IJBPAS, July, 2025, 14(7): 3812-3837

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

RP-HPLC METHOD DEVELOPMENT & VALIDATION FOR MUPIROCIN TO ASSESS FORCED DEGRADATION BEHAVIOR AND STABILITY IN AUTHORIZED DISSOLVING MEDIA

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Received 20th March 2024; Revised 24th April 2024; Accepted 16th Aug. 2024; Available online 1st July 2025

https://doi.org/10.31032/IJBPAS/2025/14.7.9203

ABSTRACT

In areas like formulation creation, manufacturing, and packaging where data on substance conduct may be used to enhance a pharmaceutical item, forced degradation investigations may aid in promoting drug improvement. Therefore, it's crucial to understand how a medicine ingredient behaves under distinct natural circumstances. Stress Testing or Forced degradation is defined as the stability testing of drug substance and drug products under conditions exceeding those used for accelerated testing. It is an integral part of the information provided to regulatory authorities in registration application dossiers [ICH Q1 A (R2)]. To investigate stability related properties of an API and to develop an understanding of degradant. This study

helps in safety toxicological, identification of possible genotoxic degradant and identification of potential metabolites and API designal discovery, determination of degradation pathway of drug substances and products; Structure elucidation of degradation products; The procedure has been evaluated for the Linearity (r^2=0.9994), Precision and System suitability %RSD was not more than 2%, in order to ascertain the suitability of analytical method. It has been proved that the method is selective and linear between concentration range 10 – 50 µg/ml for Mupirocin. In LOD and LOQ, signal-to-noise ratio found to be 0.10 and 0.30 respectively within given limit. In the Forced Degradation study various stress condition applied as, Acid Hydrolysis-Drug recovery 89.50 %. Base Hydrolysis- Drug recovery 71.08 %, Oxidative Hydrolysis – Drug recovery 58.04 % and in case of Photo-degradation, Neutral degradation there is 100% Drug recovery was found in API of Mupirocin. According to LC-MS data Mupirocin was unstable in acidic, alkaline and oxidative media but stable in neutral and photolytic condition Therefore, this method is validated as per ICH guidelines.

Keywords: Stress degradation, Mupirocin, Chromatograph, Accuracy, Precision, and Stability

INTRODUCTION:

Mupirocin categories into Topical Antibiotic, Mupirocin reversibly bind to bacterial isoleucyl t-RNA synthetase, and enzyme which promote the conversion of isoleucine & t-RNA to isoleucyl t- RNA. Result is Inhibition of Bacterial protein. At low concentration produce bacteriostatic effect and at high concentration induce bactericidal effect against Gram Positive bacteria. Mupirocin is used as a topical treatment for bacterial skin infections, for example, Furuncle, impetigo, open wounds, etc. It is also useful in the treatment of methicillin-resistant staphylococcus (MRSA), which is a significant cause of death in hospitalized patients having received systemic antibiotic therapy. It is suggested, however, that mupirocin cannot be used for extended periods of time, or indiscriminately, as resistance does develop, and could, if it becomes widespread, destroy mupirocin value as a treatment for MRSA. It may also result in overgrowth of non-susceptible organisms. In market available in proprietary name Bactroban, Centany, Plasimine, Eismycin, Turixin.

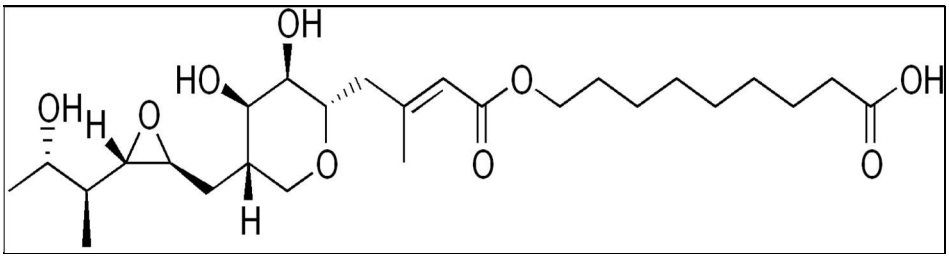


Figure 1: Chemical Structure of Mupirocin

Stress Testing or Forced degradation is defined as the stability testing of drug substance and drug products under conditions exceeding those used for accelerated testing. It is an integral part of the information provided to regulatory authorities in registration application dossiers [ICH Q1 A(R2)] **[1]**.

Significance:

Determination of degradation pathway of drug substances and products; Discernment of degradation Products in formulations that are related to drugs substances versus those that are related to non-drug substances (eg. Excipients); Structure elucidation of degradation products; Determination of the intrinsic stability of drug substance molecule in solution and solid state; and Reveal the thermolytic, hydrolytic, oxidative and photolytic degradation mechanism of the drug substance and drug product **[2]**. To investigate stability related properties of an API and to develop an understanding of degradant. This study helps in safety toxicological, identification of possible genotoxic degradant and identification of potential metabolites and API designal discovery. The information which is obtained from forced degradation studies can also be utilized in several other areas of development, including analytical method development, formulation development. In pharmaceutical companies performed stress testing during preformulation help to select compound and excipients for further development to facilitate salt selection or formulation optimization **[3, 4]**. eg. The effect of oxygen can be eliminated by the addition of antioxidants in pharmaceutical dosage form eg. Oxidation of lovastatin in aqueous solution is inhibited by antioxidants such as α -tocopherol and butylated hydroxyanisole (BHA) **[11]**. It helps to improve manufacturing process and in selection of packaging material and decide the storage condition of API and pharmaceutical dosage form **[3]**. e.g. Carbamazepine tablets containing stearic acid formed column-shaped crystals on the tablet surface during storage at high temperature **[1]** Knowledge of chemical behavior can also be used to improve the quality of drug **[3]**. e.g. Analog development to effect stabilization is the masking of reactive hydroxyl groups **[1]**. Degradation of erythromycin via 6, 9-hemiketal breakdown under acidic pH conditions is inhibited by substituting a methoxy group for the C6-hydroxyl. The acid stability of clarithromycin is 340 times greater than that of erythromycin **[5]**. It is difficult to develop a stability indicating analytical method before having key stability indicating samples generated

from purposeful degradation studies to challenge the methodology. Forced Degradation plays a key role not just in the development of stability indicating methods, but also in providing useful information about the degradation pathways and degradation products that could form during storage and this information facilitate pharmaceutical development in areas as, Formulation development, manufacturing and packaging, where knowledge of chemical behavior can be used to improve the quality of drug product.

Conditions of Force degradation [1, 2, 8]

1) Acid / Base Hydrolysis,
2) Thermal / Humidity,
3) Oxidative Degradation,
4) Photolytic Degradation,
5) Neutral Degradation.

Material and Reagents:

Mupirocin as Active Pharmaceutical Ingredient (API) was procured as a gift sample from Glenmark Pharmaceuticals, Mumbai and its claim purity was 99.8%. Chemical reagent used in the work are HPLC grade and Analytical grade (A.R.) obtained from Reliable lab as per requirements. HPLC grade methanol and water used. Also A.R. grade Hydrochloric acid, Sodium Hydroxide, Hydrogen phosphate and Orthophosphoric acid was collected from Reliable Shree Industrial Training Centre, Jalgaon India.

Instruments and Equipment's used:

U.V. Visible Spectrophotometer.

Absorbance measurements were made on U.V. visible Spectrophotometer (Analytical Technology, U.V.: model 2080), the pH meter (Chemi line) was used for measurement of pH. For sonication (Degasing) of mobile phase 'Trans-o-Sonic' Sonicator was used. Shimadzu AY 220 weighing balance was also used.

HPLC Systems:

The Young Line ACMC 9000 HPLC Gradient System consist of quaternary pump having U.V. Young Line- 370 D detector and SP -930 D pump model. Capacity 20 µl, operated at wave length 228 nm. The software used Autochrom-3000 with column Hi Q Sil C-18 (250 mm x 4.6 mm, 5 µ)

Identification of drug:

A. Appearance: White, solid powder.

B. Solubility: Soluble in Alcohol, Chloroform and in Water (>10mg/ml).

C. Melting Point: 77-78^{0}C is reported according to USP, IP & 77.5^{0}C is practically observed.

D. TLC: The solvent system: Chloroform: Methanol (90:10), Rf = 3.4/5.4=0.62

E. U.V.: The Drug sample Dissolve in methanol and U.V. abortion Spectrum maximum observe at 219,223,227,228. And Reference spectrum at 222, 228 **[4]**.

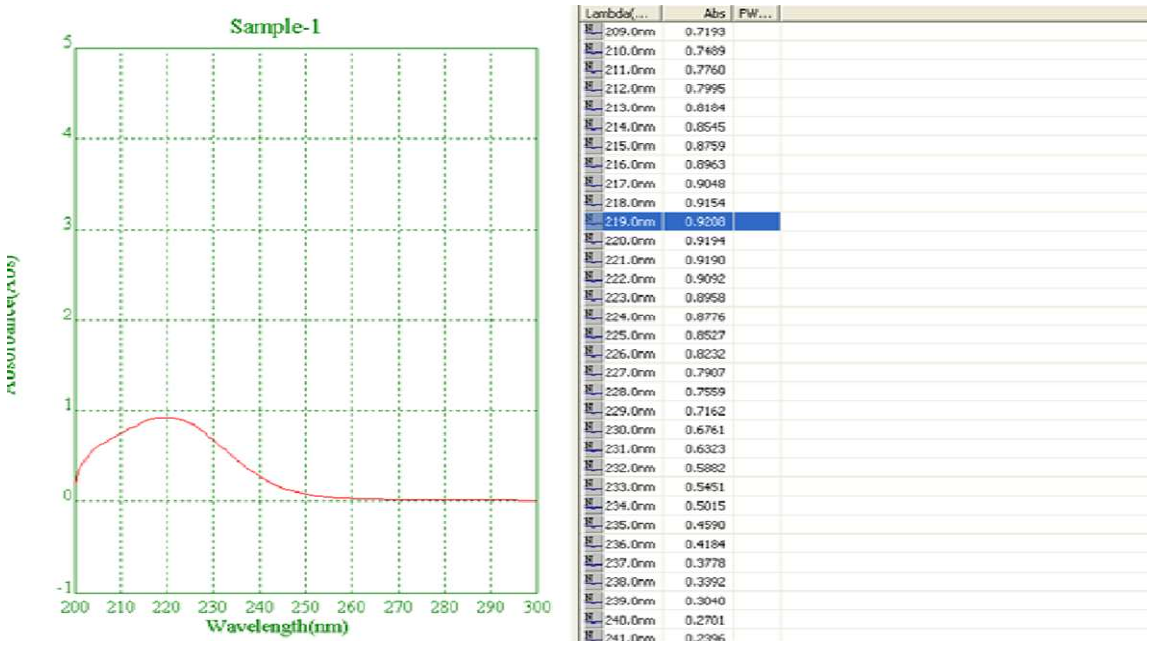


Figure 2: U.V. of Mupirocin

F. IR Spectroscopy: A Pellet of the drug prepared with KBr (Spectroscopic Grade) using hydraulic pellet press at a pressure of 7 – 10 tones. FT-IR was scanned from 400 - 4000cm-1 following peaks were observed. Referencing Principal peaks at wavenumbers 1718, 1146, 1052 cm−1.

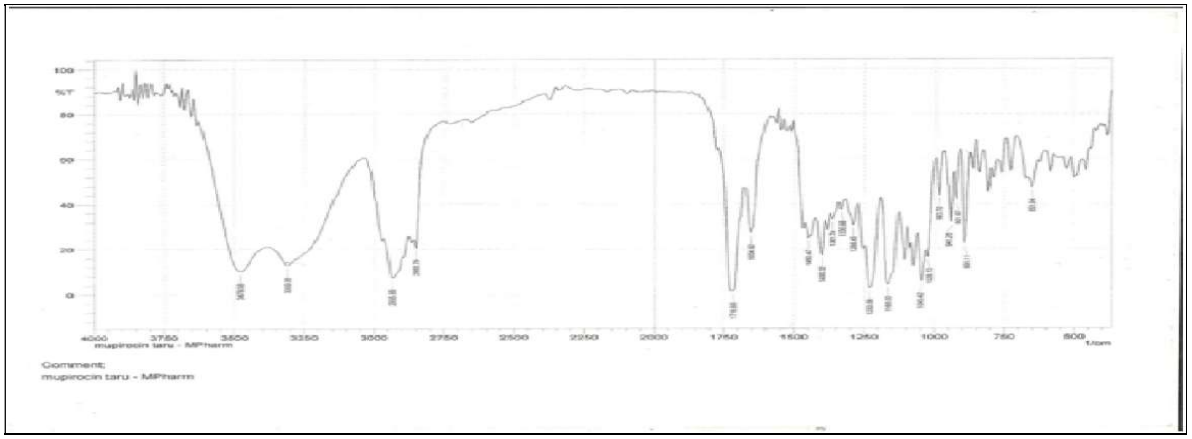


Figure 3: IR Spectroscopy of Mupirocin

Table 1: Assignment for the principal Infrared Absorption Bands of Mupirocin

Assignment	Theoretical peak value cm-1	Practical peak value cm-1
-OH	3400(m)	3479.58
>C=O	1725-1705(s)	1716.65
-C=C	1680-1600(m-w)	1654.92
-C-H(Aliphatic)	a)-CH3 (b ,m)1450-1375	1400.32
	b)-CH2(m)1465	1450.47
	c)-CH(s)3000-2850	2935.66
-C-O	1300-1000(s)	1165.00

METHOD DEVELOPMENT AND VALIDATION:

Solubility Study.

These studies were carried out to find an ideal solvent in which drugs are completely soluble. Various solvents were tried for checking solubility of Mupirocin. From solubility studies it was concluded that Mupirocin is freely soluble in Methanol.

Selection of Chromatographic Condition

Selection of detection wavelength:

For HPLC method λmax was determined from spectra of Mupirocin obtained by using UV-Vis spectrophotometer. Concentration of 100 µg/ml of Mupirocin was prepared in Methanol and scanned in UV range. Maximum absorbance of Mupirocin was found at 228 nm.

Selection of pH:

The pKa value of Mupirocin is 5. After many trials pH of mobile phase (in water by using Orthophosphoric acid) selected 6.1. Mupirocin is acidic in nature so at pH 6.1 it shows good NTP, minimize peak tailing, give sharp peak and method raggedness.

Optimization of chromatographic parameter:

The HPLC procedure was optimized with a view to develop analytical method (assay method validation) for determination of Mupirocin. This was achieved by a series of experiments in which standards and samples were run in different solvent systems. Various solvent system as mobile phase and column as Stationary Phase were used for the experiments to identify an optimum mobile phase as well as stationary phase and other chromatographic conditions such as flow rates, column temperature, and pH **[33, 34]**.

Optimization of chromatographic parameter

Table 2: Optimized chromatographic condition

Fig. No.	Column used	Mobile phase used	Flow rate, Wave length and pH	Observation	Conclusion
1	Hi Q Sil C18(250mm x 4.6mm)	Methanol: Water (70:30), pH6.1	1 ml/min, 228nm	Satisfactory	Accepted
-Repeat	Hi Q Sil C18(250mm x 4.6mm)	Methanol: Water (70 :30), pH6.1	1 ml/min, 228nm	Satisfactory	Accepted

Estimation of Mupirocin by RP-HPLC Method:

Preparation of Mobile Phase:

The Mobile Phase composition was prepared with the help of HPLC grade methanol: water (70:30) by adjusting the pH 6.1 with Orthophosphoric acid in water. Mobile Phase was filtered through 0.45 µm pore size membrane filter and sonicated by ultrasonication.

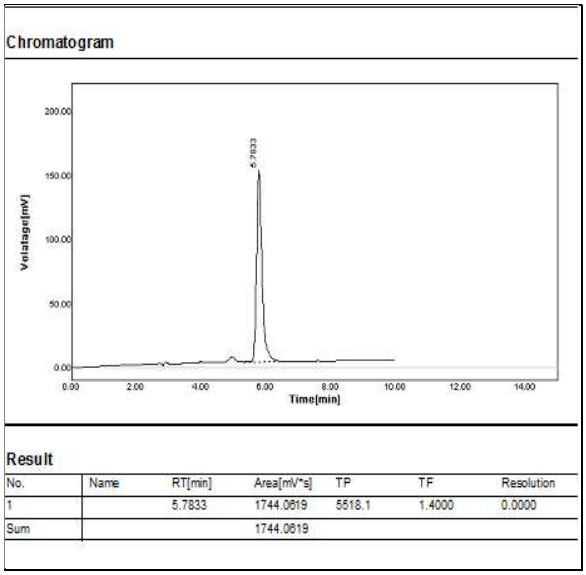
Preparation of stock solution:

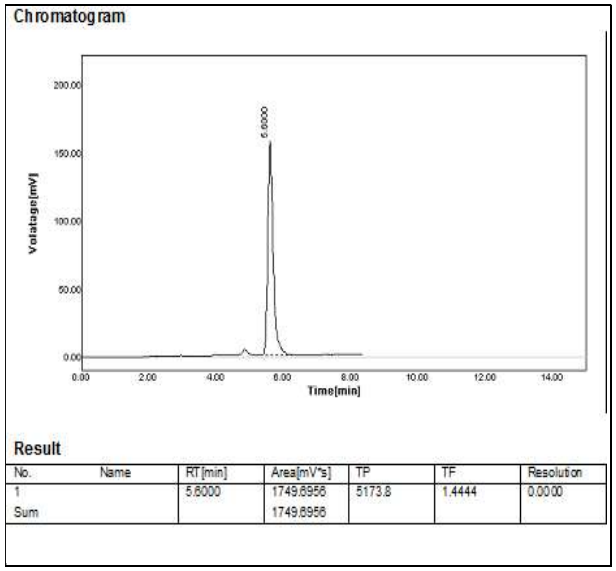
The stock solution of Mupirocin was prepared by weighing 10 mg of Mupirocin with the help of analytical weighing balance. It was

transferred into 10 ml volumetric flask and adjusted the volume (10 ml) with HPLC grade methanol, to achieve concentration of 1 mg/ml.

Preparation of dilution:

Working standard solution was prepared with the help of stock solution, from the stock solution take 1 ml, 2 ml, 3 ml, 4 ml and 5 ml solution and was transferred into 10 ml volumetric flask and volume was adjusted with the help of mobile phase, which give the concentration of 10, 20, 30, 40, and 50 µg/ml respectively, for the degassing sonication was done.





Graph 1& 2: Chromatogram showing the separation of Mupirocin

System Suitability parameter

System suitability test as per method should be performed and checked before performing any parameter. The system parameter as follows-

Table 3: System suitability as % R.S.D is less than 2%. So chromatographic conditions are suitable for study

Sr. No.	Parameters (n = 5)	Mupirocin
1	Retention Time	5.6 – 5.7 min
2	Theoretical Plate	5518.1
3	Tailing Factor	1.4
4	% RSD	0.09

METHOD VALIDATION:

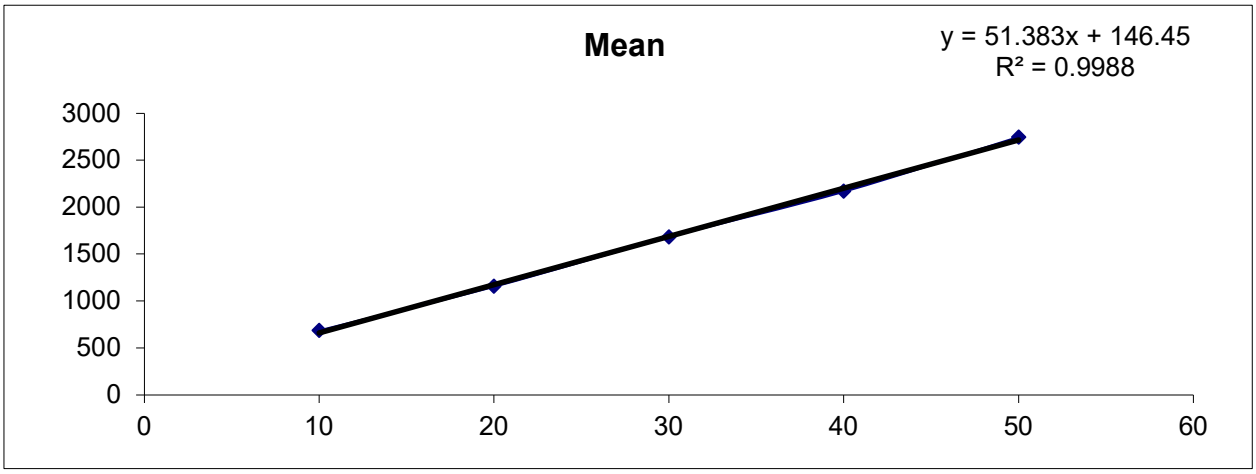
1. Linearity and Range: The linearity of analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been

demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. Five levels of working standard solutions of drugs were prepared to obtain solutions of following concentrations 10 µg/ml - 50 µg/ml. Then, system suitability parameters, linearity regression coefficient, Y-intercept calculated.

Mupirocin shows good correlation coefficient in concentration range of 10 – 50 µg /ml (r2= 0.9994).

Table 4: Linearity and range

Parameter	Mupirocin	S. No.	Conc.	Area I	II	III	Mean
Linearity range(ug/ml)	10 – 50 ug/ml	1	10	684.61	685.62	684.98	685.07
N	5	2	20	1155.34	1154.35	1156.28	1155.32
Slope	18.904	3	30	1682.52	1683.58	1682.57	1682.89
Intercept	821.13	4	40	2144.55	2178.55	2183.83	2168.97
Regration	0.9994	5	50	2740.24	2753.62	2748.25	2747.37



Graph 3: Graph shows; Area under the Curve (AUC) uv/min v/s Concentration of solution ug/ml

2. Precision:

Precision of the method was verified by repeatability and intermediate precision studies.

Repeatability studies (intra-day) were performed by analysis of 20 µg/ml, 30 µg/ml and 40 µg/ml concentration of Mupirocin respectively on the same day. Intermediate precision (inter-day) of the method was checked by repeating analysis of 20 µg/ml, 30 µg/ml and 40 µg/ml concentration of Mupirocin respectively on different day. Measurement of peak area was expressed in terms of % relative standard deviation (%R.S.D.).

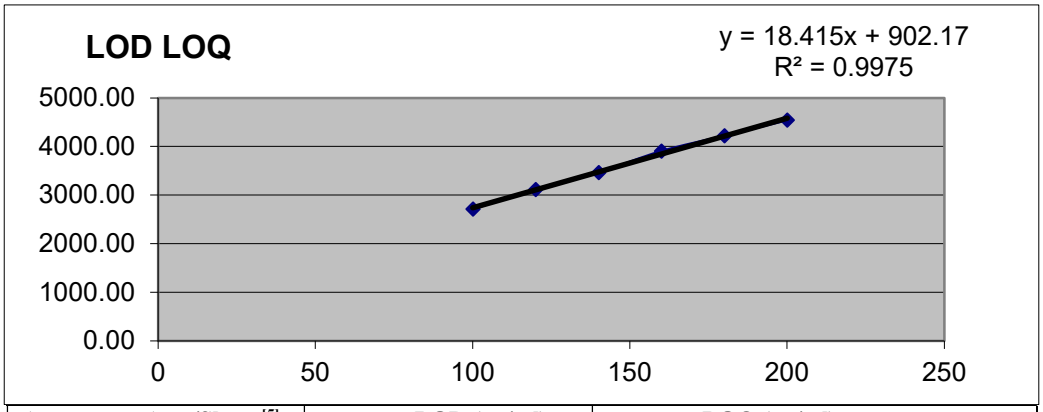
Interday							
Sr No.	**Conc.**	**Area**	**II**	**III**	**Mean**	**SD**	**RSD**
1	**20**	**1158.12**	**1156.59**	**1157.53**	**1157.41**	**0.77**	**0.07**
2	**30**	**1682.98**	**1684.11**	**1683.55**	**1683.55**	**0.57**	**0.03**
3	**40**	**2145.22**	**2153.07**	**2163.88**	**2154.06**	**9.37**	**0.43**
Intraday							
Sr No.	**Conc.**	**Area**	**II**	**III**	**Mean**	**SD**	**RSD**
1	**20**	**1161.32**	**1163.11**	**1164.33**	**1162.92**	**1.51**	**0.13**
2	**30**	**1669.12**	**1671.06**	**1670.82**	**1670.33**	**1.06**	**0.06**
3	**40**	**2144.82**	**2151.88**	**2159.45**	**2152.05**	**7.32**	**0.34**

3. Repeatability:

Repeatability is a measure of precision under the same conditions over a short period of time.

Sr No.	Conc.	Peak Area	Amt. Found	%Amt. Found
1	**30**	**1682.52**	**45.57**	**151.89**
2	**30**	**1683.11**	**45.60**	**151.99**
3	**30**	**1684.02**	**45.65**	**152.15**
		Mean	**45.60**	**152.01**
		SD	**0.04**	**0.13**
		%rsd	**0.09**	**0.09**

4. LOD and LOQ:



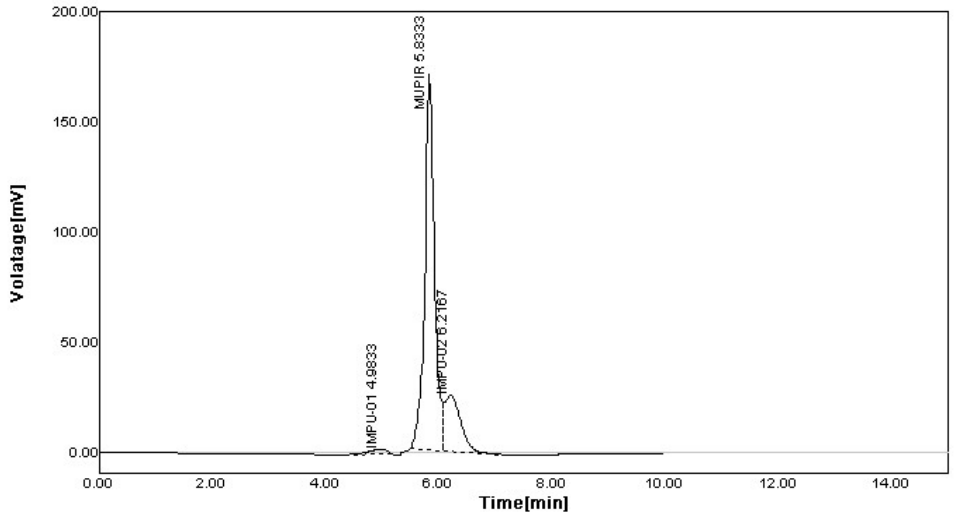
Acceptance Avg./Slope [5]	LOD (ug/ml)	LOQ (ug/ml)
3.3	**0.10**	**-**
10	**-**	**0.30**

FORCED DEGRADATION STUDY:

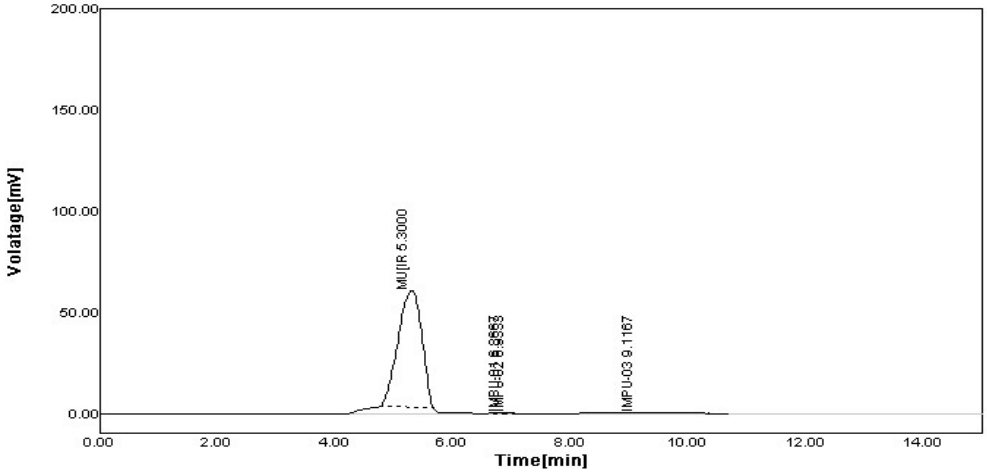
Stress Condition	Applied Condition	% Recovery.
Acid Hydrolysis	**1N HCL, 2 Hrs., 70 ^{0}C**	**89.50%**
Base Hydrolysis	**1N NaOH, 2 Hrs., 70 ^{0}C**	**71.08%**
Oxidative Degradation	**3 % H_2O_2, 24 Hrs. In Dark room.**	**58.04%**
Photo degradation	**4+4=8 Days, drug Melts.**	**100%**
Neutral Degradation	**2 Hrs., 80^{0}C**	**100%**

Chromatograms:

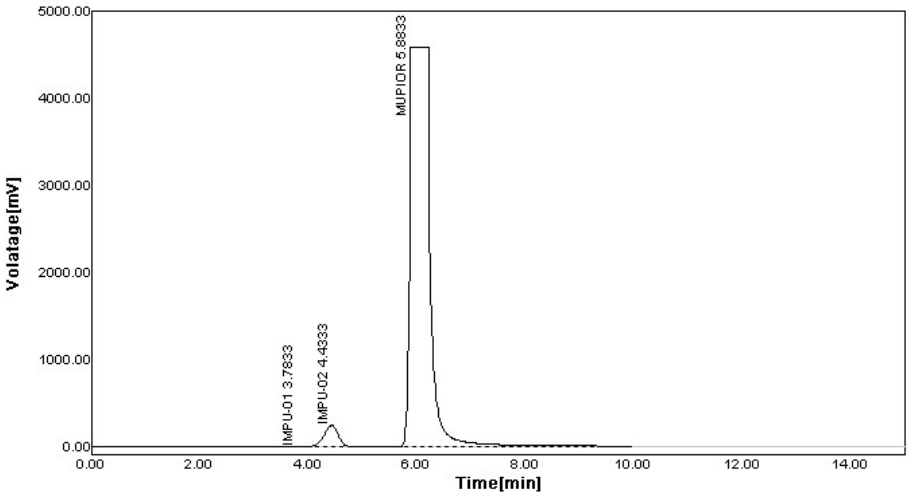
1. **Acid Hydrolysis -**



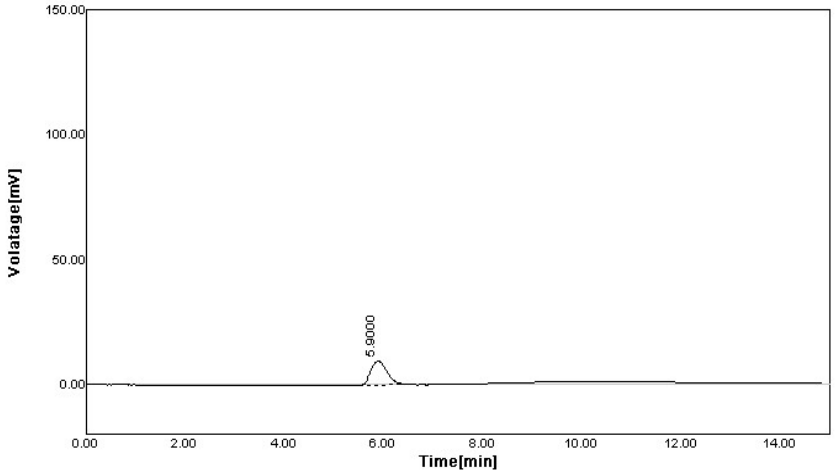
2. Base Hydrolysis-



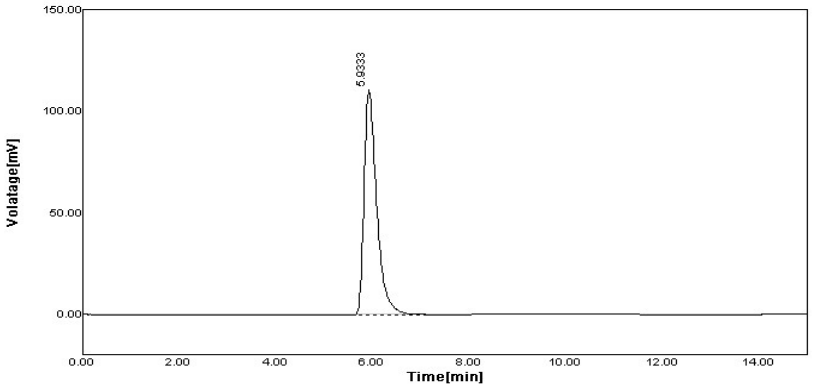
3. **Oxidative Degradation-**



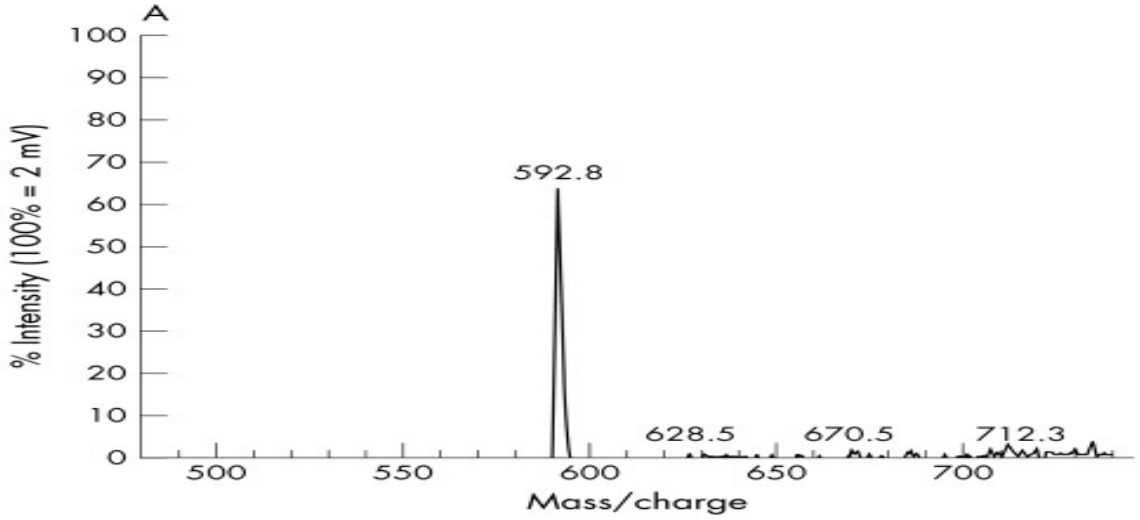
1. **Photo degradation-**



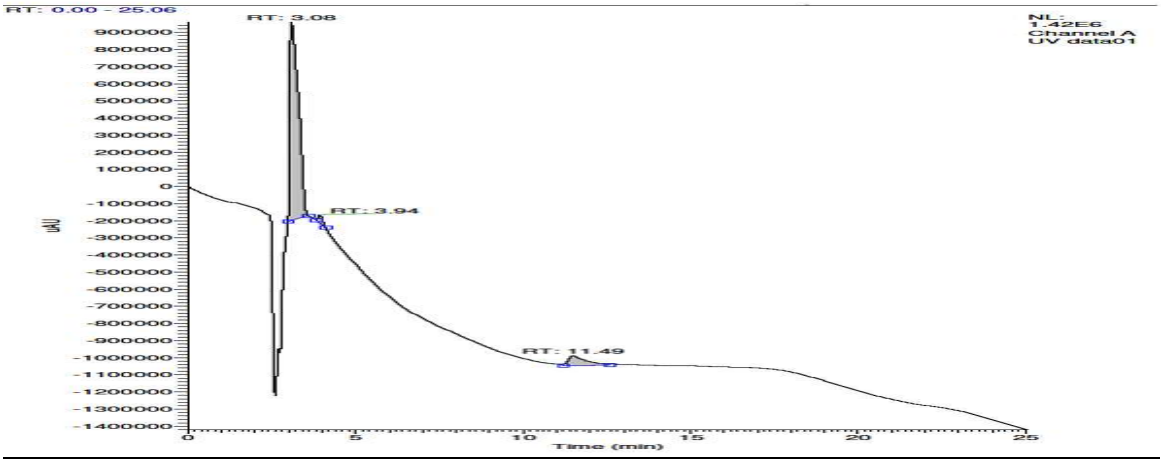
2. **Neutral Degradation-**



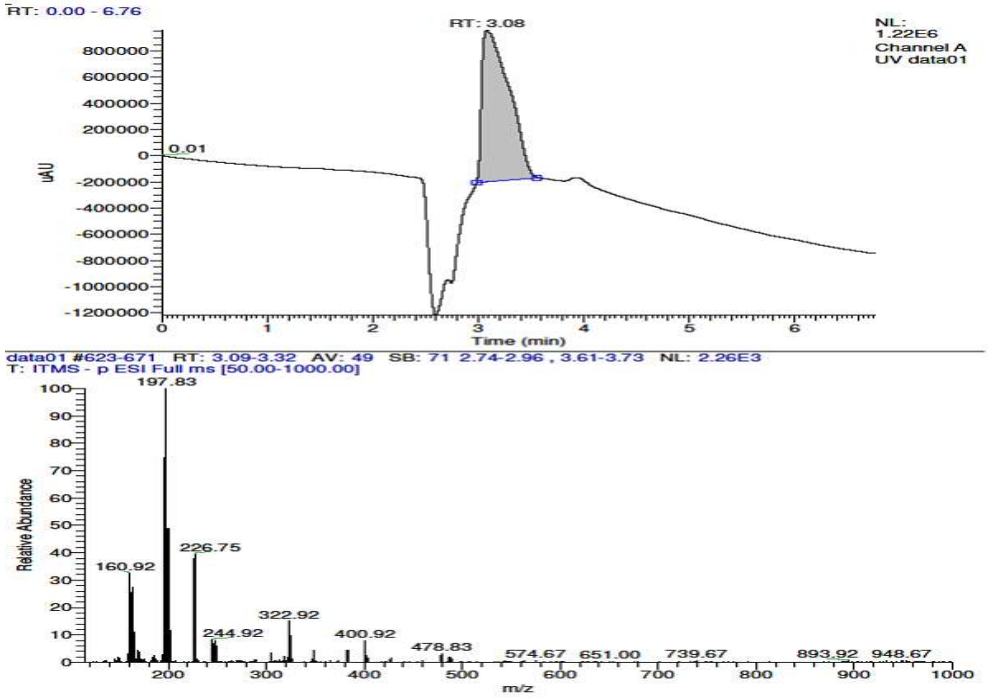
CHARACTERIZATION OF DEGRADATION PRODUCTS BY LC-MS:



LC-MS Report of Acid Degradation



LC-MS Report of First Acid Degradant of Mupirocin:



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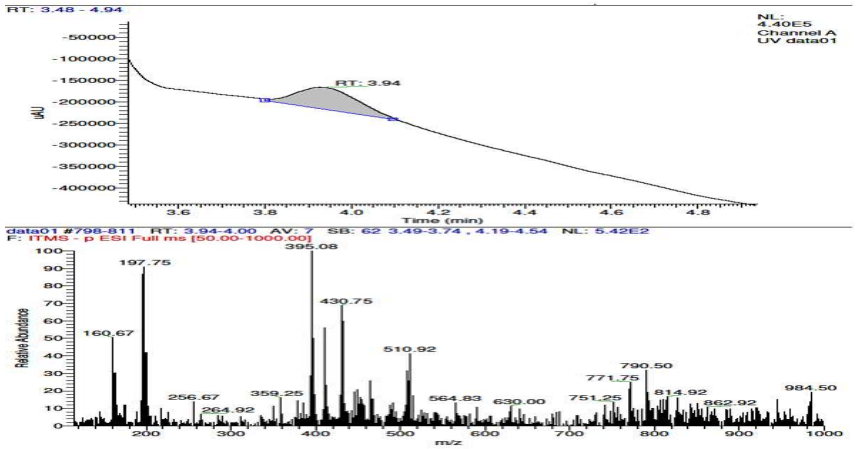
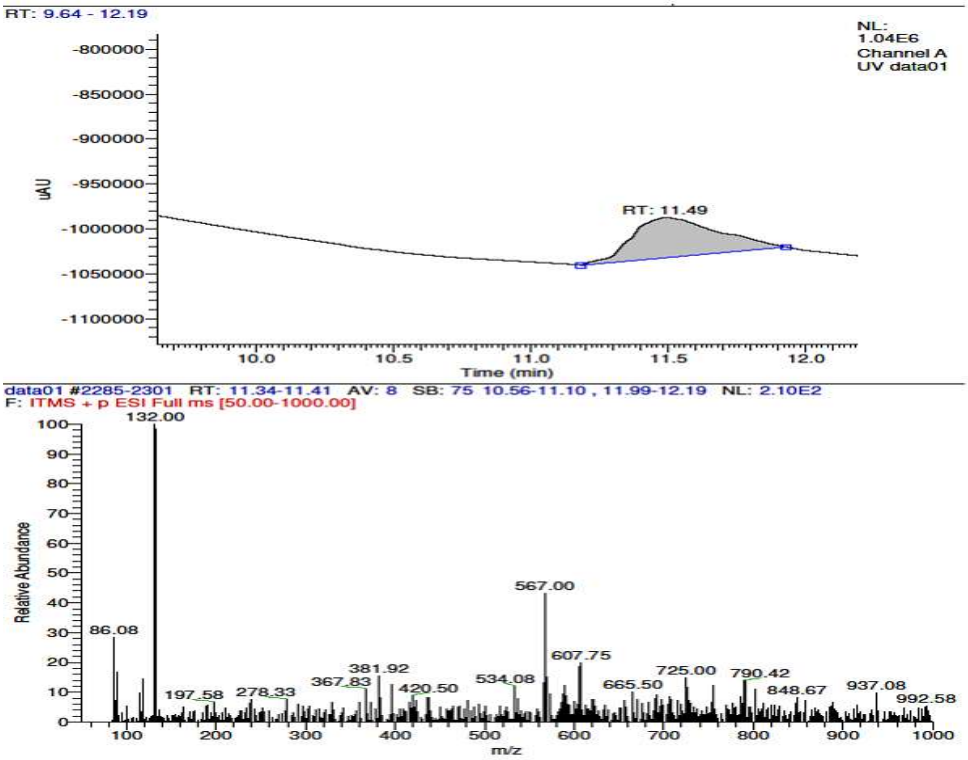
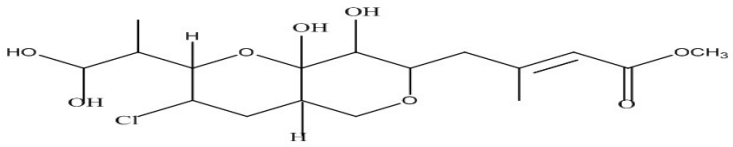


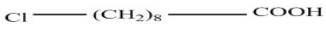
LC-MS Report of Third Acid Degradant of Mupirocin:



POSSIBILITY OF ACID HYDROLYSIS



1 - MOLECULAR WEIGHT - 395



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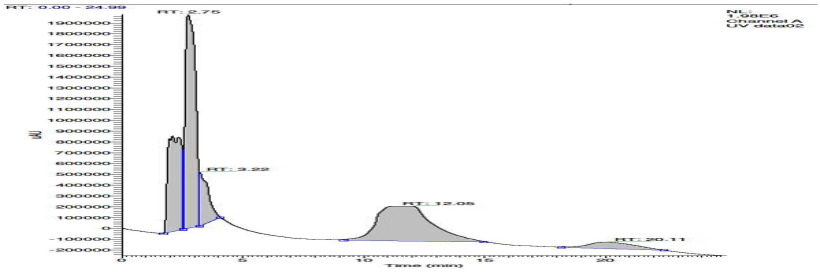
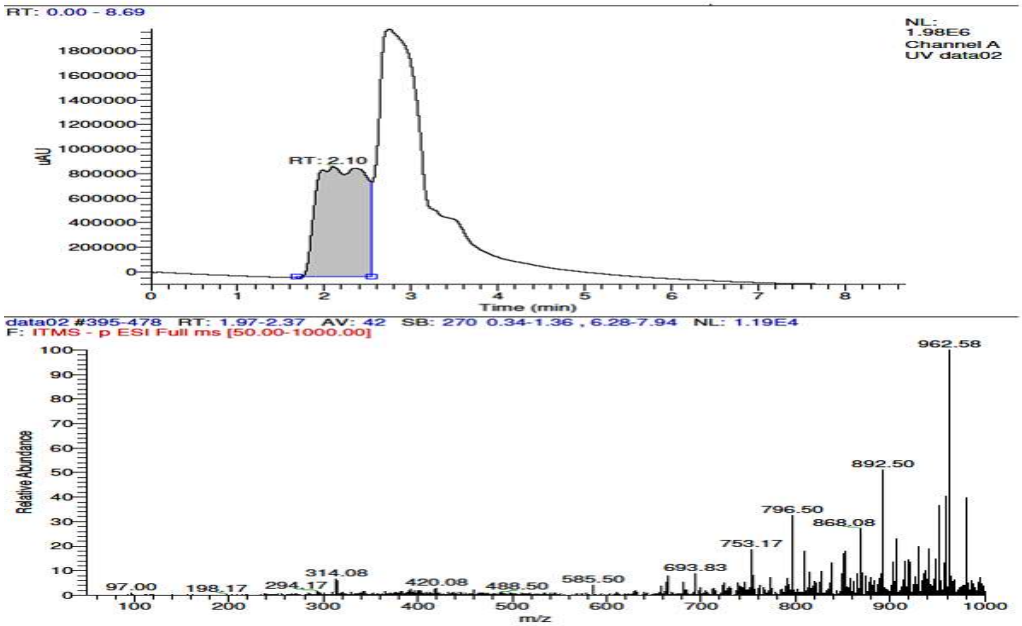


LC-MS Report of First Base Degradant of Mupirocin:



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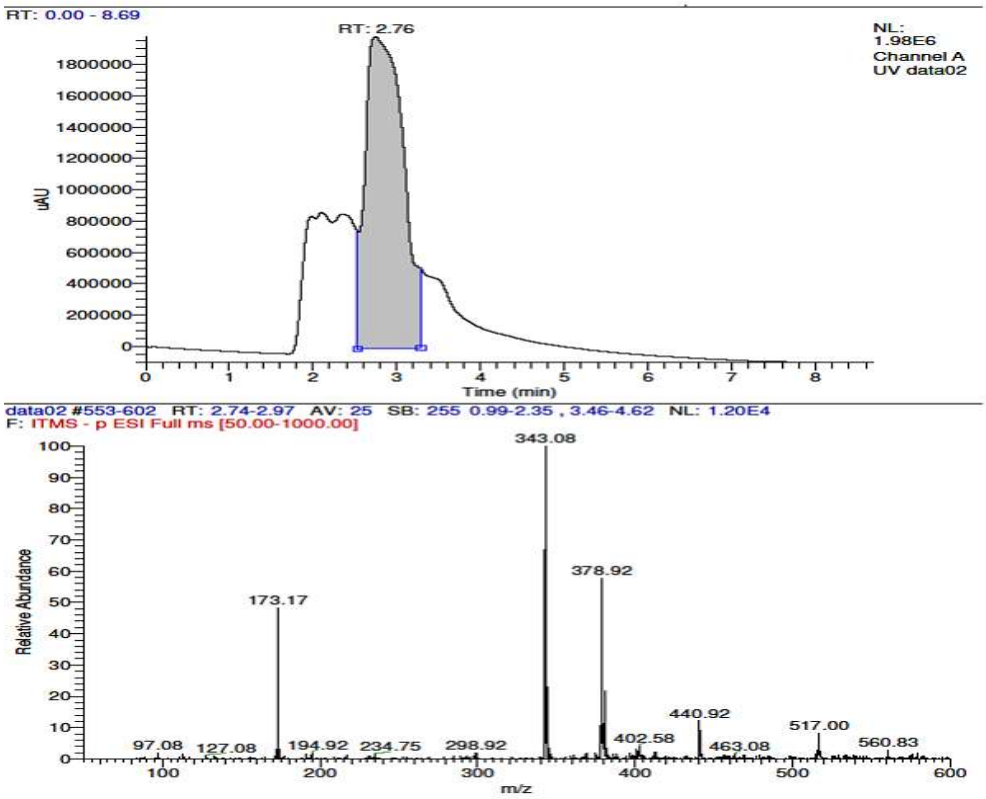
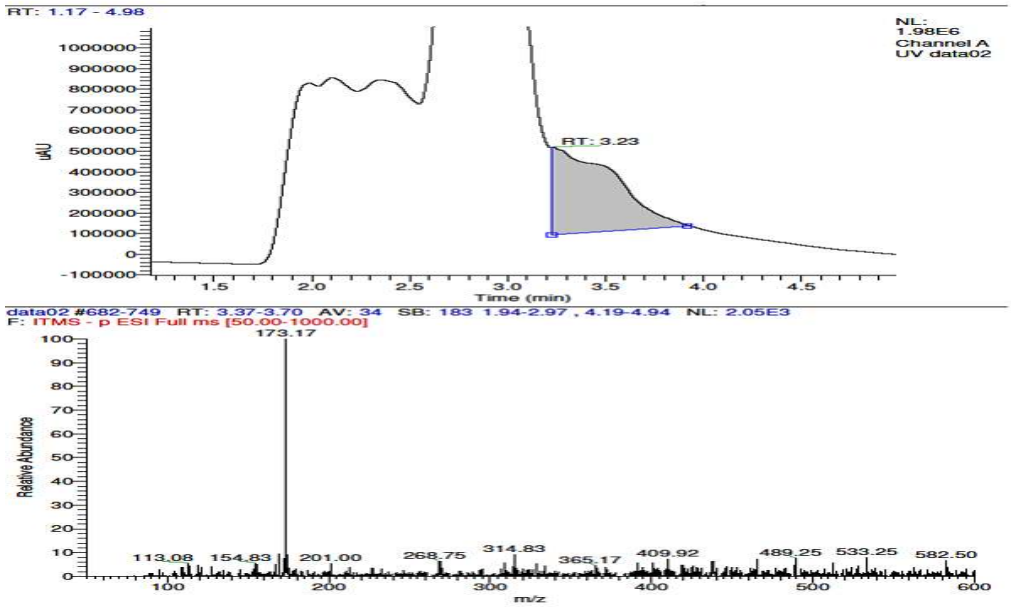


LC-MS Report of Third Base Degradant of Mupirocin:



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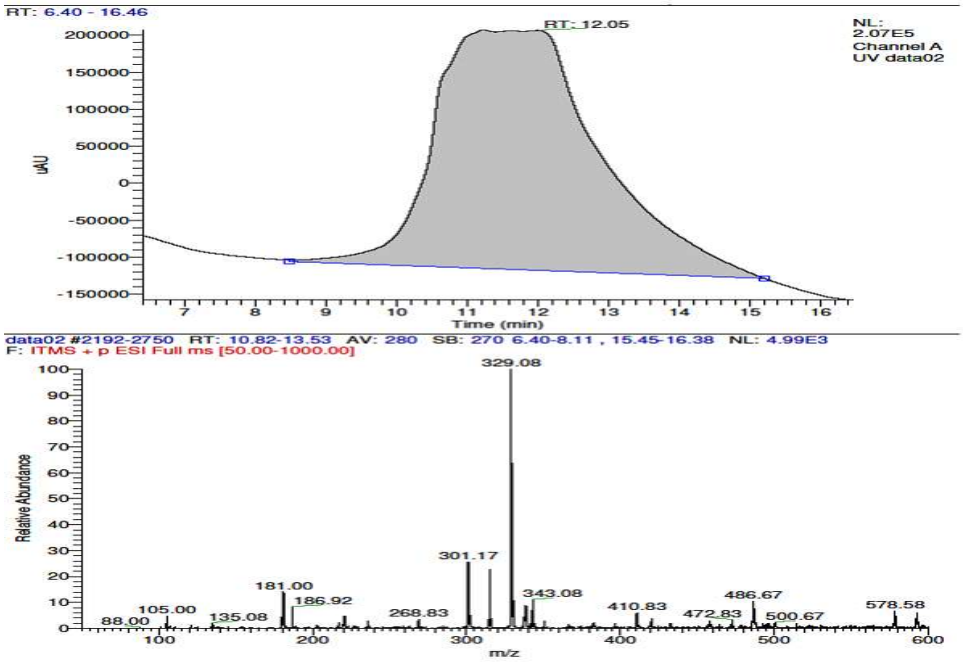

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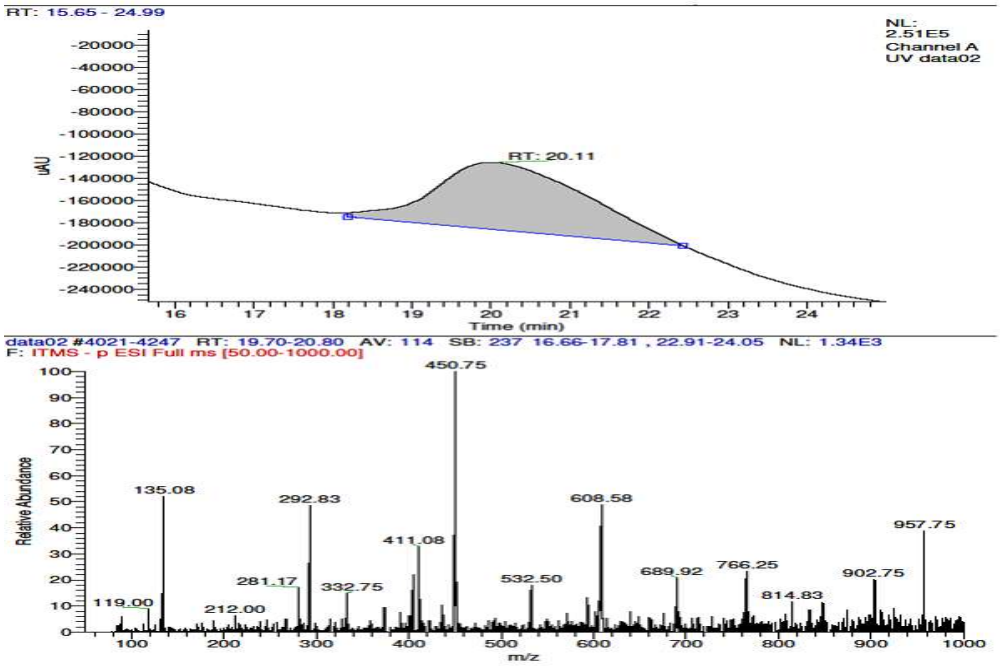
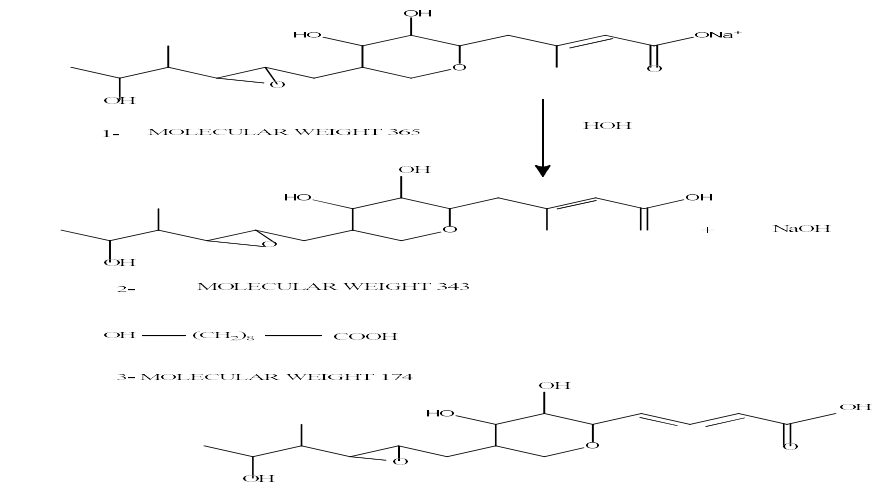


POSSIBLE BASE HYDROLYSIS:



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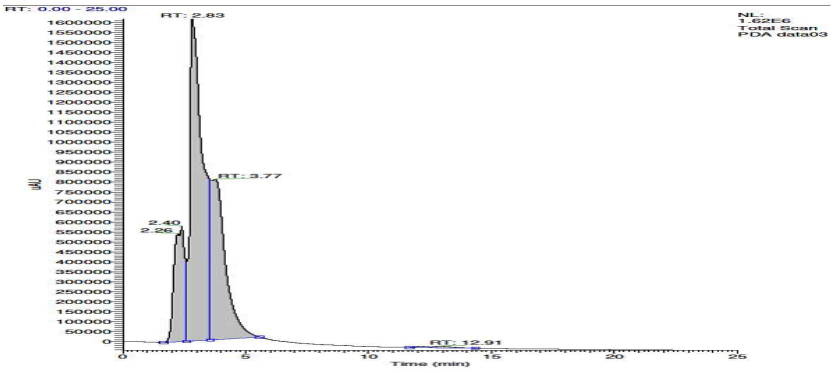

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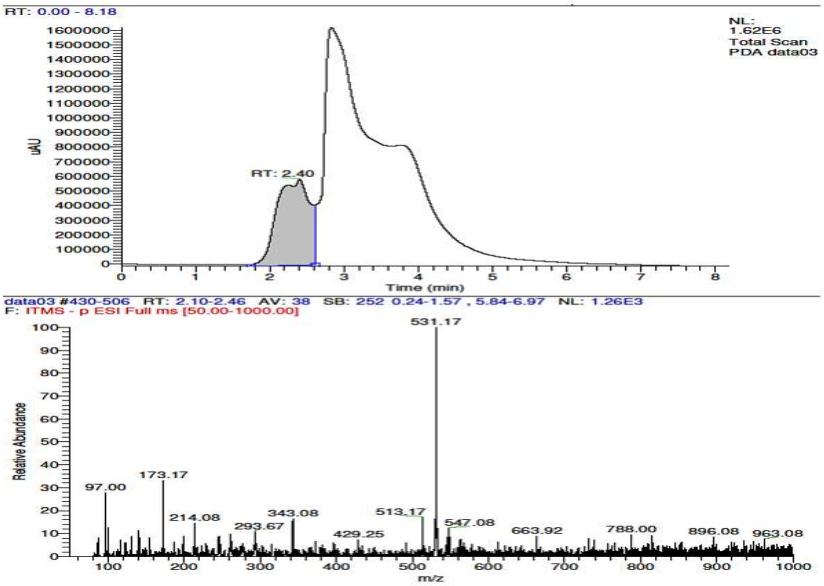

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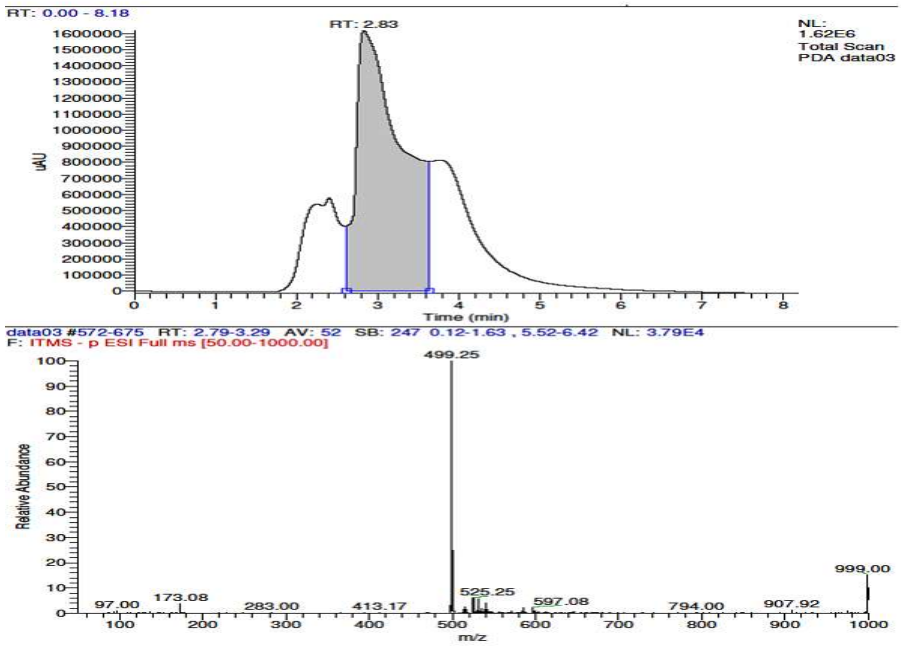

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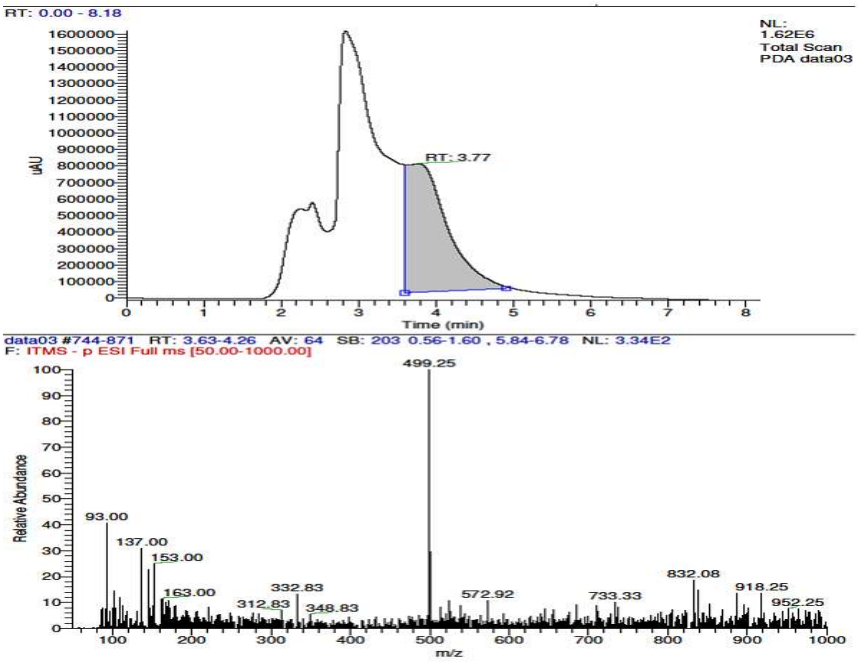

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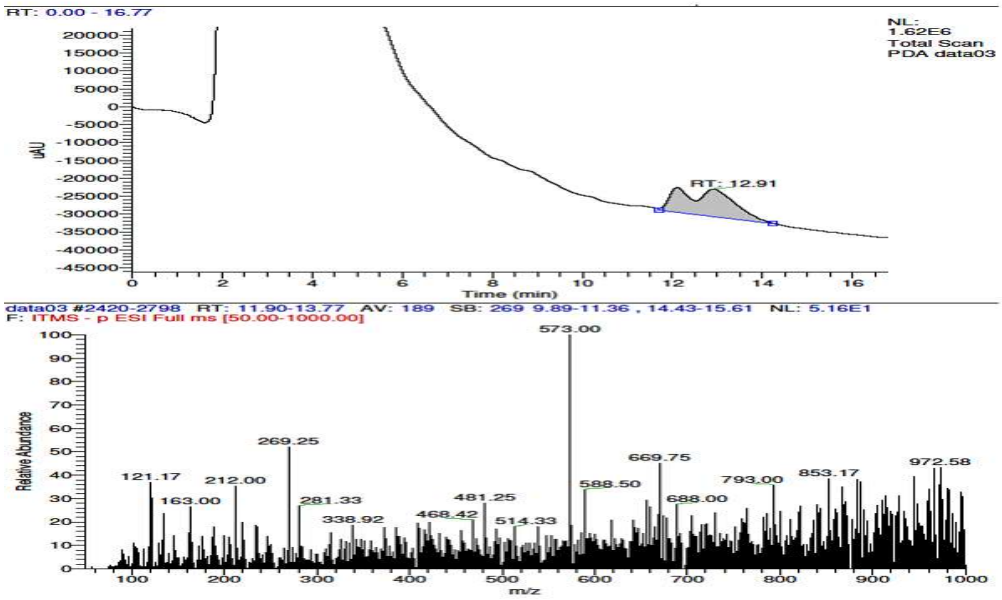
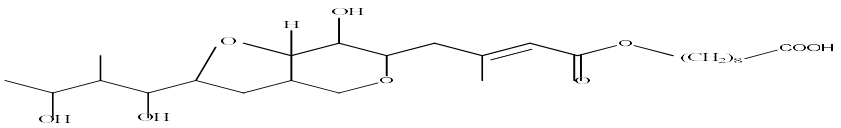


POSSIBILITY OF OXIDATIVE DEGRADATION:



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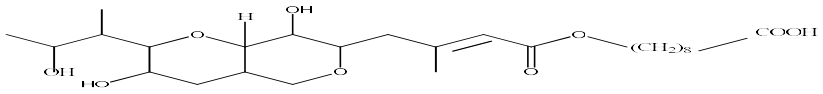

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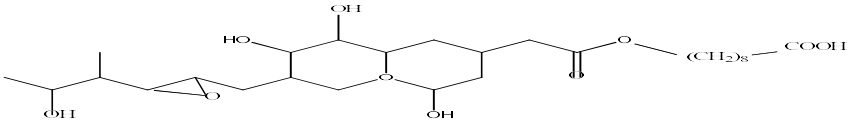

3 - MOLECULAR WEIGHT 531

RESULT AND DISCUSSION:

Identification of drug.

The identification was carried out by following test

Appearance: White, solid powder.

Solubility: Soluble in Alcohol, Chloroform and in Water (>10mg/ml).

Melting Point: 77-78^{0}C is reported according to USP, IP 77.5^0 C is practically observed.

TLC:[25] The solvent system:

Chloroform: Methanol (90:10)

Rf = 0.62

U.V. Spectroscopy: [25]

Practically found absorption spectrum maximum at, 219,223,227,228 nm.

IR Spectroscopy:

A Pellet of the drug prepared with KBr (Spectroscopic Grade) using hydraulic pellet press at a pressure of 7 – 10 tones. FT-IR was scanned from 400 - 4000cm-1 Following peaks were observed.

The reported IR spectrum of Mupirocin is identical to practically taken IR spectrum. From the data which I had observed, drug was found to be authentic.

HPLC method development:

1. Solubility study: Mupirocin is freely soluble in methanol, hence Methanol: Water (70 : 30) were selected as mobile phase.

2. Selection of detection wavelength:

After UV analysis, the wavelength is selected 228 nm for HPLC analysis.

Optimized HPLC Method:

To optimize the RP-HPLC parameters, several mobile phase composition were tried. A satisfactory separation and good peak symmetry was found in a mixture of Mupirocin: Water (HPLC grade) (70: 30) at pH 6.1 maintained by using *o*-Phosphoric acid

at 1 ml/min flow rate. The optimum wavelength for detection was set at λmax 228 nm at which much better response for drug was obtained as it was shown in fig. above. The retention time 5.6 - 5.7 min.

Method Validation: Validation was done as per ICH guideline Q2 (R1). The developed RP-HPLC methods were validated with respect to parameters such as system suitability linearity, precision, accuracy, specificity, ruggedness, robustness and solution stability.

System Suitability:

System suitability test as per method should be performed and checked before performing any parameter. The values of system suitability results obtained were recorded in Table No. 03.

Note:

I. System suitability values was taken from the first injection of five replicates of standard.
II. % RSD is calculated from five replicate injections of standard **[34-39]**.

Linearity and range:

Mupirocin shows good correlation coefficient in concentration range of 10 – 50 µg /ml (r^2= 0.9994). Five levels of working standard solutions of drug were prepared from the solutions of 100µg/ml. Triplicates of each sample were injected to obtain responses. Then, system suitability parameters, linearity regression coefficient, % RSD of response factor, %-Y-intercept calculated

Acceptance criteria:

The correlation coefficient value should not be less than 0.999 over the working range. **[34]**.

Precision

Precision of the method was verified by Intraday and intermediate precision (Interday) studies, and result was recorded

Acceptance Criteria

% RSD of method precision and intermediate precision should not be more than 2.0%. **[34]**

Repeatability:

Repeatability is a measure of precision under the same conditions over a short period of time.

Acceptance Criteria

% RSD of method Repeatability should not be more than 2.0 %. [34] The results obtained were well within the acceptance criteria. The method can therefore be termed as precise and rugged.

LOD and LOQ:

For the drugs, LOD and LOQ were determined based on the standard Deviation of response of the respective calibration curves.

In the forced degradation study the various stress condition were applied on Mupirocin

which was shown in Table No.16 and HPLC results were recorded.

In the observation found that in acid degradation the drug is degraded 10.5 % at 2 N HCl which shown in (Fig). In base degradation the drug was degraded 28.12% at 2 N NaOH, in (Fig.) and in Oxidation at 3 % H_2O_2, drug degraded 41.96 %.in (Fig.). In neutral, and photo stress condition degradation of Mupirocin was not observed in HPLC analysis which shown in (Fig.) and they show same melting point as standard drug.

CONCLUSION:

HPLC method was develop and validated as per ICH guidelines. Accurate quantitation of chromatographic compounds was observed by U.V. detection (Analytical Technology, U.V.: model 2080). The drug was analyzed by HPLC method using Hi Q Sil C-18 (250 mm x 4.6 mm, 5 µ), Gradient System consist of quaternary pump, The Mobile Phase composition was prepared with the help of HPLC grade methanol: water (70:30) by adjusting the pH 6.1 with Orthophosphoric acid in water and flow rate of 1 ml/min using U.V. detection at 228 nm. The procedure has been evaluated for the Linearity (r^2=0.9994), Precision and System suitability %RSD was not more than 2%, in order to ascertain the suitability of analytical method. It has been prove that the method is selective and linear between concentration range 10 – 50 µg/ml for Mupirocin. In LOD and LOQ, signal-to-noise ratio found to be 0.10 and 0.30 respectively within given limit. This method is validated as per ICH guidelines.

In the Forced Degradation study various stress condition applied as, Acid Hydrolysis-Drug recovery 89.50 %. Base Hydrolysis-Drug recovery 71.08 %, Oxidative Hydrolysis – Drug recovery 58.04 % and in case of Photo-degradation , Neutral degradation there is 100% Drug recovery was found in API of Mupirocin. According to LC-MS data Mupirocin was unstable in acidic, alkaline and oxidative media but stable in neutral and photolytic condition.

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