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# LC and LC/MS approach for quantification of everolimus and its degradants and application in stress analysis

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## ABSTRACT

***Objective:*** Everolimus is the 40-O-(2-hydroxyethyl) derivative of sirolimus and a potent inhibitor of mammalian target of Rapamycin (mTOR). It is used as an immunosuppressant to prevent organ rejection, treatment of renal carcinoma and as restenotic inhibitor in drug eluting coronary stents. The objective of the present study is to develop an LC and LC/MS method for quantification and structural elucidation of everolimus and its degradants in various stress conditions and strategize the stability issues in various applications. ***Method:*** Everolimus was subjected to different ICH prescribed stress conditions of thermal stress, hydrolysis and photolysis. Quantification and further structural elucidation was done by a rapid and systematic strategy based liquid chromatography–mass spectrometry (LC–MS) and liquid chromatography–tandem mass spectrometry (LC–MS–MS) sub structural technique. An acceptable separation of degradants was achieved using a C -18 column with mobile phase comprising of 70:30 acetonitrile: water with 0.1% glacial acetic acid, pH 5.4 held isocratic for 10 min.. The PDA detection wavelength was from 200nm to 800nm. The scanning range of mass was set at 100 to 1030Daltons with capillary and cone voltages at 4.0 kV and 70.0V respectively. The source temperature was set at $100^0$C and desolvation temperature at $350^0$C. ESI micro mass quadrapole detector and data management software - Mass Lynx ver. 4.1 were used. ***Result:*** Subjecting everolimus to different stress conditions resulted in degradants of different molecular weights. Molecular weights of the degradants are found to be 634, 559, 507, 437,309 Daltons (Da) in basic condition 707, 633, 549, 431, 298 Da in acidic condition and 633, 559, 429, 780, 707 Da under accelerated conditions of temperature. ***Conclusion:*** The results indicate that everolimus is highly sensitive to acid, base, light and temperature. The present method developed helped in proposing structures of degradants and further provide base for future work involving the analysis of everolimus.

**Keywords:** Degradants, Everolimus, Immunosuppressant, Stress analysis.

## 1. INTRODUCTION

Everolimus is a semi-synthetic derivative of the macrolide immunosuppressant, sirolimus. It has been demonstrated that everolimus is a potent immunosuppressant in solid organ transplant and autoimmune disease models. Large randomized clinical data demonstrated the superiority of everolimus over sirolimus as antirestinotic drug. Everolimus eluting stents showed better safety and efficacy than sirolimus eluting stents. Detailed analytical studies were conducted in our laboratory involving High Pressure Liquid Chromatography (HPLC) to quantify everolimus and its degradants produced in stress studies. To rapidly facilitate this goal as well as to provide proposed structural information about the degradants, we developed Liquid Chromatography-Mass Spectrometry (LC–MS) and Liquid Chromatography-Mass Spectrometry-Mass Spectrometry (LC–MS–MS) strategies for formulations and Active Pharmaceutical Ingredient (API). [1,2,3] Analysis combined under optimized HPLC separation conditions on-line with an electro spray MS interface to obtain molecular mass information and structural information from the tandem mass spectra (LC–MS–MS). Using these methodologies, structural and sub-structural data for degradants were obtained rapidly and systematically without prior fractionation.

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## 2. EXPERIMENTAL WORK

### 2.1 MATERIALS

Everolimus was supplied by Biocon, India used without further purification. Sodium hydroxide (NaOH), Hydrochloric acid (HCl) was obtained from Hi-Media (Mumbai, India).Acetonitrile (HPLC grade) was purchased from Merck (Mumbai, India). Double distilled Water for analytical purpose was obtained from milli-Q R-O system. (SG Analytical).

### 2.2 Instrumentation

Precision shaking water bath equipped with MV controller (Lab Tech, India) were used for degradation studies under acidic, basic and neutral conditions. Photo degradation was carried out in a photo stability

chamber (Tempo Instruments, Mumbai) equipped with a light bank consisting of two ultraviolet (UV) (OSRAM L73) and four fluorescent (OSRAM L20) lamps and capable of controlling temperature and humidity in the range of ±2$^0$C and ±5% Relative Humidity (RH), respectively. The light system complied with option 2 prescribed in the ICH guideline Q1B. The chamber was set at accelerated condition of 40$^0$C/ 75% RH during the studies. Other equipments used were an ultrasonic bath (3210, Branson Ultrasonic Corporation, Danbury, CT, USA), precision analytical balance (SE2, Shimadzu). The HPLC system consisted of a Shimadzu LC- 20AT liquid chromatographic pump, Rheodyne injection port (Rheodyne, Cotati, CA,USA) with a 20µl sample loop and SPD-M20A Photo diode array (PDA) detector (Shimadzu, Kyoto, Japan). Data collection, integration and calibration were accomplished using LC Solutions chromatography Data system. The chromatographic separation of everolimus was accomplished using 250x4.6mm Waters Xterra MS C18 5µm analytical column. The mobile phase consisted of 95:05 (acetonitrile: water) which was filtered by passing it through a 0.22µm filter and the filtrate is degassed by using bath sonicator. The mobile phase was pumped at an isocratic flow of 1ml/min at room temperature. The Photodiode Array (PDA) detection wavelength was set at 278nm. All separations were performed at ambient temperature. LC-MS studies were carried out in positive Electro Spray Ionization (ESI$^+$) mode. The MS separation of everolimus was accomplished by using 70:30 (acetonitrile: water) with 0.1% glacial acetic acid as mobile phase which was pumped at an isocratic flow of 0.3ml/min with a run time of about 3.0 mins and 10 mins for the degradants. The PDA detection wavelength was set from 200nm to 800nm. Other parameters of MS such as the scanning range of the mass were set as 100 to 1030Daltons with capillary and the cone voltage at 4.0 kilo volts (kV) and 70.0 volts (V) respectively. The source temperature was set at 100$^0$C and desolvation temperature at 350$^0$C. LC-MS system, used included a gradient module, micro mass quadrapole detector and data management software Mass Lynx ver. 4.1 (all from Waters Corporation, Milford, MA, USA).

### 2.3 Conduct of stress studies

The stress studies were carried out under the conditions of acid, base and light as defined by ICH Q1A R2 [4]. Base degradation was performed by incubating equimolar ratio of the drug solution and aqueous NaOH (0.5 N) with a pH of 7.5 for a period of 60 minutes at 37$^0$C in shaking water incubator (300 RPM). Acid degradation was performed by incubating equimolar ratio of the drug solution and aqueous HCl (1N) with a pH of 3.2 for duration of 300 minutes. The conditions were similar to base hydrolysis but the aliquots were picked up at 4 different time points (30 min, 1, 2, 3, 4 hours). The effect of temperature was studied with the combination of UV and fluorescent light to both the formulation and the drug to which the accelerated conditions of temperature and humidity of 40$^0$C/75%RH were set. A parallel set was kept under recommended storage conditions (-20$^0$C).

### 2.4 Characterization of degradation product(s)

LC-MS studies were carried out to determine mass/charge (m/z) values of the major degradation products formed under various stress test conditions.

### 2.5 Application of the developed method in stability studies of Everolimus Eluting Coronary Stents

The developed method was adopted to analyze stability of formulations containing everolimus and RELI COAT (a proprietary polymer of Relisys Medical Devices Ltd., Hyderabad, India), which is used to coat the coronary stents. The developed method is also adopted for accelerated stability studies of everolimus eluting coronary stents which was filed for a patent (Application No-62/CHE/2012) [5].

## 3.RESULTS

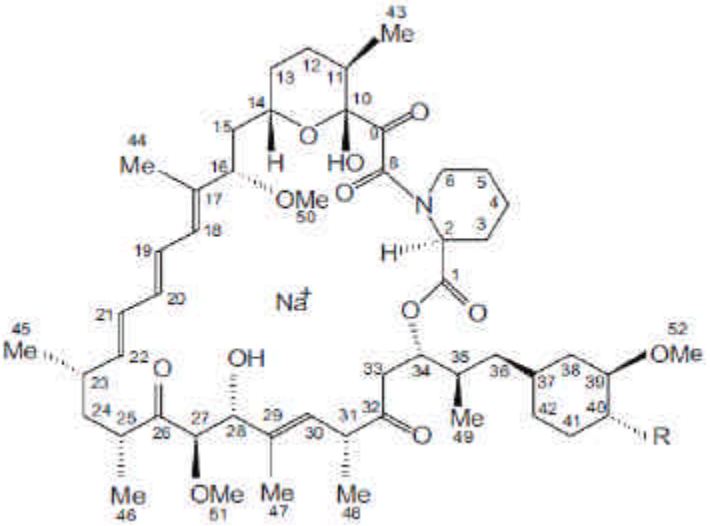
### 3.1 Degradation behavior and Characterization of degradation products

Mass chromatograms in the positive (ESI$^+$) mode for the everolimus and degradation products and the degradation behavior in individual stress conditions were outlined below.

### 3.2 Base degradation

Due to the high sensitivity of mass spectrometry, implementation of LC–MS profiling methods was found to be particularly advantageous for the rapid characterization of low level degradants. The chromatographic method used in these and other everolimus profiling studies provided good resolution using standardized HPLC conditions.

The structure and mass spectra of the everolimus was shown in Fig. 1 and 2. Based on the structure of everolimus, a favorable chemical process with basic conditions would involve hydrolysis of the enol linkages. In fact, information obtained on-line during LC–MS profile studies of the base induced degradants indicates molecular masses consistent with a ring opening at C1 and cleavage at enolic groups accompanied with the loss of water molecules (Table 1)**.**The LC–MS mass chromatograms corresponding to the molecular ions of everolimus and its base-induced proposed degradants are given along with MS spectra (Fig. 3 and 4)**.**




**R- O-CH$_2$-CH$_2$-OH**

**Fig. 1 Structure of Everolimus**

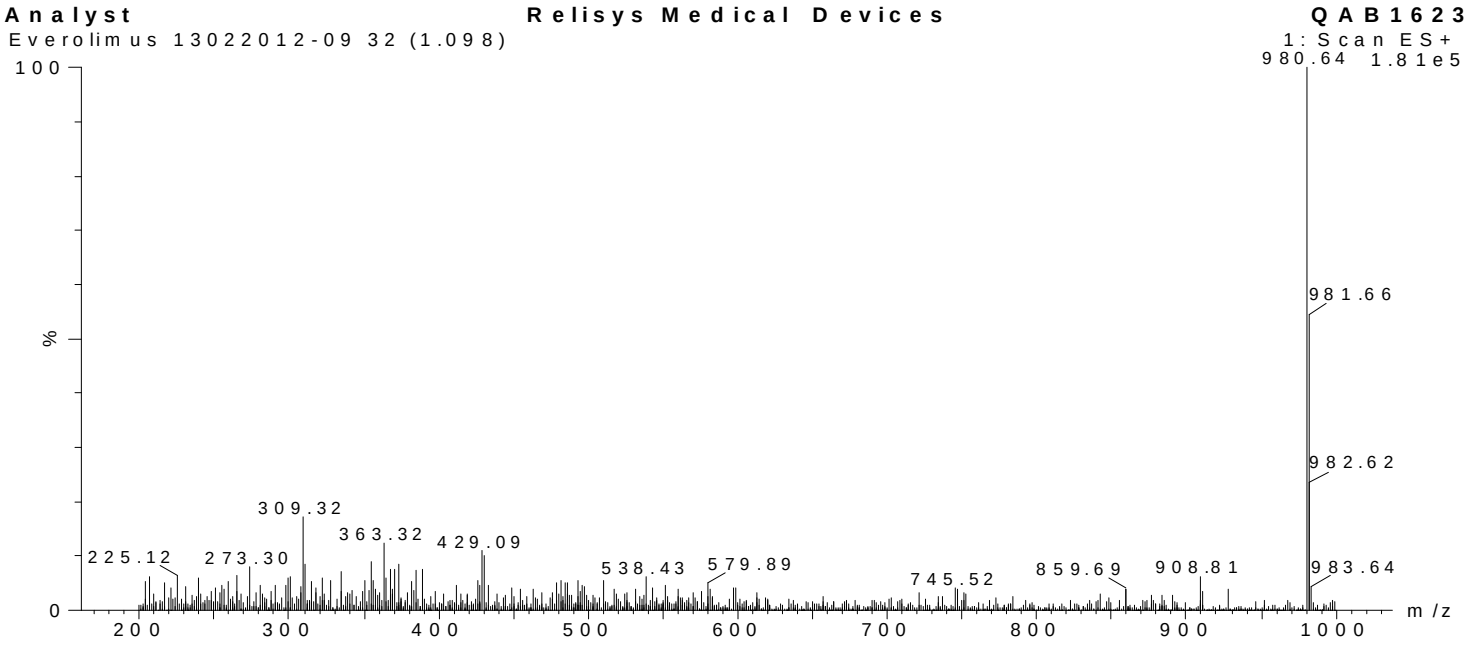
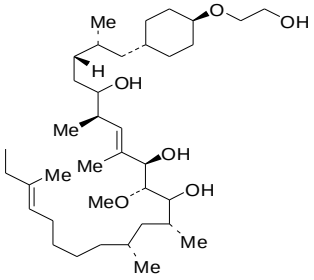
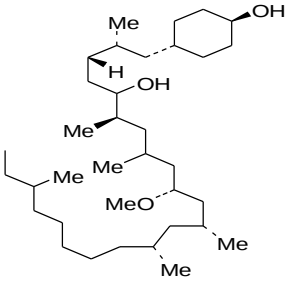
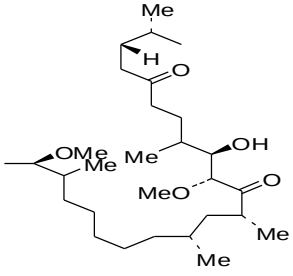
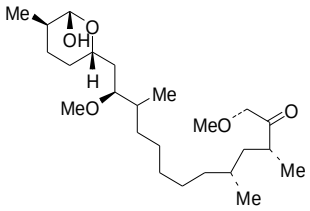
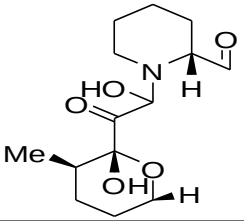



**Fig. 2 MS spectra of Everolimus**

**Table 1- Molecular formula, molecular weight, structure of the degradants of base hydrolysis**

| S.NO | Molecular formula and molecular weight [ M + $Na^+$ ] | Proposed structure | Comment |
|---|---|---|---|
| 1 | $C_{37}H_{70}O_6$ [634] |  | Cleavage at $C_{15}$ and $C_{34}$ ; Loss of $CH_2CH_2OH$ |
| 2 | $C_{35}H_{68}O_3$ [559] |  | Cleavage at $C_{15}$ and $C_{34}$ ; Reduction ; Hydrolysis |
| 3 | $C_{29}H_{56}O_5$ [507] |  | Cleavage at $C_{15}$ and $C_{34}$ ; Reduction |

| S.NO | Molecular formula and molecular weight [ M + $Na^+$ ] | Proposed structure | Comment |
|---|---|---|---|
| 4 | $C_{24}H_{46}O_3$ [437] |  | Cleavage at $C_{10}$ and $C_{27}$ ; Reduction |
| 5 | $C_{14}H_{24}NO_5$ [309] |  | Cleavage at $C_1$ and $C_{15}$ |

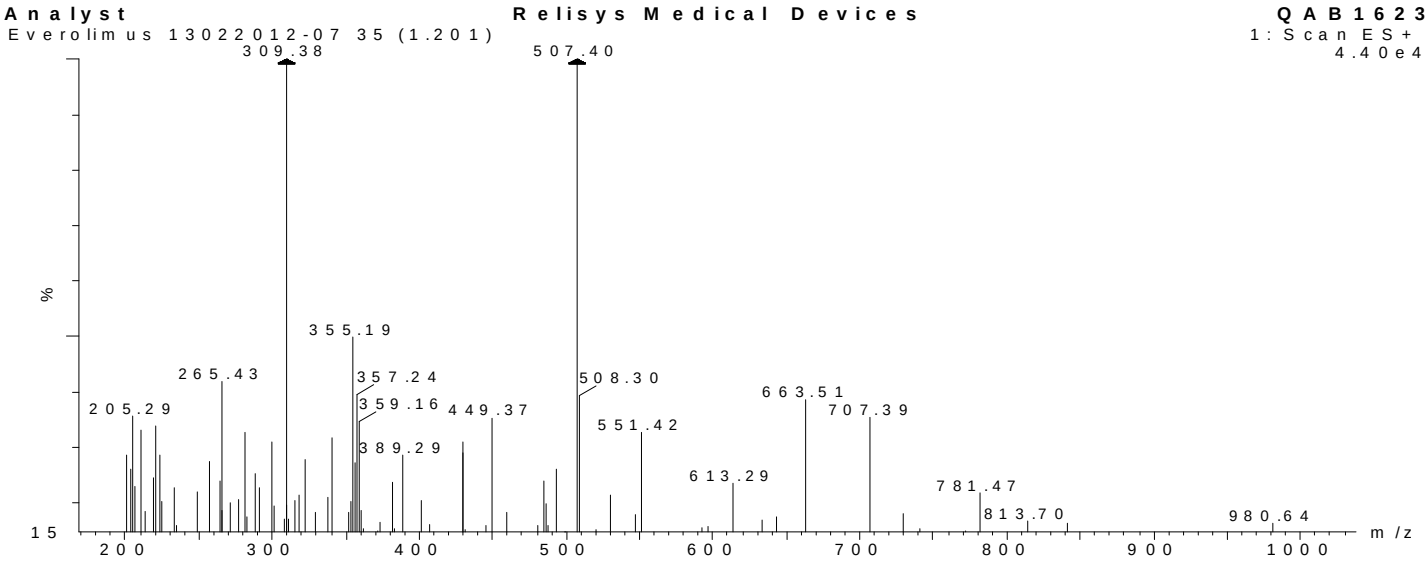



**Fig. 3 Mass spectra of degradants obtained by base hydrolysis of 1st hour**

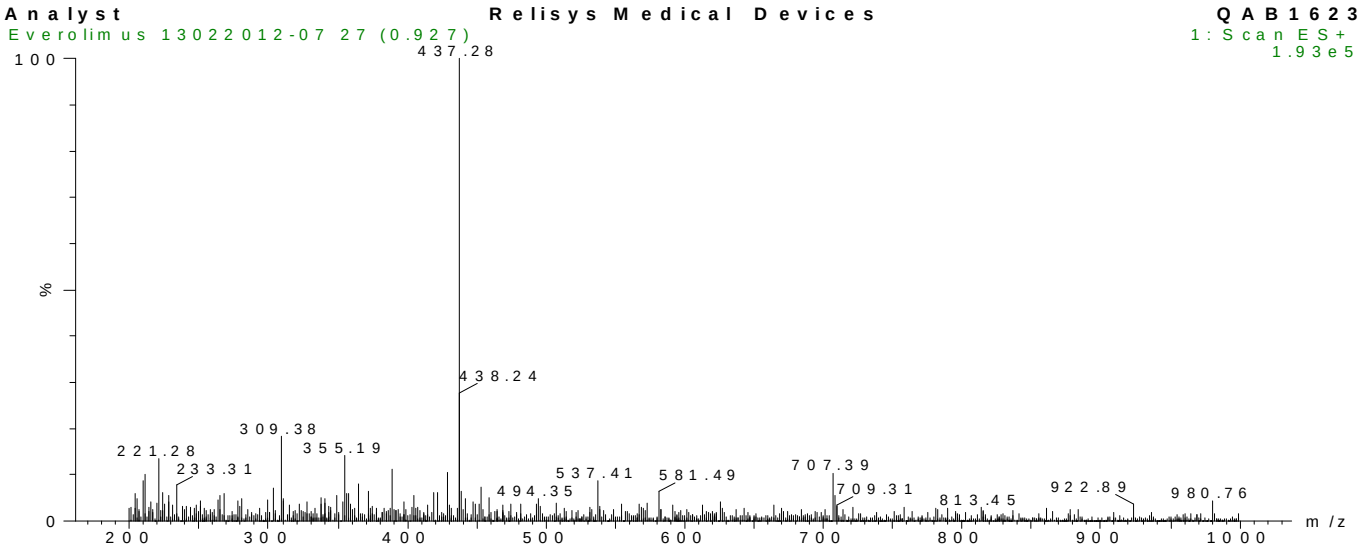



**Fig. 4 Mass spectra of degradants obtained by base hydrolysis of 1st hour.**

### 3.3 Acid degradation

The everolimus solution (1mg/ml) was made to incubate in an aqueous solution of HCl for 300 minutes. This solution was then diluted and analyzed with HPLC and LC-MS. Based on the HPLC data it was observed that there was a gradual decrease in the concentration of the everolimus at different time points (Table 2). MS data revealed that there are a few common degradants both in the acid and base hydrolysis. Mass spectra of the degradants were given in Fig. 5 and 6 and the proposed structures in Table 3.

**Table 2- Quantification of the degradants at various time points**

| S.No | Time points | % Degradation Acid hydrolysis (1N HCl) | Base hydrolysis (0.5N NaOH) |
|---|---|---|---|
| 1 | 0 min | 0 | 0 |
| 2 | 10 min | 10 | 99.01 |
| 3 | 60 min | 15 | 99.2 |
| 4 | 120 min | 20 | 99.2 |
| 5 | 180 min | 22.6 | 99.4 |

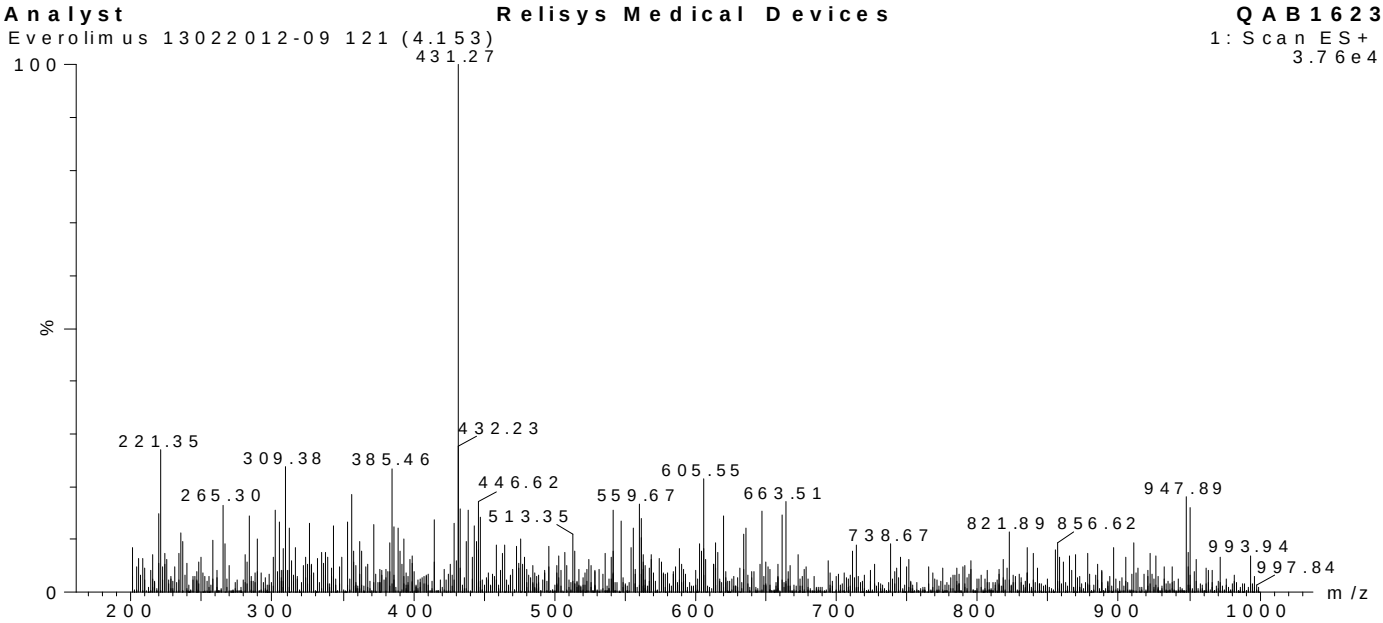



**Fig. 5 Mass spectra of degradants obtained by acid hydrolysis during 2nd hour.**

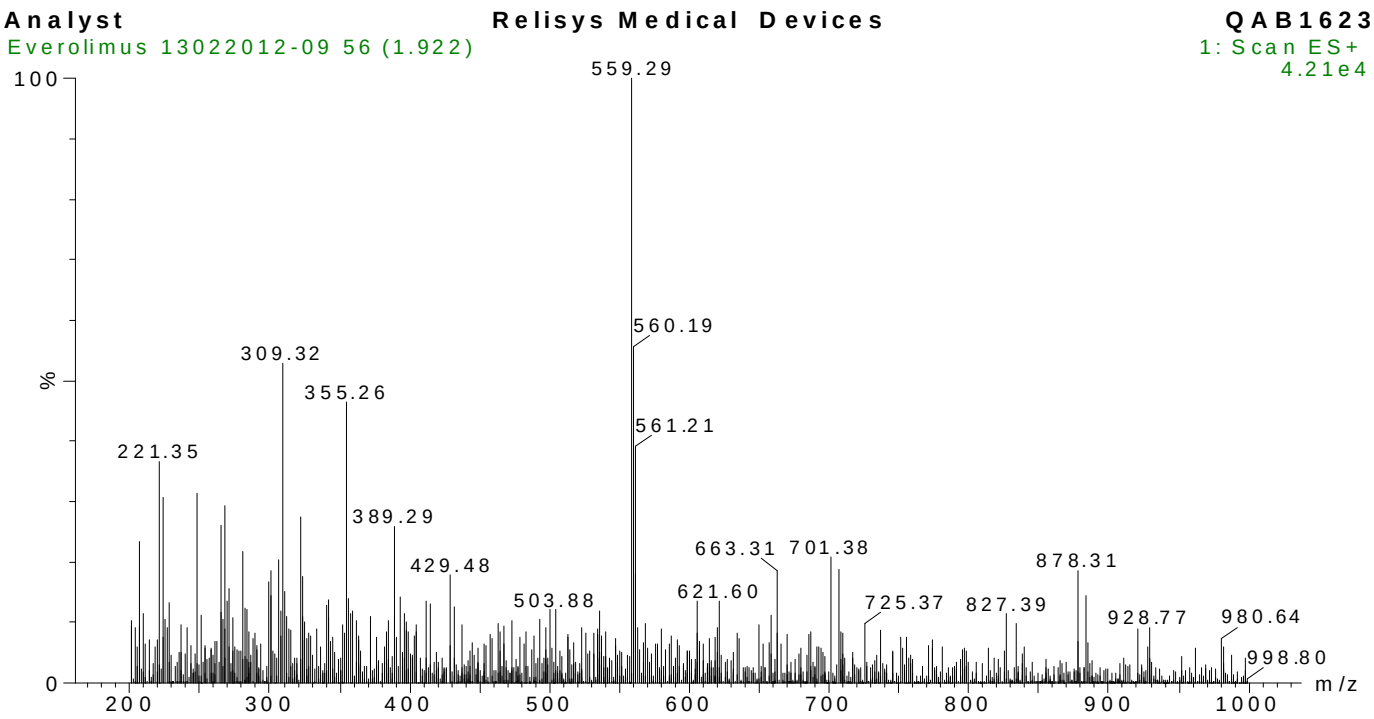
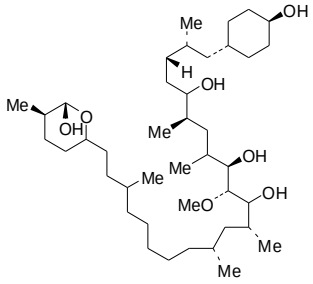
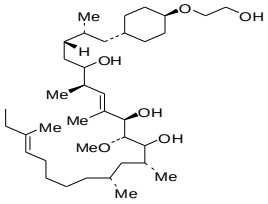
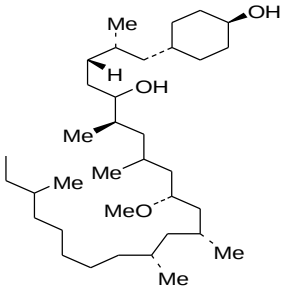
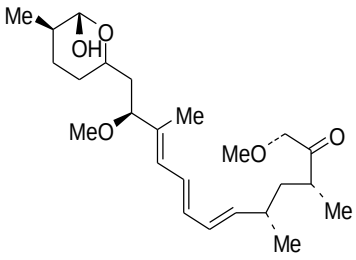
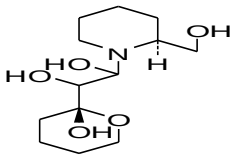



**Fig. 6 Mass spectra of degradants obtained by acid hydrolysis during 3rd hour.**

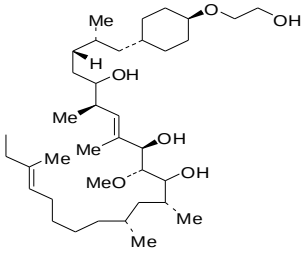
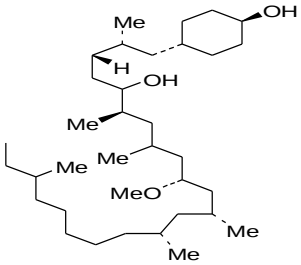
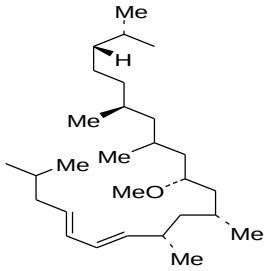
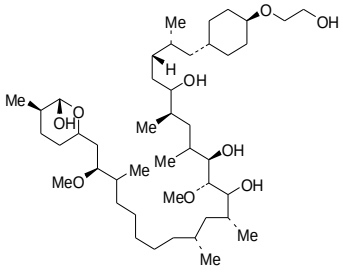
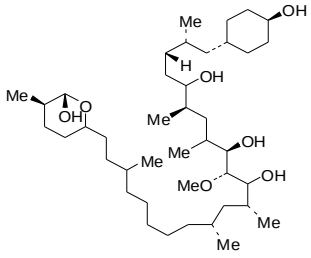
**Table 3- Molecular formula, molecular weight, structure of the degradants of acid hydrolysis**

| S.No | Molecular formula and molecular weight [ M + $Na^+$ ] | Proposed structure | Comment |
|---|---|---|---|
| 1 | $C_{24}H_{40}O_5$ [707] |  | Cleavage at $C_{15}$ and $C_{34}$ ; Loss of $CH_2CH_2OH$ ; Reduction |
| 2 | $C_{37}H_{70}O_6$ [633] |  | Cleavage at $C_{15}$and $C_{34}$;Loss of $CH_3OH$;Reduction |
| 3 | $C_{35}H_{68}O_3$ [549] |  | Cleavage at $C_{15}$ and $C_{34}$ ; Loss of $CH_2CH_2OH$ |
| 4 | $C_{24}H_{40}O_5$ [431] |  | Cleavage at $C_{20}$ and $C_{27}$ |
| 5 | $C_{13}H_{25}NO_5$ [298] |  | Cleavage at $C_1$ and $C_{15}$ ; Loss of $CH_3$; Reduction ; Hydrolysis |

### 3.4 Thermal stress

In order to find out the influence of temperature on the everolimus, it was placed at accelerated temperature conditions as mentioned in experimental work. The control sample was stored at the prescribed conditions of storage. The inference obtained from the LC and MS data suggest that there was no significant degradation of the API and the formulation under storage conditions (-20$^0$C). Structure and spectra of degradants obtained at accelerated temperature conditions (40$^0$C/75%RH) were given in Table 4 and Fig. 7, 8 and 9 respectively.

**Table 4. Molecular formula, molecular weight, structure of the degradants of Accelerated Temperature conditions (400C/70%RH)**

| S.No | Molecular formula and molecular weight [ M + $Na^+$ ] | Proposed structure | Comment |
|---|---|---|---|
| 1 | $C_{37}H_{70}O_6$ [633] |  | Cleavage at $C_{15}$ and $C_{34}$ ; Loss of $CH_3OH$ ; Reduction |
| 2 | $C_{35}H_{68}O_3$ [559] |  | Cleavage at $C_{15}$ and $C_{34}$ ; Reduction ; Hydrolysis |
| 3 | $C_{28}H_{54}O$ [429] |  | Cleavage at $C_{35}$ ,$C_{34}$ and $C_{16}$; Loss of $CH_3OH$ |
| 4 | $C_{44}H_{86}O_9$ [780] |  | Cleavage at $C_{10}$ and $C_{34}$ ; Reduction |
| 5 | $C_{41}H_{80}O_7$ [707] |  | Cleavage at $C_{10}$ and $C_{34}$ ; Reduction ; Loss of $CH_2CH_2OH$ |

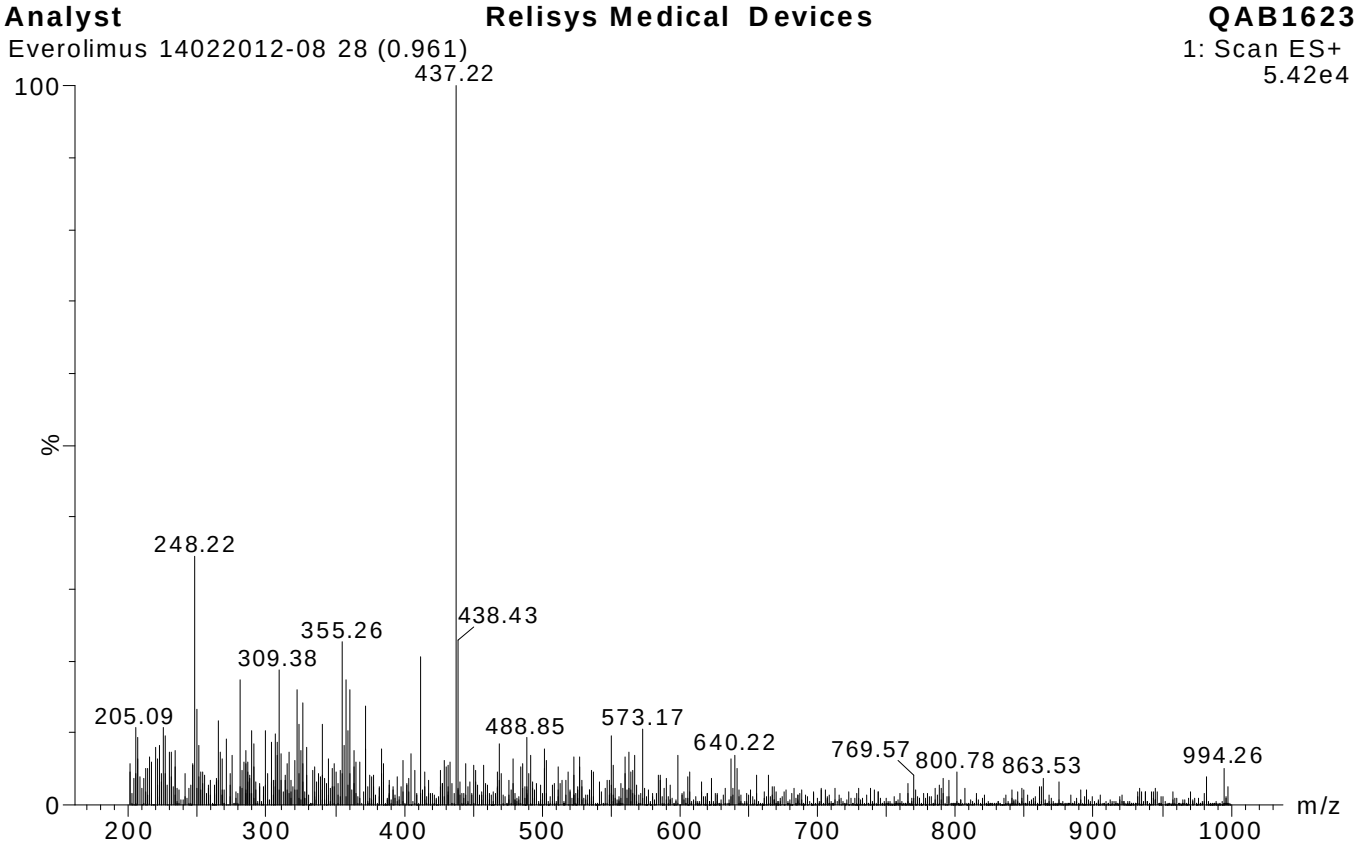



**Fig. 7 Mass spectra of degradants obtained at accelerated conditions (40$^0$C/75%RH)**

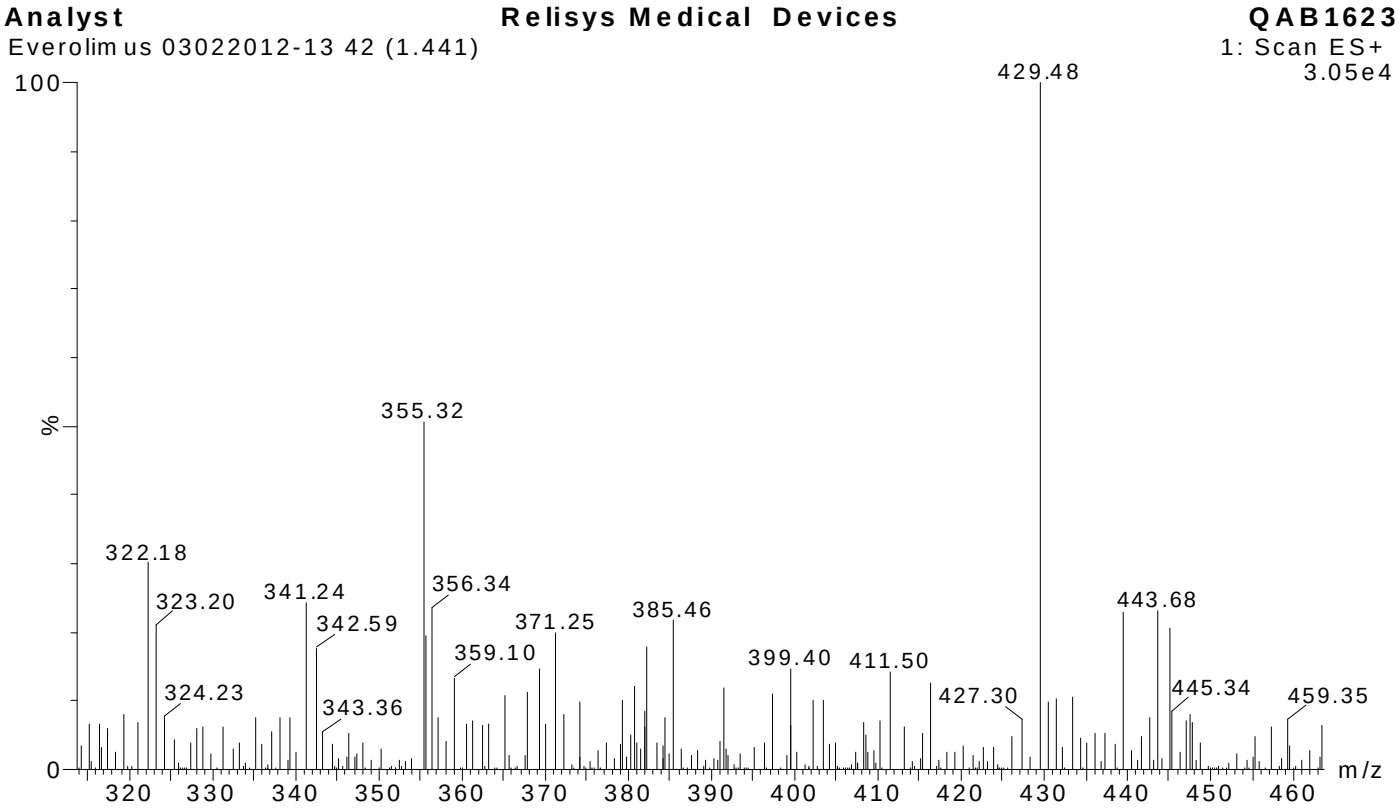



**Fig. 8 Mass spectra of degradants obtained at accelerated conditions (40$^0$C/75%RH)**

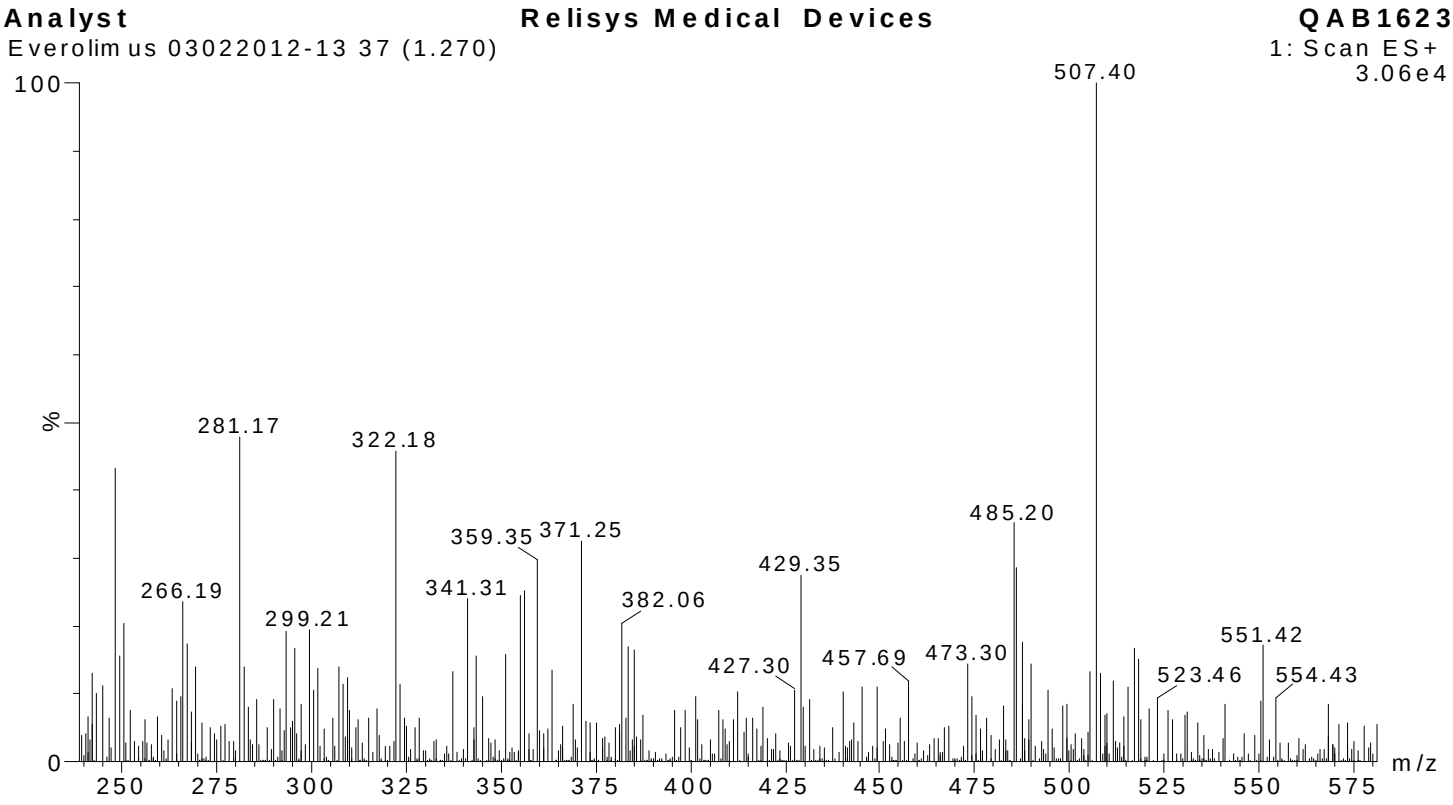



**Fig. 9 Mass spectra of degradants obtained at accelerated conditions (40$^0$C/75%RH)**

## 4. DISCUSSION

Based on the LC studies, it is clearly observed from Table 2, that everolimus quantitatively degraded under basic conditions with respect to time. The electro spray LC–MS interface utilized in these studies is an extremely soft ionization process and produces primarily pseudo-molecular ions such as $(MH)^+$ or $(M+Na)^+$ ions, providing definitive molecular mass information. Unfortunately, the lack of fragmentation information in the full scan mass spectrum is detrimental from the structure elucidation perspective. However, when electro spray ionization is coupled on-line with tandem mass spectrometer, detailed structural information for each component can be obtained. [6, 7] In addition to chromatographic retention characteristics and molecular mass information, structures of degradants were proposed based on the chemical processes such as hydrolysis, reduction, ring opening and cleavage of bonds. In comparison to the structurally related Sirolimus (913.55 Da), the ionization of everolimus was 5-fold less efficient. Hence, sirolimus yields fragments that had significantly higher ion signal intensities than Everolimus. [8]

The structures were proposed based on the common fragmentation patterns, of which the most important one is the ring opening at C1 position. Cleavage / fragmentation generally occur with the loss of water molecule or methyl group and reduction. The structures were proposed based on the molecular weight and the general fragmentation pattern using the Chemdraw 9.0 software. Molecular weights comprising of 559($C_{35}H_{68}O_3$) and 437($C_{24}H_{46}O_3$) were found to be common in base hydrolysis and at accelerated temperature conditions whereas molecular weights 633($C_{37}H_{70}O_6$) and 707($C_{24}H_{40}O_5$) were found to be common in both acid hydrolysis and at accelerated temperature conditions.

Degradation products were elucidated on the basis of their chromatographic standardized HPLC conditions, molecular mass information obtained from the full scan mass spectrum acquired during LC–MS profiling, and the product ion spectrum acquired during LC–MS–MS substructure analysis studies. Degradation products are formed upon exposure to basic conditions, acidic conditions and exposure to high intensity light. Based on these studies, an LC–MS degradants database including information on molecular structures, chromatographic behavior, and molecular mass and substructure-specific MS–MS product ions of the components has been developed.

## 5. CONCLUSION

It was possible in this study to propose degradants structures for everolimus by subjecting the drug and formulation to ICH recommended stress conditions. The drug and degradation products got well separated. The high sensitivity of mass spectrometry is particularly advantageous for application to samples which contain trace amounts of degradants, a situation frequently encountered in pharmaceutical discovery and development research. A strategy involving the use of LC–MS profiling and LC–MS–MS sub structural analy

sis has been shown to be capable of providing a highly sensitive and specific method for rapidly obtaining molecular mass and structural information about low level degradants. Using this methodology, detailed structural information is typically obtained for potential degradants in less time. Most importantly, these predictive studies will provide a foundation for future work involving the analysis of new everolimus degradation products and insight in the formulation development.

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