ANALYTICAL DEVELOPMENT AND VALIDATION FOR THE ANALYSIS OF FOSAMPRENAVIR IN BULK AND MARKETED PHARMACEUTICAL DOSAGE FORM BY USING RP-HPLC METHOD

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ABSTRACT: ***A simple, rapid, specific and accurate reverse phase high performance liquid chromatographic method has been developed for the validated of Fosamprenavir in bulk as well as in marketed pharmaceutical dosage form. This separation was performed on a Symmetry ODS (C18) RP Column, 250 mm x 4.6 mm, 5µm column with Acetonitrile, Methanol and 0.1% OPA in the ratio of 60:30:10% v/v/v as mobile phase at a flow rate of 1.0 mL min−1 with UV detection at 235 nm; the constant column temperature was Ambient. The runtime under these chromatographic conditions was less than 6.0 min. The retention time of Fosamprenavir was found to be 2.570min. The calibration plot was linear over the concentration range of 6–14 µg mL−1 with limits of detection and quantification values of 0.8 and 0.24ng mL−1 respectively. The mean % assay of marketed formulation was found to be 99.79%, and % recovery was observed in the range of 98-102%. Relative standard deviation for the precision study was found <2%.The developed method is simple, precise, specific, accurate and rapid, making it suitable for estimation of Fosamprenavir in bulk and marketed pharmaceutical dosage form dosage form.***

Keywords:Fosamprenavir, RP-HPLC, Validation, Accuracy, Precision, Robustness, ICH Guidelines.

I. INTRODUCTION

Fosamprenavir[1] is a prodrug of amprenavir, an inhibitor of human immunodeficiency virus (HIV) protease. Fosamprenavir is a prodrug form of amprenavir. In the body Fosamprenavir is metabolized to amprenavir, a synthetic derivative of hydroxy ethylamine sulfonamide that selectively binds to and inhibits human immunodeficiency virus (HIV) protease[2].Indicated in combination with other antiretroviral agents for the treatment of human immunodeficiency virus (HIV-1) infection, as well as postexposure prophylaxis of HIV infection in individuals who have had occupational or nonoccupational exposure to potentially infectious body fluids of a person known to be infected with HIV when that exposure represents a substantial risk for HIV transmission[3]. The use of Fosamprenavir is pending revision due to a potential association between the drug and myocardial infarction and dyslipidemia in HIV infected adults.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[4]. The IUPAC name of Fosamprenavir is [(3S)-oxolan-3-yl] N-[(2S, 3R)-4-[(4-aminophenyl) sulfonyl-(2-methylpropyl) amino]-1-phenyl-3-phosphono oxybutan-2-yl] Carbamate. The Chemical Structure of Fosamprenavir is shown in follows

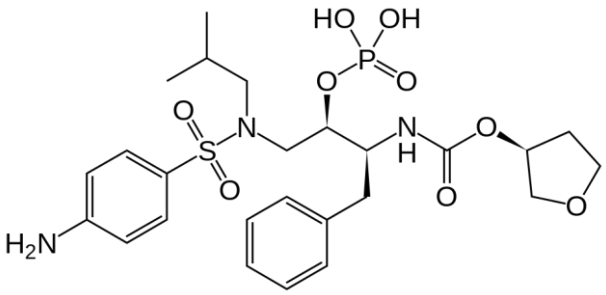

Fig.1. Chemical Structure of Fosamprenavir

HPLC is a widely used analytical technique for the separation and quantification of several mixtures. Several literatures[33-36] show different methods for the estimation of Fosamprenavir.The current work aims to develop and validate an operative RP-HPLC method for determination with good retention and resolution.

II. EXPERIMENTAL

Table-1:List of Instrument used

S. No.	Instruments/Equipments/Apparatus
1.	**HPLC** with Empower2 Software with Isocratic with UV-Visible Detector (Waters).
2.	**T60-LAB INDIA** UV – Vis spectrophotometer
3.	Electronic Balance **(SHIMADZU ATY224)**
4.	Ultra Sonicator **(Wensar wuc-2L)**
5.	Thermal Oven
6.	Symmetry ODS RP C_{18},5µm, 15mm x 4.6mm i.d.
7.	P^H Analyzer **(ELICO)**
8.	Vacuum filtration kit **(BOROSIL)**

Table-2:List of Chemicals used

S.No.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
3.	Dipotassium hydrogen orthophosphate	96%	A.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
5.	Potassium dihydrogen orthophosphate	99.9%	A.R.	Sd fine-Chem ltd; Mumbai
6.	Sodium hydroxide	99.9%	A.R.	Sd fine-Chem ltd; Mumbai
7.	Hydrochloric acid	99.9%	A.R.	Loba Chem; Mumbai.

8.	Hydrogen Peroxide	99.9%	A.R.	Loba Chem; Mumbai.

Method Development and Its Validation for Fosamprenavir by RP-HPLC

Selection of Wavelength

The standard & sample stock solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent. (After optimization of all conditions) for UV analysis[5]. Itscanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Fosamprenavir, so that the same wave number can be utilized in HPLC UV detector for estimating the Fosamprenavir. The scanned UV spectrum is attached in the following page,

Sample & Standard Preparation for the UV-Spectrophotometer Analysis

25 mg of Fosamprenavir standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase.Further dilution was done by transferring 0.5 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase[6].

Optimization of Chromatographic Conditions: The chromatographic conditions were optimized by different means. (Using different column, different mobile phase, different flow rate, different detection wavelength & different diluents for sample preparation etc.

Table-3: Summary of Process Optimization

Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	Methanol : Acetonitrile = 40 : 60	1.0ml/min	235nm	Very Low response	Method rejected
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	Methanol : Acetonitrile = 55 : 45	1.0ml/min	235nm	Low response	Method rejected
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	Acetonitrile : Water = 50:50	1.0ml/min	235nm	Tailing peaks	Method rejected
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	Methanol : Water = 70:30	1.0ml/min	235nm	Resolution was not good	Method rejected
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	ACN : Methanol: 0.1% OPA = 70:25:5	1.0ml/min	235nm	Tailing peak	Method rejected
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	ACN : Methanol: 0.1% OPA = 60:30:10	1.0ml/min	235nm	Nice peak	Method accepted

Preparation of Mobile Phase:

600ml of HPLC Grade Acetonitrile, 300ml of HPLC Grade Methanol and 100ml 0.1% OPA were mixed well and degassed in ultrasonic water bath for 15 minutes. The solution was filtered through 0.45 µm filter under vacuum filtration[7].

III. RESULTS AND DISCUSSION

Method Development

Selection of Wavelength

The standard & sample stock solutions were prepared separately by dissolving standard & sample in a

solvent in mobile phase diluting with the same solvent. (After optimization of all conditions) for UV analysis. Itscanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Fosamprenavir, so that the same wave number can be utilized in HPLC UV detector[8] for estimating the Fosamprenavir. The scanned UV spectrum is attached in the following page,

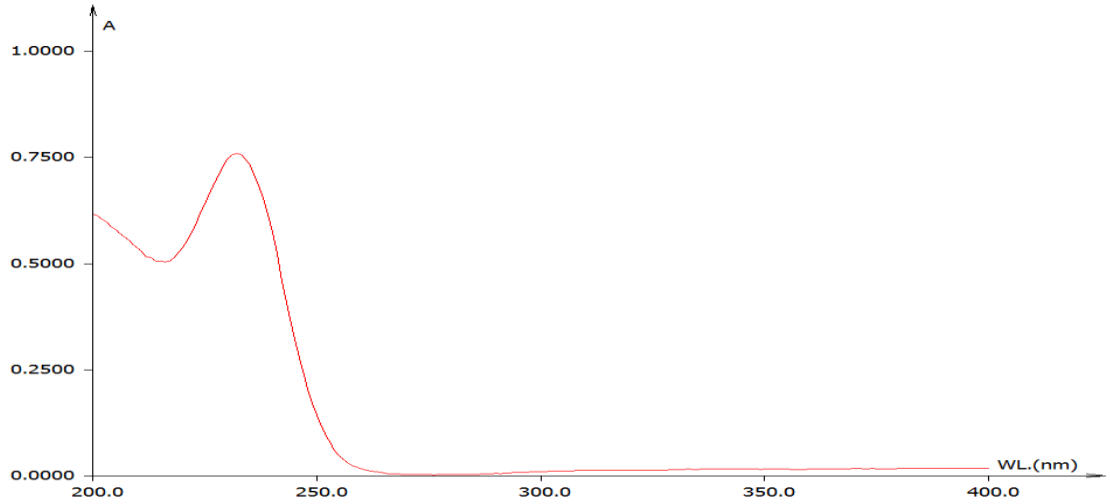

Fig.2. UV Spectrum for Fosamprenavir

Observation: While scanning the Fosamprenavir solution we observed the maxima at 235nm. The UV spectrum[9] has been recorded on T60-LAB INDIA make UV – Vis spectrophotometer model UV-2450.

Optimization of Method

Summary of Optimized Chromatographic Conditions

The Optimum Chromatographic conditions[10-13]obtained from experiments can be summarized as below:

Table-4:Summary of Optimised Chromatographic Conditions

Mobile phase	ACN : Methanol: 0.1% OPA = 60:30:10
Column	Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm
Column Temperature	Ambient
Detection Wavelength	235 nm
Flow rate	1.0 ml/ min.
Run time	06 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	10µl
Type of Elution	Isocratic
Retention time	2.570 minutes

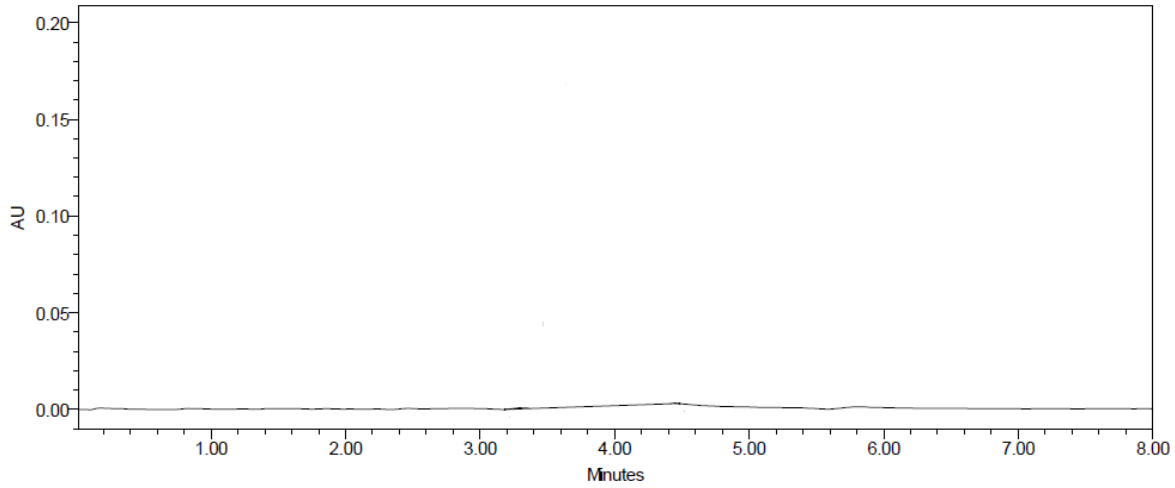

Fig.3. Chromatogram for Blank Solution

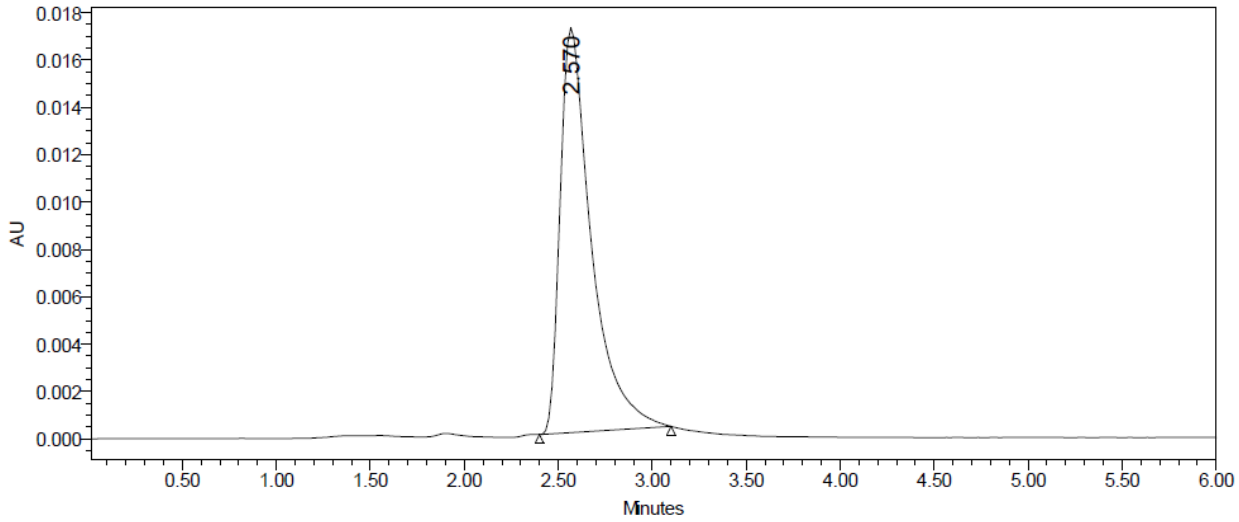

Fig.4. Chromatogram of Fosamprenavir in Optimized Condition

Conclusion: The selected and optimized mobile phase was ACN: Methanol: 0.1% OPA = 60:30:10 and conditions optimized[14] were flow rate (1.0 ml/minute), wavelength (235nm), Run time was 06 mins. Here the peaks were separated and showed better resolution, theoretical plate count and symmetry. The proposed chromatographic conditions were found appropriate for the quantitative determination of the drug.

Validation of Analytical Method

As per the ICH guidelines, method validation was performed. The method was validated for the parameters like system suitability, specificity, linearity, precision (system precision and repeatability), and accuracy, the limit of detection and limit of quantification, robustness, and assay as per ICH guidelines[19,26,27, 32].

1. Accuracy:

Recovery Study:

To determine the accuracy[15] of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Fosamprenavir were taken and 3 replications of each has been injected to HPLC system. From that percentage recovery values were calculated from the linearity equation y = 19423x + 5444.4. The results were shown in table-5.

Table-5: Readings of Accuracy

Conc. In ppm	Conc. Found	Peak Area	% Recovery
8	8.035	161523	100.437

8	8.153	163815	101.912
8	8.061	162023	100.762
		Avg.	101.037
		S.D	0.775
		%RSD	0.767046
Conc. In ppm	**Conc. Found**	**Peak Area**	% Recovery
10	9.930	198315	99.30
10	10.033	200320	100.33
10	10.044	200540	100.44
		Avg.	100.0233
		S.D	0.628835
		%RSD	0.628688
Conc. In ppm	**Conc. Found**	**Peak Area**	% Recovery
12	11.981	238151	99.841
12	12.066	239819	100.55
12	12.215	242712	101.791
		Avg.	100.7273
		S.D	0.987021
		%RSD	0.979894

2.Precision:

2.1. Repeatability

The precision[16] of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug. Fosamprenavir (API). The percent relative standard deviation was calculated for Fosamprenavir are presented in the table-6.

Table-6: Readings of Repeatability

HPLC Injection Replicates of Fosamprenavir	Retention Time (Minutes)	Peak Area (AUC)
Replicate – 1	2.572	197236
Replicate – 2	2.570	197762
Replicate – 3	2.573	195969
Replicate – 4	2.570	194724
Replicate – 5	2.574	198327
Replicate – 6	2.573	198711
Average		**197121.5**
Standard Deviation		**1515.213**
% RSD		**0.768667**

Observation: The repeatability study[17] which was conducted on the solution having the concentration of about 10µg/ml for Fosamprenavir (n =6) showed a RSD of 0.768667% for Fosamprenavir. It was concluded that the analytical technique showed good repeatability.

2.2. Intermediate Precision/Ruggedness:

2.2.1. Intra-Day & Inter-Day:

The intra & inter day variation[18] of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Fosamprenavir revealed that the proposed method is precise.

Table-7: Results of Intermediate Precision Analyst 1 for Fosamprenavir

S.No.	Peak Name	RT	Area (µV*sec)	USP Plate count	USP Tailing
1	Fosamprenavir	2.580	206587	3102	1.16
2	Fosamprenavir	2.597	206859	2986	1.18
3	Fosamprenavir	2.581	207854	3054	1.13
4	Fosamprenavir	2.573	208965	3154	1.14
5	Fosamprenavir	2.590	206547	3157	1.12
6	Fosamprenavir	2.572	209865	3268	1.18
Mean			**207779.5**		
Std.Dev.			**1381.9336**		
%RSD			**0.665**		

Table-8: Results of Intermediate Precision Analyst 2 for Fosamprenavir

S.No.	Peak Name	RT	Area (µV*sec)	USP Plate count	USP Tailing
1	Fosamprenavir	2.580	215263	3215	1.17
2	Fosamprenavir	2.597	214235	3652	1.19
3	Fosamprenavir	2.581	213254	3496	1.15
4	Fosamprenavir	2.573	212367	3258	1.16
5	Fosamprenavir	2.590	213698	3365	1.17
6	Fosamprenavir	2.572	217456	3524	1.14
Mean			**214378.8**		
Std.Dev.			**1791.516**		
%RSD			**0.835678**		

Observation: Intraday and interday studies show that the mean RSD (%) was found to be within acceptance limit (≤2%), so it was concluded that there was no significant difference for the assay, which was tested within day and between days. Hence, method at selected wavelength was found to be precise.

3. Linearity & Range:

The calibration curve showed good linearity[20-22]in the range of 6 – 14 µg/ml, for Fosamprenavir (API) with correlation coefficient (r^2) of 0.999 (Fig-5). A typical calibration curve has the regression equation of y = 19423x + 5444.4 for Fosamprenavir.

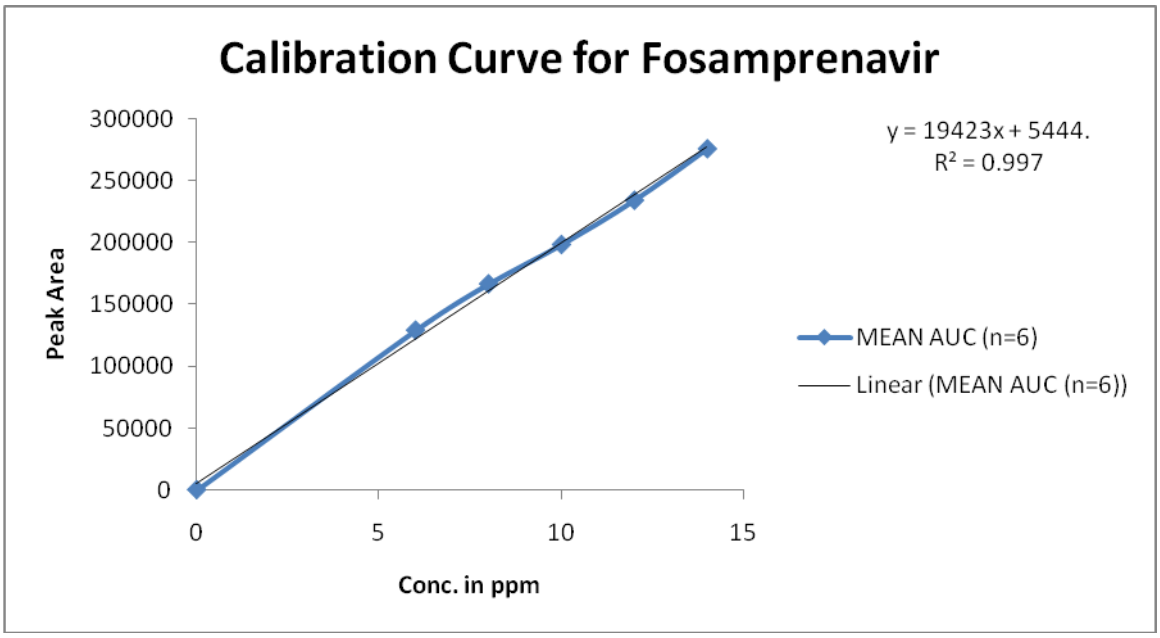

Fig-5:Calibration Curve of Fosamprenavir (API).

Table-9: Linearity Results

CONC.(µg/ml)	MEAN AUC (n=6)
0ppm	0
6ppm	129013
8ppm	166523
10ppm	198315
12ppm	234151
14ppm	275819

Linearity Plot:

The plot of Concentration (x) versus the Average Peak Area (y) data of Fosamprenavir is a straight line.

Y = mx + c

Slope (m) = 19423

Intercept (c) = 5444.4

Correlation Coefficient (r) = 0.99

Validation Criteria: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 5444.4. These values meet the validation criteria.

4. Specificity:

The system suitability for specificity[23] was carried out to determine whether there was any interference of any impurities in the retention time of the analytical peak.

5. Method Robustness: Influence of small changes in chromatographic conditions[24] such as change in flow rate (± 0.1ml/min), Wavelength of detection (±2nm) &organic phase in mobile phase (±5%) studied to determine the robustness[25] of the method are also in favour of (Table-10, % RSD < 2%) the

developed RP-HPLC method for the analysis of Fosamprenavir (API).

Table-10: Results for Robustness for Fosamprenavir

Parameter Used for Sample Analysis	Peak Area	Retention Time	Theoretical Plates	Tailing Factor
Actual Flow rate of 1.0 mL/min	203654	2.570	2915	1.16
Less Flow rate of 0.9 mL/min	265876	2.573	3652	1.19
More Flow rate of 1.1 mL/min	298653	2.631	3854	1.20
Less Organic Phase	315874	2.590	3945	1.17
More Organic Phase	326985	2.602	3487	1.19

6. LOD & LOQ:

LOD: The detection limit[28] of an individual analytical procedure is the lowest amount of analyte in a samplewhich can be detected but not necessarily quantitated as an exact value.

$$LOD = 3.3 \times \sigma / s$$

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

LOQ: The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

$$LOQ = 10 \times \sigma / S$$

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Observation: The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified[29] (LOQ) were found to be 0.08 & 0.24µg/ml respectively.

7. System Suitability Parameter: System suitability[30] was carried out with six injections of solution of 100% concentration having 10µg/ml of Avapritinib in to the chromatographic system. Number of theoretical plates (N) obtained and calculated tailing factor (T) was reported in table-11.

Table-11:Dataof System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Asymmetry	T ≤ 2	Fosamprenavir=0.23
2	Theoretical plate	N > 2000	Fosamprenavir=2987
3	Tailing Factor	T<2	Fosamprenavir=1.17

8. Estimation of Fosamprenavir in Pharmaceutical Dosage Form

Label claim: 700mg

Each tablet contains: 700mg

Twenty pharmaceutical dosage forms were taken and the I.P. strategy was taken after to decide the normal weight. Above measured tablets were at last powdered and triturated well. An amount of powder proportionate to 25 mg of medications were exchanged to 25 ml volumetric flagon, make and arrangement was sonicated for 15 minutes, there after volume was made up to 25 ml with same dissolvable. At that point 10 ml of the above arrangement was weakened to 100 ml with versatile stage. The arrangement was separated through a layer channel (0.45 □m) and sonicated to degas. The

arrangement arranged was infused in five reproduces into the HPLC framework and the perceptions were recorded.

A copy infusion of the standard arrangement was additionally infused into the HPLC framework and the peak regions were recorded. The information is appeared in Table-12.

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Avg. Wt} = \text{mg/tab}$$

Where:

AT = Peak Area of medication acquired with test arrangement

AS = Peak Area of medication acquired with standard arrangement

WS = Weight of working standard taken in mg

WT = Weight of test taken in mg

DS = Dilution of Standard arrangement

DT = Dilution of test arrangement

P = Percentage virtue of working standard

Table-12: Recovery Data for estimation of Fosamprenavir in Tablets

Brand Name of Fosamprenavir	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the Proposed Method (n=6)	Assay % (± SD)
Fosamprenavir CalciumTablets (Mylan)	700mg	699.486 (± 0.495)	99.695 (±0.752)

Result & Discussion: The amount[31] of drug in Fosamprenavir Calcium Tablets was found to be 699.486 (± 0.495)mg/tab for Fosamprenavir & % assay was 99.695 (± 0.752).

Stability Studies

Results of Stability Studies: The results of the stress studies indicated the **specificity** of the method that has been developed. Fosamprenavir was stable in thermal and photolytic stress conditions. The result of forced degradation studies[32] is given in the following table-13.

Table-13:Results of Forced Degradation Studies of Fosamprenavir API.

Stress Condition	Time	Assay of Active Substance	Assay of Degraded Products	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24Hrs.	81.36	18.64	100.0
Basic Hydrolysis (0.I M NaOH)	24Hrs.	83.37	16.63	100.0
Thermal Degradation (50 ^{0}C)	24Hrs.	98.92	1.08	100.0
UV (254nm)	24Hrs.	96.33	3.67	100.0
3 % Hydrogen peroxide	24Hrs.	89.41	10.59	100.0

IV. SUMMARY AND CONCLUSION

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Fosamprenavir, different chromatographic conditions were applied & the results observed are presented in previous chapters.Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution. In case of RP-HPLC various columns are available, but here Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm Column was preferred because using this column peak shape, resolution and absorbance were good. Mobile phase & diluent for preparation of various samples were finalized after studying the solubility of API in different solvents of our disposal (methanol, Acetonitrile, dichloromethane, water, 0.1N NaOH, 0.1NHCl). Fosamprenavir was found to be practically insoluble in water and slightly soluble, soluble in organic solvents such as DMSO and dimethyl formamide, slightly soluble in ethanol and methanol.Utilizing these solvents with suitable arrangement more current techniques can be created and approved. Discovery wavelength was chosen in the wake of examining the standard arrangement of medication more than 200 to 400nm. From the U.V range of Fosamprenavir it is apparent that a large portion of the HPLC works can be proficient in the wavelength scope of 210-300 nm helpfully. Further, a stream rate of 1.0 ml/min and an infusion volume of 10µl were observed to be the best investigation. The outcome demonstrates the created technique is amazingly, one more reasonable strategy for measure and dependability related debasement examines which can help in the investigation of Fosamprenavir in various details.

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