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Stability Indicating RP-HPLC Method for the Estimation of Everolimus in Pharmaceutical Formulations

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ABSTRACT

The present study was undertaken to develop a validated stability-indicating liquid chromatography method for estimating Everolimus in commercial tablet dosage forms. Chromatographic separation was achieved on Kromasil C_{18}column(100mm x 4.6 mm, 5µ) with mobile phase containing potassium dihydrogen orthophosphate buffer and acetonitrile taken in the ratio 75:25 v/v. The pH was adjusted to 3.0 with dilute orthophosphoric acid at a flow rate of 1.0 mL/min and the eluent was monitored at 270 nm. The developed method was validated as per International Conference on Harmonization (ICH) guidelines with respect to specificity, precision, linearity, accuracy and robustness. Linearity range was found to be between 5-30 ppm and the linear regression coefficient was not more than 0.999. The values of % RSD are less than 2% indicating that the accuracy and precision of the method are good. Statistical analysis proved that the method was precise, reproducible, selective, specific, and accurate for analysis of Everolimus. All the degradation products formed during forced degradation studies were well separated from the analyte peak.

Keywords: Everolimus, KromasilC_{18}Column, Buffer, Acetonitrile, RP-HPLC determination

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INTRODUCTION

Everolimus is a class of medications called kinase inhibitors used for treatment of different cancer. It is a 40-o-(2-hydroxyethyl) derivative of sirolimus and works similarly to sirolimus as an inhibitor of mammalian. Everolimus is (1R,9S,12S,15R,16E,18R,19R,21R,23S, 24E,26E, 28E,30S,32S,35R)-1,18-dihydroxy-12-{(1R)-2-[(1S,3R,4R)-4 2hydroxyethoxy)3methoxycyclohexyl]-1-methylethyl}-19,30 dimethoxy 15, 17,21,23,29,35-hexamethyl-11,36-dioxa-4-aza-tricyclo[30.3.1.0] hexatriaconta-16,24,26,28-tetraene-2,3,10,14,20 pentaone. The Molecular formula of Everolimus is C_{53} H_{83} NO_{14} and its molecular weight is 958.2. Its chemical structure is shown in Figure 1.

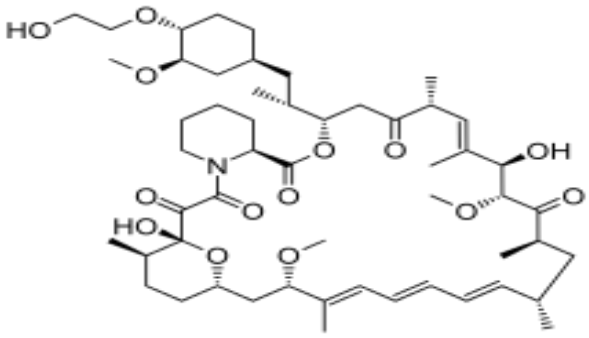

Figure 1: Chemical structure of Everolimus

The drug has been approved for treatment of advanced kidney cancer[1]. It binds to its protein receptor FKBP12 which directly interacts with mTORC1 inhibiting its downstream signalling leading to inhibition of tumour cells' growth and proliferation. It is available as tablet dosage form with different brand names Zortress (USA) and Certican (Europe and other countries). Various liquid chromatography and LC-MS methods have been described for the analysis of Everolimus either in blood samples or in combination with other drugs[2-9]. No stability indicating method has been reported individually for its determination. Hence, the present study is aimed for developing a stability indicating RP-HPLC method for the determination of Everolimus.

MATERIAL AND METHOD

Chemicals and Reagents

All experiments were performed with pharmaceutical Everolimus. The Everolimus reference and branded formulation were supplied by M/S Spectrum Labs, Hyderabad, Telangana State, India. HPLC Grade Acetonitrile was purchased from E. Merck Co, Mumbai, India and Potassium dihydrogen ortho phosphate AR Grade was purchased from SD fine Chem, Mumbai India. Solvents were filtered through 0.45 µ nylon membrane filters. All dilutions were performed in standard volumetric flasks.

Instrumentation

The determination of Everolimus was carried out on waters HPLC Model 2695 equipped with UV-Visible Detector using Data handling System-Waters alliance empower two software. Sartorious electronic balance was used for weighing the samples. Ultrasonic bath sonicator was used for degassing and mixing of the mobile phase.

CHROMATOGRAPHIC CONDITIONS:

Preparation of standard stock solutions

Accurately weighed Everolimus (5mg) was transferred into a 25 mL clean dry volumetric flask. The drug was dissolved in water with sonication and final volume was adjusted with water up to mark to prepare a 200 µg/mL stock solution. The solution was filtered through 0.45µ nylon membrane filter paper. The solution was preserved and was used as standard stock solution for the preparation of calibration curve.

Preparation of sample solutions

5 tablets were weighed and calculated the average weight of each tablet then the weight equivalent to 1(200mg) tablet was transferred into a 50mL volumetric flask, 20mL of diluent was added and sonicated for 25 minutes, further the volume made up with diluent and filtered. From the filtered solution, 1 mL was pipetted out into a 10 mL volumetric flask and made upto 10mL with diluent. Sample Everolimus concentration of 20 µg/mL was obtained. The chromatographic separation of Everolimus was carried out on a Kromasil C_{18} analytical column (100mm × 4.6 mm i.d. 5-µm particles) at a temperature of 30^{o}C under reversed-phase chromatographic conditions. Separation was carried out using a mobile phase of Buffer (pH= 3 Potassium dihydrogen orthophosphate buffer) and Acetonitrile taken in the ratio 75:25 (v/v). The mobile phase was delivered at a flow rate of 1.0mL/min in isocratic conditions. PDA detector was used for detecting the separated components. The data was analyzed on Empower 2 software version. Before analysis, the mobile phase was degassed by use of a sonicator and filtered through a 0.45µm filter. Sample solutions were also filtered through a 0.45µm filter. The system was equilibrated before each injection.

Mobile Phase

Accurately 1.36grams of potassium dihydrogen ortho phosphate was weighed out and dissolved in 1000 mL of water. The solution was filtered through 5µ membrane filter and was degassed. A freshly prepared buffer and acetonitrile in a ratio of 75:25 v/v was used as the mobile phase. Mobile phase was used as diluents for preparing the working solution of the drug.

METHOD DEVELOPMENT

Method validation

The main objective of the chromatographic method was to achieve the separation of degradation products of Everolimus. Initial trial experiments were conducted with a view to select a suitable solvent system for the accurate estimation of the drug and to achieve good resolution between the drug and the degradation products. The suitability of the mobile phase was decided on the basis of the sensitivity of the assay, suitability for stability studies, time required for the analysis, ease of preparation, and use of readily available cost-effective solvents. Optimization of method was conducted mainly by using different stationary phases like C_{18}, C_8 and cyano, different mobile phases containing buffers like phosphate, acetate and formate with different pH (2-7) and using organic modifiers like acetonitrile and methanol in the mobile phase. The developed HPLC method for the determination of Everolimus was validated for accuracy, precision, reproducibility, specificity, robustness, and detection and quantification limits, in accordance with ICH guidelines.

Forced degradation studies

Forced degradation studies were carried out for standard drug at different stress conditions like oxidation, acedic, alkaline, heat, photo stability etc. The following procedure was adopted for forced degradation studies.

Oxidation Studies

To 1 ml of stock solution of Everolimus, 1 ml of 20% hydrogen peroxide (H_2O_2)was added separately. The solutions were kept for 30 min at 60^0C. For HPLC study, the resultant solution was diluted to obtain 20µg/mL solution and 10µL were injected into the system and the chromatograms were recorded to assess the stability of sample (Figure 2).

Acid Degradation Studies

To 1 ml of stock solution of Everolimus, 1 mL of 2N Hydrochloric acid was added and refluxed for 30mins at 60^0C.The resultant solution was diluted to obtain 20 µg/mL solution and 10µL solutions were injected into the system and the chromatograms were recorded to assess the stability of sample (Figure 3).

Alkali Degradation Studies

To 1 mL of stock solution of Everolimus, 1 mL of 2 N sodium hydroxide was added and refluxed for 30mins at 60^0C. The resultant solution was diluted to obtain 20 µg/mL solution and 10 µL were injected into the system and the chromatograms were recorded to assess the stability of sample (Figure 4).

Dry Heat Degradation Studies

The standard drug solution was placed in oven at 105^0C for 6 hrs to study dry heat degradation.

For HPLC study, the resultant solution was diluted to 20µg/mL solution and10µL were injected into the system and the chromatograms were recorded to assess the stability of the sample (Figure 5)**.**

Photo Stability studies

The photochemical stability of the Everolimus drug was also studied by exposing the 120 µg/mL solution to UV Light by keeping the beaker in UV Chamber for 7 days or 200 Watt hours/m^2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 20µg/mL solution and 10µL were injected into the system and the chromatograms were recorded to assess the stability of sample (Figure 6).

Neutral Degradation Studies

Stress testing under neutral conditions was studied by refluxing the drug in water for 6 hrs at a temperature of 60ºC. For HPLC study, the resultant solution was diluted to 20µg/mL solution and 10µLwere injected into the system and the chromatograms were recorded to assess the stability of the sample (Figure 7)**.**

RESULTS AND DISCUSSION

In present study, conditions for a new analytical reversed phase stability indicating HPLC method for the determination of Everolimus in dosage form was developed. The chromatographic separation was achieved on KromasilC_{18}(100mmx4.6mm,5µ) Column. The mobile phase composition was the potassium dihydrogen ortho phosphate buffer and Acetonitrile taken in the ratio 75:25%(v/v) with pH= 3.0 adjusted with orthophosphoric acid solution. The flow rate of the mobile phase was kept 1.0 mL/minute. The column temperature was maintained at 30°C and the detector wavelength was monitored at 270 nm. Everolimus was eluted at 2.486 minutes under above optimized conditions. The run time was set for 6 minutes. The retention time of Everolimus was 2.427. The developed method is specific for the determination of Everolimus and the same was known from the blank, placebo and forced degradation studies, as no other peak was found at the retention time of Everolimus during these studies. The new HPLC method developed and validated for determination of Everolimus in pharmaceutical dosage forms and assured the satisfactory precision and accuracy and also determining lower concentration of drug in its solid combined dosage form by RP-HPLC method. The linearity range of Everolimus is 5.0 - 30.0 µg/mL, the correlation co-efficient was found to be 0.999. The percentage RSD obtained for method precision of Everolimus was 1.26. The Limit of detection (LOD) and Limit of Quantification (LOQ) values for Everolimus were 0.572 and 1.733 µg/mL respectively. The

ruggedness of the method has been verified by analyzing the six samples of same batch for method precision as per test method by different analysts using different instruments, different days. The analysts prepared six sample of the same batch on two different days and calculated %RSD for 2 different days in six samples for ruggedness results with the method precision. The system suitability was evaluated in each condition and compared the results with method precision results. The method was robust for change in flow rate and temperature. No peak was observed at the retention time of Everolimus and the developed method was found to be specific. The sample solution was injected and the amount of Everolimus present in the formulation was calculated from the calibration curve. The amount of Everolimus found in the commercial samplees as per the developed method was closed to 10 mg and the assay of Everolimus was found to be 99.8 %.

Results of forced degradation studies

Everolimus was found to degrade significantly in acid, base hydrolysis and peroxide. Everolimus was found to be stable under photolytic, thermal degradation and aqueous conditions. Assay studies were carried out for stress samples against Everolimus qualified working standard. The results of degradation studies and percentage assay were reported in Table 1.

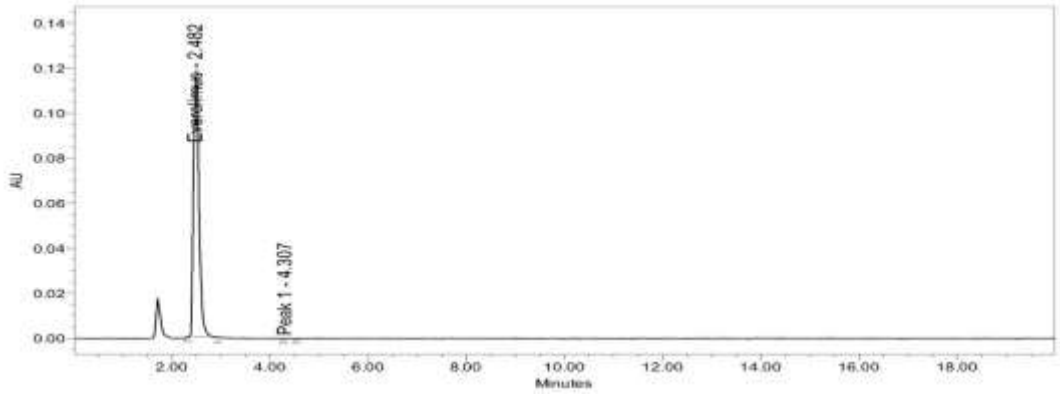

Figure 2: Chromatogram of Peroxide degradation

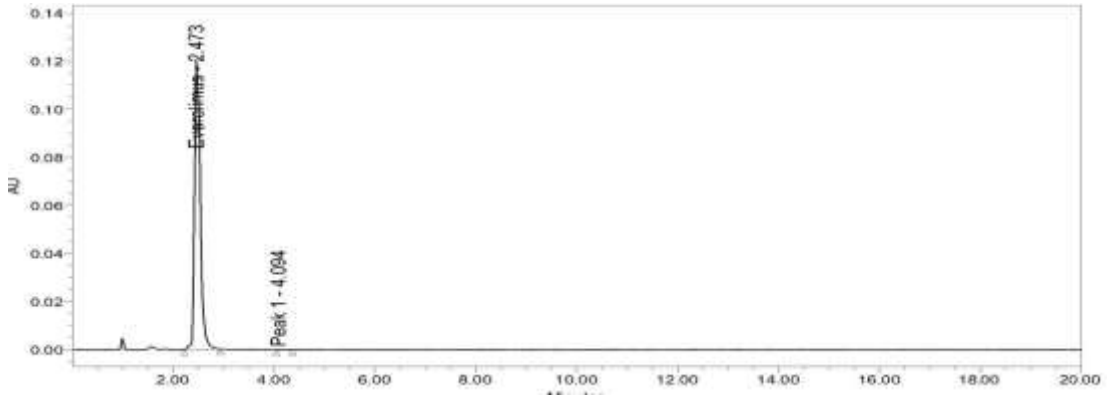

Figure 3: Chromatogram of Acid degradation

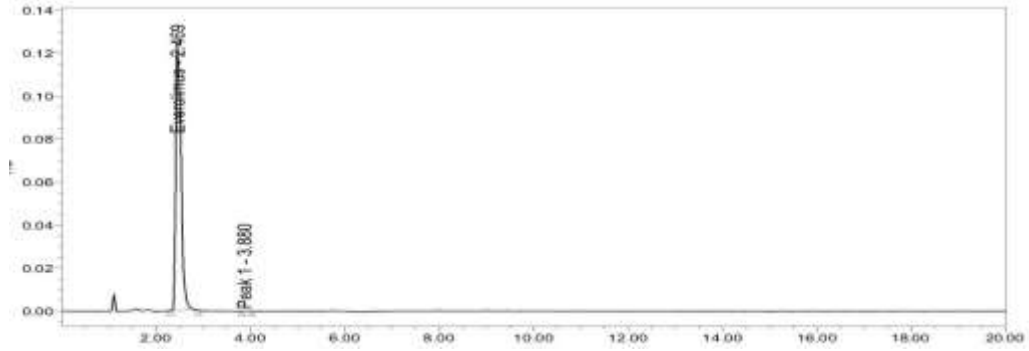

Figure 4: Chromatogram of Alkali degradation

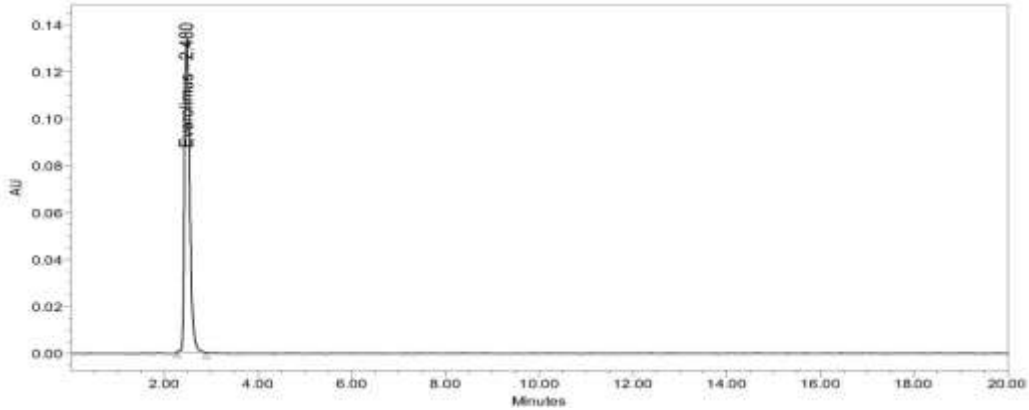

Figure 5: Chromatogram of Thermal degradation

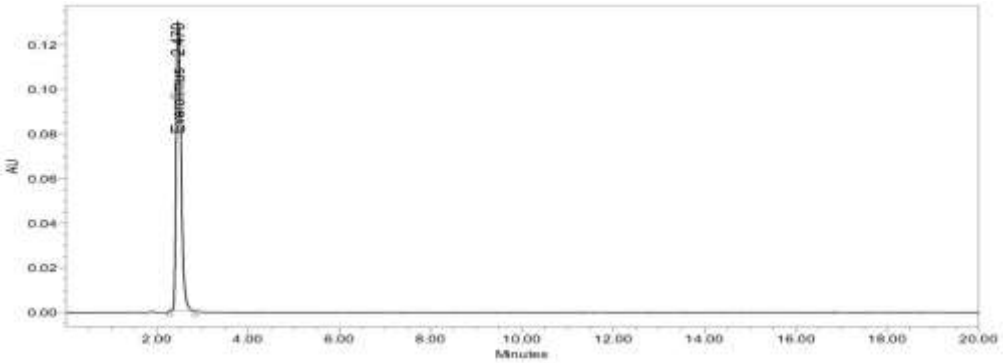

Figure 6: Chromatogram of UV degradation

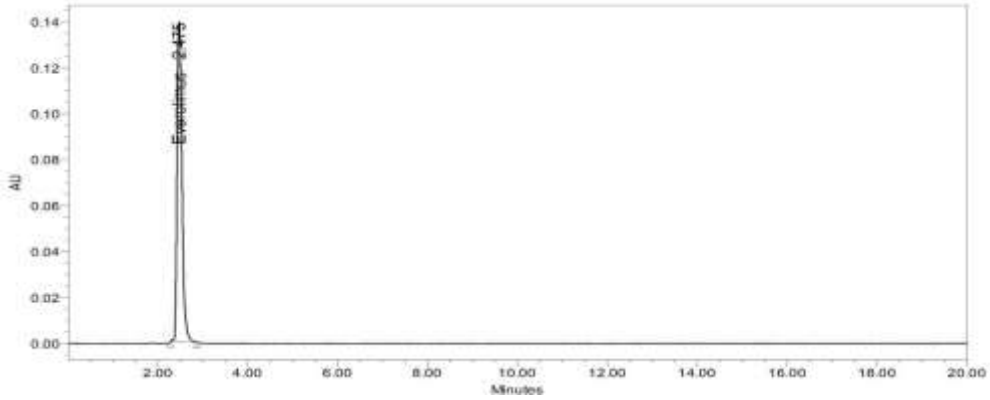

Figure 7: Chromatogram of Water degradation

Table 1: Results of stress degradation study

S.No.	Stress condition	No. of Degraded compounds	Area	Assay %
1	Standard	---	1072028	100
2	Acid	1	999621	93.06
3	Alkali	1	1011025	94.12
4	Peroxide	1	996413	92.76
5	Thermal	---	1038409	96.67
6	UV radiation	---	1046764	97.45
7	Water	---	1059291	98.61

METHOD VALIDATION

System Suitability Parameters

System suitability parameter was established to ensure that the validity of the analytical method was maintained whenever used (Table2). The retention time of Everolimus was 2.47 minutes. Efficiency and tailing factor at 5 % of the height of the main peak were determined, giving N above 2000, and tailing factor = 1.4.

Table 2: Chromatographic conditions of Everolimus

S.No	Method Parameters	Method conditions
1	Mobile phase	Buffer and Acetonitrile taken in the ratio 75:25 %v/v
2	Column	Kromasil C_{18}, 100mm x 4.6 mm, 5µ.
3	Detector wave length	270nm
4	Flow rate	1.0 mL/min
5	Injection volume	10µL
6	Diluent	Water
7	pH	3.0
8	Column temperature	30°C
9	Run time	6 min
10	Retention time	2.47min

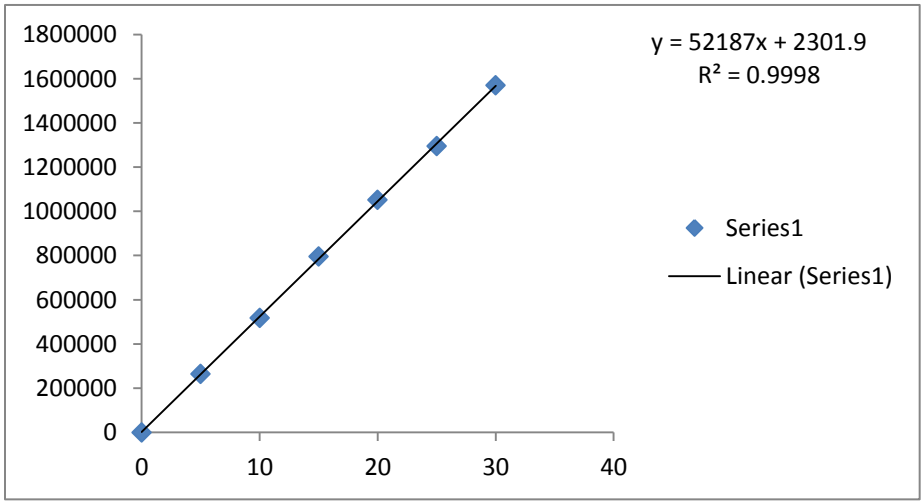

Figure 8: Calibration curve of Everolimus

Linearity and Calibration graph

Linearity was determined by preparing standard stock solutions containing 200µg/mL of Everolimus. Working solutions were prepared by diluting the stock solution with water to contain 5-30µg/ml (Table 3). These solutions were then analyzed in triplicate using the standard optimized conditions. Calibration curves were then generated by plotting peak area versus concentrations and regression equations were computed (Figure 8).

Table 3: Result of linearity

S.No	Concentration in ppm	Area
1	0	0
2	5	264753
3	10	518151
4	15	795290
5	20	1051800
6	25	1295177
7	30	1570547

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted as either a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness. In this 50, 100, and 150% of the expected analyte concentration was added to the matrix. The percentage recovery of Everolimus in assay method was within the limit (Specification limit considered as 98-102%). The percentage recovery of the Everolimus results was listed in Table.4.

Table 4: Accuracy data of Everolimus

S.No.	Everolimus		
	Area ($\mu V^2 Sec$)		
	50%	100%	150%
Injection 1	1563231	2090097	2624028
Injection 2	1574316	2088559	2580950
Injection 3	1568846	2106946	2602958
Average	1568798	2095201	2602645
Amount recovered (µg)	50.09	100.53	149.15
% recovery	99.11	99.89	99.45

System Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Repeatability of measurements, intraday and interday precision studies was conducted for 20µg/mL concentration. The %RSD for six assay values obtained was calculated (Table 5). The intermediate precision of the method was also evaluated

using different analyst and a different instrument in the same laboratory. The %RSD for assay of Everolimus during assay system precision and method precision study was within 1.0. The %RSD of assay results obtained in intermediate precision study was within 0.32% thus confirming good precision of the method.

Table 5: Result of precision

S.NO	Intraday	Interday	Ruggedness
1	1068919	1055549	1081057
2	1068157	1068157	1061930
3	1076849	1069240	1070006
4	1077309	1076864	1053501
5	1066167	1066167	1087552
6	1074768	1068553	1084518
%RSD	0.45	0.64	1.26

Analysis of Everolimus in marketed tablets

Sample solution Everolimus concentration of 20µg/mL which was obtained from tablets was analyzed by the proposed HPLC method. The possibility of interference of excipients with the analysis was studied. No interaction was observed between Everolimus and excipients present in the tablets. The RSD of the drug content was 0.64%. The low RSD indicated the suitability of this method for routine analysis of Everolimus in pharmaceutical dosage forms.

LOD and LOQ

The formulae 3.3 σ /s and 10 σ /s were used to calculate LOD and LOQ respectively. σ is the mean of standard deviation of Y intercepts of the three calibration curves and s is the mean of slopes of the calibration curves. Limit of detection (LOD) and limit of quantification (LOQ) of Everolimus were found to be 0.572 ppm and 1.732ppm respectively. Results of LOD and LOQ were presented in Table 6. The chromatograms of LOD and LOQ of Everolimus were shown in Figures 9 and 10.

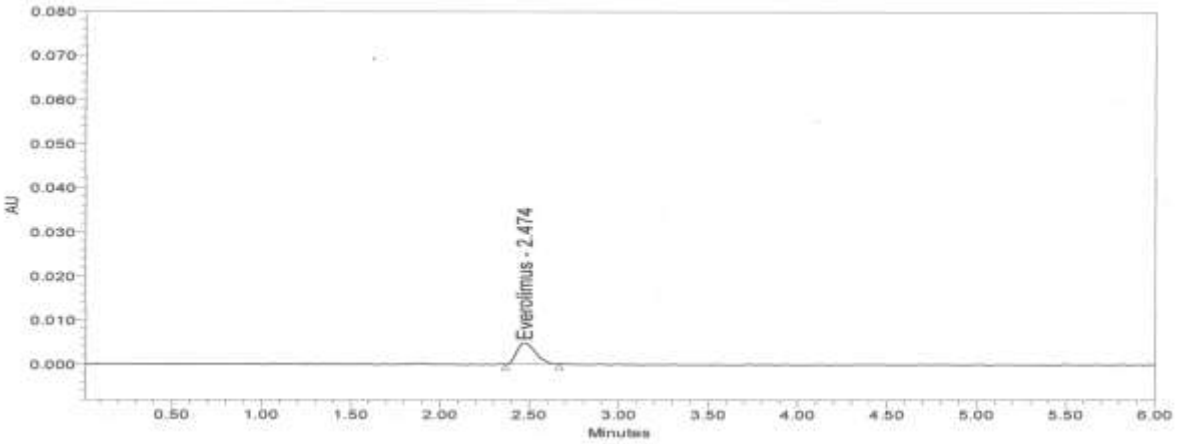

Figure 9: Chromatogram of LOD

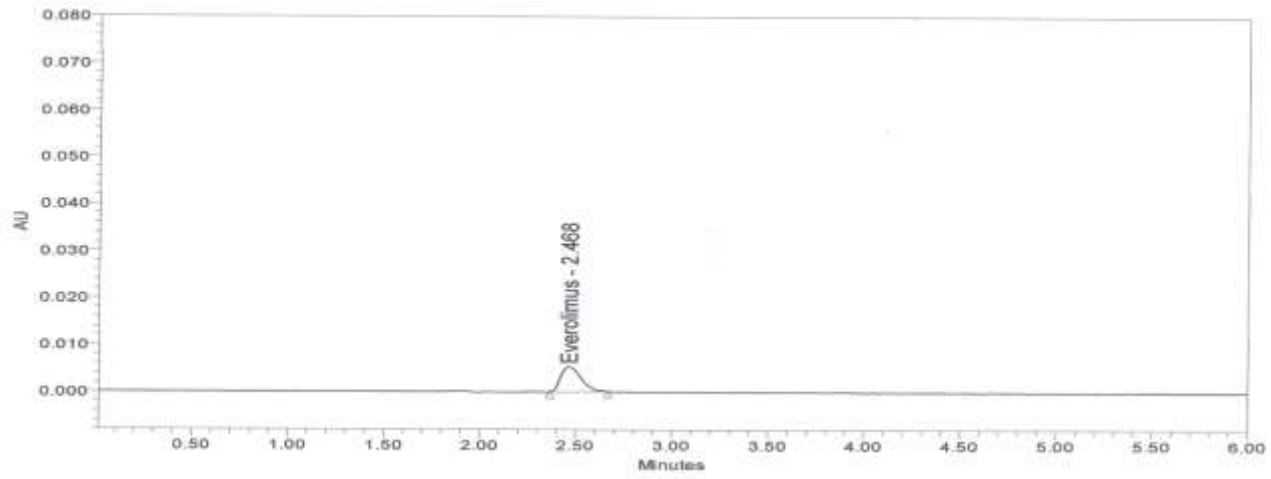

Figure 10: Chromatogram of LOQ

Table 6: Results of LOD and LOQ

S.NO	Parameter	Concentration(ppm)
1	LOD	0.572
2	LOQ	1.733

Specificity

Blank Interference

Specificity studies include application of the proposed method for blank, placebo solution, sample solution (control sample),standard solution. A study to establish the interference of blank was conducted. Diluent was injected into the chromatograph in the above defined chromatographic conditions and the blank chromatogram was recorded. Chromatogram of blank solution (Figure 11) showed no peaks at the retention time of Everolimus peak. This indicates that the Diluent solution used in sample preparation do not interfere in estimation of Everolimus in Zortress(USA) and Certican(Europe and other countries) tablets. Similarly typical representative Chromatogram of standard was also shown (Figure 12).

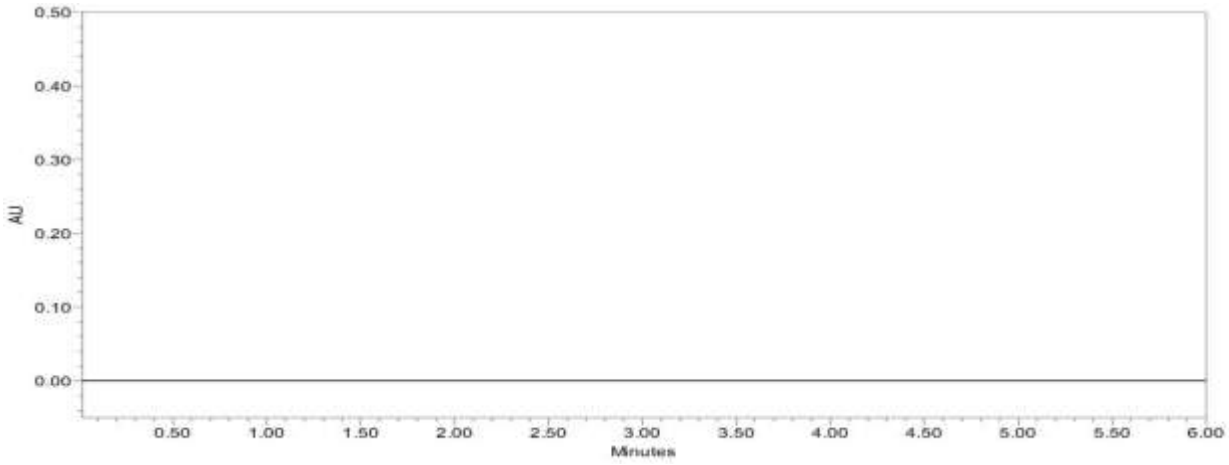

Figure 11: Chromatogram of Blank

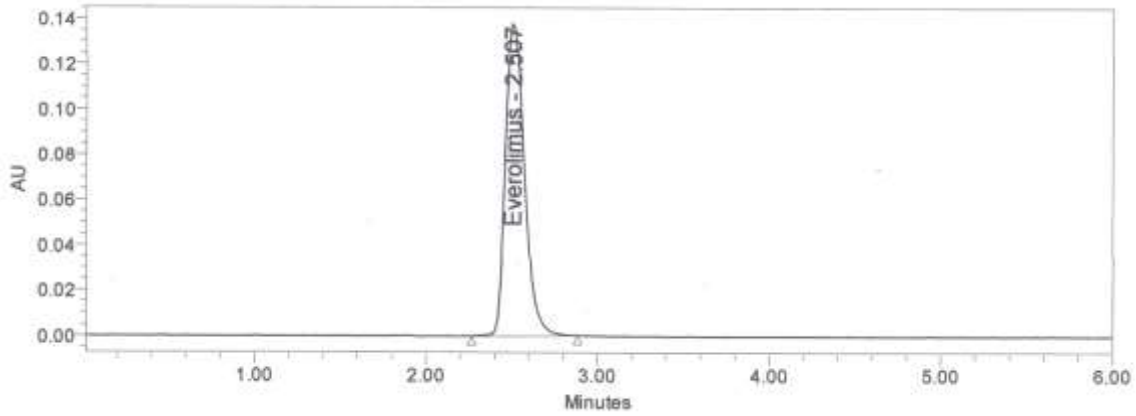

Figure 12: Chromatogram of Standard

CONCLUSION

The proposed method was completely validated as per ICH guidelines and found to be precise and accurate, as depicted by the statistical data of analysis. High values of correlation coefficients and small values of intercepts validated the linearity of the calibration plots and obedience to Beer's laws. The RSD values and the slopes and intercepts of the calibration graphs indicate the high reproducibility of the proposed method. Furthermore, the low values of LOD and LOQ indicate that the method can be employed over a wide concentration range for linearity. The stability indicating nature of the proposed method was established by performing forced degradation, which provided degradation behavior of Everolimus under various conditions. Hence, the developed stability indicating HPLC method can be used for routine analysis of production samples and also to check the stability of bulk samples of Everolimus.

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