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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING ASSAY METHOD OF EVEROLIMUS AND ITS MARKETED FORMULATION

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ABSTRACT

The High Performance Thin Layer Chromatography (HPTLC) method developed and validated according to ICH guidelines for the determination of Everolimus (Evr) in the presence of excipient and when subjected to different stress condition. Identification and determination was performed on Pre-coated silica gel G60–F254aluminium sheet (10 cm ×10 cm; thickness: 200 µm) using Toluene: Ethyl acetate: Methanol in the ration of (6:1:3) as Mobile phase at 283 nm. The linear response for Evr was observed over 50-250 ng/band (r2= 0.999) with the observed value of R_f = 0.63. Limit of Detection (LOD) = 1.707971 ng/band and Limit of Quantification (LOQ) = 5.17567 ng/band were observed. Stress degradation studies were performed to evaluate the stability indicating properties and specificity of the method. Mass spectrometry (MS), Nuclear Magnetic Resonance (NMR) and Infrared spectroscopy (IR) were studied to understand the fragmentation pattern, environment of a hydrogen atom and the functional group present in the drug. This method was used for the quantitative analysis of commercially available dosage form.

KEYWORD: Everolimus High Performance Thin Layer Chromatography; Validation, degradation pathway.

1. INTRODUCTION

Everolimus (Evr) is a 40-O-(2-hydroxy ethyl) derivative of Sirolimus which is an Antineoplastic chemotherapy drug with immunosuppressant property to prevent rejection of organ transplant. In a similar way to other mTOR inhibitor drug.[1]

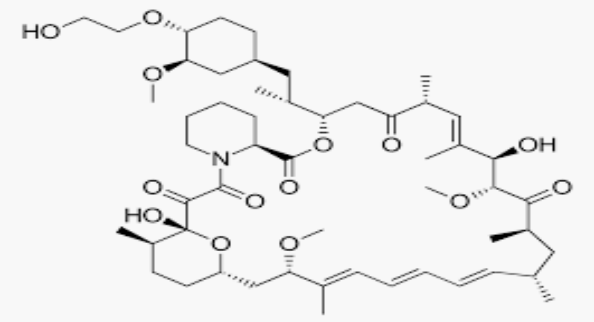

Figure 1.1: Chemical structure of everolimus.

Mechanism of this drug is it bind with high affinity to FK 506 binding protein-12, After this process drug forming a complex that inhibit the activation of mTOR.[2-8] This inhibition which leads to blockage in the process of cell from G1 phase into S phase, inducing cell growth arrest and apoptosis. Everolimus also inhibit the expression of hypoxia inducible factor which leading to a decrease in the expression of vascular endothelial growth factor. The result of everolimus inhibitor of mTOR is a reduction in cell proliferation angiogenesis and glucose intake. This drug is very potent immune suppressant used clinically in prevention of organ rejection after liver lung kidney and bone marrow transplantation.[9]

This method is very sensitive and useful for assaying everolimus for pharmacokinetic studies and therapeutic drug monitoring. Detailed analytical studies were conducted in a laboratory involving High performance thin layer chromatography to quantify everolimus and its degradant produce in stress studies. The current work includes development and validation of a simple HPTLC method for the estimation of Everolimus

2. MATERIALS AND METHODS

2.1. Instrumentation and Reagents

Evr was a gift sample from Cipla Ltd. Mumbai, India. Rolimus 5 (20 tablets) was purchased from Cipla Ltd. Mumbai, India. Analytical grade Methanol, Toluene, Glacial acetic acid, Ethyl acetate, Hydrogen Peroxide, Sodium hydroxide pellets and Conc. HCL were purchased from Merck Specialties Pvt. Ltd. Mumbai. High quality Double distilled water was used for the analysis.

The HPTLC system (Make: Camag, Model: CHF47150) was equipped with Sample Applicator (CamagLinomat V, Pressure required 3.5 bar; Dimension [360 mm x 510 mm x 410 mm {Width x Length x Height}]), Syringe (Camag 100 µL), UV – Lamp (D2 & W), Development chamber (twin-through glass chamber, 20 cm X 20 cm with stainless steel lid), TLC Scanner (Camag TLC scanner III). WinCAT's software was used to acquire and integrate data. Aluminium plates precoated with silica gel 60F254 (10 cm × 10 cm with 200 µm thickness) Merck as a stationary phase.

All the samples were weighed using Electronic weighing balance (Shimadzu AUW220D EQ-621). pH adjustment was done using pH meter (Mettler, Seven Multi) and the dissolved gas was removed using an Ultra-sonicator (Shimadzu 5.5L-150H). UV-Vis Spectrophotometer (Shimadzu UV-1800) and FT-IR (Jasco FTIR-4700) were used to determine the Absorbance/Reflectance and the various functional groups present in a drug respectively. The thermal property of the drug was determined using a TGA (Shimadzu DTG-60). Detector DLA-TGS detector for mass spectra and MS/TOF-Q mass spectrometer, 1H NMR (250 MHz, DMSO), δ (ppm) were used.

2.2. Methods

2.2.1. Preparation of sample for HPTLC analysis

Evr was weighed accurately (5 mg) and transferred in a 10.0 mL of volumetric flask, to that added 2.0 mL of methanol and sonicated for 10 minutes. Volume was makeup to 10.0 mL to obtained500 µg/mL concentration using a mobile phase (Toluene: Ethyl acetate: Methanol) in the ratio of (6: 1: 3 v/v).

2.2.2. Sample solution for assay of Evr tablet

To determine the Evr (Brand Rolimus 5, each film coated tablet contains: Evr -5mg) twenty tablets were weight to determine the average weight of the tablets and then crushed and mixed using a mortar and pestle. The powder equivalent to 5 mg Evr was weighed and transferred to10.0 ml volumetric flask, to that added 2.0 mL of methanol and sonicated for 10 minutes. Volume was makeup to 10.0 mL by using the mobile phase to obtained the 500 µg/mL concentration and filtered and spotted on HPTLC plate. The area obtained in chromatogram was correlated with regression equation and the percentage (%) label claim was calculated.

2.2.3. Development of stability indicating assay method using HPTLC for determination Everolimus in bulk and tablet dosage form

Chromatographic condition includes an Aluminium plates pre-coated with silica gel 60 F254 Merck as a stationary phase, Plate size of a 10 cm X 10 cm with a thickness of 200 µm maintained at room temperature (RT) (25 ± 5 ^{O}C), with a mobile phase Toluene: Ethyl acetate: Methanol (6:1 :3% v/v). All the samples were applied as a band with a band size of 6 mm and a saturation time of 10 minutes with ascending separation techniques and were detected at 283 nm. Nitrogen gas was purged into the system.

2.2.4. Method validation

To prove the reliability and reproducibility, the developed method validated for following validation parameters as per the ICH guidelines (Q2A (R1).[10,11]

2.2.4.1. Linearity

10 µL of different concentration of Evr was applied to HPTLC plate to deliver 50-250 ng/band. The plate was developed under optimized conditions. The linearity of the calibration curve was evaluated by linear regression analysis and the statistical data was calculated.

2.2.4.2. Accuracy

Accuracy studies were carried out by spiking known amount of Evr at 80%, 100% and 120% levels. The accuracy of the method was determined by calculating recovery of Everolimus by standard addition method. Hence 4 mg, 5 mg and 6 mg of Evr were spiked to the pre-analyzed tablet powder containing 5 mg of Evr. The sample were spotted on HPTLC plate and calculated the amount found and amount added. Further calculated the individual recovery and mean recovery values.

2.2.4.3. Precision

Precision of the method was verified by repeatability and intermediate precision studies. For repeatability, the known concentration (ng/band) for Evr was used on same days with different time interval for HPTLC studies. Intermediate precision of the method was checked by repeating the same concentrations of these studies on three consecutive days.

2.2.4.4. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ was determined by the standard deviation of the response and the slope of the calibration curve. LOD and LOQ was calculated as,

$LOD = 3.3\sigma/S$

$LOQ = 10\sigma/S$

Where,

σ = Standard deviation of the response,

S= slope of the calibration curve

2.2.4.5. Robustness

To determine the robustness of the method, the parameters such as chamber saturation time was varied in the range of (± 5 min), composition of the mobile phase i.e. toluene, ethyl acetate and methanol in the ration of 6: 1: 3. was varied in the range of (± 0.1 mL) and volume of mobile phase was varied by (± 1 mL) of the optimized method.

2.2.5. Selection of mobile phase

Aliquot portions of standard stock solutions (0.3 µL) were applied on TLC plates in the form of band (band size: 6mm). Different combination of solvent were tried

to get well separated bands i.e. Toluene, methanol in the ratio of 6:4; toluene, methanol and glacial acetic acid in the ratio of 6:4:0.1; toluene, ethyl acetate and methanol in the ratio of 6:1:3.The solvent system containing toluene, ethyl acetate and methanol in the ratio of 6:1:3 was found to obtained a good resolution.

2.2.6. Stress degradation study of Everolimus by HPTLC

Stress studies were carried out as per the ICH guidelines (Q1A (R2).[12] The Evr was subjected to different stress conditions like acidic stress; alkaline stress, oxidation; hydrolysis; photolysis and thermal stress condition.

2.2.6.1. Acidic stress degradation

In acidic stress degradation, accurately weighed 5 mg of Evr and transferred to 10 mL volumetric flask and then added 3 mL of 0.1 N hydrochloride (HCl) and kept at room temperature (RT) for 30 min. After 1 hour the solution was neutralized with 0.1 N sodium hydroxide (NaOH), and the solution was makeup to 10 ml with methanol. 0.4 µL sample was spotted on HPTLC plate under optimized conditions.

2.2.6.2. Alkaline stress degradation

In alkaline stress degradation, accurately weighed 5 mg of Evr and transferred to 10 ml volumetric flask and then added 3 mL of 0.1 N NaOH and kept at RT for 30 min. After 1 hour the solution was neutralized with 0.1 N HCl, and the solution was makeup to 10 ml with methanol. 0.4 µL sample was spotted on HPTLC plate under optimized conditions.

2.2.6.3. Oxidative stress degradation

In oxidative stress degradation, accurately weighed 5 mg of Evr and transferred to 10 mL volumetric flask and then added 3 mL of 3% H_2O_2 and kept at RT for 30 min. After 1 hour the solution was makeup to 10 mL with methanol. 0.4 µL sample was spotted on HPTLC plate under optimized conditions.

2.2.6.4. Photolytic stress degradation

In photolytic stress degradation was performed by spreading of Evr in Petri dish as a thin film separately and kept in ultraviolet chamber having a spectral distribution of 283 nm for 24 hour. After that 20 mg of Evr was added to 10 mL volumetric flask and the volume was makeup with methanol. 0.4 µl sample was spotted on HPTLC plate under optimized conditions.

2.2.6.5. Thermal stress degradation

Thermal stress degradation was performed by spreading Evr in Petri dish as a thin film separately and exposed to 80 °C for 30 min. From this 5 mg of Evr was added to 10 ml volumetric flask and volume was make up with methanol. 0.4 µL sample was spotted on HPTLC plate under optimized conditions.

2.2.6.6. Sample preparation for isolation of alkaline DP

The sample applied on HPTLC plate for isolation of DP was prepared by dissolving 20 mg of Evr in 10 mL of methanol. The prepared solution was then subjected to the alkaline stress degradation, until major degradation product was form in alkaline condition of degradation.

2.2.6.7. Isolation of major alkaline degradation product

The alkali induced degradation sample applied on precoated TLC plate in continuous band form. 2.1 µL of volume was applied. Further plates were allowed to run in mobile phase. After densitometric analysis, the major degradant was isolated from developed plates and subjected to analysis. The isolated degradant was subjected to MS analysis. The obtained spectrum was interpreted on the basis of mass to charge ratio.

2.3. Characterization

2.3.1. Fourier Transform Infrared (FT-IR) Spectroscopy

Functional groups was characterised using Fourier transform Infrared spectroscopy (JASCO FTIR—4700).[13-15] Potassium bromide (KBr) pellet was made by applying hydraulic pressure and the sample was ground along with potassium bromide. Pellet was prepared by applying pressure of 100 kpa for two min. All spectra was collected in the wavelength range of 4000—500 cm^{-1} and recorded by transmission mode with a 4cm^{-1} resolution time and 32 scans.

2.3.2. Thermo Gravimetric Analysis (TGA)

The Mass of the sample is measured over the time with temperature changes using TGA (Shimadzu, Differential Thermogravimetric DTG-60).[17] An External magnetic field is induced to manage the magnetic loss due to heating. Platinum pan was used to avoid cross-reactivity. 5mg of the sample weight is maintained. Copper sulphate was used as a reference.

2.3.3. MS/TOF and NMR studies

The mass spectra help to understand the fragmentation of the drug. The ionization source was used as Electro - Spray ionization (ESI) with range of 50 – 800 m/z. Detector DLA-TGS detector for mass spectra and MS/TOF-Q mass spectrometer were used. 10 µg mL^{-1} of concentration of sample were injected and obtained mass spectra were interpreted. Samples were also subjected to NMR analysis using same parameters with solvent chloroform-d was used, which were optimized for drug Evr.

3. RESULTS AND DISCUSSIONS

3.1. Selection and preparation of mobile phase

In order to determine best condition for the effective separation, mobile phase containing various ratios of toluene, ethyl acetate, methanol and glacial acetic acid were initially used. But Everolimus did not show proper development. Mobile phase consisted toluene: ethyl

acetate: methanol (6:1:3) showed good separation and peak shape. Everolimus showed considerable absorbance at 283nm. So the wavelength 283nm was selected for evaluation as shown in Figure 3.1 and 3.2.

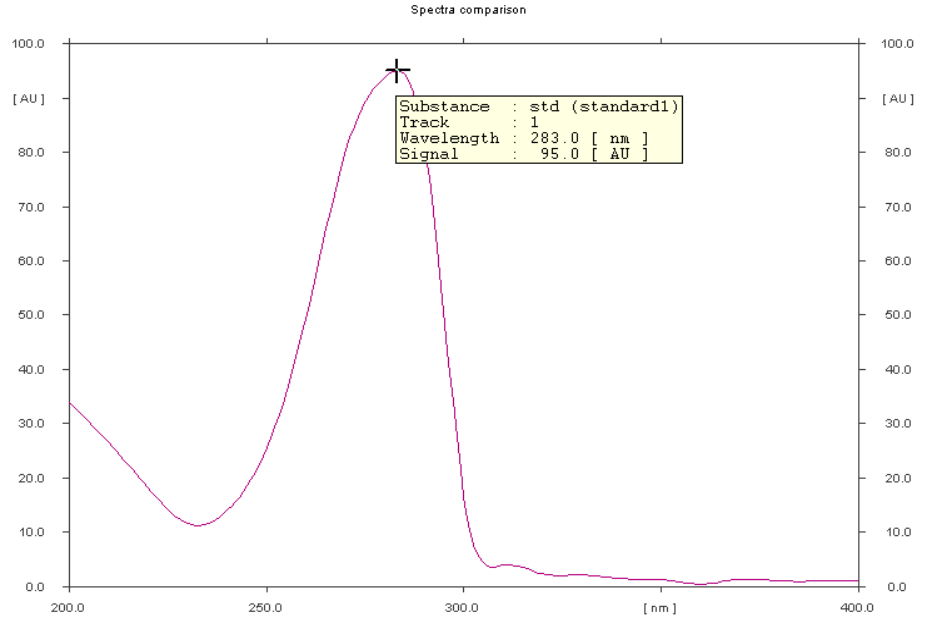

Figure 3.1: Spectra of everolimus.

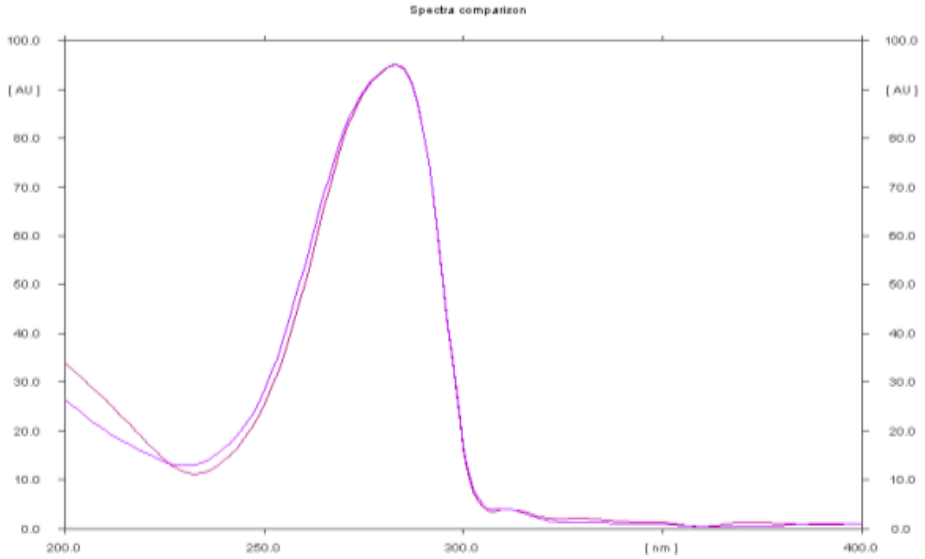

Figure 3.2: Overly spectra Standard + Sample.

3.2. Method Development and Validation

The developed method was validated for following validation parameters such as linearity, precision, accuracy, range, LOD, LOQ and robustness.

The standard curve of Everolimus was found to be in concentration range of 50—250 ng/band as shown in Figure 3.3. Correlation coefficient (r2) and slope for Everolimus was found to be 0.99919 and 14.057 respectively data is shown in (Table 3.1). The Spectra of Everolimus using Wincats software shown in Figure 3.4 and 3.5. The limits of detection and quantification were found to be 1.70797 ng/band and 5.17567 ng/band, respectively as shown in (Table 3.1). The relative standard deviation % (RSD) values for Repeatability and intermediate precision were found to be less than 2, which indicate that method is précised.

The accuracy of the method was determined by calculating the recoveries of Everolimus by the standard addition method at three concentration levels viz. 80, 100, 120 µg/mL^{-1}. The Mean recovery was found to be 100.1344 %. Shown in Table 3.2.

Table 3.1: Linearity data and LOD and LOQ.

	Concentration (ng/band)	Area	R_f
Drug Everolimus Linearity	50	680.54	0.67
	100	1456.81	0.64
	150	2149.77	0.63
	200	2679.07	0.63
	250	3483.62	0.63
Linearity range		50—250 ng/band	
Regression equation		Y = 21.436 + 14.057x	
Correlation coefficient		r2 = 0.99919	
Slope		14.057	
Intercept		21.436	
Limit of Detection Limit of Quantification		1.70797 ng/band 5.17567 ng/band	
Repeatability	% Mean	S.D. % RSD	
	99.54333	0.466333 0.468	
Intermediate Precision	% Mean 99.49333	S.D. % RSD 0.505333 0.507667	

The Robustness result of change in saturation time (±5min) of Everolimus, change in Mobile phase ratio of Everolimus and change in amount of mobile phase (±1ml) Everolimus shown in Table 3.3. These were studied to find out the robustness of the proposed method %RSD was found to be less than 2%.

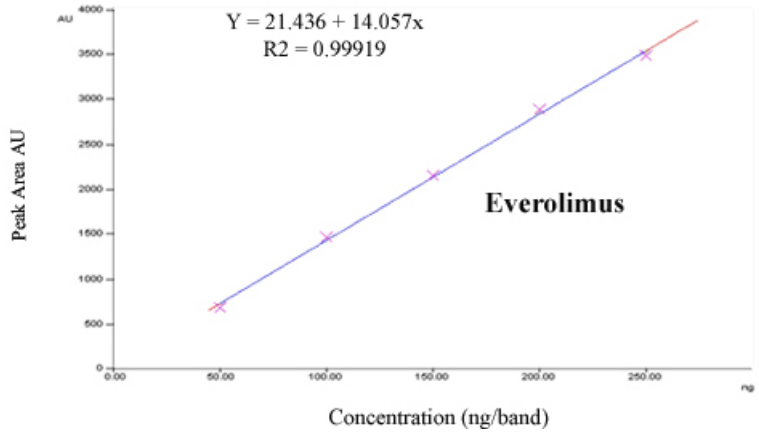

Figure 3.3: Linearity plot of everolimus.

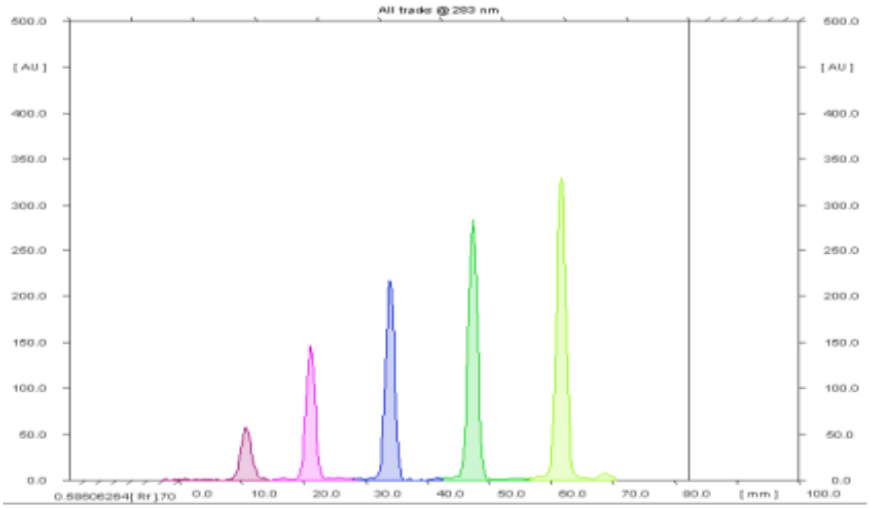

Figure 3.4: 2D Calibration linearity graph of everolimus using wincats software.

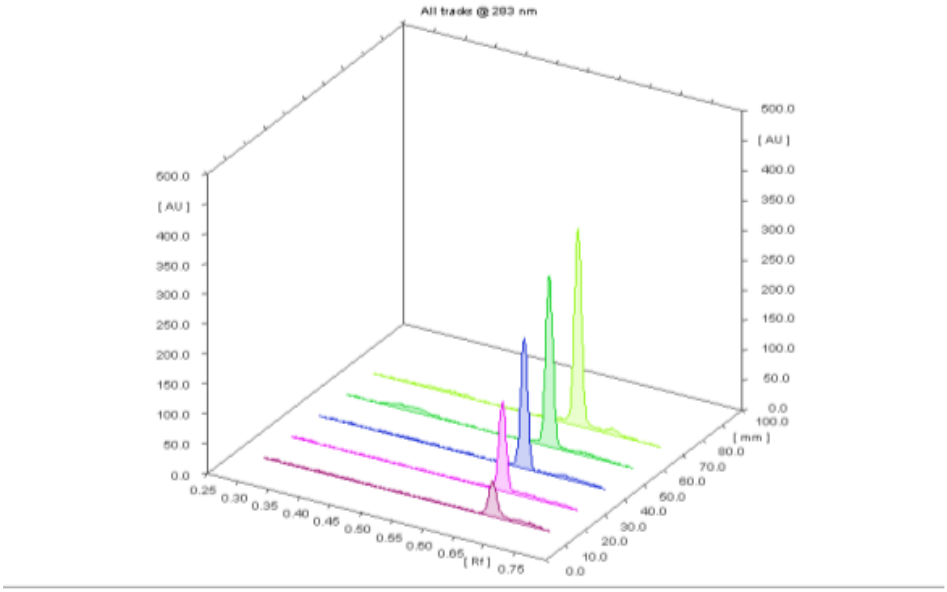

Figure 3.5: 3D Calibration linearity graph of everolimus using wincats software.

Table 3.2: Result of Accuracy and Statistical values.

Conc. Level (%)	Weight of tablet powder taken (mg)	Amount of drug added (mg)	Amount of drug recovered (mg)	% Recovery
80	150	4	4.03	99.55
	150	4	4.05	99.32
	150	4	3.99	99.00
100	150	5	5.04	100.8
	150	5	5.01	100.3
	150	5	5.02	100.4
120	150	6	6.02	100.49
	150	6	6.03	100.52
	150	6	6.05	100.83
Mean				100.1344
S.D				0.6700
% R.S.D				0.66914
S.E				0.2233

Table 3.3: Robustness result of everolimus.

Factor	Chromatographic changes		
	Level	Peak area	Rf Values
Duration of Chamber (±5min)			
5 min	-5	2041.1	0.64
10 min	0	2161.31	0.63
15 min	+5	2279.61	0.67
	RSD	0.676667	
Amount of Mobile Phase (±mL)			
09	-1	2085.40	0.54
10	0	2181.86	0.61
11	+1	2284.68	0.63
	RSD	0.66	
Mobile Phase composition			
6:0:4	±1	2141.95	0.60
6:2:2	0	2277.87	0.61
5:1:4	±1	2386.147	0.59
	RSD	0.60	

3.3. Analysis of marketed tablet formulation

The percentage label claim of Everolimus was found to be 100.38% respectively. Results of Everolimus shown in Table 3.4.

Table 3.4: Results of analysis of tablet formulation.

Brand: Rolimus 5 Each film coated tablet contains: Everolimus -5mg			
Sr. No.	Weight of tablet powder taken (mg)	Amount of pure drug estimated (mg)	% Label claim
1	150	5.04	100.82
2	150	5.01	100.24
3	150	4.96	99.37
4	150	5.04	100.90
5	150	5.01	100.62
6	150	5.03	100.38
Mean % Label Claim		100.38%	
S.D.		0.558	
% RSD		0.54	

3.4. Stress studies

The densitograms obtained for Everolimus under different stress conditions are shown in (Figure 3.6 to 3.11) and the percentage degradation is shown in Table 3.5.

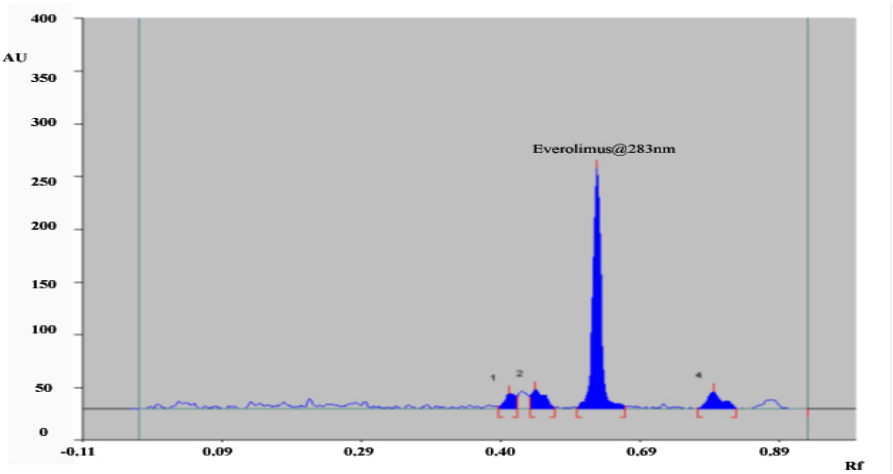

Figure 3.6: HPTLC densitogram of Everolimus in acidic condition.

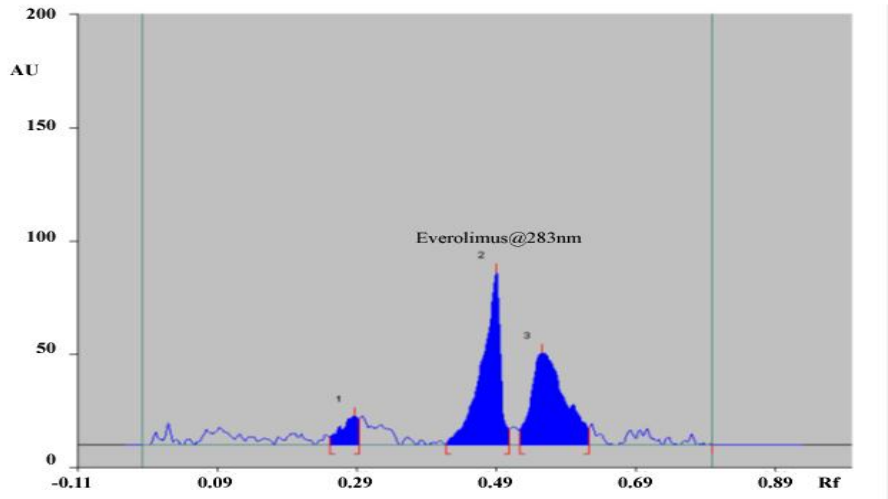

Figure 3.7: HPTLC densitogram of Everolimus in alkaline condition.

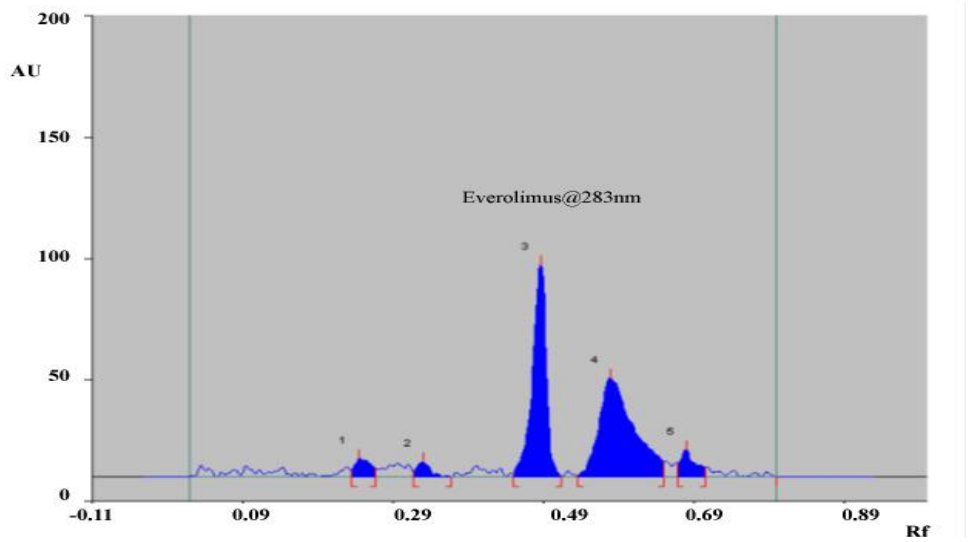

Figure 3.8: HPTLC densitogram of Everolimus in hydrogen peroxide.

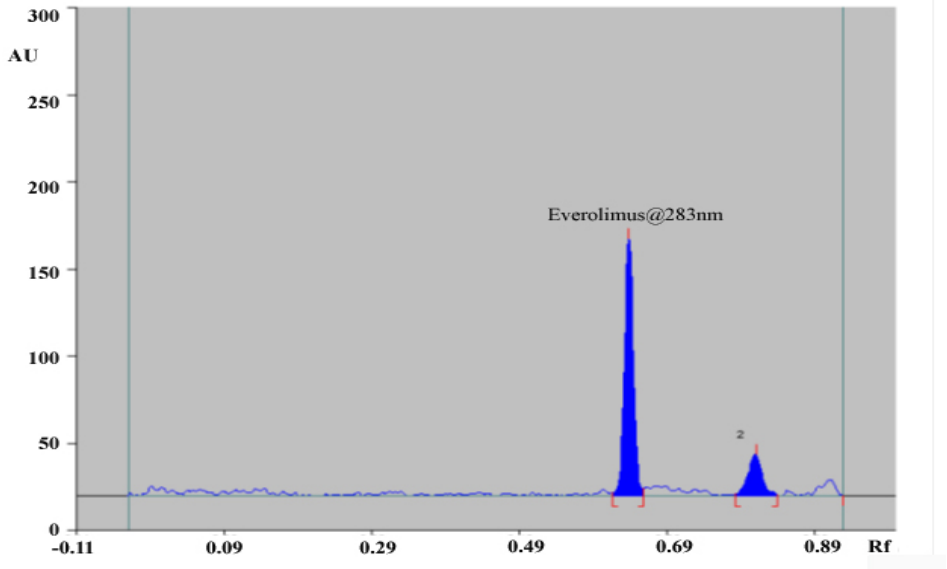

Figure 3.9: HPTLC densitogram of Everolimus in thermal condition.

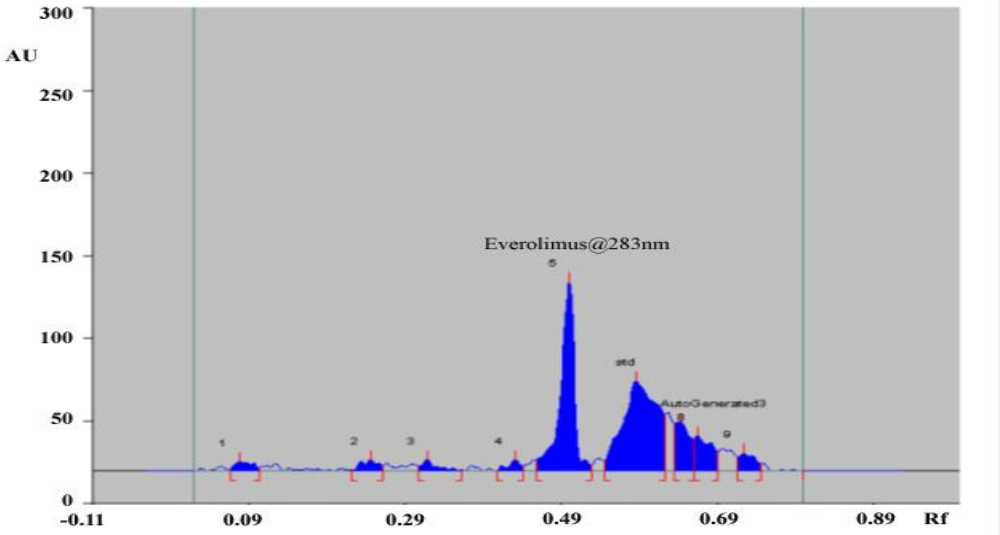

Figure 3.10: HPTLC densitogram of Everolimus in photolytic condition.

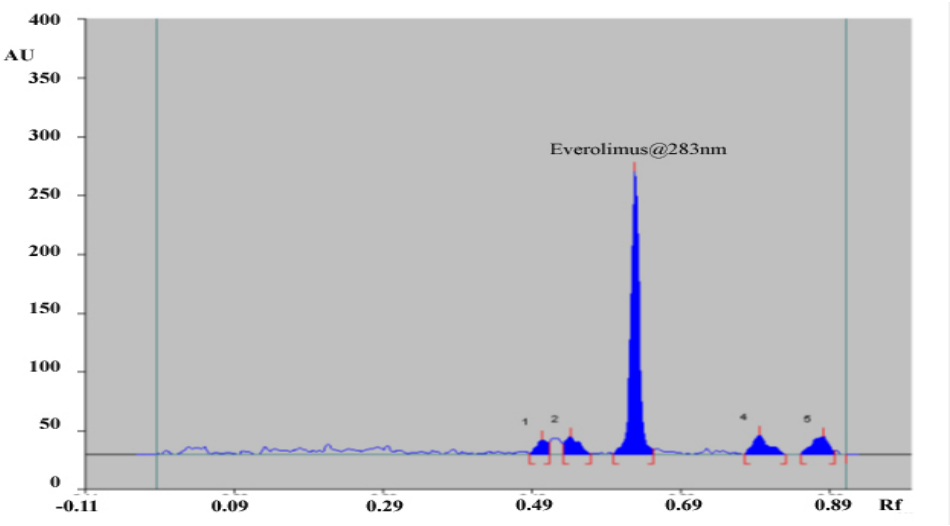

Figure 3.11: HPTLC densitogram of everolimus in neutral condition.

Table 3.5: The results of the stress degradation studies of everolimus.

Sr. no.	Stress test Conditions	Solvents	Temp.	Time	% Degradation
1	Acidic	0.1 N HCl	RT	30 min	08 %
2	Alkaline	0.1 N NaOH	RT	30 min	19.5 %
3	Oxidative	3% H_2O_2	RT	30 min	16 %
4	Thermal	--	60^{O}C	30 min	5.4 %
5	Photolytic	--	UV light	24 hr.	7.31 %
6	Hydrolytic	Distilled Water	RT	30 min	6.1 %

3.5. Thermal analysis

The drug was thermally stable up to 190.22^{O}C with the flow rate of 10^{O}C min^{-1}. The TGA curve shows that the percent weight loss was found to be 68.285% as shown in Figure 3.12.

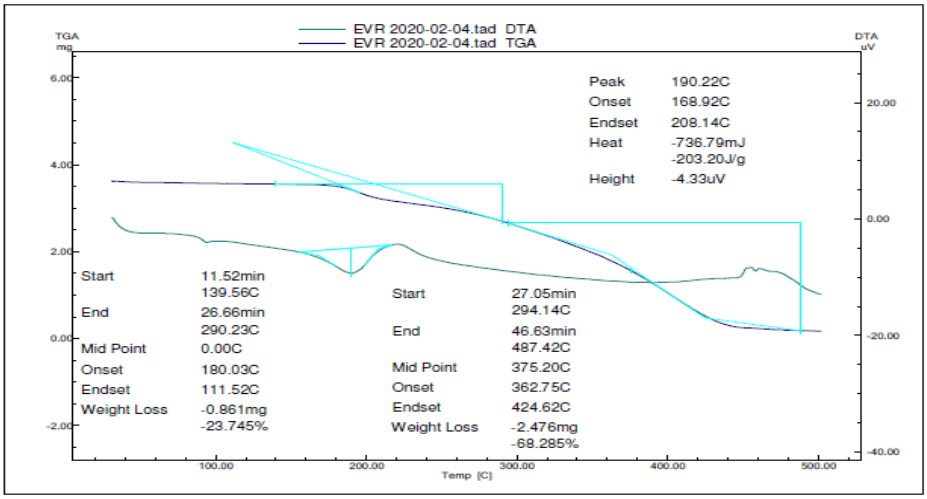

Figure 3.12: DSC and TGA curves of Everolimus.

3.6. Pathway of alkali degradation

The degradant are shown in Pathway of alkali degradation of Everolimus Figure 3.13 and Table 3.6.

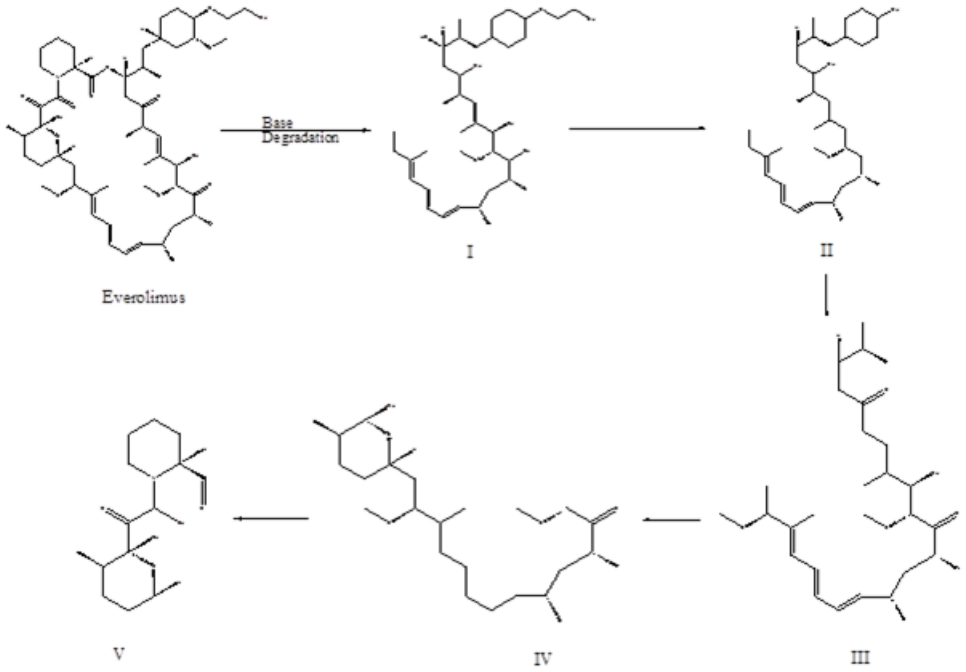

Figure 3.13: Pathway of alkali degradation of everolimus.

Table 3.6: Molecular Weight and Molecular Formula of degradant of alkali degradation.

Degradant No.	Molecular weight and Formula	Comment
I	$C_{37}H_{70}O_6$ [634]	Loss of CH_2CH_2OH {Cleavage at C_{15} and C_{34}}
II	$C_{35}H_{68}O_3$ [559]	Reduction and Hydrolysis
III	$C_{29}H_{456}O_5$ [507]	Reduction
IV	$C_{24}H_{46}O_3$ [437]	Reduction
V	$C_{14}H_{24}NO_5$ [309]	Cleavage at C_1 and C_{15}

3.7. Infrared absorption spectrophotometry; MS and NMR

The pure drug was subjected to FT-IR, the obtained spectra (Figure 3.14) which shows the characteristic functional group present in the sample mentioned in the Table 3.7

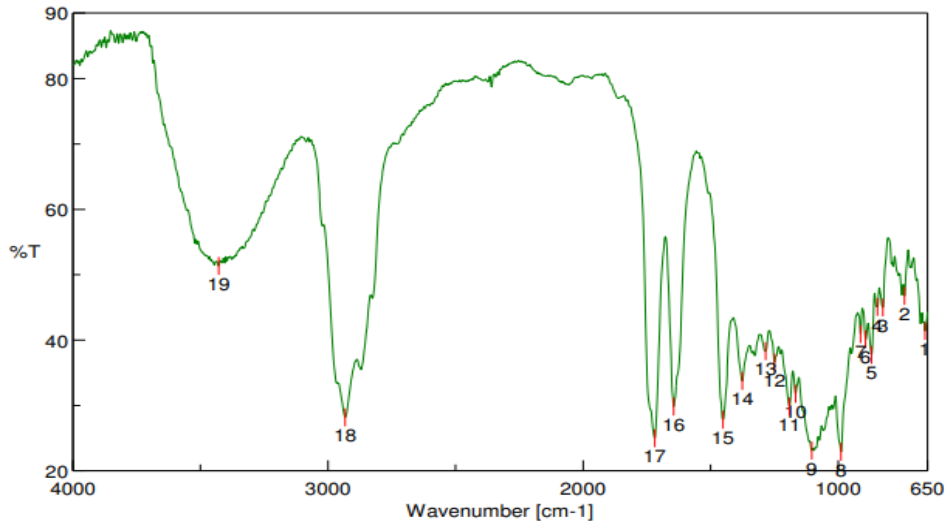

Figure 3.14: FT-IR spectrum of everolimus.

Table 3.7: Interpretation of FT-IR Spectra of everolimus.

Observed frequency (cm-1)	Functional group
3428	O-H Stretch
2933	C-H Stretch
1725	C=O Stretch
1735	C=C ring Stretch
1284	C-N Stretch

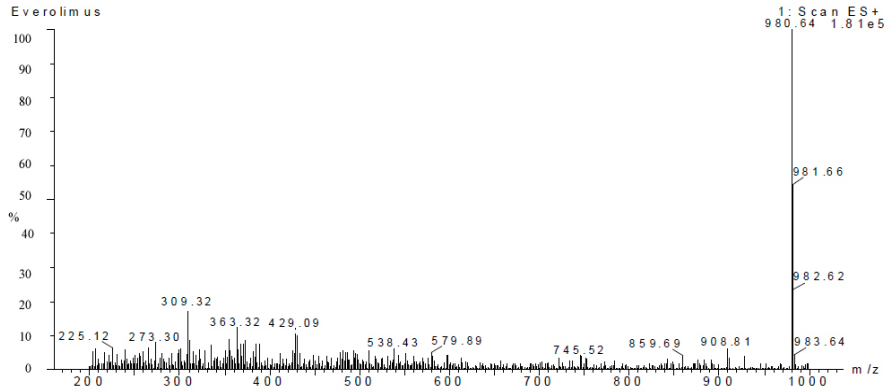

Figure 3.15: MS Spectra of everolimus.

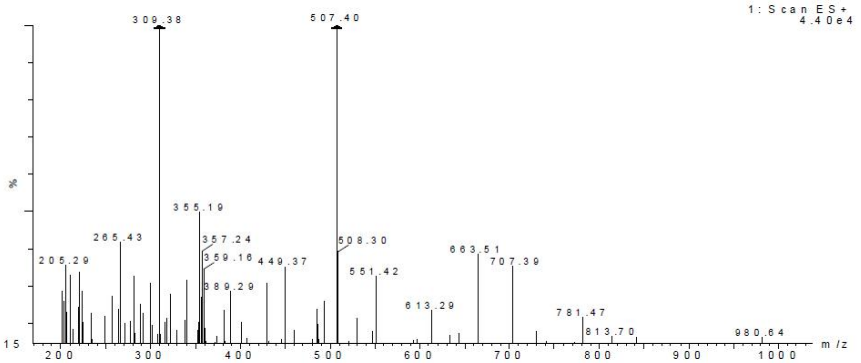

Figure 3.16: MS Spectra of degradant obtained by Alkali stress condition

The alkaline stress degradation was performed as shown in Figure 3.13 and Table 3.6, the degradant product was characterized using MS. The degradant products were identified using an m/z ratio with the comparison with standard Everolimus m/z ratio. Figure 1.1. and Figure 3.15. Shows the structure and Mass spectra of everolimus respectively. When the alkali stress condition was applied, based upon the structure hydrolysis of enol linkage occurs, ring opening and loss of water molecule as shown in Figure 3.16. and Table 3.6. The molecular weight 559 ($C_{35}H_{68}O_3$) and 437($C_{24}H_{46}O_3$) were found in base hydrolysis.

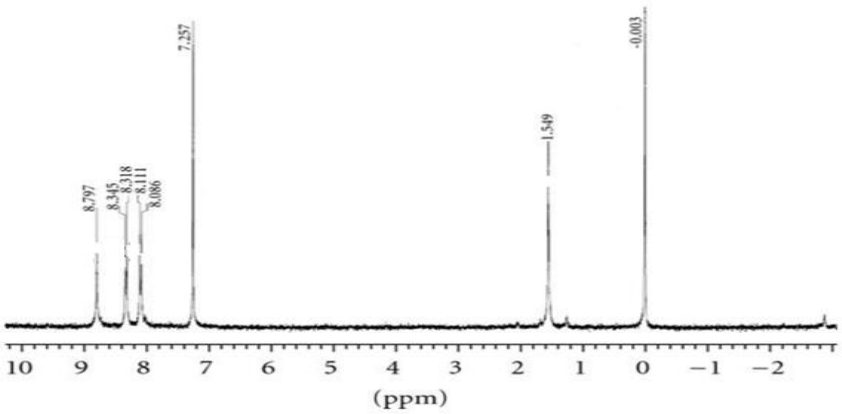

Figure 3.17: NMR Spectra of standard everolimus.

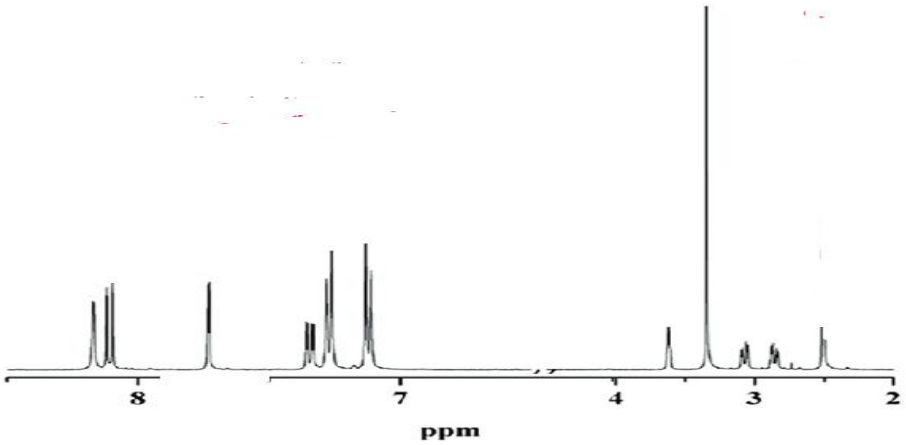

Figure 3.18: NMR Spectra of degradant obtained by Alkali stress condition.

The NMR spectra of the pure drug and degradation by alkali stress condition are shown is Figure 3.17 and 3.18. The structure elucidation was done with the help of IR, Mass and NMR data and also its degradant was studied.

4. SUMMARY AND CONCLUSION

A quick, precise and accurate method based on HPTLC has been developed for analysis of Everolimus. The method was developed and validated for the determination of Everolimus on precoated silica gel HPTLC plates using Toluene : Ethyl acetate : Methanol (6:1:3 v/v) as a mobile phase with Densitometric detection at 283 nm. The method was validated for linearity, precision, accuracy and robustness. Linearity range for Everolimus was found 50—250 ng/band Correlation coefficient was 0.99919.The developed method was precise and robust, % RSD was found less than 2% And % recovery was found to be in range of 98.-102%. LOD and LOQ were 1.70797 ng/band and 5.17567 ng/ band respectively.

Stress degradation studies were performed to evaluate the stability indicating properties and specificity of the method. Degradation study was carried out by exposing of working standard solution of Everolimus with acid (0.1N HCL at RT), base (0.1 N NaOH at RT), hydrogen peroxide (3% H_2O_2), Distilled water (H_2O) for 30 min. while one volumetric flask was exposed to (60^0 C) for 20 min. and one volumetric flask was exposed to UV light (283 nm) for 24 hours. The degradation was found to be (08%, 19.50%, 16.00%, 06.10%, 05.40% and 07.31%) respectively. To study the structure of drug and its degradant the sample was subjected to MS/TOF and NMR. Hence, it can be concluded that the developed method is accurate, precise and selective and can be employed successfully for the estimation of Everolimus in tablet formulation.

5. REFERENCES

1. Lebwohl D, Anak O, Sahmoud T, et al. Development of everolimus, a novel oral mTOR inhibitor, across a spectrum of diseases. *Ann N Y Acad Sci*, 2013; 1291: 14–32.
2. Pape L, Offner G, Kreuzer M, Froede K, Drube J, Kanzelmeyer N, *et al*. *De novo* therapy with everolimus, low-dose ciclosporine A, basiliximab and steroid elimination in pediatric kidney transplantation. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg, 2010; 10: 2349–54.
3. Bemelman FJ, de Fijter JW, Kers J, Meyer C, Peters-Sengers H, de Maar EF, *et al*. Early conversion to prednisolone/everolimus as an alternative weaning regimen associates with beneficial renal transplant histology and function: the randomized-controlled MECANO trial. Am J Transplant, 2017; 17: 1020–30.
4. Budde K, Becker T, Arns W, Sommerer C, Reinke P, Eisenberger U, *et al*. Everolimus-based, calcineurin-inhibitor-free regimen in recipients of *de-novo* kidney transplants: an open-label, randomised, controlled trial. Lancet, 2011; 377: 837–47.
5. Kacar S, Gurkan A, Karaca C, Varılsuha C, Tilif S. Low-dose calcineurin inhibitor regimen combined with mammalian target of rapamycin inhibitors preserves kidney functions in renal transplant recipients without allograft nephropathy. Transplant Proc, 2010; 42: 3513–6.
6. Ferraresso M, Belingheri M, Ginevri F, Murer L, Dello Strologo L, Cardillo M, *et al*. Three-yr safety and efficacy of everolimus and low-dose cyclosporine in *de novo* pediatric kidney transplant patients. Pediatr Transplant, 2014; 18: 350–6.
7. Lin M, Mittal S, Sahebjam F, Rana A, Sood GK. Everolimus with early withdrawal or reduced-dose calcineurin inhibitors improves renal function in liver transplant recipients: a systematic review and meta-analysis. Clin Transplant, 2017; 31: e12872.
8. de Fijter JW. Cancer and mTOR inhibitors in transplant recipients. Transplantation, 2017; 101: 45–55.
9. Atkins MB, Yasothan U, Kirkpatrick P. Everolimus. Nat Rev Drug Discov, 2009; 8: 535-6.
10. ICH. Guidance for industry: Q2B validation of analytical procedures: methodology. International

Conference on Harmonisation of Technical Requirements for Registration Tripartite Guideline, (November), 1996; 13.
11. Food and Drug Administration. Guidance for Industry: Bioanalytical Method Validation. U.S. Department of Health and Human Services, (May), 2001; 4–10.
12. ICH. Q1A (R2) Harmonised Tripartite Guideline. Stability Testing of New Drug Substances and Products. Geneva: International Conference on Harmonization, 2003; 1-18.
13. Berthomieu, Catherine & Hienerwadel, Rainer. Fourier transforms infrared (FTIR) spectroscopy. Photosynthesis research, 2009; 101: 157-70. 10. 1007/s11120-009-9439-x.
14. Ayala I, Range K, York D, Barry BA Spectroscopic properties of tyrosyl radicals in dipeptides. J Am Chem Soc, 2002; 124: 5496–5505.
15. Barth A Infrared spectroscopy of proteins. Biochim Biophys Acta, 2007; 1767: 1073–1101.
16. Schick, Christoph. Differential scanning calorimetry (DSC) of semicrystalline polymers. Analytical and bioanalytical chemistry, 2009; 395: 1589-611. 10.1007/s00216-009-3169-y.
17. Ng, H.M. & mohamad saidi, Norshahirah & Omar, Fatin & Kasi, Rameshkasi & T subramaniam, Ramesh & Baig, Shahid. Thermogravimetric Analysis of Polymers, 2018; 1-29. 10.1002/0471440264.pst667.