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DUAL ANALYTICAL EVALUATION OF EVEROLIMUS 2% BHT: HPLC METHOD VALIDATION AND LC-MS IDENTIFICATION OF UNCHARACTERIZED IMPURITIES

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ABSTRACT
Creating a robust and precise reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the quantitative analysis of Everolimus 2% BHT API. The chromatographic separation was performed using a C18 column which is 250mm length × 3.0 mm internal diameter size and 5 µm of particle size, with an inflow rate of 1.1 mL/min. The discovery was carried out at 210 nm, using a 10 µL injection volume, and the total runtime was 55 minutes.
The mobile phase consisted of two components: Mobile Phase A, a 60:40 (v/v) mixture of 0.27 g/L potassium dihydrogen phosphate buffer and acetonitrile, and Mobile Phase B, which was 100% acetonitrile and also used as the diluent. Both standard and sample concentrations were 10 mg/mL. The retention time (Rt) for Everolimus and BHT were found to be 15.52 minutes and 18.51 minutes, respectively.
System suitability parameters such as %RSD, resolution, theoretical plates, and tailing factor complied with ICH Q2(R1) guidelines. During accelerated stability testing, an unknown impurity was detected at Rt 8.01 minutes (RRT 0.53), which was identified via LC-MS as Seco-Everolimus acid, a product of acid degradation. All validation parameters, including specificity, linearity, precision, accuracy, sensitivity, robustness, and forced degradation studies, were within acceptable limits, confirming the method's reliability for routine analysis.

KEYWORDS: Everolimus, Butylated Hydroxytoluene, Validation, RP-HPLC, Method Development, Relative Retention Time, Forced Degradation, LC-MS.

INTRODUCTION

Everolimus is a chemically or structurally modified form of Rapamycin (Sirolimus). Sirolimus is manufactured by fermentation process. This chemical modification significantly enhances the drug's solubility and bioavailability, making it more effective in clinical use.[1] Like its parent compound, Everolimus acts as an mTOR inhibitor, selectively targeting the mTORC1 complex while leaving the mTORC2 complex unaffected.[2] This selective inhibition plays a key role in regulating cell growth, proliferation, and survival.[3] Clinically, Everolimus is generally suppresses immune system, which is predominantly used during the organ transplantation process.[4] It also an antineoplastic agent. It received approval from the U.S. Food and Drug Administration (FDA) in 2009.[5]
In terms of physical characteristics, Everolimus appears as an amorphous powder. It is practically insoluble in water and heptane, yet freely soluble in anhydrous ethanol.[6] The compound is also light-sensitive, hygroscopic, and exhibits optical activity.[7] Despite showing polymorphic behavior, the drug is consistently manufactured and utilized in its amorphous form, due to its superior stability and processability.[8]
For any drug substance especially one as sensitive and clinically important as Everolimus analytical methods must be carefully designed to ensure accurate and reproducible results.[9] In this project, High-Performance Liquid Chromatography (HPLC) served as the core analytical technique for the estimation of Everolimus 2% BHT.[10] HPLC was chosen for its high sensitivity, precision, and ability to separate the active pharmaceutical ingredient from its antioxidant (BHT) and potential impurities.[11] Method development involves optimizing various chromatographic parameters such as the choice of mobile phase, stationary phase (column), flow rate, and detection wavelength.[12] These conditions are fine-tuned to achieve efficient separation of the drug from its impurities, degradation products, and excipients.[13] Once a method is developed, it must undergo validation in accordance with international regulatory standards, particularly the ICH Q2(R1) guidelines 2005.[14] LC-MS (Liquid Chromatography–Mass Spectrometry) is an advanced analytical technique that integrates the separating power of liquid chromatography (LC) with the identification accuracy of mass spectrometry (MS).[15] This combination allows precise separation and structural elucidation of compounds in a complex mixture, making LC-MS a vital tool in pharmaceutical analysis.[16]
In the context of this project on Everolimus 2% BHT, LC-MS was crucial for identifying an unknown impurity peak. While HPLC confirmed the presence of an impurity, its exact identity remained unclear. LC-MS enabled the detection of the molecular mass and fragmentation pattern of this impurity.[17]

MATERIALS AND METHODS

Everolimus standard and Seco Everolimus acid standard was purchased from Sigma Aldrich. Water make is J.T. Baker (HPLC Grade), Potassium dihydrogen phosphate make is Biosolve (HPLC Grade), Acetonitrile make is Honeywell (HPLC Grade), Glacial acetic acid make is Honeywell (HPLC Grade) were used.

Chromatographic separation was performed using a Reverse Phase–High Performance Liquid Chromatography (RP-HPLC) system (Agilent 1100 Series) equipped with a UV detector. Empower 2 software was used to sequencing and integration of the chromatograms. The analysis was carried out on a Hypersil BDS C18 column (250 × 3.0 mm, 5 µm particle size) maintained at a temperature of 50 °C. The mobile phase A was 60:40 (v/v) mixture of 0.27 g/L potassium dihydrogen phosphate buffer with pH 4.97 adjusted with glacial acetic acid and acetonitrile, and Mobile Phase B, which was 100% acetonitrile delivered at a flow rate of 1.1mL/min using a gradient elution program with a total runtime of 55 minutes. Detection was carried out at a wavelength of 210 nm, and the injection volume was 10 µL. Acetonitrile (100%) was used as the diluent. Mettler Toledo analytical balance was used for weighing.

Mobile phase preparation:

Optimized mobile phase was prepared after the series of trials. After the series of weights, pH adjustments and composition of Potassium dihydrogen phosphate and Acetonitrile, finally the perfect composition of mobile phase with perfect gradient elution was created to get the clear peak shape of Everolimus 2%BHT which perfectly attains the system suitability criteria and validation parameters. The optimized gradient elution program was given in table.no.1. The mobile phase was filtered through 0.45µ membrane filter and degassed it using sonicator for 10minutes. Before starting the instrument, the system was purged with warm water for 30minutes, then it was flushed in Water, Acetonitrile, Mobile phase A and mobile phase B in each pump for 5 minutes. This procedure is known as equilibration of system. The chromatographic condition is given in table.no.2

Table.no.1 Optimized Gradient Program:

Time (min)	0	2	7	25	33	43	43.10	45	55
%A	100	100	72	60	0	0	70	100	100
%B	0	0	28	40	100	100	30	0	0

Table.no.2 Chromatographic Conditions:

Column	Hypersil BDS C18 (250 x 3.0) mm 5 µ
Column temperature	50°C
Flow rate	1.1 mL/min
Wavelength	210 nm
Injection volume	10 µl
Runtime	55 minutes
Elution	Gradient
Diluent	Acetonitrile (100%)

Note: BDS – Base Deactivated Silica

Preparation of 0.27g/l solution of potassium dihydrogen phosphate buffer-0.27 g of Potassium dihydrogen phosphate is weighed in analytical balance which was transferred into 1000 ml of Water in a standard glass beaker, then it was sonicated to dissolve for 10minutes and pH adjusted to 3.98 with glacial acetic acid. Mobile phase A is the mixture of 600 ml of buffer and 400 mL of Acetonitrile into 1000 mL mobile phase bottle, shake well to mix and sonicated to degas for 10minutes. 100% Acetonitrile is used as both mobile phase B as well as diluent.

Standard Preparation and Sample preparation:

Perfectly weighed 100mg of Everolimus standard into 10 mL volumetric flask and added 5ml of diluent, then sonicated to dissolve for 1minute and made up to volume with diluent. Resulting concentration is 10mg/ml.

Perfectly weighed quantity of 100.63mg of Everolimus API sample into 10 mL volumetric flask and added 5ml of diluent, then sonicated to dissolve for 1minute and made up to volume with diluent. Resulting concentration is 10mg/ml. The resulting sample chromatogram with retention time was shown in figure.no.1.

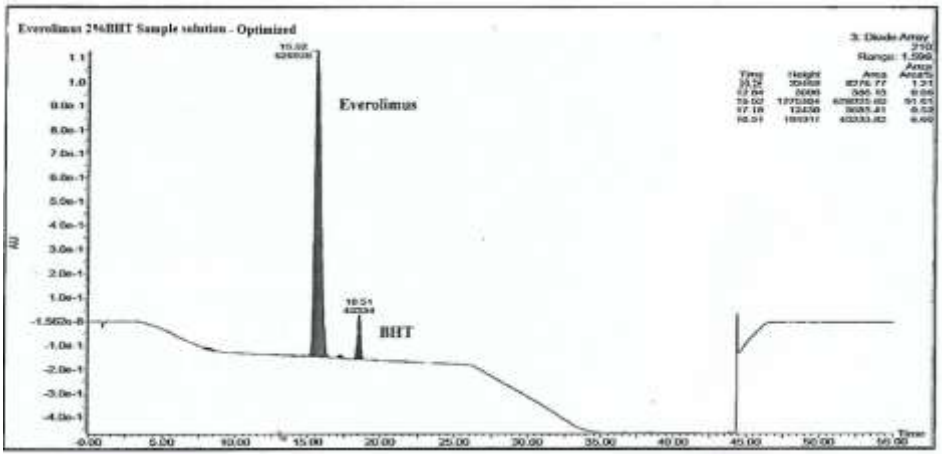


Fig.No:1 Sample Chromatogram of Everolimus 2%BHT

Calculation:

$$\%\text{Assay} = \frac{\textbf{\textit{Average Sum area of Everolimus \& Everolimus tautomer in test solution X Wt of std sol }}(mg)\textbf{\textit{X}}\ \mathbf{50}\textbf{\textit{XP }}(std)\textbf{\textit{X}}\mathbf{100}}{\textbf{\textit{Avg Sum area of Everolimus \& Everolimus tautomer in std sol X}}\mathbf{50}\textbf{\textit{XWt of spl}}(mg)\textbf{\textit{X}}\ \mathbf{100} - (\textbf{\textit{WC}} + \textbf{\textit{BHT}})}$$

Method Validation:

System suitability

System suitability testing is a crucial step performed before sample analysis to ensure the HPLC system's performance is acceptable. This involves injecting six replicates of the standard and evaluating parameters such as retention time, peak area, theoretical plates, resolution, tailing factor, and %RSD. The method is considered suitable if %RSD ≤ 2.0%, resolution ≥ 2.0, tailing factor ≤ 2.0, and theoretical plates ≥ 2000.

Linearity

Linearity is assessed to confirm that the test results are directly proportional to the analyte concentration within a specified range. Standard solutions were prepared from a stock solution of 10 mg/mL (100 mg in 10 mL) by diluting to achieve concentrations ranging from 80% to 120% (i.e., 8, 9, 10, 11, and 12 mg/mL). A calibration curve was plotted using peak area versus concentration. Acceptable limit as per ICH guideline is, the correlation coefficient (r^2) should be ≥ 0.999. Slope, intercept, and residual sum of squares are also evaluated for accuracy of the fit.

Precision and Sensitivity (LOD &LOQ)

Precision measures how closely repeated results agree under the same conditions. It includes system precision, method precision (repeatability), and intermediate precision (ruggedness). System precision is checked by injecting the same standard six times, while method precision involves six sample preparations from the same batch by the same analyst on the same day. Intermediate precision evaluates consistency across different days, analysts, or instruments. A low %RSD in all cases confirms the method's reliability and robustness. %RSD ≤ 2.0% is the acceptable limit.

LOD is the lowest amount of analyte that can be detected, while LOQ is the lowest that can be quantified with accuracy. They are calculated using: LOD = 3.3 × (σ/S) and LOQ = 10 × (σ/S) or based on S/N ratios of 3:1 (LOD) and 10:1 (LOQ). A clear peak at LOQ with %RSD ≤ 10% confirms method sensitivity. The resulting chromatogram was mentioned in figure no.2.

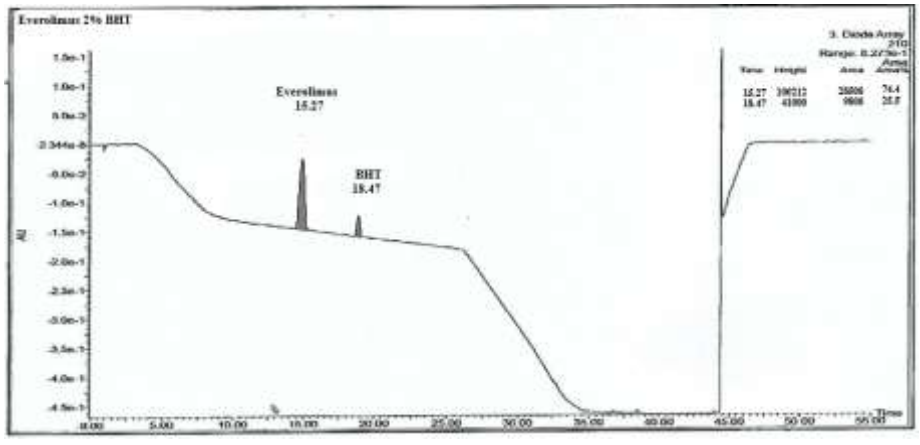


Fig.No:2 Limit of Detection chromatogram

Accuracy

Accuracy compares the amount of concentration injected and the amount of concentration obtains in the result. In HPLC, it is evaluated through recovery studies by spiking known amounts of analyte into placebo at 50%, 100%, and 150% of the target concentration. Each level is tested in triplicate. Mean recovery should be 98.0% – 102.0% with %RSD ≤ 2.0%, confirming method accuracy. The result was shown in table no.3

Table No:3 Accuracy result

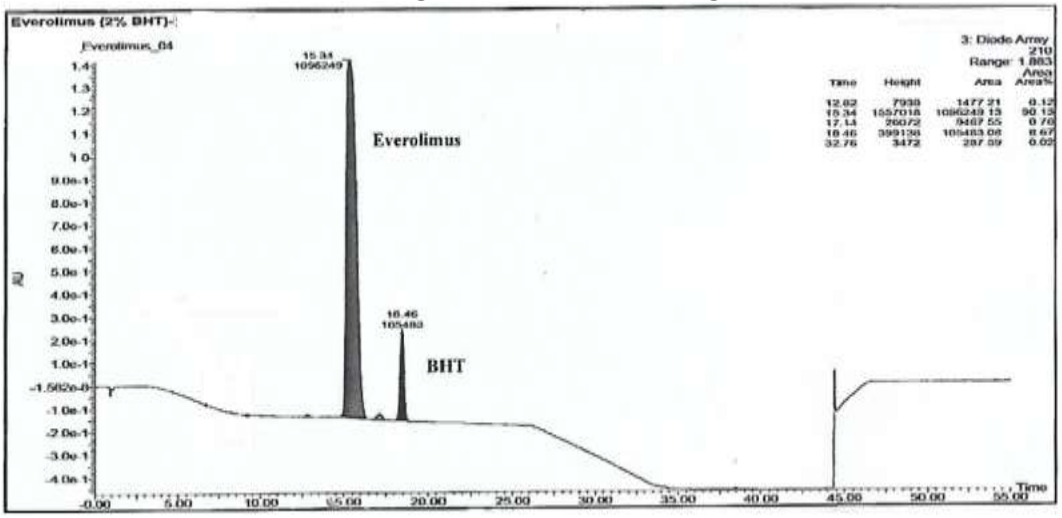
Level		Spiked Amount (µg/mL)	Measured Amount (µg/mL)	% Recovery	Mean Recovery (%)	%RSD
LOQ	1	0.4163 (0.004163)	0.4154	99.8%	99.8%	0.058%
	2	0.4163 (0.004163)	0.4156	99.8%		
	3	0.4163 (0.004163)	0.4160	99.9%		
50%	1	5,000 (0.5%)	4898	98%	98.5%	1.03%
	2	5,000 (0.5%)	4987	99.74%		
	3	5,000 (0.5%)	4899	97.98%		
100%	1	10,000 (1%)	9898	98.98%	99%	0.079%
	2	10,000 (1%)	9884	98.84%		
	3	10,000 (1%)	9897	98.97%		
150%	1	15,000 (1.5%)	14,989	99.9%	99.4%	0.39%
	2	15,000 (1.5%)	14,893	99.2%		
	3	15,000 (1.5%)	14,884	99.2%		

Note: %RSD – Percentage Relative Retention Time, LOQ – Limit of Quantification

Specificity

It is assessed in HPLC by injecting blank, placebo, standard, and spiked samples. Blank and placebo should show no interfering peaks at the analyte's retention time. Spiked samples help confirm peak resolution and identity. No interference at analyte or impurity RT; resolution ≥ 2.0 is the acceptable criteria. The resulting chromatograms has been shown in figure no.3,4,5.

Fig.No:3 Blank chromatogram



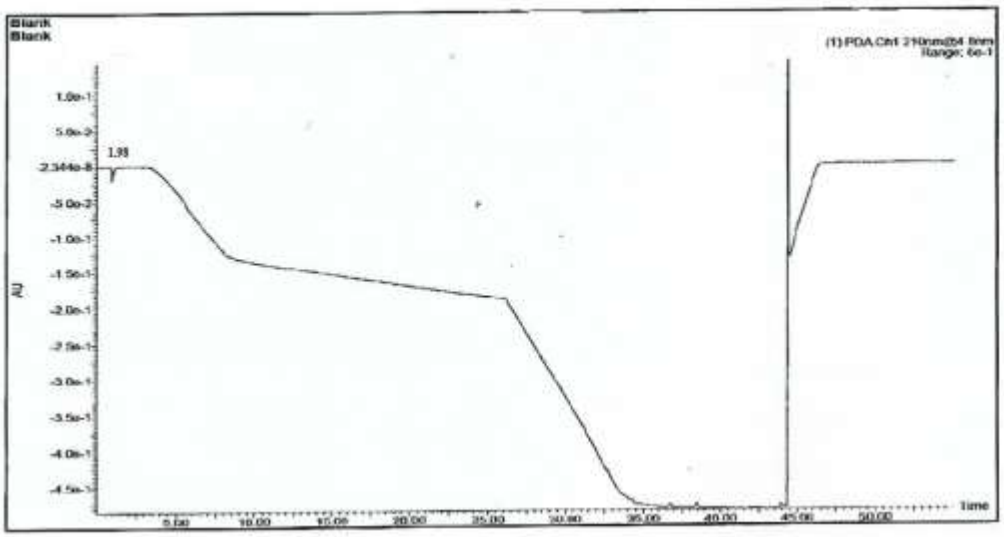


Fig.No:4 Placebo chromatogram

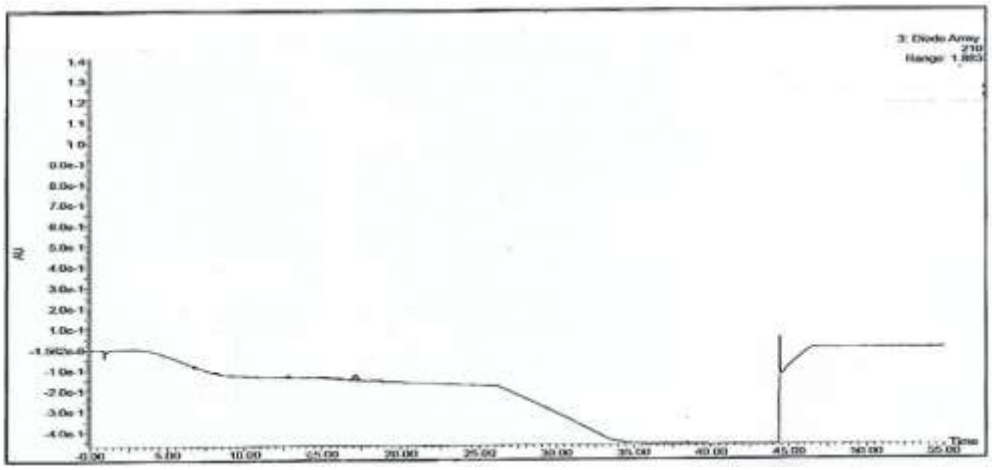


Fig.No:5 Spiked sample- Everolimus 2% BHT chromatogram

Forced degradation under acidic, basic, oxidative, thermal, and photolytic conditions further ensures the method can separate the analyte from its degradation products. The forced degradation data result was shown in table no.4.

Table No:4 Forced degradation study data

Stress condition	**RRT**									
	0.526	0.538	0.546	0.564	0.582	0.59	0.615	0.633	0.648	0.663
Sample exposed at 25oC/60%RH for 72hrs	-	0.202	-	0.048	-	0.059	0.196	0.003	0.024	0.020
Sample exposed at 25oC/90%RH for 72hrs	-	-	0.002	0.037	-	0.047	0.143	0.002	0.017	0.017
Sample exposed at 40oC/75%RH for 72hrs	-	0.051	-	0.001	-	0.002	0.003	0.004	-	0.002
0.001N HCl 0th Hour	-	0.013	0.007	0	0.001	0.003	0.001	0.002	-	0.001
0.001N NaOH 2Hour	-	0.074	-	-	-	-	0.002	0.029	-	0.003

10%H_2O_2 24th Hour	-	-	0.152	0.654	-	0.725	-	-	-	-

Note: RRT – Relative Retention Time, RH – Relative Humidity, N – Normality, HCl – Hydrochrloric acid, NaOH – Sodium Hydroxide, H2O2 – Hydrogen peroxide

Robustness

Robustness tests a method's reliability under small changes in conditions like flow rate, temperature, pH, wavelength, and mobile phase composition. Acceptance: Resolution ≥ 2.0, tailing factor ≤ 2.0, and %RSD ≤ 2.0%. The results are shown in table no.5.

Table No:5 Robustness result

Variation	Condition Tested	Mean Assay (%)	%RSD	Theoretical plates	Resolution	Tailing factor
Flow rate (+0.1 mL/min)	1.2 mL/min	99.6	0.35%	70180	15.48	0.99
Flow rate (−0.1 mL/min)	1.0 mL/min	99.7	0.38%	69800	16.48	1.09
Wavelength (+2 nm)	212 nm	99.8	0.30%	68997	16.25	0.98
Wavelength (−2 nm)	208 nm	99.6	0.32%	69900	16.01	1.09
Mobile phase +2% organic	48:52 Buffer:ACN	99.5	0.42%	69897	15.56	0.95
Mobile phase −2% organic	52:48 Buffer:ACN	99.4	0.40%	69945	15.89	0.96

Note: *%RSD – Percentage Relative Standard Deviation, ACN - Acetonitrile*

Accelerated stability study

An accelerated stability study was conducted on the Everolimus 2% BHT API sample over a period of three months to evaluate its stability under stress conditions (40 ± 2 °C and 75 ± 5% relative humidity), as per ICH Q1A(R2) guidelines. Samples were withdrawn at regular intervals initial (0 month), 1 month, 2 months, and 3 months for analysis. At each time point, the sample was evaluated for physical appearance, assay by a validated RP-HPLC method, degradation products, and impurity profiling. The assay was expected to remain within 98% - 103% of the initial concentration, and the appearance should show no significant change. The resulting chromatogram was shown in figure.no. 6.

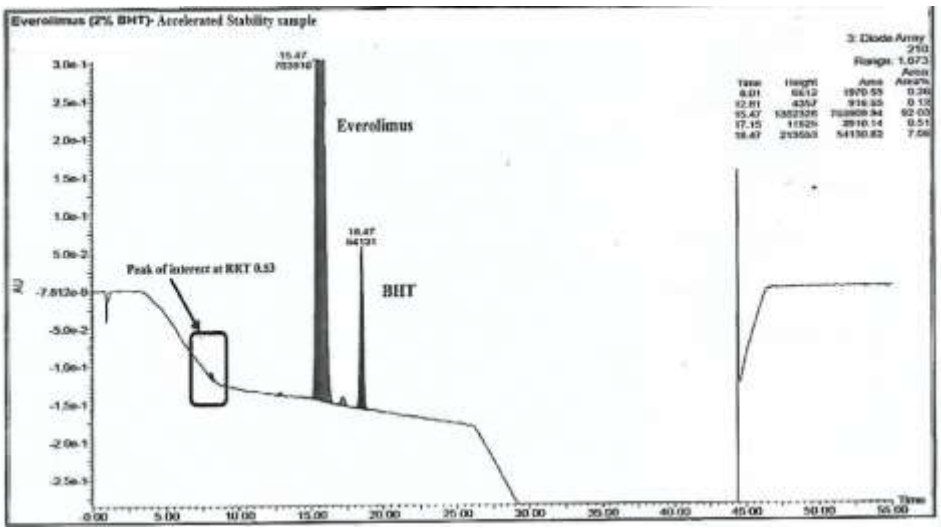


Fig.No:6 Accelerated stability sample chromatogram of Everolimus 2%BHT

Impurity profiling by LC-MS ANALYSIS (Waters -make, model -ACQUITY LC with SQ Detector)
The same chromatographic conditions, mobile phases preparation, sample preparation, diluent were used for LC-MS impurity profiling to detect the unknown impurity peak that has been observed at Rt 8.12. Mass spectrometric conditions were shown in table.no.6.

Table.No:6 Mass Spectrometric conditions:

Scan mode	**Q 1 MS**
Source	Electro Spray Ionization (ESI)
Polarity	Positive and Negative
Capillary (kV)	3.0 kV
Cone (V)	30 V
Source Temperature (°C)	150°C
Desolvation Temperature (°C)	500°C
Desolvation Gas Flow (L/Hr)	1000 L/Hr
Cone Gas Flow (L/Hr)	50 L/Hr
Scan range	50 to 2000 Da
Scan time	0.2 seconds

Note: *Q 1 MS- Quadrant 1 – Mass Spectrometry, kV – Kilo volt, oC – Degree Celsius, L/Hr – Litre/Hour, Da - Dalton*

Spiked Sample Preparation: 1.0 mL of freshly prepared Everolimus sample was transferred to an HPLC vial, and 10 µL of Everolimus Seco acid standard (1 mg/mL) was added. The added contents were mixed well or vortexed and placed into the LC-MS.

Impurity Standard: Everolimus Seco acid standard was prepared at a concentration of 1 mg/mL. Diluent is used as blank. The resulting chromatogram was shown in figure.no.7.

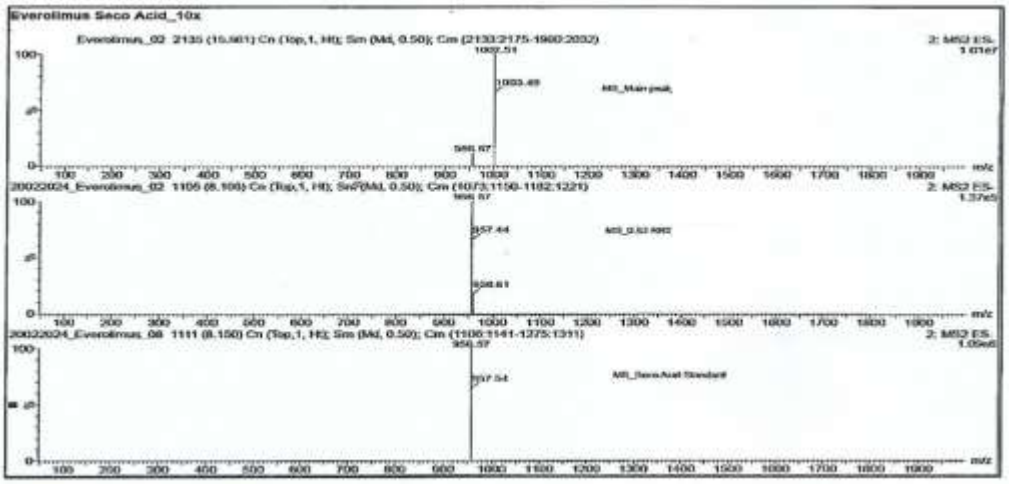


Fig.No:7 Comparison of MS Spectra of Everolimus peak Vs RRT 0.53 Impurity Vs Everolimus seco acid

Result and discussion
The developed new HPLC method for determining Everolimus 2% BHT perfectly attains all system suitability criteria. The %RSD of six multiple run of Everolimus standard was observed as 0.45%, which indicates uniformity. A resolution of 16.44 was observed between critical peaks, with a tailing factor of 1.07 and a theoretical plate count (N) of 67,890, demonstrating good column efficiency and peak symmetry. Following

this, the assay of the sample was performed and yielded an excellent result of 99.7%, confirming the method's suitability for accurate quantification.

The initial and important step in validation is checking the linearity. A calibration curve was plotted using various concentrations of Everolimus 2% BHT against their corresponding peak areas. It showed linear straight graph with correlation coefficient (r^2) value of 0.998. Precision has three steps of verification: system, method, and intermediate precision. The %RSD values for system precision, method precision, and intermediate precision were 0.04%, 0.28%, and 0.30%, respectively all well within the acceptable limit of ≤2.0%, confirming the method's reproducibility and reliability.

LOD and LOQ for sensitivity calculation uses the standard deviation value from precision study and slope from the linearity study. The LOD was calculated as 0.1372 µg/mL, and the LOQ was 0.4163 µg/mL. At the LOQ level, the %RSD was 0.044%, and for LOD, it was 0.02%, both showing excellent precision. The chromatogram at the LOD level displayed a clear, well-defined peak, further confirming the method's sensitivity.

Accuracy was demonstrated through recovery studies, with the mean recovery values falling within the acceptable range of 98–102%, as shown in Table No. 3. Specificity was confirmed by the absence of any interfering peaks at the retention times of Everolimus and BHT in both blank and placebo chromatograms, indicating that the method can reliably distinguish the analyte from excipients and impurities.

Robustness study was assessed by changing certain chromatographic parameters, like flow rate, pH, and wavelength. In all conditions tested, the %Assay values remained within the acceptable range (98–102%), %RSD values were below 2.0%, resolution exceeded 2.0, and tailing factors remained under 2.0, which indicates that the method is robust.

During the accelerated stability study, an impurity peak was observed at a retention time (Rt) of 8.01 minutes, with a relative retention time (RRT) of 0.53. Based on the forced degradation data, the impurity was suspected to be Seco Everolimus acid, a known acid degradation product. To confirm this, a standard solution of Seco Everolimus acid was spiked into a freshly prepared Everolimus 2% BHT sample. As we suspect, the retention time of the standard seco Everolimus peak is same as impurity peak. Further confirmation was achieved by comparing the LC-MS spectra of the Everolimus peak, the unknown impurity peak, and the Seco Everolimus acid standard. All three spectra displayed matching fragmentation patterns, conclusively identifying the impurity as Seco Everolimus acid.

CONCLUSION

The developed RP-HPLC method for the quantitative estimation of Everolimus 2% BHT demonstrated excellent performance in terms of system suitability, precision, linearity, accuracy, specificity, and robustness, aligning well with ICH guidelines. The method showed high reproducibility and sensitivity, with a well-defined linear calibration curve, low %RSD values, and accurate recovery results. The absence of interfering peaks and the method's resilience to minor variations in analytical conditions further confirmed its reliability for routine analysis.

Additionally, the method proved effective in impurity profiling during the accelerated stability study. A degradation peak observed at RRT 0.53 was successfully identified as Seco Everolimus acid through LC-MS analysis and comparison with a spiked standard. This confirmed the method's capability to distinguish and characterize degradation products, making it suitable for both assay and stability studies. Overall, the method provides a scientifically sound and regulatory-compliant approach for quality control of Everolimus 2% BHT API.

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