LC-ESI-MS/MS Method for the Fosamprenavir Quantification Bioanalytical Method Development and Validation for the Quantification Fosamprenavir in Human Plasma by LC-ESI-MS/MS

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ABSTRACT

Objectives: The main goal of the current research was to develop a bioanalytical technique for the quantification of fosamprenavir in plasma by LC-ESI-MS/MS. **Materials and Methods:** Chromatographic elution was attained thru a Zorbax (3.5 μm; 50 × 4.6 mm) analytical C_{18}-column with isocratic system by methanol, 0.1%v/v formic acid and acetonitrile in the ratio of 60:10:30 V/V as mobile phase with flowrate of 0.70 ml/min. Liquid-liquid extraction was executed for the drug separation with an ethyl acetate solvent. Parent and product ions were monitored at *m/z* 586.19 /57.0 for Fosamprenavir and 590.0/61.0 for Fosamprenavir-D4 on MRM. **Results:** Linearity plot of fosamprenavir was rectilinear over the concentration of 4.0- 1600.0 ng/ml with correlation coefficient (*r²*) value of more than 0.99. The developed procedure has fine recovery with percentage recovery findings of HQC, MQC and LQC standards were present between 89.65% to 95.61%. The % RSD findings were < 6.30% for intra-day and inter-day accuracy and precision. **Conclusion:** 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: Fosamprenavir, Protease inhibitor, LC-MS/MS, FDA guidelines, Acuuracy.

INTRODUCTION

Fosamprenavir chemically 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 molecular formula $C_{25}H_{36}N_3O_9PS$ (Figure 1) and molecular weight 585.608 g/mol. When it is combined with any other kind of antiretroviral agent, it will usefull in the management of infection caused by human immuno deficiency virus(HIV-1). This drug also utilized in post-exposure prevention of HIV-infected peoples, who have had any kind of occupational experience to potential contagious body fluids of an individual known to be diseased with HIV when that experience represents a substantial risk for communication of HIV. Fosamprenavir is a prodrug and it will hydrolyzed by cellular phosphatases to amprenavir in the gut-epithelium. Amprenavir inhibits HIV-1 protease and acts as anticancer agent.[1,2] During HIV reproduction, HIV-protease splits polypeptide components of virus like Gag-Pol and Gag genes into structural protein form of virion core and important enzymes of virus. Amprenavir inhibits this progression by bound HIV-protease which acts as active site, thereby inhibits the progression of virus Gag-Pol and Gag polyprotein precursors, results in the development of undeveloped non-infected particles of virus.[3,4]
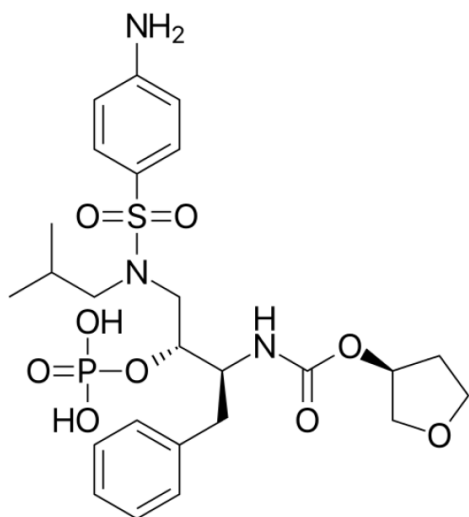
Figure 1: Structure of Fosmprenavir.

Drug literature review discloses that only few analytical quantification methods for the fosamprenavir in bulk, formulations and biological matrices. The reported analytical techniques were HPTLC, spectroscopic[5] and HPLC.[6-10] Goal of the current research was to progress a precise and specific LC-MS/MS technique for the quantitaation of fosamprenavir in plasma samples and application of method validation as per regulatory guidelines.

MATERIALS AND METHODS

Chemicals and reagents

Fosamprenavir (Purity:99.84%) was obtained from MSN Labs, India. Internal standard (Fosamprenavir-D4) of 99.86% was acquired from Hetero drugs PVT. LTD, Hyderabad, India. Methanol (MeOH), Acetonitrile (ACN) of HPLC-grade and formic acid of analytical grade were bought from J.T.Baker, Hyderabad, India. In the present research work water used from Milli-Q water purification system installed in the lab obtained from Bangalore, India.

Liquid chromatographic-MS/MS system

A modular Liquid Chromatographic (LC) system (Shimadzu, Japan) equipped with a DGU- A3-20 solvent degassification, binary LC-AD-20 prominence pump, CTO-ASVP-oven for column and high-through put SILHTC-auto sampler were utilized for present research. Chromatography was attained on a Zorbax (3.5 μm; 50×4.6 mm) C_{18}-column with isocratic separation by methanol, 0.1%v/v formic acid and ACN in proportion of 60:10:30 V/V as movable phase with flowrate of 0.70 ml/min. Fosamprenavir and Fosamprenavir-D4 internal

standard were separated in the total runtime of 5 min. The autosampler temperature and analytical Column temperatures were monitored at 5.0°C and 30.0°C respectively.

The eluents of the liquid chromatographic system were infused into the Electro Spray Ionization (ESI) source operated with positive ionization method. Starting 0.5 min eluent was avoided from the chromatographic system to evade unnecessary impurities from the various salts existed in the human plasma samples. In the mass system following settings were applied; gas-1, N_2 at 50 psi; gas-2, N_2 (45 psi); temperature of ion source, 500°C; curtain-gas, N_2 at 20 psi; voltage of ion spray, 4500 V. Parent and product ions were executed at m/z 586.19/57.0 for Fosmprenavir and 590.2/61.0 for Fosamprenavir-D4 on MRM. The mass conditions were presented in Table 1.

Sample Preparation

To 200μl of spiked plasma, 50μl of Fosamprenavir-D4 (1μg/ml) was mixed and sonicated for 15 sec. To the resulting solution, 500 μl of ethyl acetate was added and vortexed for five min, followed by centrifugateion at 4500 rpm upto 25.0 min at 5.0°C. The organic phase was dried in a lyophiliser. The final residue was solubilized in 500 μl mobile phase and relocated into autosampler vials and infused into an LC-MS/MS system.

Preparation of Standard stock and calibration standards

Fosamprenavir and IS stock solutions were processed in 90% MeOH at concentration level of 1.0 mg/ml. Quality control(QC) and calibration standard(CC) solutions were processed by spiking blank human plasma sample from the fosamprenavir stock solution. CC solutions of eight concentration levels were prepared to produce the final concentrations of 4.0, 16.0, 80.0, 200.0, 400.0, 800.0, 1200.0 and 1600.0 ng/ml. LQC standard, MQC standard and HQC standards were QC sample solutions and were prepared to produce the concentrations of 16, 400 and 1500 ng/ml respectively. All the stock, QC and CC solutions were maintained at -20°C till the method of analysis.

Validation

The method of analysis was assessed by validation parameters like sensitivity, precision, linearity, recovery, dilution integrity, accuracy, matrix effect and stability. Three QC samples of LQC, MQC and HQCs as well as LLOQ (4.0 ng/ml) were employed and analyzed in method validation.[11-14]

Table 1: Mass parameters for fosamprenavir and internal standard.							
Component	Parent ion	Product ion (m/z)	CEP (V)	CXP (V)	DP in V	EP (V)	CE (V)
Fosamprenavir	m/z 586.19	m/z 57.0	20	5	50	9	75
Fosamprenavir-D4	m/z 590.2	m/z 61.0	24	5	52	9	75

Potential at entrance: EP; Declustering potential: DP; Collision cell entrance potential: CEP; Collisional cell exit potential CXP; Collisional energy: CE.

Accuracy and precision

Inter-day and intra-day accuracy and precision were examined as a part of precision and accuracy (PA) parameter. Intra-day PA was evaluated by injecting QC solutions (16, 400 and 1500 ng/ml) and LLOQ (4.0 ng/ml) in 5 replicates in a day arbitrarily. Inter-day PA was evaluated by injecting the same QC and LLOQ solutions once in a day for 5 different days. The % RSDs for LQC, MQC and HQCs should be ≤ 20.0% for LLOQ QC and ≤15.0% for the remaining control levels.[15]

Linearity

CC standards (Non-zero) of 8 different concentrations at 4.0, 16.0, 80.0, 200.0, 400.0, 800.0, 1200.0 and 1600.0 ng/ml solutions were prepared and processed in 3 different runs. Linearity curve (peak area fraction of fosamprenavir and fosamprenavir-D4 peaks against original concentrations) were plotted by least squares linear regression and reciprocal of the squared concentration ($1/x2$) utilized as a weighting factor. Deviation should be within ±20.0% for LLOQ and ±15.0% for remaining control levels.

Specificity and selectivity

Method selectivity was analyzed by equating the chromatograms acquired from blank and spiked samples. Method specificity was analyzed by infusing 6 dissimilar lots of blank plasma solutions to confirm no endogenous compounds interfere with fosamprenavir and internal standard (IS).

Recovery and Matrix Effect (ME)

Fosamprenavir recovery was assessed by paralleling the average peak response of extracted and un-extracted solutions at HQC, MQC and LQC standard levels. At each concentration level percentage recoveries was calculated and finally overall mean recovery was calculated. The ME was analyzed by paralleling the un-extracted samples with post-extracted samples.[16,17]

% Recovery of fosamprenavir

$$= \frac{\text{Mean fosamprenavir peak response in extracted samples}}{\text{Mean fosamprenavir peak response in un-extracted samples}} \times 100$$

Stability

Stability was analysed at HQC, MQC and LQC quality control levels. It includes bench-top, freeze and thaw, autosampler and long-term stabilities. Bench-top stability was assessed for 5 h at ambient temperature (25°C). Freeze and thaw stability was analyzed by montoring the quality control solutions at -70°C for at least 3 h and for thaw cycle keep the solutions at room temperature. Repeat the freeze and thaw cycles for 3 times. The autosampler stability was analyzed by placing the QC solutions in an autosampler at 10.0 degree centigrade for 8.0 h. Long term stability was assessed by keeping the QC solutions in a freezer at -70°C for three months.[18,19]

Dilution Integrity

The sample solution more than the upper calibration limit was prepared and evaluated for PA parameters. The percentage nominal concentration must be ±15 %.

RESULTS AND DISCUSSION

The LC-MS/MS peaks of fosamprenavir blank, HQC, MQC, LQC and LLOQ concentration levels were shown in Figures 2-5.

Method validation

Specificity

From the Figures 2 and 3, system chromatographic conditions were clearly separating fosamprenavir and internal standard from endogenous and other plasma substances. The fosamprenavir-LLOQ peak response is more than 20% the interference peak response and methyl indinavir peak response is more than 5% from the interference peak response.

Accuracy and precision

Fosamprenavir inter-day and intra-day accuracy and precision were analyzed and the %RSD values were calculated for the same and were tabulated in the Table 2.
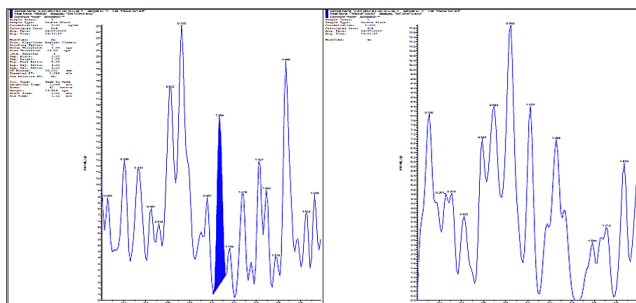

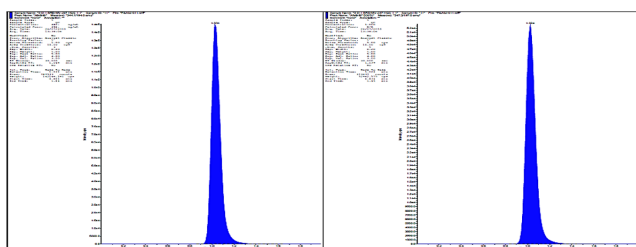**Figure 2: Fosamprenavir blank chromatogram.**

Figure 3: Fosamprenavir HQC-chromatogram.

Linearity

Fosamprenavir calibration graph was rectilinear in concentration over 4 to 1600 ng/ml with regression equation of Y = 0.2684 X + 2.4102. The regression coefficient (r^2) value is more than 0.99 which was acceptable as per the FDA regulatory guidelines.[19]

Recovery and Matrix Effect (ME)

The developed technique has nice recovery and the recovery findings were 89.65%, 92.56% and 95.61% for LQC, MQC and HQC quality control samples respectively. The data for fosamprenavir recovery were tabulated in Table 3. The matrix effect was evaluated at HQC, MQC and LQC level and the calculated % CV values were 1.33%, 1.38% and 5.59%, respectively.

Dilution Integrity

Dilution integrity of fosamprenavir was performed and evaluated. The percentage nominal was within the limit (± 15%) and the estimated precision was less than or equals to 15%. It shows that the drug can be dilute to twenty times and the results will be reproducible.

Stability

All the QC standards were exposed to different stability conditions and evaluated to analyse the stability of fosamprenavir. From evaluated % CV stability data, fosamprenavir was more stable at different environments like bench-top stability (< 11.4%), freeze-thaw stability (< 12.1%), autosampler stability (< 10.3%) and long term stability (< 8.7%) and the values were represented in Table 4.

CONCLUSION

A simple and specific LC-MS/MS technique for the fosamprenavir was developed and validated by utilizing fosamprenavir-D4 as IS. This method has excellent

Table 2: Fosamprenavir accuracy and precision data.				
Nominal concentration	**Intraday**		**Interday**	
	%Accuracy	**%RSD^n**	**%Accuracy**	**%RSD^n**
4.0 ng/ml	97.5	3.5	92.6	4.2
16.0 ng/ml	94.6	4.6	103.5	2.9
400.0 ng/ml	108.5	5.2	95.6	6.2
1500.0 ng/ml	102.3	4.3	98.1	5.7

n=6 replicates (for precision)

Table 3: Recovery data for fosamprenavir.						
	LQC		**MQC**		**HQC**	
SL.No	**Extracted area fraction**	**Unextracted area fraction**	**Extracted area fraction**	**Unextracted area fraction**	**Extracted area fraction**	**Unextracted area fraction**
1	0.015	0.023	0.568	0.924	8.245	12.456
2	0.017	0.025	0.579	0.954	8.125	12.561
3	0.016	0.024	0.589	1.006	8.231	12.891
4	0.017	0.025	0.576	0.956	7.982	12.095
5	0.015	0.023	0.589	0.98	8.012	12.564
Mean	0.016	0.024	0.5802	0.964	8.119	12.5134
SD	0.001	0.001	0.008	0.027	0.108	0.255
%CV	5.59	3.73	1.38	2.85	1.33	2.039
% Mean recovery	89.65		92.56		95.61	

		Bench-top		Autosampler		Freeze and thaw		Long-term	
Drug	**Concentration (ng/mL)**	**Mean±SD (ng/mL)**	**CV(%)**	**Mean±SD (ng/mL)**	**CV(%)**	**Mean±SD (ng/mL)**	**CV(%)**	**Mean±SD (ng/mL)**	**CV(%)**
Fosamprenavir	16	16 ± 0.85	7.8	16 ± 0.61	5.4	16 ± 0.94	8.4	16 ± 0.71	4.8
	400	400 ± 19.6	8.7	400 ± 14.2	6.2	400 ± 20.1	12.01	400 ± 16.4	5.8
	1500	1500 ± 22.9	11.4	1500 ± 18.2	10.2	1500 ± 24.1	11.2	1500 ± 21.9	8.6

Table 4: Stability for Fosamprenavir.
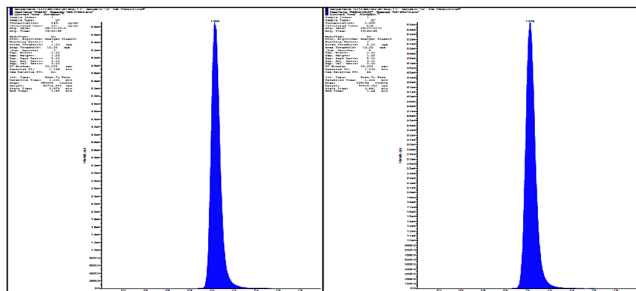
Figure 4: Fosamprenavir MQC-chromatogram.
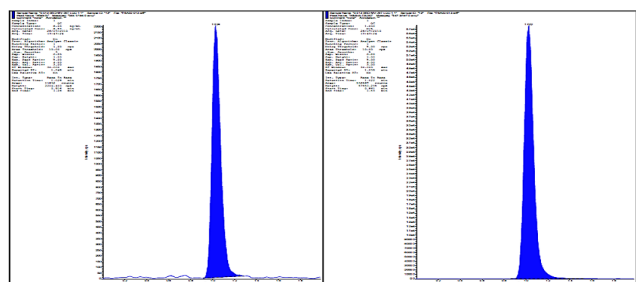
Figure 5: Fosamprenavir LQC-chromatogram.

recovery, accuracy and precision compared with existed methods for the analysis of drug in plasma samples. The drug was subjected for extraction with ethyl acetate from human plasma samples by LLE. The drug was eluted within 5 min using a Zorbax (3.5 μm; 50 ×4.6 mm) C_{18}-analytical column with isocratic system by methanol, 0.1%v/v formic acid and ACN in the proportion of 60:10:30 V/V as movable phase with flowrate of 0.70 ml/min. The developed technique was validated as per the FDA regulatory guidelines and all the parameters of validation were within standard limit. The established technique was effectively applied to routine analysis of fosamprenavir in plasma samples.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

**LC-ESI-MS/MS:** liquid chromatographic-electrospray-ionisation tandem mass spectrometry, **LLE:** liquid-liquid extraction, **FDA:** Food and Drug Administration; **QC:** quality control; **SD:** standard deviation, **LQC:** low quality control; **MRM:** multilple reaction monitoring; **LLOQ:** lower limit of quantification; **HQC:** high quality control; **MQC:** median quality control; **CV:** coefficient of variation.

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PICTORIAL ABSTRACT
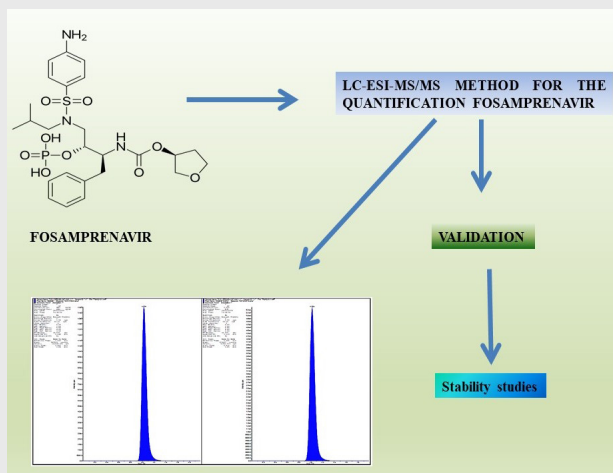
SUMMARY

Fosamprenavir is useful in the management of contagion caused by human immuno deficiency virus by the inhibition of protease enzyme. The goal of the current work was to establish an LC-MS/MS technique for the quantitaation of fosamprenavir in plasma and its validation as per the regulatory guidelines. The drug was eluted within 5 min using a Zorbax (3.5 μm; 50×4.6mm) analytical C_{18}-column with isocratic system by methanol, 0.1%v/v formic acid and acetonitrile in the ratio of 60:10:30 V/V as movable phase. The drug was isolated by LLE method from plasma samples with ethyl acetate as an extraction solvent. The research work was recctilinear over 4 to 1600 ng/ml with regression equation of $Y = 0.2684 X + 2.4102$ and regression coefficient value more than 0.99. The developed technique has nice recovery and the recovery findings were 89.65%, 92.56% and 95.61% for LQC, MQC and HQC quality control samples respectively. Matrix effect was evaluated at HQC, MQC and LQC level and the calculated % CV values were 1.33%, 1.38% and 5.59%, respectively.

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