CHAPTER 5

STRESS DEGRADATION STUDY AND IMPURITY PROFILING OF RIFABUTIN

5.1 Selection of drug[1-4]

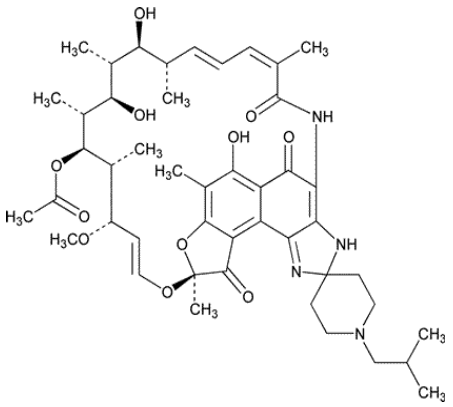
Rifabutin belongs to rifamycin antibiotic category which is natural or semi synthetic antibiotics produced from *Amycolaptosis Rifamycinica.* U. S Food and drug administration approved rifabutin in the year 1992 and [4] Central Drugs Standard Control Organization approved Rifabutin in the year 2007. [3] U. S Food and drug administration approved rifabutin in the year 1992 and [4] Central Drugs Standard Control Organization approved Rifabutin in the year 2007. The rifabutin is indicated for the use in HIV associated with MAC (*Mycobacterium Avium Complex*) syndrome; the bacterial infection caused by the *Mycobacterium* e.g. TB. In recent years the circumstances of HIV associated with other microorganisms or bacterial infections are heavily rising due to lifestyle and carelessness towards health. For the treatment of such associated HIV, rifampin was prescribed in the early years; rifampin has a rapid pharmacokinetic half-life so frequent dosing was required, while the new molecule synthesized from the same source of *A. Rifamycinica,* rifabutin has a slow pharmacokinetic half-life (Approximately 35 hrs.) that can reduce the frequency of dosing and was more effective against bacterial infection as rifabutin is bactericidal, therefore rifabutin became the choice of drug to treat HIV associated with MAC. Rifabutin is considered as the replacement therapy of rifampin. The sufficient analytical and stability data; impurity and degradation products data of rifabutin was not published in the public domain (Section 5.2) so the study was essential to identify and explore the unexplored area of rifabutin.

5.2 Literature Review[5-24]

An extensive literature survey showed that analytical data reported for rifabutin includes UV spectrophotometric methods [5-6], RP-HPLC methods [7-15], LC/MS/MS [16,17], LC/MS [18] simultaneous estimation with other drugs [22-24] are reported in literatures. The stability studies are published by HPTLC [19] and HPLC [20-21]. The published methods and studies include only quantification of rifabutin by these methods; it does not show impurity or degradation products' identification, isolation, or characterization. There was no published data in terms of significant stability study and impurity profiling of rifabutin at the time of the study; being an analytical scientist it was an opportunity as well as a challenge to identify impurities, degradation products in rifabutin and report the stability study with impurity profiling of rifabutin in the public domain.

5.3 Drug Profile [25]

The Rifabutin is approved drug by USFDA and CDSCO; the physical-chemical properties of rifabutin are mentioned in Table 5.1.

Drug property	Inference
CDSCO Approval	July, 2007.
Drug Category	Anti Mycobacterium (Rifamycin)
Mechanism of action	RFT acts on DNA- dependent RNA Polymerase enzyme which is required for RNA Synthesis for *Mycobacterium*, thus RFT exhibits bactericidal activity.
Marketed Formulation	Ributin ® (Lupin) contains 150mg of RFT.
Chemical structure [5]	
IUPAC Name	(7S,9E,11S,12R,13S,14R,15R,16R,17S,18S,19E,21Z)-2,15,17-trihydroxy-11-methoxy-3,7,12,14,16,18,22-heptamethyl-1'-(2-methylpropyl)-6,23,32-trioxo-8,33-dioxa-24,27,29-triazaspiro[pentacyclo[23.6.1.1^{4},^{7}.0^{5},^{31}.0^{26},^{30}]tritriacontane-28,4'-piperidin]-1(31),2,4,9,19,21,25,29-octaen-13-yl acetate
Molecular Weight	847.0047g/Mol
Molecular Formula	$C_{46}H_{62}N_4O_{11}$
Physical Appearance	Red-Violet solid amorphous powder

Solubility	Soluble in methanol, chloroform, very slightly soluble in water.
Pka	6.9
Log P	4.1

Table 5.1 Physicochemical properties of rifabutin

5.4 Bulk Drug identification [26-27]

Rifabutin was procured from Lupin Pharmaceuticals, Ankleshwar, Gujarat, India as a gift sample. The sample was analyzed against any changes that may have occurred during the transport of rifabutin. The identification of the sample was done by Infra-Red study, Ultra-Violet spectrophotometric study, melting point study, and solubility study. These studies can confirm the identification of the sample by comparing it with reported data.

5.4.1 IR spectroscopy [26]

The instrument and its specification for IR spectroscopy study are same as described in section 3.4.1. IR study shows the presence of functional groups in the solid samples by analysis of atomic vibrations. Each sample has a definite different set of chemical groups present in it, which helps to identify or differentiate it from others. The IR spectrum is shown in Fig. 5.1

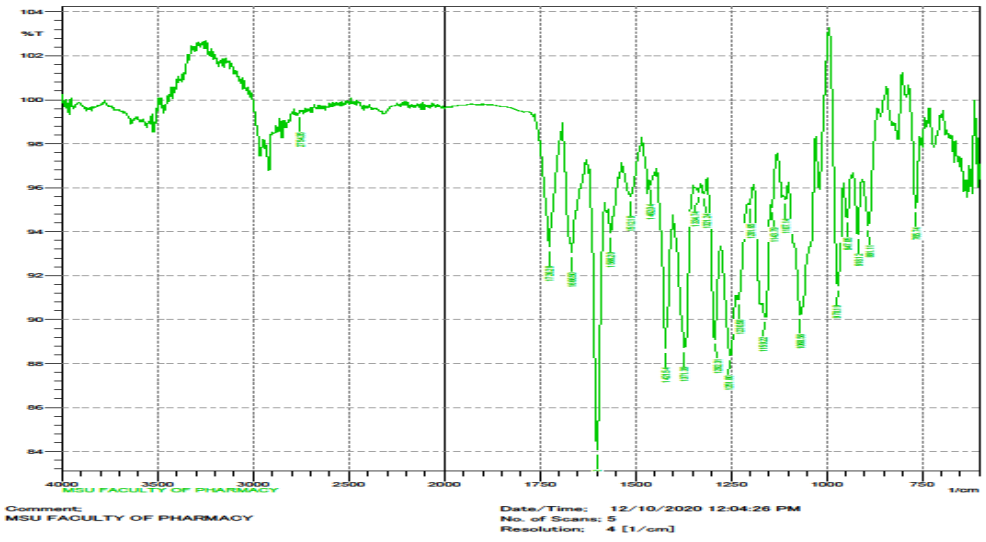


Fig. 5.1 IR spectrum of rifabutin

The analysis of the IR spectrum is shown in Table 5.2 for assigned wave number and obtained wave number, against chemical group or atoms.

Group	Obtained wave number (Cm^{-1})	Assigned wave number (Cm^{-1})
-CH bending, (Mono substituted/ 1,2 di substituted/ 1,2,3, tri substituted)	764.74	780 ± 20
-C=C bending, (Alkene (Di substituted ,Cis))	891.11	885-895
C=C bending, (Alkene (di substituted, trans))	970.19	960-980
OH, (Primary alcohol)	1068.56	1050-1085
C-O stretching, (Secondary alcohol)	1107.14	1124-1087
C-O stretching, (Aliphatic ether)	1143.79 1159.22	1085-1150
C-O stretching, (Tertiary alcohol)	1201.65	1124-1205
C-N stretching,(amine)	1230.58	1020-1250
C-O stretching, (alkly aryl ether)	1251.80	1200-1275
C-N stretching, (aromatic amine)	1292.31	1266-1342
O-B bending (phenol)	1321.24,1334.72, 1371.39	1310-1390
C-H bending (methylene group)	1462.04	1465
C=C stretching ,(cyclic alkene)	1566.20,1601,02	1566-1650
C=C stretching (tri substituted alkene)	1666.50	1665-1675
C=O stretching (α,β unsaturated ester)	1726.29	1715-1730
C-H stretching (aldehyde)	2754.35	2695-2830

Table 5.2 IR spectrum analysis of rifabutin sample

The IR spectrum analysis shows the primary group of rifabutin as alcohol, amine, and ether which are also present in the chemical structure of rifabutin. Based on this analysis the sample can be identified as rifabutin.

5.4.2 UV spectrophotometric study [27]

The instrument and its specification for UV spectrometric study is same as described in section 3.4.2. Each molecule has a particular absorbance wavelength in the UV range (190-400nm), and at the maximum absorbance of UV, the sample shows a high-intensity peak. The UV spectrophotometric curve for 90.0 µg/ml of rifabutin solution was taken to analyze the UV maxima of rifabutin. (Fig. 5.2)

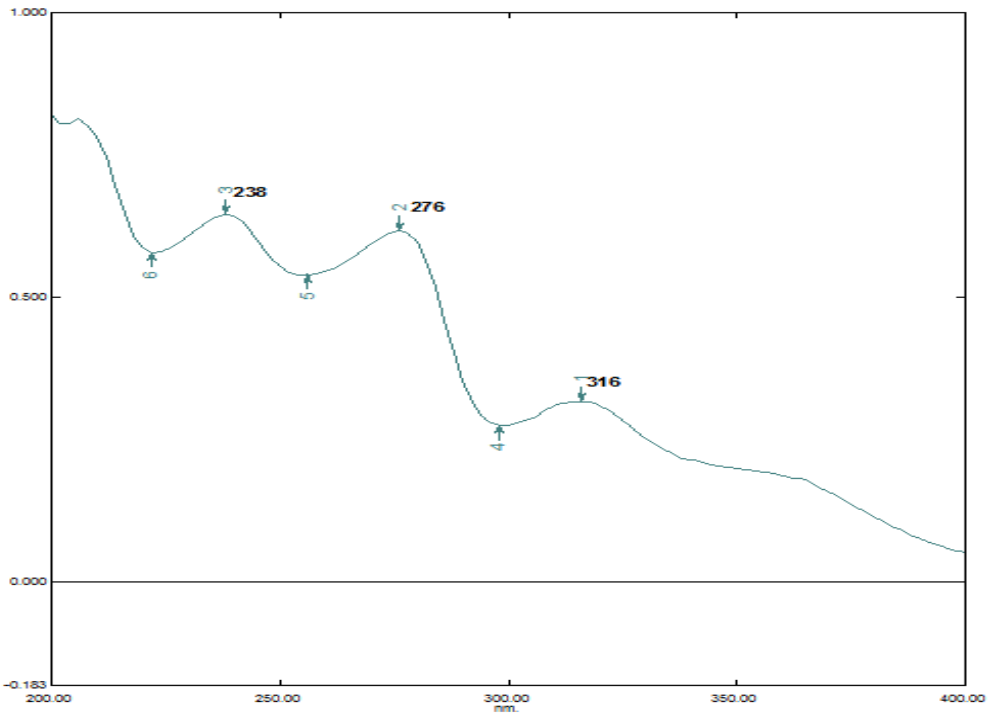


Fig. 5.2 UV spectrophotometric curve for rifabutin (90.0µg/ml)

The UV spectrophotometric curve shows three UV maxima for rifabutin which are 238, 276 and 316nm; the reported UV maxima for rifabutin in literature are 236,275 and 315nm. Therefore the sample can be identified as rifabutin without any change in structure.

5.4.3 Melting point study [27]

The molecule requires a particular temperature to change state from solid to liquid and this temperature can be referred to as the melting point. Each molecule has established range of melting point. To find out the melting temperature range of rifabutin DSC (differential scanning calorimetry) was performed. The instrument and its specifications are same described in section 3.4.3. The DSC thermogram is shown in Fig 5.3.

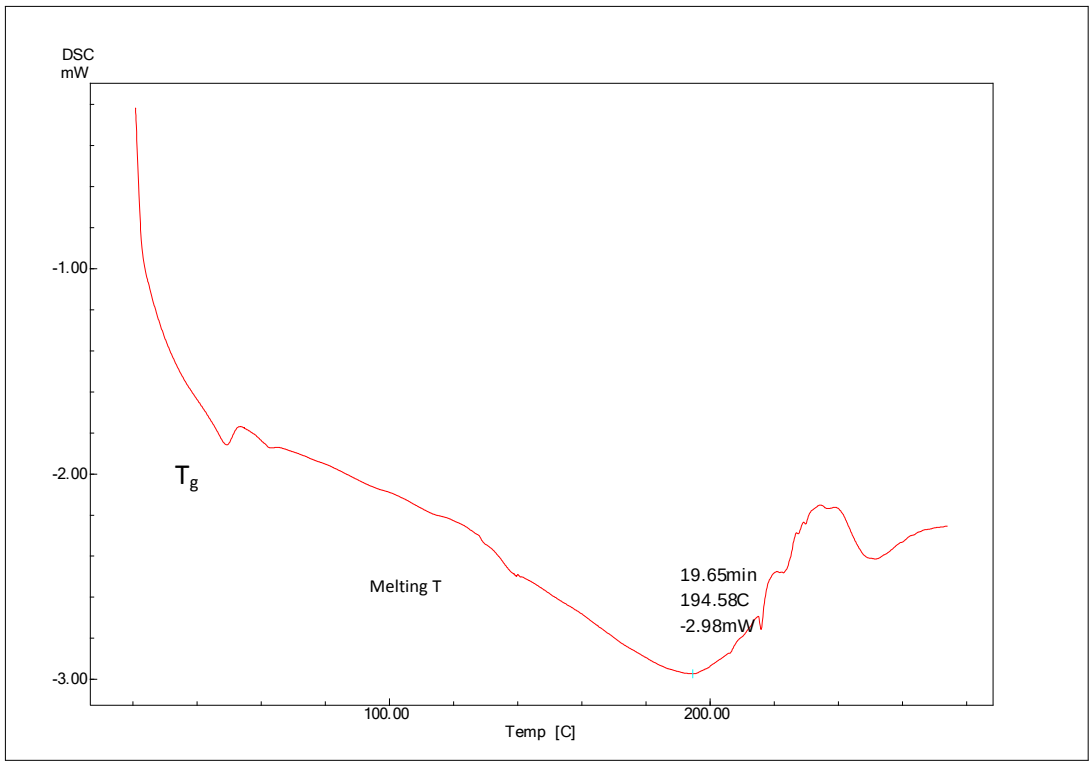


Fig. 5.3 DSC thermogram for melting point analysis of rifabutin

The DSC thermogram shows that rifabutin shows transition temperature (t_g) at 49.5^0C and melting point at $145\text{-}149^0$C; the reported melting point for rifabutin is $149\text{-}151^0$C. The reported and obtained melting points are close enough to identify the sample as rifabutin.

5.4.4 Solubility study [27]

The solubility study was completed in different solvents by adding accurate 1mg of rifabutin in 1ml of solvent. The result showed that rifabutin is freely soluble in methanol, soluble in chloroform and partial soluble in water. From this study the organic solvent for dissolving and dilution purpose, selected was methanol.

PART-A

5.5 Development and validation of stability-indicting method and stress degradation study of rifabutin

5.5.1. Experimental

5.5.1.1. Chemicals and reagents

The chemicals and reagents utilized in these studies were same as described in section 3.5.1.1.

Rifabutin was obtained as gift sample from Lupin pharmaceuticals Ltd. (Ankleshwar, Gujarat, India).

5.5.1.2. Equipments and chromatographic conditions

The equipments for analytical studies used are same as described in section 3.5.1.2.

For method development, the separation was accomplished on a Thermo scientific RP-C_{18} column (250×4.6mm, 5µm) at wavelength 275nm. The analysis was performed at ambient temperature with manual sample loading of 20µl. The mobile phase was filtered through 0.22µm disposable filters (Ultipore®, PALL Life sciences, USA), and degassed with provisional ultra sonicator prior to use.

Stability-indicating method: Stability indicating method was developed to achieve separation between degradation products and rifabutin bulk drug using **A)** 0.01M ammonium acetate buffer pH 4.2±0.5 with glacial acetic acid and **B)** acetonitrile as mobile phase in isocratic mode (% V/ V: 50/50 %).

5.5.1.3. Analytical sample preparation

Preparation of Stock, Sample and Buffer solutions

The buffer preparation for chromatographic analysis was same as described in section 3.5.1.3.

Sample preparation for linearity: Stock solution of rifabutin was prepared by dissolving 10mg of rifabutin in 1 ml methanol and remaining volume was made up with methanol to produce final 10ml volume to achieve 1mg/ml stock concentration. The working standards were prepared in methanol to produce 50.0-250.0 µg/ml of rifabutin by taking aliquots of 0.5,

1.0, 1.5, 2.0, 2.5, 3.0ml and diluted to 10ml with methanol separately in 10ml volumetric flask.

Preparation of stress degradation samples

A preliminary stability of rifabutin was evaluated in organic solvent (methanol) and for solid bulk drug at room temperature to gather some basic information about the stability of the bulk drug at room temperature and in organic solvent. Two samples were generated for every stress condition samples;

i) The blank solution(Without API) subjected to stressed condition in the same manner as the API

ii) The API solution (with degradant) subjected to stressed condition.

Sample for stress degradation study: Rifabutin (accurately weighed 500mg) was dissolved in 5ml methanol and sonicated for 15minutes with provisional shaking; 50ml final volume was achieved using 30% hydrogen peroxide, 0.5N HCl, 0.1N NaOH and water (final concentration 100mg/ml) individually. At regular time interval, aliquot of 2ml was withdrawn from the 0minute to till the stability study was completed, sample was kept in dark and diluted to 10ml with methanol (20mg/ml); filtered through 0.45 µ Pall syringe filter (procedure was carried out in dark to partial darkness) and injected in chromatographic system described in section 5.5.1.2 for RP-HPLC data acquisition and analytical purpose.

Rifabutin was stressed to maximum condition where 5-100% decreases in peak area of rifabutin bulk drug noticed. Marketed formulation of Rifabutin (Ributin, Lupin Pharma Pvt. Ltd.) was treated with same degradation conditions as performed for bulk drug using equivalent weight to 10mg from formulation to determine formation of any DPs due to drug excipient interaction.

Preparation for Assay of formulation

Ributin is the proprietary marketed formulation of Lupin Pharmaceuticals, Ankleshwar, Gujarat which contains Rifabutin in 150mg dose. It is available in the form of soft gelatin capsule. 20 capsules of Ributin was emptied, mixed and for assay, accurately weighed powder equivalent to 10mg rifabutin was dissolved in 10ml methanol to produce 1mg/ml stock solution, 1ml aliquot was taken in 10ml volumetric flask and volume was made up with methanol to get 100µg/ml. sample was injected in HPLC chromatographic system to perform HPLC assay method.

5.5.1.4. Method development

Preliminary investigations

Preliminary investigations were executed to study the effect of various chromatographic parameters e.g. buffer pH, organic modifier, organic ratio and flow rate on chromatographic separation. Various trials were executed to optimize HPLC method for rifabutin. Optimization of HPLC method is shown in Table 5.3.

Detection wavelength and Polarity: The UV Spectrophotometric graph in Fig. 5.2 showed maximum absorbance at 276nm therefore RP-HPLC detection was carried out at 275nm Rifabutin is mid-Polar in nature with Pka value of 6.9. DPs are even more polar in nature than rifabutin, so equal ratio of buffer and organic solvent was used to increase retention time (Rt) of rifabutin peak and to resolve the DPs.

Buffer pH: The pH of buffer showed high impact on drug peak shape and retention time while it showed less effect on DPs' separation, therefore pH selection was contributed for peak symmetry. Lower buffer pH 2.5 to 3 contributed in broadening of API peak and decrease in retention time, while increasing pH to 4.7 provided peak symmetry, more increase in pH 5.0 lead to drug peak fronting and increase in retention time. pH had no effect in separation of degradation products.

Choice of organic solvent: organic solvent selection depends on solubility of bulk drug in solvent. As per literature report and practical experience, rifabutin has high solubility in methanol and sparingly soluble in chloroform. Therefore for solubility and dilution purpose methanol was used as an organic solvent. Rifabutin is mid-polar in chemical nature; as a result methanol was a choice of organic solvent. The standard solution of rifabutin was prepared in methanol before stress study to dissolve the bulk drug. As DPs formed were polar in nature and rifabutin bulk drug has limited solubility, acetonitrile was preferred for mobile phase in terms of DP resolution, to optimize R_t, sensitivity and theoretical plates and maintain the polarity of mobile phase.

5.5.1.5. Validation of stability-indicating method [28]

The method validation is described in section 3.5.1.5.

5.5.2. Result and discussion

5.5.2.1. Determination of suitable wavelength

The overlay of UV spectrophotometric curves is shown in Fig 5.4. The concentration range for the overlay is 10.0-60.0µg/ml, the samples were scanned over UV range 190-400nm.

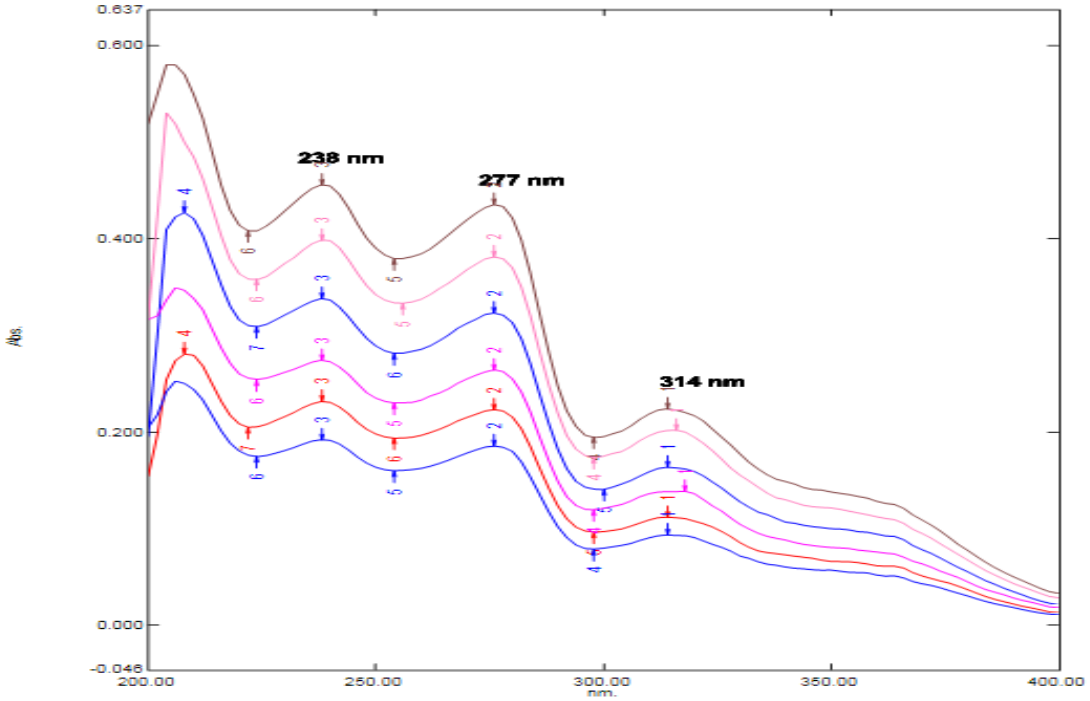


Fig. 5.4 The overlay spectrophotometric graph for rifabutin (10.0-60.0µg/ml)

The spectrophotometric graph showed UV maxima at 238, 277 and 314nm, during HPLC studies DPs and rifabutin showed good absorption at UV maxima 275nm therefore 275nm was selected as detection wavelength.

5.5.2.2. Method development and optimization

The initial method development trials were taken using different ratio of aqueous and organic solvent but the buffer and acetonitrile in % 50:50 ratio was best suited to separate rifabutin peak from DPs. Trials and optimized mobile phase is shown in Table 5.3.

Mobile Phase	Ratio	Result
1. Water: methanol	10:90	No repeatability of results.
2. Water: methanol	50:50	No repeatability of results.
3. Water: acetonitrile	50:50	Broad peak.
4. 0.01M ammonium acetate pH 5.0: methanol	50:50	Broad Peak Obtained
5. 0.01M ammonium acetate pH 5.0: acetonitrile	50:50	Peak shape needed correction.
6. 0.01M ammonium acetate pH 4.2±0.5: acetonitrile	**50:50**	**Good peak shape with long retention time.**

Table 5.3 Method development trials and optimization for rifabutin

5.5.2.3. Stress degradation studies [29,30]

The stress degradation studies were completed in hydrolytic solutions, oxidizing media, photolytic degradation and thermal condition. The mathematical formula for calculation of % drug and % degradation is shown in section 3.5.2.3. The %degradation of rifabutin is shown in Table 5.4. The chromatogram for each stress condition is shown in Fig 5.5.

The stress degradation conditions are;

Hydrolytic degradation condition

Acid degradation condition: The sample was prepared as described in section 5.5.1.3. For stress degradation 0.5N HCl was used as stressor, the sample was kept at RT (room temperature) for 24hrs.

Alkali degradation condition: The sample for stress degradation study was prepared in freshly prepared 0.1N NaOH and solution was allowed to stand in dark at room temperature for 2hours.

The aliquot of 2ml was neutralized with respective concentration of acid or alkali; filtered through 0.45µ Pall syringe filter, before injecting in chromatographic system of HPLC.

Neutral degradation condition: The sample for stress degradation was prepared in water and solution was kept at 80^0C for 24hrs.

Oxidative degradation condition

For oxidative degradation condition, the sample was prepared in 30% H_2O_2 and solution was kept at 80^0C in dark for 3 hours.

Photolytic Degradation

For light induced degradation, solid API was spread in approximately 1mm thickness on a Petri plate and exposed to 5382 LUX and 144UW/cm^2 in photo stability chamber for 28 days.

Dry heat induced (Thermal) Degradation

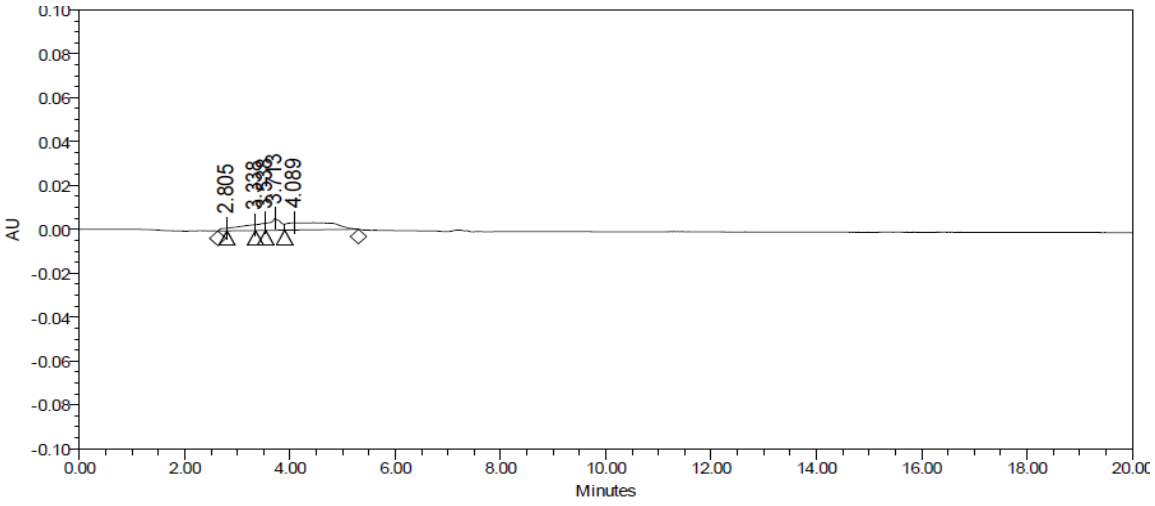
For thermal degradation, solid API was spread on a Petri dish with approximately 1mm thickness and placed in oven at 80^0C for 28 days under dry heat condition in dark.

Stressor Type	**Stressor Conc.**	**Time**	**DPs formed with RT**	**%Deg.**	**%Deg. of formulation**
Acid	0.5N HCl/RT	24hrs	Peak-1: 4.3 Peak-2: 4.5 Peak-3: 5.6 **Peak-4: 6.3** Peak-5: 7.5 Peak-6: 7.8 Peak-7: 8.5 API: 11.91 Peak-8:11.1 Peak-9:22.6 Minor peaks: 1.99 to 3.1	74.23	74.09
Alkali	0.1N NaOH/RT	1hrs.	**Peak-2:6.9** Peak-3:10.5 API: 13.117	88.86	89.01
Neutral	$H_2O/80^0$C	24hrs.	-	0.05	0.043
Oxidative	30% $H_2O_2/80^0$C	3hrs.	Peak-1: 2.4 API: 12.9	5.02	4.56
Thermal	80^0C	28 days	-	0.012	0.021

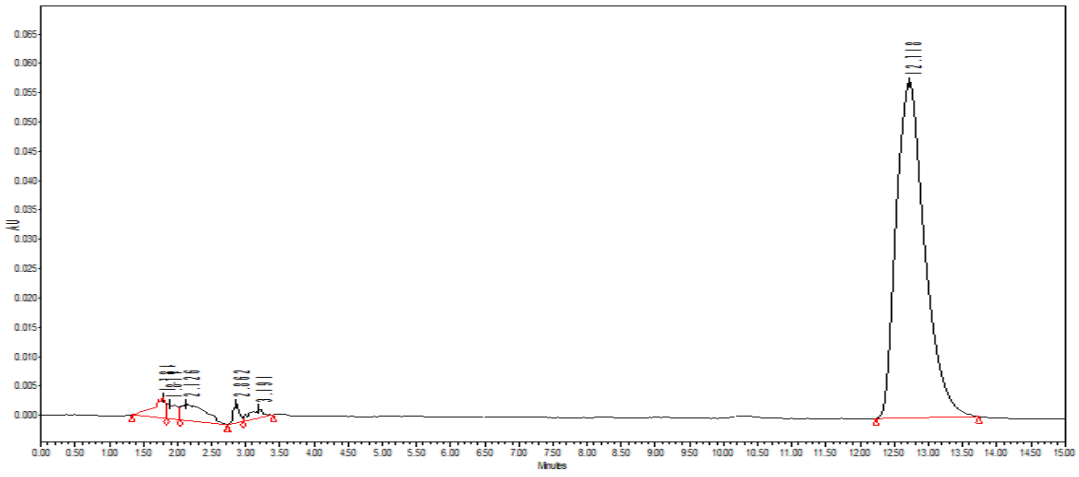
Photolytic	-	28 days	-	1.2	1.3

Table 5.4 summary of stress degradation study of rifabutin bulk drug and formulation

The blank chromatogram showed specificity of method, while chromatogram showing rifabutin bulk drug shows the selectivity of method for rifabutin. The acid chromatogram shows degradation products among which one major degradation product was formed. The chromatogram for alkali degradation showed one major degradation product. The oxidative degradation condition showed degradation of rifabutin but the degradation product was not detected in chromatogram.



A



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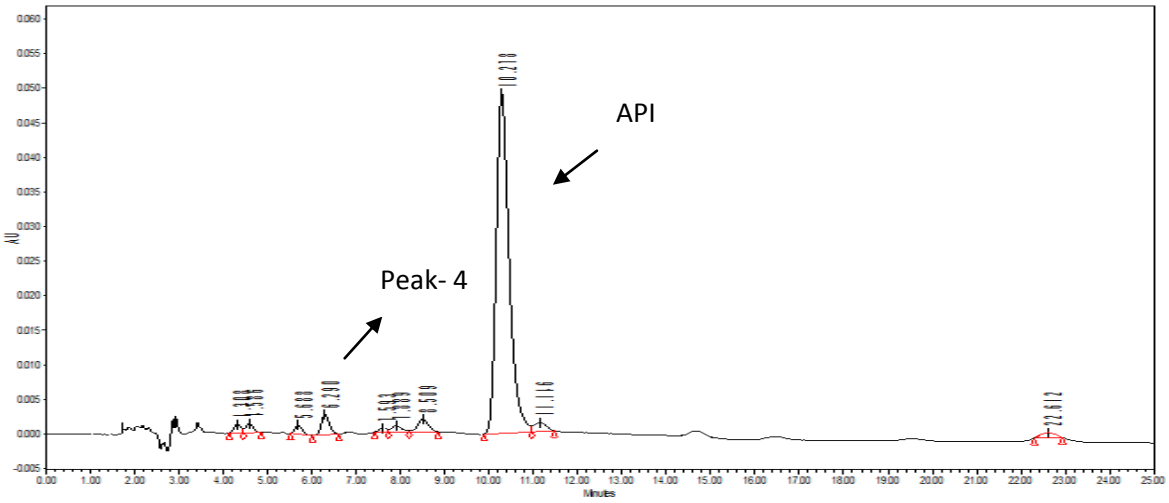

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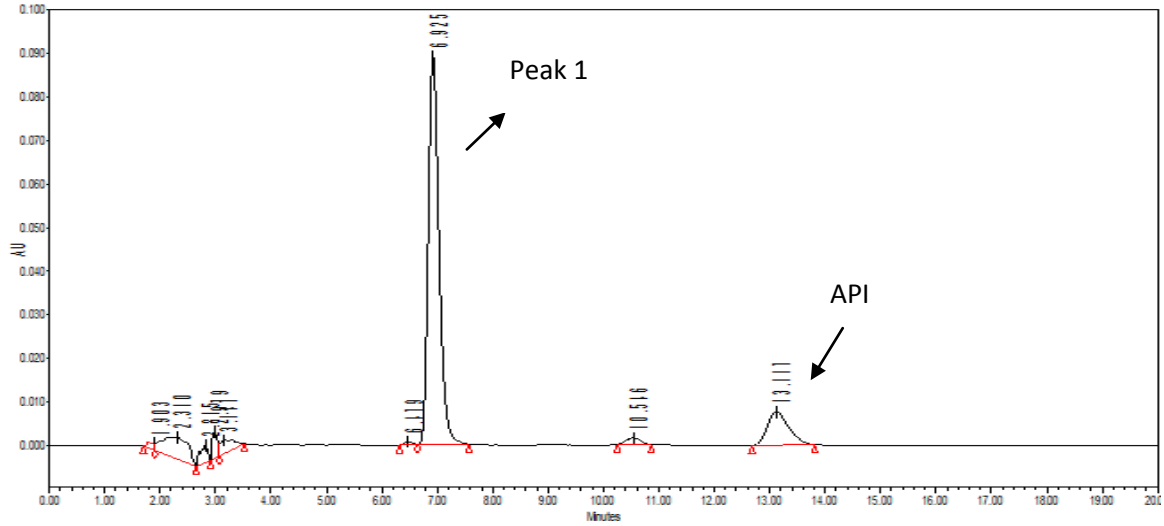

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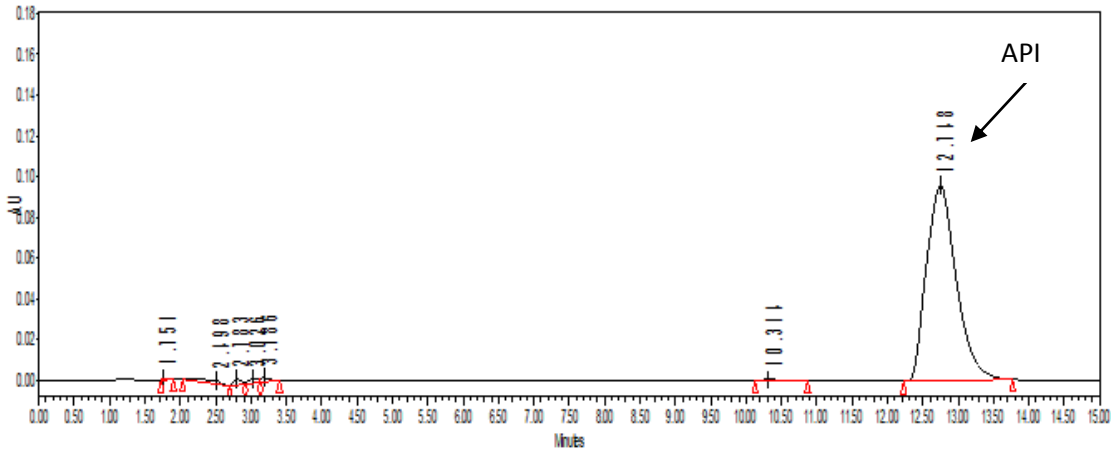

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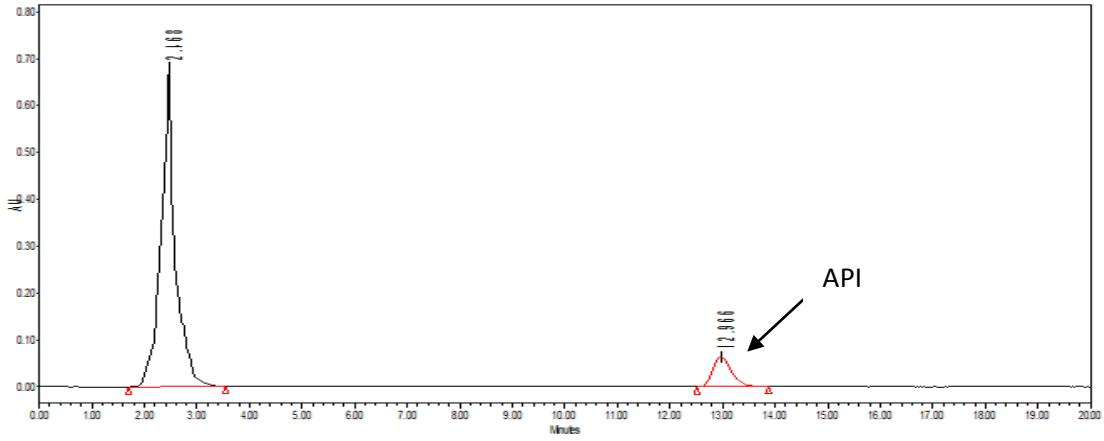

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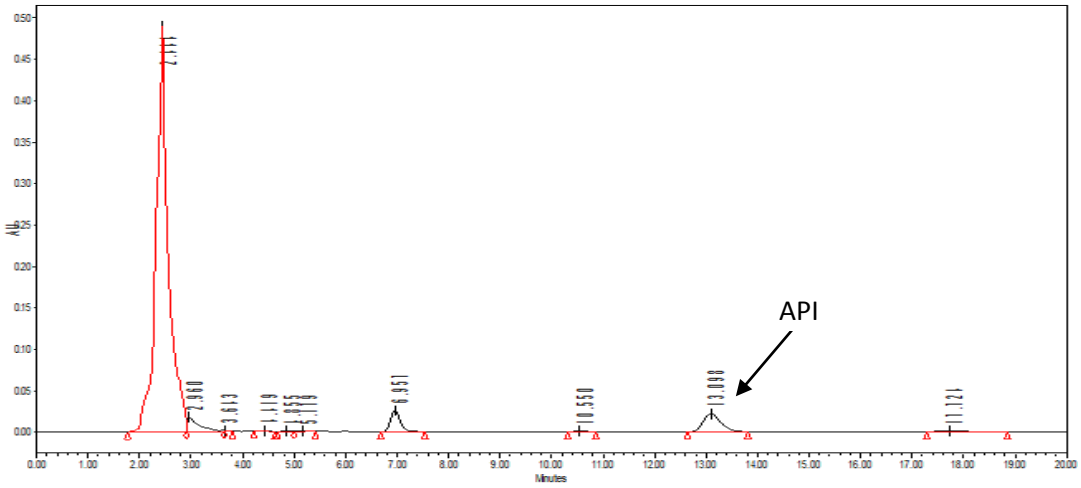

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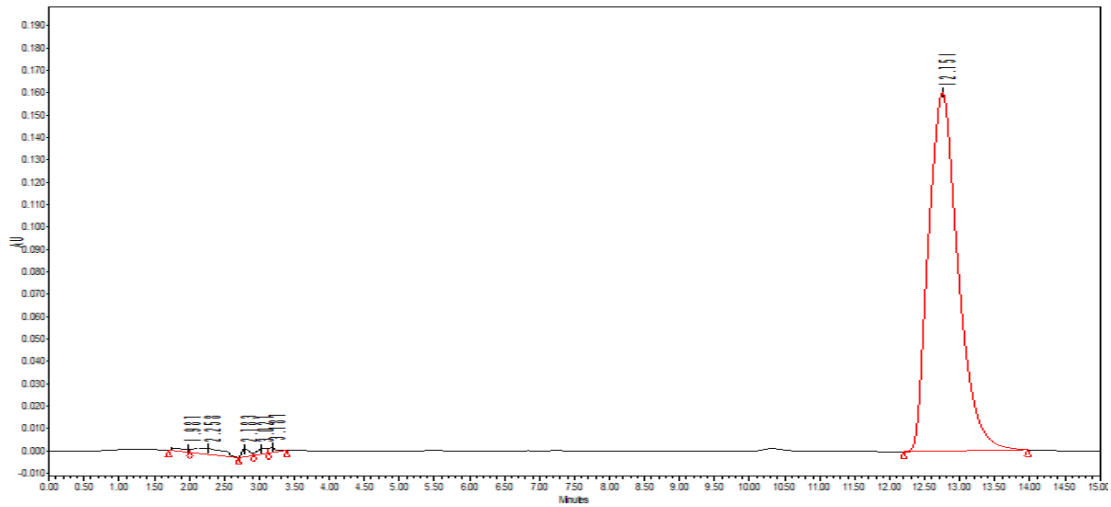

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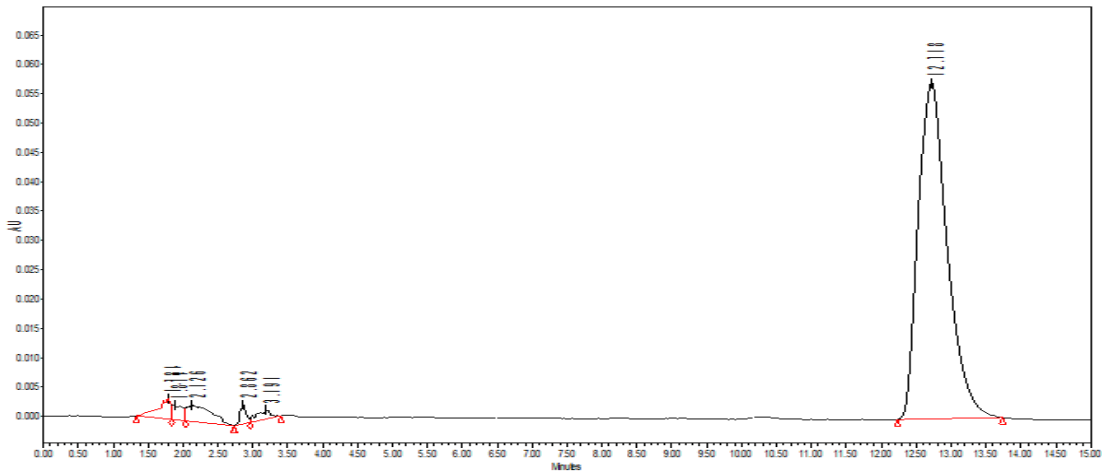


I

Fig. 5.5 Chromatograms of A) Specificity B) Selectivity C) Acid degradation D) Alkali Degradation E) Neutral Degradation F) Oxidative Degradation G) mixture chromatogram H) Thermal degradation I)Photolytic degradation of Rifabutin

There was no degradation observed in thermal, photolytic condition, and thermal/humidity induced degradation. The DPs in sufficient quantity to be identified or reported are reported and identified by UPLC/ESI-MS and NMR (^{1}H NMR, ^{13}C NMR and APT).

Peak purity test: Peak purity tests were performed for the DPs classified under to be identified and /or reported, characterized. The peak purity tests were performed to know whether any co-eluting DP with bulk drug or DP peak is obtained or not. It was performed for chromatogram shown in Fig.5.5 (B), (C), and (D). The result for peak purity is shown in Table 5.5 with purity angle which should not be exceeding the purity threshold.

DPs and R_t	Purity angle	Purity threshold	Pass/Fail
(B) Rifabutin bulk drug			
API-12.71	0.075	0.242	Pass
(C) Rifabutin acid degradation sample			
Peak1: 4.3	2.360	2.549	Pass
Peak 2:4.5	2.362	2.545	Pass
Peak3:5.6	2.889	3.262	Pass
Peak 4:6.2	1.226	1.512	Pass
Peak 5:7.5	4.368	4.951	Pass
Peak 6:7.8	3.116	3.403	Pass
Peak 7:8.5	2.090	2.303	Pass
API:10.2	0.157	0.278	Pass
Peak 8:11.1	2.147	2.318	Pass
Peak 9:22.6	3.746	4.111	Pass
(D) Rifabutin alkali degradation sample			
Peak 1:6.9	0.164	0.240	Pass
Peak 2 : 10.5	2.5	3.2	Pass
API:13.1	0.730	0.992	Pass
(F) Rifabutin oxidative sample			
Peak1:2.4	0.298	0.318	Pass
API: 12.9	0.013	0.284	Pass

Table 5.5 Peak purity results for chromatogram B, C, D and F

The peak purity for rifabutin bulk drug chromatogram is shown in Fig. 5.6 with low purity angle.

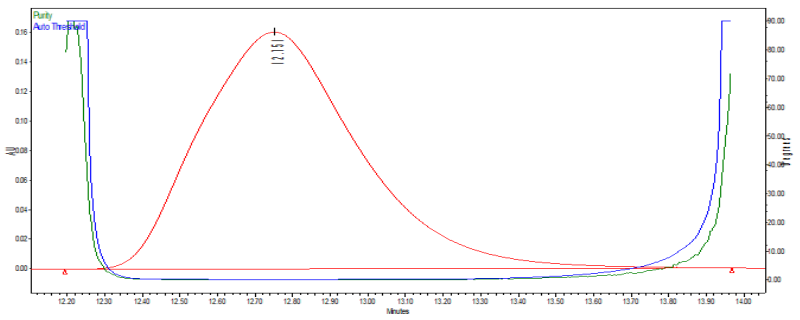
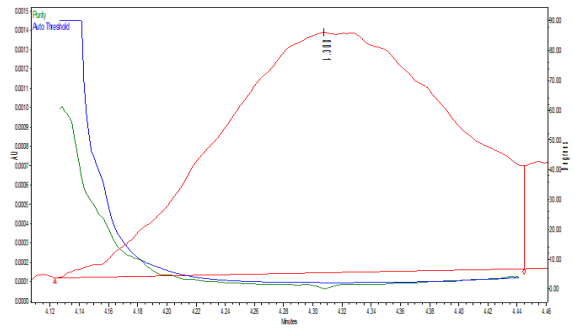
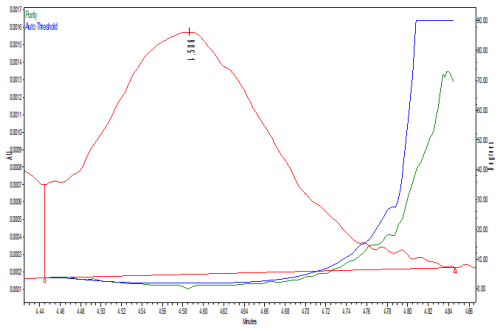


Fig. 5.6 Peak purity for rifabutin bulk drug

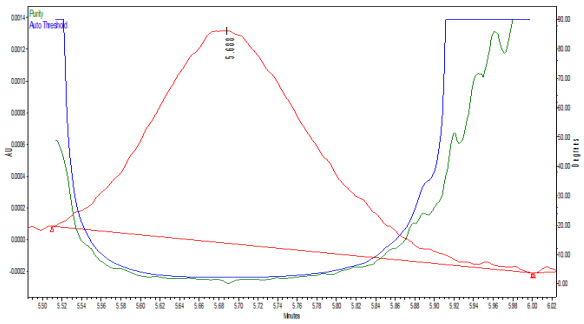
Peak purity plot for acid degradation sample chromatogram is shown in Fig 5.7 for DPs and API.



A



B



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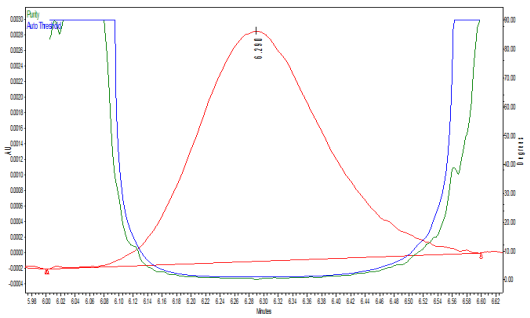

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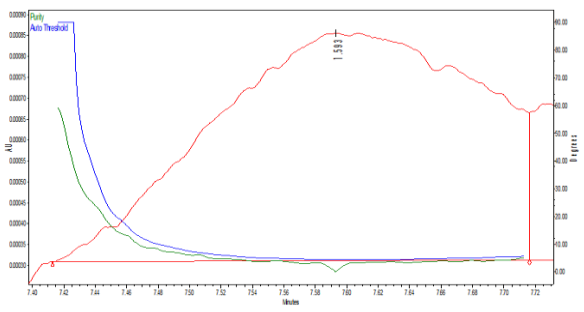

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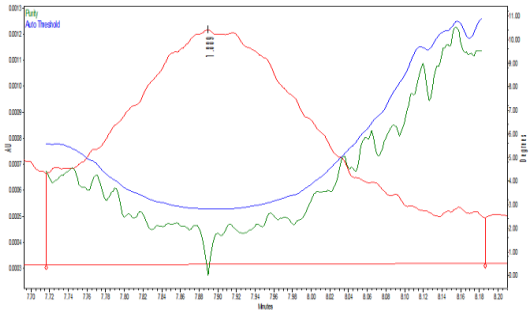

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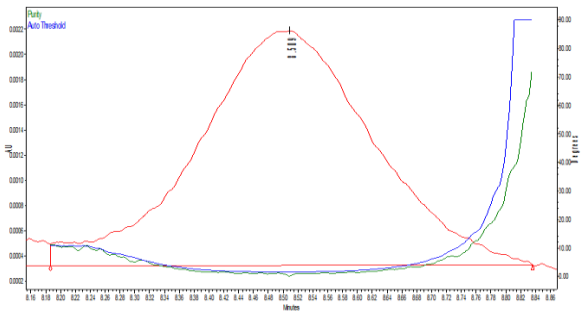

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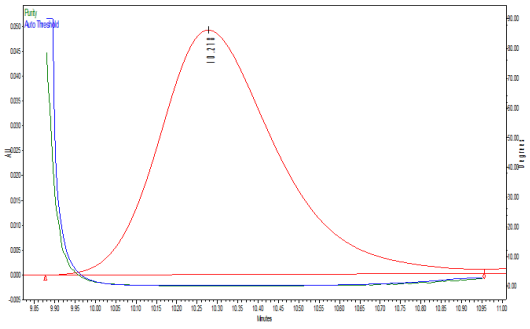

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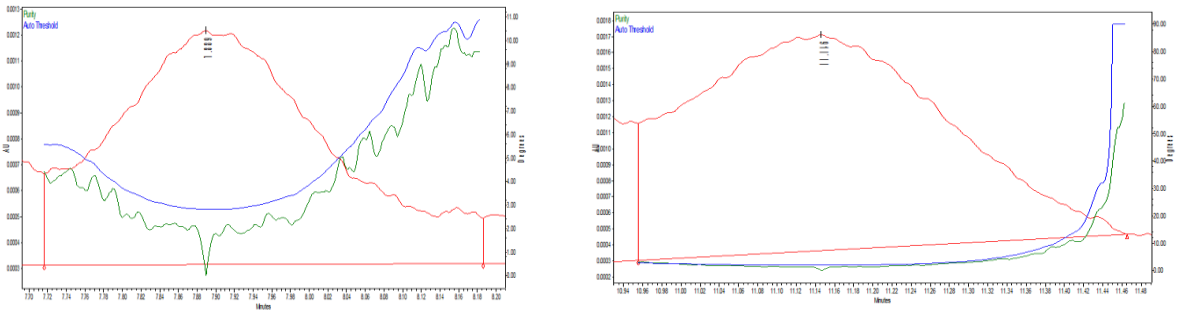
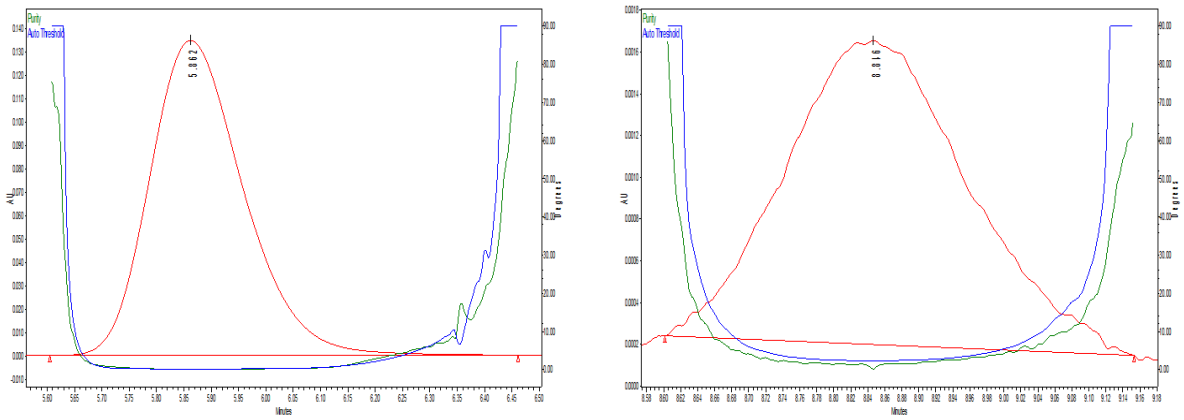


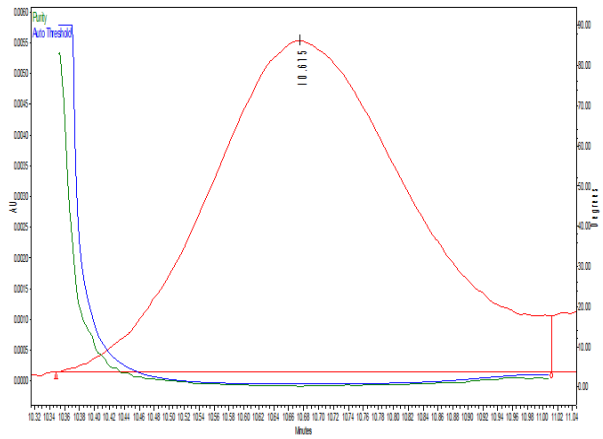
I **J**

Fig. 5.7 Peak purity test for acid degradation chromatogram of rifabutin A) peak 1 B) peak 2 C) peak 3 D) Peak 4 E) Peak 5 F) Peak 6 G) Peak 7 H) API I) Peak 8 J)Peak 9

The peak purity for alkali degraded sample chromatogram is shown in Fig 5.8 for DPs and API.



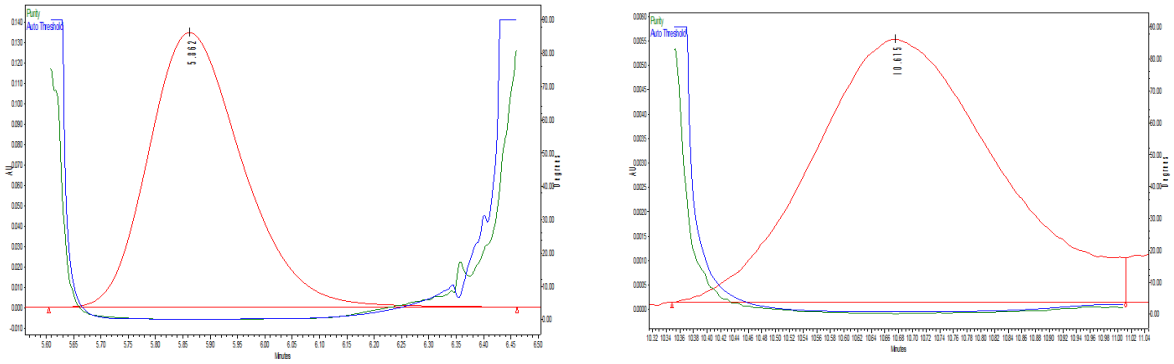
A **B**



C

Fig. 5.8 Peak purity plot for chromatogram showing alkali degradation of rifabutin A) peak 1 B) peak 2 and C) API

The peak purity plot for oxidative condition chromatogram is shown in Fig. 5.9 for bulk drug and peroxide peak.



A **B**

Fig. 5.9 Peak purity plot for peroxide degraded sample chromatogram of rifabutin A) Peroxide peak B) API

The peak purity shows that a single peak is eluted without the interference of any seen or unseen peak of the chromatogram. The bulk drug peak is pure in every chromatogram shows that there was no interference of DP with API peak.

5.5.2.4. Validation of stability indicating method [28]

The stability-indicating method was developed and validated as per ICH Q2 (R1) guidelines. The method chromatographic condition is shown in section 5.5.1.2. The stress degradation chromatogram is shown in Fig 5.5. The validation of method is completed in terms of linearity, range, precision, accuracy, detection limit and quantitation limit.

Linearity and Range

Linearity test solutions for rifabutin assay method were prepared from drug stock solution of 1mg/ml in methanol at concentration level 50.0-250.0 µg/ml in triplicate. The calibration curve was constructed by plotting concentrations versus peak area of rifabutin. The regression equation was calculated and was found to be linear in the selected concentration range with correlation coefficient value 0.998 and regression equation y = 16300x + 59118. The linearity of method was established in accordance with ICH Q2 (R1) guideline. Overlay chromatogram and calibration curve showing linearity of RFT is shown in Fig. 5.10.

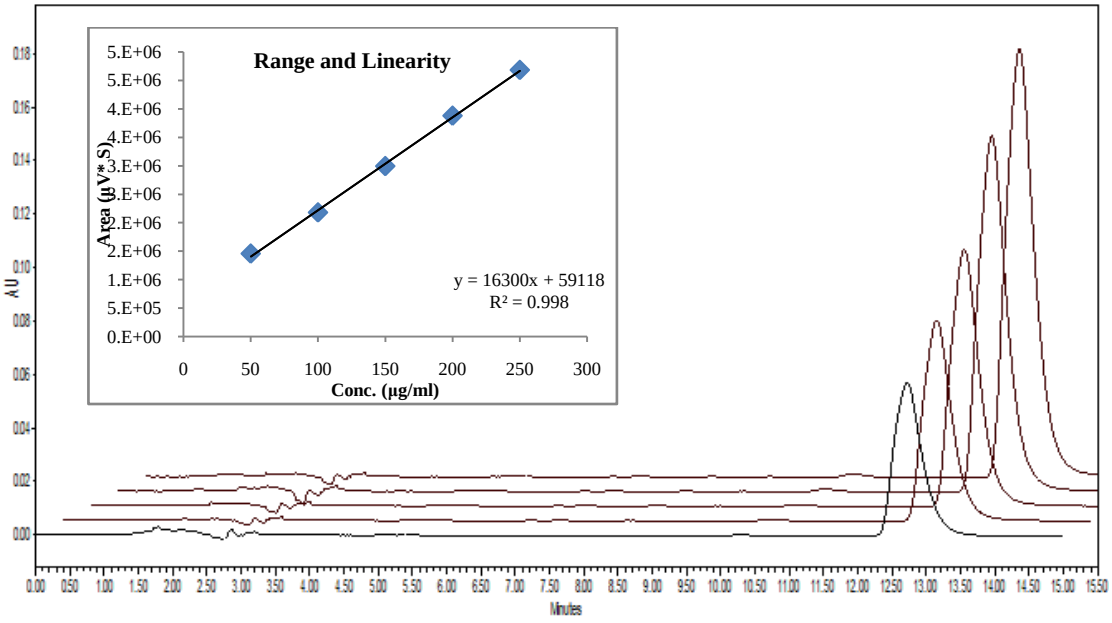


Fig. 5.10 The overlay chromatogram and correlation plot for rifabutin linearity and range

The concentration range and average peak area with relative standard deviation (RSD) for rifabutin linearity is shown in table 5.6. The RSD value < 2.0% and correlation coefficient 0.998 shows that good linearity was obtained in given range.

Conc. (µg/ml)	*Mean peak area (µV*S) ± RSD
50.0	1454719 ± 0.013
100.0	2177750 ± 0.016
150.0	2991607 ± 0.018
200.0	3876533 ± 0.167
250.0	4680333 ± 0.279

*mean peak area is average of three readings

Table 5.6 Range and mean peak area of rifabutin linearity study

Precision study

The precision study for validation of method was performed to gather knowledge about how precisely the method can detect or quantify rifabutin peak. The precision study was completed by three studies; inter day precision, intra-day precision and repeatability study.

Inter day study measures the deviation of result at different day, while intra-day study measures the deviations within a day. The repeatability study shows that the same result can be obtained by injecting the same sample in the same chromatographic condition again and again. The result of precision study is shown in Table 5.7.

Repeatability			
Conc. (µg/ml)	***Avg. Area (µV*S)**	**SD**	**% RSD**
100.0	2135713	19487	0.0091
Intermediate Precision			
Inter day precision			
50.0	1504218	16457	0.0109
100.0	2157558	20191	0.0093
150.0	2896403	20589	0.0071
Intraday precision			
50.0	1474115	22745	0.0154
100.0	2167359	23795	0.0109
150.0	3091201	24789	0.0080

Table 5.7The result of precision study of rifabutin

The result shows that the RSD value is < 2.0% indicates that the method is reliable to perform the same experiment with repeated same results.

Accuracy study

The recovery study was performed to establish that the developed method can quantify the concentration of rifabutin, thus the accuracy of the method can be proven. The accuracy study is shown in Table 5.8.

Level (%)	**Conc. from formulation (µg/ml)**	**Std Conc. Spiked (µg/ml)**	***Mean Conc. Recovered (µg/ml)**	**Mean % recovered ± RSD**
50	100.0	50.0	149.0	99.3±0.0010
100	100.0	100.0	201.0	100.5±0.0021
150	100.0	150.0	248.0	99.2±0.0027

*mean of three replications

Table 5.8 Accuracy study and result

The accuracy study showed RSD < 2.0% and good recovery (99.2-100.5%) was obtained. The method was accurate for the measurement of quantification of rifabutin in bulk drug and formulation.

Detection limit and quantification limit

The detection limit and quantification limit was studied from standard deviation of response and slope of regression equation of linearity plot. For given range detection limit (LOD) was 4.08µg/ml and quantification limit (LOQ) was 12.3µg/ml. The method is very sensitive to detect the rifabutin in a very trace limit.

Assay of formulation

The formulation (Ributin) assay was performed and recovery of sample concentration was calculated; 100.5% sample recovery was observed for rifabutin in capsule formation. (Limit for formulation recovery is 98-102%)

Robustness

The robustness study shows how steady the method towards small but deliberate changes in method, which describes the robustness of the method. The robustness results are shown in Table 5.9.

Chromatographic Changes	**Observations***			
	Area (%RSD)	**Rt (%RSD)**	**Tailing factor (%RSD)**	**Theoretical Plates (%RSD)**
Flow rate ± 0.1 (ml/min)				
0.9	0.0019	0.00081	0.0024	0.0178
1.0	0.0019	0.00080	0.0026	0.0189
1.1	0.0019	0.00082	0.0028	0.0184
pH ± 0.5				
3.5	0.0027	0.00098	0.0035	0.0168
4.0	0.0026	0.00095	0.0032	0.0160
4.5	0.0029	0.00097	0.0036	0.0172
Detection wavelength ± 10 (nm)				
270	0.0023	0.00083	0.0030	0.0170
275	0.0020	0.00082	0.0028	0.0167
280	0.0025	0.00085	0.0034	0.0179

*average of three replication

Table 5.9 Result of robustness study

The robustness study was completed for 100.0 µg/ml concentration. The robustness study RSD value < 2.0%indicates that method was steady towards changes in chromatographic conditions. Average theoretical plates for linearity samples were more than 2000 and tailing factor was less than 2.0 indicate that good peak symmetry was obtained in given method.

5.5.2.5. Application of stability-indicating method for analysis of bulk drug and formulation

The developed method was used to analyze stress degraded samples of formulation containing rifabutin as well as bulk drug. Stress degradation was carried out for rifabutin formulation under the same condition as specified for bulk drug and analyzed in the same way and in the same chromatographic condition. The degradation products were discernible and well separated. As represented in Table 5.5 the same degradation pattern was observed for formulation as it was observed in bulk drug. It depicted that there was no interference or interaction with capsule cell in degradation of rifabutin. The developed stability indicating method can be applied for routine analysis in clinical and pre-clinical studies of rifabutin. The method has detection limit of 4.0 µg/ml indicates that method can detect low concentration of impurities in rifabutin samples. The average tailing factor for rifabutin peak was ≤2.00 and average theoretical plated were ≥2000.00. The stability study results can be applied to formulation for shelf life study, and for suggested storage condition of rifabutin formulation.

PART-B

5.6 Degradation kinetic study of rifabutin by conventional method

The degradation kinetic study for rifabutin was completed in acid, alkali and peroxide conditions, where sufficient degradations were observed.

5.6.1 Experimental

5.6.1.1 Chemicals and reagents

The chemicals and reagents utilized for this study is same as described in section 5.5.1.1.

5.6.1.2 Equipments and chromatographic conditions

The equipments and chromatographic conditions are same as described in section 5.5.1.2.

5.6.1.3 Analytical sample preparation

The sample preparation and buffer preparation is same as described in section 5.5.1.3., except in terms of stressor concentrations and temperatures.

Acid degradation study: For acid degradation study samples were prepared with stressor concentrations 0.1N, 0.5N and 1N HCl; temperatures were 25, 40 and 60^0C, aliquot of 2ml was withdrawn from 0minutes to the 50minutes (6 points).

Alkaline degradation study: For alkaline degradation study samples were prepared with stressor concentrations 0.01N, 0.05N and 0.1N NaOH; study was completed at RT, aliquot of 2ml was withdrawn from 0minutes to the 60minutes (7 points).

Oxidative degradation kinetics: For oxidative degradation study samples were prepared with stressor concentrations 7%, 15% and 30% H_2O_2; temperatures were 25, 50 and 80^0C, aliquot of 2ml was withdrawn from 0minutes to the 30minutes (7 points).

5.6.1.4 Degradation kinetic parameters

The degradation kinetic parameters were calculated using equation 1 to 7 for enthalpy of activation, entropy of activation, activation energy, rate constant, half life and shelf life respectively. The effect of stressor, temperature and time can be evaluated by analysis of degradation kinetics parameters. The order of reaction was evaluated by generating plot of % drug versus time, *ln C* versus time and 1/C versus time to analyze the strongest correlation coefficient.

5.6.2 Results and discussion

The study of degradation kinetics was completed in chromatographic conditions and by preparing the samples described in section 5.6.1.2., and 5.6.1.3., respectively.

5.6.2.1 Acid degradation kinetics

The acid degradation kinetic study for rifabutin was completed using the parameters and sample preparation described in section 5.6.1.3. The results obtained from the chromatographic run were evaluated for identification of the order of the reaction. Therefore the plots were constructed for *%C versus time, ln C versus time,* and *1/C versus time*, to find out the strongest correlation coefficient among these plots. The correlation coefficient and regression equation for zero order, first order and second order reaction is shown in Table 5.10.

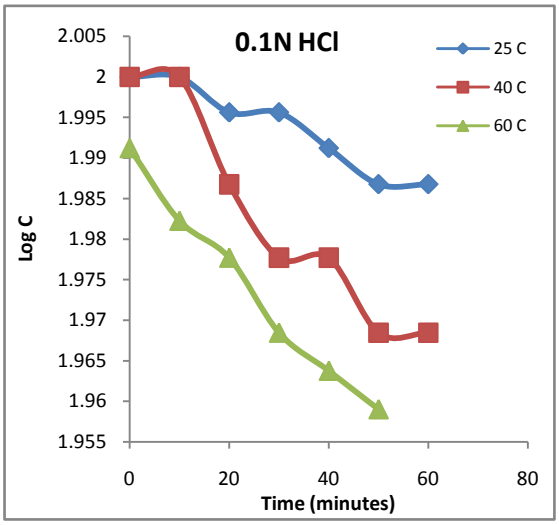
		Correlation coefficient ($r^{2)}$			Regression Equation		
Conc. HCl	**Temp**	**Zero Order**	**First Order**	**Second Order**	**Zero Order**	**First Order**	**Second Order**
0.1N	25	0.933	**0.940**	0.935	y = -0.057x + 100.2	y = -0.0002x + 2.00	y = 0.000x + 0.499
	40	0.925	**0.927**	0.920	y = -0.132x + 100.1	y = -0.0006x + 2.00	y = 0.0002x + 0.499
	60	0.984	**0.986**	0.985	y = -0.15x + 99.5	y = -0.0007x + 1.98	y = 0.000x + 0.500
0.5N	25	0.970	**0.973**	0.969	y = -0.117x + 99.53	y = -0.0005x + 1.99	y = 0.0001x + 0.500
	40	0.927	**0.931**	0.903	y = -0.160x + 98.82	y = -0.0007x + 1.9951	y = 0.000x + 0.501
	60	0.898	**0.908**	0.898	y = -0.182x + 97.75	y = -0.0009x + 1.9903	y = 0.000x + 0.502
1N	25	0.970	**0.972**	0.960	y = -0.178x + 99.35	y = -0.0008x + 1.99	y = 0.00021x + 0.50
	40	0.984	**0.985**	0.982	y = -0.235x + 99.5	y = -0.0011x + 1.99	y = 0.000x + 0.500
	60	0.982	**0.989**	0.985	y = -0.357x + 98.42	y = -0.0018x + 1.9944	y = 0.000x + 0.501

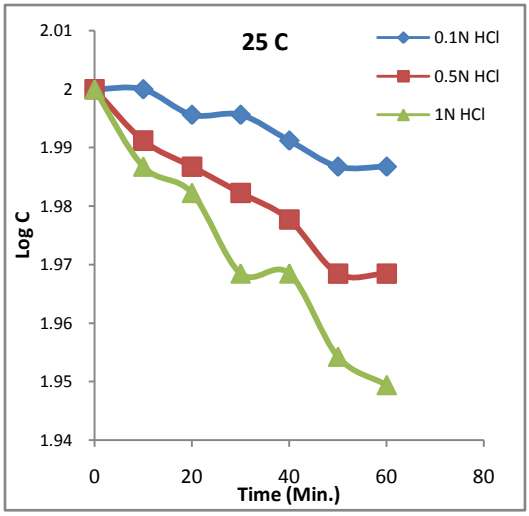
Table 5.10 correlation coefficient and regression equations for order of reaction

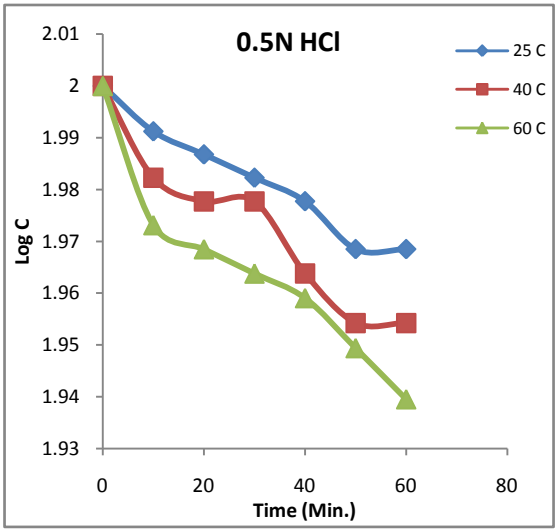
R^2 value ≥ 0.900 indicated linear and continues process of degradation was observed. As it can be seen in table 5.10 that strongest correlation coefficients were observed *in ln C versus time* plot indicates that reaction followed first order kinetics.

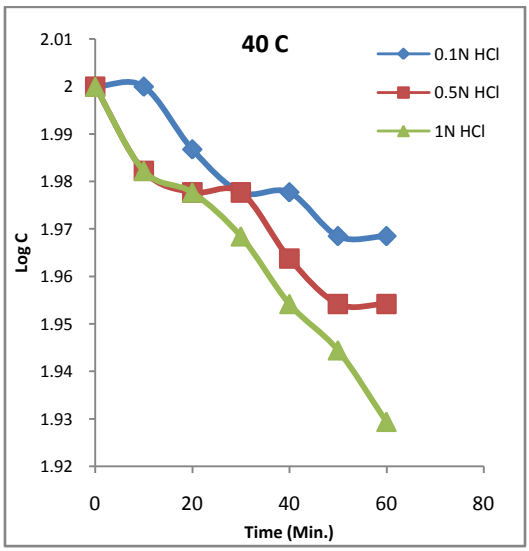
Effect of stressor concentration and temperatures on degradation of rifabutin

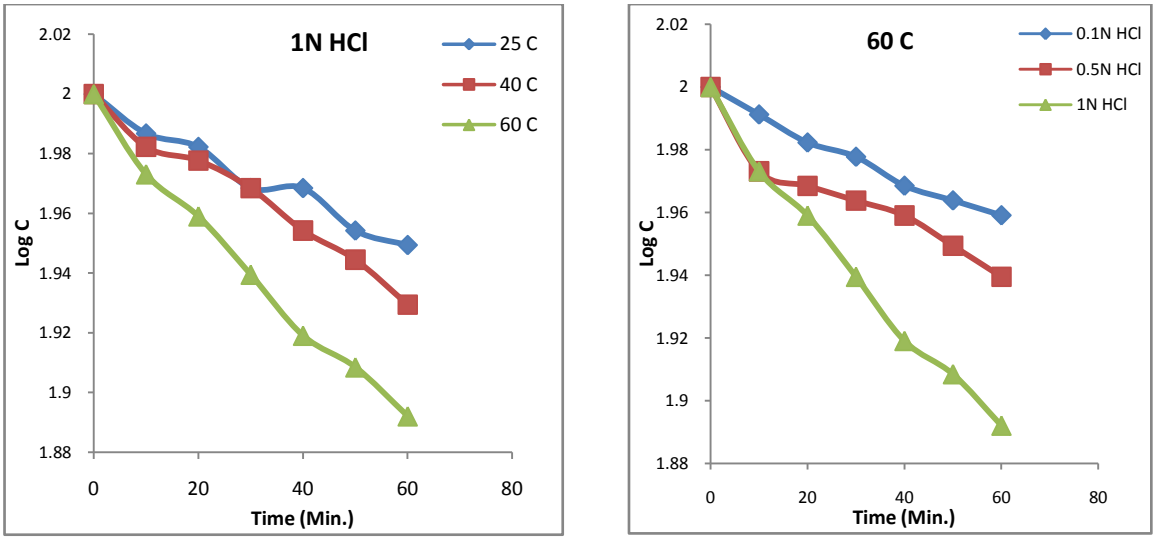
The effect of stressor concentration and temperatures on rifabutin degradation in acid condition was evaluated by plotting different concentrations of stressor at particular temperatures and plotting different temperature response at particular concentration, respectively. The plots are shown in Fig 5.11











A **B**

Fig. 5.11 The effect of A) different temperatures B) stressor concentrations on rifabutin degradation

The plot shows that HCl concentration and temperature both have an emergent effect on rifabutin degradation. In lower temperature and lower HCl concentration, the rifabutin seems stable in acid media while high temperature and high HCl concentration provoke the degradation of rifabutin.

Effect of temperature and stressor concentration on kinetic parameters

The kinetic parameters were calculated using equations and plots were constructed in 3D to know the effect of both the parameters simultaneously. The plots are shown in Fig 5.12.

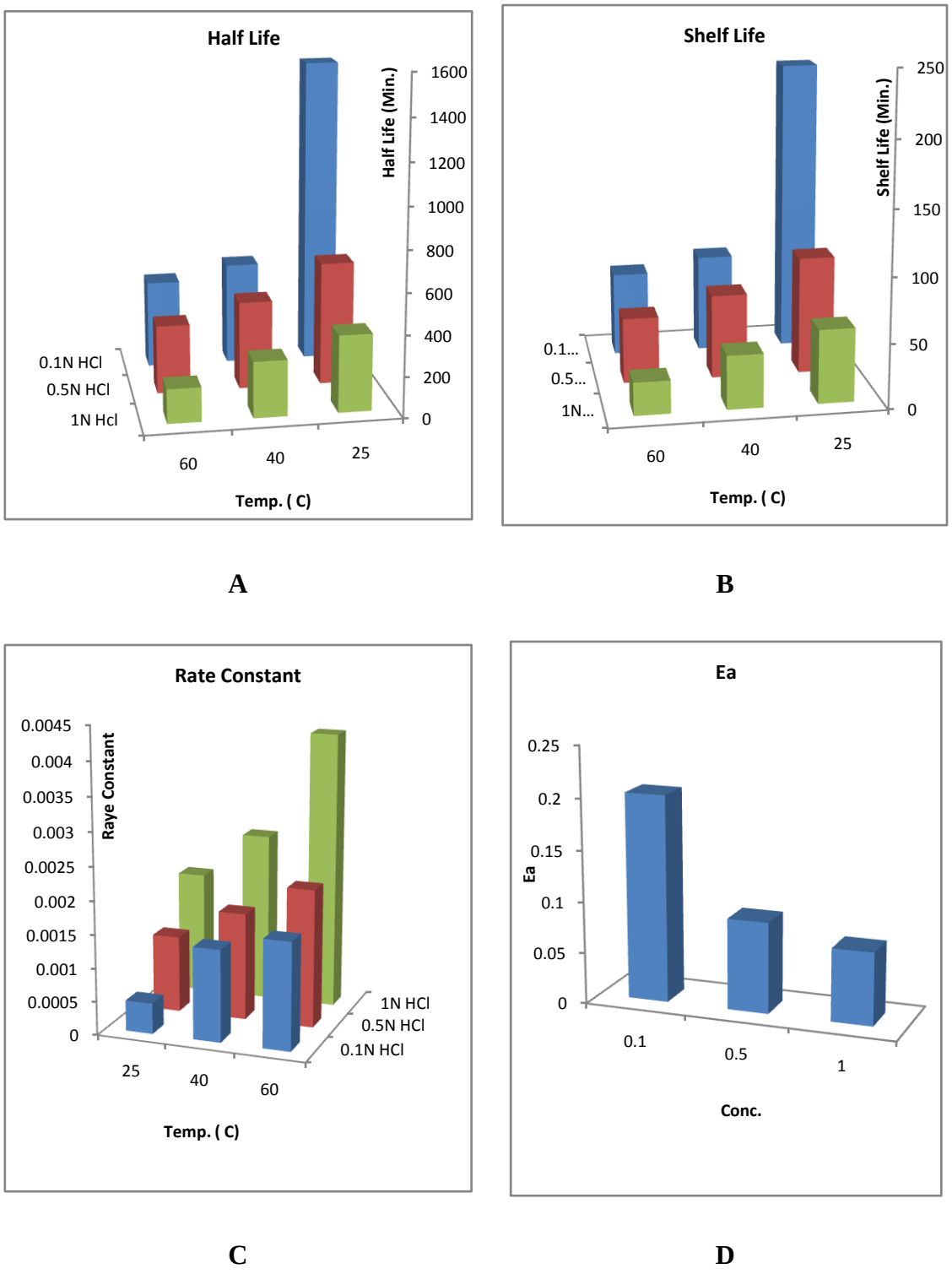


Fig. 5.12 Effect of stressor concentration and temperature on A) Half life B) Shelf life C) Rate constant D) Activation energy

The plot shows effect of stressor concentration on all the kinetic parameters; half life, shelf life and activation energy decreases with increases in HCl concentration while rate constant has direct proportional relation to HCl concentration. The temperature factor showed

inversely proportion relation with half life, shelf life and activation energy, while rate constant has direct proportional relation to temperature.

The degradation kinetic parameters were calculated and shown in Table 5.11.

		From Graph			From Equation			
Conc. (HCl)	**Temp. (^{O}C)**	**Slope**	**k**	**Log k**	**k**	**Log k**	**t_{50} [min]**	**t_{90} [min]**
0.1N	25	0.0002	0.000461	-3.34	0.000482	-3.31	1504.56	227.96
	40	0.0006	0.001382	-2.86	0.001472	-2.83	501.52	75.99
	60	0.0007	0.001612	-2.79	0.001713	-2.76	429.87	65.13
0.5N	25	0.0005	0.001152	-2.94	0.001032	-2.98	601.82	91.19
	40	0.0007	0.001612	-2.79	0.001682	-2.77	429.87	65.13
	60	0.0009	0.002073	-2.68	0.002176	-2.66	334.35	50.66
1N	25	0.0008	0.001842	-2.73	0.001782	-2.74	376.14	56.99
	40	0.0011	0.002533	-2.60	0.002462	-2.60	273.56	41.45
	60	0.0018	0.004145	-2.38	0.004167	-2.38	167.17	25.33

Table 5.11 Degradation kinetic parameters for rifabutin

Arrhenius Plot and activation parameters for degradation of rifabutin

The Arrhenius plot was constructed for *1/T versus ln K;* the slope of linear regression equation was used to calculated activation energy. The Arrhenius plot is showed in Fig 5.13.

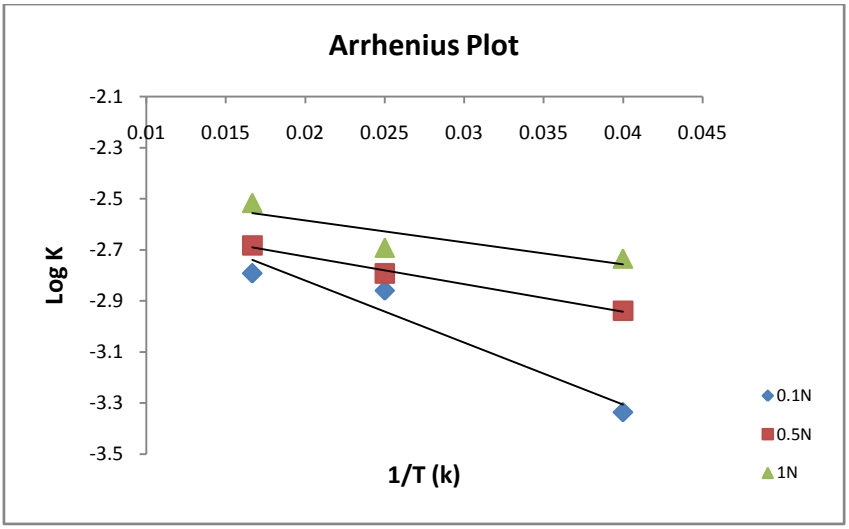


Fig. 5.13 Arrhenius plot for degradation kinetic of rifabutin

The linear line in Arrhenius plot shows that slope can be incorporated for calculation of activation energy. The activation energy was further used to calculate the enthalpy and entropy of reaction. The activation parameters are shown in Table 5.12.

Acid	Activation	r	Ea(kj/mol)	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$
0.1N	y = -372.7x + 0.448	0.978	0.2021	2.27	-23.07
0.5N	y = -297.8x + 0.308	0.900	0.0897	2.27	-23.07
1.0N	y = -111.9x - 0.100	0.995	0.0713	2.27	-23.07

Table 5.12 Activation parameters for degradation kinetics of rifabutin

The positive enthalpy value indicates that the system has lower energy than the environment; while negative and close values of entropy value indicates that the level of disarrangement in system was low.

5.6.2.2 Alkali degradation kinetics

Rifabutin was highly sensitive for degradation in alkaline medium, therefore degradation kinetic study for rifabutin was carried out in 0.01N, 0.05N and 0,1N NaOH at RT for 60 minutes. The plot of *% C versus time, ln C versus time* and *1/C versus time* constructed to know the order of reaction by comparing correlation coefficients. The Table 5.13 shows correlation coefficient and regression equation for zero order, first order and second order reaction.

		R^2 Value			Regression equation		
Conc. [NaOH]	**Temp (^{0}C)**	**Zero order**	**First order**	**Second order**	**Zero order**	**First order**	**Second order**
0.01N		0.986	**0.987**	0.985	y = -0.585x + 100	y = -0.003x + 2.004	y = 0.000x + 0.498
0.05N	25	0.986	**0.988**	0.968	y = -1.096x + 98.17	y = -0.007x + 2.017	y = 0.002x + 0.489
0.1N		0.982	**0.984**	0.948	y = -1.432x + 97.67	y = -0.009x + 2.052	y = 0.005x + 0.466

Table 5.13 correlation coefficient and regression equation for alkali degradation kinetic of rifabutin

The table shows strongest correlation coefficient in first order of reaction in degradation of rifabutin in alkaline medium.

Effect of stressor concentration on degradation of rifabutin

The NaOH concentrations and temperatures effect on degradation of rifabutin was evaluated by constructing plot of *ln C versus time* at RT. (Fig. 5.14)

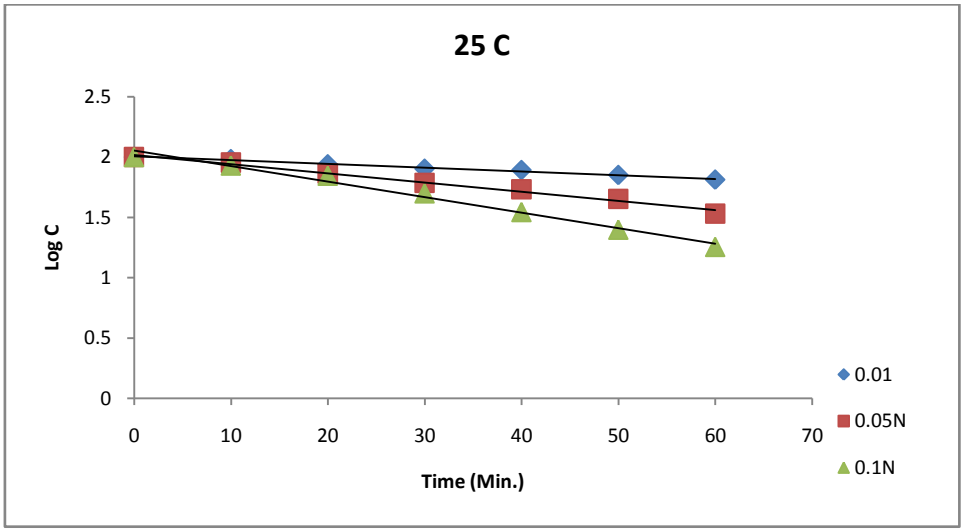


Fig. 5.14 effect of NaOH concentration on degradation of rifabutin

The plot shows that at increase in NaOH concentration the degradation rate increase, highest degradation was observed in 0.1N NaOH after 60minutes.

Effect of stressor concentration on kinetic parameters

To study the effect of kinetic parameters on kinetic parameters, 3D plots were constructed shown in Fig 5.15.

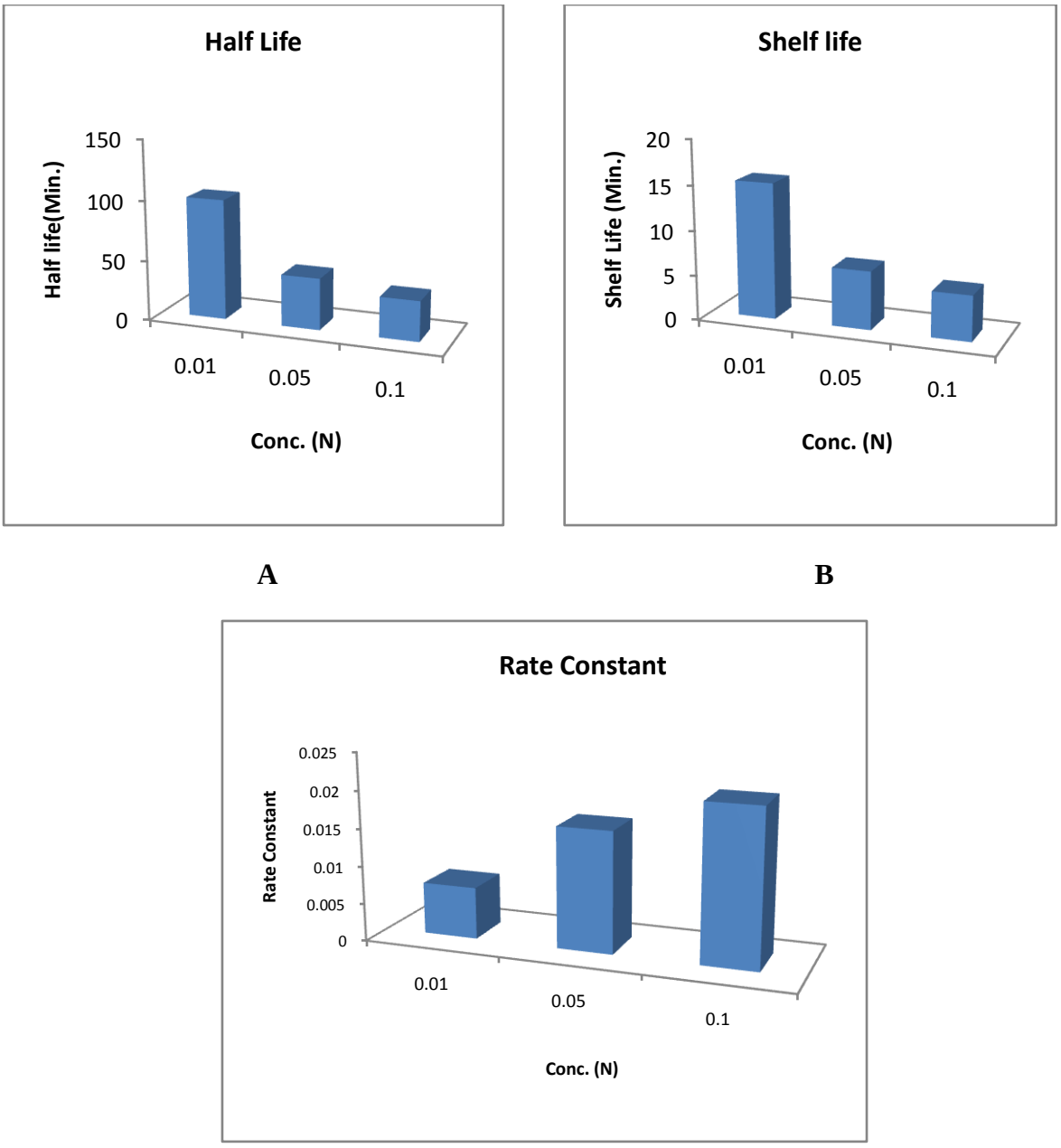


Fig. 5.15 3D plot for effect of stressor concentration on A) half life B) shelf life and C) rate constant

The plot shows that in low concentration of NaOH, the time required for 50% degradation of rifabutin is higher than it requires in 0.1N NaOH, this indicates the low degradation rate in 0.1N NaOH shown in plot (C). The kinetic parameters are shown in Table 5.14.

		From Graph			From Equation			
Conc. (NaOH)	**Temp. (^{O}C)**	**Slope**	**k**	**Log k**	**k**	**Log k**	**t_{50} [min]**	**t_{90} [min]**
0.01N		0.003	0.0069	-2.16	0.007	-2.15	100.30	15.19
0.05N	25	0.007	0.0161	-1.79	0.016	-1.79	42.98	6.51
0.1N		0.009	0.0207	-1.68	0.021	-1.67	33.43	5.06

Table 5.14 Degradation kinetic parameters for rifabutin

Arrhenius Plot and activation parameters for degradation of rifabutin

Rifabutin in 0.1N NaOH at RT 88.86% degraded within 60minutes, adding more concentration of NaOH would provoke instant 100% degradation of rifabutin or that might be result in degradation of primary DPs. Rifabutin is highly sensitive for degradation in alkaline medium at room temperature therefore temperature factor was not included. Activation energy for alkali degradation kinetic of Rifabutin was not calculated as calculation requires different temperatures and here study was carried out at room temperature only.

5.6.2.3 Peroxide induced degradation kinetics

Peroxide induced degradation kinetics for rifabutin was calculated in 7%, 15% and 30% H_2O_2 at 25, 50 and 80^{0}C for 30minutes. Rifabutin was degraded in oxidative medium but degradation product was not detected in chromatogram. The plot for zero order, first order and second order kinetics were constructed and correlation coefficient and regression equations are shown in Table 5.15.

		R^2 Value			Regression equation		
H_2O_2	T (^{0}C)	Zero order	**First order**	Second order	Zero order	**First order**	Second order
7%	25	0.987	**0.989**	0.988	y = -1.964x + 100.6	y = -0.000x + 2.00	y = 0.0023x + 0.499
	50	0.988	**0.993**	0.992	y = -2.357x + 99.35	y = -0.0002x + 1.99	y = 0.0029x + 0.500
	80	0.988	**0.996**	0.995	y = -3.142x + 98.85	y = -0.0004x + 1.99	y = 0.0041x + 0.500
15%	25	0.982	**0.985**	0.984	y = -3.678x + 98.46	y = -0.0001x + 1.99	y = 0.004x + 0.501
	50	0.975	**0.989**	0.983	y = -4.000x + 97.71	y = -0.0003x + 1.99	y = 0.005x + 0.501
	80	0.984	**0.989**	0.981	y = -4.785x + 97.78	y = -0.0004x + 1.99	y = 0.006x + 0.502
30%	25	0.993	**0.998**	0.997	y = -6.357x + 98.21	y = -0.0001x + 1.99	y = 0.009x + 0.499
	50	0.98	**0.981**	0.969	y = -8.678x + 95.89	y = -0.0003x + 1.99	y = 0.016x + 0.498
	80	0.979	**0.981**	0.944	y = -11.82x + 93.89	y = -0.0005x + 2.00	y = 0.033x + 0.487

Table 5.15 correlation coefficient and regression equation for order of reaction

The comparison of correlation coefficient showed that strongest correlation coefficient was observed in first order reaction. Thus, rifabutin degradation followed first order kinetics in oxidative media.

Effect of stressor concentration and temperatures on degradation of rifabutin

The stressor concentration and temperature effect was studied by plotting *ln C versus time* at different temperature and at different concentration. The plots are shown in Fig 5.16.

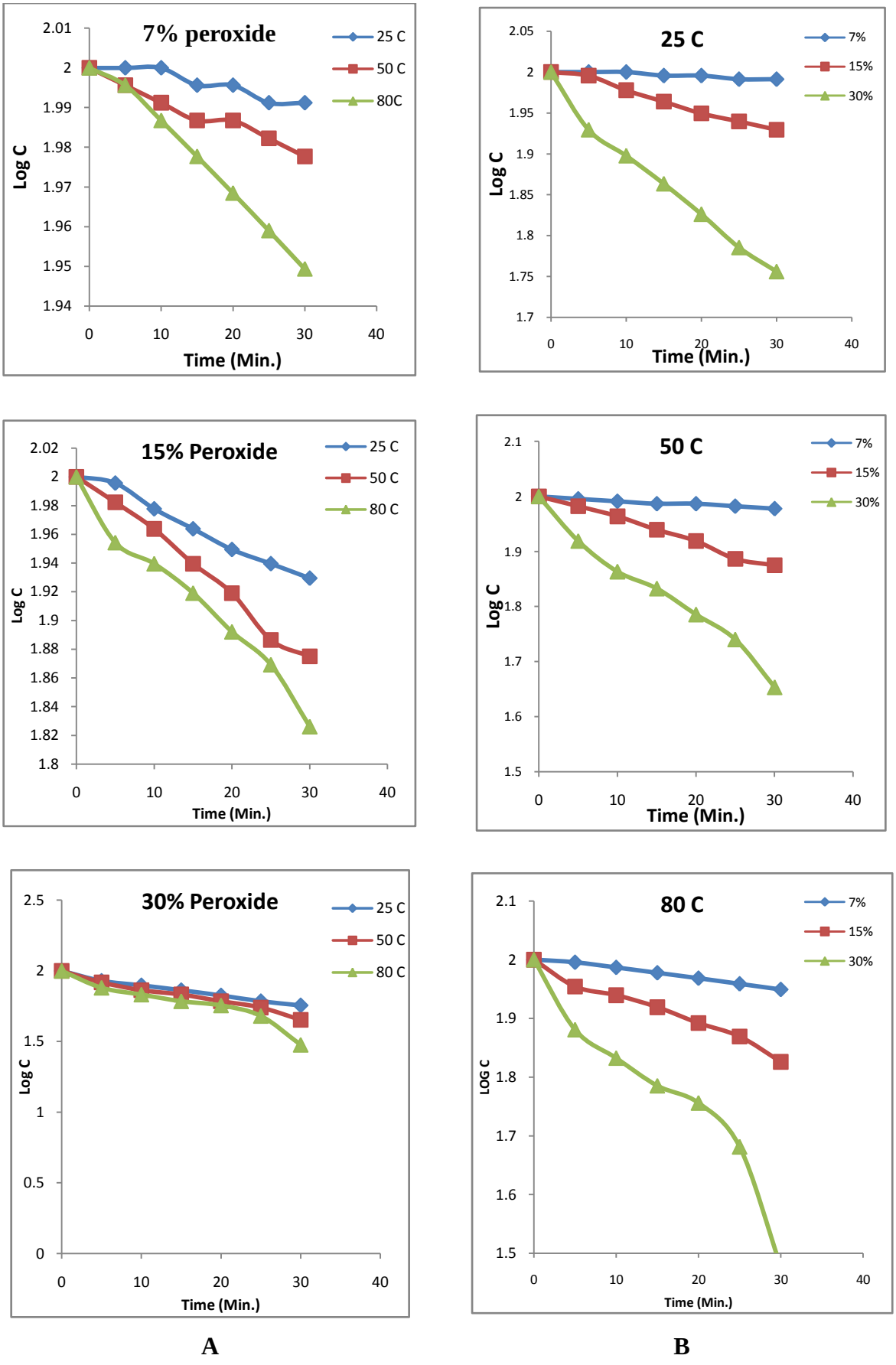
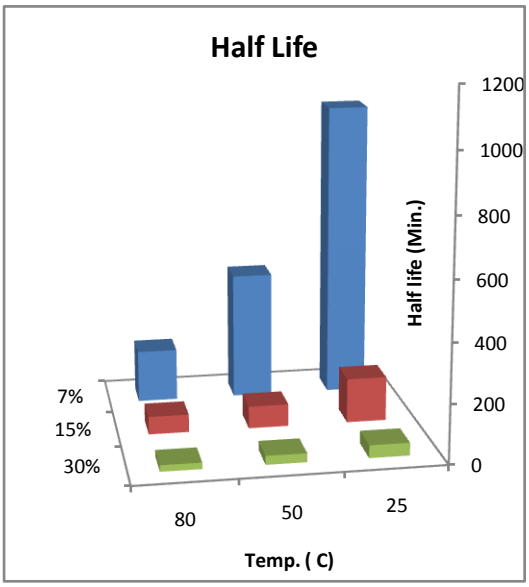


Fig. 5.16 Effect of A) temperatures B) stressor concentration on degradation of rifabutin

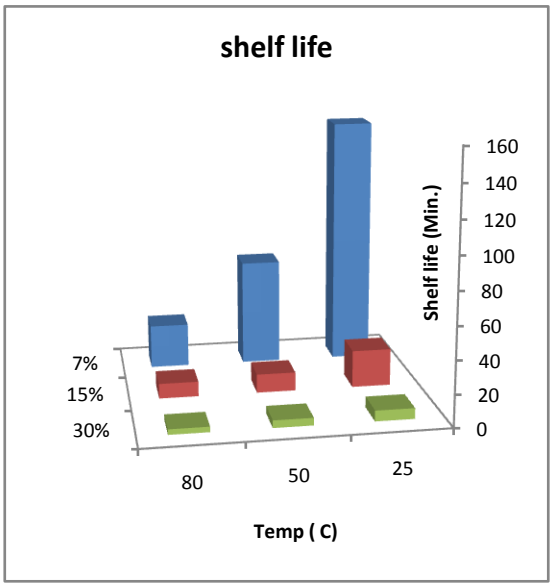
The plot shows that highest degradation was observed in 30% H_2O_2 at 80^0C while at lower concentration and lower temperature, the degradation of rifabutin was slower.

Effect of temperature and stressor concentration on kinetic parameters

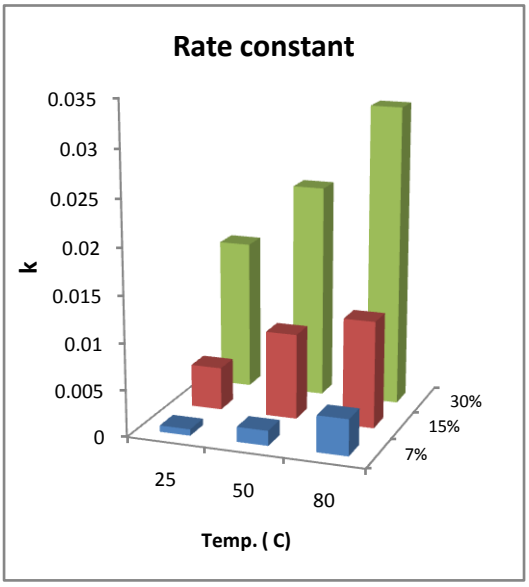
The effect of temperature and stressor concentration was evaluated by constructing 3D plot to know the simultaneous effect of temperatures and stressor concentration. The plots are shown in Fig 5.17.



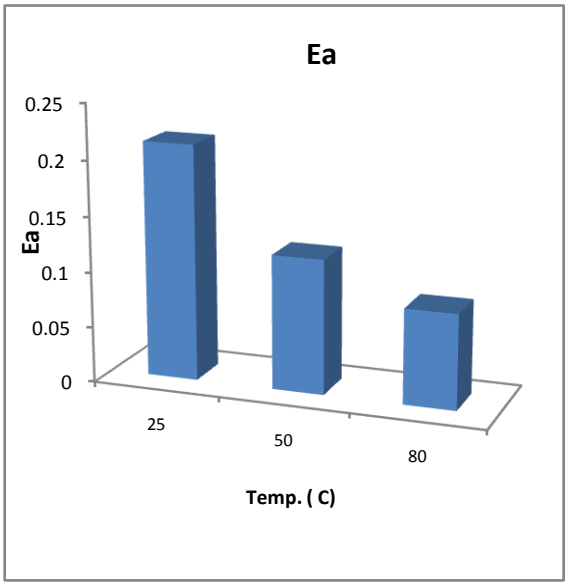
A



B



C



D

Fig. 5.17 Effect of stressor concentration and temperature on A) Half life B) Shelf life C) Rate constant and D) Activation energy

The highest rate constant in 30% H_2O_2 shows that degradation of rifabutin was spontaneous at higher concentration of oxidative agent containing solution. The half life and shelf life of rifabutin was decreasing with increase in oxidative agent concentration and temperature. The activation energy plot shows that reaction was endothermic.

The kinetic parameters values used to construct plot is shown in Table 5.16.

		From Graph			**From Equation**			
Conc. (%)	**Temp. ^oC**	**Slope**	**k**	**Log k**	**k**	**Log k**	**t_{50} (min.)**	**t_{90} (min.)**
7% H_2O_2	25	0.0003	0.000691	-3.16	0.0008	-3.09	1003.03	151.97
	50	0.0007	0.001612	-2.79	0.0015	-2.82	429.87	65.13
	80	0.0017	0.003915	-2.40	0.004	-2.39	177.00	26.81
15% H_2O_2	25	0.002	0.004606	-2.33	0.0045	-2.34	150.45	22.79
	50	0.004	0.009212	-2.03	0.0093	-2.03	75.22	11.39
	80	0.005	0.011515	-1.93	0.0114	-1.94	60.18	9.11
30% H_2O_2	25	0.007	0.016121	-1.79	0.016	-1.79	42.98	6.51
	50	0.01	0.02303	-1.63	0.024	-1.61	30.09	4.55
	80	0.014	0.032242	-1.49	0.031	-1.50	21.49	3.25

Table 5.16 Degradation kinetics parameters for rifabutin

Arrhenius Plot and activation parameters for degradation of rifabutin

The Arrhenius plot was constructed to calculate the activation parameters for degradation of rifabutin in oxidative medium. (Fig. 5.18)

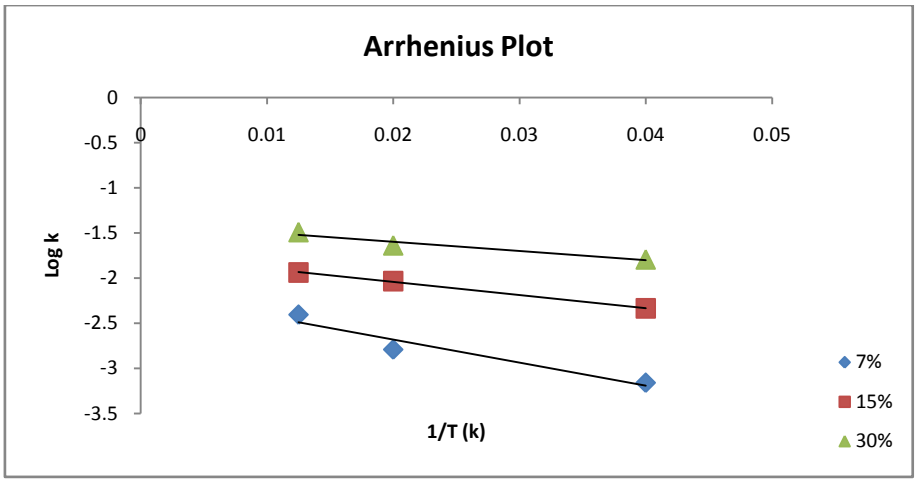


Fig. 5.18 Arrhenius plot for activation parameters of rifabutin degradation

The straight line of Arrhenius plot indicates that slope of regression equation can be used to calculate the activation energy and other activation parameters.

The activation parameters are shown in Table 5.17.

H_2O_2	Activation	R^2	Ea(kj/mol)	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$
7%	y = -25.57x - 2.169	0.928	0.0212	2.48	-31.399
15%	y = -14.58x - 1.751	0.999	0.1212	2.39	-31.398
30%	y = -10.28x - 1.392	0.943	0.0854	2.35	-31.398

Table 5.17 Activation parameters for rifabutin

- **Conclusion**

The degradation kinetic study of rifabutin showed that it degraded noticeably in acid medium, alkaline medium and oxidative medium. The degradation rate was higher at higher stressor (acid, alkali or peroxide) concentration while addition of temperature increased the degradation rate of rifabutin. Rifabutin is stable in thermal condition (stable at 80^0C for > 28 days) indicates that degradation of rifabutin was not the result of temperature, but temperature increased the degradation reaction rate by providing environmental activation energy to the reactions.

PART-C

5.7 Multi-factorial tool for study of degradation kinetic of rifabutin

The degradation kinetics of rifabutin was studied by conventional method and the kinetics parameters obtained were used to evaluate the effect of stressor concentration and temperatures. The conventional kinetics study requires the more utilization of time and solvent; to reduce the time and solvent utilization multi-factorial tool was applied. The tool creates the design, analyzes the responses and variants to generate a formula that can predict the response value in a 3D manner for a maximum of two factors while keeping other factors constant. To study this tool Design of Experiment (DoE) approach was used.

5.7.1 Experimental

5.7.1.1 Chemicals and reagents

The chemicals and reagents used for the study were same as described in section 5.5.1.1.

5.7.1.2 Equipments and chromatographic conditions

The equipments and chromatographic conditions were same as described in section 5.5.1.2. The multi-factorial tool was applied using software Design Expert ™ (StateEase, USA).

5.7.1.3 Analytical sample preparation

The samples were prepared same as described in section 5.5.1.3.

5.7.1.4 Multi-factorial design generation

The Factorial Experiment was designed using 2 × 3 factorial designs where 2 levels and 3 factors were included in the design. The levels were -1 and +1 indicated for the lower limit and upper limit of design respectively; moreover the central point can be added manually if a researcher wants to study the response of center level of factor. The factors included for the study were stressor concentration, temperature, and time, levels were the upper limit and lower limit of factors; one center point was added in each factor level to ease the analysis of data. Table 5.18 shows the factor and level of multi factorial design.

Factor	Name	Units	Type	Minimum	Maximum	Coded Low	Coded High	Mean
A	Acid concentration	N	Numeric	0.1000	1.0000	-1 ↔ 0.10	+1 ↔ 1.00	0.5444
B	Temperature	C	Numeric	25.00	60.00	-1 ↔ 25.00	+1 ↔ 60.00	42.22
C	Time	Minutes	Numeric	0.0000	60.00	-1 ↔ 0.00	+1 ↔ 60.00	30.00

Table 5.18 Factors and limits for design of experiment

The responses of factors and levels were selected as kinetic parameters, five responses were selected; R1: % Drug (%), R2: Rate constant (-), R3: Half life (min.), R4: Shelf life (min.), and R5 Activation energy (Kj/mol*K).

5.7.1.5 Analysis of responses and data generation

The responses obtained against the factor and level was performed and data was gathered in Design Expert © 12 software. The analysis of each response was done using the same software. The actual values (calculated in MS Excel 2007) were gathered by performing the experiment (RP-HPLC) and were compared with the predicted value obtained from the software.

5.7.2 Result and discussion

Design generation and suitability of design

Design for acid, alkali and oxidative degradation kinetic was generated using 2×3 factorial design; suitability of design was restrained by FDS plot. Standard error mean term was taken in consideration for suitability of design.

Significance of factors for design

The relative significance of factors is shown by Perturbation plot, the effect of one factors while keeping other factors constant. For acid, alkali, and oxidative degradation design the midpoint was obtained for each factor indicates that factors were significant for study.

5.7.2.1. Hydrolytic degradation kinetics of rifabutin

Acid degradation kinetics

Analysis of response

The run obtained for given level of factor was practically performed and responses were entered into the design. The responses were analyzed against each factors and level. Analysis of response for each factor was confirmed by *half normal plot* and *Pareto Chart*. Both the plot can be analyzed for positive or negative effect of factors on response and to identify significant factor for particular response. The analysis of half normal plot and Pareto chart (Fig.5.19) for responses shows following results:

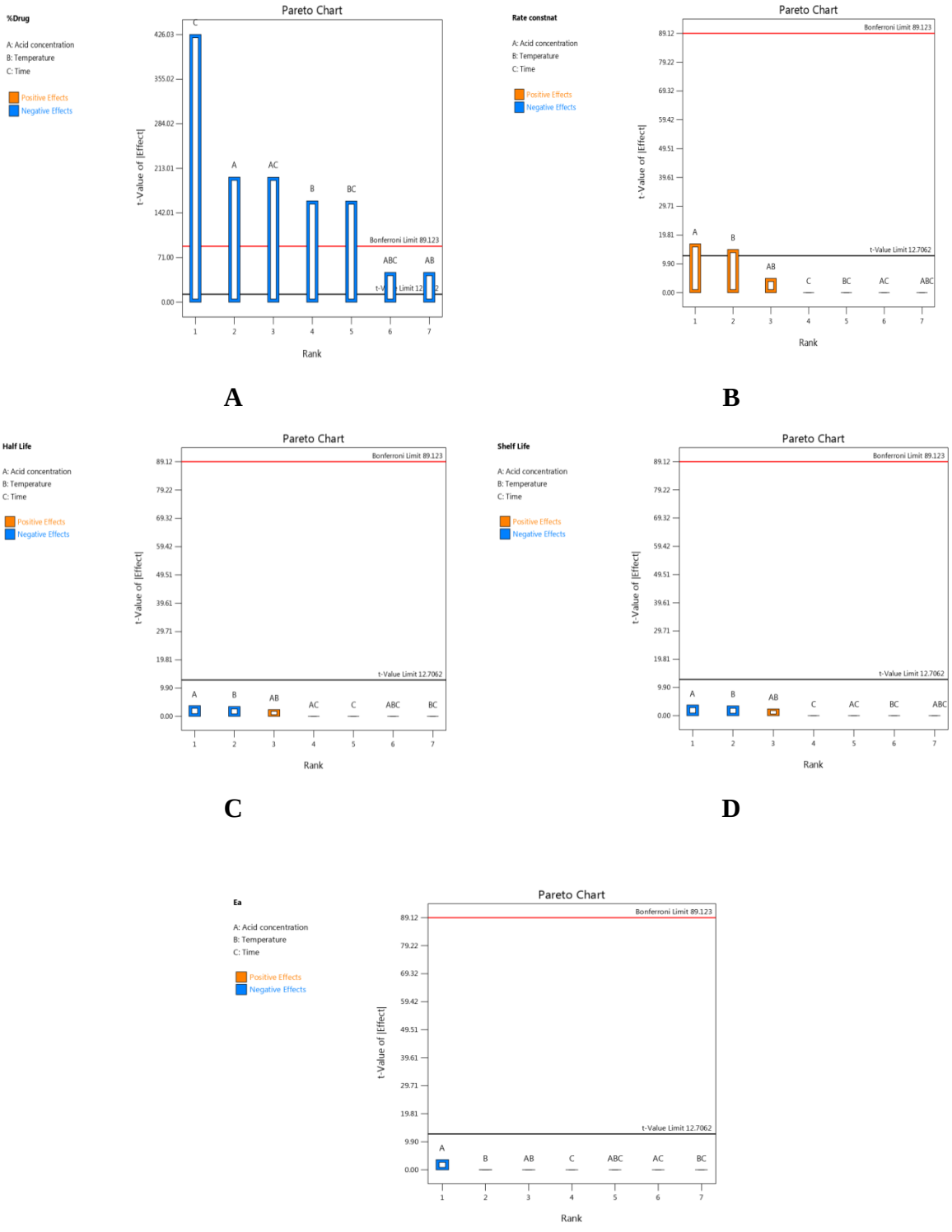


Fig. 5.19 Pareto chart for the analysis of significant factors for **A)** % Drug, **B)** Rate Constant, **C)** Half life, **D)** Shelf life and **E)** Activation energy.

The stressor concentration and temperature showed negative effect on responses % drug, half life, shelf life and activation energy while it show positive effect on rate constant. It makes the same conclusion as drawn from conventional kinetic study method in section 5.6.2.1.

Analysis of Variants

ANOVA is a mathematical term used to analyze effect of factor (either positive or negative) on response. For acid degradation study of rifabutin mathematical terms are generated for each response;

R1 (%drug): +94.38 -2.63A -2.13B -5.62C -0.6249AB -2.63AC -2.13 BC -0.6250 ABC

R2 (Half life): +0.0020 +0.0010A +0.0009B +0.0000C +0.0003AB +0.0000AC +0.0000BC +0.0000ABC

R3 (Shelf life): +588.72 -344.37A -316.52B +0.0000C +215.94AB +0.0000AC +0.0000BC +0.0000ABC

R4 (Rate constant): = +89.20 -52.18A -47.96B +0.0000C +32.72AB +0.0000AC +0.0000BC +0.0000ABC

R5 (Activation energy): +0.1307 -0.0647A +0.0009B +0.0000C -0.0001AB +0.0000AC +0.0000BC +0.0000ABC

These mathematical equations can generate a predicted value of response. By putting the desired factors value, an analyst can get the value for response. These values can be directly obtained in 2D and 3D plot of responses.

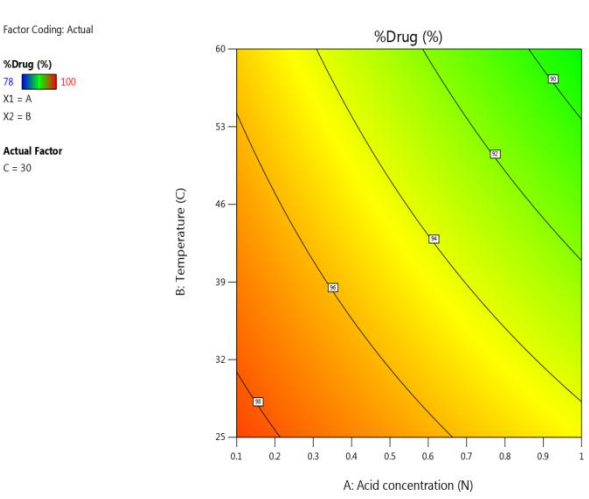
Interaction of factors

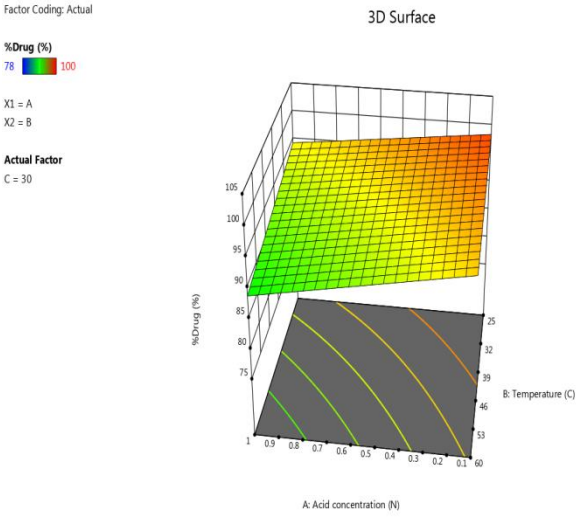
The factors interacting with each other can reduce the factors' effect on responses. Interaction plot showed that if any factor interacted and produced changes in response. To evaluate the interaction between two factors, one factor was kept constant and other factors are studied simultaneously. For rifabutin acid degradation kinetic study, factor C (time) was kept constant as factor C had less effect on responses, while factor A and Factor B was studied. Factor A and B show no interaction in any responses, the lines are parallel indicates that both factors have their individual effect on response.

Contour plots and 3-Dimentional study

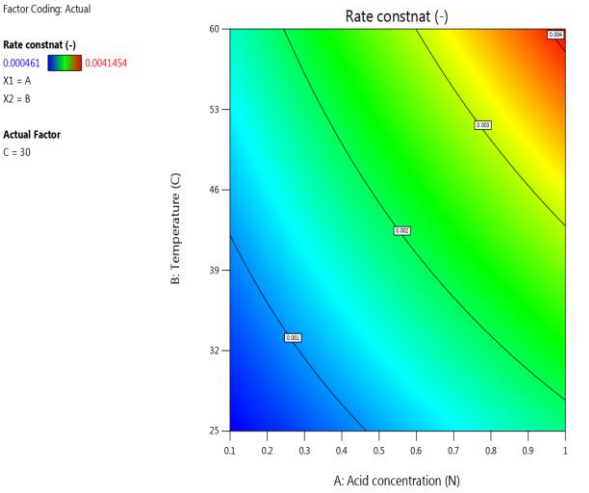
Contour plots are 3D-graphical representation of effects of three factors on response. The plots in 2D and 3D format are shown in Fig. 5.20 for each response. The contour plot is taken

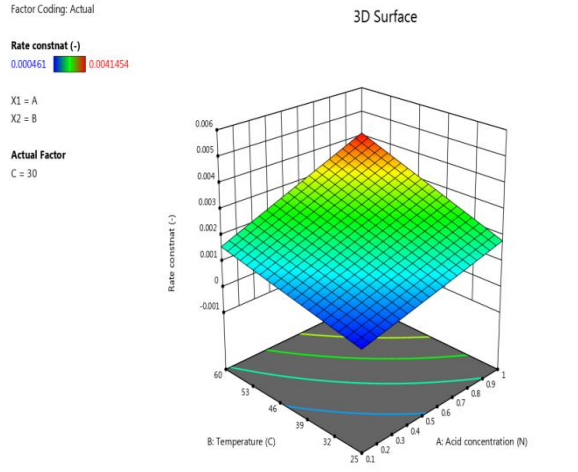
by keeping Factor C (time at 30min.) constant so that Factor A and Factor B effect can be analyzed.



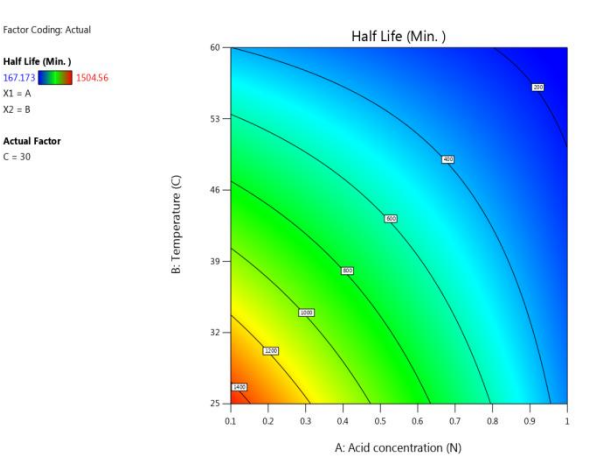


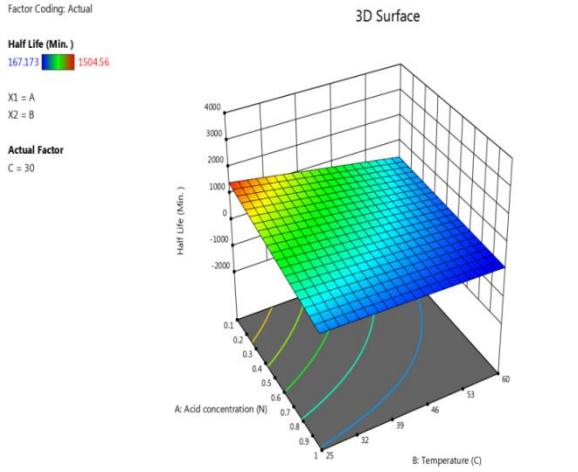
A_1 **A_2**



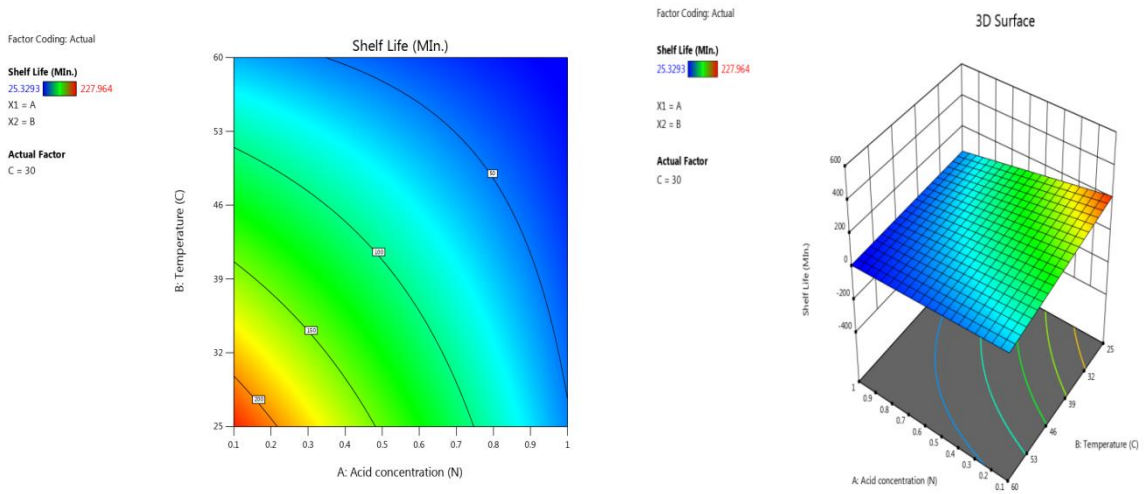


B_1 **B_2**

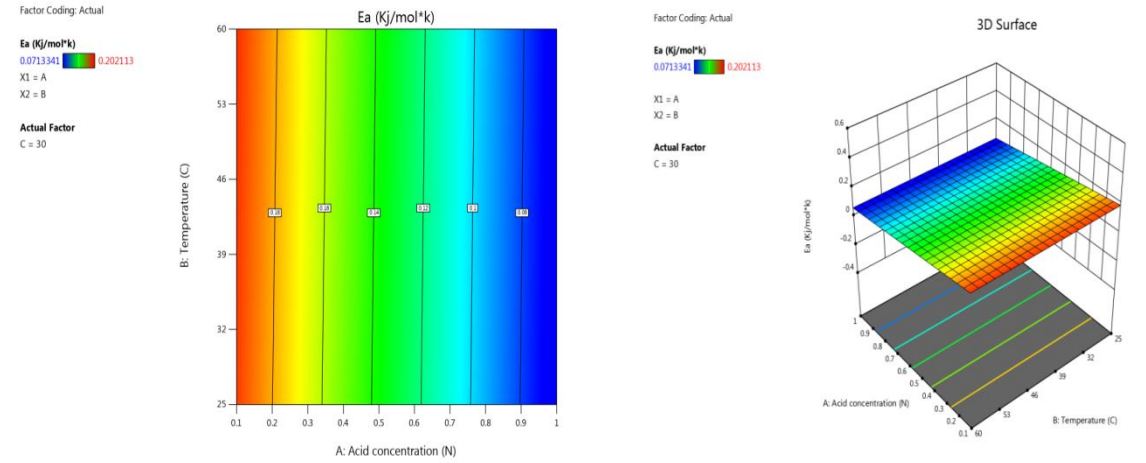




C_1 **C_2**



D_1 **D_2**



E_1 **E_2**

Fig. 5.20 2D contour for **A_1)** % Drug, **B_1)** Rate Constant, **C_1)** Half life, **D_1)** Shelf life and **E_1)** Activation energy and 3D plot for **A_2)** % Drug, **B_2)** Rate Constant, **C_2)** Half life, **D_2)** Shelf life and **E_2)** Activation energy

The contour plots showed three color schemes for showing effect of factor, the red color indicates no effect; green color indicated mild effect and blue color indicates severe effect of factor. The %drug response shows mild change in %drug of rifabutin, while in other responses moderate to severe effects were observed.

Prediction of kinetic parameters using Design Expert ^{TM} software

The Design Expert software can generate mathematical equation for each response. The equations are shown in *ANOVA* section. The application of these equations are shown here in 2D plot and flagged in it. (Fig. 5.21)

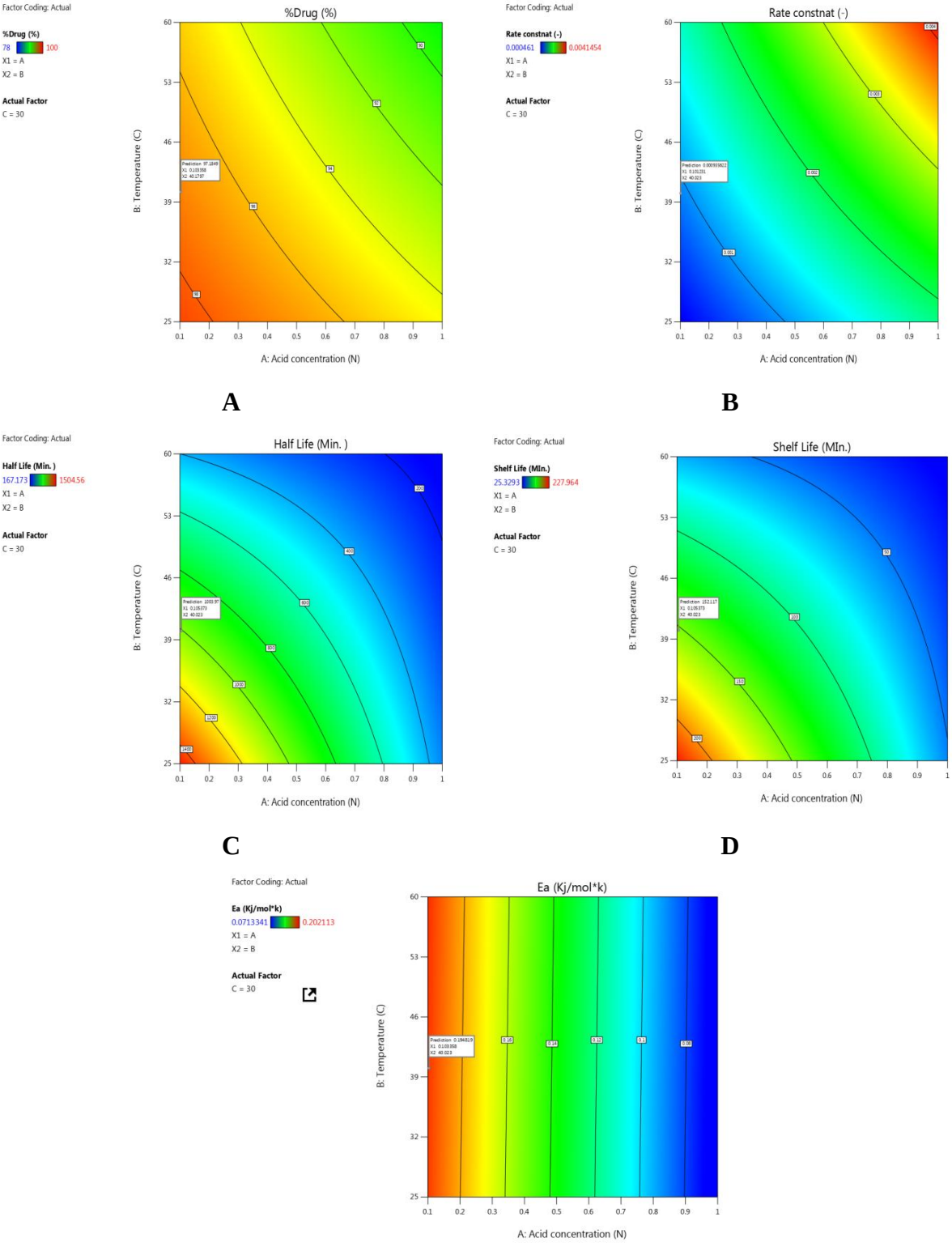


Fig. 5.21 Prediction of kinetic parameters for degradation sample of 0.1N HCl at 40^0C for 30minutes

Application of multi factorial tool

The application of this study was to analyze that whether prediction feature of multi-factorial tool can be applied for degradation kinetic study or not. The kinetic parameter predicted value is flagged in Fig 5.21 and practical value was obtained by conventional kinetic study. (Table 5.19)

0.1N HCl	Multi-factorial value	Conventional study value
%Drug (%)	97.18	95
Rate constant	0.0014	0.0013
Half life (Min.)	508.97	501.5
Shelf life (Min.)	79.11	75.99
Ea (kj/mol. k)	0.194	0.202

Table 5.19 Degradation kinetic parameters by conventional and multi factorial method

The values in table indicates that both the values are corresponding to each other with < 10.0% deviation, therefore the DoE approach can be used for degradation kinetic study of rifabutin in acid medium.

Alkaline degradation kinetics

The degradation of rifabutin in alkaline medium by conventional study is described in section 5.6.2.2. To study alkaline degradation kinetics of rifabutin by multi-factorial tool 2×3 factorial design was chosen, the limit and level of design is shown in Table 5.20.

Factor	Name	Units	Type	Minimum	Maximum	Coded Low	Coded High
A	alkali Conc.	N	Numeric	0.0100	0.1000	-1 ↔ 0.01	+1 ↔ 0.10
B	Time	min	Numeric	0.0000	60.00	-1 ↔ 0.00	+1 ↔ 60.00

Table 5.20 Multi factorial design criteria for alkali degradation kinetics

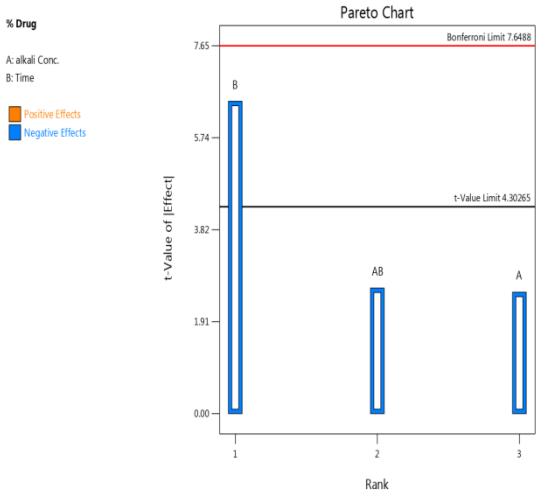
The responses for the above factors selected are shown below in Table 5.21.

Response	Name	Units	Analysis
R1	% Drug	%	Factorial
R2	Rate Constant	-	Factorial
R3	Half Life	Min	Factorial
R4	Shelf Life	MIn	Factorial

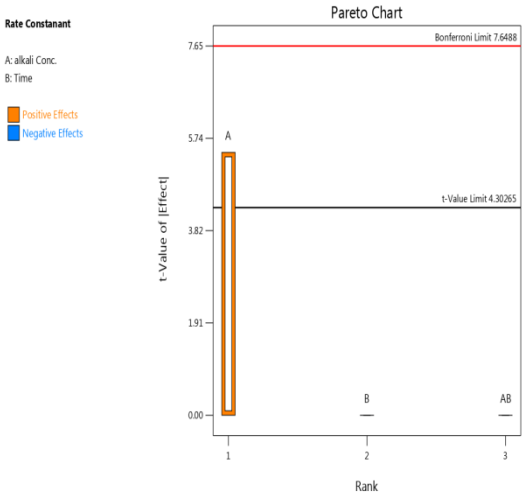
Table 5.21 Responses for alkaline degradation kinetics of rifabutin

Analysis of responses

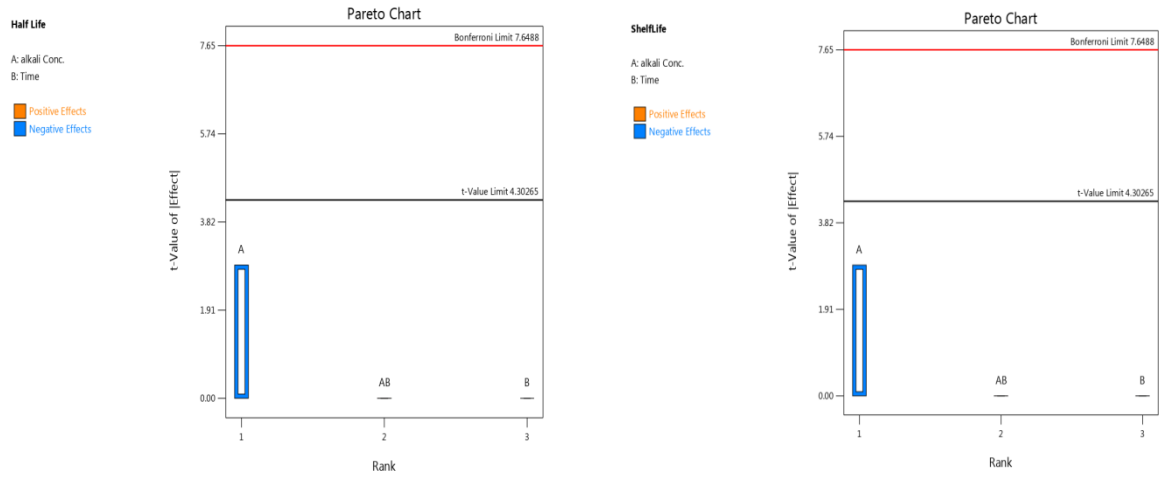
The responses are analyzed against factors and their levels, the run obtained in design were practically imposed to get the data for addition in response block. After completion of collection of data, the responses were analyzed for effect of factors on it. The Pareto chart and half normal plots show the factors' effect in the form of chart. The Pareto chart for each responses are shown in Fig. 5.22.



A



B



C **D**

Fig.5.22 Pareto chart for **A)** % Drug, **B)** Rate Constant, **C)** Half life, **D)** Shelf life

The Pareto chart shows that alkali concentration is the only significant factor that hugely affects the degradation of rifabutin. For response R1 (%drug) both factor (A and B) and combined effect of both factors (A+B) showed negative effect on it.

Analysis of Variants (ANOVA)

The ANOVA study helps to identify the significant difference in mean of one or two parameters and to identify the impact of factors on response. It is an alternative way of t-test to identify the significant difference of mean in the study.

The equations generated from ANOVA can be used later to predict the values of response in the form of plot. The equations generated for each response are;

R1 (% drug): +67.08 -11.34A -29.25B -11.75AB

R2 (Rate constant): +0.0148 +0.0068A +0.0000B +0.0000AB

R3 (Half life): +57.71-32.42A +0.0000B +0.0000AB

R4 (Shelf life): +8.74 -4.91A +0.0000B +0.0000AB

By putting the values of the factors, for which response value is to be needed and calculating the equation will give the response value for that factor. Using these equations, the predicated values are calculated in 2Dand 3D plots.

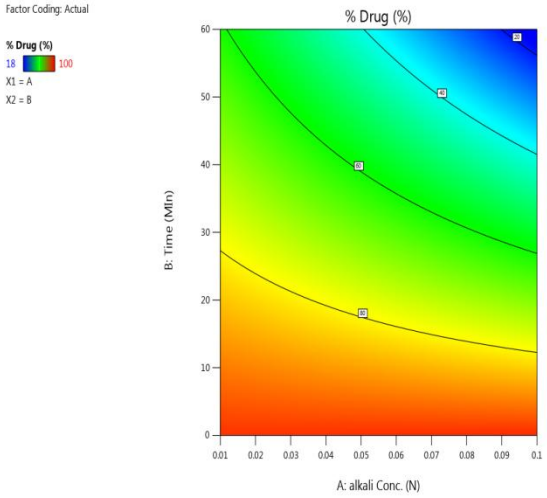
Interaction of factors

The factors can interact and can diminish the effect of each other on response; as a result different response can be obtained. To identify any interaction between factors, interaction study was carried out by Design Expert software. The plot showed a parallel line for each

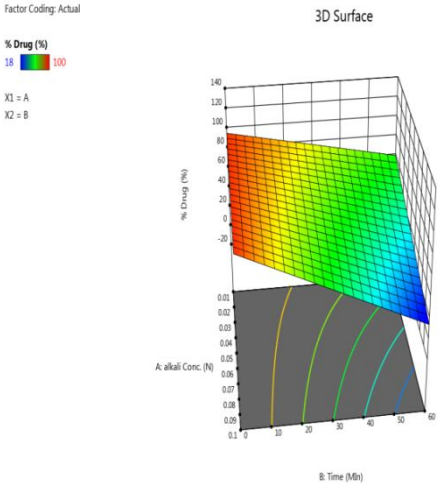
factor indicates that there was no interaction between factors and the effect on response was of individual factor.

Contour plots and 3-Dimentional study

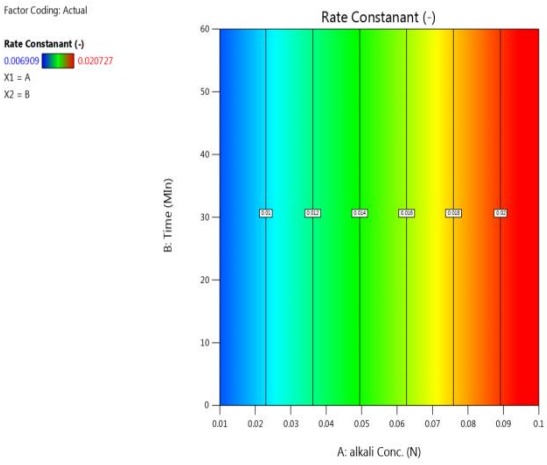
The contour plots and 3D plots are generated to show the effect of every factor on response in a single plot. Both the plots are shown in Fig 5.23.



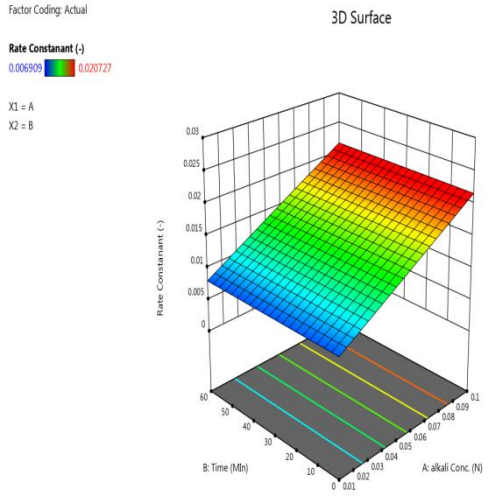
A_1



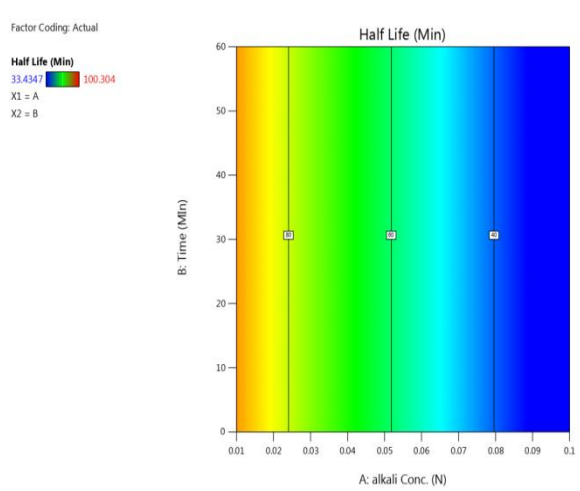
A_2



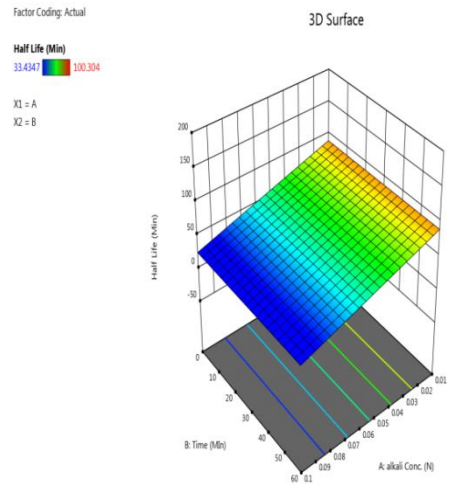
B_1



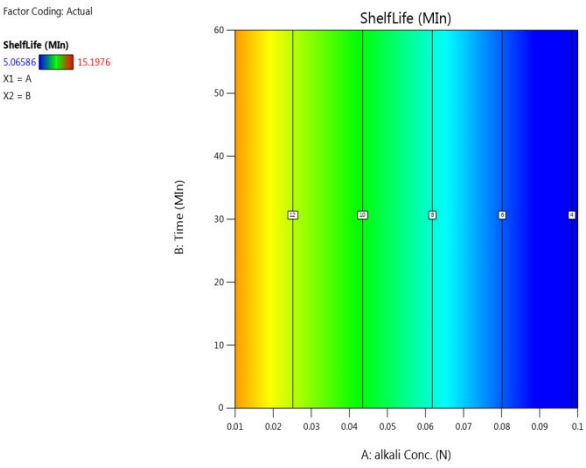
B_2



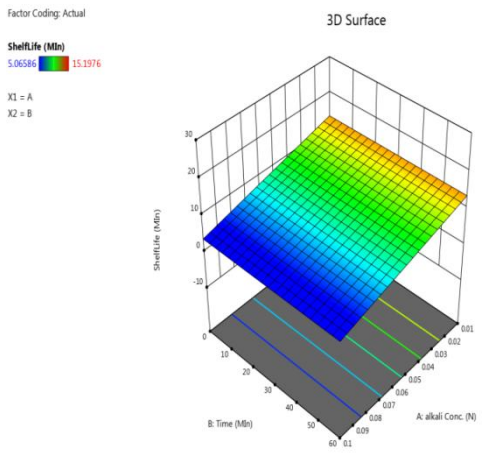
C_1



C_2



D_1



D_2

Fig.5.23 2D contour plot for **A_1)** % Drug, **B_1)** Rate Constant, **C_1)** Half life, **D_1)** Shelf life and 3D plot for **A_2)** % Drug, **B_2)** Rate Constant, **C_2)** Half life, **D_2)** Shelf life

The contour plots and 3D plots are showing the effect of factors on the response, it can be seen that with an increase in time and stress concentration each factor decreases except rate constant. The increase in rate constant shows that degradation rate increases with increase in time and alkali concentration.

Prediction of kinetic parameters using Design Expert ^{TM} software

The kinetic parameters values were predicted using the ANOVA equation by Design Expert software. The values are predicted in 0.01N NaOH after 30minutes at RT shown in Fig 5.24, the values are flagged in 2D plot.

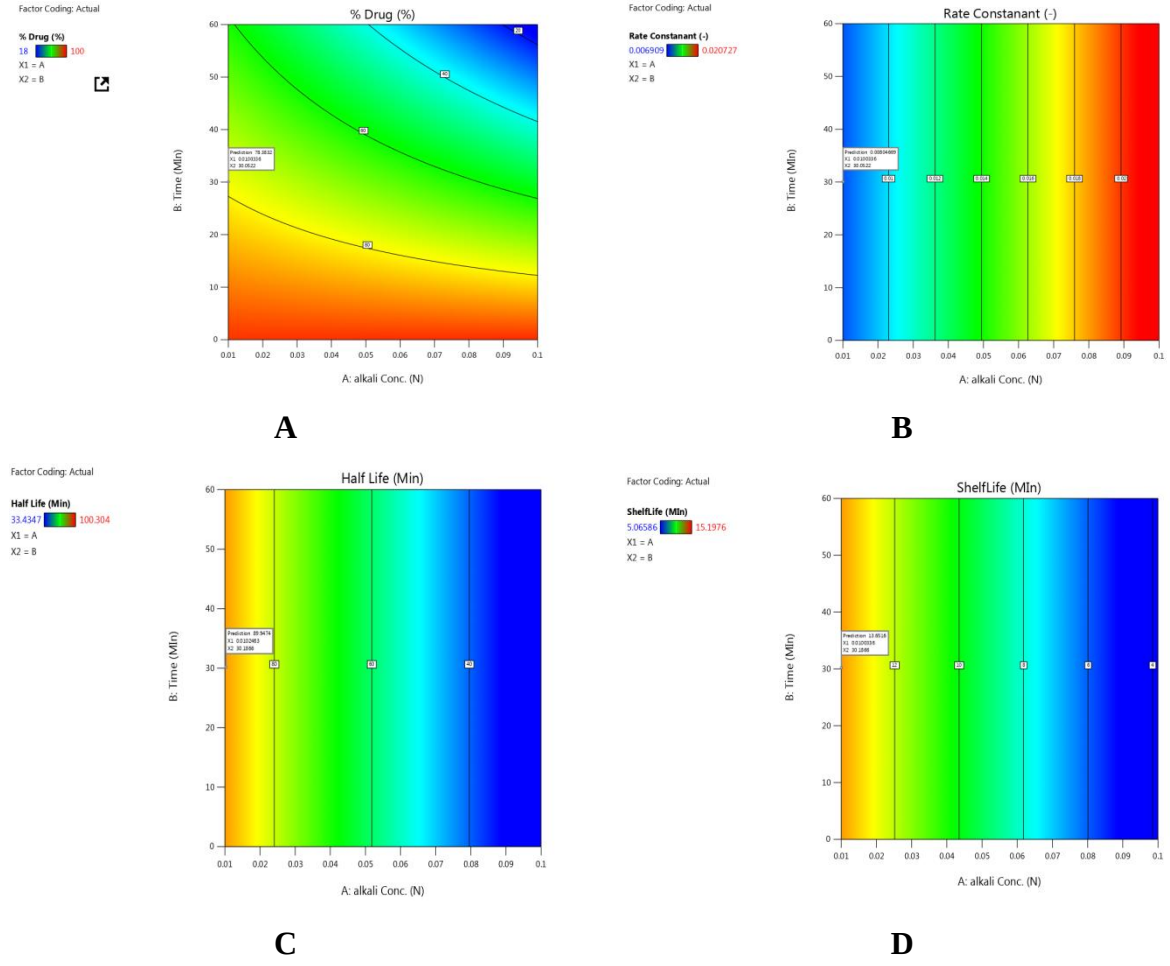


Fig. 5.24 Predication of alkali degradation kinetic parameters using Design Expert ™ software A) % drug B) Rate constant C) half life D) shelf life

Application of multi factorial tool

The multi-factorial tool was applied to predict the degradation kinetic parameter and compare the obtained value with practical conventional method value. The comparisons of both the values are shown in Table 5.22.

0.01N NaOH	Multi-factorial tool value	Conventional study value
%Drug (%)	78.38	80
Rate constant	0.0080	0.0069
Half life (Min.)	89.94	100.30
Shelf life (Min.)	13.65	15.19

Table 5.22 comparison of degradation kinetic parameters value obtained by multi-factorial tool and conventional study

The table shows that deviation in values obtained by both the methods is < 20.0% and therefore the multi factorial tool can be used to study the degradation kinetics of rifabutin.

5.7.2.2. Oxidative degradation kinetics of rifabutin

The oxidative degradation kinetic study of rifabutin was studied by 2×3 factorial design using the factors and responses shown in Table 5.23 and 5.24 respectively.

Factor	Name	Units	Type	Minimum	Maximum	Coded Low	Coded High
A	Peroxide Conc.	%	Numeric	7.00	30.00	-1 ↔ 7.00	+1 ↔ 30.00
B	Temperature	C	Numeric	25.00	80.00	-1 ↔ 25.00	+1 ↔ 80.00
C	Time	Min	Numeric	0.0000	30.00	-1 ↔ 0.00	+1 ↔ 30.00

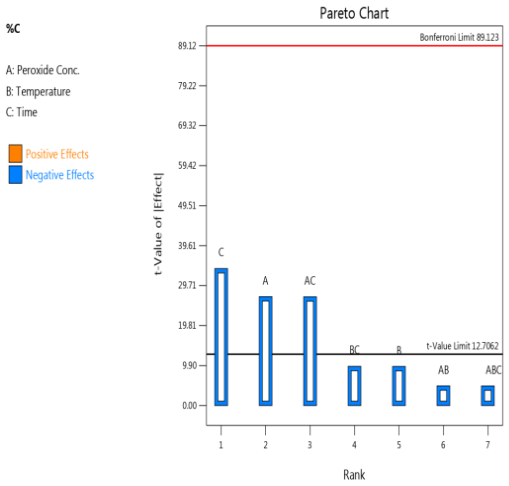
Table 5.23 factors and levels for oxidative degradation kinetics

Response	Name	Units	Observations	Analysis
R1	R1	%C	9.00	Factorial
R2	R2	K	9.00	Factorial
R3	R3	Half Life	9.00	Factorial
R4	R4	Shelf Life	9.00	Factorial
R5	R5	Ea	9.00	Factorial

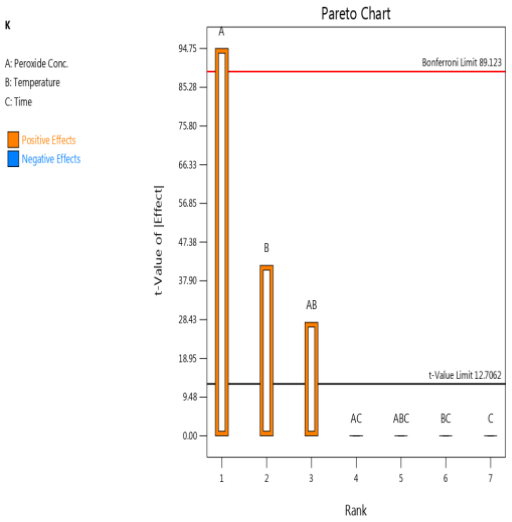
Table 5.24 Response for the design of oxidative degradation kinetics

Analysis of responses

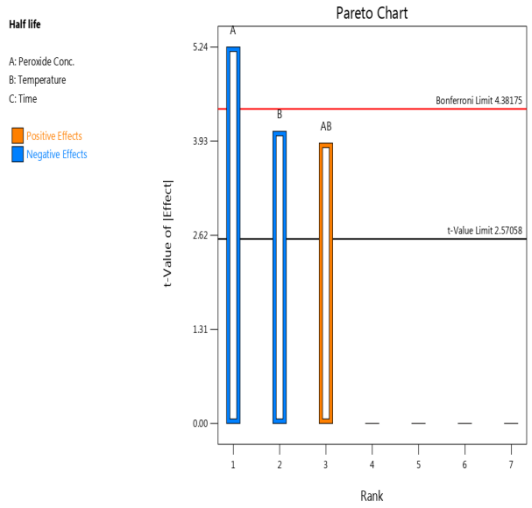
The obtained response for each run was analyzed and significant factor for each response was evaluated. The significant factors were identified by half normal plots and by Pareto chart study. The Pareto charts are shown in Fig 5.25.



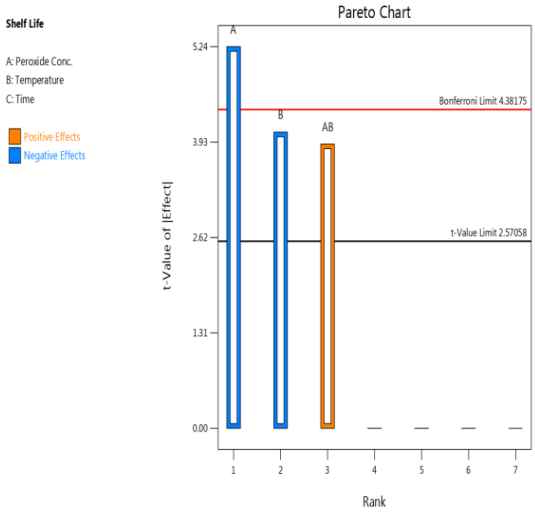
A



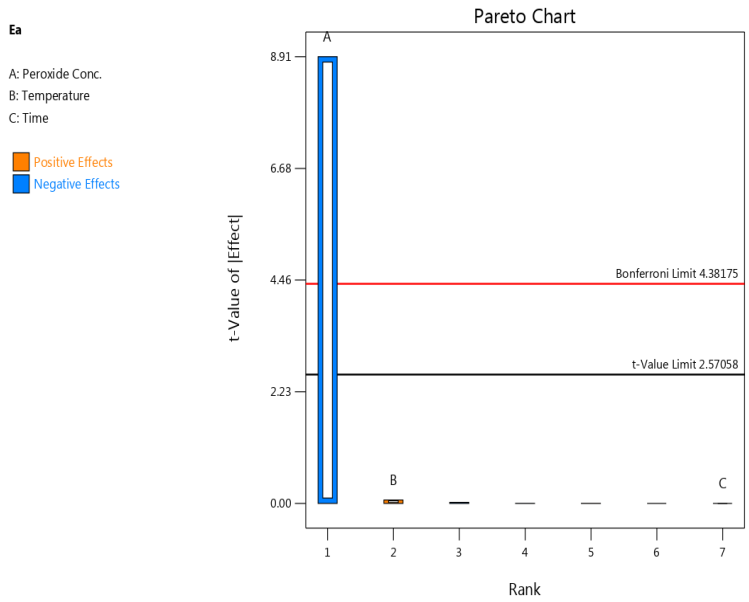
B



C



D



E

Fig.5.25 Pareto chart for response to evaluate significant factors

The analysis of Pareto chart shows that input A, B and AB had desirable (positive effect in rate constant) and undesirable (negative effect on % drug, half life, shelf life and activation energy) effects on responses. The factors approaching to bonferroni limit and t-limit value are having true effect on responses and should be considered as significant factors.

Analysis of Variants (ANOVA)

The ANOVA study used to know significant difference of mean of dependant and independent variables. The equation generated from ANOVA can be used to know the value of response using desired factor value in equation. The equation for each factor is shown below;

R1 (% drug): +84.10 - 12.45A - 4.49B - 15.75C - 2.25AB - 12.50AC - 4.50BC - 2.25ABC

R2 (Rate constant): +0.0132 + 0.0110A + 0.0048B + 0.0000C + 0.0032AB + 0.0000AC + 0.0000BC + 0.0000ABC

R3 (Half life): +273.16 -267.33A -208.43B +0.0000C +200.08AB + 0.0000AC +0.0000BC +0.0000ABC

R4 (Shelf life): +41.39 -40.51A -31.58B +0.0000C +30.32AB + 0.0000AC +0.0000BC +0.0000ABC

R5 (Activation energy): +0.1438 -0.0620A +0.0005B +0.0000C + 0.0000AC +0.0000BC +0.0000ABC

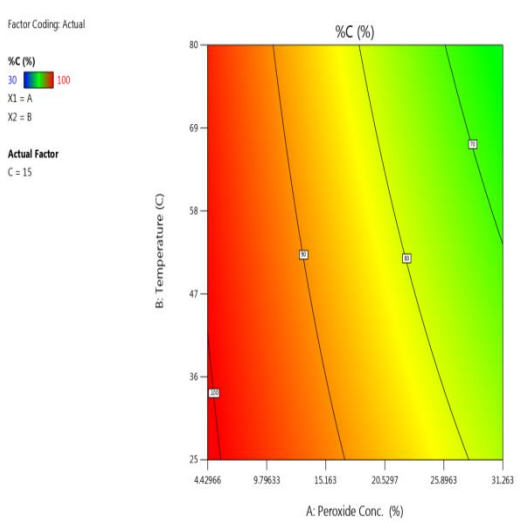
The equations are used to calculate the response value for prediction in contour plots.

Interaction of factors

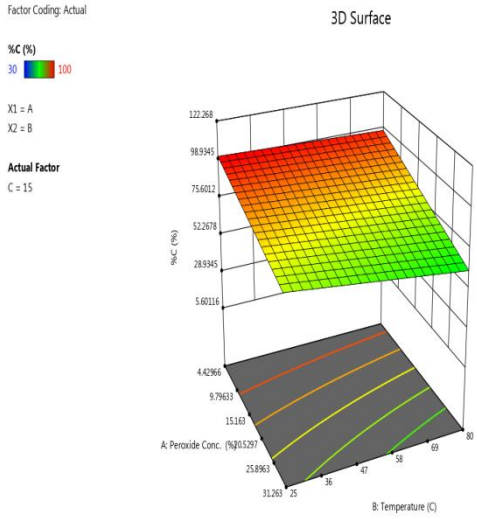
The interaction plots can show if any interaction between factors that can affect the response value. The parallel line of factors showed that there was no interaction between factors and the effect of factors on each response is separate.

Contour plots and 3-Dimentional study

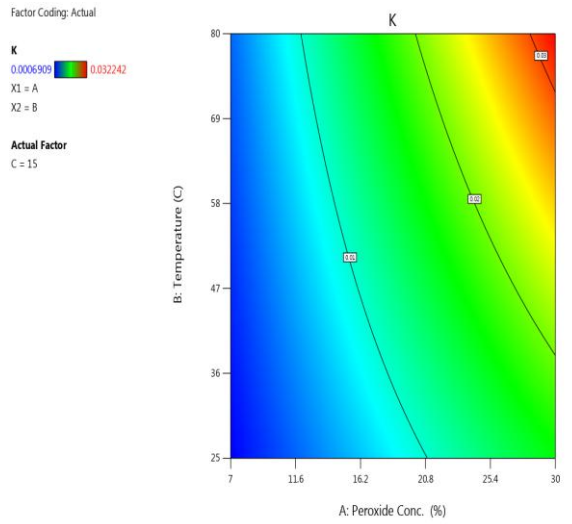
The contour plots and 3D plots can show the effect of factors on response in a single plot with effect rate. The contour and 3D plots are shown in Fig 5.26.



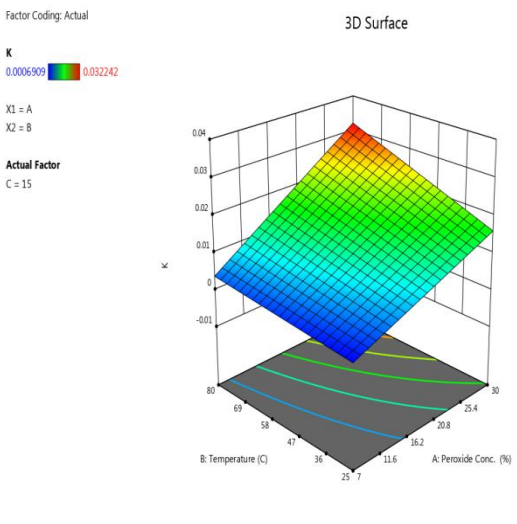
A_1



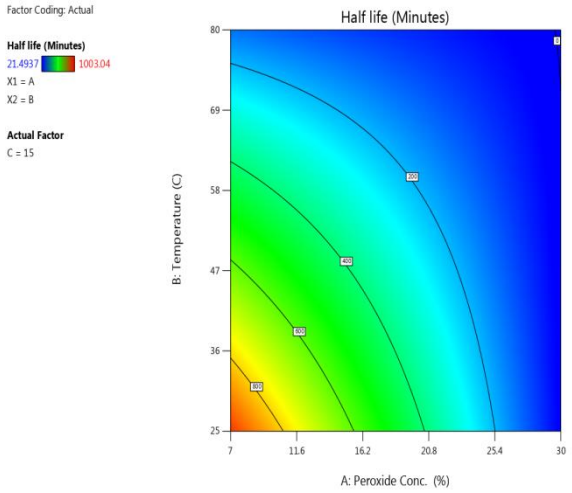
A_2



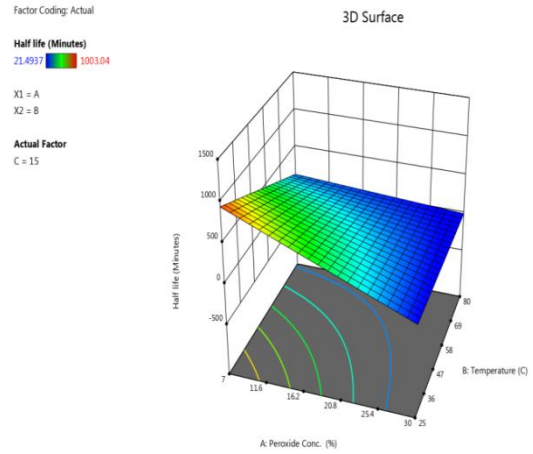
B_1



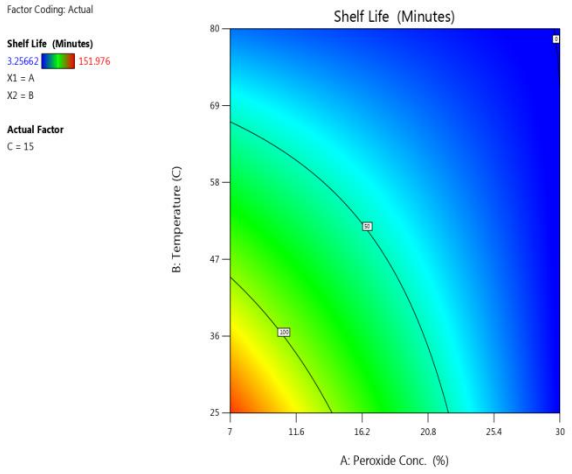
B_2



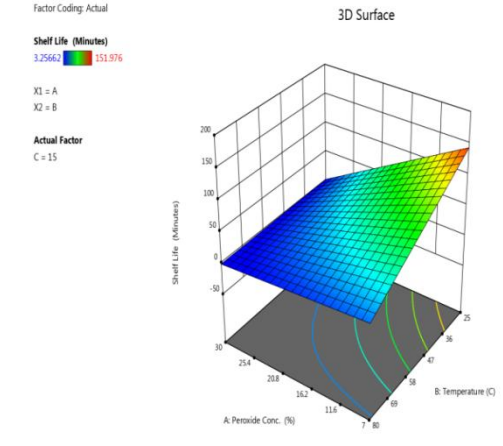
C_1



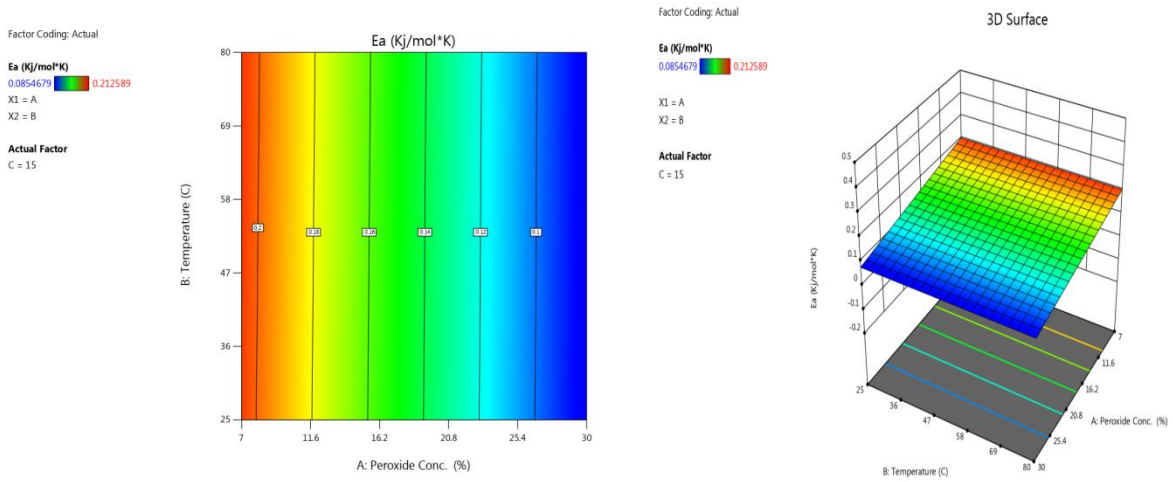
C_2



D_1



D_2



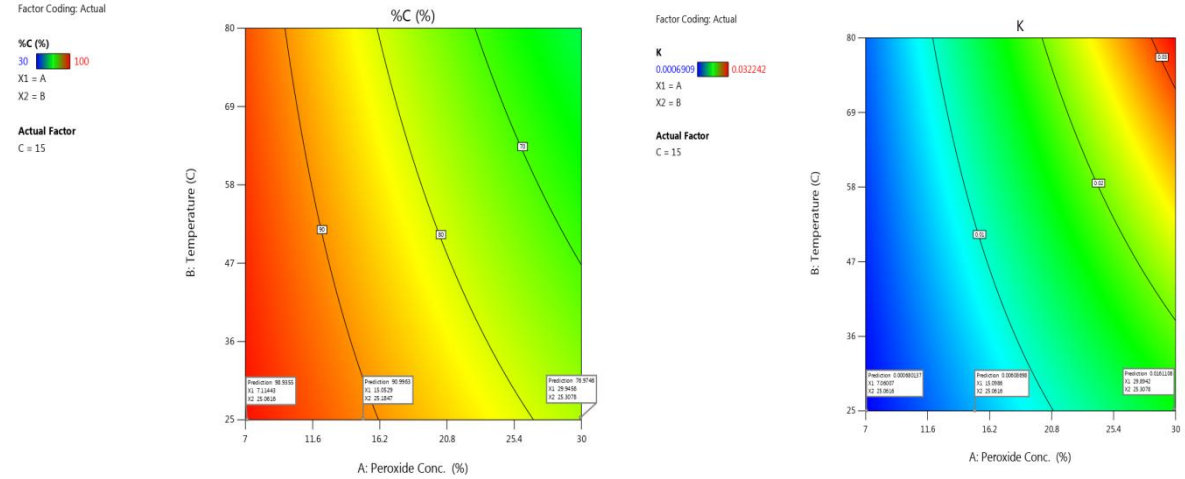
E_1 **E_2**

Fig. 5.26 2D contour plot for **A_1)** % Drug, **B_1)** Rate Constant, **C_1)** Half life, **D_1)** Shelf life and **E_1)** Activation energy and 3D plot for **A_2)** % Drug, **B_2)** Rate Constant, **C_2)** Half life, **D_2)** Shelf life and **E_2)** Activation energy

The plot showed that stressor concentration and temperatures had negative effect on response R1, R3, R4 and R5 while positive effect on response R2.

Prediction of kinetic parameters using Design Expert ^{TM} software

The predictions of kinetic parameters were done using contour plots and ANOVA equations. The contour plots are shown in Fig 5.27.



A **B**

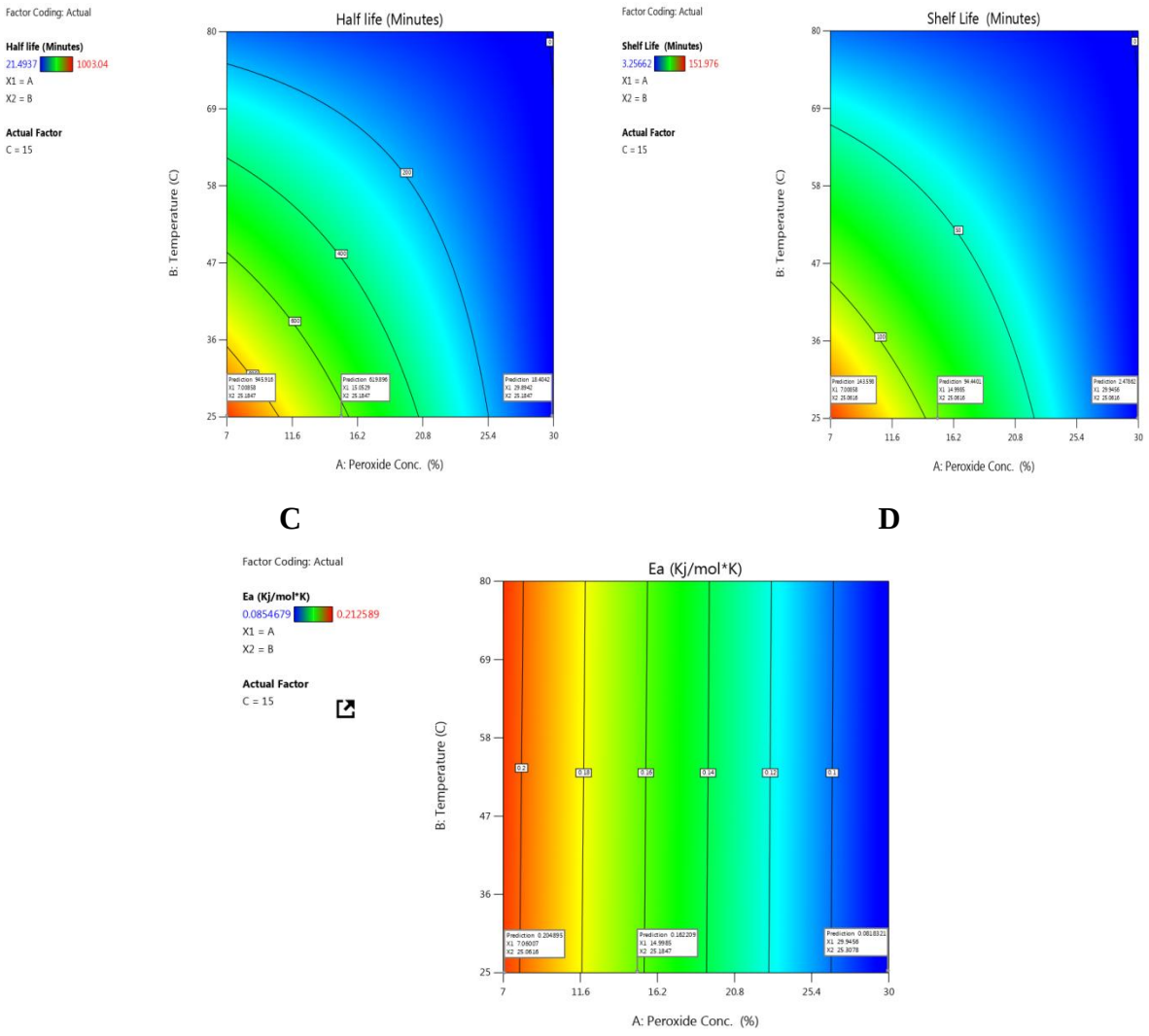


Fig. 5.27 Predication of degradation kinetic parameters A) % drug B) rate constant C) half life D) shelf life E) activation energy

Application of multi factorial tool

The contour plots were used to predict the response value for 30% H_2O_2 at RT for 15minutes. (Table 5.25)

30% Peroxide	Multi-factorial study value	Conventional study value
%Drug (%)	76.97	73
Rate constant	0.016	0.016
Half life (Min.)	48.40	42.98
Shelf life (Min.)	6.47	6.51
Ea (kj/mol. k)	0.081	0.085

Table 5.25 Degradation kinetic parameters value by multi-factorial tool and conventional study

The table showed that deviations of both the values are < 10.0% and co relational values are obtained suggests that degradation kinetics can be studied by both the methods.

- **Conclusion**

The rifabutin degradation kinetics in acid, alkali and oxidative condition was studied by conventional approach using three different concentration of stressor (higher, middle and lower concentration), using three temperatures (higher , moderate and mild temperature) at different time interval (0 to 4 or 5 points). The responses were recorded as kinetic parameters. The effect of factors and relation between factor and responses were evaluated using the values at different factor level.

The other approach to study degradation kinetics was Design of Experiment by multi-factorial tool. The data generated in conventional study was used to fill the responses in design and compare the values predicted by tool.

The predicted values and conventional study values are close with average < 20.0% deviation. The significant differences between means were calculated by *ANOVA* by software Design Expert. The significant overall effects of factors were studied by Pareto chart for each response. The comparisons of values by both the methods for degradation kinetic parameters were showing corresponding value with each other indicates that both the methods can be used promptly for prediction of value and to study the degradation kinetic parameters.

PART-D

5.8. ISOLATION AND IDENTIFICATION OF MAJOR DPs

5.8.1 EXPERIMENTAL

5.8.1.1 Chemicals and Reagents

The chemicals and reagents are same as described in section 5.5.1.1. The UPLC grade chemicals utilized for study were same as described in section 4.8.1.1.

5.8.1.2 Equipments and chromatographic conditions

The Equipments are described in section 5.5.1.2.

UPLC-MS: UPLC/ESI-MS study was completed using mobile phase **A)** 0.1 % formic acid in Milli Q water (pH= 2.70) and **B):** 0.1%formic acid in Milli Q water: acetonitrile (10:90). Gradient elution program was set to T = 0 min (97% A); T = 0.75 min (97% A); gradient to T = 2.7 min (2% A); gradient to T = 3 min (0% A); T = 3.5 min (0% A); gradient to T= 3.51 min (97% A); end of run at T = 4 min (97% A), Flow rate: 1.0 ml/min, analysis time 4 min. Mass probe (Probe temperature 400^0C) was set as source for electro spray ionization in positive mode (temp. 120^0 C) with cone voltage 10 and 30V and capillary voltage 3.25kV. Cone gas flow and desolvation (400^0C) gas flow was 100 and 800L/hr, respectively. Column and auto sampler temperature was set to 35^0 and 5^0C, respectively.

Flash HPLC: The isolation of major DP was completed using Bioteg® Selekt systems, using flash chromatography, two channels, extended collection bed, UV-Vis detector, and 240ml tray.

Isolation of major stress degradation product was completed using stationary phase YMC 80G ($C_{18,}$ 250×19mm, 50) column set to ambient temperature. Mobile phase consist of **a)** water and **b)**: Acetonitrile with flow rate 80ml/min, PDA detection at 275nm. Gradient elution program was set as follows; run time 25minutes; T (min) =% v/v **a) %** v/v **T=** 0.01(90), **T=** 20 (10), **T** = 20.01 (0), **T=** 22.0 (0), **T=** 22.01 (90) and stop command after **T=**25 (90). The data acquisition and analysis was done using software Bioteg® Selekt Spektra software.

NMR: ^{1}H NMR, ^{13}C NMR and APT were performed on Bruker 400MHz NMR spectrometer using deuterated di methyl sulphoxide (DMSO-d6) as solvent. Chemical shifts were recorded in ppm (δ Scale) and coupling constants in Hertz concerning TMS (0 δ ppm) as an internal standard. For data analysis Top spin software 3.2 was used.

5.8.1.3 Sample preparation

Analytical sample preparation, stress degradation sample and buffer preparations are same as described in 5.5.1.3.

Enrichment of DPs for identification and isolation

High concentration sample was prepared for isolation; accurately weighed 5gm of rifabutin was dissolved separately in 15ml methanol, sonicated for 15minutes with provisional shaking; 50ml final volume was achieved using 0.5N HCl, 0.1N NaOH or 30% hydrogen peroxide, the solution was kept in specified stability condition in dark (acid: 0.5N HCl at RT for 18 hrs, 0.1N NaOH at RT for 60minutes and Oxidative: 30% H_2O_2 at 80^0C for 5hrs). The 3ml aliquot +1ml acetonitrile + 1ml water was filtered through whatman filter paper prior to filtering with 0.45µ Pall syringe filter. The sample was injected in described chromatographic condition of preparative HPLC for isolation of degradation impurity with purity <95.0%.

5.8.2 RESULT AND DISCUSSION

Isolation and Characterization of major DPs

5.8.2.1. Alkali degradation impurity

Alkali degraded sample of rifabutin showed three degradation impurities, the stress degradation conditions are described in section 5.5.2.2. The chromatogram is shown in Fig 5.5. The peak purity results are shown in table 5.5 and Fig 5.8. For isolation the sample was prepared as described in section 5.8.1.3. The major degradation product was identified by area occupied by peak in chromatogram. DP-AL6 (from DP-AL1 to AL10) was identified as major degradation product in HPLC chromatogram at Rt 5.5.± 0.05minutes (68.14% area).

Isolation and Purification of DP-AL6

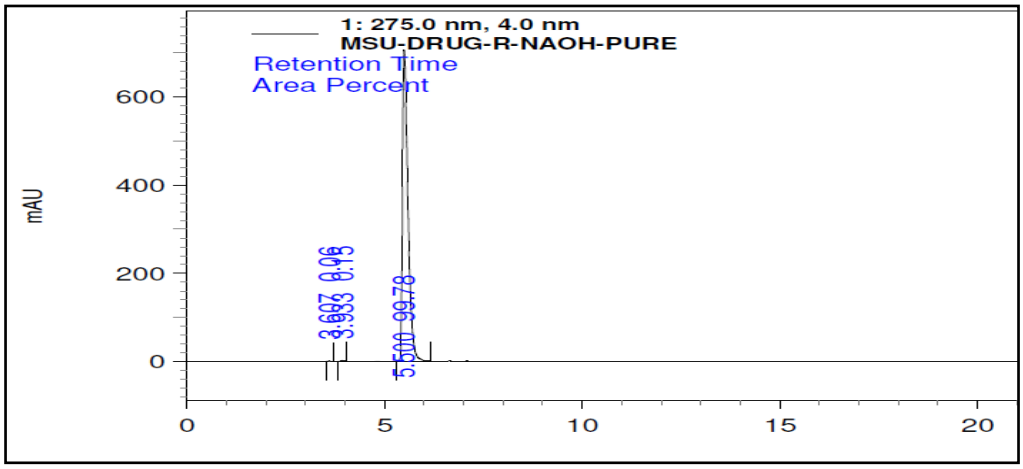
The isolation of DP-AL6 was carried out using flash chromatography instrument and chromatographic condition described in section 5.8.1.2.

The isolated fraction was confirmed in HPLC conditions described in section 5.5.1.2 to precede the purification of fraction. The organic solvent was evaporated using Rotavapour and the fraction was washed with water to remove excess of buffer in fraction. The lyophilization process was carried out to get solid mass degradation product. The obtained DP was pale violet to light purple in color. The DP was further used for NMR analysis.

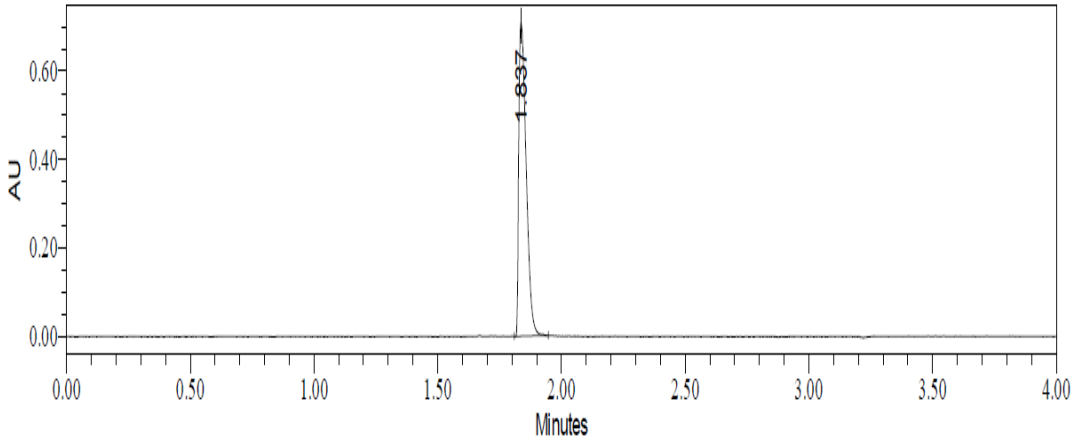
Confirmation of DP-AL6

The isolated DP was confirmed by RP-HPLC and UPLC chromatogram and ESI/MS spectrum (Fig 5.28) to carry out NMR and IR study and data analysis.

A



B



C

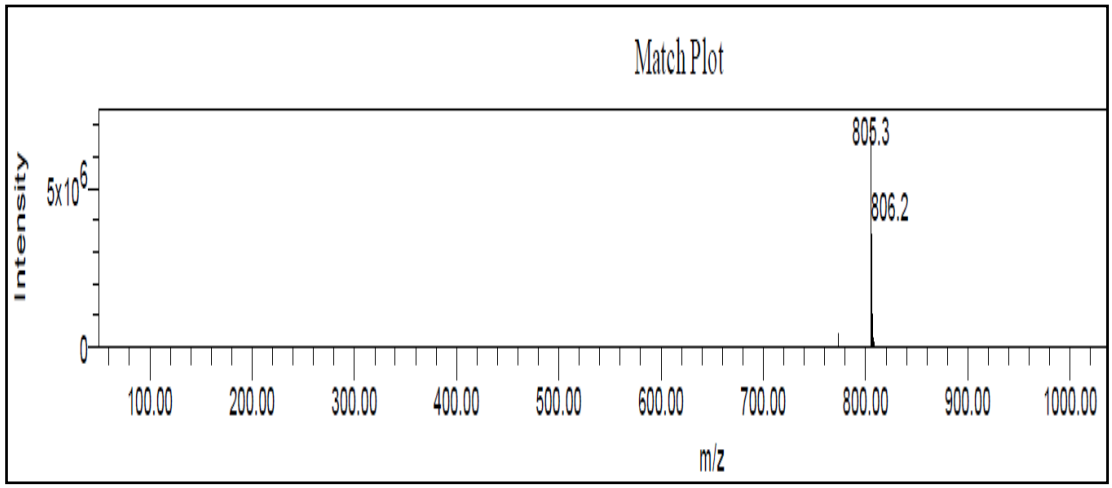


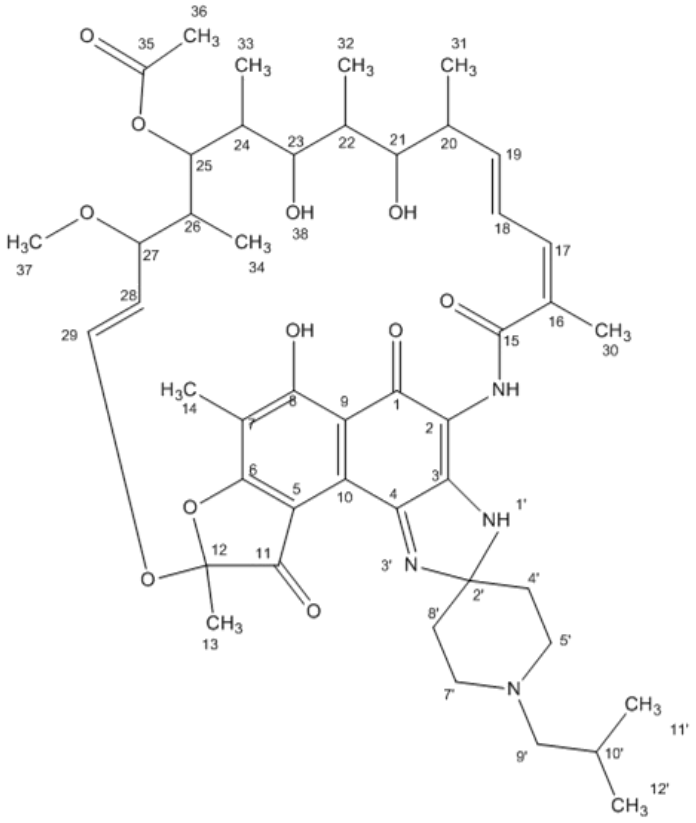
Fig. 5.28 DP-AL6 confirmation by A) RP-HPLC B) UPLC and C) ESI/MS spectrum

The chromatogram and spectrum showed absence of other peaks confirms that desired DP was isolated with desired purity (>98.0%). Further characterization was carried out using NMR.

Characterization of DP-AL6

The isolated DP with purity >98.0% was characterized by NMR studies; proton NMR, Carbon-13 NMR and Attached proton test was performed for DP-AL6 characterization. The chemical structure of rifabutin bulk drug and DP-AL6 is shown in Fig 5.29 with atom number for assignment of NMR.

A)



B)

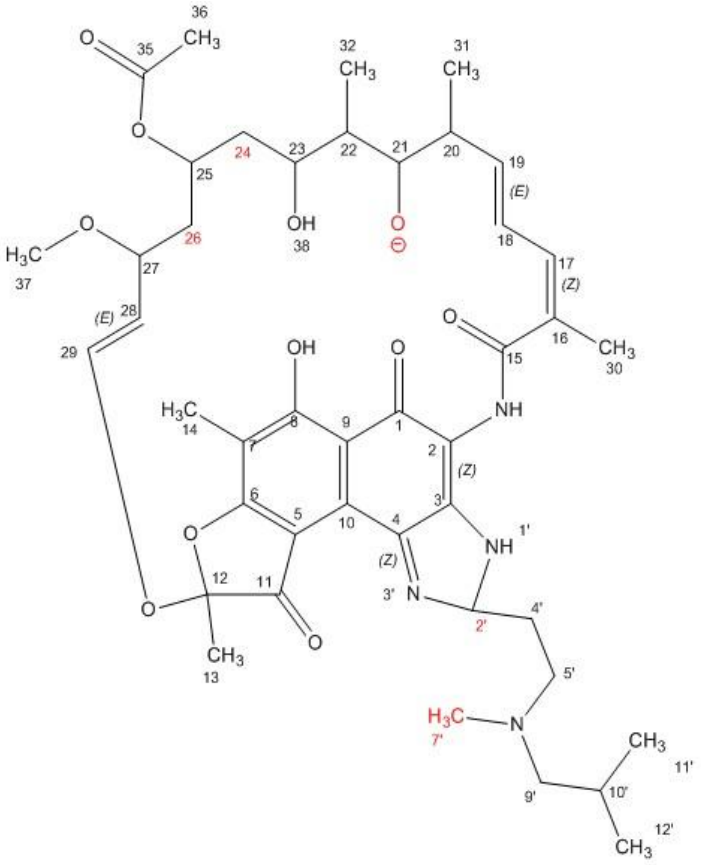
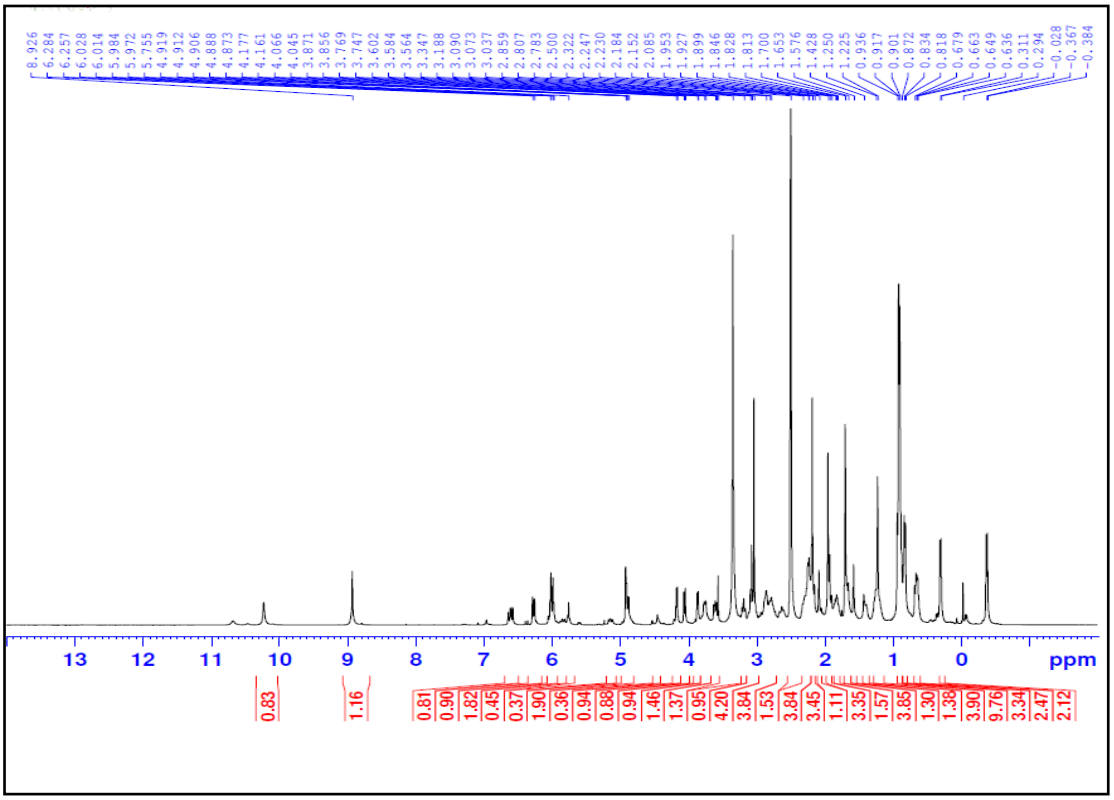


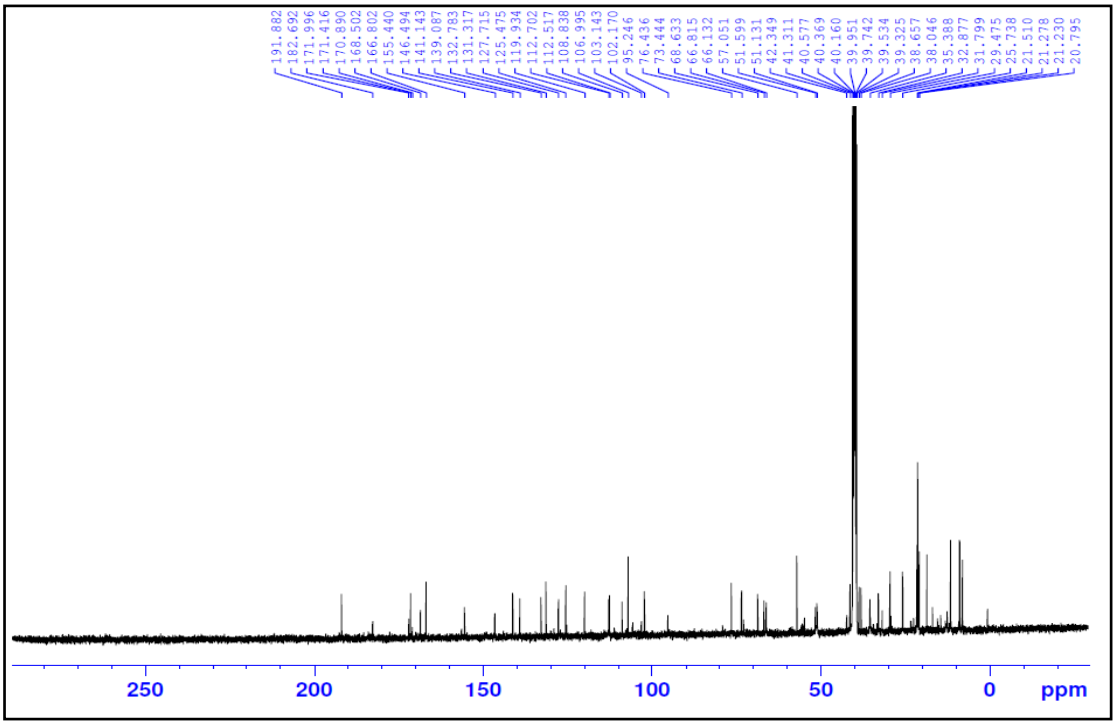
Fig. 5.29 Chemical structure of A) rifabutin and B) DP-AL6 with atom number

The chemical changes occurred in DP-AL6 are shown with atom number in Fig 5.29. These chemical changes were observed in NMR spectrum of DP-AL6. The NMR spectra are shown in Fig 5.30.

A



B



C

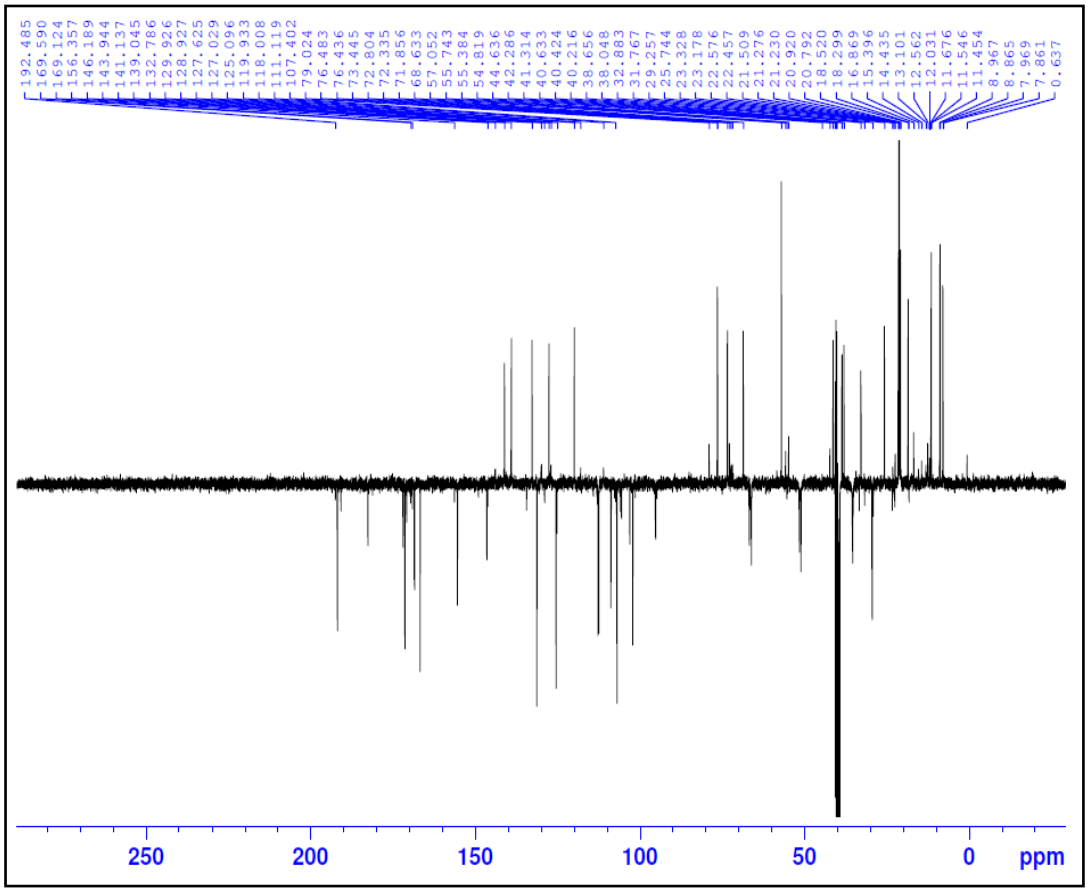


Fig. 5.30 NMR spectrum for A) proton NMR B) Carbon-13 NMR and C) APT NMR

The NMR spectrum assignment with atom number is shown in Table 5.26 and it confirms the proposed structure of DP-AL6 shown in Fig 5.29.

	Rifabutin		**DP-AL6**			
Sr.no	*Proton NMR*	*Multi.*	*Proton NMR*	*Multi.*	*C^{13} NMR*	*APT*
1	-	-	-	-	182.6	*(-C-)lw*
2	-	-	-	-	141.1	*(-C-)lw*
3	-	-	-	-	103.1	*(-C-)lw*
4	-	-	-	-	155.4	*(-C-)lw*
5	-	-	-	-	125.4	*(-C-)lw*
6	-	-	-	-	171.4	*(-C-)lw*
7	-	-	-	-	112.7	*(-C-)lw*
8	-	-	-	-	168.5	*(-C-)lw*
9	-	-	-	-	112.5	*(-C-)lw*
10	-	-	-	-	108.8	*(-C-)lw*
11	-	-	-	-	191.8	*(-C-)lw*
12	-	-	-	-	107.4	*(-C-)lw*
13	1.706	*s*	1.700	*s*	21.5	*($-CH_3$) up*
14	2.224	*s*	2.322	*s*	7.9	*($-CH_3$) up*
15	-	-	-	-	168.5	*(-C-) lw*
16	-	-	-	-	131.3	*(-C-) lw*
17	6.262	*dd*	6.284	*dd*	132.7	*(-CH-) up*
18	6.289	*dd*	6.028	*dd*	125.1	*(-C-)lw*
19	6.025	*dd*	5.984	*dd*	139.0	*(-CH-) up*
20	2.326	*m*	2.247	*m*	38.0	*(-CH-) up*
21	4.168	*dd*	*3.871	*m*	38.0	*(-CH) up*
22	1.7-1.8	*m*	1.7-1.89	*m*	72.3	*(-CH) up*
23	3.345	*ddd*	3.584	*ddd*	76.4	*(-CH) up*
24	1.463	*ddd*	*1.95	*m*	*29.4	*($-CH_2$) lw*
25	4.147	*dd*	4.177	*dd*	73.4	*(-CH) up*

26	1.7-1.8	*m*	*1.899	*m*	*39.9	*(-CH_2) lw*
27	3.694	*ddd*	3.602	*ddd*	79.0	*(-CH) up*
28	4.863	*ddd*	4.888	*ddd*	119.9	*(-CH) up*
29	5.152	*ddd*	4.919	*ddd*	143.9	*(-CH) up*
30	2.192	*d*	2.184	*d*	20.7	*(-CH_3) up*
31	0.809	*d*	0.872	*d*	18.5	*(-CH_3) up*
32	0.848	*d*	0.818	*d*	11.5	*(-CH_3) up*
33	0.691	*d*	*-	-	*-	-
34	-0.244	*d*	*-	-	*-	-
35	-	-	-	-	171.9	*(-C-) lw*
36	2.091	*s*	1.953	*s*	21.2	*(-CH_3) up*
37	3.074	*s*	3.073	*s*	57.0	*(-CH_3) up*
2'	-	-	*5.115	*dd*	*79.0	*(-CH-) up*
4'	1.959	*m*	2.0	*m*	35.3	*(-CH_2) lw*
5'	2.78	*m*	2.7	*m*	51.1	*(-CH_2) lw*
7'	2.24	*m*	*1.250	*s*	*54.8	*(-CH_3) up*
8'	2.0	*m*	-	-	*-	-
9'	2.804	*d*	2.807	*d*	66.1	*(-CH_2) lw*
10'	1.882	*m*	1.861	*m*	25.7	*(-CH_2) lw*
11'	0.901	*d*	0.917	*d*	20.7	*(-CH_3) up*

12'	0.901	*d*	0.917	*d*	20.7	*(-CH_3) up*
8 OH	16.17	*s*	16.128	*s*	-	-
21 OH	4.433	*br s*	*-	-	-	-
23 OH	4.168	*d*	4.161	*d*	-	-
NH	10.225	*d*	10.225	*s*	-	-
NH'	10.705	*d*	10.269	*s*	-	-

*Multi.-multiplicity *change observed in NMR spectrum *lw-lower field *up-upper filed*

Table 5.26 Assignment for ^{1}H, ^{13}C and APT NMR spectrum for rifabutin and DP-AL6

The DP-AL6 was major degradation product and it formed by removal of -3 CH_3 and $–H^+$ molecules under the alkali catalyzed reaction. The removal of methyl group (β-position 33) from α-position 24 was confirmed by proton NMR and C^{13} NMR by absence of 3H and –C atom peak in proton and C^{13} NMR; while $–CH_2$ peak was observed in APT NMR in lower field (detects –C and $–CH_2$, $-CH_4$). The loss of $–CH_3$ group (β-position 34) from α-position 26 was confirmed by absence of proton and carbon peaks in proton and C^{13} NMR while $–CH_2$ peak was observed for position 26 in lower field of APT. Removal of $–CH_3$ from position 8' was confirmed by absence of $–CH_2$ peak in APT and C^{13} NMR while presence of $–CH_3$ group at position 7' was confirmed by C^{13} and APT NMR by peak in Upper field (-detects –CH and $–CH_3$). The H^+ removal from –OH group attached to α-position 21 was confirmed by absence of broad singlet in proton NMR. The compilation of all the changes in NMR resulted in DP-AL6 structure; further confirmation was obtained from *m/z* ion of ESI/MS spectrum which matched with proposed structure. The suggested chemical name for DP-6 can be (2Z, 4E, 6S, 7R, 9R, 11S, 12S, 13S)-11-acetoxy-1-(((2S,9S)-6,9-dihydroxy-2-(-2isobutyl(methyl)amino)ethyl)-7,9-dimethyl-5,10-dioxo-3,5,9,10-tetrahydro-2H-furo[2',3':7,8] napthol[1,2-d]imidazol-4-yl)amino)-9-hydroxy-13-methoxy-2,6,12-trimethyl-1-oxopentadeca-2,4,14-trien-7-olate.

IR Confirmation of DP

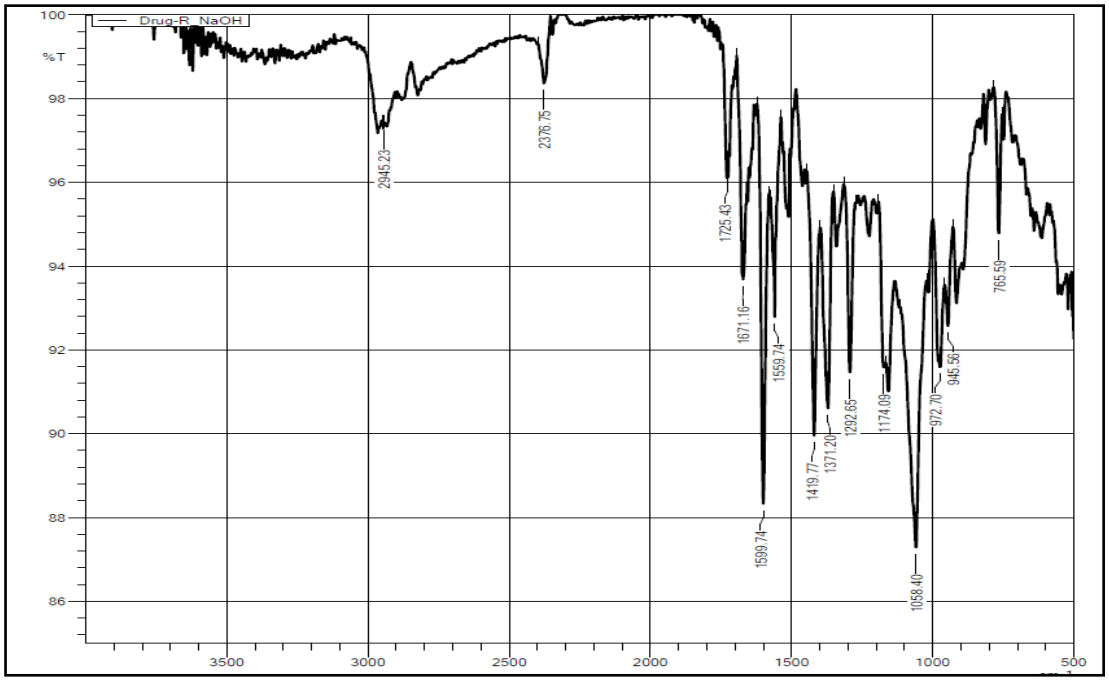


Fig. 5.31 IR spectrum of alkali DP of rifabutin

Assigned Wave number	Obtained Wave number	Comments
2800-3000	2945.3	-N-H stretching
1740-1720	1725.4	C=O stretching
1690-1640	1671.1	C=N stretching
1650-1580	1599.7	N-H bending
1440-1395	1371.2, 1419.7	O-H bending
1342-1266	1292.6	C-N stretching
1205-1124	1174.0	C-O stretching
1085-1050	1058.4	C-O stretching
980-960	972.7	C=C bending
755±20	765.5	C-H bending

Table 5.27 IR spectrum analysis for alkaline DP

5.8.2.2. Acid degradation impurity

Rifabutin formed 9 DPs in acid medium which were named as DP-A1 to A9 among which DP-A5 was major DP which was isolated and identified using UPLC/ESI-MS, Tandem MS and IR analysis.

Isolation and Purification of DP

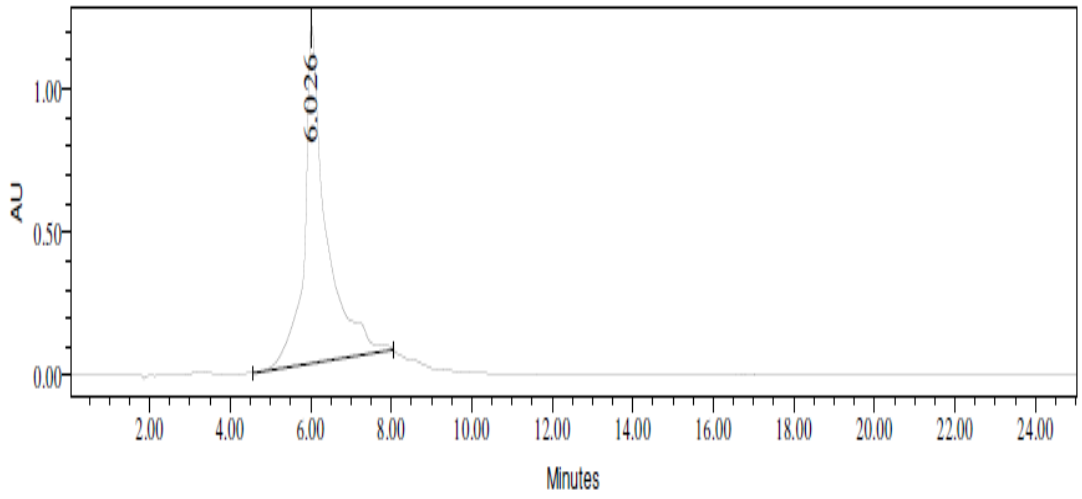
The isolation of major DP was carried out using flash chromatography instrument and chromatographic condition described in section 5.8.1.2.

The isolated fraction was confirmed in HPLC conditions described in section 5.5.1.2 to precede the purification of fraction. The organic solvent was evaporated using rotavapour and the fraction was washed with water to remove excess of buffer in fraction. The lyophilization process was carried out to get solid mass degradation product. The obtained DP was pale violet to light purple in color. The DP was further used for MS/MS study.

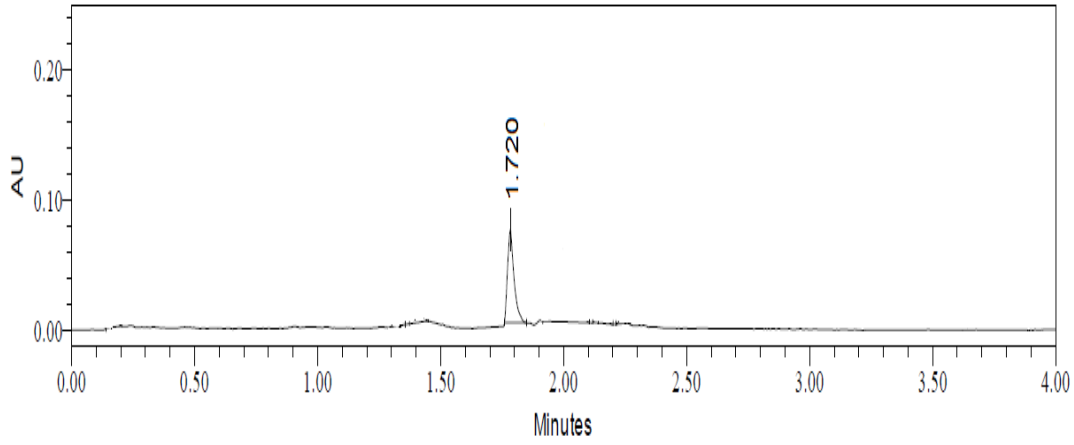
Confirmation of DP-A5

The isolated DP –A5was confirmed by UPLC, HPLC and ESI/MS to confirm that desired DP was isolated. The HPLC Rt was matched with the Rt obtained during stress degradation study (Fig. 5.5/ Rt 6.2±0.5 minutes). The UPLC/ESI-MS result data combination showed that if any other *m/z (*for adduct, ionization or extra peak) present with isolated DP. The confirmation data is showed in Fig 5.32.

A



B



C

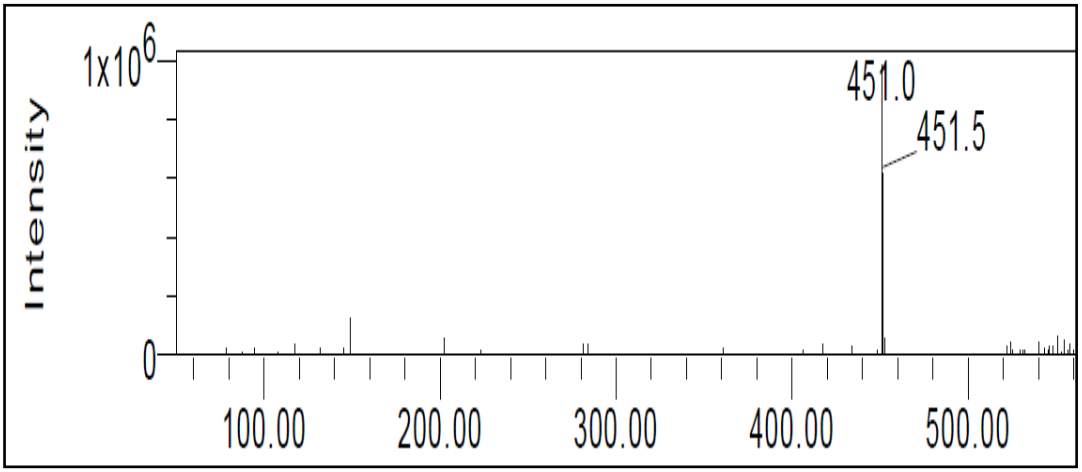


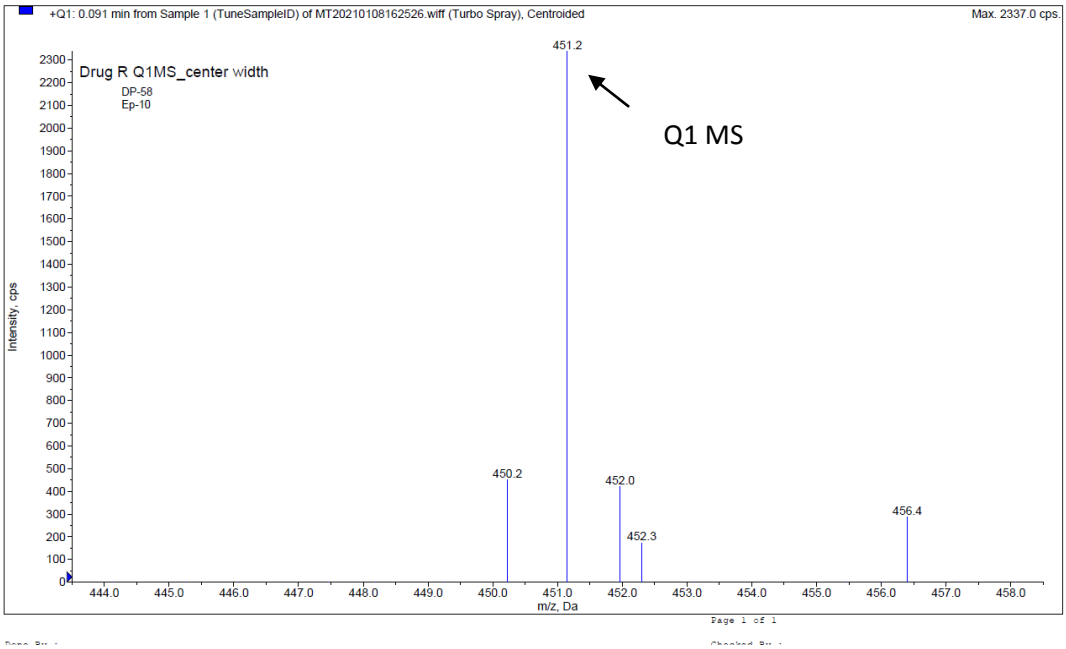
Fig. 5.32 DP confirmation by A) RP-HPLC B) UPLC chromatogram and C) ESI/MS spectrum

The isolated DP was confirmed by HPLC, UPLC and ESI-MS spectrum. Isolated DP had purity >98.0 %. The ESI spectrum showed ionization of DP with *m/z* 451.0. The isolated DP was characterized by MS/MS.

Characterization of DP-A5

The isolated DP was subjected to MS/MS to evaluate the fragmentation pattern for the DP. The DP with *m/z 451.0* is shown in Q1 MS spectrum in Fig 5.34 while Q3 MS spectrum shows the fragmentation in *m/z* 390 which is daughter peak and 251.2 *m/z* which is daughter-daughter peak. MS/MS spectrums are shown in Fig.5.34. The fragmentation pathway is shown in Fig.5.33.

A



B

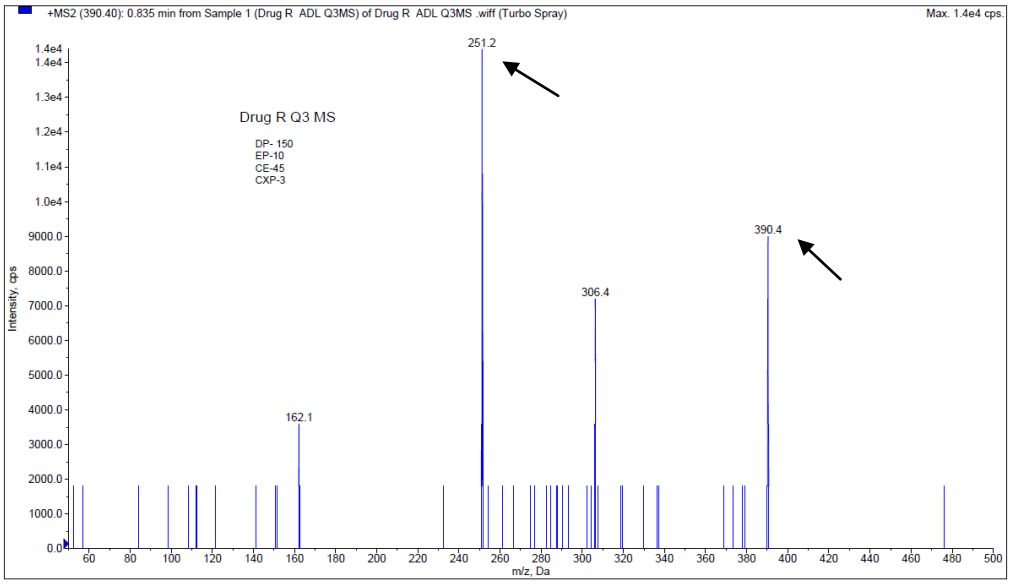


Fig. 5.33 A) Q1 Ms spectrum for DP B) Q3 MS spectrum for acid DP-A5 of rifabutin

Fragmentation pathway is shown in Fig. 5.34.

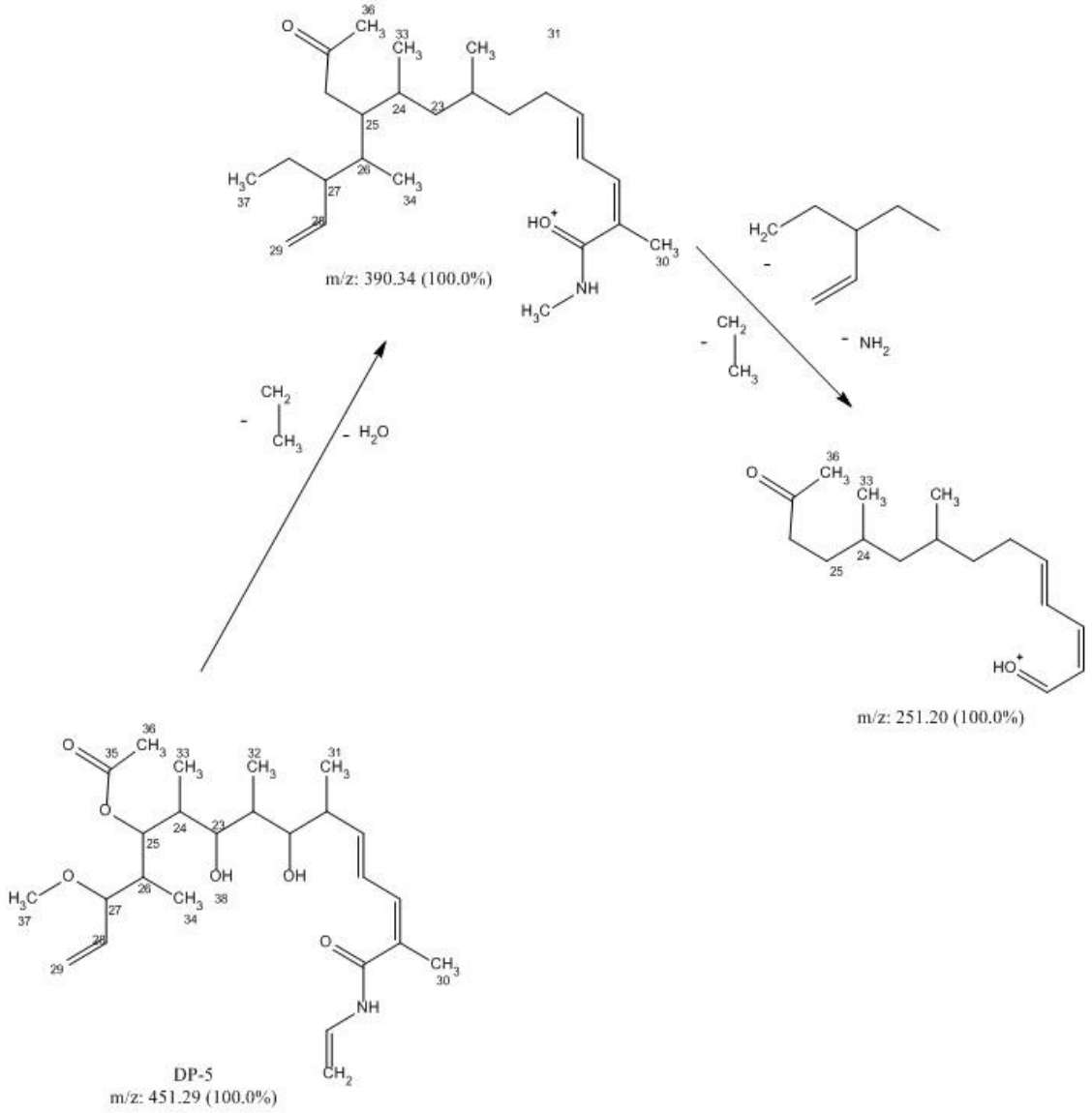
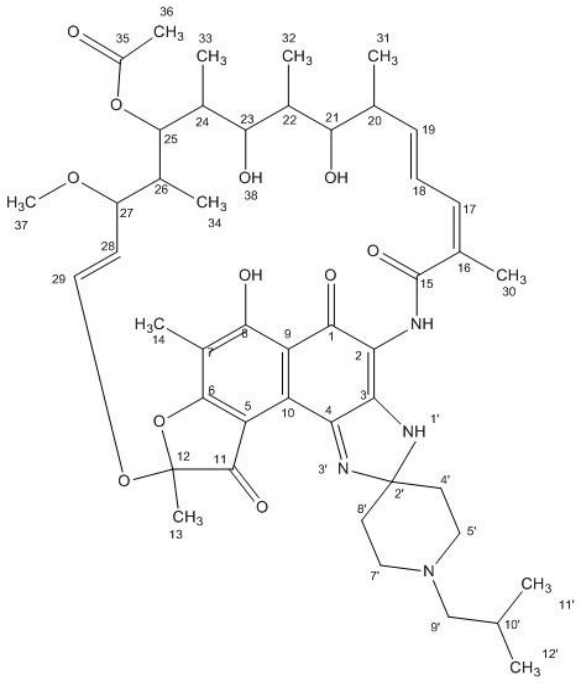


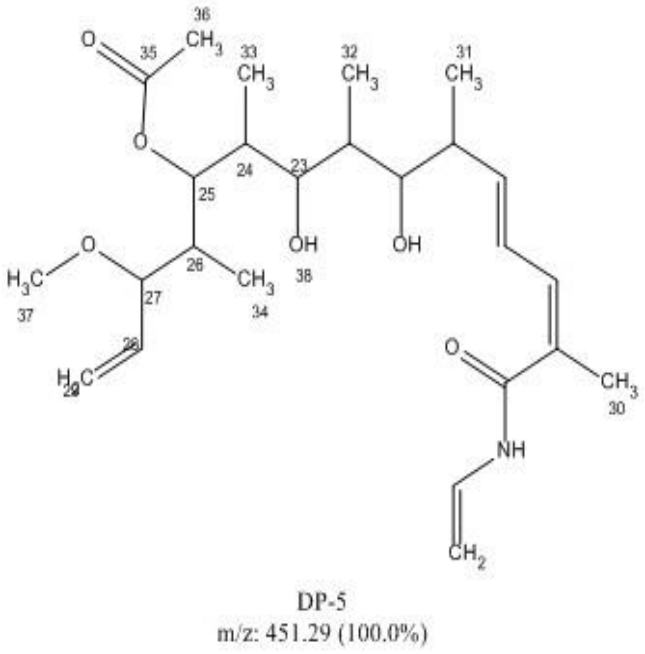
Fig.5.34 Fragmentation pathway for acid DP of rifabutin

Acid major DP-A5 showed *m/z* 451 in Q1 MS spectrum, in Q3 MS spectrum DP fragmented into *m/z 390* and further fragmentation showed in *m/z* 251. The pathway shows that lost of $–C_2H_5$ and $–H_2O$ formed daughter peak (*m/z* 390) and further fragmentation in daughter-daughter peak was due to loss of $–C_2H_5$, $-NH_2$, $-C_7H_{13}$. The rifabutin chemical structure and major DP structure is shown in Fig. 5.35.

A



B



DP-5
m/z: 451.29 (100.0%)

Fig. 5.35 Chemical structure of A) rifabutin and B) DP A5 with atom number

IR analysis of isolated DP

The isolated DP-A5 was confirmed for chemical groups present in it by IR analysis. The IR spectrum for isolated DP is shown in Fig. 5.36.

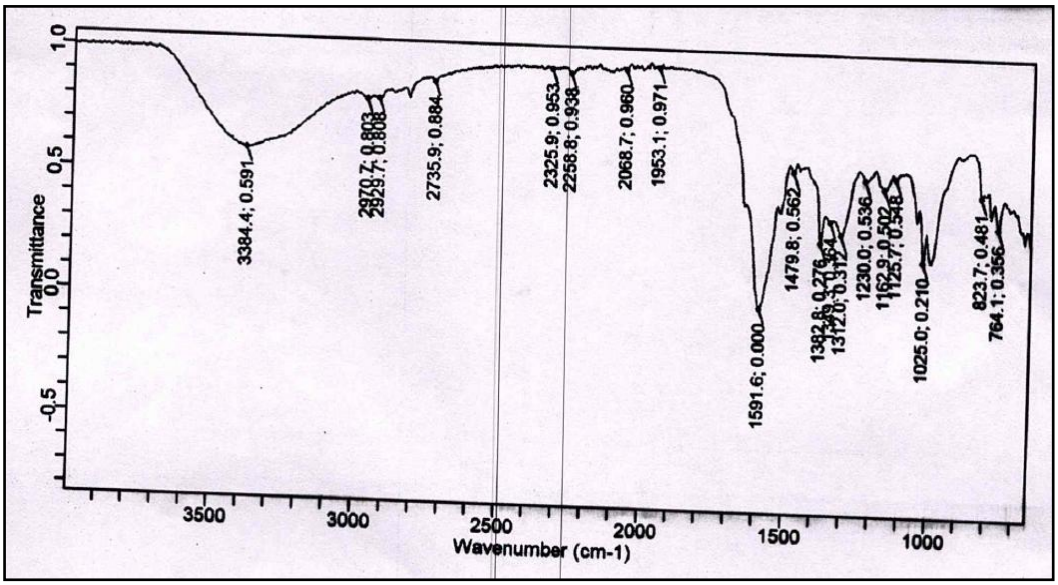


Fig.5.36 IR spectrum for isolated DP A5 of acid of rifabutin

The analysis of IR spectrum shown in Fig.5.36 is shown in table 5.28 with assigned group and wave number.

Assigned groups	Obtained wave number (cm^{-1})	Assigned wave number (cm^{-1})
C-H bending	764.1	780±20
C=C bending	823.7	840-790
C-O stretching	1125.7	1150-1085
C-N stretching	1025.0, 1162.9	1250-1020
C-O stretching	1230.0	1275-1200
O-H bending	1312.0	1390-1310
O-H bending	1349.0,1382.8	1420-1330
N-H bending	1591.6	1650-1580
C-H bending	1953.1	2000-1650
N-C=O stretching	2325.9	2275-2250
O-H stretching	2735.9	3200-2700
N-H stretching	2929.7	3000-2800
O-H stretching	2970.7	3200-2700
O-H stretching	3384.4	3550-3200

Table 5.28 IR spectrum analysis for isolated acid DP A5 of rifabutin

The IR spectrum analysis result, supports the groups supposed to be present in elucidated structure.

PART-E

5.9. IMPURITY PROFILING OF RIFABUTIN

The degradation products were identified and major degradation product was isolated and characterized using sophisticated instruments.

5.9.1 Experimental

5.9.1.1. Chemicals and reagents

The chemicals and reagents used for the stability studies were same as described in section 5.8.1.1.

5.9.1.2. Equipments and chromatographic condition

The equipments and chromatographic conditions used were same as described in section 4.8.1.2.

5.9.1.3. Sample preparations

Sample and buffer preparation for stress degradation studies are same as described in section 5.5.1.3.

5.9.2. Result and Discussion

5.9.2.1. Analysis of rifabutin

Rifabutin UPLC/ESI-MS and proton NMR (for alkaline DP) was completed to study any process related impurity and compare the NMR data for degradation impurity study.

Chromatographic identification

The chromatographic identification was done using chromatographic equipment and chromatographic condition described in section 5.5.1.2. The chromatogram for rifabutin is shown in Fig. 5.37.

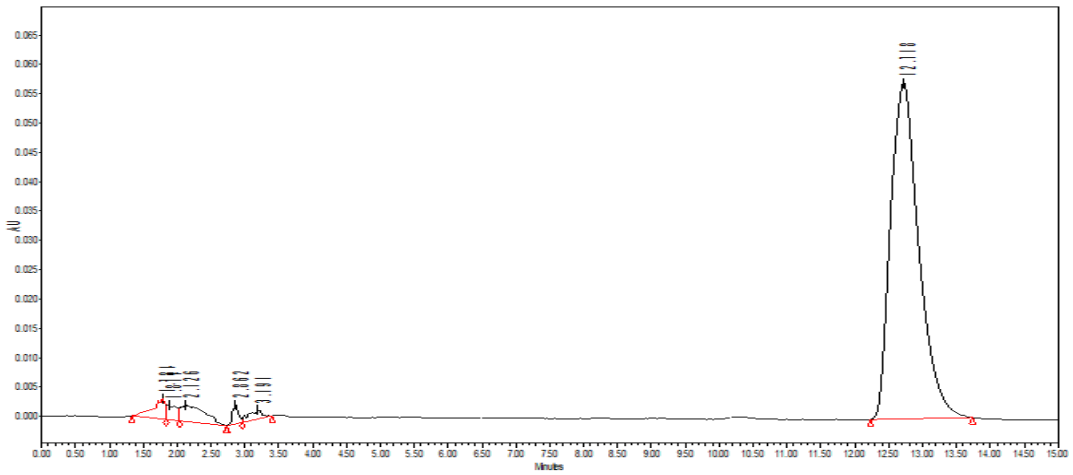


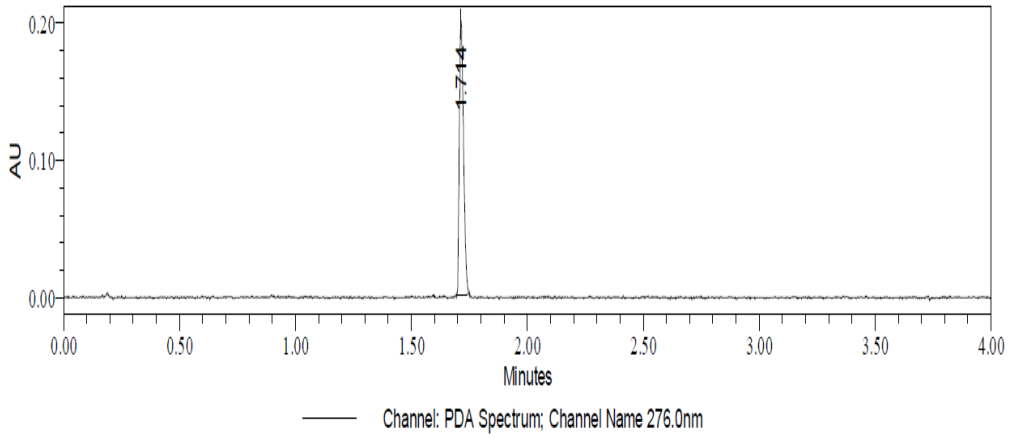
Fig. 5.37 Rifabutin bulk drug chromatogram

Chromatogram shows absence of any additional peak except void volume peaks are indicating absence of any process related impurity. To confirm this interpretation LC-MS data was compared with HPLC chromatogram.

LC/ESI-MS identification

The UPLC/ESI-MS study was carried out to confirm the absence of any process related or inherent impurity in rifabutin. The UPLC chromatogram and ESI-MS spectra is shown in Fig 5.38.

A



B

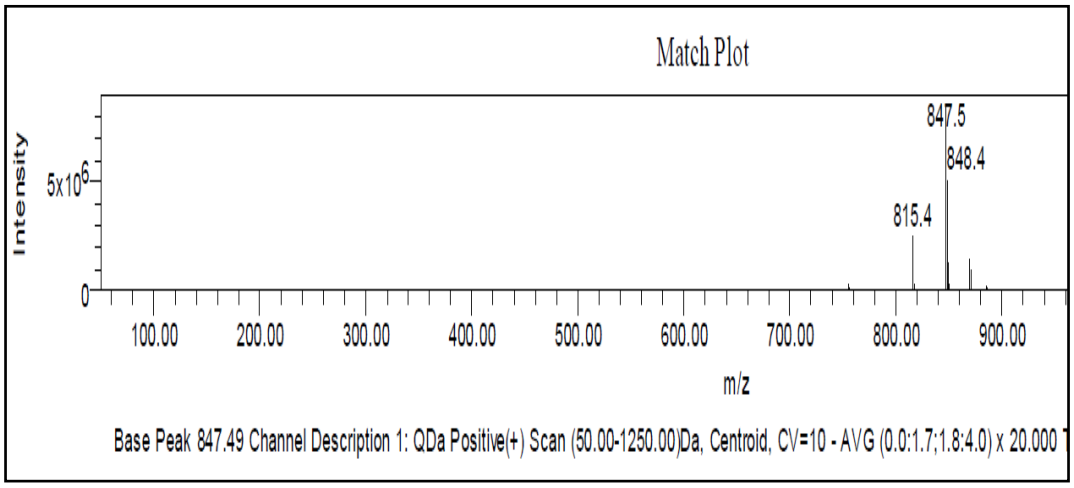


Fig. 5.38 A) UPLC chromatogram B) ESI-MS spectrum of rifabutin

The chromatogram and spectrum for rifabutin showed rifabutin molecular mass 847 amu $[M+H]^+$ as base peak indicates that no process related impurity was present in rifabutin. The ionization of rifabutin in MS process is shown in Fig 5.39.

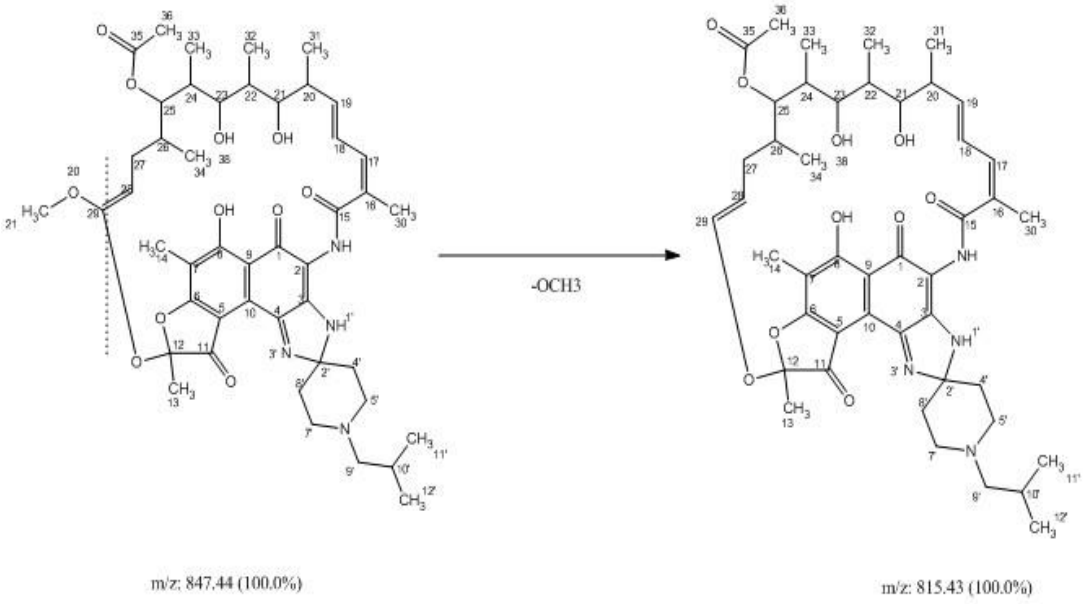


Fig.5.39 MS ionization of rifabutin

NMR identification of rifabutin bulk drug

The proton NMR spectrum was generated for rifabutin in deuterated DMSO using TMS as standard. The proton NMR spectrum for rifabutin is showed in Fig. 5.40 and the assignment of NMR is shown in Table 5.29.

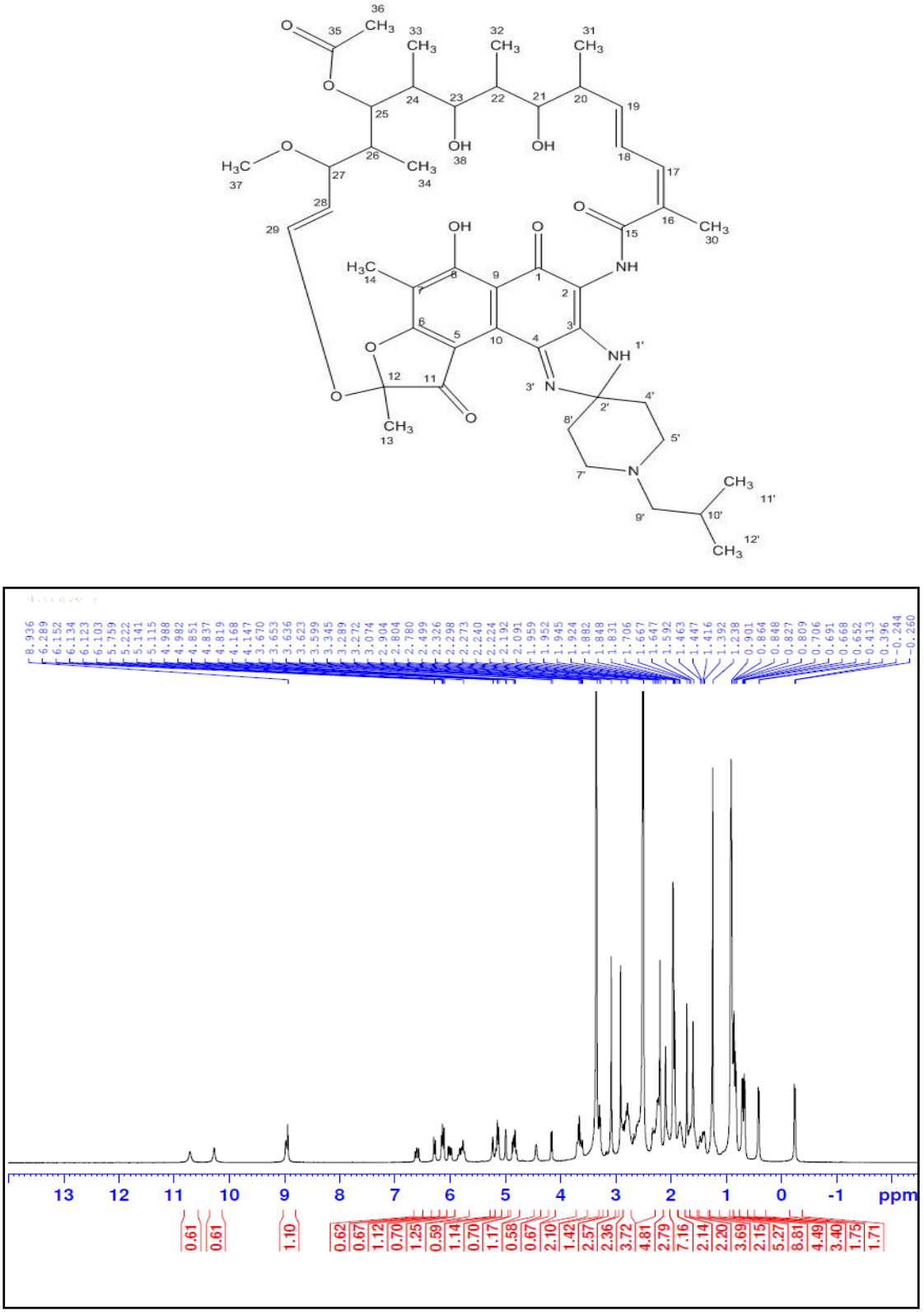


Fig. 5.40 Proton NMR of rifabutin bulk drug

The analysis of proton NMR for rifabutin bulk drug is shown in Table 5.29.

Atom No.	*Proton NMR*	*Multi.*
1	-	-
2	-	-
3	-	-
4	-	-
5	-	-
6	-	-
7	-	-
8	-	-
9	-	-
10	-	-
11	-	-
12	-	-
13	1.706	*s*
14	2.224	*s*
15	-	-
16	-	-
17	6.262	*dd*
18	6.289	*dd*
19	6.025	*dd*
20	2.326	*m*
21	4.168	*dd*
22	1.7-1.8	*m*
23	3.345	*ddd*
24	1.463	*ddd*
25	4.147	*dd*
26	1.7-1.8	*m*
27	3.694	*ddd*
28	4.863	*ddd*
29	5.152	*ddd*
30	2.192	*d*
31	0.809	*d*
32	0.848	*d*

33	0.691	*d*
34	-0.244	*d*
35	-	-
36	2.091	*s*
37	3.074	*s*
2'	-	-
4'	1.959	*m*
5'	2.78	*m*
7'	2.24	*m*
8'	2.0	*m*
9'	2.804	*d*
10'	1.882	*m*
11'	0.901	*d*
12'	0.901	*d*
8 OH	16.17	*s*
21 OH	4.433	*br s*
23 OH	4.168	*d*
NH	10.225	*d*
NH'	10.705	*d*

Table 5.29 Proton NMR analysis of rifabutin bulk drug

The proton NMR confirmed the number of protons present in rifabutin chemical structure, this spectrum data was used for the analysis of DPs. The rifabutin bulk drug NMR spectrum and DP NMR spectrum was compared and analyzed for the changes observed in spectrum which indicated the changes in proton occurred during degradation of rifabutin.

5.9.2.2. Alkali degradation product

For alkali degradation, the stress degradation condition and chromatogram is shown in section 5.5.2.3 (Fig. 5.5(D)). The degradation products formed were identified and major degradation product (in terms of area in chromatogram) was isolated and characterized using LC/ESI-MS and NMR.

Degradation behavior of rifabutin in acid media

Rifabutin is highly prone to degradation in alkaline media. For stress degradation study 0.1N NaOH was used as a stressor and study was competed at RT for 60minutes. The area

occupied by each peak in chromatogram was used to calculate the % degradation of rifabutin. The summary of area, %area and calculated degradation of rifabutin is shown in Table 5.30.

	Name	Retention Time	Area	% DP and bulk drug
1	Mix.DP-1to5	2.310	161577	9.29
2	Mix.DP-1to5	2.815	38818	2.23
3	Mix.DP-1to5	2.979	41273	2.37
4	Mix.DP-1to5	3.147	52158	3.76
5	DP-6	6.479	7638	0.44
6	DP-6	6.925	1192968	68.60
7	DP-7	10.546	26144	2.15
8	API	13.117	205653	11.16

Table 5.30 Rifabutin degradation behavior in alkaline medium

Identification of degradation impurities

The identification of DPs was carried out using UPLC/ESI-MS spectrum, $[M+H]^+$ values showed the *m/z* ion of DP. The UPLC chromatogram for mixture of alkaline DPs is shown in Fig 5.41.

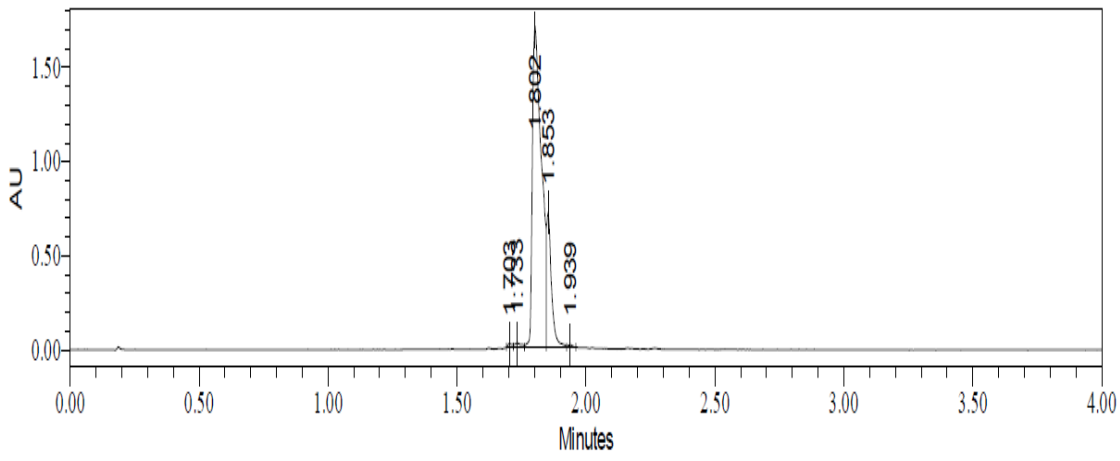
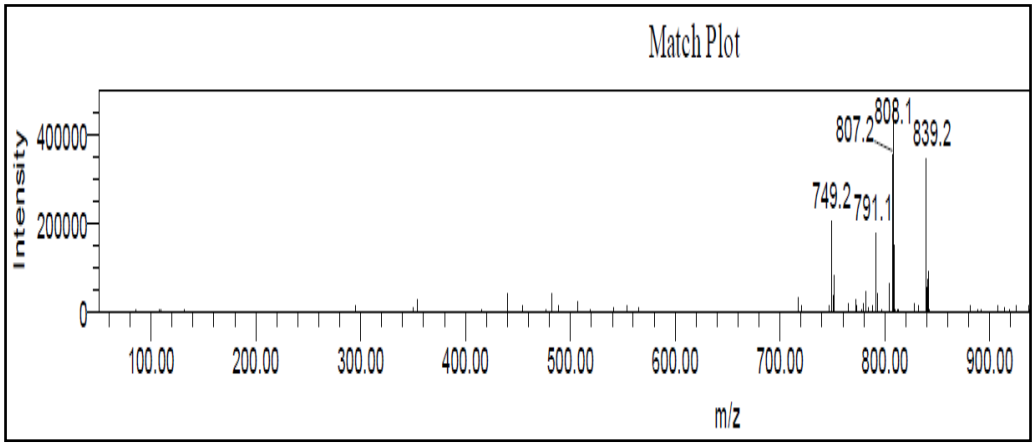


Fig. 5.41 UPLC chromatogram for mixture of alkaline degradation products

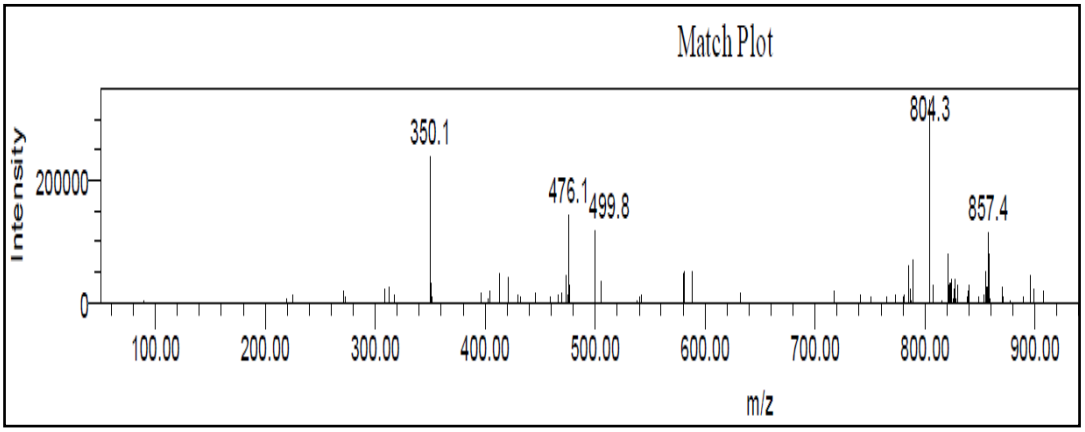
The chromatogram showed one major peak and other minor peaks. The ESI/MS spectrum was obtained for this chromatogram showed that at 1.20 to 1.73 minutes, co-elution of DPs were observed while at 1.80 to 1.85 minutes major DP was eluted and at 1.93minutes co-

elution of major DP and one minor DP was observed. The identification and characterization of major DP was completed by structure elucidation. It can be seen from chromatogram that other DPs were quantitatively not in sufficient % to identify and report the DPs as per ICH guidelines. The DPs were named as per the *m/z* ion in ascending order. The MS scan of chromatogram showed elution at 1.72, 1.75, 1.82, 1.88 and 1.97 minutes for *m/z ion* of 749.2, 791.1, 808.1 and 839.2 (Co-elution), 350.1, 476.1, 499.8, 857.4 (Co-elution), 805.1 (single peak), 805.1 (Single peak) and 805.1 and 820.1 (Co-elution), respectively. The DPs are named as 350.1 (DP-AL1), 476.1 (DP-AL2), 499.8 (DP-AL3), 749.2 (DP-AL4), 791.1 (DP-AL5), 805.1 (DP-AL6), 808.1 (DP-AL7), 820.1 (DP-AL8), 839.2 (DP-AL9) and 857.4 (DP-AL10). Here 'AL' stands for alkaline medium. The major DP was $[M+H]^+$ ion of *m/z* 805.1 (DP-AL6), it was isolated and characterized while DP-AL7 (808.1) was identified by proposing the structure; while other DPs were reported by chromatogram. The ESI/MS spectrum for minor DPs are shown in Fig 5.42.

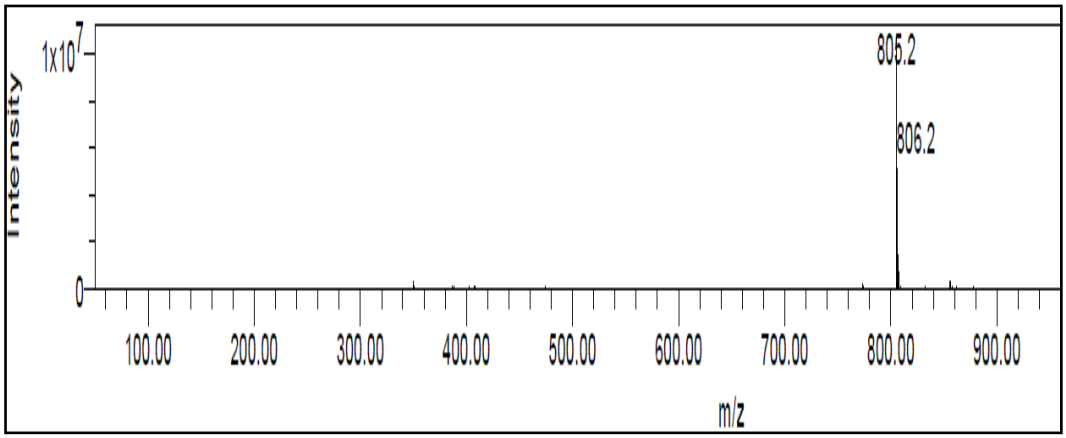
A



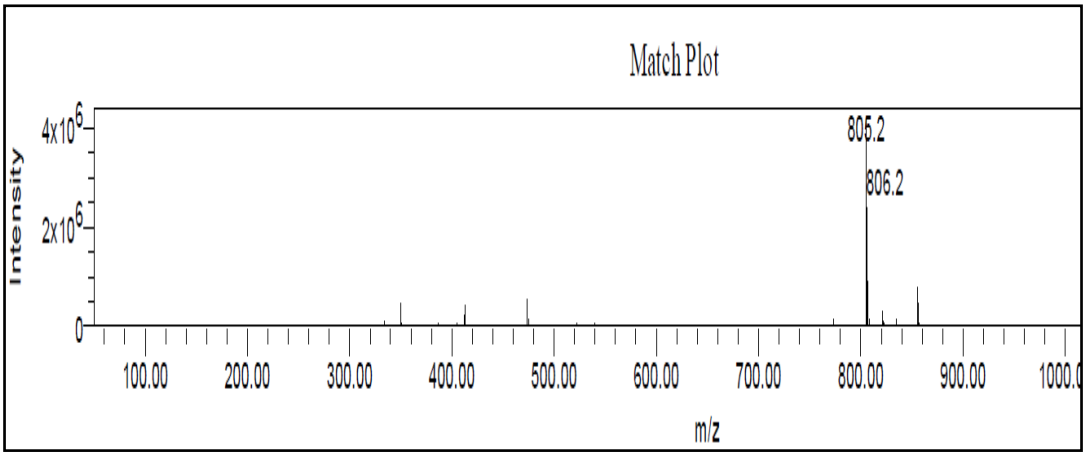
B



C



D



E

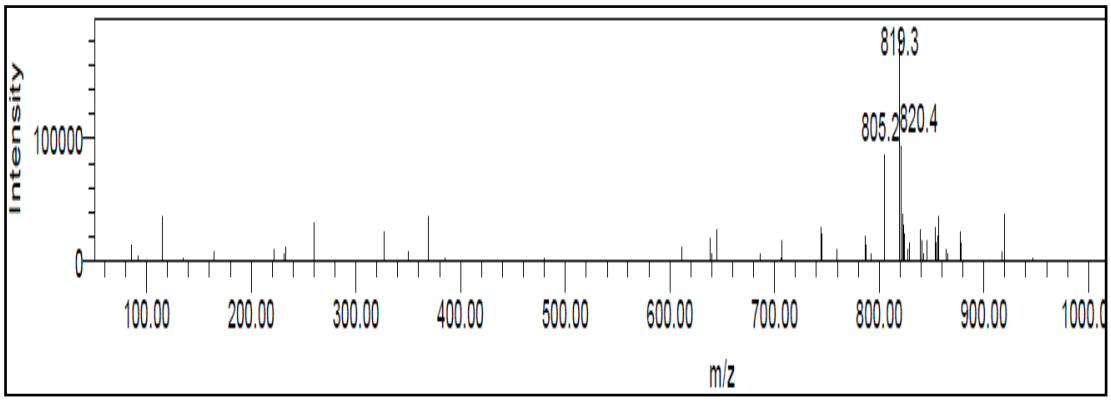


Fig. 5.42 ESI/MS spectrum of rifabutin DPs in alkaline medium in order of elution

The DPs eluted in < 0.5% was reported in UPLC and ESI/MS spectrum while DP eluted > 0.5% was identified by proposing the probable structure using *m/z* ion of mass spectrum.

Minor DPs

The HPLC chromatogram shown in Fig 5.5 showed that mixtures of DPs were eluted in 1.9 to 3.1 minutes. The UPLC chromatogram showed that at 1.70 and 1.72 minutes cluster of DPs were eluted. The ESI/MS spectrum showed *m/z* ion for mixture of DPs eluted at 1.70 and 1.72 minutes. The DPs 350.1 (DP-AL1), 476.1 (DP-AL2), 499.8 (DP-AL3), 749.2 (DP-AL4), 791.1 (DP-AL5), 839.2 (DP-AL9) and 857.4 (DP-AL10) were minor DPs and reported in above chromatograms and spectrums. The DPs eluted in very minute quantity and in co-elution with other DPs; therefore structure was not proposed for above DPs.

DP-AL6

The DP-AL6 has *m/z* ion of $[M+H]^+$ 805.1 amu; the DP was eluted in major quantity with peak purity in chromatogram shown in Fig 5.5, UPLC chromatogram (Fig 5.30) and ESI/MS (Fig 5.31) spectrum. The DP-AL6 has 42amu less than rifabutin bulk drug amu 847.1 indicates removal of groups. The complicated structure of rifabutin needs structure confirmation by sophisticated instrument as *m/z* was not sufficient to elucidate the structure. The further confirmation was taken by NMR (^{1}H proton, ^{13}C NMR and APT) and IR analysis.

DP-AL7 and DP-AL8

DP-AL7 (808.1 *m/z*) and DP-AL8 (819.2 *m/z*) were eluted in 3.86% and 0.76% respectively. The DPs are supposed to be congeners of DP-6. The analysis of ESI-MS data is shown in Table 5.31.

DP No	*m/z*	Rt	Chemical formula
alkaline degradation products			
DP-6	805.2 (*806.0)	1.72	$C_{45}H_{63}N_3O_{10}$
DP-7	808.1 (*809.0)	1.82	$C_{45}H_{66}N_3O_{10}^+$
DP-8	819.3 (*820.0)	1.88	$C_{45}H_{63}N_4O_{10}^+$
API	846.1 (*847.0)	1.97	$C_{46}H_{62}N_4O_{11}$

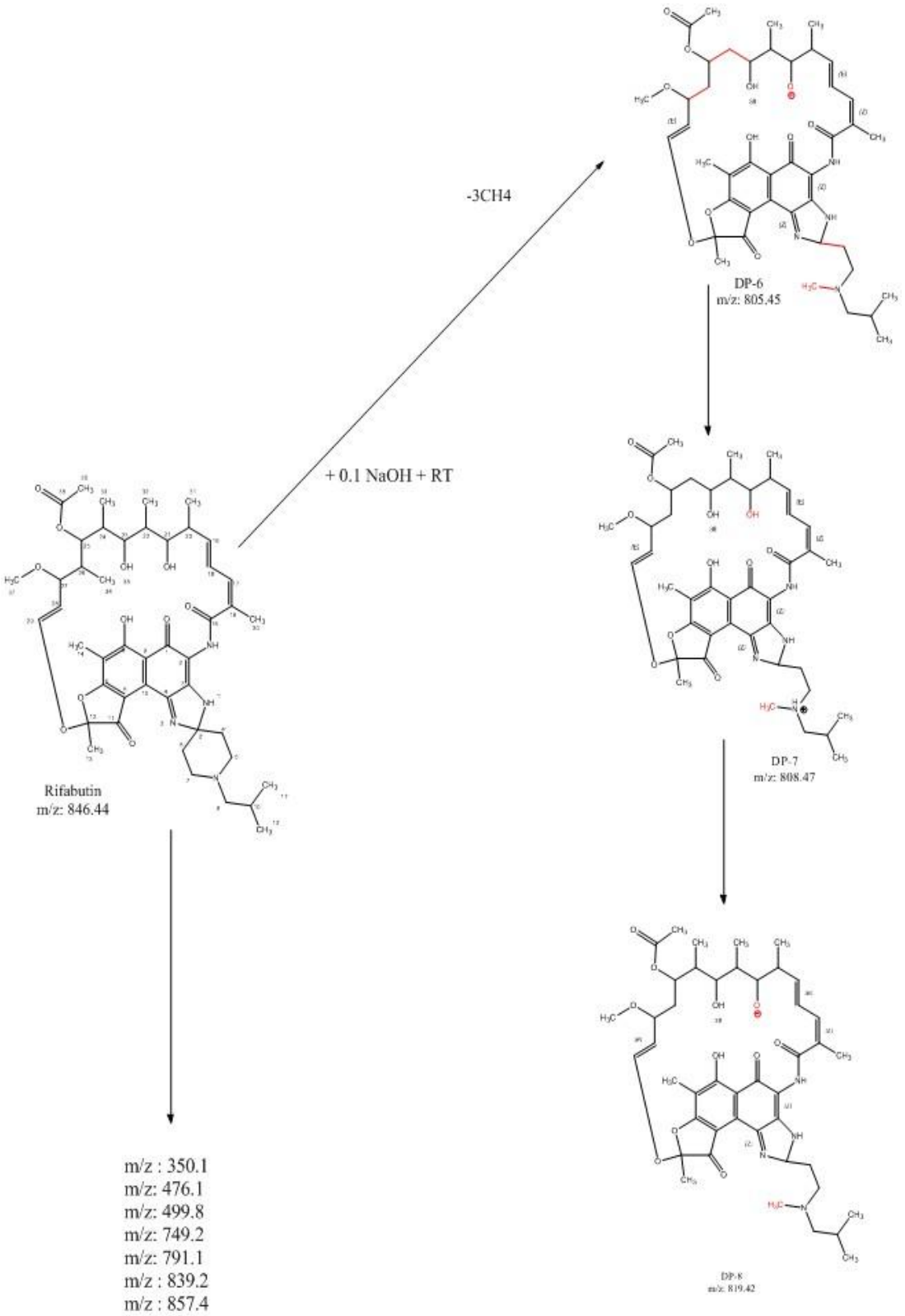
*****Exact molecular mass

Table 5.31 LC/ESI-MS data for alkaline degradation products of rifabutin

Mechanism for formation of DPs

Rifabutin was treated with 0.1N NaOH at room temperature for 1hr; after 1hr the % of rifabutin in sample was 11%. The major DP covered majority of area in chromatogram was DP-AL6. The mechanism for formation of major DP can be explained as de-methylation by alkali. In alkaline condition β-elimination reactions are common for degradation which was occurred with rifabutin degradation. The methyl groups in β-position were eliminated while H^+ ion conjugated with O^- atom to form –OH group was also eliminated. In DP-AL7 –OH group was intact and additional H^+ ion was attached to N atom attached with 7' position. The DP-AL8 can be precursor of DP-AL6 as $–CH_3$ (β-position 34) and –OH molecule was intact in structure.

The proposed degradation pathway is shown in Scheme-1.



Scheme-1.Proposed degradation pathway for rifabutin in alkaline media

5.9.2.3. Acid degradation product

For acid degradation of rifabutin, stress degradation conditions and chromatogram is shown in section 5.5.2.3 (Fig. 5.5 (C)). Rifabutin formed fractions of DPs which were identified by ESI/MS.

Degradation behavior of rifabutin in acid media

The Rifabutin showed several DPs in HPLC chromatogram, the DP and rifabutin bulk drug behavior in HPLC chromatogram is shown in Table 5.32.

	Name	Retention Time	Purity1 Angle	Purity1 Threshold	Area	% Area
1	A1	4.308	2.360	2.549	13922	1.19
2	A2	4.586	2.362	2.545	16199	1.38
3	A3	5.688	2.889	3.262	15022	1.28
4	A4	6.290	1.226	1.512	36195	3.09
5	A5	7.593	4.368	4.951	6481	0.55
6	A6	7.889	3.116	3.403	14545	1.24
7	A7	8.509	2.090	2.303	30574	2.61
8	API	10.278	0.157	0.278	995536	85.04
9	A8	11.146	2.147	2.318	25428	2.17
10	A9	22.612	3.746	4.111	16827	1.44

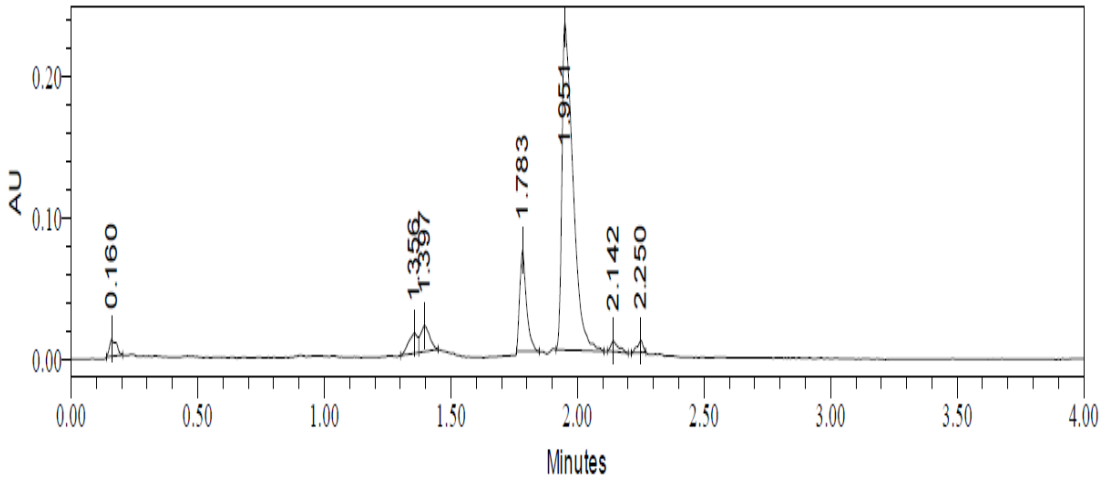
Table 5.32 Rifabutin degradation behavior in acidic medium

As seen in Table 5.32, several DPs are formed in acid medium; the major DP was identified and characterized while other minor DPs were reported.

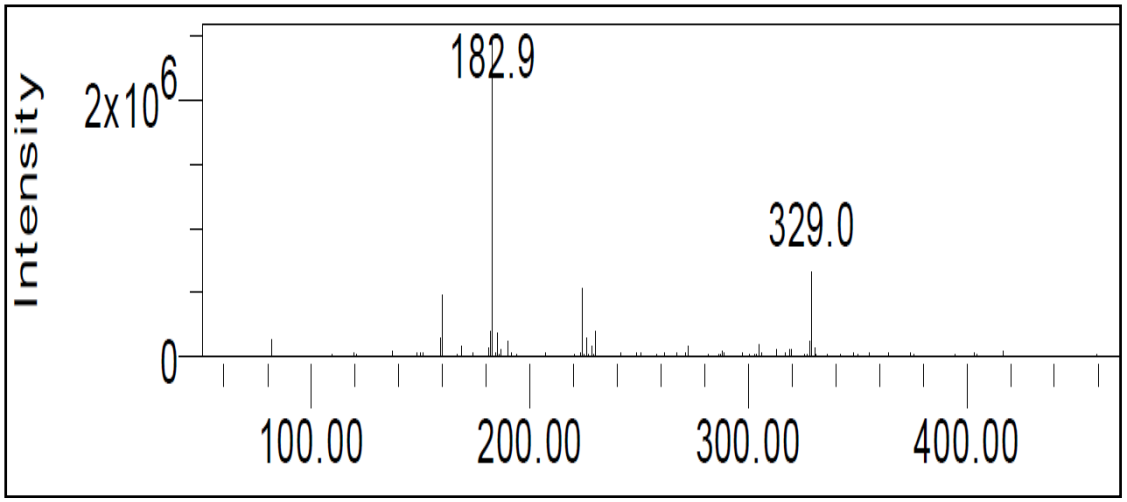
Identification of degradation impurities

Identification of DPs were completed by ESI/MS for rifabutin in acid medium, the DPs at Rt 1.35 are reported while structures for other DPs were evaluated for identification of DPs. The UPLC chromatogram for rifabutin in acid medium is shown in Fig. 5.43 (A) and ESI/MS spectrum for DPs in order of Rt is shown in Fig. 5.42 (B) to (H).

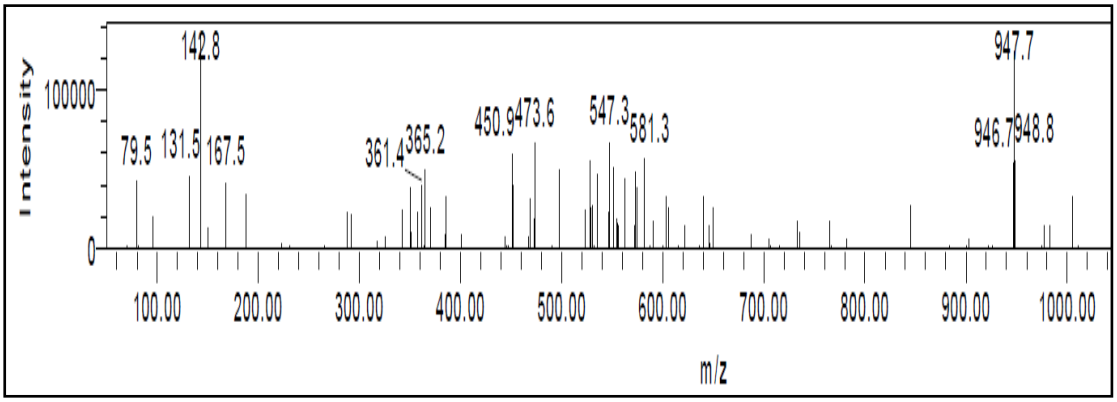
A


AU
0.20
0.10
0.00
0.160
1.356
1.397
1.783
1.951
2.142
2.250
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
Minutes

B


Intensity
2x10^6
0
182.9
329.0
100.00
200.00
300.00
400.00

C


Intensity
100000
0
142.8
947.7
79.5
131.5
167.5
361.4
365.2
450.9
473.6
547.3
581.3
946.7
948.8
100.00
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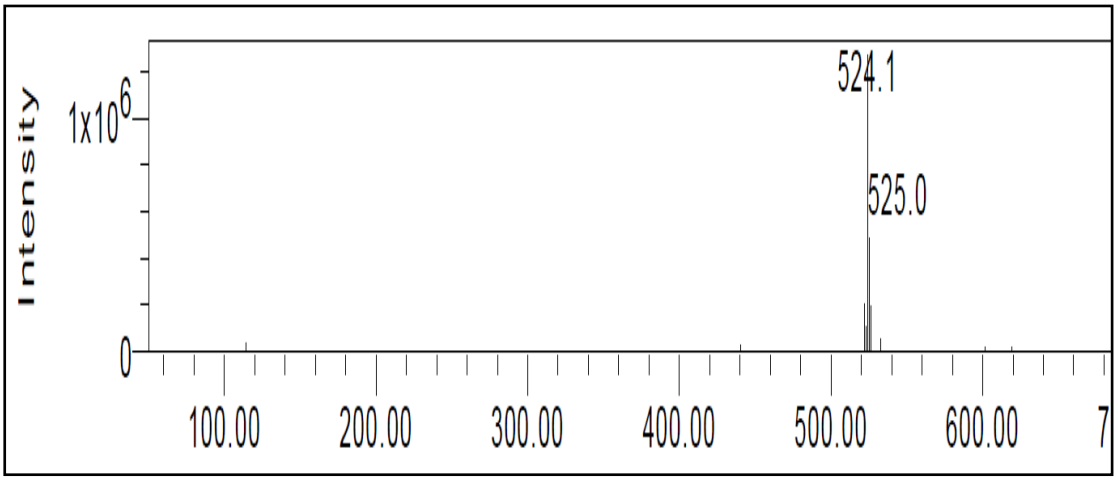
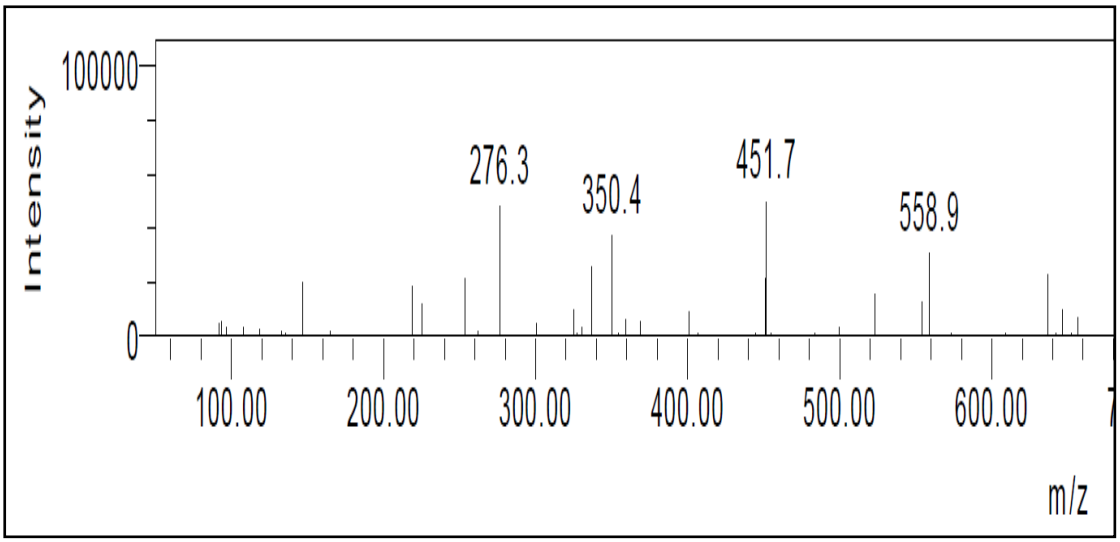
300.00
400.00
500.00
600.00
700.00
800.00
900.00
1000.00
m/z

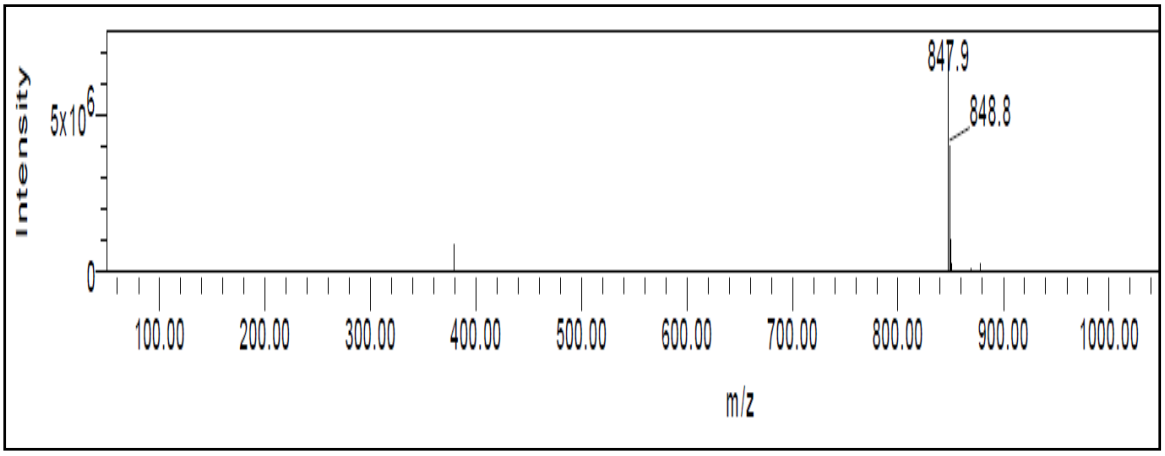
D


Intensity
1x10^6
0
524.1
525.0
100.00
200.00
300.00
400.00
500.00
600.00

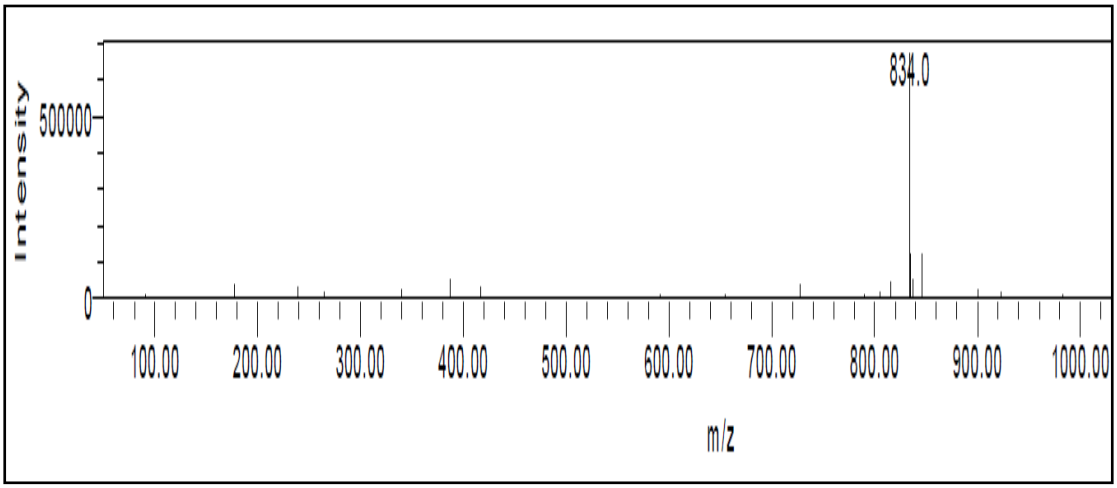
E


Intensity
100000
0
276.3
350.4
451.7
558.9
100.00
200.00
300.00
400.00
500.00
600.00
m/z

F


Intensity
5x10^6
0
847.9
848.8
100.00
200.00
300.00
400.00
500.00
600.00
700.00
800.00
900.00
1000.00
m/z

G



H

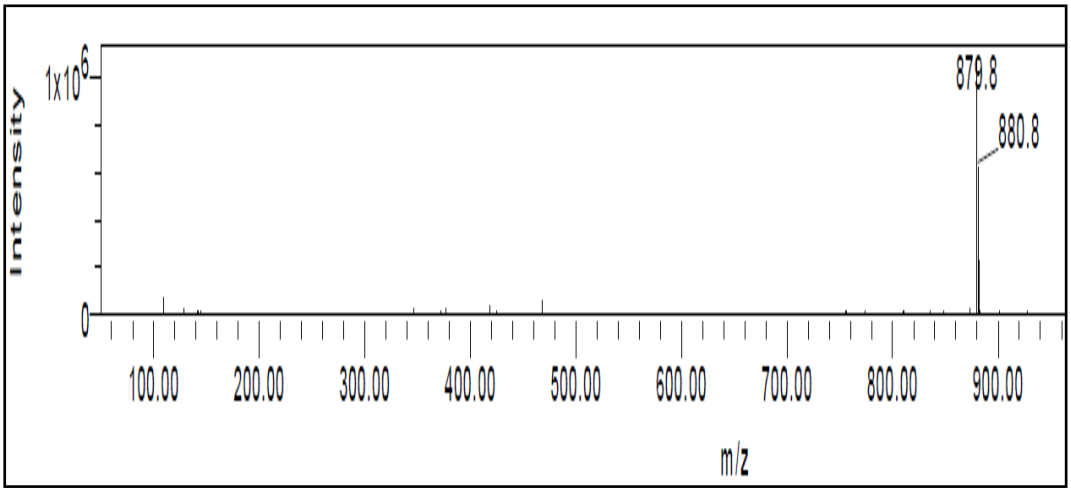
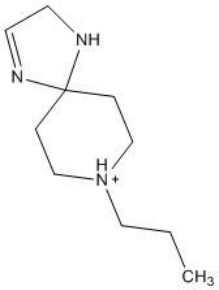
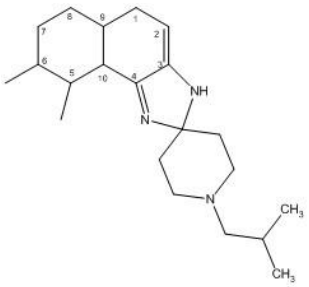
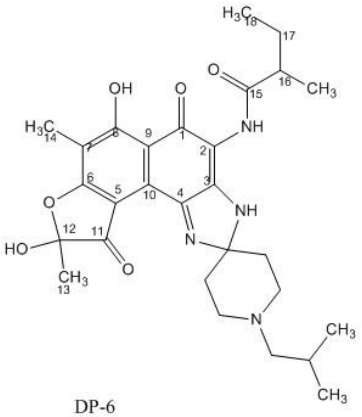
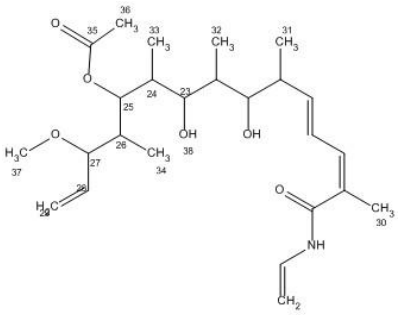
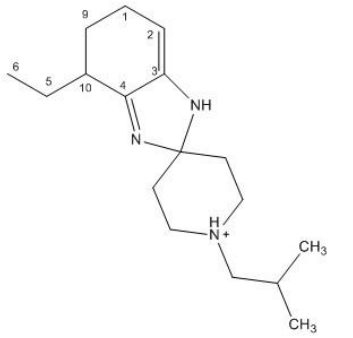
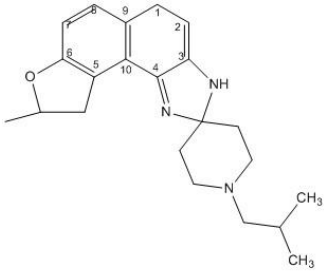
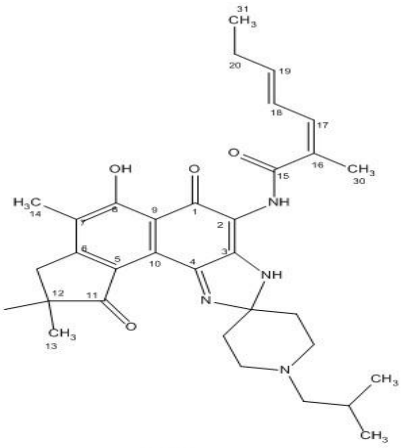


Fig. 5.43 A) UPLC chromatogram of mixture of DPs in acidic medium B) ESI/MS spectrum DPs at Rt 0.16 C) DPs at Rt 1.35 D) DPs at Rt 1.39 E) DPs at Rt 1.7 F) rifabutin bulk drug at Rt 1.9 G) DP at Rt 2.14 H) DP at Rt 2.25

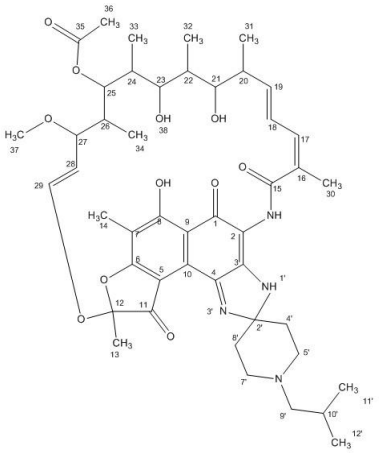
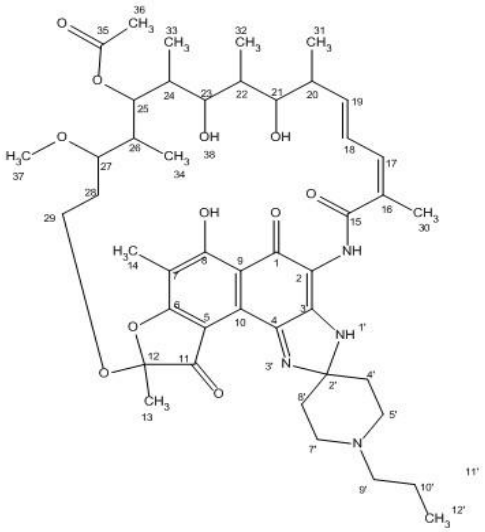
Total 20 DPs were reported for acid condition of rifabutin among which 9 DPs were identified for structure elucidation while DPs at Rt 1.35minutes were not identified for structure elucidation. (Table 5. 33)

Rt (min.)	No. of DPs	*m/z* (assigned DP num)	Chemical structure	Chemical formula
0.160	2	182.9 (DP-1) 329.0 (DP-3)	 DP-1 m/z: 182.17 (100.0%)  DP-3 m/z: 329.28 (100.0%)	$C_{10}H_{20}N_3$ $C_{21}H_{35}N_3$
1.35	11	79.5 131.5 142.1 167.5 351.4 355.2 450.5 473.6 547.3 581.3 947	-	-

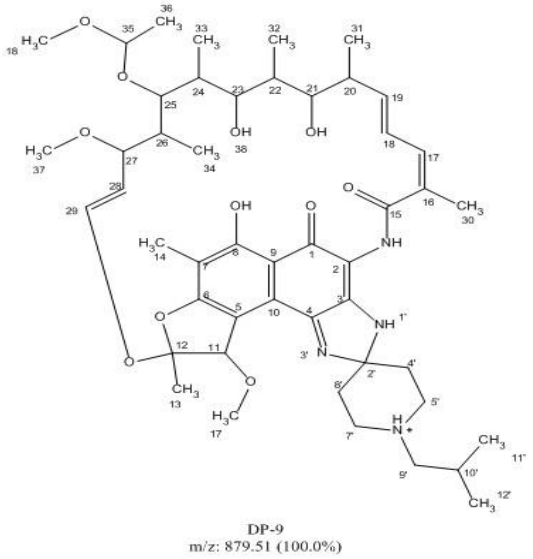
1.39	1	524.1 (DP-6)	 DP-6 m/z: 524.26 (100.0%)	$C_{28}H_{36}N_4O_6$
1.78	4	276.3 (DP-2) 350.4 (DP-4) 451.7 (DP-5) 558.9 (DP-7)	 DP-5 m/z: 451.29 (100.0%)  m/z: 276.24 (100.0%), 277.25 (18.4%), 278.25 (1.6%), 277.24 (1.1%)  DP-4 m/z: 351.23 (100.0%)	$C_{17}H_{30}N_3$ (DP-2) $C_{22}H_{29}N_3O$ (DP-4) $C_{25}H_{41}NO_6$ (DP-5) $C_{33}H_{42}N_4O_4$ (DP-7)



DP-7
m/z: 558.32 (100.0%)

1.95	API	847.9 (API)		$C_{46}H_{62}N_4O_{11}$
2.14	1	834.0 (DP-8)		$C_{45}H_{62}N_4O_{11}$

DP-8
m/z: 834.44 (100.0%)

2.25	1	879.8 (DP-9)		$C_{48}H_{71}N_4O_{11}$

DP-9
m/z: 879.51 (100.0%)

Table 5.33 LC/ESI-MS data for acidic degradation products of rifabutin

The DPs are numbered in ascending order of *m/z* obtained in ESI/MS spectrum, Rt/DP (DP no.); 0.160/182.9 (DP-A1), 329.0 (DP-A3), 1.78/276.3 (DP-A2), 350.4(DP-A4), 451.7 (DP-A5), 1.39/524.1 (DP-A6), 1.78/558.9 (DP-A7), 2.14/834.0 (DP-A8) and 2.25/879.8 (DP-A9). Here 'A' stands for acid media. The DPs are discussed in detail.

DP-A1 (*m/z 182.9)*

The DP-A1 can be chemically named as 8-propyl-1,4,8-triazaspiro[4.5]dec-1-en-8-ium and chemical formula is $C_{10}H_{20}N_3$. Source for DP-A1 is DP-A4 and DP-A1 is formed by loss of $C_{10}H_{17}O$ group from DP-A4 with *m/z* 182.1.

DP-A2 (*m/z 276.3)*

DP-A2 can be chemically named as 4-ethyl-1'-isobutyl-1,4,5,6-tetrahydrospiro[benzo[*d*]imidazole-2-4'-piperdin]-1'-ium and chemical formula is $C_{17}H_{30}N_3$. Source of formation of DP-A2 is DP-A4; the formation was occurred by loss of C_5H_9O with *m/z* 276.3.

DP-A3 (*m/z 329.0)*

DP-A3 can be chemically named as 1'-isobutyl-8,9-dimethyl-3,5,5a,6,7,8,9,9a-octahydrospiro[napthol[1,2-*d*]imidazol-2,4'-piperdine and chemical formula is $C_{21}H_{35}N_3$. Source of formation of DP-A3 is DP-A4; the DP-A3 was formed by loss of C_3H_8O with *m/z* 329.0.

DP-A4 ***(m/z 350.4)***

The chemical name for DP-A4 can be 1'-isobutyl-9-methyl-3,5,9,10-tetrahydrospiro[furo[2',3':7,8]napthol[1,2-*d*]imidazol-2,4'-piperdine and chemical formula is $C_{22}H_{29}N_3O$. The DP-A4 was formed from rifabutin bulk drug and formation was result of loss of $C_{23}H_{39}NO_6$.

DP-A5 ***(m/z 451.7)***

The chemical name forDP-A5 can be (11*E*,13*Z*)-7,9-dihydroxy-3-methoxy-4,6,8,10-pentamethyl-15-oxo-15-(vinylamino)pentadeca-1,11,13-trien-5-yl-acetate and chemical formula is $C_{25}H_{41}NO_6$. The DP-A5 was formed from rifabutin bulk drug and formation was result of loss of $C_{21}H_{29}N_3O_5$.

DP-A6 (*m/z 524.1)*

The DP-A6 can be chemically named as *N*-(6,9-dihydroxy-1'-isobutyl-7,9-dimethyl-5,10-dioxo-3,5,9,10-tetrahydrospiro[furo[2',3':7,8]napthol[1,2-*d*]imidazol-2,4'-piperdin]-4-yl)-2-methylbutanamide and chemical formula is $C_{28}H_{36}N_4O_6$. The DP-A6 was formed from DP-A7 by loss of C_2H_5 from DP-7.

DP-A7 ***(m/z 558.9)***

DP-A7 can be chemically named as (2*Z*,4*E*)-*N*-(6-hydroxy-1'isobutyl-7,9,9'-trimethyl-5-,10-dioxo-3*H*-spiro[cyclopenta][7,8]napthol[1,2-*d*]imidazoe-2,4'-piperdin]4-yl]-2-methylhepta-2,4-dienamide and chemical formula is $C_{33}H_{42}N_4O_4$. DP-A7 was formed from rifabutin bulk drug by loss of $C_{30}H_{36}N_4O_6$.

DP-A8 ***(m/z 834.0)***

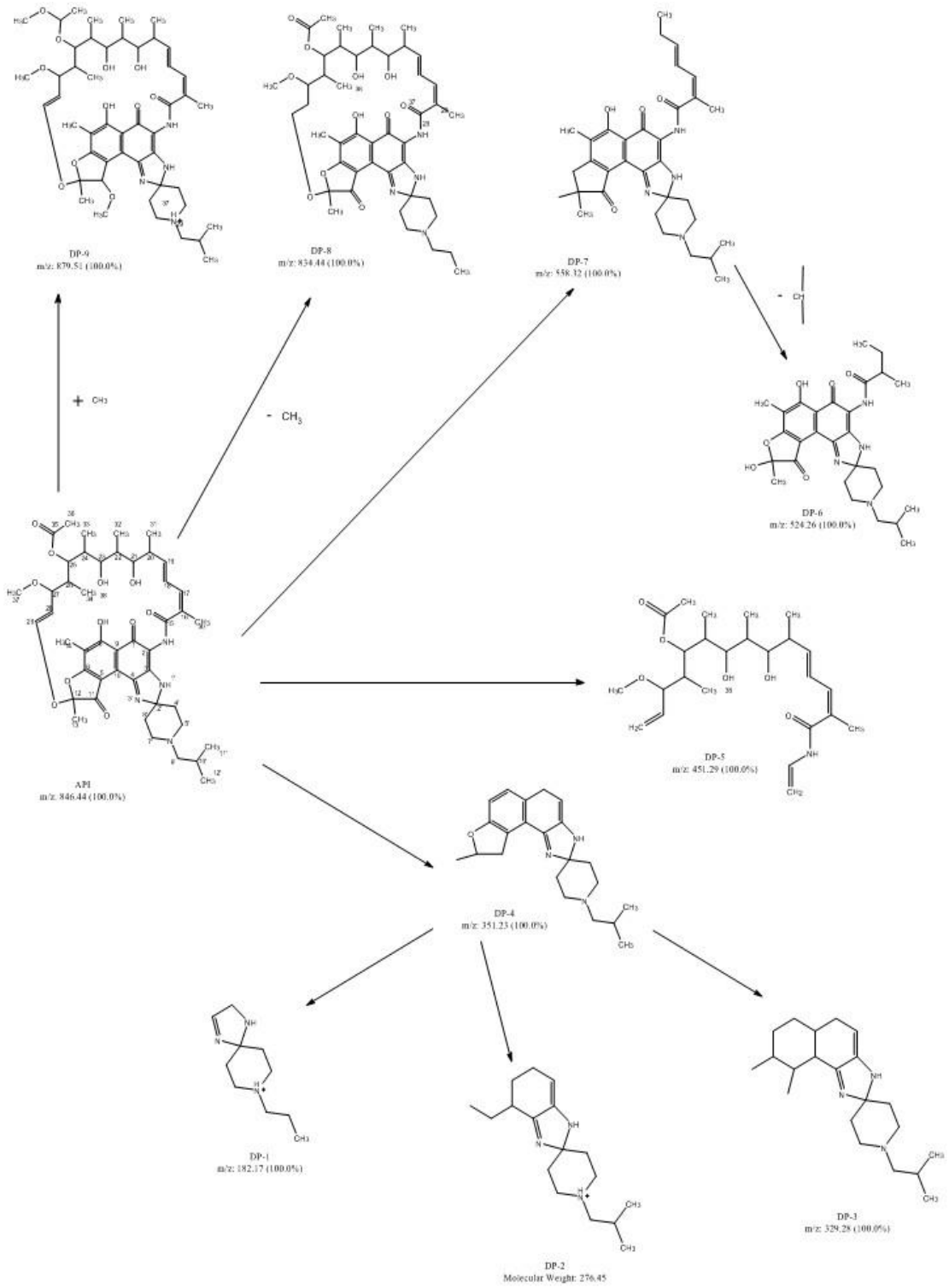
The DP-A8 can be chemically named as (11*E*,13*Z*)-15-((6,9-dihydroxy-7,9-dimethyl-5,10-dioxo-1'-propyl-3,5,9,10-tetrahydrospiro[furo[2',3':7,8]naptho[1,2-*d*]imidazol-2,4'-piperdin]-4-yl)amino)7,9-dihydroxy-3-methoxy-4,6,8,10,14-pentamethyl-15-oxopentadeca-11,13-dien-5-yl-acetate and chemical formula is $C_{45}H_{62}N_4O_{11}$. DP-A8 was formed from rifabutin bulk drug by loss of methyl ($-CH_3$) group.

DP-A9 ***(m/z 879.8)***

DP-A9 can be chemically named as 4-((2*Z*,*4E)*-7,9-dihydroxy-13-methoxy-11-(1-methoxyethoxy)-2,6,8,10,12-pentametylpendadeca-2,4,14-trienamido)-6,9-dihydroxy-1'-isobutyl—10-methoxy-7,9-dimethyl-5-oxo-3,5,9,10-tetrahydrospiro[furo[2',3':7,8]naptho[1,2-*d]*imidazol-2,4'-piperdin]-1'-ium and chemical formula is $C_{48}H_{71}N_4O_{11.}$ DP-A9 was formed from rifabutin bulk drug by addition of methyl group.

Mechanism for formation of DPs

The DPs are formed by removal of attached groups or fractions of rifabutin bulk drug and few DPs are formed from DP also. DP-A1, A2, A3 were formed from DP-A4as a source by removal groups and DP-A6 was formed from source of DP-A7; While other DPs were formed from rifabutin bulk drug. The common mechanism for formation of DPs was demethylation although DP-A9 was formed by attachment of methyl group. The bonds in rifabutin chemical structure were broken to from the fractions (DP-A4,A5,A7). The acid catalyzed reaction formed 9 DPs which were identified and structures are proposed in scheme-2 by showing proposed degradation pathway.



Scheme-2.Proposed degradation pathway for rifabutin in acid media

5.9.2.4. Oxidative degradation product

Degradation behavior of rifabutin in oxidative media

Rifabutin was kept in 30% hydrogen peroxide at room temperature for >3hrs, there was no peak observed other than rifabutin bulk drug peak. Although bulk drug peak intensity was not

much affected indicated that there may be no or very slight degradation occurred. The calculation of bulk drug peak intensity showed 2.0% decrease but absence of DP peak led to conclusion that rifabutin is stable in presence of oxidative agent. The 2.0% decrease is reported in next section. The rifabutin behavior in HPLC chromatogram is shown in Table 5.34.

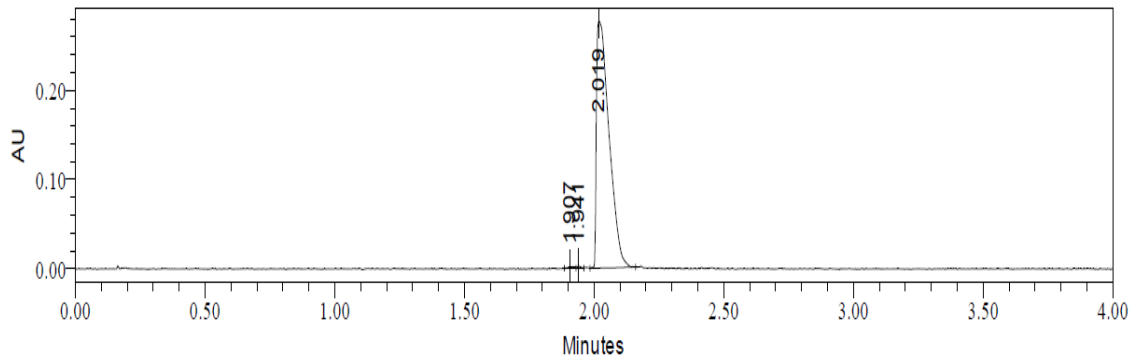
	Name	Retention Time	Purity1 Angle	Purity1 Threshold	Area	% Area
1	Peak-1	2.468	2.672	1.343	12600074	88.92
2	API	12.966	0.078	0.248	1570370	11.08

Table 5.34 Rifabutin behavior in oxidative medium

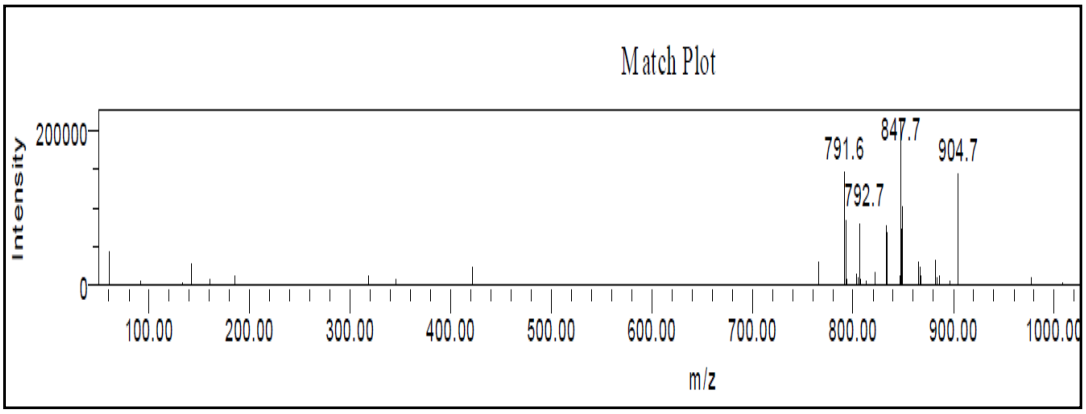
Identification of degradation impurities

The DPs are reported which were formed in oxidative medium, the UPLC chromatogram and ESI/MS spectrum is shown in Fig. 5.44.

A



B



C

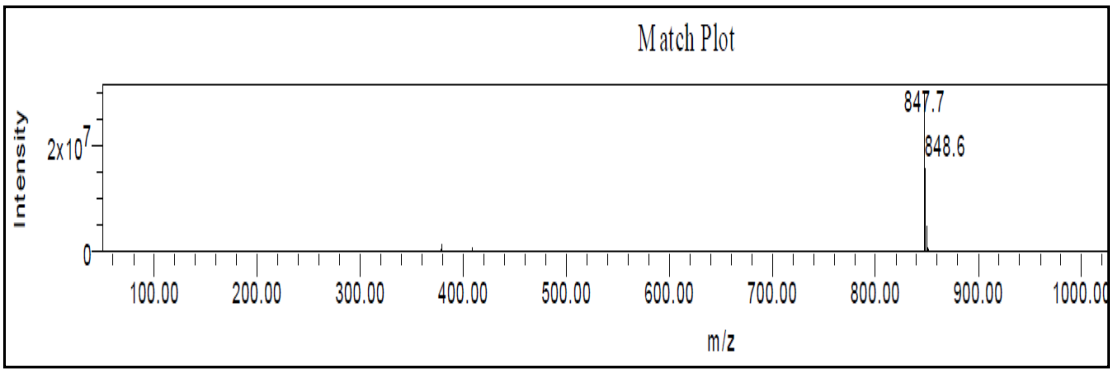


Fig.5.44 A) UPLC chromatogram B) ESI/MS spectrum of oxidative sample DP mixture

As seen in Fig. 5.39 two new *m/z* of $[M+H]^+$ ion are observed but intensity and area covered by these two peaks were <5.0%, therefore they are reported but not identified or characterized. The LC/ESI-MS data are shown in Table 5.35.

Peak	***m/z***	**% Area in UPLC**
Peak-1 (Rt-1.90-1.94)	791.6 847.7 904.7	3.5%
API(Rt-2.05)	847.7	96.50

Table 5.35 ESI/MS data for rifabutin in oxidative medium

Degradation pathway is not generated for oxidative degradation of rifabutin as DPs formed in presence of oxidative agent are covering very less area (<1.0%) in chromatogram.

Conclusion

The identification of rifabutin was done by various tests and sample was confirmed as rifabutin. The kinetic study for rifabutin was completed in acid, alkali and oxidative conditions for degradation. Rifabutin followed first order kinetics in degradation reaction of acid solution, alkaline solution and in presence of oxidative agent. The degradation kinetic parameters were evaluated which suggested that increase in temperature and stressor concentration led to increase in degradation rate of rifabutin. The multi-factorial tool for rifabutin degradation kinetic study was successfully applied to predict the degradation kinetics parameters. The stability study method was developed by using the physicochemical properties data of rifabutin. Stability study was completed by stress degradation of rifabutin in hydrolytic solutions (acid, alkaline and neutral) and oxidative solution, under UV light and in thermal condition to check the stability strength of rifabutin. The rifabutin could not hold the stability in acid and alkaline condition; while in oxidative condition slight degradation was observed. DPs were identified using ESI/MS while major DPs were isolated using preparative HPLC, identified using ESI/MS and characterized by NMR (^{1}H NMR, ^{13}C NMR and APT). In acid media, rifabutin degraded and formed 9 DPs among which the major DP was identified from HPLC and Tandem MS data, the isolation product was not sufficient to produce other data as rifabutin was not degrading further and using harsh condition was resulted in formation of secondary degradation product (further degradation of primary DPs). In alkaline media 15 DPs of rifabutin were reported among which three DPs were identified based on UPLC/ESI-MS data and one major DP was isolated and characterized using sophisticated instruments. There was no process related impurity present in rifabutin. The DPs are reported for the first time in literature.

References

1. Horne D.J., Spitters C., and Narita M., Experience with Rifabutin replacing Rifampin in the treatment of Tuberculosis., *Int J TubercLung Dis.* 2011; 15 (11), 1485.

2. Maddix D.S., Tallian K.B., and Mead P.S., Rifabutin: a review with emphasis on its role in the prevention of disseminated Mycobacterium avium complex infection., *Ann Pharmacother*, 1994; 28 (11), 1250-64.

3. U.S Food and Drug Administration (Internet) , FDA Approved Drugs, Available on:https://www.accessdata.fda.gov/scripts/cder/daf/index.cfm?event=overview.process&ApplNo=050689

4. Central Drugs standard control organization (Internet), Home, Drugs; Available on: https://cdscoonline.gov.in/CDSCO/Drugs

5. Kishore M., Development of new Spectrophotometric methods for the quantitative determination of Rifabutin in Pharmaceutical formulations, *Int J Phar Res Dev.* 2010; 2(10), 49.

6. Bhusari S., Aamer A. I., and Wakte P., Development and validation of UV-Visible spectrophotometric method for estimation of Rifabutin in bulk and formulation. *Int J Pharm Biol Sci* , 2019; 9(1), 1157-62.

7. Lewis R.C., Hatfield N.Z., and Narank P.K., A sensitive method for quantitation of Rifabutin and its desacetyle metabolites in human biological fluids by high performance liquid chromatography (HPLC). *Pharmaceutical Reseracg*, 1991; 8, 1434-40.

8. Bartels H., and Bartels R., Determination of Rifabutin by high performance liquid chromatography using on-line concentration and column-switching. *J Chrom B Bio Sci*, 1996; 686 (4), 235-40.

9. Patil Y. D., and Banerjee S.K., RP-HPLC method for the estimation of Rifabutin in bulk dosage form. *Int J Drug Dev & Res*, 2012; 4(2), 294-97.

10. Hemanth Kumar A., Sudha V., & Ramachandran G., Simple and Rapid Liquid Chromatography Method for Determination of Rifabutin in Plasma. *SAARC Journal of Tuberculosis, Lung Diseases and HIV/AIDS*, 2013; *9*(2), 26-29.

11. Singh G. and Srivastva A.K., High-performance Liquid chromatography method validation and development strategy for Rifabutin. *Int J Pharm Sci & Res*, 2018; 9(9):3903-07.

12. Mankar S. S., Shelke P. A., Wanjari D. S., Daware S. and Mundhada D. R., Method development and validation of Rifabutin by RP-HPLC Technique. *WJPPS,* 2019; 8(7), 1210-16.

13. Sajini R. J., Prema S., Niveditha S., Nithya S., Pavithra G. M., and Nivetha V., HPLC method development and validation for estimation of Rifabutin in bulk and capsule dosage form. *Int J Pharm Sci & Res,* 2020; 11(1), 297-0.

14. Bhusari S., Ansari I., Wakte P., Development and Validation of High-Performance Thin Layer Chromatography Method for Estimation of Rifabutin in Bulk and Formulation. *Asian J. Pharm. Ana.* 2020; 10(1), 32-36.

15. Bartels H, Bartels R. Determination of rifabutin by high-performance liquid chromatography using on-line concentration and column switching. *J Chromatogr B Biomed Appl.* 1996; 15; 686(2):235-40.

16. Winchester L. C., Podany A. T., Baldwin J.S., Robbins B. L. and Fletcher C. V., Determination of Rifamycin antibiotics Rifabutin, Rifampin, Rifapentine and their major metabolites in human plasma via simultaneous extraction coupled with LC/MS/MS. *J Pharm Biomed anal,* 2015; 104, 55-61.

17. Jaiswal S, Sharma A, Shukla M and Lal J., Simultaneous LC-MS/MS determination of Lopinavir and Rifabutin in human plasma. *J Chromatogr Sci.* 2017; 55(6), 617-24.

18. Wesley A, Waldrof B, Rao G, Stiles B, Griffis J, Salata R and Robert B., Development and validation of an LC/MS method for the simultaneous determination of Bedaquiline and Rifabutin in human Plasma. *J Pharma Biomed Anal,* 2019; 176, 112775.

19. Avachat A.M., and Bhise S. B., Stability indicating validated HPTLC method for simultaneous analysis of Rifabutin and Isoniazid in pharmaceutical formulation. *J Plan Chromtgr* , 2010; 23(2), 123-28.

20. Sanghshetty J. N., Hingankar S., Waghule S. and Shinde D.B., Stability indicating Liquid chromatographic method for the determination of Rifabutin in bulk drug and in pharmaceutical dosage form. *Afr J Pharm Pharmacol,* 2011; 5(3), 298-05.

21. Haslam J. L., Egodage K. L., Chen Y., Rajewski R. A., Stella V., Stability of rifabutin in two extemporaneously compounded oral liquids. *Am J Health Syst Pharm*. 1999; 15; 56(4):333-6.

22. Maria Á., Goberna B., Durgbanshi A., Bose D., Mishra P., Albiol-Chiva J, Esteve-Romero J., Peris-Vicente J., Quantification of rifampicin and rifabutin in plasma of tuberculosis patients by micellar liquid chromatography, *Microchemical Journal*, 2020; 157, 104865.

23. Kandula R, Sundararajan R. Novel Development of RP-HPLC Method to Quantify Amoxicillin, Omeprazole and Rifabutin in Combination. *IJPI*, 2020; 10(4):531-6.

24. Jain V, Jain N. K., Analytical Method Development and Validation for Simultaneous Determination of Amoxicillin, Omeprazole and Rifabutin in Bulk and in a Synthetic Mixture by UV Spectroscopy. *JDDT*, 2019; 9(4):788-94.

25. Chemical Entities of Biological Interest (ChEBI); CHEBI:45367-rifabutin, Available on: https://www.ebi.ac.uk/chebi/searchId.do?chebiId=CHEBI:45367 (Last accessed : 6 June,2019)

26. Larkin Peter: IR and Raman spectroscopy, Principles and spectral interpretations by, 1st edition, 2011, Elsevier, USA.

27. (Internet) Pfizer, Product monograph, Rifabutin, Available on, https://www.pfizer.ca/sites/default/files/201802/MYCOBUTIN_PM_E_209545_6Feb2018.pdf

28. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Harmonized Tripartite Guidelines, Impurities in New Drug Substances Q2 (R1), 2006.

29. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Harmonized Tripartite Guidelines, Impurities in New Drug Substances Q3 A (R2), step 4, 2006.

30. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Harmonized Tripartite Guidelines, Impurities in New Drug Substances Q1 A (R2), 2006.