenzene) sulfonamido]-3-({[(3*S*)-oxolan-3-yloxy]carbonyl}amino)-4-phenylbutan-2-yl]oxy} phosphonic acid with a molecular weight and formula of 585.608 **[5-8]** and $C_{25}H_{36}N_3O_9PS$ **(Figure 1)**.

Literature on fosamprenavir reveals that, one dissolution method **[9]**, electrochemical evaluation method **[10]**, ultraviolet, high-performance thin-layer chromatography **[11]**, and stability indicating high-performance liquid chromatography (HPLC) method **[12, 13]** and HPLC method for the estimation of drug from pharmaceutical formulations **[14–19]**. The development of specific method like LC-MS/MS is highly essential for the quantification of fosamprenavir in biological matrices.

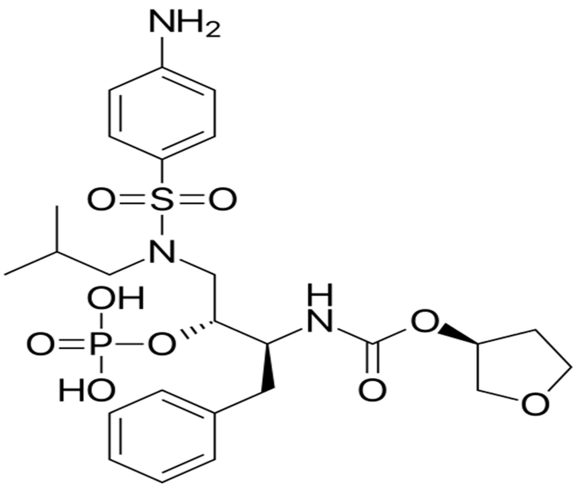

Figure 1: Structure of Fosamprenavir

MATERIALS AND METHODS

Chemicals and reagents

Fosamprenavir (99.87% purity) was acquired from Hetero drugs, Hyderabad, India. Dolutegravir (Internal standard) **o**f 99.92% was received from MSN Labs, Hyderabad, India. Methanol of HPLC-grade and formic acid of analytical grade were procured from J.T.Baker, Hyderabad, India. Water of Milli-Q was utilized during the processing of movable phase and washing solvents, acquired from in-built Milli-Q®RO system.

Liquid chromatographic-MS/MS system

An LC-MS/MS SCIEX API4000 instrument furnished with a positive Electro-spray ionization (ESI) source and Shimadzu prominence HPLC comprising of SIL-HTC autosampler, binary pumps and column oven was utilized in the present work. Analyte quantitation, acquisition of data and it's integration were processed by utilizing analyst software-1.6.3 version.

Liquid chromatography

Chromatographic elution was attained on a Phenomenex C_{18} stationary column having the dimensions of 50 × 4.6 mm and particle size of 5 µm. Column oven temperature was monitored at 30.0 °C and 0.7 mL/min flow rate. Injection volume was 5 µL. Isocratic elution was processed with 0.1%V/V formic acid and methanol in the ratio of 25:75 V/V as mobile phase. Fosamprenavir and dolutegravir internal standard were separated in the total runtime of 3 minutes. The autosampler temperature and analytical Column temperatures were kept at 5.0 °C and 30.0 °C respectively.

Mass system conditions

The mass spectrometer was operated under multiple reactions monitoring (MRM) mode in positive ion mode for both Fosamprenavir and internal standard (IS). The adjusted mass system parameters for Fosamprenavir and IS were: both of drying gas and sheath gas temperatures were 400 °C. Nebulizer pressure was monitored at 30.0 psi. The sheath gas and drying gas flow rates were 10.0 L/min and 5.0 L/min, respectively. Capillary voltage was set at 2.5.0 kV. Collision energy and fragmentor voltage were 15 eV and 115 V for Fosamprenavir, 15 eV and 105 V for IS. The transitions (precursor to product) monitored were m/z 586.19 → 57.07 for Fosamprenavir and 420.1 →136.0 for IS. The dwell time was 200 ms for each transition.

Calibration standards

A stock solution of 1mg/mL solution of fosamprenavir was prepared freshly by dissolving 100mg of drug in 100mL of mobile phase. Calibration standards of eight different concentration levels were processed by spiking blank plasma with fosamprenavir standard solution to get the concentrations of 1.0, 3.0, 60.0, 180.0, 480.0, 950.0, 1500.0 and 2100.0ng/mL.

Quality Control Standards

These solutions were prepared at three different levels of lowest quality control(LQC), standards, median quality control(MQC) standards and highest quality control(HQC) standards. These quality control(QC) samples were prepared according to calibration standards to get the concentration of 3.0, 950.0 and 1500 ng/mL for LQC, MQC and HQC respectively. Prepared solutions were store at -20^{0}c till the time of analysis.

Sample Preparation Method

Sample solution was prepared by transferring 200 µl of plasma and 100µl of IS (1µg/mL) in to a polypropylene tube and vortexed for 2 min. Fosamprenavir and IS were extracted with 4mL of ethyl acetate and acetonitrile (3:2) as solvent system followed by centrifugation of the solution at 5000 rpm/min for 25min. After centrifugation, organic phase was separated and it was dried using lyophiliser. The resulting product was made solubilize in 250µl of mobile phase and then it transferred into a pre-labeled vials. The vials were kept in an auto-sampler and infused into LC-MS/MS system.

Method validation

The developed method was validated for the parameters: specificity, selectivity, linearity, stability, matrix effect, recovery, precision and accuracy **[20, 21]**.

RESULTS

Mass system conditions

For the mass determination, we applied the MRM in positive mode with fosamprenavir to achieve the higher sensitivity and better specificity. ESI was likely to be the best source of ionization for LC–MS/MS and the precursor ions and product ions were ascertained by infusing the standard solutions into the mass instrument through a syringe pump. The product ions mass spectra of fosamprenavir and IS were obtained at m/z 57.07 and 136.0, which were selected as detecting ions. Meanwhile, the parameters of mass spectrum environments (capillary voltage, ION-spray oltage and temperature, heater gas, nebulizer gas, collision gas and curtain gas and so on) were optimized to acquire higher mass response.

Internal standard selection

In the present study, dolutegravir was selected as the IS because it had similar chromatographic behaviors, ionization, extraction efficiency and retention action as the fosamprenavir and there was no obvious interferences found at the retention times of analytes and IS according to the method validation results.

Method validation

Specificity

Blank plasma samples obtained from six different batches of human plasma was spiked with the fosamprenavir at LLOQ and IS to assess the specificity. As **Figure 2** showed, the retention times of fosamprenavir and IS were found to be 2.5 and 1.18 min, respectively. There was no noticeable intrusion of matrix substance and internal standard on fosamprenavir analytical data, and the response of all interfering peaks were less than 20% of LLOQ samples **[21, 22]**.

Sensitivity and linearity

The method linearity was established for fosamprenavir and excellent linearity was exhibited in the concentration range of 1.0 to 2100.0 ng/ml **(Table 1)**. The linearity graphs were produced by the peak area ratios (y) of the fosamprenavir to the IS versus the concentrations (x) with the $1/C^2$ weighting factor. The developed method linearity equation of the calibration graphs was $y = 0.0021x - 0.002$ with a correlation coefficient (r^2) value of 0.9997. The LLOQ of fosamprenavir was 1.0 ng/ml (signal-to-noise ratio > 10) assessed by 5 replicate sample solutions which were adequate for reliable quantitation of fosamprenavir in the plasma samples study **[22-25]**.

Accuracy and precision

The intra-batch and inter-batch precision and accuracy were evaluated by six spiked samples of plasma with fosamprenavir at LLOQ, LQC, MQC and HQC levels in a batch and in 3 succeeding batches, respectively. The results of precision and accuracy for quantitation of fosamprenavir were represented in the **Table 2**. The percentage relative standard deviation values of inter and intra-batch precision were present in the range of 0.87 to 3.01. The deviation of relative error in the accuracy of inter and intra-batch values were present in between -4.55 to 2.63 **[26, 27]**.

Extraction recovery

The biological samples were effectively pretreated before the analysis. The extraction recovery was evaluated by calculating the peak area ratio of LQC, MQC and HQC level solutions (n=6) of fosamprenavir to the extracted spiked samples at respective concentration levels. In same manner, extraction recovery of IS was assessed by calculating the peak area ratio of quality control plasma sample solutions (n=6) to spiked human plasma samples at respective concentration levels. The mean extraction recovery of fosamprenavir was 97.35%, 98.61 and 102.65% at low, medium and high QC levels respectively. The mean extraction recovery of IS was 98.12% at the concentration of 250 ng/L. The results were represented in the **Table 3** and **Figure 3 to 5** **[21, 25]**.

Matrix effect

Co-eluting matrix constituents can reduce or increase ionization process in mass system, which may not produce a detectable response in blank matrix due to accuracy of the method. Hence, the different matrix related component ions dominance was assessed by estimating the IS normalized matrix factor in eight individual sources (consisting 2-hemolytic and 2-lipemic batches) of human plasma. The average IS normalized matrix factor for all the analytes were present in between 0.96 and 1.05 with a %RSD of ≤4.91 and the results were shown in the **Table 4** **[27]**.

Dilution integrity

The dilution integrity was processed at twice the concentration ULOQ (upper limit of quantification) for fosamprenavir. After the 1:4 dilution the average back calculated drug content for dilution quality control samples were within 85–115% of the nominal value with a % RSD of ≤4.5.

Stability

Stability of fosamprenavir was evaluated in both matrix and aqueous based samples. Fosamprenavir and IS in stock solutions were not affected at 1–10°C for 70 days and stock solutions in diluent were not affected at 1–10°C for 48 h. Matrix stability was assessed at −70 and −20°C for 60 days. Matrix stability was analysed against freshly prepared spiked linearity standards. The findings of stability data were represented in **Table 5**. Drug was not degraded up to 20 h at the bench-top at a temperature of below 10°C and after six freeze-thaw cycles. The prepared sample solutions were stable at 10°C for 72 h in the auto-sampler **[23-25]**.

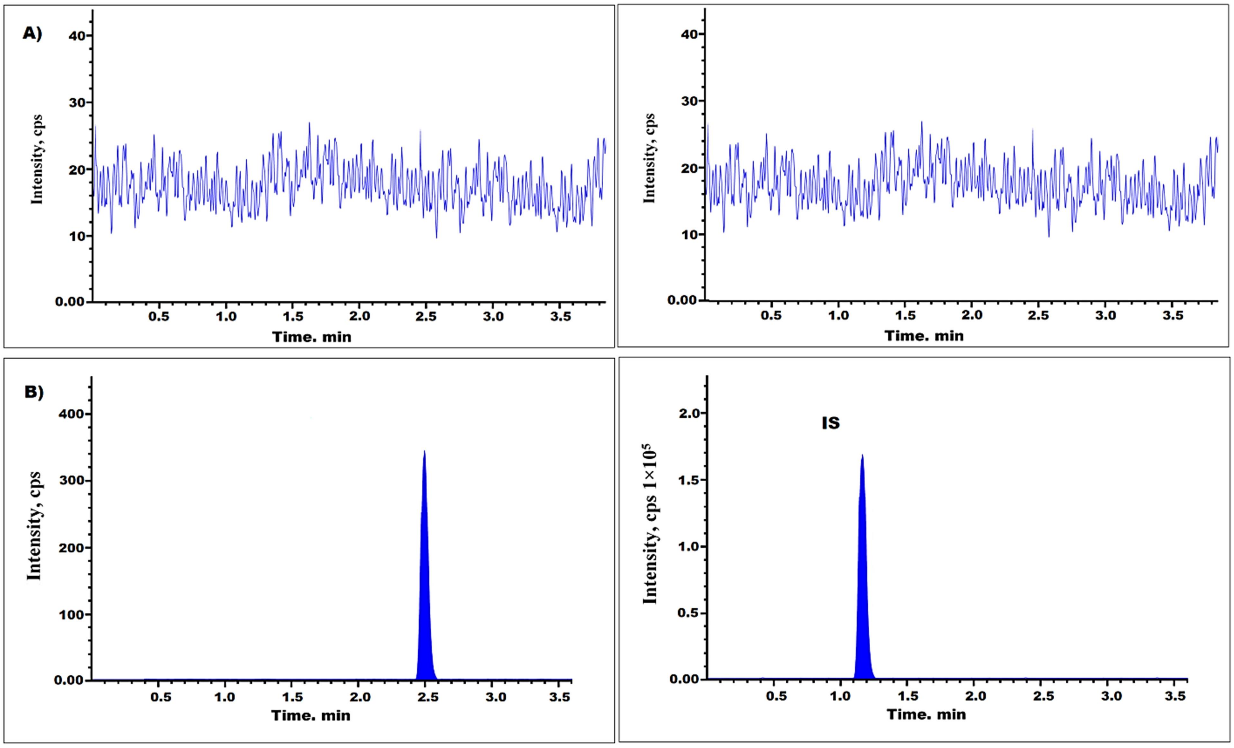

Figure 2: Chromatograms of blank plasma (A) and LLOQ (B) samples

Table 1: Calibration standards for fosamprenavir

CS-ID	Concentration (ng/ml)	Mean area	IS area	Area ratio
CS-1	1	346	165451	0.002091
CS-2	3	1138	165724	0.006867
CS-3	60	20760	165295	0.125594
CS-4	180	62954	165562	0.380244
CS-5	480	168080	165324	1.01667
CS-6	950	318845	165399	1.927732
CS-7	1500	509127	165845	3.069897
CS-8	2100	726600	165165	4.399237

CS: Calibration standard

Table 2: Intra-batch and Inter-batch Accuracy and Precision

		Intra-batch			Inter-batch		
Concentration level	Nominal concentration (ng/ml)	Amount found^a (ng/ml)	%RSD	%RE	Amount found^b (ng/ml)	%RSD	%RE
LLOQ	1.0	0.979	1.94	2.1	0.987	1.92	1.3
LQC	3.0	3.081	2.43	-2.7	2.921	2.56	2.63
MQC	950.0	984.04	2.12	-3.58	993.24	3.01	-4.55
HQC	1500.0	1472.37	0.87	1.842	1562.16	2.93	-4.144

RSD: Relative standard deviation; RE: Relative error;

Table 3: Extraction Recovery of fosamprenavir and IS

Concentration level	A	B	% Recovery	% Mean recovery	%RSD
LQC	1108	1078	97.35	99.54	2.27
MQC	328794	324223	98.61		
HQC	519128	532884	102.65		
IS	165324	162215	98.12		

RSD- Relative standard deviation; A, mean recoveries of un-extracted samples; B, average recovery of extracted samples

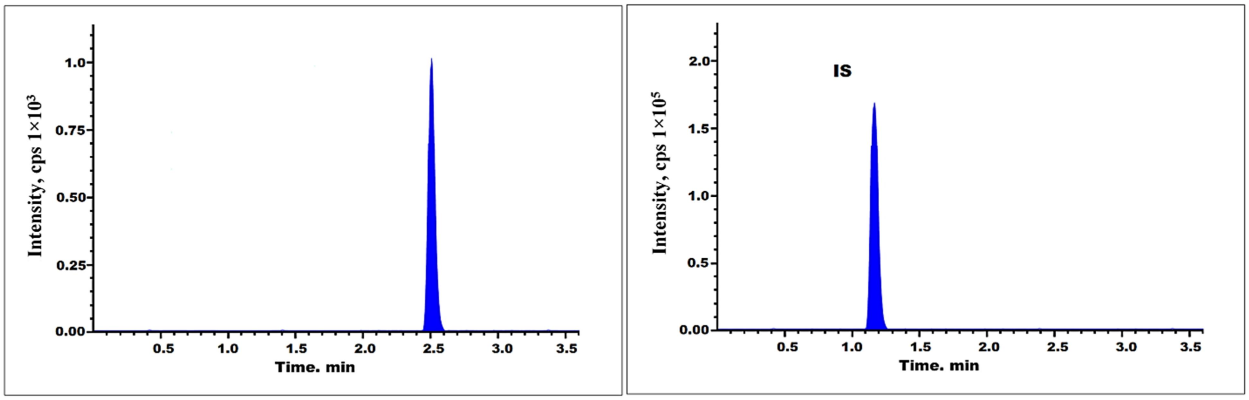

Figure 3: Chromatograms of fosamprenavir at LQC level

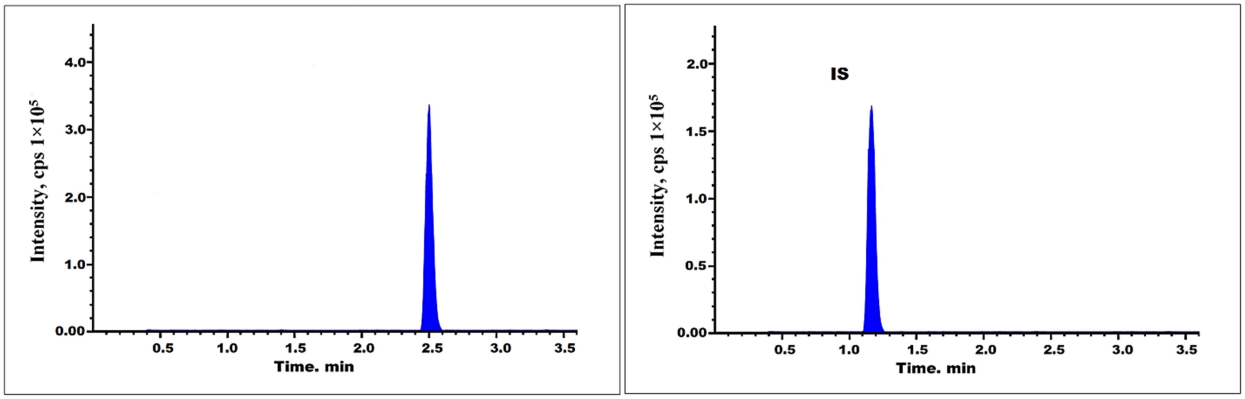

Figure 4: Chromatograms of fosamprenavir at MQC level

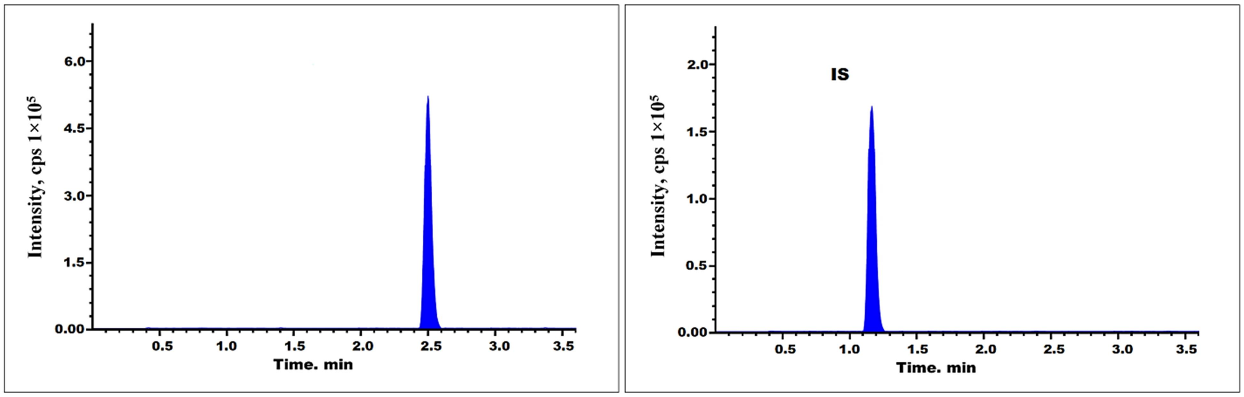

Figure 5: Chromatograms of fosamprenavir at HQC level

Table 4: Matrix Effect Results

	LQC level			HQC level		
Fosamprenavir	**Analyte MF**	**IS MF**	**IS normalized MF**	**Analyte MF**	**IS MF**	**IS normalized MF**
L-1	**1.02**	**1.03**	**0.99**	**1.13**	**1.07**	**1.05**
L -2	**1.11**	**1.07**	**1.04**	**1.07**	**1.04**	**1.03**
L -3	**1.1**	**1.01**	**1.03**	**1.1**	**1.12**	**0.98**
L -4	**1.07**	**1.08**	**0.99**	**1.12**	**1.02**	**1.05**
L -5[a]	**1.03**	**1.02**	**1.01**	**1.08**	**1.06**	**1.02**
L -6[a]	**1.09**	**1.02**	**1.07**	**1.04**	**1.12**	**0.96**
L -7[b]	**1.08**	**1.13**	**0.97**	**1.06**	**1.03**	**1.03**
L -8[b]	**1.02**	**1.09**	**0.96**	**1.01**	**1.05**	**0.97**
Mean		**1.00**			**1.016**	
SD		**0.049**			**0.045**	
%RSD		**4.91**			**4.46**	

a, Hemolyzed lot; b, Lipemic lot; MF, matrix factor; RSD, Relative standard deviation

Table 5: Stability Results of fosamprenavir

Parameter	QC level	X	Y	%RSD	%Stability
Freeze and thaw stability	**LQC**	**3**	**2.91**	**3.65**	**97.018**
	HQC	**1500**	**1435.14**	**4.12**	**95.67**
Stability in refrigerator (1–10°C for 48 h)	**LQC**	**3**	**2.83**	**2.95**	**94.39**
	HQC	**1500**	**1429.22**	**3.27**	**95.28**
Bench-top stability (at <10°C for 20 h)	**LQC**	**3**	**3.10**	**1.98**	**103.49**
	HQC	**1500**	**1397.783**	**3.29**	**93.18**
Long term stability (60 days at −20°C)	**LQC**	**3**	**2.87**	**4.25**	**95.97**
	HQC	**1500**	**1492.29**	**3.44**	**99.48**
In-injector stability (at 10°C for 72 h)	**LQC**	**3**	**2.87**	**4.77**	**95.70**
	HQC	**1500**	**1472.34**	**3.94**	**98.16**
Long-term stability (60 days at −70°C)	**LQC**	**3**	**2.84**	**1.38**	**94.75**
	HQC	**1500**	**1466.91**	**2.71**	**97.79**

X, nominal concentration (ng/ml); Y, mean concentrations (ng/ml) of analytes

CONCLUSION

In the present research work, a sensitive and accurate LC-MS/MS technique was developed and validated for the successful determination of fosamprenavir in human plasma. This method exhibited excellent specificity, linearity, accuracy, precision and stability. The linearity equation and correlation coefficient (r^2) findings were y = 0.0021x - 0.002 and 0.9997 respectively. The %RSD of intra and inter-day precision of the developed technique was found between 0.87

to 3.01% for the QC-samples (1.0, 3.0, 950.0and 1500.0 ng/ml). Hence, the validated method can be applicable for the pharmacokinetic and toxicokinetic studies in the clinical and forensic analysis of fosamprenavir in different kinds of biological matrices successfully.

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