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Original Article

STABILITY INDICATING HIGH-PERFORMANCE THIN LAYER LIQUID CHROMATOGRAPHIC METHOD FOR EVEROLIMUS

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ABSTRACT

Objective: Developing and validating a stability-indicating method for everolimus by HPTLC and depiction of degradation product of in alkaline conditions by LC-MS.

Methods: The chromatographic separation was performed on aluminium plates pre-coated with silica gel 60 F_{254} as the stationary phase using Toluene: Methanol: Ethyl Acetate (6:2:2*v/v/v*) as the mobile phase. The evaluation was carried out at 277 nm. For the developed stability indicating method, the ICH Q2 (R1) guidelines were used for validation. Stress degradation studies like hydrolysis under different pH conditions, photolytic degradation, thermal degradation and oxidative degradation as per ICH Q1A (R2) and Q1B guidelines were performed. LC-MS analysis was carried out for the standard everolimus and its alkaline degradation sample using TOF analyser and the degradation pathway was proposed for each degradation product.

Results: The Rf value of everolimus was found to be 0.63±0.03. The response was quite linear over the concentration range of 100-500 ng/band, with the regression coefficient value of 0.9921. Under alkaline hydrolytic conditions, everolimus was analyzed using Liquid Chromatography-Mass Spectrometry (LC-MS). The Retention Time (RT) and prominent mass fragmentation (m/z) for the everolimus standard were observed at 9.46 min with m/z values of 980.56 and 908.54. For the degradation products, DP-1 showed an RT of 8.88 min with m/z values of 349.23, 403.24, 574.33, and 646.35, while DP-2 exhibited an RT of 9.10 min with m/z values of 926.55, 614.32, and 542.30. These data were used to propose the structures of the degradation products.

Conclusion: The proposed method can conveniently be applied for quantitative analysis of everolimus on routine basis and for stability testing under different stress environments.

Keywords: Everolimus, HPTLC, LC-MS, Forced degradation, Stability indicating

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INTRODUCTION

Everolimus is a rapamycin (sirolimus) derivative. Similar to rapamycin, it suppresses mTOR (mammalian target of rapamycin). Everolimus is a yellowish powder that has a molecular weight of 958.22 g/mol. Its melting point is between 189 and 190 C, and water barely slightly dissolves it. It dissolves readily in acetone, methanol, and ethanol. It is a substance that needs to be kept in a container that is tightly closed and protected from light and moisture. Chemically, everolimusis (1S,9R,15R,16E,18R,19R,21S, 23R,24E,26E,30S,32R,35S)-1,18-dihydroxy-12-[(2S)[(1S,3R,4R)-4-(2-hydroxyethoxy)-3-methoxycyclohexyl]propan-2-yl]-19,30-dimethoxy-15,17,21,23,29,35,-hexamethyl-11,36-dioxa4azatricyclo [30.3.1.04,9]hexatriaconta-16,24,2–tetraene-2,3,10,14,20-pentone and empirical formula is $C_{55}H_{83}NO_{14}$[1,2].

The drug everolimus is in present date applied in organ transplant treatment rejection serving role of an immunosuppressant. Everolimus has the same effect as other mTOR inhibitors in that it only affects the mTORC1 protein. The prime disease for everolimus administration is kidney cancer [3]. Everolimus binds to FKBP-12 and inhibits mTOR, which stops cell proliferation and induces apoptosis. Additionally, it reduces angiogenesis, cell proliferation, and glucose uptake by decreasing the expression of hypoxia-inducible factor and vascular endothelial growth factor [4, 5]. In context to breast cancer, the USFDA had authorized everolimus in a mixture with exemestane after letrozole or anastrozole treatment failure [6]. Therefore, aim of the study was to develop a method for the rapid and accurate estimation of everolimus and access its stability using high-performance thin-layer liquid chromatography.

MATERIALS AND METHODS

Instrumentation

The HPTLC system CAMAG, which includes the TLC Scanner III, the Linomat 5 applicator, the software WinCATS (version 1.4.3), the Microliter syringes Hamilton (100 l), the TLC plates (Merck's aluminium TLC plate pre-coated with silica gel 60F254), and the Twin trough glass chamber, are the instruments used in this approach. Additional equipment includes a UV-visible spectrophotometer (JASCO, Model-V730), an electronic balance (Shimadzu, Model AXT224R), a sonicator (PRAMA, Model SM15 US), a hot air oven (BIOMEDICA), and a photo-stability chamber (Newtronic, Model-IC DAC version 1.2). Agilent 1260 (Infinity II) with Agilent 6540 UHD Accurate-Mass Q-TOF LCMS was the system used for LC/MS. It contained an Agilent (HPH-C18) column with measurements of 4.6 x 100 mm and 2.7 micron.

Chemicals

Everolimus was a gift sample from NATCO Pharmaceuticals; Methanol (AR and HPLC grade), chloroform (AR grade), ethyl acetate (AR grade), HCl (AR grade), sodium hydroxide (AR grade), and 30% v/v H_2O_2 (AR grade), are bought from LOBA CHEMIE Pvt. Ltd., Mumbai.

Preparation of solutions

Everolimus was soluble in methanol. Hence, methanol was selected for preparing different sample solutions.

- **Preparation of standard stock solution**

About 10 mg of everolimus was weighed and then put into a 10 ml

volumetric flask. To make stock solution standard of 1000 µg/ml, it was dissolved in methanol and the volume was adjusted.

- **Preparation of standard working solution**

The final solution of 20 µg/ml was properly diluted from the everolimus sock solution standard of 1000 µg/ml.

Selection of analytical wavelength

The solution of everolimus (20 µg/ml) was prepared using methanol and UV spectrum was recorded. It showed maximum absorbance at 277 nm. UV spectrum is shown in fig. 1.

Optimization of chromatographic conditions

On aluminium plates (10 cm 10 cm with a 250 m layer thickness silica gel 60 F254), pre-coating chromatographic separation of the medication everolimus was carried out. Using a 100 l syringe and linomat applicator, samples were spot-applied to the plate as a band of 4 mm in width. Toluene: Methanol: Ethyl Acetate (6:2:2 v/v/v) made up the mobile phase. For the linear ascending development of a TLC plate with a 10 min saturation time and a 70 mm migration distance, a twin trough glass chamber (dimensions 10cm* 10cm) was used. At 277 nm, densitometric scanning was carried out using the WinCATS software and a deuterium lamp as the radiation source. Fig. 2 displays a typical densitogram for everolimus.

Forced degradation studies

The degradation conditions were as per ICH guidelines Q1A (R2). Factors such as reagent strength and the exposure time were optimized to obtain 10-30 % degradation. The optimized conditions are as follow [13, 14].

Acid hydrolysis

Everolimus working solution (200 µg/ml), 1 ml, 1 ml of 0.1 N HCl, and 10 ml of methanol were mixed to create the final volume. After 30 min at room temperature, the 20 µg/ml resultant solution of volume 10 µl was spotted on a TLC plate and developed using the best mobile phase.

Alkali hydrolysis

At first the working solution of 200 µg/ml concentration showed no peaks at Rf of drug and used to get consumed instantly; hence a ten times higher concentration was prepared. To obtain final solution of 10 ml, a ten times of 200 µg/ml dilution was made with 1 ml of 2000 µg/ml everolimus solution was combined with 1 ml of 0.1 N NaOH and methanol. Instantly, at room temperature, 10 µl volume of this solution was applied on TLC plate and developed using the mobile phase.

Oxidative degradation

Everolimus working solution 1 ml of strength 200 µg/ml was combined with 1 ml of 3% H_2O_2 *v/v* to make a total volume of 10 ml. From the resulting (20 µg/ml) solution applied 10 µl on to a TLC plate and scanned at 277 nm after 30 min reaction time at room temperature.

Neutral degradation

A 200 µg/ml everolimus solution was divided into 1 ml, 1 ml of water, and 10 ml of methanol. 10 µl of the resulting (20 µg/ml) solution were spotted on a TLC plate after 30 min at room temperature and developed using the best mobile phase.

Photolytic degradation

A solid everolimus sample was exposed to UV light till exposure of 200 watt hours per square metre (UV degradation) and to cool white fluorescent light up to 1.2 million lux h (Fluorescence degradation) in a photo stability chamber in accordance with the ICH Q1B Guidelines. Methanol was used to generate a 20 µg/ml everolimus solution for both. HPTLC was used to analyse the resultant solutions.

Thermal degradation

To put the bulk drug under thermal stress, it was placed in an oven set at 60 °C for 1 h. This was selected as the forced degradation condition. The sample was removed from the oven, allowed to cool to room temperature, weighed, and then diluted with methanol to a final concentration of 20 µg/ml of 10 µl. It was then placed on a TLC plate and submitted to an analysis under the optimum chromatographic conditions.

Validation of HPTLC method for everolimus

According to ICH Q2 (R1) recommendations, the developed HPTLC technique for everolimus was validated by means of linearity and range, accuracy, specificity, limit of detection, limit of quantitation, repeatability, and intermediate precision and robustness [15].

Specificity

Peak purity profiling tests and assays were conducted to assess the method's specificity. WinCATS software's spectral detection was used to determine the peak purity under all deterioration scenarios.

Linearity and range

Everolimus standard (20 µg/ml) was applied on the TLC plate of the volume 5, 10, 15, 20 and 25µl, thus leading to spotted amounts. The range was 100-500 ng/band. After being saturated in an optimised mobile phase for 10 min, the plate was developed, and this process was repeated five times. For linearity, the 3D densitogram was displayed in fig. 8. Calibration curve here was created by graphing the peak area in fig. 9 against the amount of drug observed (ng/band).

Assay

Everolimus is offered by Novartis Pharmaceuticals Corporation under the brand names Afinitor (2.5 mg, 5 mg, and 10 mg Tablets) and Zortress (0.25%, 0.5 mg, 0.75 mg, and 1 mg Tablets). A boosted blend was created because the advertised composition was unavailable in the local market.

Preparation of spiked blend: About 450 mg starch and 450 mg lactose was mixed in the mortar pestle. Then 100 mg of everolimus was mixed with the above excipients. From this spiked blend, 100 mg of blend which is to 10 mg of drug was weighed and diluted to 10 ml with methanol to obtain a solution (1000 µg/ml). The solution was sonicated and then filtered using whatman filter paper. Further diluted to get 20 µg/ml working solution. Application of sample solution individually was done to a TLC plate in a 10 µl volume, and later the developed plate with mobile phase was scanned at 277 nm, with the peak area recorded.

Accuracy

Recovery study was done at 80%, 100% and 120% level applying standard addition method. The standard drug everolimus was added to the pre-analysed sample solution at three levels. A 200 ng/band strength of everolimus was chosen. The peak areas obtained after the development of plate were extrapolated from standard linearity to calculate recovered amount.

Precision

Studies on repeatability and intermediate precision helped develop the precision approach. In repeatability precision, the application of six replicates of standard solution of strength 20 µg/ml was spotted on TLC plate on the same day after some time interval. In intermediate precision, the application of six replicates of standard solution of strength 20 µg/ml was spotted for 3 consecutive days on TLC plate.

Limit of detection and limit of quantitation

LOD=3.3 σ/S and LOQ=10 σ/S, respectively, where σ is the standard deviation of the responses at lowest concentration and S is calibration curve slope, were used to compute the LOD and LOQ. The formula approach was used in the calculation.

Robustness

By making small, deliberate modifications to the mobile phase ratio, saturation duration, and detecting wavelength, the new method's resilience was examined. With the intention of creating Toluene: Methanol: Ethyl Acetate in the ratios of 5.8:1.8:2.4 and 6.2:2:1.8

v/v/v, saturation time was between 5 and 15 min. The detection wavelength was changed between 275 nm and 279 nm, or by around 2 nm. The factors were changed one at a time. The application to development time and development to scanning time were taken into account for robustness right away and after two hours. Everolimus was utilized at a concentration of 200 ng/band in order to examine the impact of all variables on the drug's peak area.

RESULTS

The methanolic solution showed maximum absorbance at 277 nm. Fig. 1 represents the UV spectrum of everolimus.

Chromatographic conditions like saturation time, band length, detection wavelength, stationary phase and mobile phase were optimized and summarized in table 1. Fig. 2 depicts the densitogram of standard everolimus (200 ng/band).

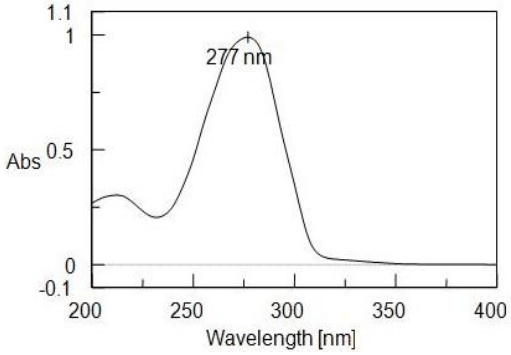

Fig. 1: UV spectrum of everolimus (10 µg/ml)

Table 1: Optimized chromatographic parameters

Parameters	Details
Stationary Phase	Merck's TLC plate Silica Gel 60 F_{254}precoating
Mobile Phase	Toluene: Methanol: Ethyl acetate (6:2:2*v/v/v*)
Development Chamber	Twin-Though Glass Chamber (10X10 cm)with stainless steel lid
Saturation time	10 min
Bandwidth	4 mm
Detection Wavelength	277 nm
Separation Technique	Ascending
Scanning Mode	Absorbance/Reflectance

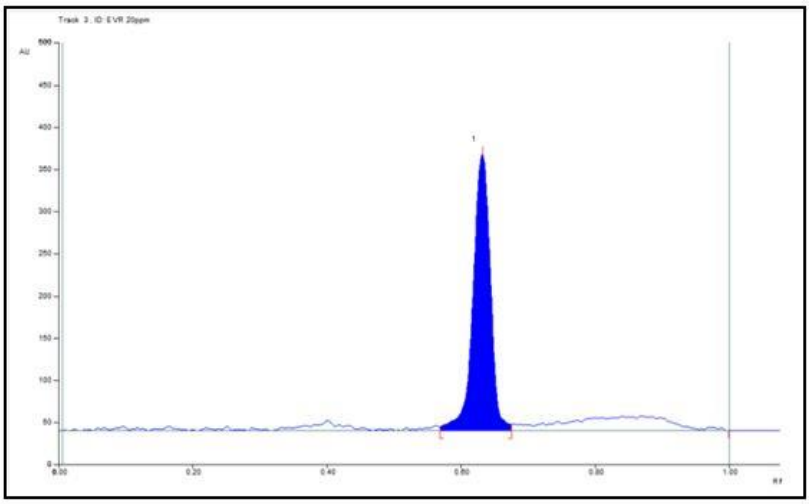

Fig. 2: Everolimus representative densitogram (200 ng/band, Rf =0.63±0.03)

Forced degradation studies

The degradation circumstances were in consideration of ICH Q1A(R2) [13, 14]. To achieve a 10-30% degradation, the reagent strength and exposure time were tuned. The results of are table 2. The following are the optimized stress conditions. Only in basic hydrolytic condition we got degradation products at Rf of DP 1-0.02, DP 2-0.15, DP 3-0.41 densitogram is shown in fig. 3a. The spectral scanning overlay is fig. 3b.

Table 2: Summary of degradation parameters for everolimus

S. No.	Stress condition	Temperature and time	% Recovery	Rf of degradation product	Peak Purity	
					Purity front	Purity tail
1	Acid hydrolysis	0.1 N HCl for 30 min	80.7	_	0.996	0.998
2	Alkali hydrolysis	0.1N NaOH instantly	78.3	DP 1-0.02, DP 2-0.15, DP 3-0.41	0.985	0.991
3	Oxidative degradation	3%v/v H_2O_2 for 30 min	81.8	_	0.998	0.996
4	Neutral degradation	Water for 30 min	72.8	_	0.994	0.997
5	UV degradation	200-watt h meter sq	82.8	_	0.999	0.999
6	Thermal degradation	60 °C for 1 h	92.6	_	0.998	0.996
7	Fluorescence degradation	1.2 million lux h	95.8	_	0.999	0.997

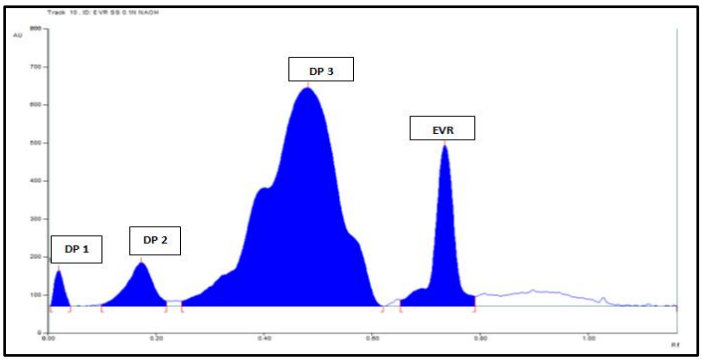

Fig. 3a: EVR after basic degradation, Rf of EVR = 0.63, Rf's of DP 1-0.02, DP 2-0.15, DP 3-0.41 (Amount Spotted: 2000 ng/band)

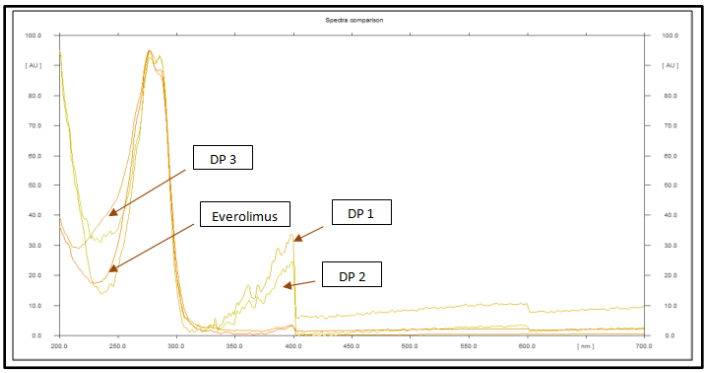

Fig. 3b: Spectral overlay for base degradation having products DP-1, DP-2, DP-3

LC-MS studies

The standard everolimus and degradation samples under alkaline conditions are subjected to LC-MS analysis. From the obtained results of LC-MS analysis possible fragmentation and degradation pathways are proposed.

LC-MS instrument details and chromatographic conditions

The following conditions and composition of the gradient mobile phase were used:

0.1% formic acid was used in mobile phase A and 0.1% formic acid was used in mobile phase B. There was a 0.5 ml/min flow.

An ESI ion source was used for the LC-MS study of everolimus and the alkali degradation products it produces. The mass range for detection was set at m/z 50 to 1700, and the polarity of the ion source was adjusted to positive. For effective analysis, many ion source characteristics were optimised. The gas flow rate was set to 8 l/min, gas temperature was maintained at 300 °C. The material was nebulized at a pressure of 35 psig in the nebulizer. A 350 °C was the temperature specified for the sheath gas, and an 11 l/min flow rate was used. The nozzle voltage was set to 1000 V, while the capillary voltage remained at 3500 V. The analytes were then fragmented with the help of a 175 V fragment or voltage.

The samples everolimus (standard) and everolimus, when treated with sodium hydroxide (EVR-alkaline) at zero time, were checked for their masses and masses of corresponding degradation product peak.

Preparation of solutions

1. Everolimus standard solution (EVR-STD): A standard of everolimus 20 µg/ml in methanol.

2. Everolimus alkaline degradation sample (EVR-AL): About 1 ml (0.5 N) NaOH was treated to 1 ml (200 ppm, that is 10 times of standard) everolimus stock. The final injected solution was 20 ppm.

The fragment ions observed in the mass spectrum exhibited characteristic peaks corresponding to the cleavage of specific functional groups within the molecule. The major fragment ions observed were consistent with the loss of various moieties, such as methyl groups, methoxy groups, and carbonyl groups. The mass fragmentation results provided important information for the structural elucidation and identification of everolimus standard, contributing to the comprehensive understanding of its degradation pathway.

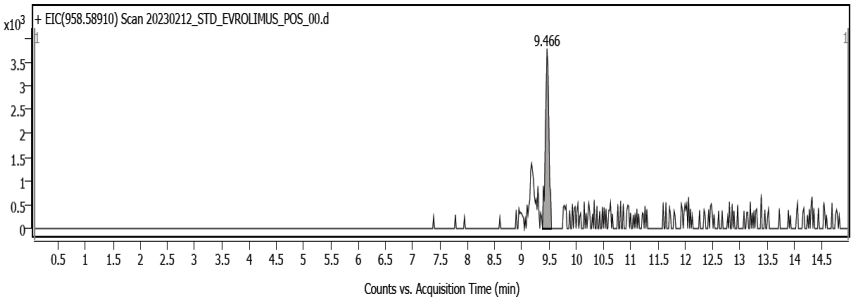

Fig. 4a: LC-MS chromatogram of standard everolimus (RT: 9.46 min)

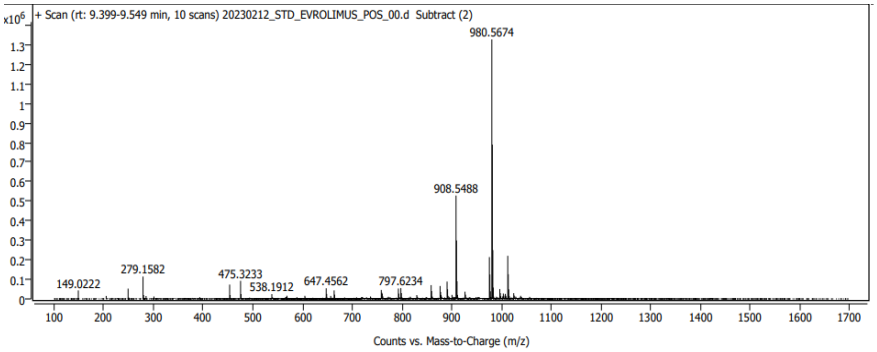

Fig. 4b: LC-MS mass spectra of standard everolimus (RT: 9.46 min)

LC-MS study under alkaline condition

Both the everolimus standard and the everolimus exposed to alkaline hydrolytic conditions showed findings for mass fragmentation. For the everolimus standard, multiple significant pieces were seen. The sodium adduct ion of everolimus has a m/z value of 980.56, which is the value of the most noticeable fragment. The mass fragmentation pattern did, however, significantly change when everolimus was subjected to alkaline hydrolysis. Two notable degradation products were present. The sodium adduct ion peak at m/z 980.56 was significantly diminished, showing everolimus breakdown in an alkaline environment. Peaks were seen at various m/z values, which suggested that degradation products were formed. The m/z values for DP-1 and DP-2 at their respective RT are shown in table 3. These mass fragmentation results provide insights into the stability and degradation pathways of everolimus under alkaline hydrolytic conditions.

Table 3: Mass fragmentation

Everolimus (under alkaline conditions)	RT (Retention time)	Prominent mass fragmentation obtained in LC-MS (m/z)
Everolimus Standard	9.46 min	980.56 and 908.54
Degradation Product 1 (DP-1)	8.88 min	349.23, 403.24, 574.33 and 646.35
Degradation Product 2 (DP-2)	9.10 min	926.55, 614.32 and 542.30

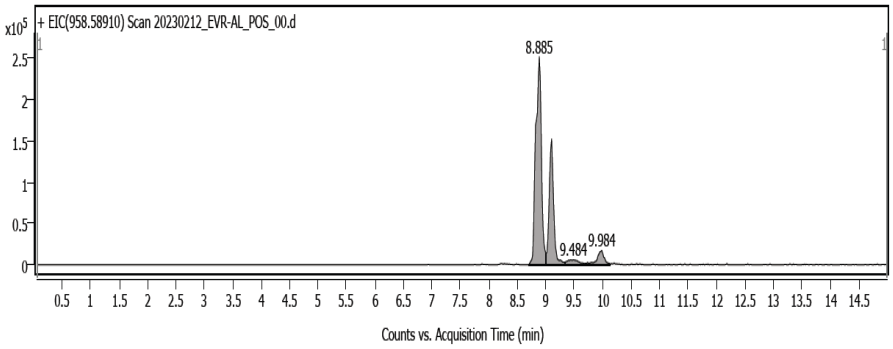

Fig. 5: LC-MS chromatogram, alkali-treated everolimus (RT of DP 1-8.88 min; DP 2-9.10 min)

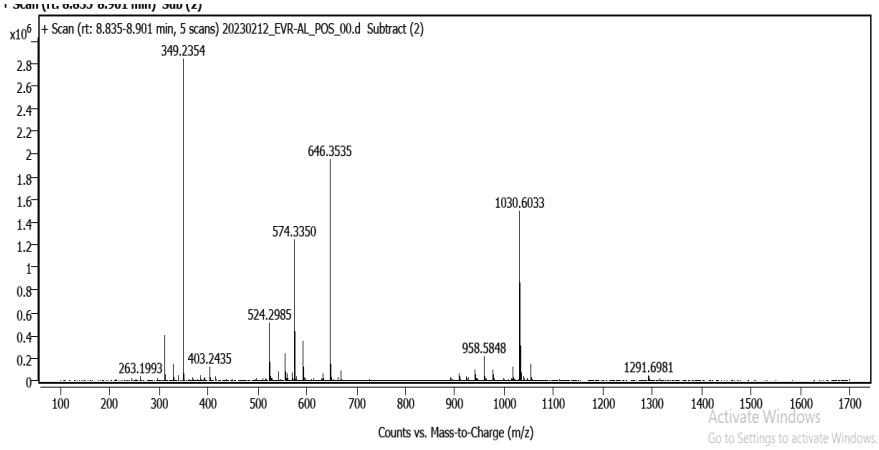

Fig. 6a: LC-MS mass spectrum of alkali-treated EVR (RT – 8.88 min)

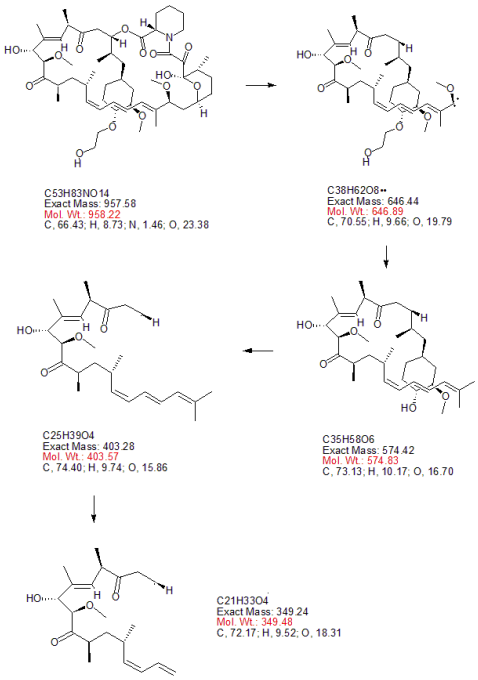

Fig. 6b: Degradation pathway of DP1 (RT – 8.88 min)

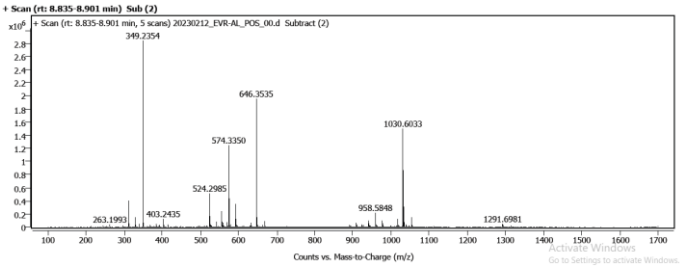

Fig. 7a: LC-MS mass spectrum of alkali-treated EVR (RT – 9.10 min)

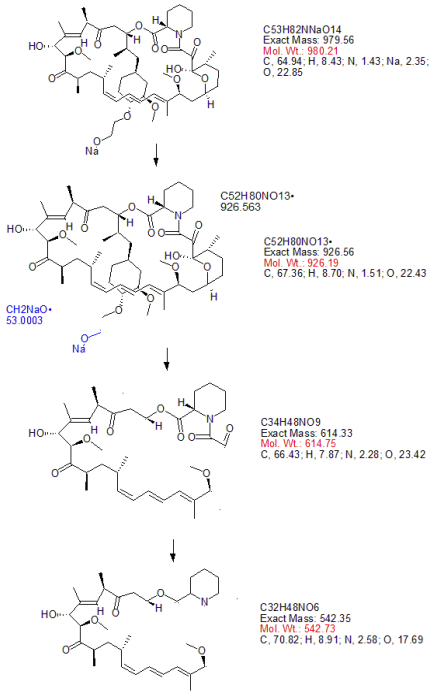

Fig. 7b: Degradation pathway of DP 2 (RT – 9.10 min)

Method validation

a) Specificity

Specificity was monitored by peak purity studies for both standard and sample**.** The peak purity was found 0.9998 under stress conditions, showing that there were no interferences. The method was found specific.

b) Linearity

A standard solution of everolimus (20 µg/ml) applied was of the volume 5, 10, 15, 20 and 25µl, thus leading to spotted amounts. The range was 100-500 ng/band. Development of plate was achieved after saturation in optimized mobile phase for 10 min and this was repeated over 5 times. The correlation coefficient was found 0.9921 with equation of y = 13.735x+2171.4. The 3D densitogram was shown in the fig. 8 for linearity. We got the calibration curve by plotting amount of drug spotted (ng/band) vs peak area shown in the fig. 9. Testing the residual is one of the simplest linearity tests suggested for HPTLC method. The fig. 10 shows no tendency behavior and thus linearity of calibration curve [16].

c) Assay: After an assay, it was discovered that the drug content was 99.98 1.1539 (SD). Table 4 displays the outcomes.

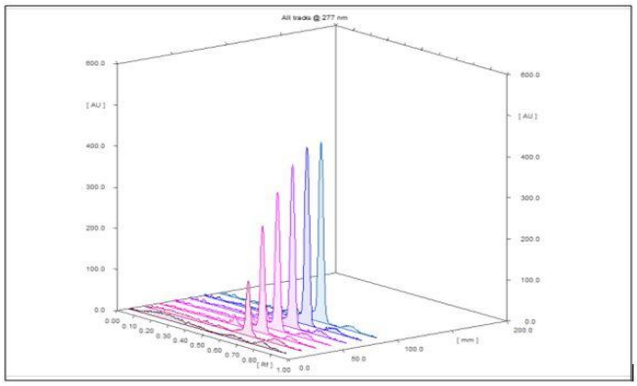

Fig. 8: 3Ddensitogram of linearity (100-500ng/band)

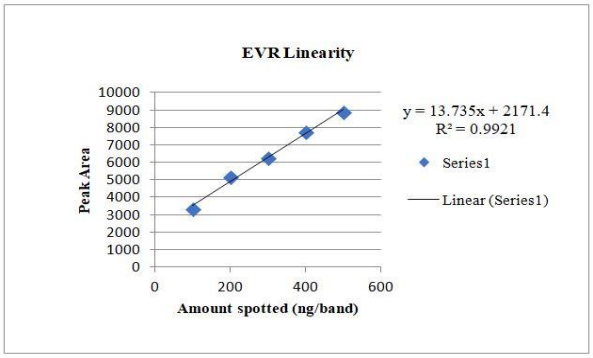

Fig. 9: Calibration curve (100-500ng/band)

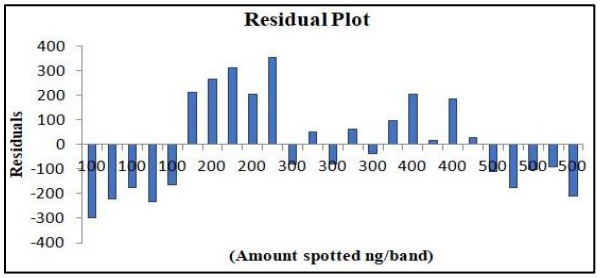

Fig. 10: Residual plot for linearity

Table 4: Assay

Drug	Amount recovered (ng/band)	% Drug content	Mean±SD
Everolimus	191.22	99.16	99.98±1.1539
	201.60	100.8	

(n=2, assay was performed twice for the mean±SD)

d) Accuracy

Recovery was determined to be 101.34%, 100.58%, and 98.97% at 80%, 100%, and 120% levels, respectively. This demonstrated that the suggested method is reliable for estimating medicine dosage in tablet form. The outcomes are displayed in table 5. The fig. 11 displays the representative densitogram for the assay and accuracy.

e) Precision

The % RSD was calculated and values were less than 2%. Table 6 depicts the results.

f) Limit of detection and limit of quantitation

LOD and LOQ measurements are 12.59 and 38.16 ng/band, respectively. Table 7 mentions the results.

g) Robustness

One factor at a time was altered to test for robustness, and when it was discovered that the peak area's % RSD was less than 2%, it was determined that the procedure was indeed robust. Table 8 provides the robustness results.

Table 5: Accuracy studies

S. No.	Amount from marketed formulation (ng/band)	Standard amount	Overall amount of EVR (ng/band)	Recovered amount	% Recovery
1	200	160	360	364.83	101.34
2	200	200	400	402.34	100.58
3	200	240	440	435.40	98.97

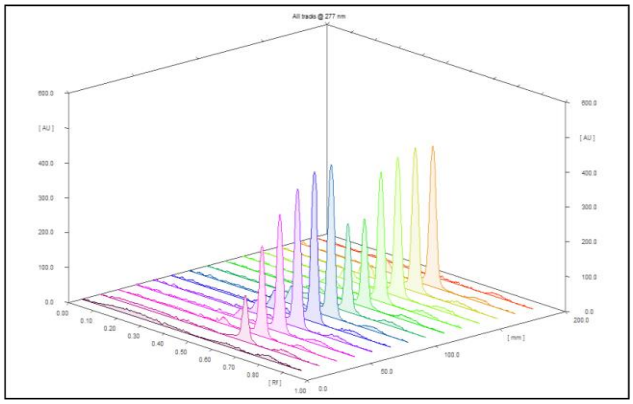

Fig. 11: Assay and Accuracy for everolimus (Track 2-6 are of linearity, track 7 and 8 are of assay, Track 9 is of 80%, Track 10 is of 100%, Track 11 and 12 is of 120%)

Table 6: Precision studies

S. No.	Peak area	
	Intraday precision	Interday precision
1	3246	3303.5
2	3278.2	3265.1
3	3272.7	3304.8
4	3274.4	3258.1
5	3296.4	3296.1
6	3282.2	3302.5
Mean	3274.9	3288.4
SD	16.53	21.05
%RSD	0.52	0.64

(n=3, for interday precision 6 replicates for consecutive 3 d)

Table 7: LOD and LOQ study

S. No.	Parameter	Spotted amount (ng/band)
1	LOD	12.59
2	LOQ	38.16

Table 8: Robustness studies

Parameter	Condition	% RSD
Mobile phase composition	5.8:1.8:2.4 *v/v/v*	0.81
(Toluene: Methanol: Ethyl acetate, 6:2:2 *v/v/v*)	6.2:2:1.8 *v/v/v*	0.61
Saturation time (10±5 min)	5 min	1.30
	15 min	1.35
Application to development time	Instant	1.31
	After 2 h	1.51
Development to scanning time	Instant	1.40
	After 2 h	1.05
Change in wavelength (277±2 nm)	275 nm	1.80
	279 nm	0.80

DISCUSSION

According to a literature review, numerous analytical techniques, including UV, HPLC, UPLC MS/MS, and one HPTLC approach, have been utilised to determine the presence of everolimus in bulk drugs or formulations [1-7, 10]. Except for the alkaline degradation condition, a very low percent deterioration of less than 10% was indicated in previous methods and in the proposed approach, in contrast to the previous method, the neutral degradation was also explored [5]. A forced degradation of 10–30% for each condition was thus accomplished in accordance with standards, leading to the development of an enhanced stability indicating technique using HPTLC. The devised HPTLC method for everolimus estimate was found to be exact, accurate, and specific. Referring to ICH Q2 (R1) standards, the procedure was validated. The method is found linear. Therange is100-500 ng/band (R^2= 0.9921). The stress conditions were optimized in terms of concentration of the reagent and time period. Everolimusis sensitive to mostly alkaline, oxidative, acidic and photolytic conditions. Degradation product peaks were observed at alkaline hydrolysis only. LC-MS Study for Degradation pattern of everolimus was done to identify possible degradation products and to propose a detailed fragmentation pattern of everolimus as and when subjected to basic hydrolytic conditions. The proposed and developed Chromatographic method may be used for analysis and optimization of formulation/packaging aspects of everolimus.

CONCLUSION

The literature review highlighted various analytical techniques for everolimus, emphasizing the need for stability indicating methods. A developed HPTLC method addressed existing shortcomings, demonstrating accuracy, precision, and compliance with regulatory standards. Validation according to ICH principles confirmed its reliability. Alkaline hydrolysis was identified as a significant degradation process, with optimized stress conditions facilitating forced degradation. LC-MS analysis provided a fragmentation pattern, enhancing understanding of degradation pathways and aiding in stability assessment. This comprehensive approach ensures the quality control of everolimus for safe pharmaceutical use.

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ABBREVIATIONS

HPTLC (High-Performance Thin Layer Chromatography), TLC (Thin Layer Chromatography), UV (Ultraviolet), LC-MS (Liquid Chromatography – Mass Spectrometry), EVR (Everolimus), DP (Degradation Product), ICH (International Council for Harmonisation of Technical Requirements of Pharmaceuticals for Human Use)

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AUTHORS CONTRIBUTIONS

MCD designed the work. PYK contributed for the analysis and data. Collection parts of the work. MCD and PYK contributed to the interpretation of the results.

CONFLICT OF INTERESTS

Declared none

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