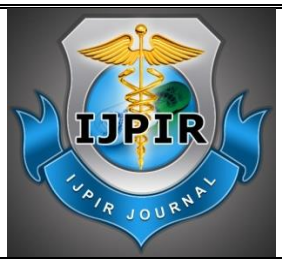
Research Article



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Analytical method development for the estimation of everolimus by rp-hplc

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ABSTRACT

In reversed phase systems the strong attractive forces between water molecules arising from the 3-dimentional inter molecular hydrogen bonded network, from a structure of water that must be distorted or disrupted when a solute is dissolved. Only higher polar or ionic solutes can interact with the water structure. Non- polar solutes are squeezed out of the mobile phase and are relatively insoluble in it but with the hydrocarbon moieties of the stationary phase.
A sensitive & selective RP-HPLC method has been developed & validated for the analysis of Everolimus API. Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility.

Keywords: RP-HPLC, Everolimus, Method development, Method validation

INTRODUCTION

Reversed Phase Chromatography Since 1960's chromatographers started modifying the polar nature of silanol group by chemically reacting silica with organic silanes. The objective was to make less polar or non polar so that polar solvents can be used to separate water-soluble polar compounds. Since the ionic nature of the chemically modified silica is now reversed i.e. it is non-polar or the nature of the phase is reversed. The chromatographic separation carried out with such silica is referred to as reversed- phase chromatography.

As a general rule the retention increases with increasing contact area between sample molecule and stationary phase i.e. with increasing number of water molecules, which are released during the adsorption of a compound. Branched chain compounds are eluted more rapidly than their corresponding normal isomers.

In reversed phase systems the strong attractive forces between water molecules arising from the 3-dimentional inter molecular hydrogen bonded network, from a structure of water that must be distorted or disrupted when a solute is dissolved. Only higher polar or ionic solutes can interact with the water structure. Non- polar solutes are squeezed out of the mobile phase and are relatively insoluble in it but with the hydrocarbon moieties of the stationary phase.

Adsorption Chromatography or Normal Phase Chromatography[2,3]

In normal phase chromatography, the stationary phase is a polar adsorbent and the mobile phase is generally a mixture of non-aqueous solvents. Chromatographic methods can be classified most practically according to the stationary and mobile phases, as shown in the table-1.

Classification of Chromatographic methods

Stationary phase	Mobile phase	Method
Solid	Liquid	Adsorption column, thin-layer, ion exchange, High performance liquid chromatography.
Liquid	Liquid	Partition, column, thin-layer, HPLC, paper chromatography.
	Gas	Gas – Liquid Chromatography.

The importance of Chromatography is increasing rapidly in pharmaceutical analysis. The exact differentiation, selective identification and quantitative determination of structurally closely related compounds are possible with chromatography. Another important field of application of chromatographic methods is the purity testing of final products and intermediates (detection of decomposition products and by-products). As a consequence of the above points, chromatographic methods are occupying an ever-expanding position in the latest editions of the pharmacopoeias and other testing standards.

The various components of a HPLC system are here with described:

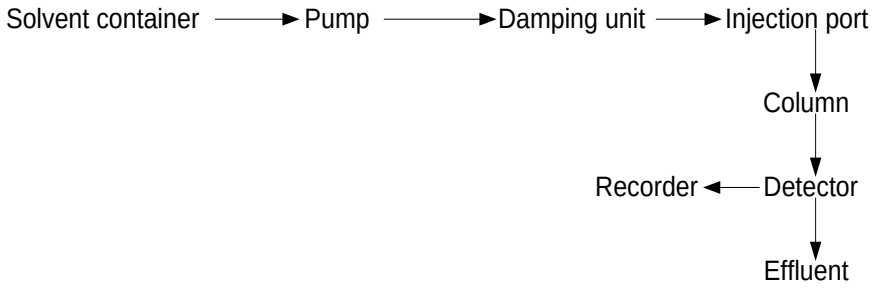
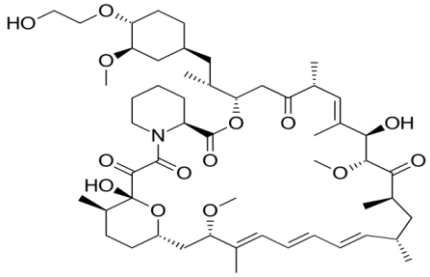


Fig 1: Various Components of HPLC

DRUG PROFILE

Name: EVEROLIMUS

Structure:



Molecular Weight: 958.24

Chemical Formula: $C_{53}H_{83}NO_{14}$

Indication: Everolimus is indicated for the treatment of postmenopausal women with advanced hormone receptor-positive, HER2-negative breast cancer (advanced HR+ BC) in combination with exemestane, neuroendocrine tumors of pancreatic origin (PNET) with unrespectable, locally advanced or metastatic disease. renal cell carcinoma (RCC). renal angiomyolipoma and tuberous sclerosis complex (TSC), tuberous sclerosis complex (TSC) for the treatment of subependymal giant cell astrocytoma (SEGA) that requires therapeutic intervention but cannot be curatively resected.

METHOD DEVELOPMENT

Materials and Instruments:

Equipments :

A suitable HPLC having isocratic system equipped with manual injector with UV detector.

Analytical Balance, capable of measuring the 0.01mg, Sonicator, Usual laboratory glass ware of class-A

Chemicals and Reagents:

S.N.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	HPLC grade water	----	----	Sd fine-Chem ltd; Mumbai
2.	Methanol	99.9%	A.R.	Loba Chem; Mumbai.
3.	Dipotassium hydrogen orthophosphate	96%	L.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
5.	Potassium dihydrogen orthophosphate	99.9	L.R.	Sd fine-Chem ltd; Mumbai

6.	Ortho phosphoric acid	99.9	L.R.	Sd fine-Chem ltd; Mumbai
7.	3% Hydrogen Peroxide	99.9%	A.R.	Vijaya Enterprises

Solubility

REAGENTS	SOLUBILITY
DMSO	Soluble
Ethanol	Soluble
Dimethyl Formamide (DMF)	Soluble
Aqueous Buffers	Sparingly Soluble
Acetonitrile	Insoluble
Water	Insoluble

HPLC Instrumentation & Conditions: The HPLC system employed was **HITACHI L2130** with D Elite 2000 Software with Isocratic with UV-Visible Detector (L-2400).

Mobile phase preparation: The mobile phase used in this analysis consists of a mixture of Methanol and Acetronitrile in a ratio of 30:70.

Sample & Standard Preparation of solutions: Working concentration should be around 10 µg/ml.Accurately weighed around 10mg of everolimus working standard, taken into a 100 ml volumetric flask, then dissolved and diluted to volume with the mobile phase to obtain a solution having a known concentration of about 1000 mcg/ml. Further dilutions has been made to get the final concentration of 10 µg/ml Diluted quantitatively an accurately measured volume of label claim solution with diluents to obtain a solution containing about a linear range.

Table 1: Trials For Method Development and Optimized Condition

Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
C18 Develosil ODS HG-5 RP 150mm x 4.6mm 5µm particle size	Methanol : Water = 70 : 30	1.0 ml/min	272nm	Low response	Method rejected
C18 Develosil ODS HG-5 RP 150mm x 4.6mm 5µm particle size	ACN only	0.5 ml/min	272nm	Very low response	Method rejected
C18 Develosil ODS HG-5 RP 150mm x 4.6mm 5µm particle size	ACN: water = 70 : 30	1.0 ml/min	272nm	Peak broken	Method rejected
C18 Develosil ODS HG-5 RP 150mm x 4.6mm 5µm particle size	ACN: acetate buffer = 70 : 30	1.0 ml/ min	272nm	Broad Peak	Method rejected
C18 Develosil ODS HG-5 RP 150mm x 4.6mm 5µm particle size	Methanol: Acetonitrile = 30:70	1.0 ml/min	272nm	Good sharp peak	Method accepted

Optimized Chromatographic Conditions

Column : C_{18} Develosil ODS HG-5 RP 150mm x 4.6mm 5µm particle size
Mobile Phase : Methanol : Acetonitrile (30:70)
Flow Rate : 1.0ml/minute
Wave length : 272 nm
Injection volume : 10 µl
Run time : 10 minutes
Column temperature : Ambient
Sampler cooler : Ambient

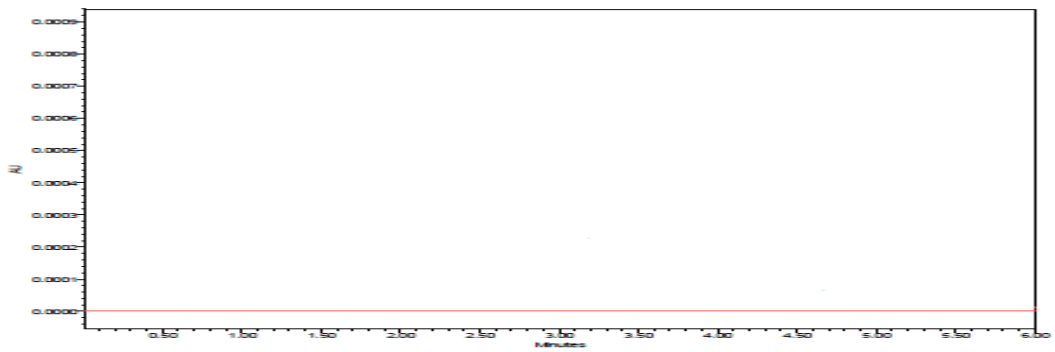
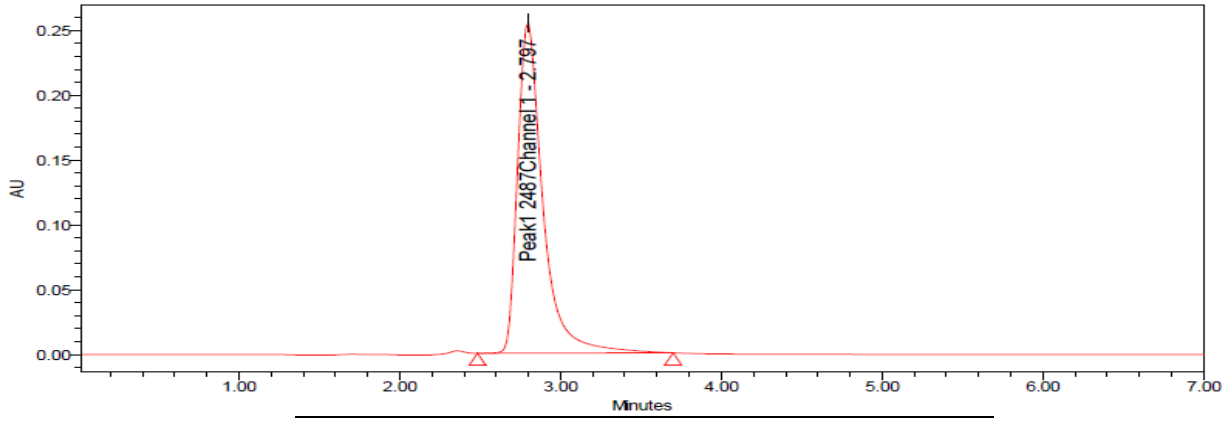


Fig 2: Chromatogram for Blank



Drug Name	RT	Peak Area	Tailing Factor	Plate count
Everolimus	2.79	2785133	1.08	2892

Fig 3: Chromatogram for Optimised Condition (Everolimus) (Rt 2.79)

METHOD VALIDATION

Accuracy: Recovery study

To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of EVEROLIMUS were taken and added to the pre-analyzed formulation of concentration 10µg/ml. From that percentage recovery values were calculated. The results were shown in table-2.

Table 2: Accuracy Readings

Sample ID	Concentration (µg/ml)		Peak Area	% Recovery of Pure drug	Statistical Analysis
	Pure drug	Formulation			
S_1 : 80 %	8	8.03	385224	100.3	Mean = 100.9333%
S_2 : 80 %	8	8.08	387234	101	S.D. = 0.602771
S_3 : 80 %	8	8.12	389166	101.5	% R.S.D.= 0.59719
S_4 : 100 %	10	10.04	491354	100.4	Mean = 100.6666%
S_5 : 100 %	10	10.07	491423	100.7	S.D. = 0.251661
S_6 : 100 %	10	10.09	491854	100.9	% R.S.D.= 0.24999
S_7 : 120 %	12	12.02	526491	100.1	Mean = 100.3666%
S_8 : 120 %	12	12.05	542354	100.4	S.D. = 0.503322
S_9 : 120 %	12	12.08	561426	100.6	% R.S.D.= 0.50148

Precision: Repeatability

The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of five replicates of a fixed amount of drug. Everolimus (API) The percent relative standard deviation were calculated for Everolimus are presented in the table 3.

Table 3: Repeatability Results

HPLC Injection Replicates of Everolimus	Area
Replicate – 1	162359
Replicate – 2	166447
Replicate – 3	170251
Replicate – 4	167368
Replicate – 5	170088
Replicate – 6	169387
Average	167650
Standard Deviation	3008.243
% RSD	1.79435908

Intermediate precision: Intra-assay & inter-assay

The intra & inter day variation 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 Everolimus revealed that the proposed method is precise.

Table 4: Results of Intra-Assay & Inter-Assay

Conc. Of Everolimus (API) (µg/ml)	Observed Conc. Of Everolimus (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
8	8.08	0.96	8.03	0.97
10	10.04	0.40	10.29	0.42
12	12.97	0.33	12.52	0.14

Linearity & Range

The calibration curve showed good linearity in the range of 0-14 µg/ml, for Everolimus (API) with correlation coefficient (R^2) of 0.999. A typical calibration curve has the regression equation of y = 45801x - 17108 for Everolimus.

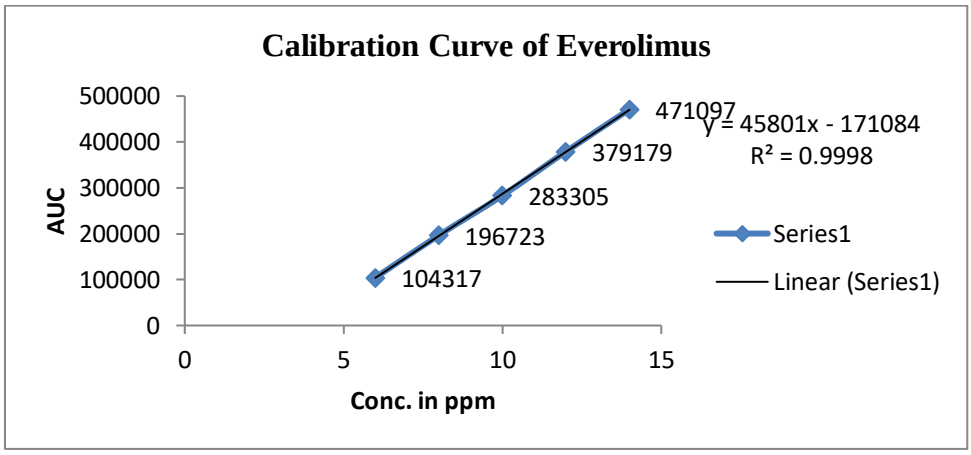


Fig 4: Calibration Curve for Everolimus

Method Robustness

(Table-5.24, % RSD < 2%) the developed RP-HPLC method for the analysis of Everolimus (API).

Table 5: Result of method robustness test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.02
Flow (0.9 ml/min)	0.08
Temperature (27^0C)	0.04
Temperature (23^0C)	0.16
Wavelength of Detection (274 nm)	0.05
Wavelength of detection (270 nm)	0.07

LOD & LOQ

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.04 & 0.12 µg/ml respectively.

System Suitability Parameter

The System Suitability parameters examined. Finally system suitability test parameters are established. The obtained data is shown in the following table 6.

Table 6: Results of System Suitability Parameter

S. No	Parameter	Everolimus
1	Retention time	2.79
2	Theoretical plates	2596
3	Tailing factor	1.07
4	Peak Area	218965
5	Resolution	3.72

ASSAY OF EVEROLIMUS IN DOSAGE FORM

Estimation of Everolimus in Tablet Dosage Form

Table 7: The assay of Afinitor Tablet tablets containing was found to be 99.4 %.

Brand name of tablets	Labelled amount of Drug (mg)	Mean (±SD) amount (mg) found by the proposed method (n=6)	Mean (± SD) Assay (n = 6)
Afinitor Tablet	10	9.94 (±0.05)	99.4 (±0.48)

FORCED DEGRADATION STUDIES

The result of forced degradation studies are given in the following table-7.6.

Table 8 : Results of force degradation Everolimus studies of API.

Stress condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	96.44	3.56	100
Basic Hydrolysi (0.IN NaOH)	24Hrs.	97.11	2.89	100
Thermal Degradation (50 ^{0}C)	24Hrs.	95.77	4.23	100
UV (254nm)	24Hrs.	96.25	3.75	100
3% Hydrogen peroxide	24Hrs.	96.79	4.21	100

RESULT & DISCUSSION

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Everolimus, 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 develosil C_{18}, 5µm, 150 x 4.6 mm i.d. 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). The drug was found to be highly soluble in DMSO, Ethanol and Dimethyl Formamide (DMF), sparingly soluble in Aqueous Buffers . Drug was insoluble in Acetonitrile and water. Using these solvents with appropriate composition newer methods can be developed and validated. Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Everolimus it is evident that most of the HPLC work can be accomplished in the wavelength range of 272 nm conveniently. Further, a flow rate of 1 ml/min & an injection volume of 10 µl were found to be the best analysis. The result shows the developed method is yet another suitable method for assay, stability and purity which can help in the analysis of Everolimus in different formulations.

CONCLUSION

A sensitive & selective RP-HPLC method has been developed & validated for the analysis of Everolimus API. Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility.

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