CHAPTER 3

STRESS DEGRADATION STUDY AND IMPURITY PROFILING OF TERIZIDONE

3.1 Selection of Drug [1-4]

Tuberculosis is contagious disease spread through *Mycobacterium Tuberculosis* bacteria. Combination of effective antibiotics course is only treatment. Terizidone (TRZ) is the second line drug in treatment of drug resistant TB along with other second line Anti TB drugs. TRZ can be given in dose of 15 to 20mg/kg along with 50mg Pyridoxine for 250mg of TRZ to reduce adverse effect of TRZ. TRZ inhibits enzymes which are essential for cell wall synthesis of bacteria thus TRZ acts as bacteriostatic drug. CDSCO (Central drugs standard control organization) approved TRZ in June, 1981 in India. WHO includes TRZ in essential medicine list. TRZ has been used by patients for TB treatment since 1981; although few stability data on impurity is available in the literature, it was an opportunity as well as the challenge to study the degradation behavior of TRZ, to light up on the unreported impurity of TRZ, and degradation kinetics studies.

3.2 Drug Profile [1-6]

Some important parameters for TRZ are mentioned in Table 3.1.

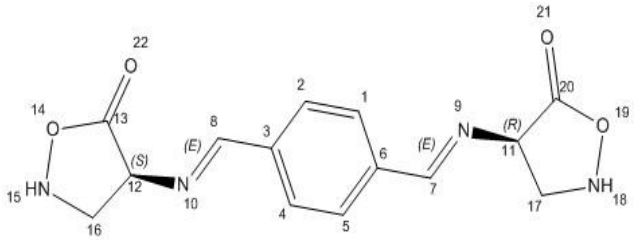
Drug property	Inference
CDSCO Approval	June, 1981.
Drug Category	Anti Tuberculosis
Mechanism of action	TRZ inhibits enzymes L –alanine racemace and D-alanine ligase which are essential for bacterium cell wall synthesis; thereby peptidoglycan formation is inhibited.
Marketed Formulation	Tericox® (Macleods Pvt. Ltd, Gujarat) contains 250mg of Terizidone in tablet formulation.
Chemical structure [5]	
IUPAC Name	4-[[4-[(3-oxo-1,2-oxazolidin-4-yl)iminomethyl]phenyl]methylideneamino]-1,2-oxazolidin-3-one
Molecular Weight	302.29 g/mol
Molecular Formula	$C_{14}H_{14}N_4O_4$
Physical Appearance	Solid white amorphous powder
Solubility	Soluble in DMSO and partially soluble in methanol
pKa	3.54(Strongest Acidic) and 2.75 (Strongest Basic).
Log P	0.17

Table 3.1 Drug Profile of Terizidone

3.3 Literature Review

As the TRZ is widely used for TB treatment since 1981, analytical and stability studies are explored by authors; these includes UV [7-8], HPLC-UV [9], stability study by HPLC [10] stability study by HPTLC [11, 12], Pharmacokinetic study [13] and analytical technique review [14]. There was no reported method for isolation and characterization of degradation products for Terizidone, Reported literature and current study is compared in section 3.5.2.6.

3.4 Bulk drug Identification

The TRZ was furnished by Macleods Pharmaceutical Pvt. Ltd., Gujarat, India as a gift sample. The sample was identified by Infrared spectroscopy, Differential Scanning Calorimetric and UV spectroscopy.

3.4.1 IR spectroscopy [15]

The Infrared spectroscopy was performed on Shimadzu IRaffinity 1S, Miracle ATR-IR instrument, with maximum resolution 0.5 cm^{-1} in a range of 4000-700 cm^{-1}, analyzed by Lab-Solution IR software.

The reference IR spectrum for TRZ is not available in literature therefore available chemical groups in TRZ chemical structure and groups identified in IR spectrum were compared. Table 3.2 shows the spectrum analysis for TRZ and spectrum is shown in Fig. 3.1.

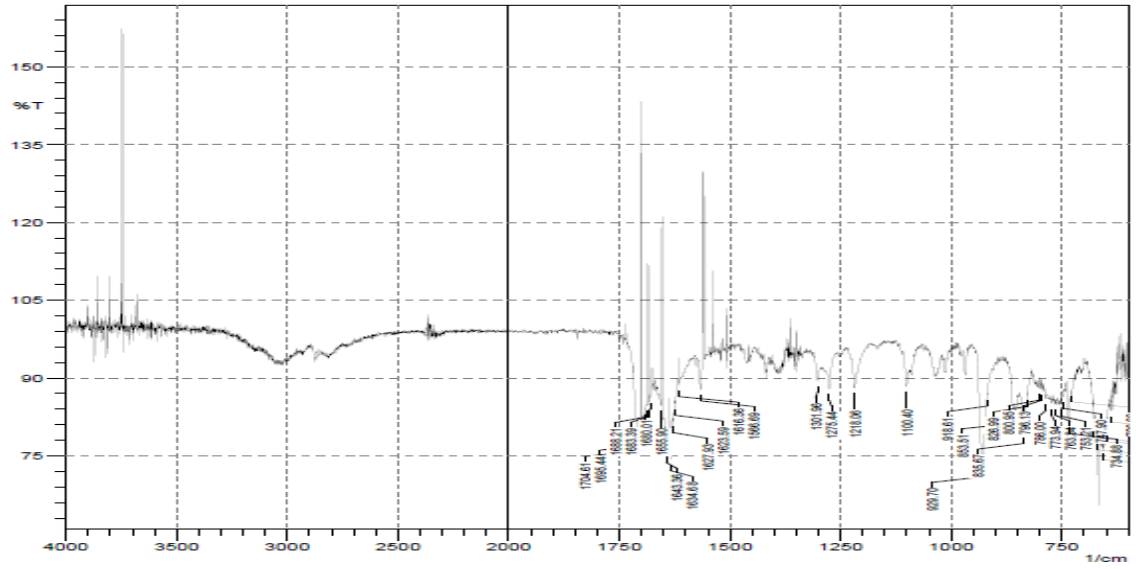


Fig. 3.1 IR spectrum of TRZ

Group	Obtained wave number (Cm^{-1})	Assigned wave number (Cm^{-1})
C=O	1680	1710-1680
C-N	1275	1250-1020
NH	1655	1650-1580
C-O	1218, 1275	1275-1200
C=C	1634,1655	1648-1638

Table: 3.2 IR spectrum inferences for TRZ [15]

The analysis of the IR spectrum shows that the groups available in the TRZ structure are found in the IR spectrum. The DSC and UV data was obtained to confirm identity of sample.

3.4.1 Melting Point study [17]

The sample was studied for Differential Scanning Calorimetry (DSC) for melting point analysis. The study was carried out using Shimadzu DSC instrument with TC 60 detector and temperature controller and the analysis of data was done DSC T 60 software. Nitrogen was used as inlet gas for maintaining instrument sensitivity and heat measurement efficiency. The study was started at room temperature (25 ± 5^0C) and continued till 300^0C with heating rate 10^0C/min. The thermogram is shown in Fig. 3.2 and the exothermic peak was observed at 207^0C.

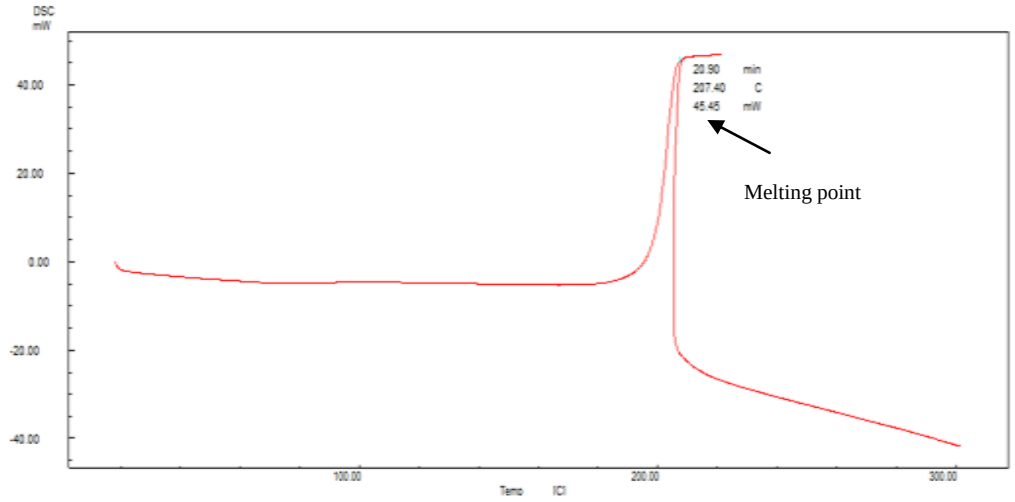


Fig. 3.2 DSC thermogram of TRZ

The exothermic peak can be considered as melting point due to physical state of sample changed at 207^0C, the explanation of exothermic peak can be considered as; when heat is applied, reactant bonds will break down and release the stored energy of bonds. TRZ bonds were broken down and released energy at 207 ^0C as an exothermic peak, the reported melting

point range for TRZ is 205-210 ^{0}C [17]. Though reference thermogram of TRZ was not available for comparison but melting point data matches with TRZ reported data and the sample was identified as TRZ.

3.4.3. Solubility study

The solubility test confirmed that TRZ is highly soluble in DMSO (Di Methyl Sulfoxide) (500.0µg/ml), sparingly soluble in methanol (300.0µg/ml) and very low solubility in water (20.0µg/ml). Therefore for sample preparation, initially TRZ bulk drug was dissolved in minimum required quantity of DMSO, final volume was achieved using different stressor (acid, alkali, oxide and water) or diluent (methanol). [17]

3.4.4. UV Spectrophotometric study

UV spectrophotometric study was completed using Shimadzu 1700 instrument. The sample for analysis under UV spectrophotometer was prepared by accurately weighing 1mg of TRZ bulk drug and dissolved in 1ml of DMSO (Di Methyl Sulph Oxide) and further diluted to 10ml with methanol to get 0.1mg/ml of stock solution, from this stock solution 1ml aliquot was diluted to 10ml with methanol to get 100.0µg/ml. This solution was scanned under UV range 190-400nm to find out λ_{max} of TRZ. The UV spectrum obtained for 0.01mg/ml solution is shown in Fig. 3.3.

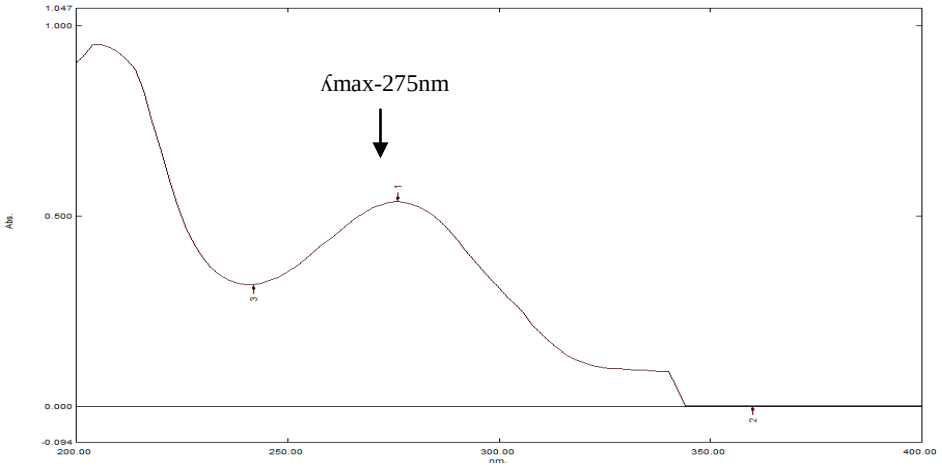


Fig. 3.3 UV Spectrophotometric curve for TRZ (100µg/ml)

The UV Spectrophotometric curve showed maximum absorbance at 275nm which is corresponding to the reported UV λ_{max} for TRZ is 270-275nm [17]. This result identified the sample as TRZ.

Result and discussion; The results of Identification tests for TRZ (IR, melting point and UV spectrophotometric test) matched with reported data or analyzed data so based on these results further method development and stability studies were started.

PART- A

3.5 DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD AND STRESS DEGRADATION STUDIES FOR TRZ

3.5.1 Experimental

3.5.1.1 Chemicals and Reagents

TRZ bulk drug was furnished by Macleods Pharma Pvt. Ltd. (Gujarat, India) as a gift sample. HPLC grade methanol and acetonitrile was purchased from Rankem Chemicals, Gurugram, India. Ammonium acetate buffer was purchased from Loba Chemicals Pvt. Ltd, Mumbai, India. Glacial acetic acid (>99.0% purity) was purchased from Sigma-Aldrich.

Unless and otherwise specified, all solutions were filtered through a 0.45µm Nylon 6,6 membrane filter, Ultipore® N66® From Pall Life Sciences, USA; prior to use. Analytical grade Hydrochloric acid (HCl) and Sodium hydroxide (NaOH) were procured from SD Fine Chem. Ltd., Mumbai. Hydrogen peroxide (H_2O_2) was procured from Fischer Ltd., India. The water for studies was prepared by deionization of water in laboratory.

3.5.1.2 Equipment and chromatographic conditions

Precision Water/Oil bath (EIE, India) with temperature controller equipment was used for stress degradation studies to maintain temperature of samples.

Photolytic degradation study was carried out in a photo –stability chamber (Thermolab Scientific Equipments Pvt Ltd, Vadodara) Equipped with a light back consisting of four UV (OSRAM L73) and Fluorescent (OSRAM L20) lamps, that compiled with specifications prescribed in the ICH guideline Q1B. The system is capable of controlling specific temperature and humidity ($\pm 2^0$C and ± 5% RH).

For thermal and Humidity study; thermal –humidity chamber (S.R Labs. Instruments, Maharashtra, India) was used and set accelerated condition of 40^0C/ 75% RH.

Other equipment used were an Ultrasonic bath (Analab Scientific Instruents Pvt Ltd, Vadodara), Precision analytical balance (AX 120, by Shimatzu Corporation analytical and measuring instruments division, Kyoto, Japan.), pH Meter (Lab India Instruments Pvt Ltd., Navi Mumbai). The analysis of data and Calculations were done using Microsoft Excel-2007.

UV Spectrophotometer: The suitable wavelengths for estimation of drugs were identified by scanning over the range of 200-400nm with a Shimadzu UV-1700 double beam Spectrophotometer (Shimadzu Japan) which contains double beam optics with scanning range 190-800nm with maximum stray light of 0.04%and wavelength accuracy ±0.3nm, spectral band width was 1nm, scanning speed approx. 3000nm/min with silicone photodiode detector.

Liquid Chromatographic system: The HPLC (High Performance Liquid Chromatographic) system consisted of manual injector, low pressure gradient flow control valve, solvent delivery module, photodiode array(PDA) detector, and system controller with Empower-2 Software (all from Waters Acquity Corporation, Milford, MA, USA).

For method development, the separation was accomplished on a Thermo scientific RP-C_{18} column (250×4.6mm, 5µm) at wavelength 270nm. 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 TRZ bulk drug using

A) 0.01M ammonium acetate buffer pH 4.7 with glacial acetic acid and

B) Acetonitrile as mobile phase ran in gradient mode.

TRZ bulk drug peak was obtained at Rt 28.8 ±0.5 minutes with acceptable peak purity.

The gradient method used for the separation is shown in Table 3.3.

Time	%B	Flow rate
0	5	1
10	5	1.2
50	30	1.2
55	5	1
60	5	1

Table: 3.3. Gradient flow scheme for stability indicating method of TRZ

3.5.1.3 Analytical sample preparation

Preparation of Stock, Sample and Buffer solutions

Sample preparation for linearity: Stock solution of TRZ was prepared by dissolving 10mg TRZ in 1 ml of DMSO (Di methyl sulfoxide) 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 50.0-300.0µg/ml of TRZ 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.

Ammonium acetate buffer (0.01M) was prepared by dissolving 0.770 gm of ammonium acetate buffer in a 900ml of double distilled water and adjusted to pH 4.7 using glacial acetic acid, remaining volume was added to make final volume of 1l, which was finally filtered with 0.2 µm nylon membrane filter and degassed by ultra sonicator for 5 minutes.

Preparation of stress degradation samples

A preliminary stability of TRZ was evaluated in organic solvent (DMSO) 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.

Samples for stress degradation study: TRZ (accurately weighed 500mg) was dissolved in 3ml DMSO and sonicated for 15minutes with provisional shaking; 50ml final volume was achieved using 6% hydrogen peroxide, 0.5N HCl, 0.5N 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, all the samples except for photo-stability were kept in dark and diluted to 10ml with methanol (200µg/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 3.5.1.2 for RP-HPLC for data acquisition and analytical purpose.

TRZ was stressed to maximum condition where 5-80% decreases in peak area of TRZ bulk drug noticed. Marketed formulation of TRZ (Tericox, Macleods Pharma Pvt. Ltd.) was

treated under similar degradation conditions as performed for bulk drug using equivalent weight to 10.0mg from formulation to determine formation of any DPs due to drug excipient interaction.

Preparation for assay study

Terizidone is marketed by Macleods Pharmaceutical Ltd., Gujarat under the proprietary name Tericox (250.0mg) in the form of tablets were taken, 20 tablets were powdered, mixed and for Assay, accurately weighed tablet powder equivalent to 10.0mg TRZ was dissolved in 3ml DMSO to produce 1.0mg/ml stock solution, 1ml aliquot was taken in 10ml volumetric flask and volume was made up with methanol to get 100.0µg/ml. Sample was injected in HPLC chromatographic system to perform assay.

3.5.1.4. 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 TRZ. Optimization of HPLC method is shown in Table 3.4.

Detection wavelength and Polarity: The UV Spectrum of TRZ (Fig. 3.3) showed maximum absorbance at 270nm therefore RP-HPLC detection was carried out at 270nm.

Buffer pH: The pH of buffer showed high impact on drug peak shape while it showed less effect on DPs' separation. Lower buffer pH 2.5 to 3 contributed in broadening of API peak, while increasing pH to 4.7 provided peak symmetry, more increase in pH 5.0 lead to drug peak fronting. 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, TRZ has high solubility in DMSO and sparingly soluble in methanol. TRZ was initially dissolved in minimum amount of DMSO (1-5ml) then diluted with methanol to prepare all the TRZ solutions. TRZ is mid-polar in chemical nature; as a result methanol and DMSO were a choice of organic solvent. The standard solution of TRZ was prepared in DMSO before stress study to dissolve the bulk drug. As DPs formed were polar in nature and TRZ 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.

3.5.1.5. Method Validation as Per ICH Q2 (R1) guideline.

The present method was validated as per ICH Q2 (R1) guideline taking in consideration linearity, range, accuracy, precision, robustness, LOD (Limit of detection) and LOQ (Limit of quantification).

Range of concentration was selected to get **linearity** of TRZ, mean of repeated results in triplicate are taken in consideration to prove repeatability and robustness of method. Regression square (R^2) value showed the linearity of method and regression equation was used to calculate recovery of concentration during accuracy study.

Precision was determined by intraday, inter day and repeatability study. Samples were analyzed by HPLC in same day and different days to check sample stability as well as method reproducibility. Repeatability was done by analyzing samples of same concentration for six times to prove method was reproducible with RSD < 2.

Accuracy of method was performed to check recovery of samples. Standard addition method was used for accuracy study. Mean of repeated samples was used to calculate recovered concentration and RSD value should be < 2.

Robustness study was performed to prove that method was robust within small deliberated variation in method parameters like organic ratio, pH, and wavelength or flow rate. RSD value for robustness data should be < 2.

Sensitivity of developed method [14] was studied by LOD and LOQ data. Limit of detection (LOD) is the minimum concentration that can detect apart from zero line. Limit of quantitation is the minimum concentration that can be quantified with repeatability and accuracy. The LOD and LOQ were calculated by equation;

$$\boldsymbol{LOD = 3.3 \times \sigma \div b}$$

$$\boldsymbol{LOQ = 10 \times \sigma \div b}$$

Where,

σ = the standard deviation of response

b= the slope of the calibration curve

3.5.2 Results and discussion

3.5.2.1 Determination of suitable wavelength

The UV spectrum of TRZ was extracted from 200-400nm and it is shown in Fig. 3.4 for 10.0-70.0µg/ml. The spectrum indicates that 270-276 nm may show high sensitivity for the DPs and TRZ so 270nm was selected as the detection wavelength for all the chromatographic studies of TRZ. (The DPs showed sufficient absorption at the selected wavelength)

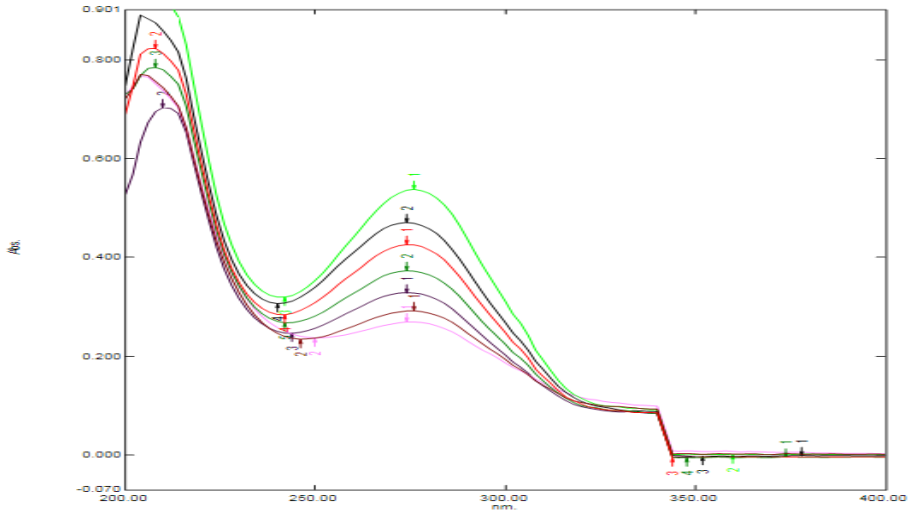


Fig.3.4 UV spectra of TRZ bulk drug (10.0-70.0µg/ml)

3.5.2.2 Method development and optimization

As per TRZ physical-chemical properties the method development was started using different buffer based on its Pka value and methanol, as TRZ has very low solubility in water. Buffers of different pH (range 3.7-5.0) were taken in trial; Pka of TRZ is 3.54 (strongest acid) and 2.75 (strongest basic) therefore buffer ranges in pH (±2.0 of Pka) 3.75 and 4.54 were taken in trials; ammonium acetate buffer with pH 3.7-4.7 showed good system suitability for TRZ peak and DPs. The pH above 4.8 showed peak broadening of TRZ peak. DPs are even more polar in nature than TRZ, so higher ratio of buffer was used to increase retention time (Rt) of TRZ peak and to achieve maximum resolution the DPs peaks. pH of buffer did not play role in separation of DPs and TRZ bulk drug peak. After trying various ratios of mobile phase, some random trials are shown in Table 3.4 and the final gradient program used for separation with acceptable purity of peaks in chromatogram is shown in Table 3.2.

Sr.	Mobile Phase	Ratio	Result
1	Water: acetonitrile	50:50	Distorted Peak
2	Water: acetonitrile	30:70	Not good peak shape.
3	Water: methanol	50:50	Very less peak height and not good peak shape.
4	0.01M ammonium acetate pH 3.8: acetonitrile	50:50	good peak height but tailing observed
5	0.01M ammonium acetate pH 4.0: acetonitrile	50:50	Tailing of peak observed.
6	**0.01M ammonium acetate pH 4.7: acetonitrile**	**50:50**	**Good peak shape and intensity.**
7	**0.01M ammonium acetate pH 4.7: acetonitrile**	**Gradient**	**To separate DPs and TRZ bulk drug peaks.**

Table: 3.4.Optimization of analytical method of TRZ

3.5.2.3. Stress degradation studies

The stress degradation studies were completed in acid, alkali, neutral (water), oxidative medium, under UV light (photolytic) and thermal condition. The degradation of TRZ was observed in different stress condition and listed in Table 3.5. The TRZ degradation was observed in acid, alkaline, neutral and oxidative medium, chromatogram for these conditions are shown in Fig. 3.5.

The %deg. was calculated by the formula:

$$\%Drug = \ Reduced\ area\ of\ Treated\ solution\ of\ drug\ \times 100 \div Initial\ area\ of\ untreated\ solution$$

$$\%Degradation = 100 - \%Drug$$

The specific stress conditions applied are as follow:

Hydrolytic degradation (Acidic/Alkali/Neutral)

Hydrolytic degradation studies were performed in acidic, alkali and neutral conditions if required with heating and refluxing. Stability conditions are as described below;

For acid hydrolysis, freshly prepared stress degradation sample in 0.5N HCl was kept at room temperature in dark for 8 hours

For alkali hydrolysis, freshly prepared stress degradation sample of TRZ in 0.5N NaOH was allow to stand in dark at room temperature for 120min.

For neutral hydrolysis, Solution of TRZ in water was kept at room temperature in dark for 12hrs.

Peroxide induced (Oxidative) Degradation

For oxidative degradation study, sample prepared in 6% H_2O_2 was kept at room temperature in dark for 24 hours.

Photolytic Degradation

For light induced degradation, bulk drug of TRZ in solid state 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, bulk drug of TRZ in solid state was spread on a petri plate with approximately 1mm thickness and placed in oven at 80^0C for 28 days under dry heat condition in dark.

In these stressed samples, 5-100% degradation was observed in RP-HPLC chromatogram.

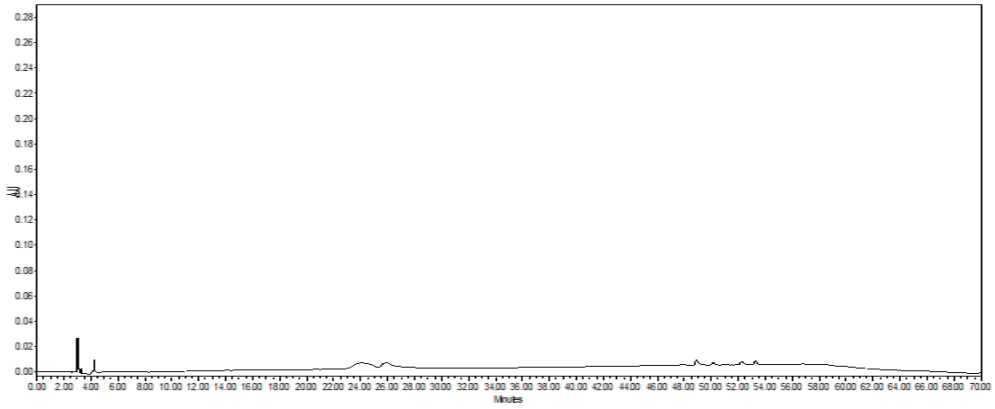
The stressor conditions applied to the bulk drug were also applied to the marketed formulation of TRZ (Tericox 250mg, Macleods Pvt. Ltd. Gujarat).

TRZ was highly sensitive and unstable in alkali condition, although it also degraded at significant level in acidic, neutral and oxidative condition. Very slight degradation (<5%) occurred under photolytic condition and under dry heat induced condition. Also peak purity plots for individual stress degradation condition were evaluated. The stress degradation conditions and TRZ behavior is shown Table 3.5 and degradation chromatograms are shown in Fig. 3.6 (A to F).

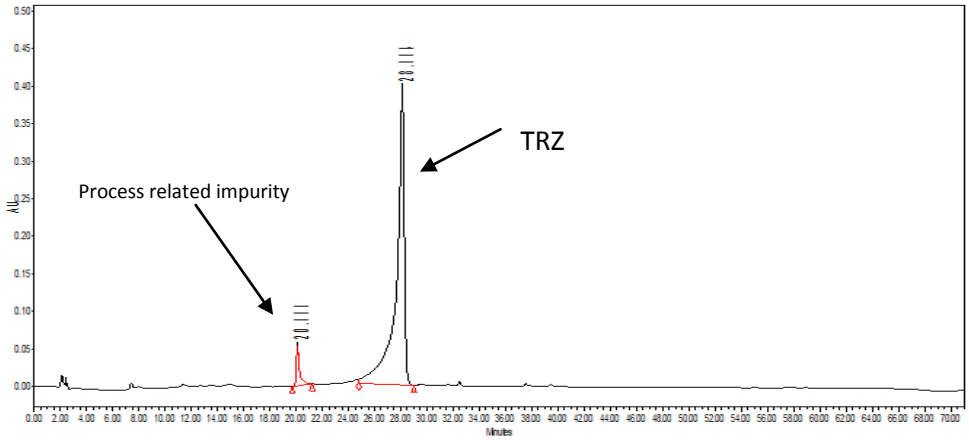
Stressor Type	**Stressor Conc.**	**Time**	**DPs formed with RT (min.)**	**TRZ %Deg. Bulk drug**	**TRZ %Deg. Formulation**
Acid	0.5N HCl/RT	8hrs.	Peak-1: 8.1 **Peak-2: 15.3** Peak-3: 15.9 API: 26.11	51.8%	49.2%
Alkali	0.5N NaOH/RT	2hrs.	Peak-1:28.6 Peak-2:29.6 Peak-3:33.6 Peak-4:34.7	99.1%	98.5%
Neutral	H_2O/RT	12hrs.	Peak-1: 10.9 **Peak-2: 16.2** Peak-3: 24.1 API: 26.16 Peak-4: 35.9 Peak-5: 37.0	55.4%	52.7%
Oxidative	6% H_2O_2/RT	24hrs.	Peak-1: 8.0 **Peak-2:15.2** API:26.16	94.6%	93.7%
Photolytic	-	28days	-	3.2%	3.6%
Dry heat	80^0C	28days	-	2.7%	2.1%

Table: 3.5 Summary of stress degradation of TRZ (bulk drug and Formulation)

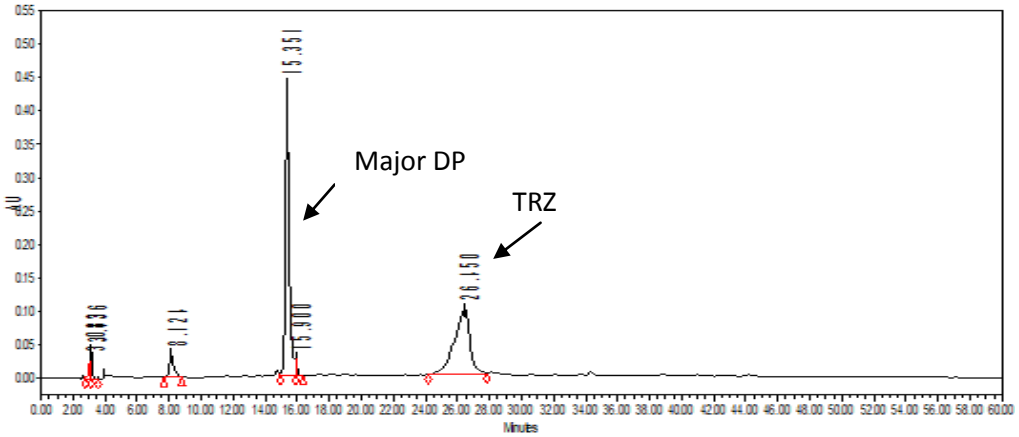
A


AU
Minutes

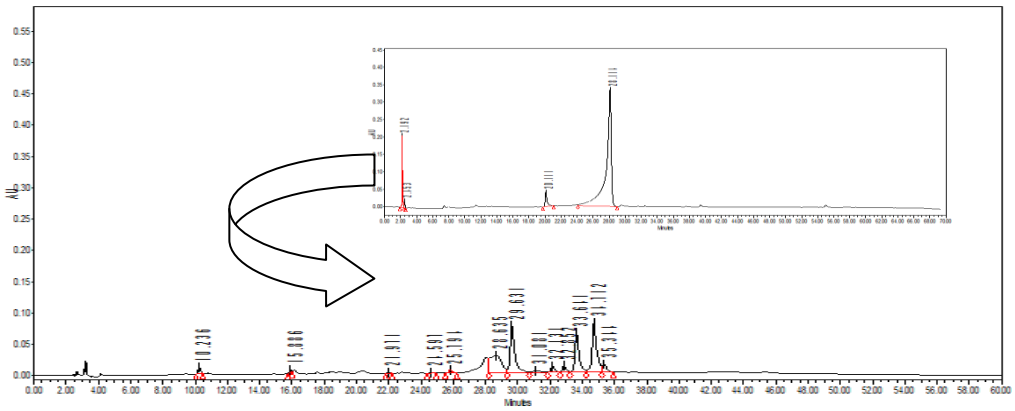
B


TRZ
Process related impurity
AU
Minutes

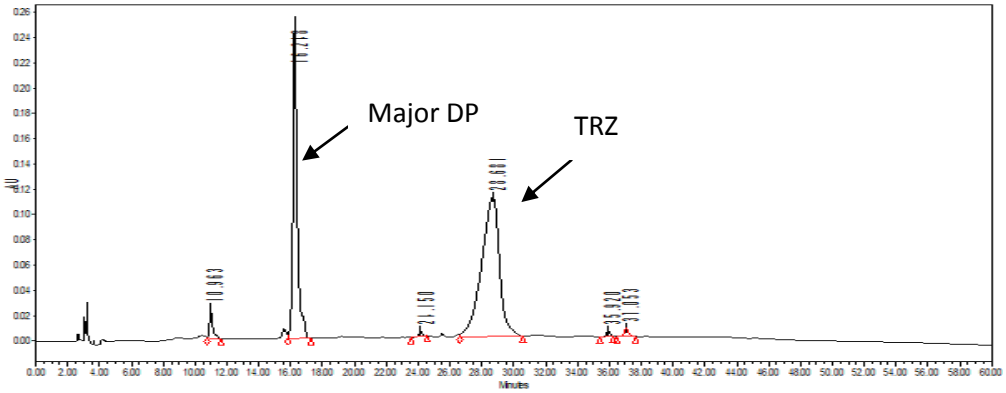
C


Major DP
TRZ
AU
Minutes

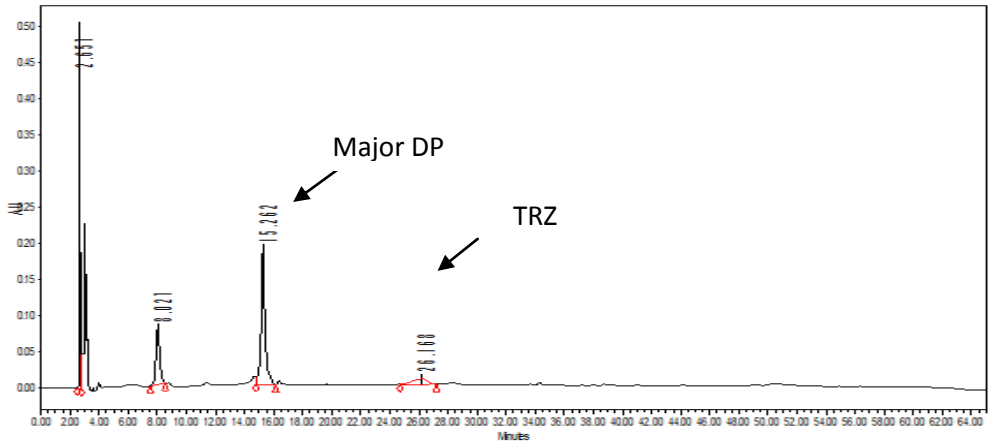
D



E



F



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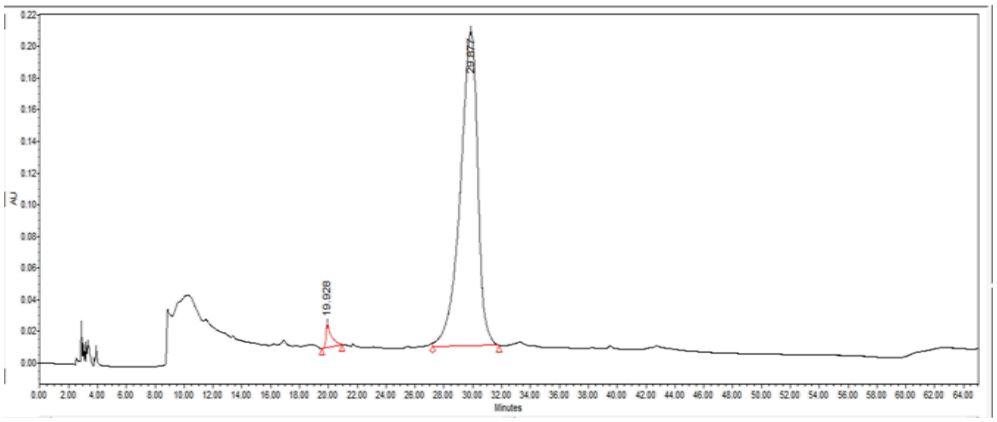


H

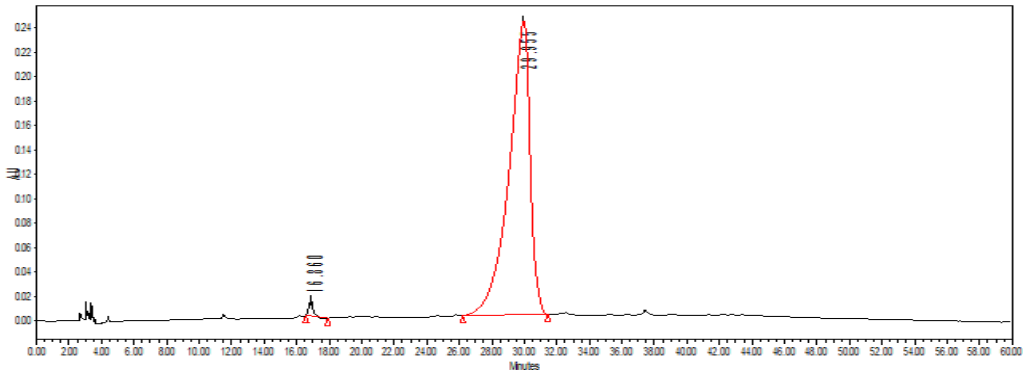


Fig.: 3.5 Chromatograms of A) chromatogram for blank sample B) Chromatogram showing selectivity of method C) Acid degradation D) Alkali Degradation E) Neutral Degradation F) Oxidative Degradation G) photolytic degradation H)Thermal degradation

TRZ did not show any degradation under thermal and photolytic condition for more than 28days so it can be concluded that it is stable under UV light and dry heat.

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.3.6 (B), (C), (E) and (F). The results for peak purity are shown in Table 3.6 with purity angle which should not be exceeding the purity threshold.

DPs and R_t	Purity angle	Purity threshold	Pass/Fail
(B) TRZ bulk drug			
Peak: 20.11 (Impurity-1)	0.168	0.565	Pass
API: 26.11	0.117	0.246	Pass
(C) TRZ acid degradation sample			
Peak1: 8.1 (DP Mix.: A1-A7)	1.415	0.281	Fail
Peak 2:15.3 (Major DP: A4)	**0.133**	**0.248**	**Pass**
Peak 3:15.9	0.241	0.359	Pass
API:26.11	0.211	0.303	Pass
(D) TRZ neutral degradation sample			
Peak1:10.9	0.241	0.373	Pass
Peak2: 16.2 (Major DP: N3)	**0.104**	**0.241**	**Pass**
Peak3 : 24.9 (Mixture of DPs)	1.868	1.711	Fail
API:26.16	0.188	0.274	Pass
Peak4: 35.9	1.155	1.300	Pass
Peak5: 37.0	0.310	0.947	Pass
T(E) TRZ alkali degradation sample			
Peak1: 28.6	0.086	0.355	Pass
Peak2:29.6	0.063	0.245	Pass
Peak3:33.6	0.213	0.287	Pass
Peak4 :34.7	0.257	0.267	Pass
(F) TRZ oxidative degradation sample			
Peak1: 8.02 (Mixture of DPs)	1.945	1.115	Fail
Peak2: 15.26 (Major DP: O7)	**0.237**	**0.248**	**Pass**
API: 26.16	0.244	0.248	Pass

*Major DPs are highlighted with bold fonts

Table 3.6 Peak purity result for chromatogram B, C, E and F

Peak purity test for Fig. 3.5 (B) TRZ bulk drug chromatogram is shown in Fig. 3.6. The chromatogram showed process related impurity and TRZ bulk drug peak; both with peak purity and non-co eluting peak. The process related impurity is discussed in Part-D.

A **B**

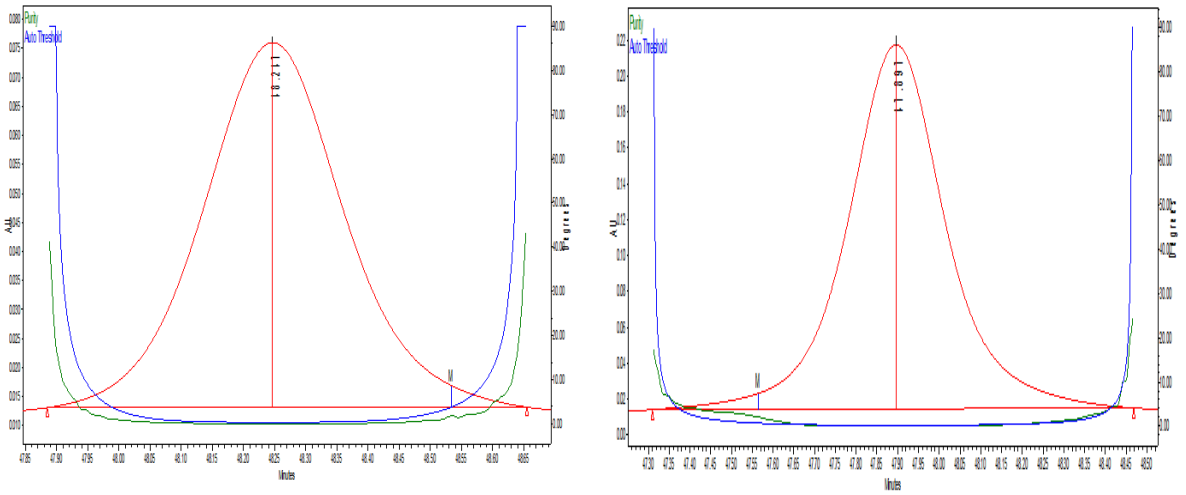
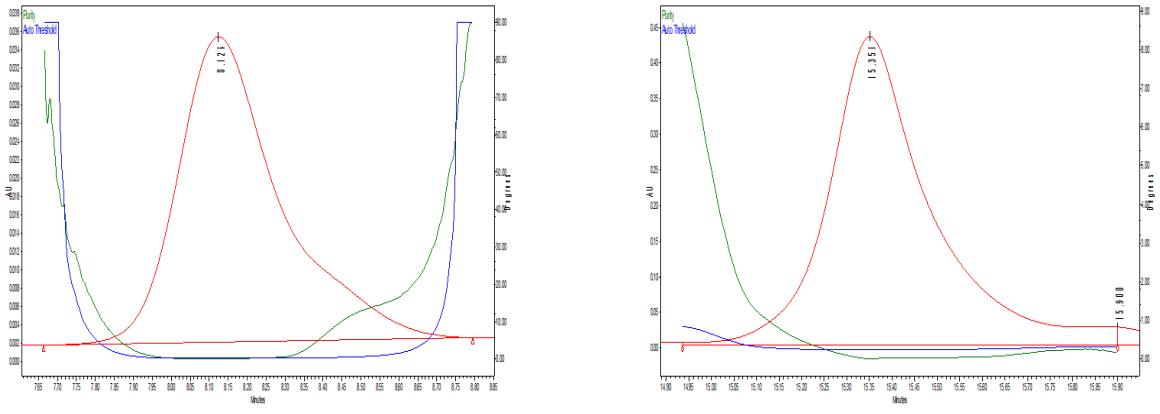
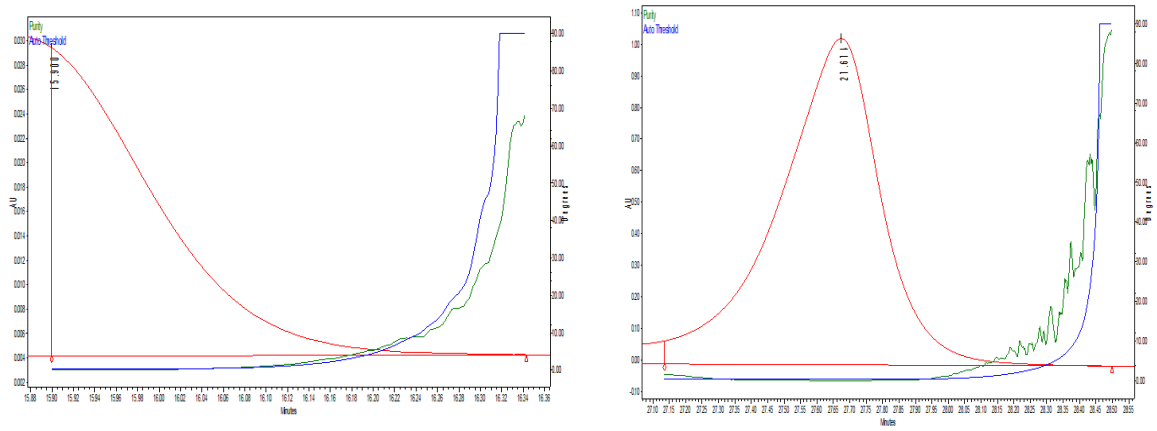


Fig. 3.6 Peak purity for A) Process related impurity and B) TRZ bulk drug chromatogram shown in Fig.3.5

The peak purity test for chromatogram shown in Fig 3.5 (C) acid degraded sample of TRZ is shown in Fig. 3.7 with three degradation peaks and TRZ peak. The peaks were found to be pure by purity test and the DP obtained at Rt 15.3minutes was treated as major DP from acid hydrolysis.



A **B**

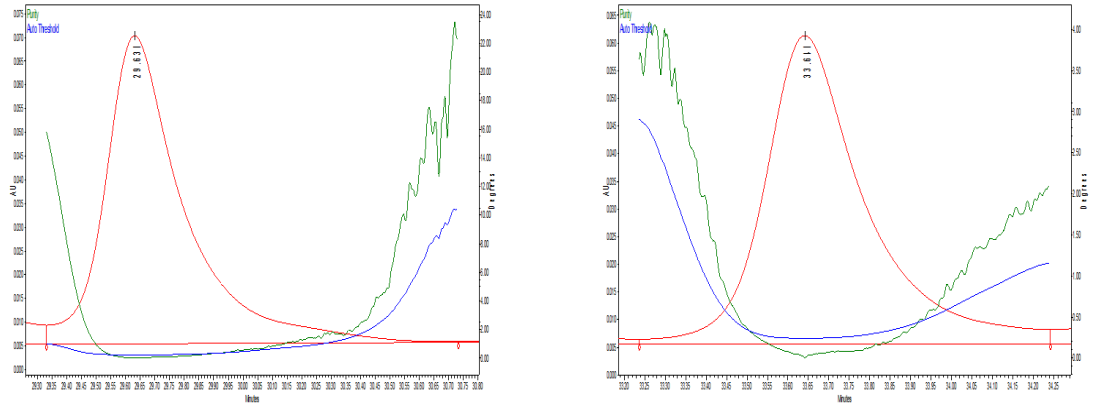


C **D**

Fig. 3.7 Peak purity for acid degraded sample A) Peak-1 B) Peak-2 C) Peak-3 and D) API

The peak purity test for chromatogram shown in Fig 3.5 (D) alkali degraded sample of TRZ is shown in Fig 3.8 with four DPs peak; although cluster of several DPs were obtained, the four DPs peaks were obtained with peak purity.

A **B**



C **D**

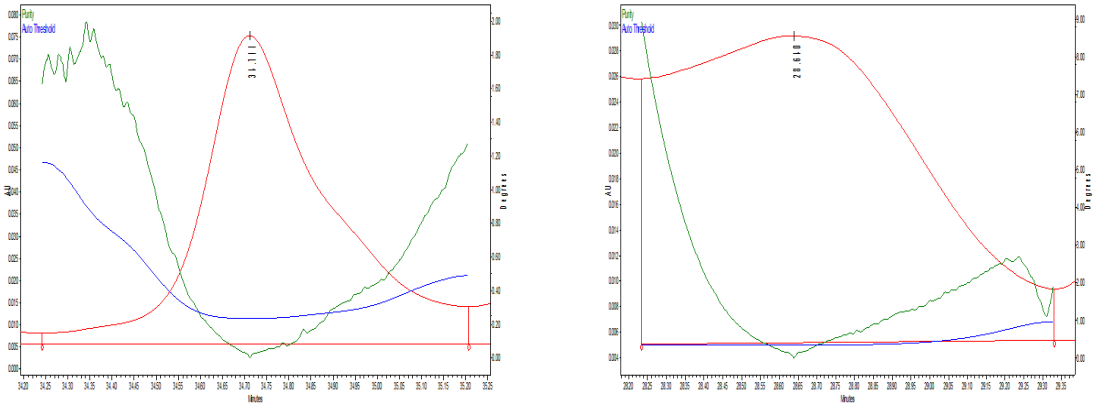
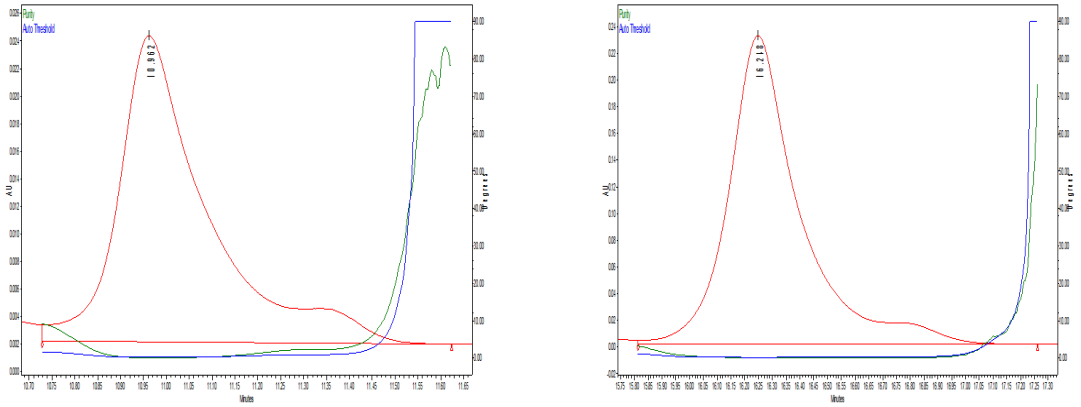


Fig. 3.8 Peak purity for alkali degraded sample chromatogram of TRZ A) Peak-1 B) Peak-2 C) Peak-3 D) Peak-4

The peak purity for neutral degradation chromatogram of TRZ (Fig. 3.5 (E)) is shown in Fig 3.9. The chromatogram showed five DPs peaks among which the peak at Rt 16.02 minutes was treated as major DP.

A **B**



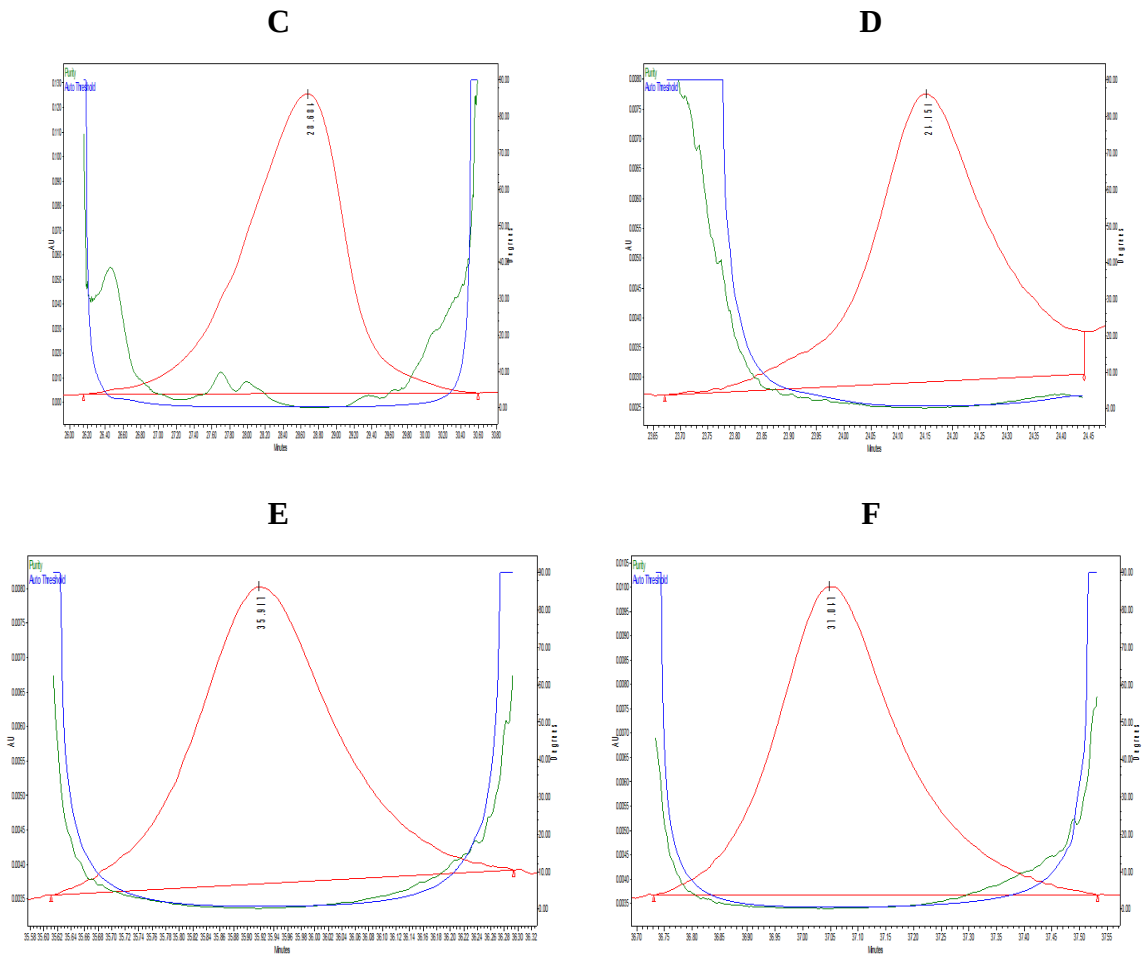


Fig. 3.9 peak purity for neutral degradation chromatogram of TRZ A) Peak-1 B) Peak-2 C) Peak-3 D) API Peak E) Peak-4 F) Peak-5

The peroxide degraded chromatogram (Fig. 3.5 (F)) and purity plot for it is shown in Fig 3.10. The Chromatogram showed three DPs along with API peak, the major DP was at Rt 15.26minutes from oxidative degradation.

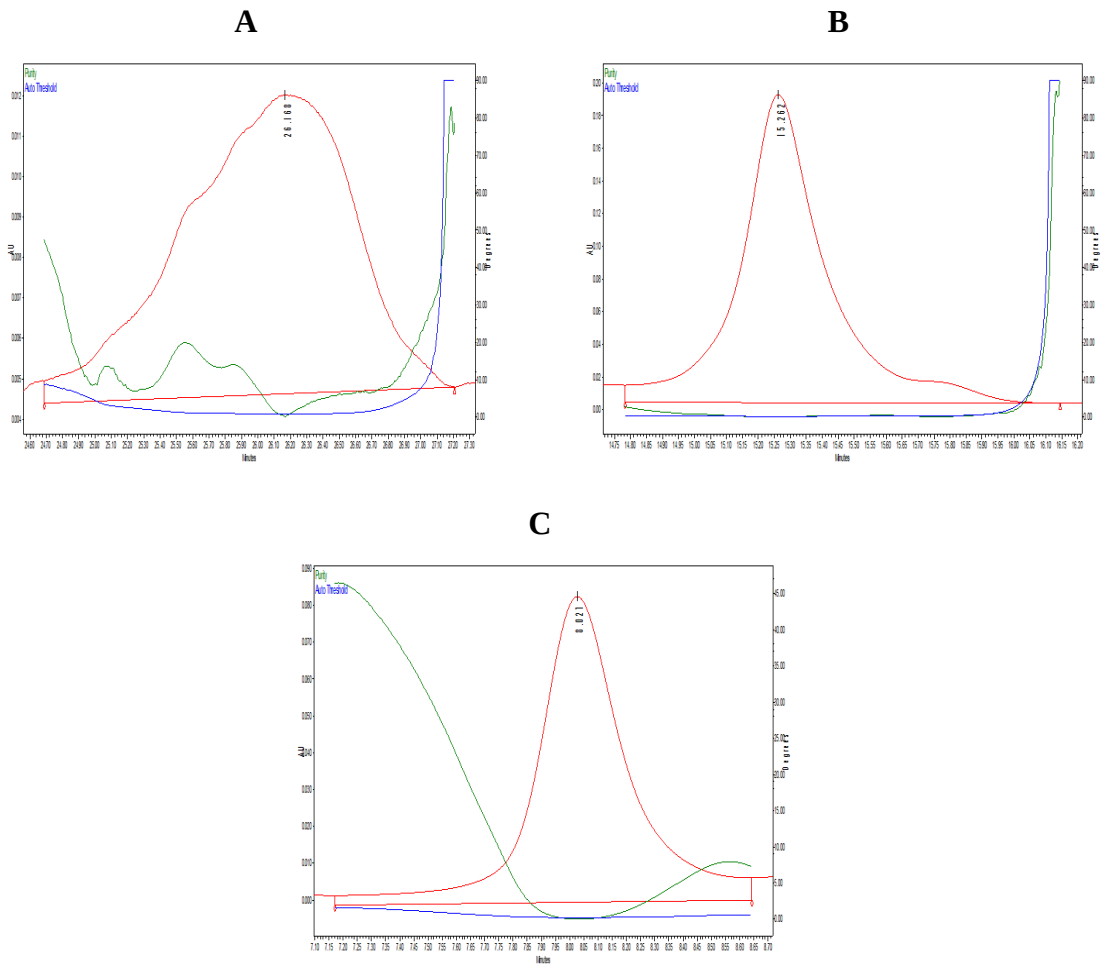


Fig.3.10 Peak purity for oxide degraded sample chromatogram of TRZ A) Peak-1, B) Peak-2 C) API Peak

From the evaluation of chromatogram and peak purity plots, three major DPs (one in every condition; acid, neutral, and oxidative) and one process-related impurity was identified. The identification and characterization are briefly explained in Part- C and D.

3.5.2.4. Validation of stability-indicating method [18]

The stability-indicating method was developed to achieve separation between DPs and bulk drug peak in the chromatogram. The numbers of DPs generated in the degradation of TRZ interfered with each other's peak purity of DP therefore to achieve peak purity run time of the stability-indicating method was extended. The gradient program of method is shown in Table 3.2. Validation of the method was performed in terms of the parameters linearity, range, precision, accuracy, and LOD, LOQ.

Linearity and Range

The linearity of TRZ sample was studied for the range 50.0-300.0µg/ml, the sample preparation for analysis is described in section 3.5.1.3. The strong correlation coefficient (r^2) of 0.997 with regression equation y = 57453x + 6E+06 showed a good linearity for the range. The overlay chromatogram for TRZ range is shown in Fig 3.11.

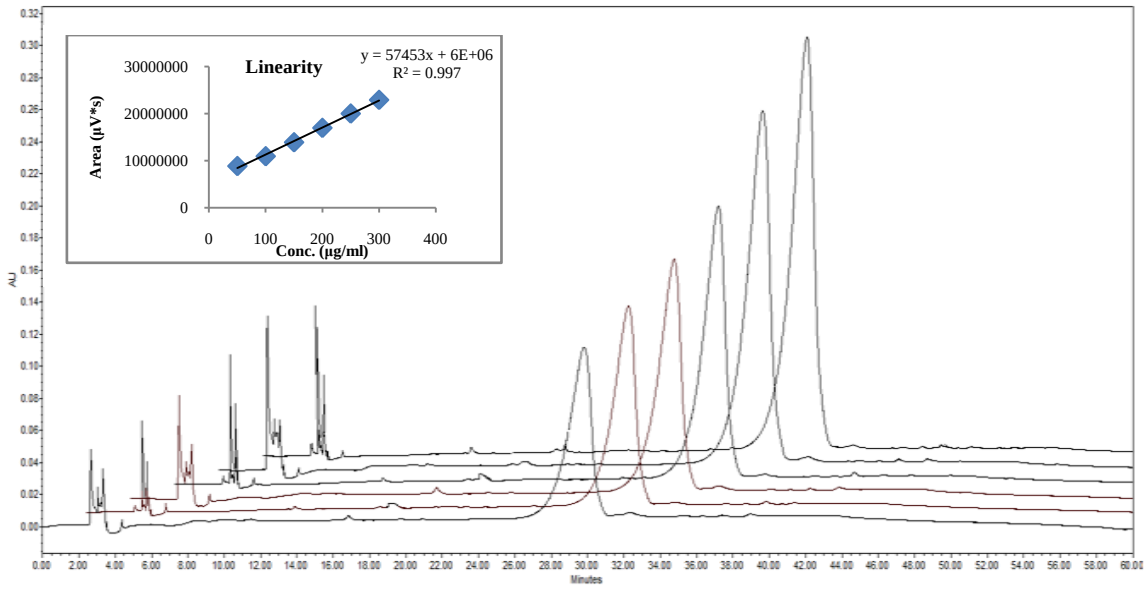


Fig. 3.11 Overlay chromatogram and linearity plot for TRZ bulk drug (50.0-300.0µg/ml)

The linearity of method was established in accordance with ICH Q2 (R1) guideline. The average peak area of the experiment performed in triplicate with respective concentrations is shown in Table 3.7.

Conc. (µg/ml)	*Mean peak area (µV*S) ± RSD
50.0	8883500 ± 0.781
100.0	10966068 ± 0.650
150.0	13930364 ± 0.824
200.0	16991347 ± 0.851
250.0	20034539 ± 0.795
300.0	22938678 ± 0.679

Table 3.7 Mean peak area and respective concentration of TRZ bulk drug

The RSD value for average reading was < 2.0% indicates that good linearity was obtained.

Precision

The precision data show that how precisely the present method can measure TRZ for qualitative and quantitative study. The result of precision data is shown in Table 3.8; RSD value for precision study is < 2.0 % indicates that developed method is precise enough measure the TRZ in RP-HPLC studies.

Repeatability			
Conc. (µg/ml)	***Avg. Area (µV*S)**	**SD**	**% RSD**
200.0	16826347	165378.4	0.982
Intermediate Precision			
Inter day precision			
50.0	8913500.3	51916.52	0.582
100.0	10999401.3	57735.02	0.524
150.0	13853697.3	109696.55	0.791
Intraday precision			
50.0	8920166.6	63508.52	0.711
100.0	10932734.6	100000.0	0.920
150.0	13897030.6	115470.05	0.832

*Average value of three replicates for intermediate study and six replicates for repeatability study

Table 3.8 Intermediate precision and repeatability study of TRZ

Accuracy

Accuracy study shows that method is capable to determine the substance quantitatively. The recovery study results are shown in Table 3.9; RSD values < 2.0% indicated that variation in results are not observed.

Level (%)	Conc. from formulation (µg/ml)	Std Conc. Spiked (µg/ml)	*Mean % Conc. recovered	Mean % recovered ± RSD
50	200.0	100.0	100.5	100.57±0.252
100	200.0	200.0	98.9	98.91 ± 0.399
150	200.0	150.0	98.4	98.44 ± 0.374

*Mean of three replicates

Table 3.9 Recovery result of TRZ bulk drug and formulation

LOD and LOQ

Limit of detection and quantification shows minimum limit of the concentration that can be detected and quantified by an instrument in the range of linearity. The LOD and LOQ were calculated using the standard deviation of responses and slope of the calibration curve of average response values. The LOD of range was 4.23µg/ml and LOQ of range was 13.2µg/ml.

TRZ Assay results

The TRZ formulation assay was performed by HPLC method; the assay results showed that 99.5% sample concentration was recovered. (Limit for assay recovery-98-102%).

Robustness

The robustness method is shown in Table 3.10 with variation in flow rate (±0.1ml), pH (±0.2) and detection wavelength (±5nm). The robustness study was carried out using 200.0µg/ml solution of TRZ.

Variation	Observations*			
	Area (%RSD)	**Rt (%RSD)**	**Tailing factor (%RSD)**	**Theoretical Plates (%RSD)**
Flow rate (ml/min)				
0.9	0.967	0.005	0.008	0.041
1.0	0.863	0.006	0.007	0.041
1.1	0.764	0.006	0.007	0.042
pH				
4.5	0.863	0.006	0.007	0.040
4.7	0.863	0.006	0.007	0.041
4.9	0.862	0.006	0.008	0.041
Detection wavelength (nm)				
270	0.860	0.005	0.006	0.042
275	0.863	0.006	0.007	0.041
280	0.863	0.005	0.007	0.041

*average value of three replicates

Table 3.10 Robustness study of stability-indicating method of TRZ

The small but deliberate changes in stability indicating method did not affect the results of TRZ system suitability. The RSD value was < 2.0 % indicates that these changes are acceptable.

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

The developed method was used to analyze stress degraded samples of formulation containing TRZ. Stress degradation of TRZ formulation was carried out under same conditions as specified for bulk drug and analyzed in the same way under the same chromatographic conditions. The degradation products were discernible and well separated. As represented in Table 3.6 the same degradation pattern was observed for formulation as it was observed in bulk drug and it can be inferred that there was no interference of excipient in degradation of TRZ.

The developed stability indicating method can be applied for routine quality analysis in various studies of TRZ. The method has detection limit of 4.23 µg/ml indicates that method can detect low concentration of impurities in TRZ samples. The stability study results can be

applied to formulation for shelf life study, and for suggested storage condition of TRZ formulation.

3.5.2.6.Comparison with reported Stability indicating method

There are three stability indicating methods reported for TRZ; the reported method and the current study are different as following;

1. The method reported by author Gandhi S. V. and et.al includes stability indicating method for TRZ by RP-HPLC but in the study DPs are neither identified, neither separated nor reported formed by forced degradation study of TRZ. [10]
2. The HPTLC method reported by author Bhole R.P and et.al includes degradation product identification using MS/MS data among which some of the reported DPs are solvent peaks and adduct peaks; while the DP with *m/z* 241 is also reported in this study as minor DP. [11]
3. The HPTLC method reported by Patil P. and Alalaiwe A. identified two DPs with *m/z* 370 and 378 amu. No such DPs are identified in this study of TRZ. [12]

The current study is novel which identified and characterized major DP of TRZ in different stress degradation condition by LC/MS, NMR and IR data. None of the above described study created impurity profiling and identified the DPs reported in this study so it was thought of interest to develop the stability indicating method for TRZ and study the stress degradation behavior of TRZ, identify the degradation impurities and isolate and characterize the major degradation impurity.

PART- B

3.6. DEGRADATION KINETIC STUDY OF TERIZIDONE

The degradation kinetics of TRZ was studied in acid, alkali, neutral and oxidative conditions with different stressor concentration, temperatures and time. The degradation kinetics study gather knowledge about the energy changes at the molecular level with amount of the energy requirements.

3.6.1 EXPERIMENTAL

3.6.1.1 Chemicals and Reagents

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

3.6.1.2 Equipments and Chromatographic conditions

The equipments and chromatographic conditions are as described in section 3.5.1.2.

3.6.1.3 Preparation of stock, sample and buffer solution

The sample preparation for degradation kinetics study was same as described in 3.5.1.3 except the stressor concentrations and temperatures from stress degradation study.

Acid degradation study: For acid degradation study samples were prepared with stressor concentrations 0.1N, 0.3N and 0.5N HCl; temperatures were 25, 40 and 60^0C, aliquot of 2ml was withdrawn from 0minutes to the 40minutes (4 points).

Alkaline degradation study: For alkaline degradation study samples were prepared with stressor concentrations 0.1N, 0.3N and 0.5N NaOH; temperatures were 25, 40 and 50^0C, aliquot of 2ml was withdrawn from 0minutes to the 20minutes (4 points).

Oxidative degradation kinetics: For oxidative degradation study samples were prepared with stressor concentrations 1%, 3% and 6% H_2O_2; temperatures were 25, 40 and 60^0C, aliquot of 2ml was withdrawn from 0minutes to the 40minutes (4 points).

Neutral degradation study: For acid degradation study samples were prepared with water; temperatures were 25, 40 and 60^0C, aliquot of 2ml was withdrawn from 0minutes to the 120minutes (4 points).

3.6.1.4 Degradation kinetic parameters

The degradation kinetic parameters were calculated using equation 1 to 7 (ch.1. sec.1.2.6) 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.

3.6.2. Result and discussion

The study of degradation kinetics for TRZ was completed by preparing the samples as described in section 3.6.1.3 and calculating the parameters as described in section 3.6.1.4. The results for each condition are shown below;

3.6.2.1. Acid degradation kinetics

Acid degradation kinetics was studied in different acid concentration and temperatures at different time. The plots of % drug versus time, *ln C* versus time and 1/C versus time created and strongest correlation coefficients were observed in *ln C* versus time plot. The correlation coefficient and regression equation for zero order, first order and second order reaction is shown in Table 3.11.

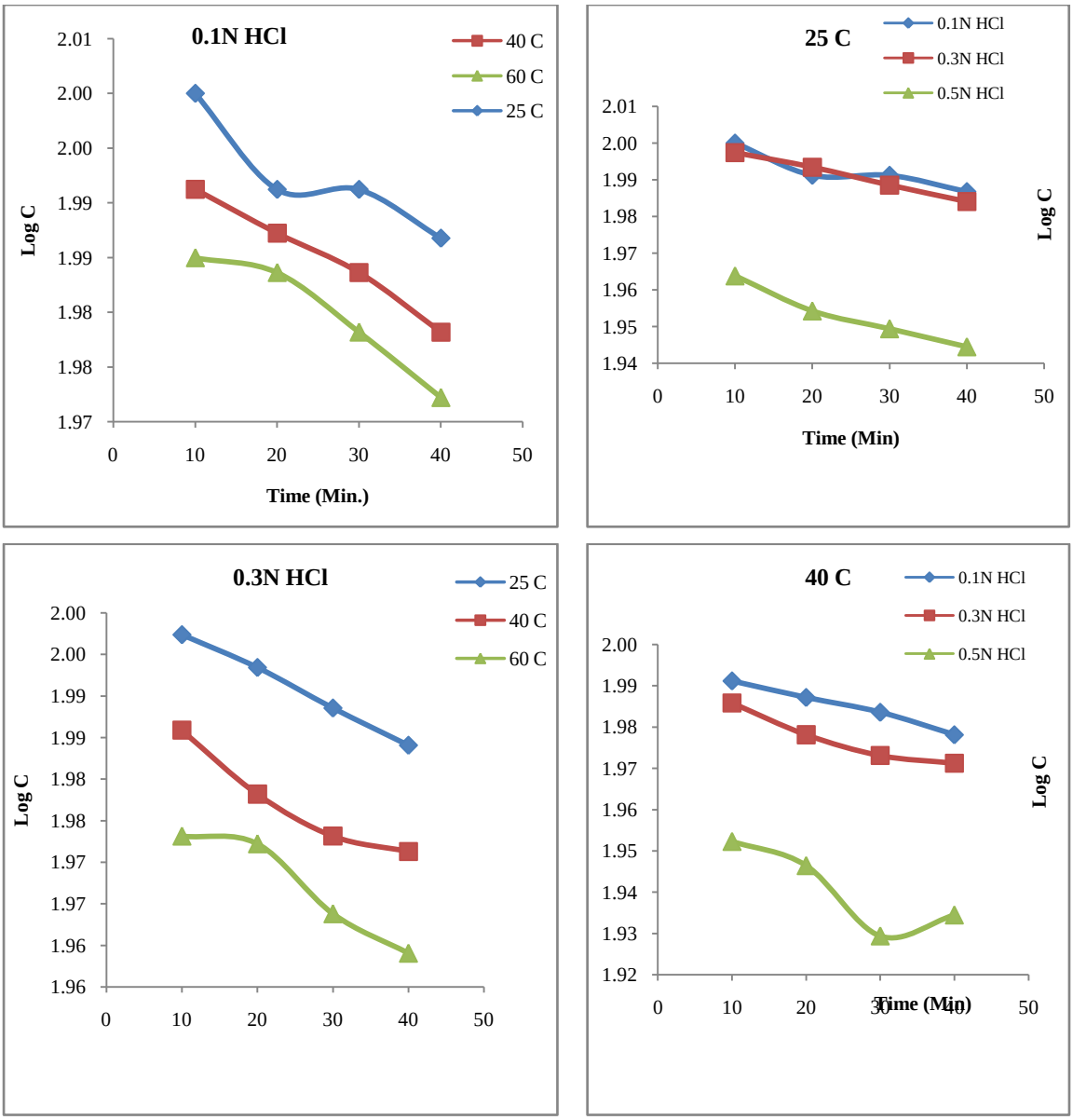
		R^2 Value			Regression equation		
Conc. HCl	**Temp. (^{0}C)**	**Zero order**	**First order**	**Second order**	**Zero order**	**First order**	**Second order**
0.1 N	25	0.852	**0.854**	0.853	y = -0.09x + 100.5	y = -0.0004x + 2.00	y = 0.0000x + 0.01
	40	0.989	**0.991**	0.990	y = -0.095x + 99.0	y = -0.00043x + 1.99	y = 0.0000x + 0.010
	60	0.939	**0.941**	0.940	y = -0.096x + 97.85	y = -0.00044x + 1.99	y = 0.0000x + 0.010
0.3N	25	0.997	**0.998**	0.997	y = -0.101x + 100.4	y = -0.00045x + 2.00	y = 0.0000x + 0.01
	40	0.931	**0.933**	0.930	y = -0.107x + 97.55	y = -0.00049x + 1.98	y = 0.0000x + 0.01
	60	0.928	**0.930**	0.929	y = -0.108x + 95.4	y = -0.00051x + 1.97	y = 0.0000x + 0.01
0.5N	25	0.965	**0.968**	0.967	y = -0.93x + 93.0	y = -0.00063x + 1.96	y = 0.0000x + 0.01
	40	0.746	**0.748**	0.747	y = -0.142x + 90.8	y = -0.00070x + 1.95	y = 0.0000x + 0.011
	60	0.869	**0.870**	0.868	y = -0.19x + 89.0	y = -0.00097x + 1.94	y = 0.0000x + 0.01

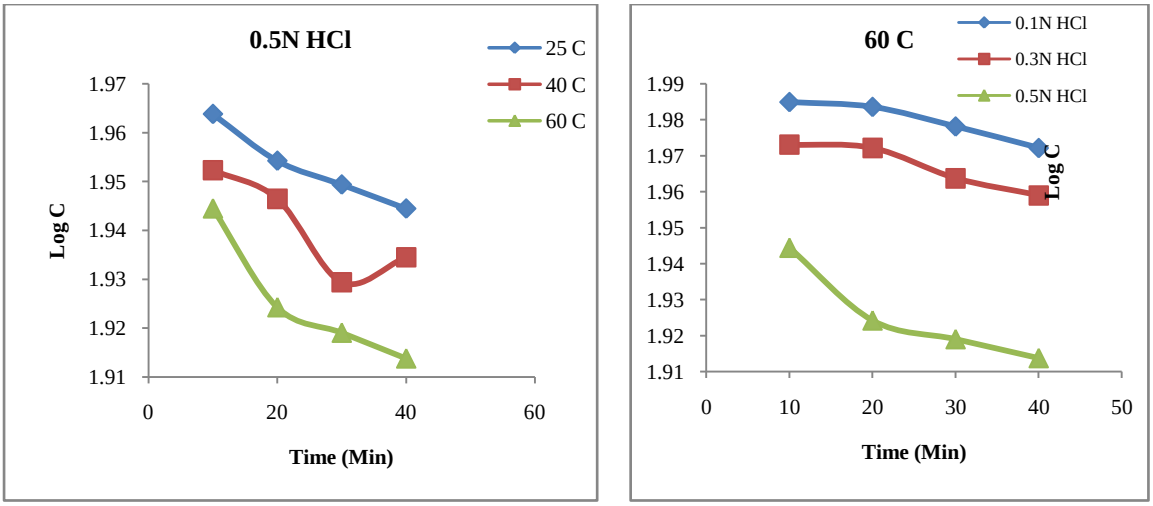
Table 3.11 Regression equations and r^2 value for acid degradation kinetic

It can be seen from Table 3.12 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

The effect of stressor concentration and temperature is shown in Fig 3.12 (A) and (B) respectively.





A **B**

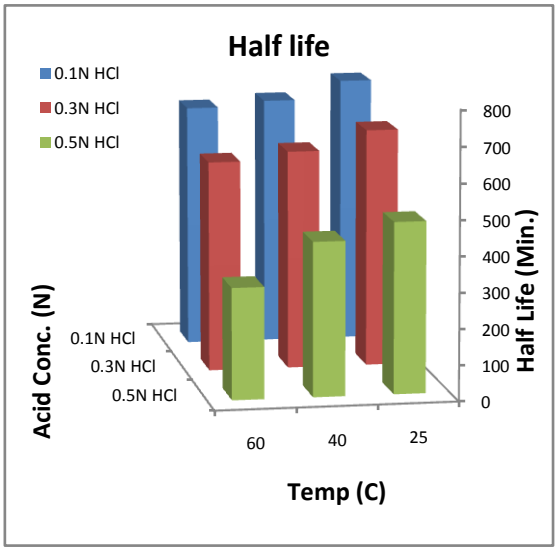
Fig. 3.12 First order of reaction showing (A) effect of temperatures (B) effect of stressor concentration

The Fig 3.12 shown the effect of stressor concentration and temperatures; it can be seen from plots that in 0.1N HCl degradation rate was slow while in 0.5N HCl maximum degradation was observed. The degradation rate was slow at low temperatures while maximum at high temperature.

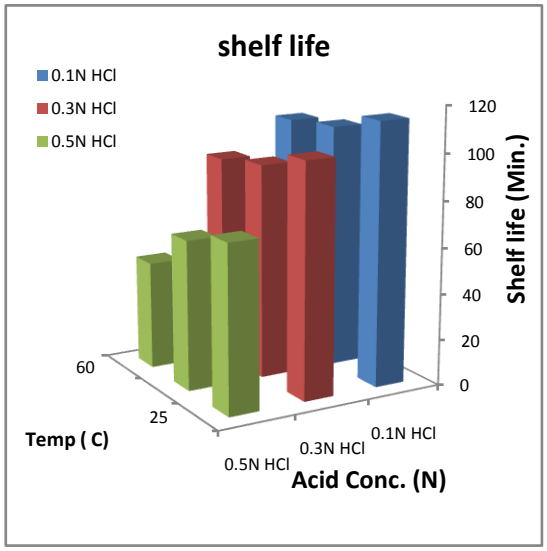
Effect of temperature and stressor concentration on kinetic parameters

The degradation kinetic parameters were calculated for first order reaction; the parameters were rate constant (k), half life (t_{50}), shelf life (t_{90}), and activation energy (E_a), enthalpy of activation ($\Delta H^{\ddagger}$) and entropy of activation ($\Delta S^{\ddagger}$). The effects of temperature and stressor concentrations were studied on these parameters. It is shown in Fig. 3.13; (A) a 3D plots show the effect of temperature and acid concentration simultaneously on half life of TRZ in acid medium. The longest half life was observed in 0.1N HCl at 25^0C, as the temperature and concentration of acid increased the half life of TRZ was decreased in acid medium. (B) Shelf life of TRZ decreased in solution as the temperature and concentration of acid was increased. (C) Rate Constant was calculated by equation 4 and 5 (section 1.2.6), equation and graphical method respectively. Degradation rate constant increased with temperature and stressor concentration indicates that reaction rate was increased with increment in acid concentration and temperature. The reaction was temperature and acid- catalyzed. (D) Energy of activation: Activation energy of reaction was calculated from slope (m) of regression equation of Arrhenius Plot (*ln k* versus 1/t (K)) using equation 3 (Section 1.2.6).

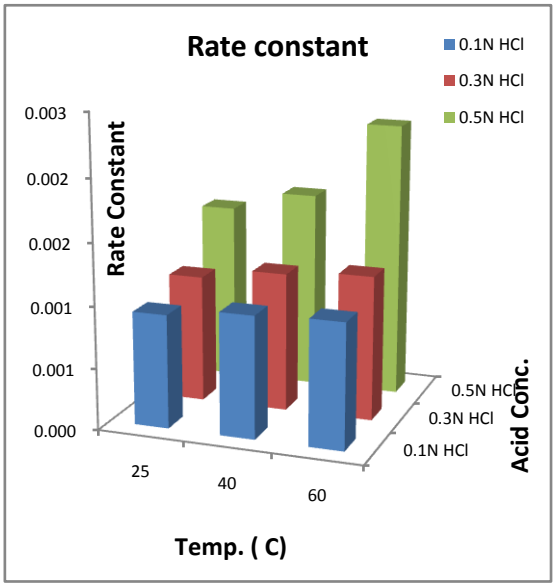
The Activation energy of the reaction decreased with increase in these two factors indicates that temperature and stressor concentration provided external energy that reduced the internal energy of reaction.



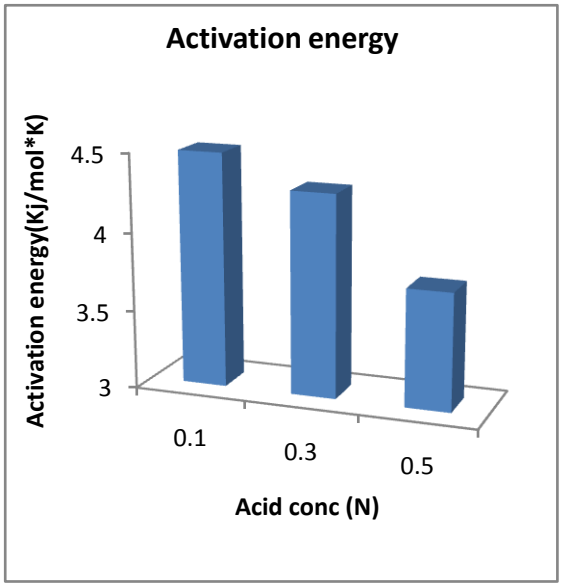
A



B



C



D

Fig.3.13 Effect of temperature and stressor concentration on **A)** Half life **B)** shelf life **C)** Rate constant, and **D)** Activation energy of TRZ acid degradation kinetics

The degradation kinetic parameters were calculated using equations for first order of reaction. The rate constant was calculated using equation 4 and 5 for equation and graphical method,

the half life was calculated using equation 6 and shelf life was calculated using equation 7. The effect of temperature and concentration of acid on kinetic parameters are discussed. The kinetic parameters are shown in Table 3.12.

Parameters			**From Graph**	**From Equation**		
Conc. (N HCl)	**Temp. (^{O}C)**	**Slope**	**k**	**k**	**t_{50} [Min]**	**t_{90} [Min]**
	25	0.00040	0.00092	0.00094	752.27	113.98
0.1	40	0.00043	0.00099	0.0010	699.79	106.02
	60	0.00044	0.00101	0.0011	683.89	103.01
	25	0.00045	0.00103	0.00129	668.69	101.3
0.3	40	0.00049	0.00112	0.00130	614.10	93.0
	60	0.00051	0.00117	0.00132	590.02	89.3
	25	0.00063	0.00145	0.00149	477.63	72.3
0.5	40	0.00070	0.00161	0.0017	429.87	65.1
	60	0.00097	0.00223	0.0021	310.21	47.0

Table 3.12 Arrhenius Plot and activation parameters of TRZ in different acid concentrations

Arrhenius Plot and activation parameters for degradation of TRZ

Arrhenius plot (Fig.3.14) was constructed for *ln k* versus 1/Temperature (unit Kelvin) to study the effect of temperature, energy of activation and degradation rate of TRZ degradation. A straight line indicates that reaction was thermally activated and can be used to calculate energy of activation for the reaction.

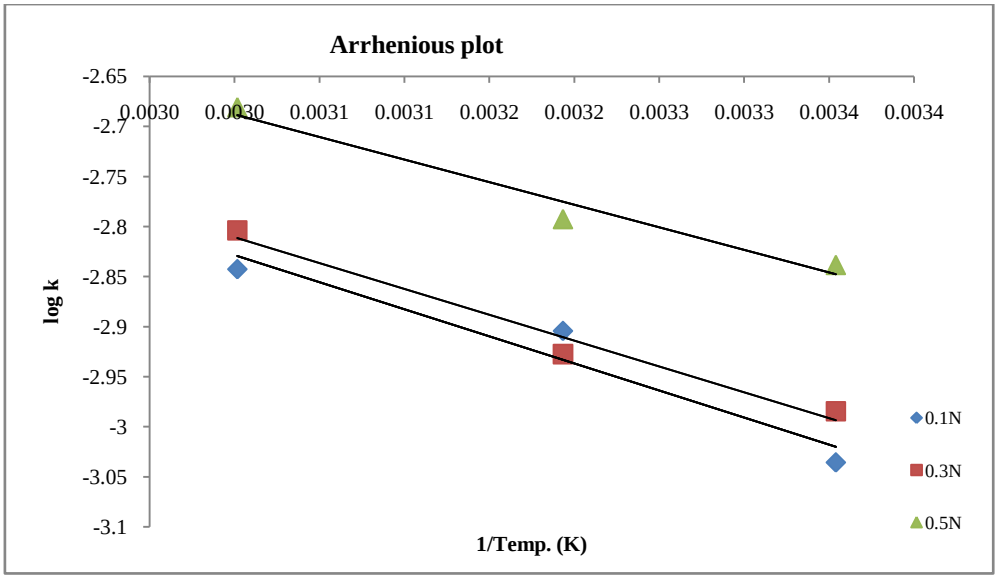


Fig. 3.14 Arrhenius plot for degradation kinetics of TRZ

The activation parameters were calculated from Arrhenius plot. The activation parameters were; enthalpy of activation ($\Delta H^{\updownarrow}$), entropy of activation ($\Delta S^{\updownarrow}$) and energy of activation for reaction were calculated using equation 1, 2 and 3 respectively and shown in Table 3.13. The reaction in system brings out rearrangement of molecules and lead disarrangements in system, this disarrangement of system at start and end of reaction can be measured by entropy. The negative value of entropy shows less disarrangement in system. The linear equations of Arrhenius plot has correlation coefficient r>0.90 for the range of temperature 25-60^{0}C, slope of the equations in Arrhenius equation (Ea/R) were large this indicated TRZ molecule was stable at lower pH (acid) and this statement is reliable in concern of effect of pH on stability of TRZ. The enthalpy values are positive indicating that the reaction was endothermic and absorbed the externally applied energy to speed up the reaction as system enthalpy was less than the environmental enthalpy.

Acid	**Activation**	**r**	**Ea(kJ/mol)**	**$\Delta H^{\updownarrow}$**	**$\Delta S^{\updownarrow}$**
0.1N	y = -516.4x - 1.261	0.975	4.29	2.02	-49.58
0.3N	y = -540.9x - 1.205	0.935	4.49	2.22	-48.83
0.5N	y = -451.1x - 1.334	0.965	3.75	1.48	-51.57

Table 3.13 Arrhenius Plot and activation parameters of TRZ in different acid solutions

3.6.2.2.Alkali degradation kinetics

Alkaline degradation kinetics study was completed in same manner of acid degradation study. The stressor concentration was 0.1N, 0.3N and 0.5N NaOH at 25, 40 and 50^{0}C for 20minutes with 5minutes interval.

The correlation coefficients and regression equations for zero order, first order and second order reaction is shown in Table 3.14.

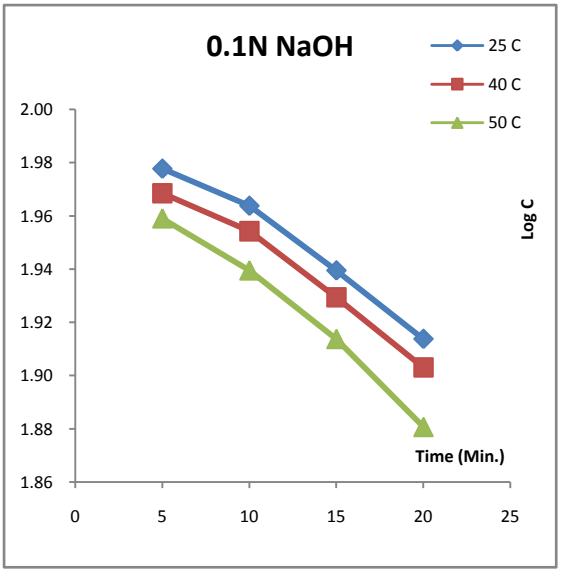
		R^2 Value			Regression equation		
Conc. [N NaOH]	**Temp. (^{0}C)**	**Zero order**	**First order**	**Second order**	**Zero order**	**First order**	**Second order**
0.1	25	0.983	**0.987**	0.979	y = -0.088x + 100.5	y = -0.0043x + 2.00	y = 0.0000x + 0.01
	40	0.983	**0.987**	0.978	y = -0.086x + 98.0	y = -0.0044x + 1.99	y = 0.0000x + 0.010
	50	0.982	**0.986**	0.980	y = -0.082x + 96.5	y = -0.0052x + 1.98	y = 0.0000x + 0.010
0.3	25	0.979	**0.986**	0.977	y = -1.12x + 96.0	y = -0.0059x + 1.98	y = 0.0000x + 0.01
	40	0.996	**0.997**	0.994	y = -1.12x + 93.5	y = -0.0061x + 1.97	y = 0.0000x + 0.01
	50	0.993	**0.997**	0.995	y = -1.08x + 90.5	y = -0.0062x + 1.96	y = 0.0000x + 0.01
0.5	25	0.867	**0.870**	0.868	y = -1.32x + 89.5	y = -0.0077x + 1.95	y = 0.0000x + 0.01
	40	0.865	**0.869**	0.866	y = -1.30x + 87.5	y = -0.00079x + 1.94	y = 0.0000x + 0.011
	50	0.894	**0.895**	0.892	y = -1.28x + 84.5	y = -0.0081x + 1.98	y = 0.0000x + 0.01

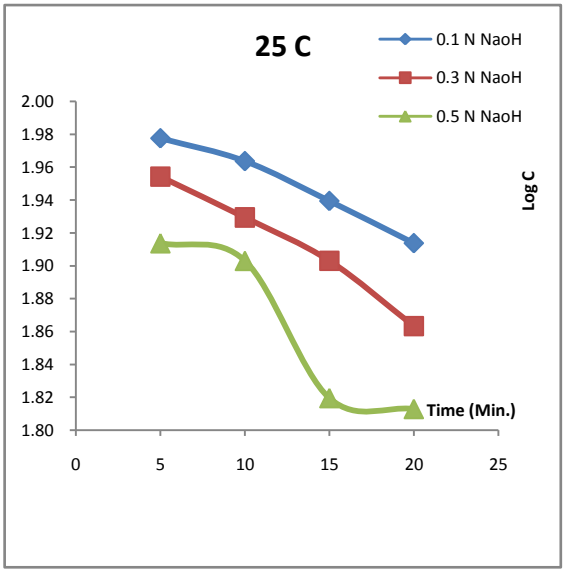
Table 3.14 Regression equations and correlation coefficients for alkali degradation kinetics of TRZ

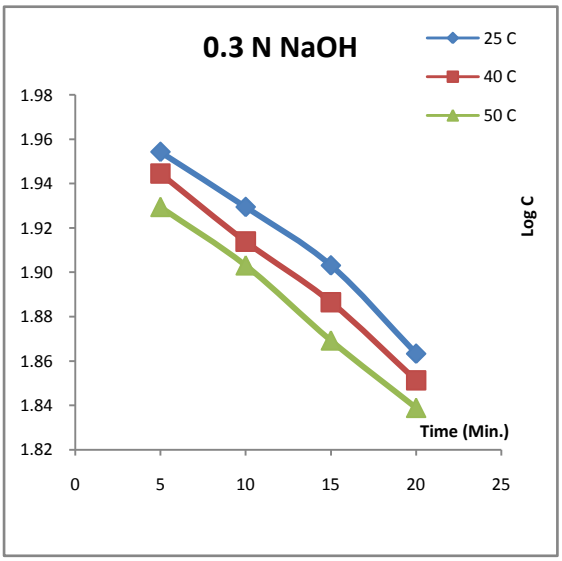
The Table 3.14 shows that strongest correlation coefficient was observed for first order of reaction. This indicates that TRZ in alkaline media followed first order of kinetics.

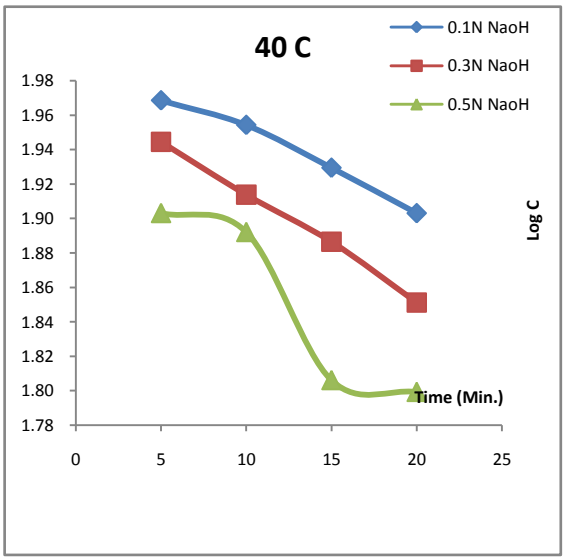
Effect of temperature and stressor concentration on TRZ degradation

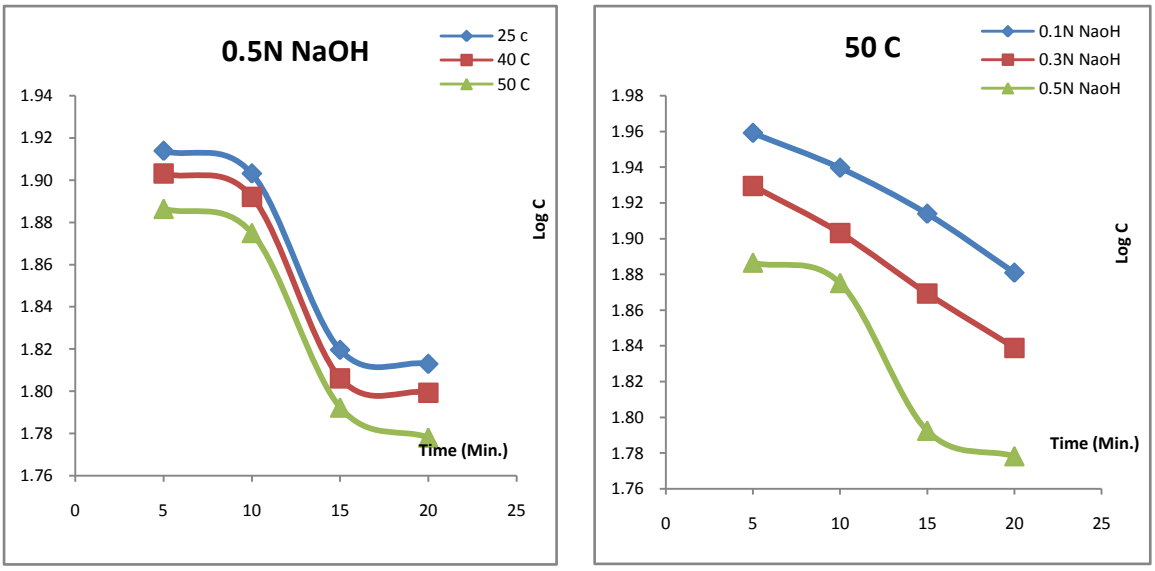
The effect of temperature and stressor concentration on TRZ degradation in alkaline medium is shown in Fig 3.15.











A **B**

Fig. 3.15 Effect of (A) temperatures (B) alkali concentration on TRZ degradation

The analysis of Fig 3.14 shows that TRZ is sensitive to alkaline media, at low concentrations (0.1N and 0.3N) the degradation rate was slow and steady while in 0.5N NaOH there is a steep fall after 10minutes at 25, 40, or 50^0C. The chromatogram of TRZ in alkaline media (0.1N, 0.3N, and 0.5N NaOH after 20min.) shows that there was not a single major degradation product but only three small peaks passed purity test. These might be because of the total rupture of the TRZ chemical structure. There is huge effect of alkali on TRZ and addition of temperature might have boosted the reaction rate.

Effect of temperature and stressor concentration on kinetic parameters

The effect of stressor concentration and temperature on kinetic parameters was evaluated by 3D plot of kinetic parameters at different temperatures and different alkali concentration shown in Fig 3.16.

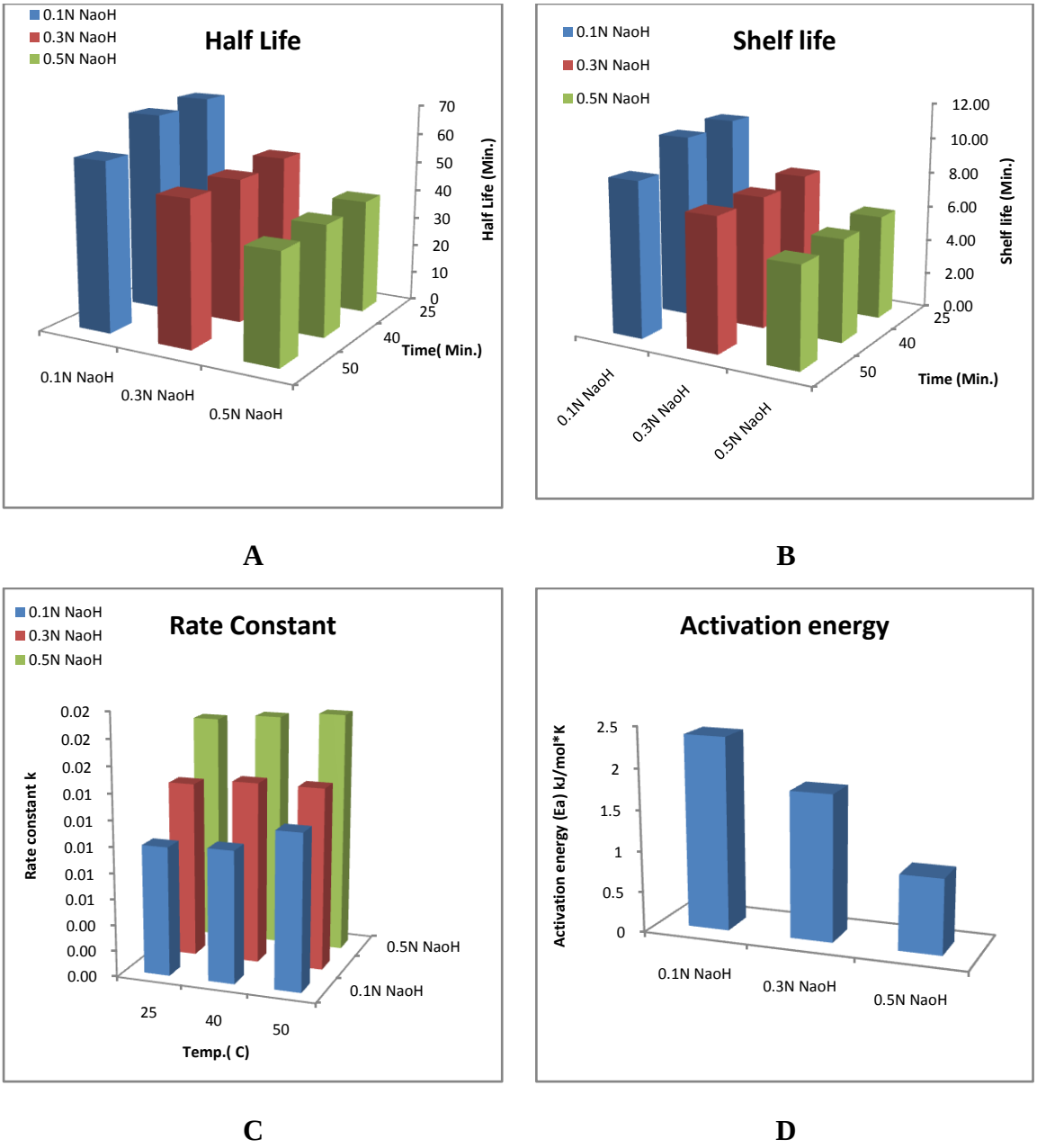


Fig. 3.16 Effect of stressor concentration and temperature on (A) Half life (B) Shelf life (C) Rate constant (D) activation energy

The analysis of Fig 3.16 shows that half-life and shelf life decreased with an increase in temperatures and stressor concentration; this clearly indicates that increasing stressor concentration and temperature can increase the rate of degradation of TRZ. The rate constant increases with increasing the factors (temperatures and stressor concentrations); this might be because of increase in degradation rate of TRZ. The decrease in activation energy of reaction with factor increase indicates that the reaction system had the low energy system than atmosphere but factors provided external energy to boost the reaction rate. The kinetic

parameters calculated for alkaline degradation of TRZ is shown in Table 3.15. The parameters calculated for TRZ alkali degradation were rate constant (equation and graphical method), half life, shelf life, and activation energy.

		From Graph			From Equation			
Conc. NaOH	**Temp. (^{O}C)**	**Slope**	**k**	**Log k**	**k**	**Log k**	**t_{50} [min]**	**t_{90} [min]**
	25	0.0043	0.0099	-2.00	0.00990	-2.00	69.65	10.55
0.1N	40	0.0044	0.0101	-1.99	0.01150	-1.93	68.07	10.31
	50	0.0052	0.0119	-1.92	0.01370	-1.86	57.86	8.76
	25	0.0059	0.0135	-1.86	0.0157	-1.80	51.00	7.72
0.3N	40	0.0061	0.0140	-1.85	0.0171	-1.76	49.33	7.47
	50	0.0062	0.0142	-1.80	0.0185	-1.73	49.33	7.47
	25	0.0077	0.0177	-1.75	0.0215	-1.66	39.07	5.92
0.5N	40	0.0079	0.0181	-1.74	0.0231	-1.63	38.09	5.77
	50	0.0081	0.0186	-1.72	0.0255	-1.59	37.15	5.62

Table 3.15 Degradation kinetic parameters for TRZ in alkaline medium

The kinetic parameters and effect of factors are already discussed under Fig 3.15.

Arrhenius Plot and activation parameters for TRZ alkaline degradation

Arrhenius plot was constructed using *ln k* versus 1/temperature (kelvin) for three temperatures 25, 40 and 50^{0}C and three alkali concentrations 0.1N, 0.3N and 0.5N NaOH. (Fig. 3.17)

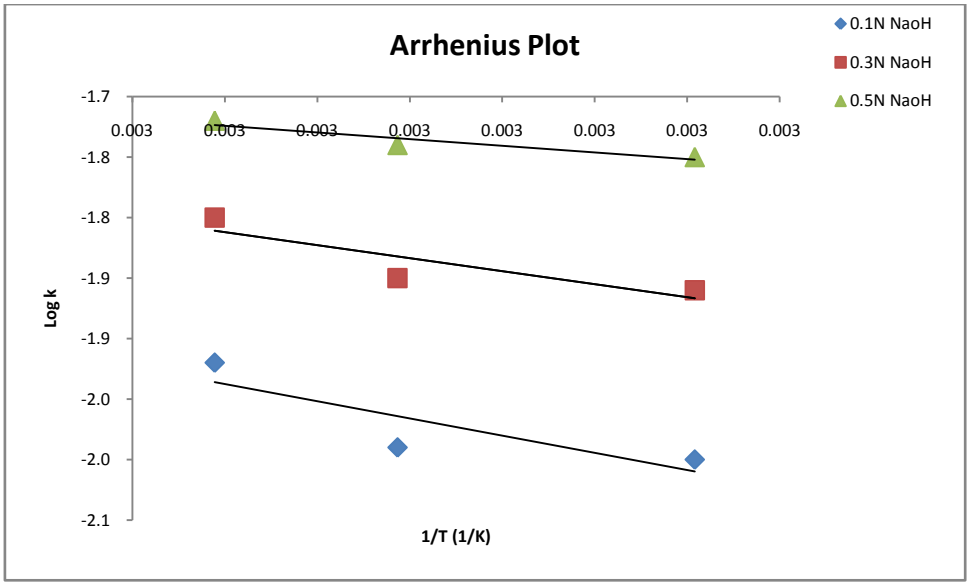


Fig. 3.17 Arrhenius plot for alkaline degradation of TRZ

The straight line of plot indicates that reaction was linear and capable to calculate the activation energy of reaction. The activation parameters for TRZ alkaline degradation is shown in Table 3.16.

Acid	Activation	r	Ea(kJ/mol)	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$
0.1N	y = -284.5x - 1.055	0.766	2.36	9.52	-52.11
0.3N	y = -214.9x - 1.145	0.730	1.78	4.83	-54.23
0.5N	y = -110.4x - 1.381	0.896	0.91	1.35	-57.42

Table 3.16 Arrhenius plot and activation parameters for TRZ in alkaline medium

The table shows enthalpy of reaction and entropy of reaction. Higher the difference in entropy value indicates the more disarrangement in system while lower difference indicates the fewer atoms of group movements in system. The positive enthalpy value indicates that system energy was lower than environmental energy status.

3.6.2.3.Neutral degradation kinetics

The degradation of TRZ in neutral pH (water) and its degradation products (chromatogram, degradation products, and major degradation product and peak purity) are shown in section 3.5.2.3. For degradation kinetic study stressor was water foe neutral pH and temperatures selected for study were 25, 40 and 60^0C for 120minutes (4 points).

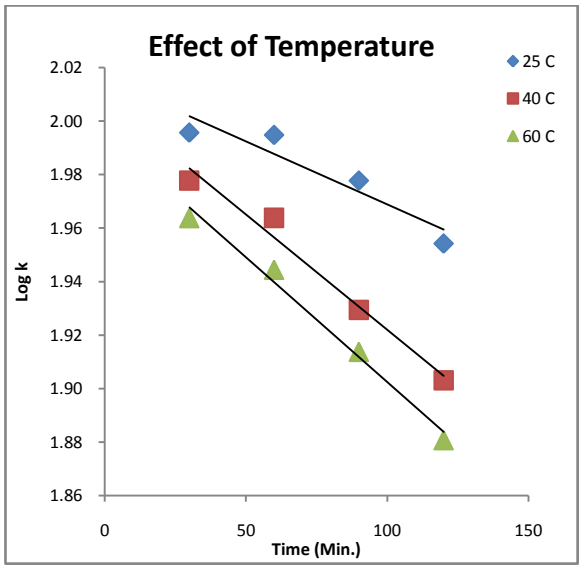
The regression equations and correlation coefficients are shown in Table 3.17 for zero, first and second order of reaction.

		R^2 Value			Regression equation		
	T (^{0}C)	Zero order	First order	Second order	Zero order	First order	Second order
H_2O	25	0.861	**0.864**	0.863	y = -0.102x + 103.4	y = -0.00047x + 2.01	y = 0.000x + 0.009
	40	0.974	**0.976**	0.975	y = -0.173x + 101.0	y = -0.00086x + 2.00	y = 0.004x + 0.01
	60	0.986	**0.987**	0.985	y = -0.186x + 98.0	y = -0.00093x + 1.99	y = 0.003x + 0.01

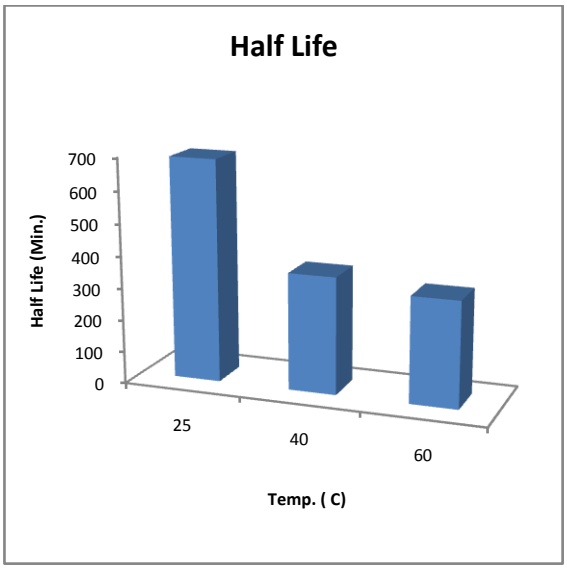
Table 3.17 Regression equations and correlation coefficients for neutral degradation kinetics of TRZ

The effect of stressor and temperatures on degradation of TRZ and kinetic parameters

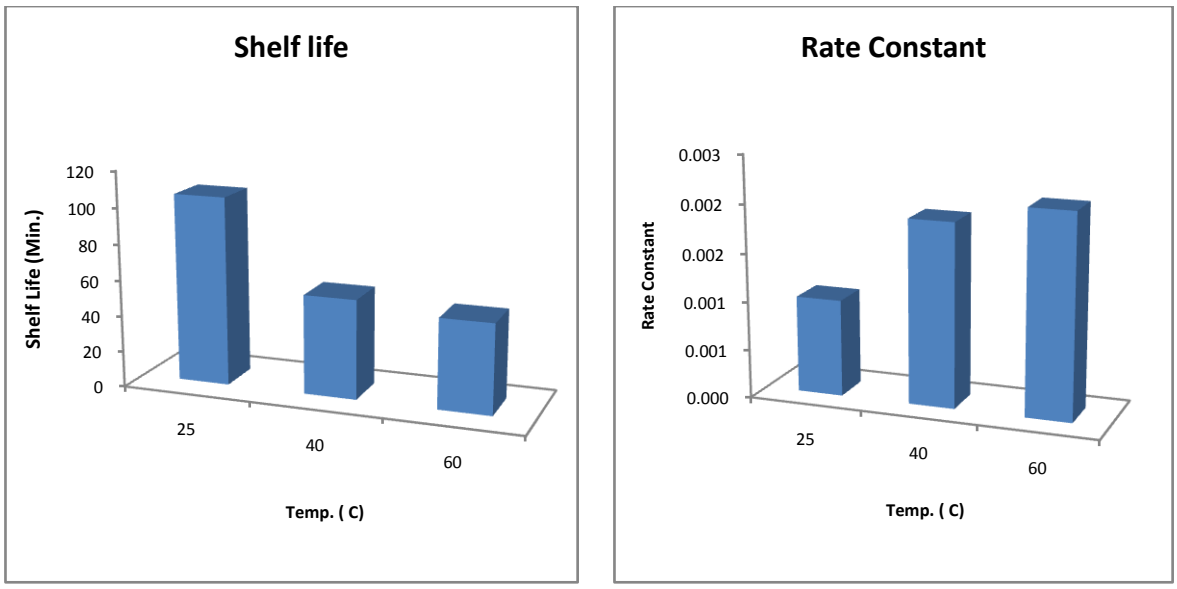
The effect of stressor and temperatures on TRZ degradation and kinetic parameters are shown in Fig 3.18.



A



B



C **D**

Fig. 3.18 stressor and temperatures effects on (A) TRZ degradation (B) half life (C) shelf life and (D) rate constant

The Fig. 3.18 (A) shows that degradation of TRZ in water was linear but increasing the temperature resulted in decrease in the stability, half life and shelf life of TRZ. The increase in rate constant value shows that temperature increment would increase the degradation rate of TRZ. Energy of activation was not calculated as different stressor concentration factor was not available for this study.

The degradation kinetic parameters for TRZ degradation in neutral condition are shown in Table 3.18.

	Graphical method			Equation method			
Temp. (^{0}C)	**Slope**	**K**	**Log K**	**K**	**Log K**	**t_{50} min.**	**t_{90} min.**
25	0.00047	0.001	-3.000	0.0011	-2.959	693.00	105.00
40	0.00086	0.0019	-2.721	0.0021	-2.678	364.74	55.26
60	0.00093	0.0021	-2.678	0.0024	-2.620	330.00	50.00

Table 3.18 Degradation kinetic parameters for TRZ

Arrhenius Plot and activation parameters for TRZ alkaline degradation

Arrhenius plot for neutral sample was constructed for *ln k* versus 1/temperature (kelvin) shown in Fig 3.19.

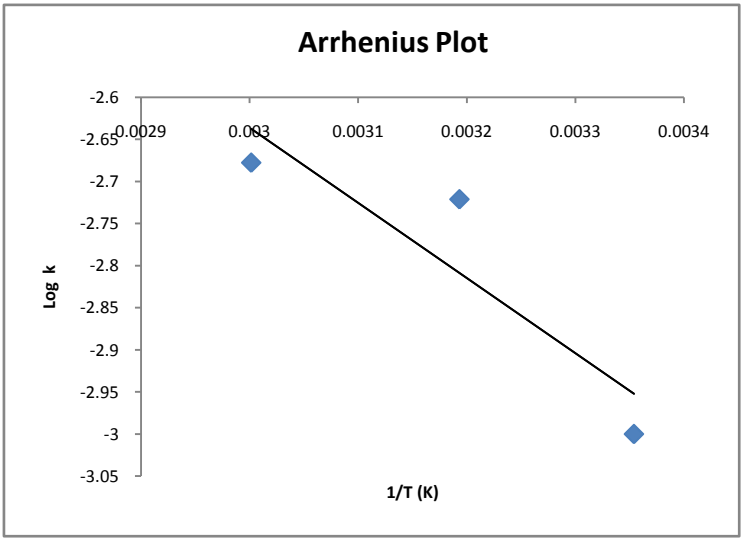


Fig. 3.19 Arrhenius plot for neutral degradation of TRZ

The activation energy for TRZ degradation at all temperature was 7.420 kj/mol*k. The enthalpy and entropy of system is shown in Table 3.19. The positive enthalpy value indicates that system energy was lower than the environmental energy; the energy source was temperature in the reaction. Moreover entropy value was negative indicates less molecular movement.

Stressor	Activation	r	Ea(kJ/mol)	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$
Water	y = -892.5x + 0.041	0.811	7.42	51.50	-38.13

Table 3.19 Arrhenius plot and activation parameters of TRZ neutral degradation

3.6.2.4.Oxidative degradation kinetics

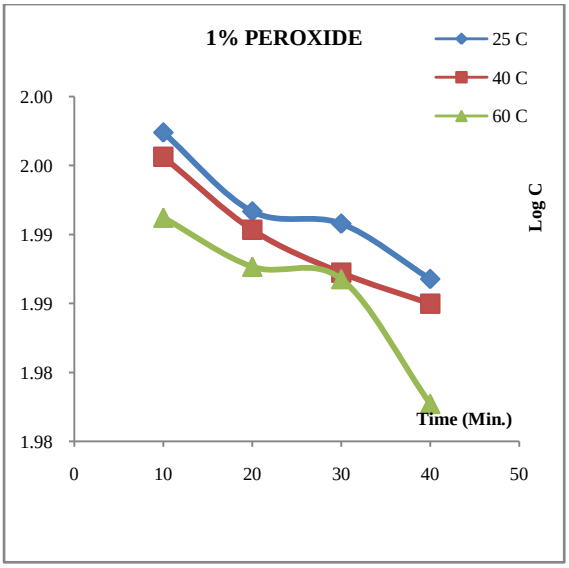
The degradation of TRZ in presence of oxidative agent is shown in section 3.5.2.3 with chromatogram of peroxide degraded TRZ sample and peak purity results. For oxidative degradation, selected stressor concentrations were 1%, 3% and 6% oxidizing agent at temperature 25, 40 and 60^0C for 40minutes (4 points). The analysis of order of reaction was carried out by correlation coefficient method. Plot of % drug versus time, *ln C* versus time and 1/C versus time were constructed and correlation coefficients were observed. Strongest correlation coefficients were noticed in *ln C* versus time plots. The data is shown in Table 3.20.

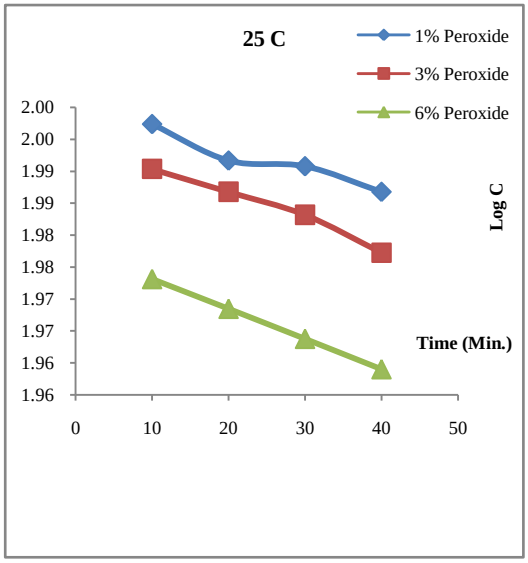
		R^2 Value			Regression equation		
% H_2O_2	T (^{0}C)	Zero order	**First order**	Second order	Zero order	**First order**	Second order
1%	25	0.931	**0.933**	0.932	y = -0.074x + 99.9	**y = -0.00033x + 1.99**	y = 0.000x + 0.0100
	40	0.960	**0.963**	0.962	y = -0.079x + 99.6	**y = -0.00035x + 1.99**	y = 0.000x + 0.0100
	60	0.864	**0.867**	0.862	y = -0.092x + 99.1	**y = -0.00041x + 1.99**	y = 0.000x + 0.0100
3%	25	0.981	**0.983**	0.982	y = -0.095x + 98.5	**y = -0.00043x + 1.99**	y = 0.000x + 0.01
	40	0.835	**0.839**	0.837	y = -0.094x + 94.3	**y = -0.00045x + 1.97**	y = 0.000x + 0.01
	60	0.811	**0.816**	0.813	y = -0.095x + 91.35	**y = -0.00047x + 1.96**	y = 0.000x + 0.01
6%	25	0.980	**0.981**	0.980	y = -0.145x + 95.0	**y = -0.00047x + 1.97**	y = 0.000x + 0.01
	40	0.825	**0.826**	0.821	y = -0.118x + 93.4	**y = -0.00057x + 1.97**	y = 0.000x + 0.01
	60	0.986	**0.987**	0.985	y = -0.161x + 91.6	**y = -0.0008x + 1.96**	y = 0.000x + 0.01

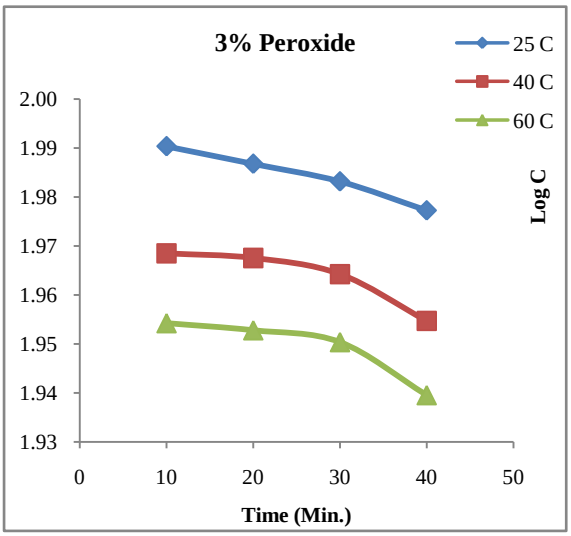
Table 3.20 correlation coefficients and correlation equations for oxidative degradation of TRZ

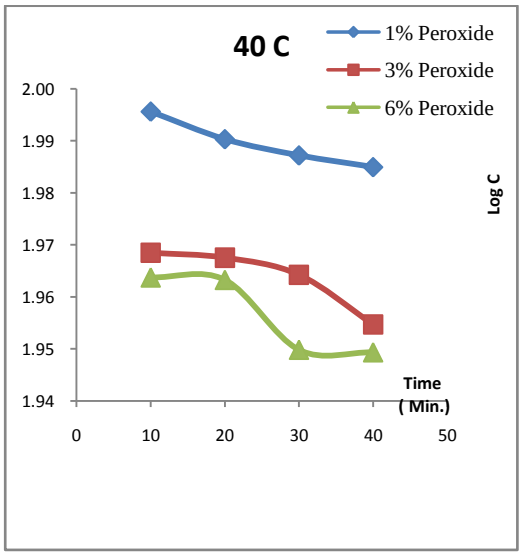
Effect of temperature and peroxide concentration on TRZ degradation

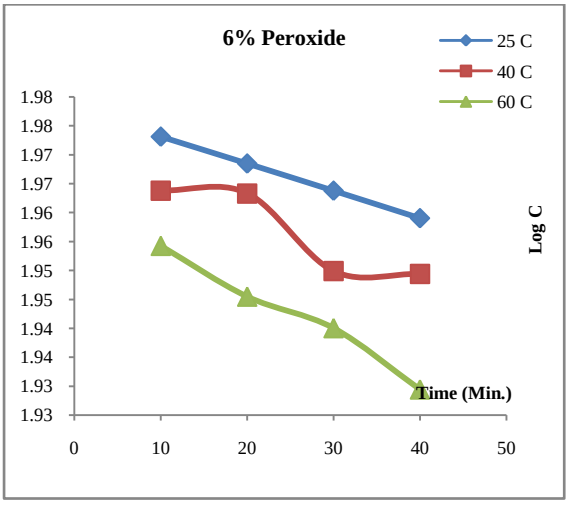
The effect of hydrogen peroxide concentrations on TRZ degradation by oxidizing agent was illustrate by plotting temperature at different concentration while to know the temperature effect, plot was constructed for concentration at different temperatures. The plots are shown in Fig 3.20.

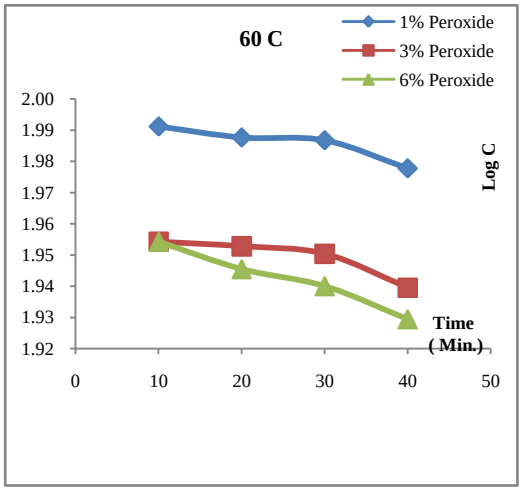












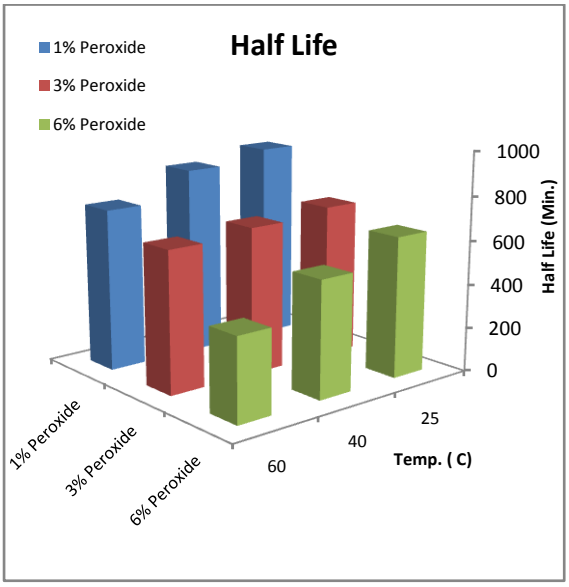
A **B**

Fig. 3.20 Effect of (A) temperatures (B) stressor concentration on TRZ degradation in peroxide medium

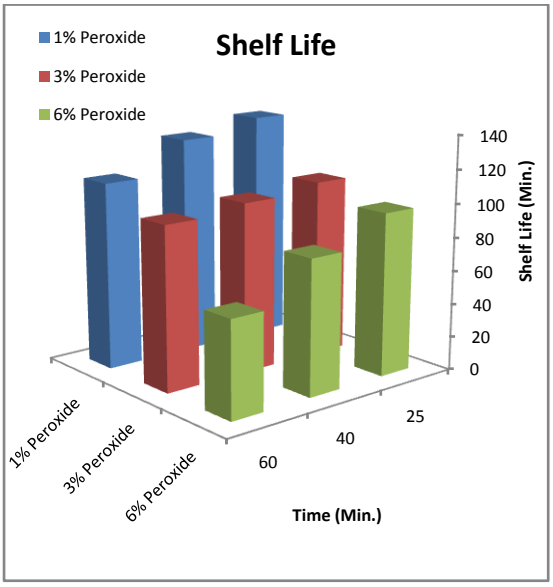
Fig 3.20 shows that degradation rate of TRZ increased with increase in stressor concentration, and increase in temperature also affected the degradation rate of TRZ. The degradation rate was slow at 25^0C (room temperature) in all stressor concentration while increasing the temperature speed up the degradation of TRZ.

The effect of stressor concentrations and temperatures on kinetic parameters

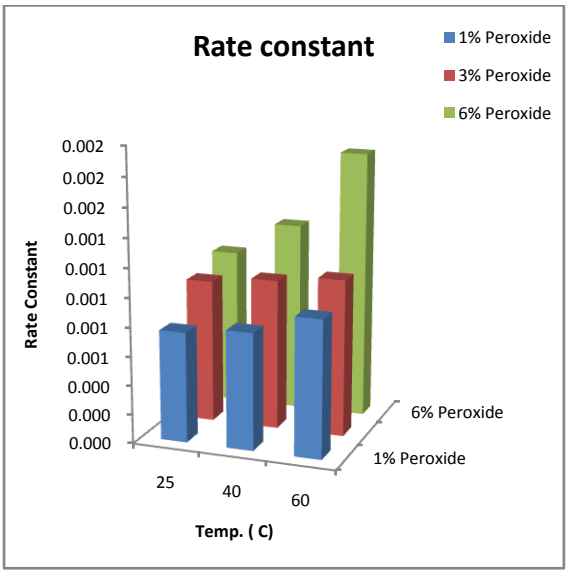
The stressor concentration and temperature effect on kinetic parameters are shown in Fig 3.21.



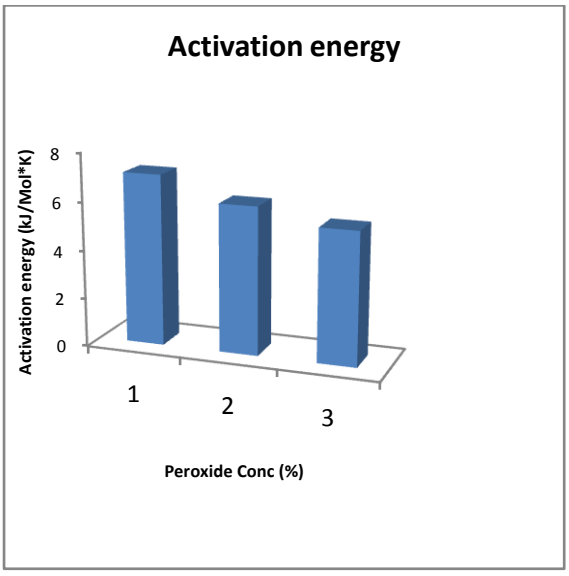
A



B



C



D

Fig.3.21 The effect of temperatures and stressor concentrations on (A) Half life (B) Shelf life (C) Rate constant (D) Activation energy

The analysis of figure clearly indicates that temperature and stressor concentration together had the negative effect on kinetic parameters such as half life and shelf life. While it showed positive effect on rate constant indicates the increase in degradation rate and formation of DPs with structural changes/rupture of TRZ physical and chemical stability. Decrease in activation energy shows that the environmental energy was source of energy to boost the degradation reaction of TRZ in presence of oxidizing agent. The degradation kinetic parameters are shown in Table 3.21.

		From Graph			**From Equation**			
Conc. (%)	**Temp. ^{o}C**	**Slope**	**k**	**Log K**	**K**	**Log K**	**t_{50} (Min.)**	**t_{90} (Min.)**
1% H_2O_2	25	0.00033	0.00076	-3.119	0.0008	-3.094	911.8	138.1
	40	0.00035	0.00081	-3.094	0.0008	-3.070	859.7	130.2
	60	0.00041	0.00094	-3.025	0.0009	-3.014	733.9	111.2
3% H_2O_2	25	0.00043	0.00099	-3.104	0.0010	-2.984	699.7	106.2
	40	0.00047	0.00103	-2.984	0.0010	-2.974	699.7	101.3
	60	0.00051	0.00108	-2.965	0.0011	-2.947	668.6	97.0
6% H_2O_2	25	0.00047	0.00108	-2.965	0.0010	-2.965	640.2	97.0
	40	0.00057	0.00131	-2.881	0.0013	-2.874	640.2	79.9
	60	0.00080	0.00184	-2.734	0.0018	-2.729	527.9	56.9

Table 3.21 Degradation kinetic parameters for TRZ oxidative degradation

Arrhenius Plot and activation parameters for TRZ oxidative degradation

The Arrhenius plot for degradation of TRZ in presence of oxidizing agent was created using *ln k* versus 1/temperature (kelvin) to study the linearity of degradation of TRZ and calculate the activation energy. (Fig. 3.22)

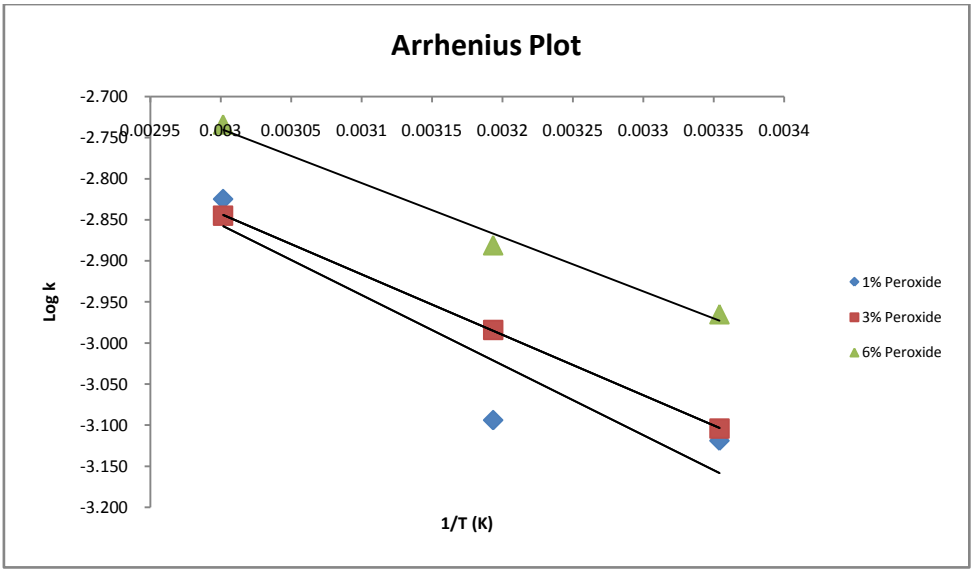


Fig.3.22 Arrhenius plot and activation parameters for TRZ degradation in oxidative medium

The linear trade line for Arrhenius plot shows that degradation of TRZ was in linear manner and can be used to calculate the activation energy using slope of regression equations. The enthalpy and entropy were also calculated for the reaction system to understand the amount of disarrangement and energy source of reaction. (Table 3.22)

Stressor H_2O_2	**Activation**	**r**	**Ea(kj/mol)**	**$\Delta H^{\ddagger}$**	**$\Delta S^{\ddagger}$**
1%	y = -852.4x - 0.299	0.851	7.08	11.75	-45.24
3%	y = -734.7x - 0.639	0.991	6.10	14.05	-42.94
6%	y = -659.1x - 0.762	0.988	5.47	17.64	-39.35

Table 3.22 activation parameters for TRZ oxidative degradation

The positive enthalpy value indicates that reaction was endothermic and energy was obtained from environment; similar conclusion was noticed in activation energy study. The entropy values are close in three stressor concentration indicates that less atomic movements were occurred in reaction.

- **Conclusion**

The degradation kinetic study was completed using different temperatures and different stressor concentrations. The *ln C versus t* plot showed strongest correlation coefficients and TRZ followed first order kinetics for acid, alkaline, neutral and oxide induced reactions. TRZ is stable under the thermal conditions for more than 28 days indicates that hydrolytic and oxidative reactions of TRZ were the simultaneous effect of stressor and temperature. Stressor alone can induce degradation of TRZ but temperature pushes the reaction to speed up. The activation energy and activation parameters showed that stressor induced reaction were endothermic. The kinetic parameters such as half life and shelf life showed highest value at lower temperature and lower stressor concentration while lowest value was observed at higher temperature and stressor concentration, while opposite condition was observed for degradation rate constant, lowest value for higher temperature and stressor concentration and highest value for lower temperature and stressor concentration. The kinetic study suggests that TRZ is prone to degradation under acidic, alkaline, neutral and peroxide conditions; proper precautions should be taken for storage of TRZ.

PART-C

3.7. ISOLATION AND CHARACTERIZATION OF MAJOR DPs

3.7.1 Experimental

3.7.1.1 Chemicals and reagents

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

UPLC/ESI-MS (Ultra Performance Liquid Chromatography/ Electron Spray Ionization-Mass Spectrometer) grade acetonitrile was purchased from Fisher Scientific chemicals, India. Formic acid was purchased from Merck, USA. Ammonia was purchased from sigma-Aldrich, USA. NMR grade deuterated Di Methyl Sulph Oxide (DMSO) was purchased from Sigma-Aldrich (USA).

3.7.1.2 Equipments and chromatographic conditions

The equipments and chromatographic conditions are described in section 3.5.1.2.

The HPLC run was taken on Shimadzu LC-20Prominenece series HPLC system, consist of binary pump with PDA detector (SPD M20 A), manual rheodyne injector (20µl) and data acquisition was done by LC Solution software (Shimadzu, Kyoto, Japan).

UPLC-MS: Study was carried out on Waters Acquity UPLC with quaternary solvent manager with PDA detector, column oven, Acquity ESI performance mass detector and auto sampler. Mass analysis was carried out on single quad mass spectrometer equipped with Waters jet stream Electron Spray Ionization (ESI) source with positive mode (Waters corp. USA). Mass Lynx software was used for the UPLC-PDA and UPLC/ESI-MS data acquisition and analysis of bedaquiline and its DP.

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,) flow : 0.8 ml/min; 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 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.

Preparative HPLC: Isolation of major acid stress degradation was performed on preparative HPLC; Waters 2489, binary, auto sampler and auto fraction collector, high pressure mixing chamber, and UV detector. Rheodyne injector was used to load sample in chromatographic system.

Isolation of major acid stress degradation product was completed using stationary phase YMC actus triart ($C_{18,}$ 250×20mm, 5) column set to ambient temperature. Mobile phase consist of **a)** 0.05% ammonia (NH_3) in water and **b)**: Acetonitrile + 20% mobile phase A with flow rate 18.0ml/min, PDA detection at 270nm. Gradient elution program was set as follows; run time 23minutes; T (min) =% v/v **a) %** v/v**. T=** 0.01(100), **T=** 2 (100), **T** = 17.0 (90), **T=** 17.01 (2), **T=** 20 (2), **T**=20.1 (100) and stop command after **T=**23 (100).

MS/MS was completed on system (Thermoscientific, USA) with MS plus pump and auto sampler. TSQ Quantum access max mass spectrometer was used for analysis. The MS system used spray electron ionization in positive mode with voltage 3500V, Capillary temperature was 300^0C. The Mass transition was observed for M1 and M2 level with collision energy 34 eV for both transition. Xcalibur Software was used for data analysis (Thermo Fisher, USA).

NMR: ^{1}H NMR was performed on Bruker 400MHz NMR spectrometer using deuterated di methyl sulphoxide (DMSO-d6) and D_2O (deuterated water) 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.

3.7.1.3 Sample preparation

Analytical sample, stress degradation sample and buffer preparations were same as described in section 3.5.1.3.

Enrichment of DPs for isolation

High concentration sample was prepared for isolation; accurately weighed 5gm of TRZ was dissolved separately in 15ml DMSO with provisional shaking for 15mintes by sonication; 50ml final volume was achieved using stressor reagent e.g. 0.5N HCl, water or 6% H_2O_2, the solution was kept in specified stability conditions in dark (0.5N HCl at RT for 24hrs, water at RT for 24hrs and 6% H_2O_2 at RT for 5hrs). The 3ml aliquot +1ml acetonitrile+1ml water was filtered through whatman filter paper prior to filtering by 0.45µ Pall syringe filter. The sample was injected under described chromatographic condition of preparative HPLC for isolation of degradation impurity with purity >95.0%

3.7.2 Result and Discussion

3.7.2.1. Spectral Analysis of TRZ bulk drug

The IR, DSC and UV data results for TRZ are discussed in section 3.4 and other studies e.g. UPLC/ESI-MS and proton NMR are discussed in this section. Instrumental details and chromatographic conditions are described in section 3.7.1.2.

UPLX/ESI-MS: The UPLC/ESI-MS spectrum of TRZ bulk drug showed process related impurity along with bulk drug peak. The spectrum is discussed in section 3.8.2.1 of Part-D.

NMR Characterization of TRZ: The proton NMR study was performed for TRZ sample characterization. The NMR spectrum is shown in Fig. 3.23 and analysis of spectrum is shown in Table 3.23.

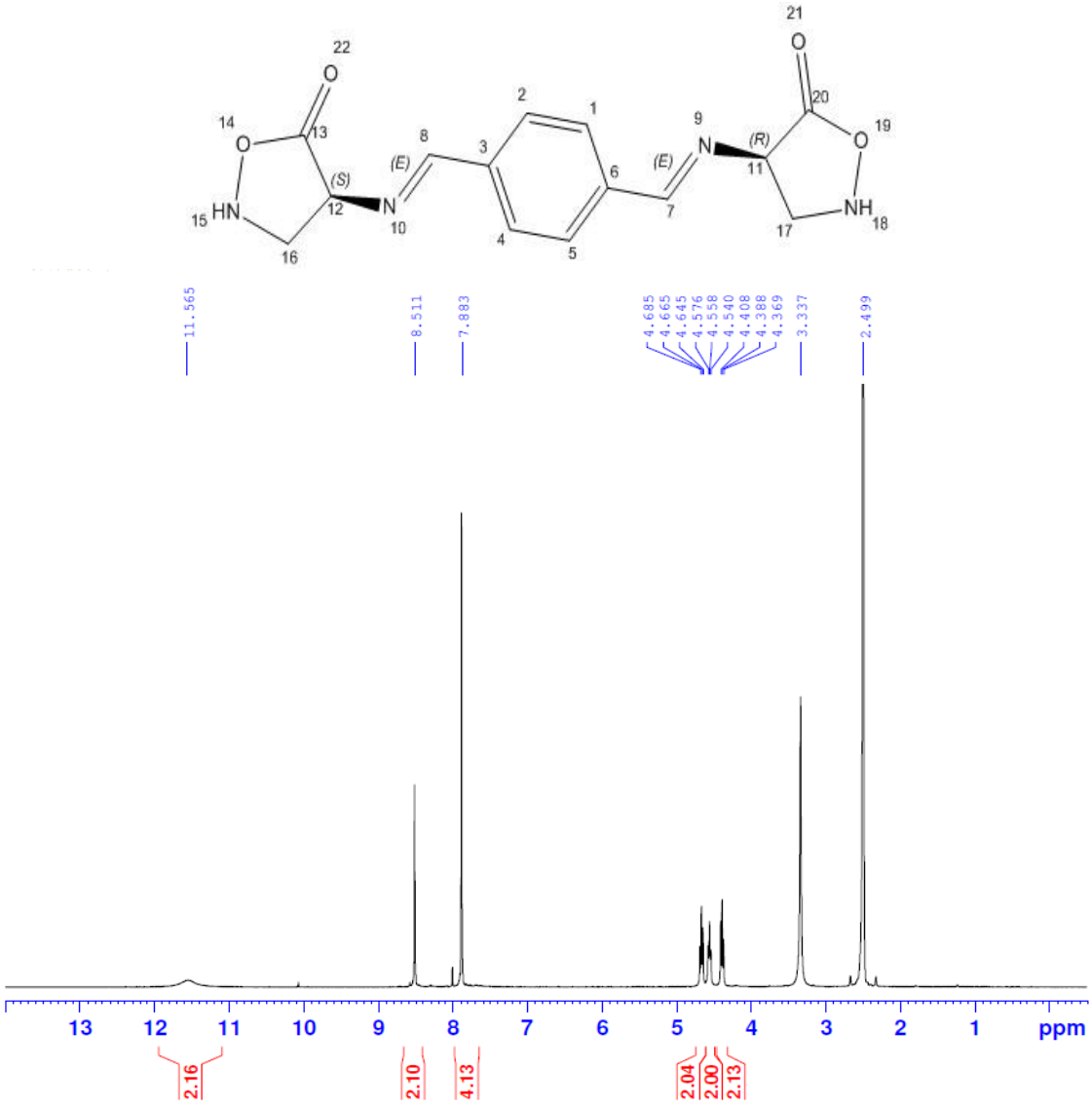


Fig. 3.23 Proton NMR spectrum of TRZ bulk drug

The analysis of TRZ bulk drug proton NMR spectrum is shown in Table 3.23 with α-position and chemical shift.

α-position	**Chemical shift (ppm)**
1	7.88*(m)*
2	7.88*(m)*
3	-
4	7.88*(m)*
5	7.88*(m)*
C-6	-
7	8.5*(s)*
8	8.5*(s)*
N-9	-
N-10	-
11	4.6*(m)*
12	4.6*(m)*
C-13	-
O-14	-
N-15	-
16	4.4,4.5 *(t)*
17	4.4, 4.5*(t)*
N-18	-
O-19	-
C-20	-
O-21	-
O-22	-

Table 3.23 Proton NMR spectrum analysis of TRZ bulk drug

The reference spectrum of TRZ for comparison was not available but analysis of spectrum showed and allocation of proton was found in match with chemical structure of TRZ. The NMR spectrum and analysis of spectrum of TRZ is useful in degradation product (DP) identification and characterization.

3.7.2.2. Acid degradation impurity (DP-A4)

The acid degraded sample of TRZ showed three DPs among which one major DP was formed at Rt 15.3minutes. The major DP was isolated by preparative HPLC, identified by ESI-MS and LC/MS/MS and characterized by proton NMR and IR data.

The name of the DPs are mentioned in the increasing order of *m/z* observed in UPLC/ESI-MS spectrum for DPs; DP-A1(*m/z 417)*, DP-A2 *(m/z 161* co-elution with *m/z 116* Impurity-1*)*, DP-A3*(m/z 170)*, **DP-A4** ***(m/z 237.2)***, DP-A5*(m/z 279)*, DP-A6 *(m/z 283)* and DP-A7 *(m/z 455)*. Here 'A' stands for acid DPs.

Isolation and purification of DP

The DP-A4 was identified as major DP as it covers highest area (46.79%) in HPLC chromatogram with peak purity after TRZ peak. The degradation impurity was isolated using preparative HPLC, instrumental and chromatographic specifications are described in section 3.7.1.2.

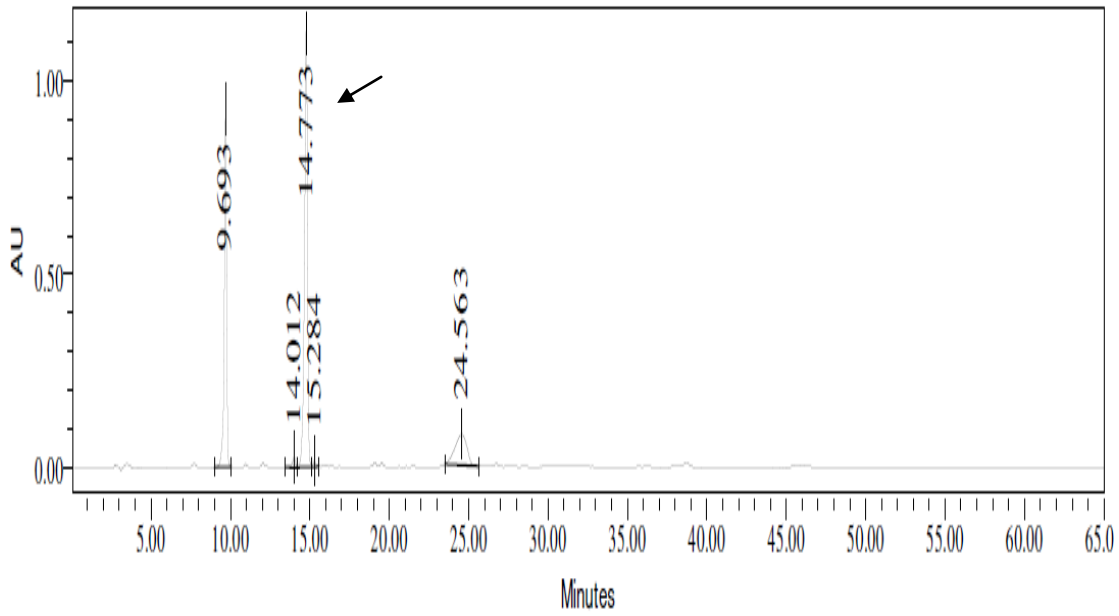
Aliquot of 2.6ml acid degraded sample (0.6ml vial sample+1ml water+1ml methanol)was injected in described chromatographic condition of preparative HPLC, The fractions were collected separately and gathered to concentrate the fraction by removing organic solvent on rotavapor and washing with water to remove buffer used in mobile phase. The concentrated sample was again injected in system to check against purity and for other co-eluting substance presence.

The lyophilization process: The concentrated sample was freeze dried to get in solid state after confirmation of purity >98.0 % by HPLC. This final product was analyzed by UPLC/ESI-MS, IR and NMR.

Confirmation of DP-A4

The confirmation and identification of DP-A4 was done using HPLC, UPLC and ESI-MS. The HPLC confirmation was done by comparing the chromatogram of isolated DP with chromatogram of sample degraded by HCl (section 3.5.1.2). The chromatograms are shown in Fig 3.24.

A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40.00
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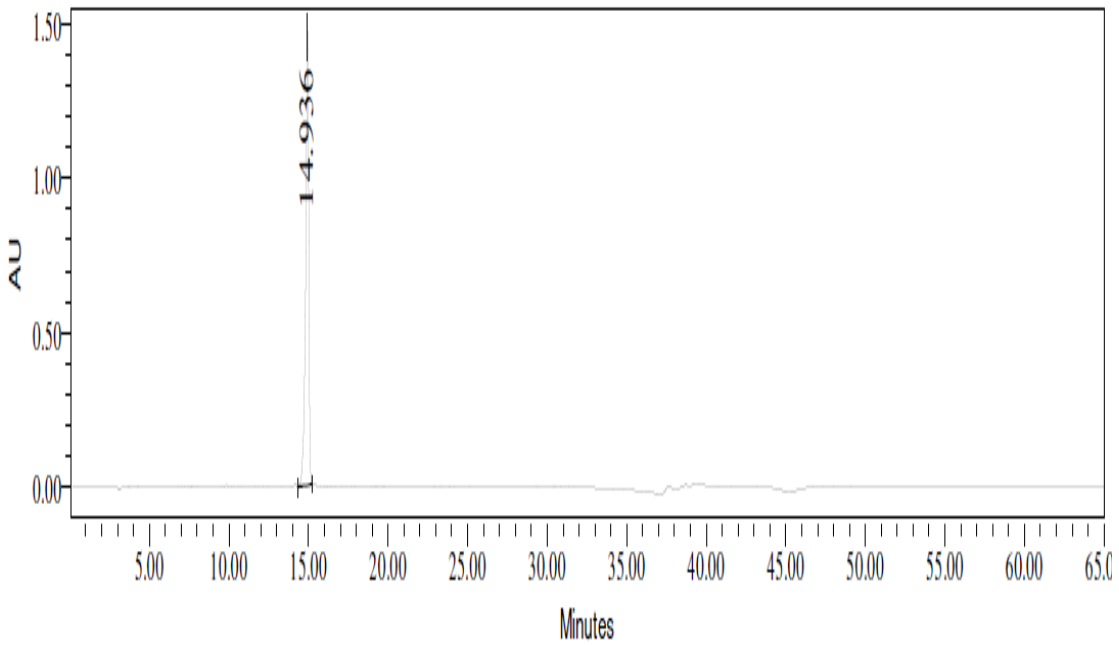
50.00
55.00
60.00
65.0
Minutes

B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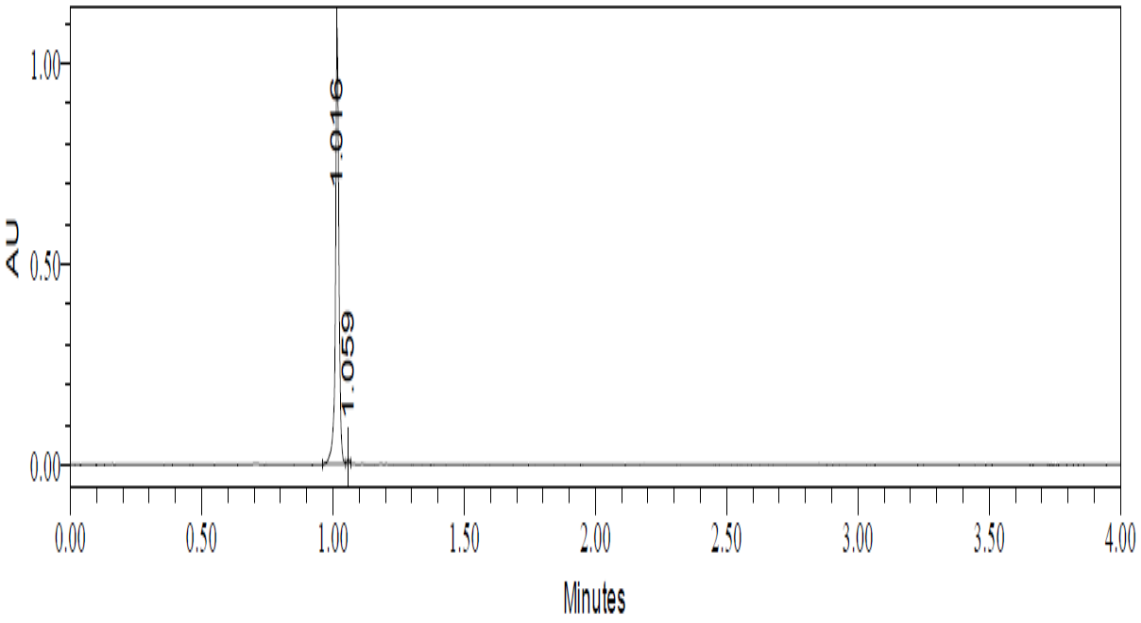
Minutes

C



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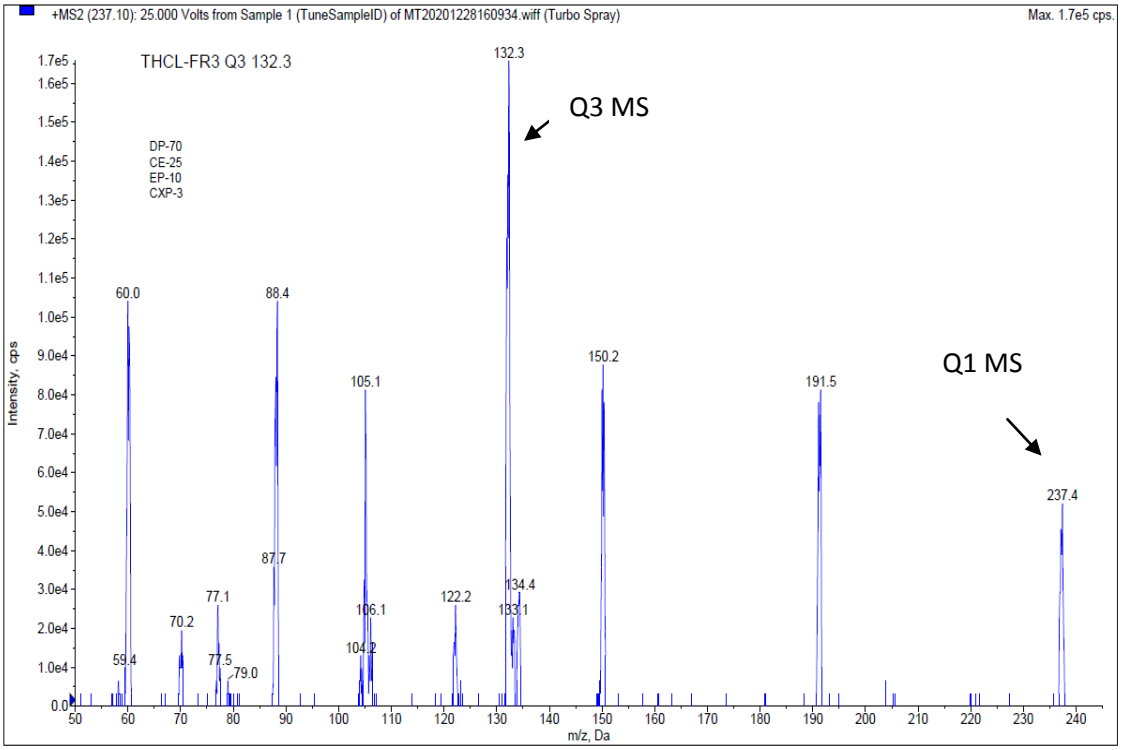


Fig. 3.24 Confirmation by A) HPLC chromatogram of HCl degraded sample of TRZ B) HPLC chromatogram of isolated DP-A4 C) UPLC chromatogram of DP-A4 D) MS/MS spectra of DP-A4

The Fig 3.24 confirms that desired major DP was isolated as no other interfering peaks were observed in HPLC chromatogram and ESI-MS spectrum, while Rt of DP was matching with the chromatogram of sample of TRZ degraded by acid. The *m/z* for $[M+H]^+$ ion for DP-A4 were 237.3 which suggest the removal of atoms and or group from parent molecule. Further fragmentation was observed in MS/MS spectrum.

Characterization of DP-A4

The isolated DP-A4 (*m/z* 237) was further characterized by proton NMR, IR spectrum and LC/MS/MS. The proton NMR spectrum for DP-A4 is shown in Fig 3.25.

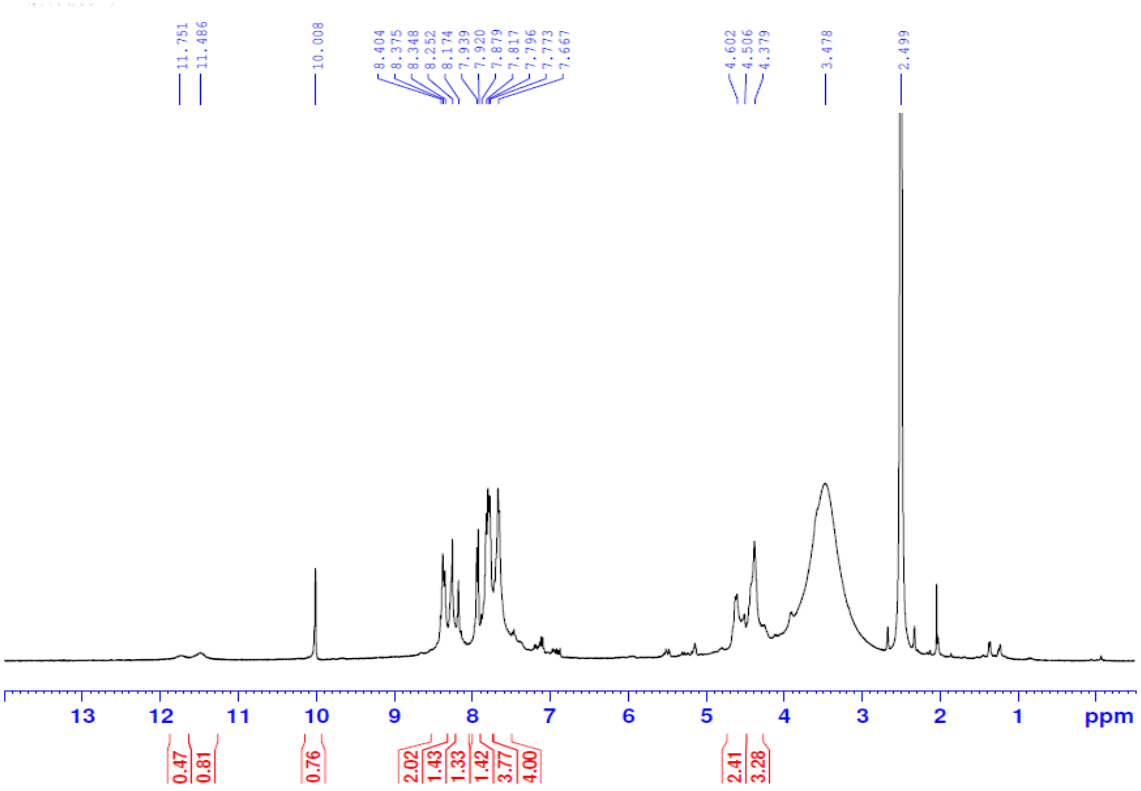


Fig. 3.25 characterization of DP by Proton NMR of DP-A4

The assignment for different protons by NMR spectrum is shown in Table 3.24 for TRZ bulk drug and DP-A4 for structure elucidation. The DP-A4 displayed *m/z* of ion $[M+H]^+$ 237 which are 64 amu less than the TRZ bulk drug amu 302.02.

	Chemical shift (∂, ppm)(Multiplicity)	
α-position	**TRZ API**	**DP-A4**
1	7.88*(m)*	7.8*(m)*
2	7.88*(m)*	7.6*(m)*
3	-C	-C
4	7.88*(m)*	7.8*(m)*
5	7.88*(m)*	7.6*(m)*
6	-C	-C
7	8.5*(s)*	8.4*(s)*
8	8.5*(s)*	8.4*(s)*
9	-N	-N
10	-N	-N
11	4.6*(m)*	4.3*(m)*
12	4.6*(m)*	4.3*(m)*
13	-C	-
14	-O	*11.7*(brs)*
15	-N	*10.0*(s)*
16	4.4,4.5 *(t)*	4.5*(m)*
17	4.4, 4.5*(t)*	4.5*(m)*
18	-N	*1.5*(s)*
19	-O	-
20	-C	-
21	-O	-
22	-O	-

*Changes observed in DP-A4 proton NMR spectrum

Table 3.24 Proton NMR assignment for TRZ bulk drug and DP-A4

The DP-A4 displays 64 amu less than the TRZ bulk drug amu 302.02; after analysis of proton NMR it was observed that five-member ring was attacked by acid, that resulted in removal of –OH and =O groups. The chemical structure elucidated by NMR analysis is shown in Fig 3.26. The ring A and B in chemical structure of TRZ bulk drug is referred to as D-cycloserin ring which is attachment and/or cleavage site for reaction in TRZ.

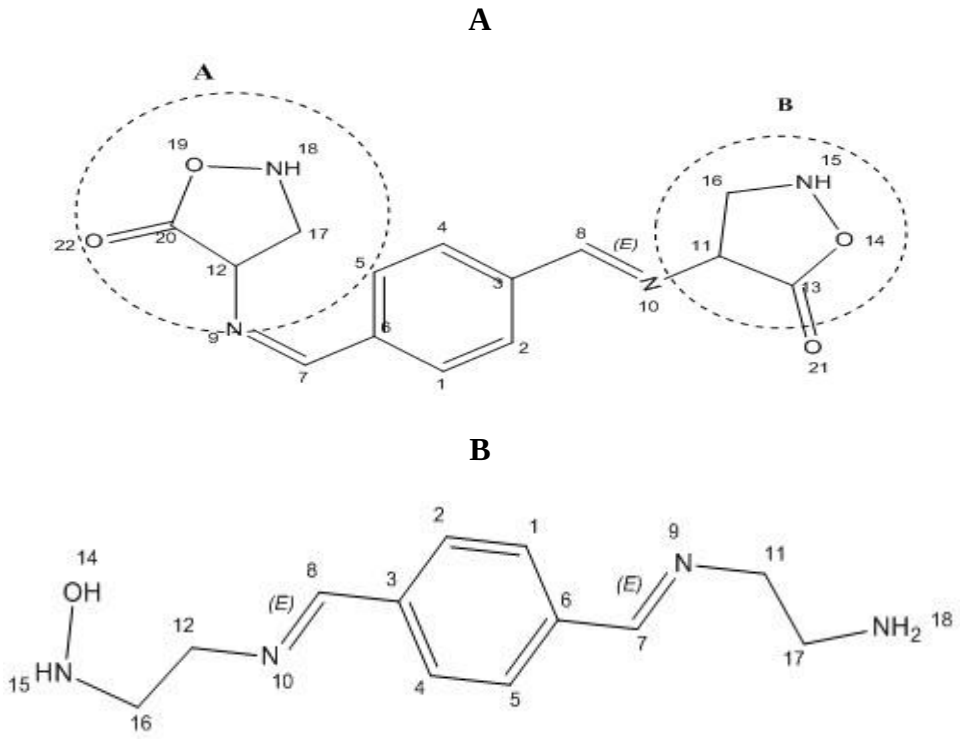


Fig. 3.26 Chemical structure of A) TRZ API and B) DP-A4

LC/MS/MS analysis

The LC/MS/MS analysis of DP-A4 showed that fragmentation of DP-A4 was observed in *m/z* of 132 amu. The loss of 105 amu in fragmentation of DP-A4 was observed. The fragmentation pathway is shown in Fig 3.29.

IR analysis

The IR spectrum of isolated DP-A4 of acid is shown in Fig. 3.27, and analysis of IR spectrum is shown in Table 3.25.

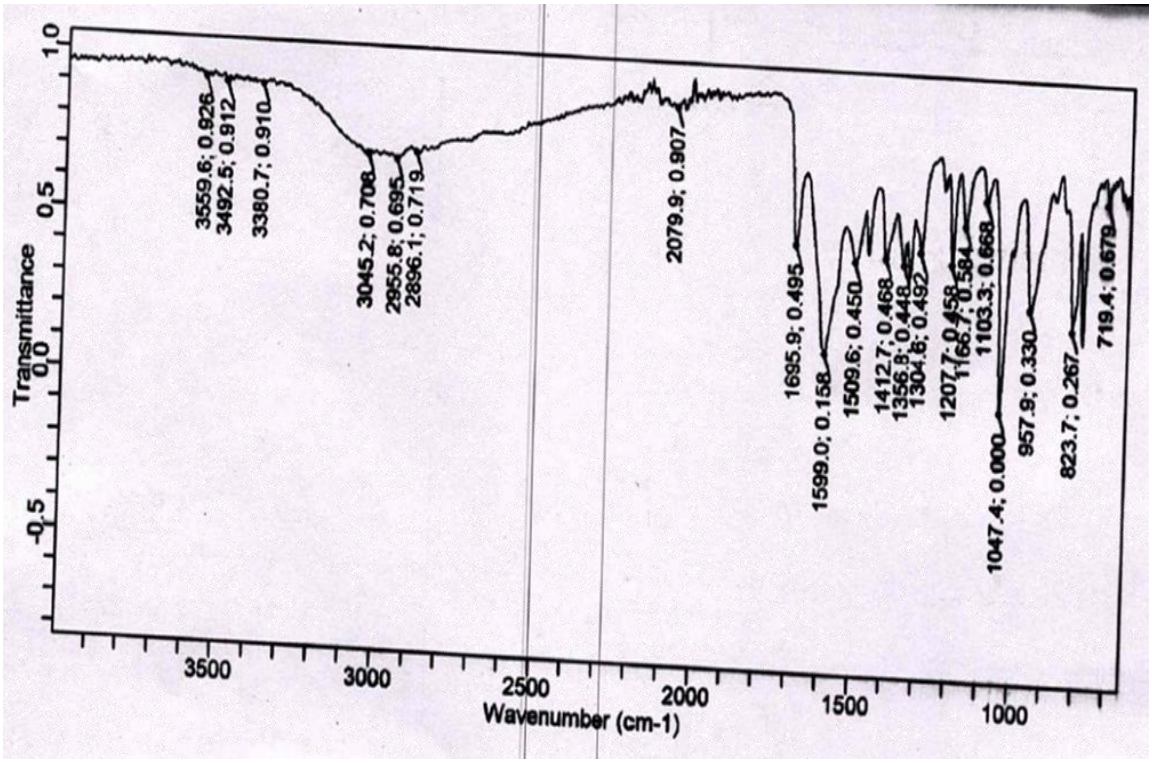


Fig. 3.27 IR spectrum of DP-A4 of acid degradation for TRZ

Observed Group	Observed Wave number	Assigned Wave number
C=C alkene bending	719.4	730-665
	823.7	840-790
Primary alcohol	1047.4	1050-1085
	1103.3	
C-N stretching	1166	1020-1250
	1207	1266-1342
	1304.6	
O-H bending	1356.8	1330-1420
	1412.7	
N-H bending	1599.0	1580-1650
C=N stretching	1695.9	1690-1640
N-H stretching	2896.1	2800-3000
	2955.8	

C-H stretching	3045.2	3100-3000
Aliphatic primary amine	3380.7	3300-3400
O-H stretching	3492.5	3550-3200
N-H stretching	3559.6	3500

Table 3.25 IR spectrum analysis for DP-A4 of TRZ

The elucidated structure matched with IR spectrum and confirmed the groups present in DP-A4 of acid condition for TRZ.

3.7.2.3. Neutral degradation impurity (DP-N3)

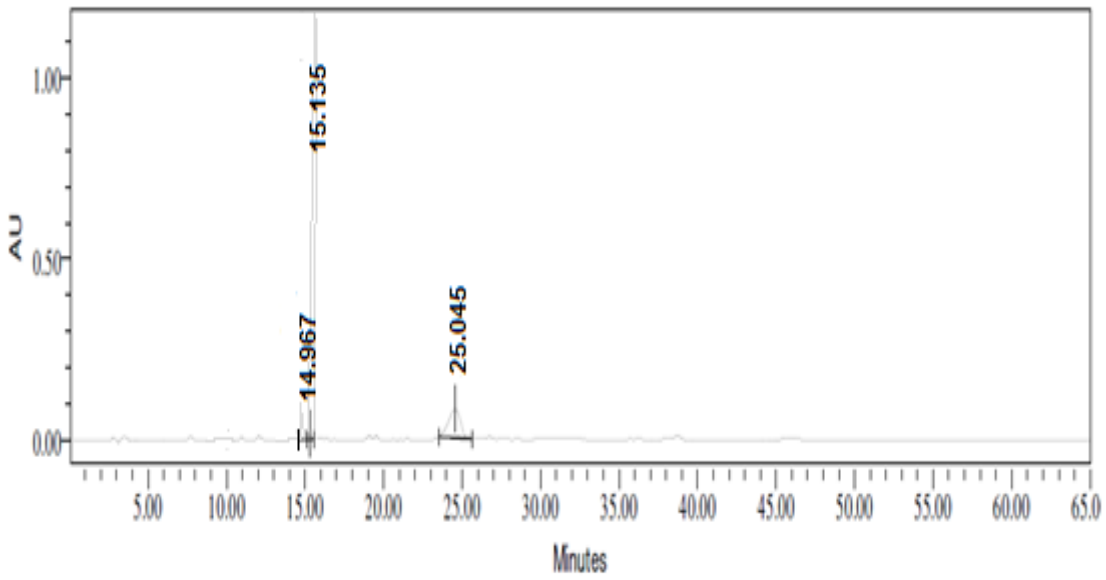
Isolation and purification of DP

The isolation and purification method is described in section 3.7.2.1. The DP at Rt 15.1min was identified as major DP (32.99%) and named as DP-N3, where 'N' stands for neutral condition.

Confirmation and Characterization of DP-N3

The confirmation of DP was carried out by HPLC, UPLC and MS/MS spectrum. The structure was elucidated based on LC/MS/MS results (Fig. 3.28) and confirmed by NMR and IR spectrum data.

A



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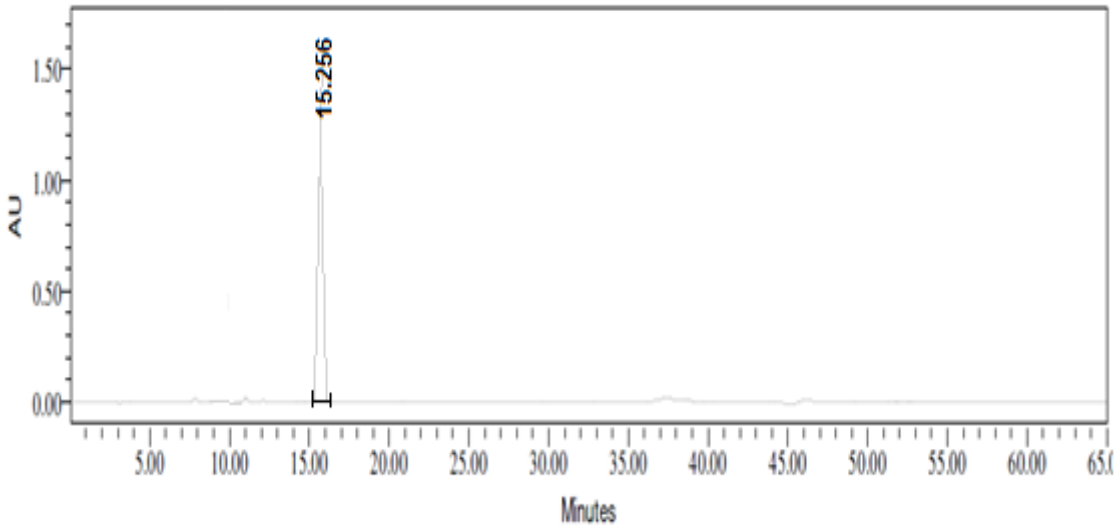
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10.00
15.00
20.00
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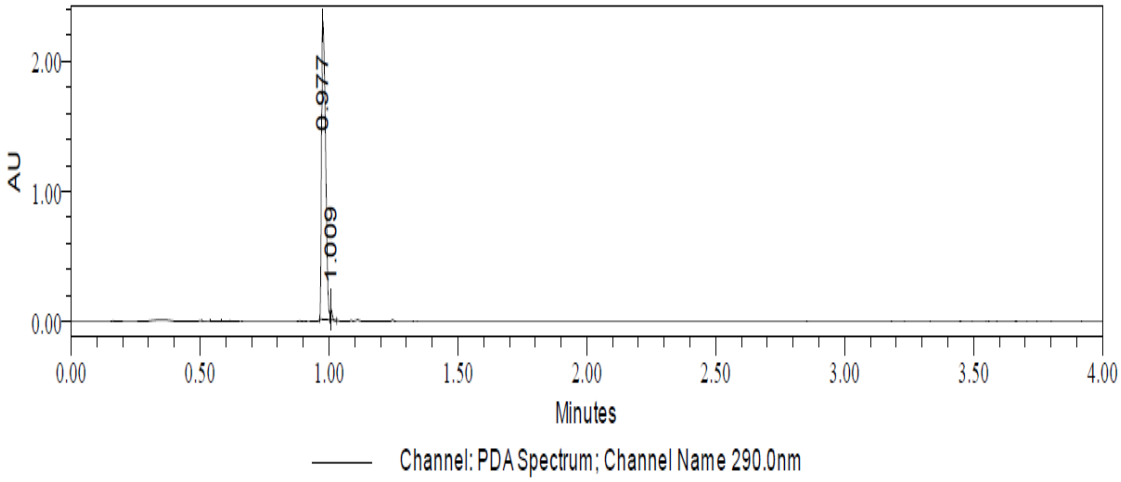
35.00
40.00
45.00
50.00
55.00
60.00
Minutes

C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2.00
1.00
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0.00
0.50
1.00
1.50
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2.50
3.00
3.50
4.00
Minutes
Channel: PDA Spectrum; Channel Name 290.0nm

D

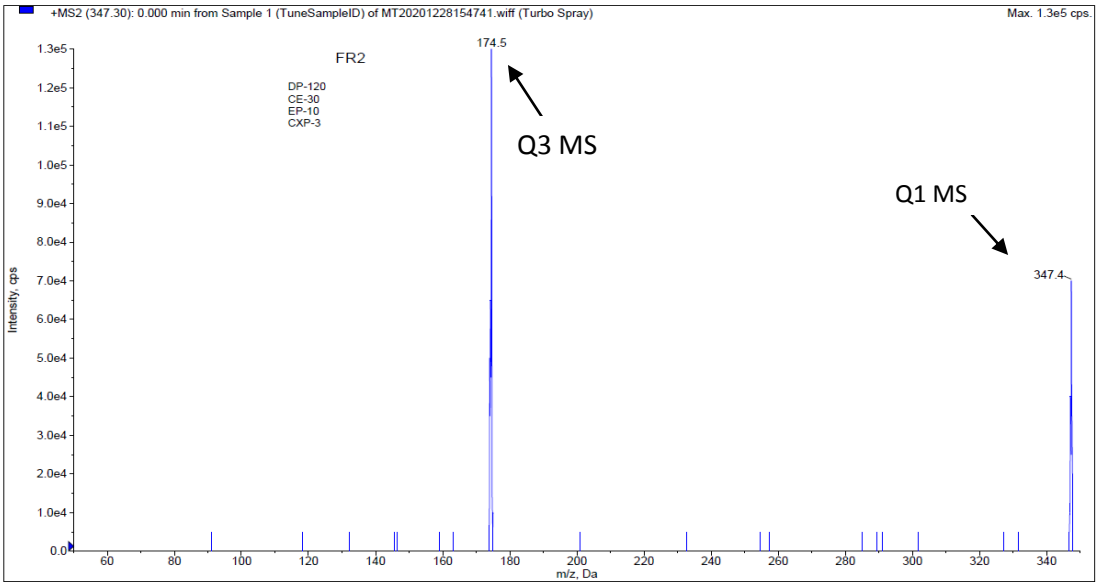


Fig. 3.28 A) HPLC chromatogram of water degraded TRZ bulk sample B) HPLC chromatogram of isolated DP-N3 C) UPLC chromatogram of isolated DP-N3 D) LC/MS/MS spectrum of DP-N3

The DP-N3 was isolated and confirmed against the presence of other substance by HPLC chromatogram and MS/MS spectrum. The chromatogram and MS/MS spectrum showed that desired major impurity DP-N3 with purity ≥98.0% was isolated. The isolated degradation impurity of neutral condition of TRZ showed *m/z* ion of 347, which is 45 amu higher than TRZ bulk drug amu 302.2; this suggest that atomic groups are attached to TRZ chemical structure that made the amu higher than the TRZ amu. The fragmentation pathway is shown in Fig 3.29.

The fragmentation pathway for acid degradation impurity and neutral degradation impurity is shown in Fig.3.28.

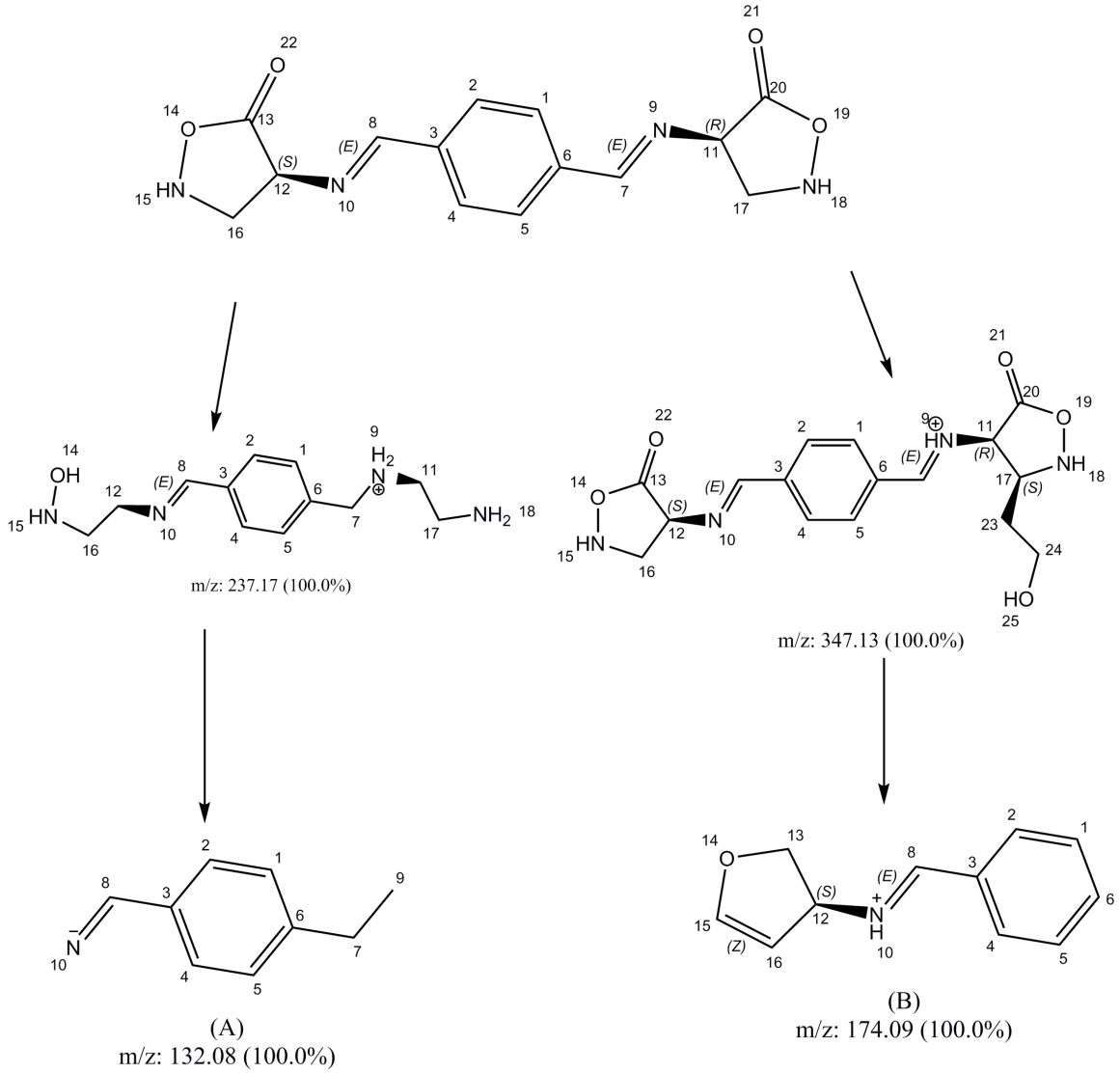
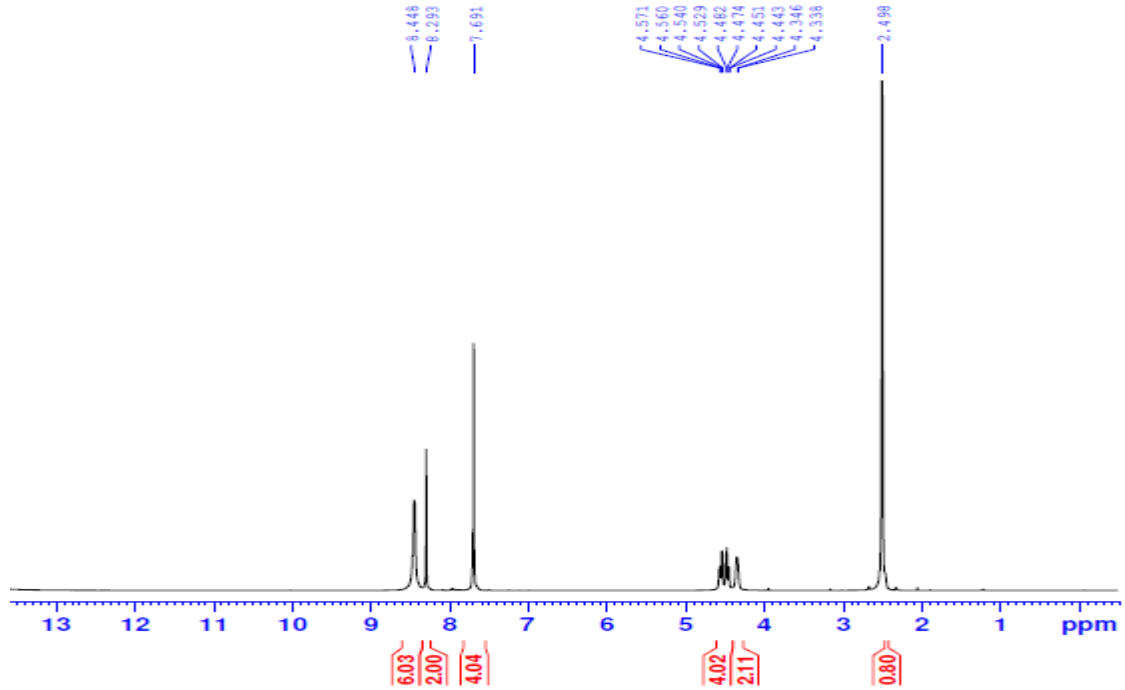


Fig.3.29 Fragmentation pathway for A) Acid degradation impurity (DP-A4) and B) Neutral degradation impurity (DP-N3) of TRZ

NMR Characterization of DP-N3

The Proton, C^{13} and APT NMR were obtained for neutral degradation product DP-N3. The NMR spectra are shown in Fig 3.30.

A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2.498
13
12
11
10
9
8
7
6
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4
3
2
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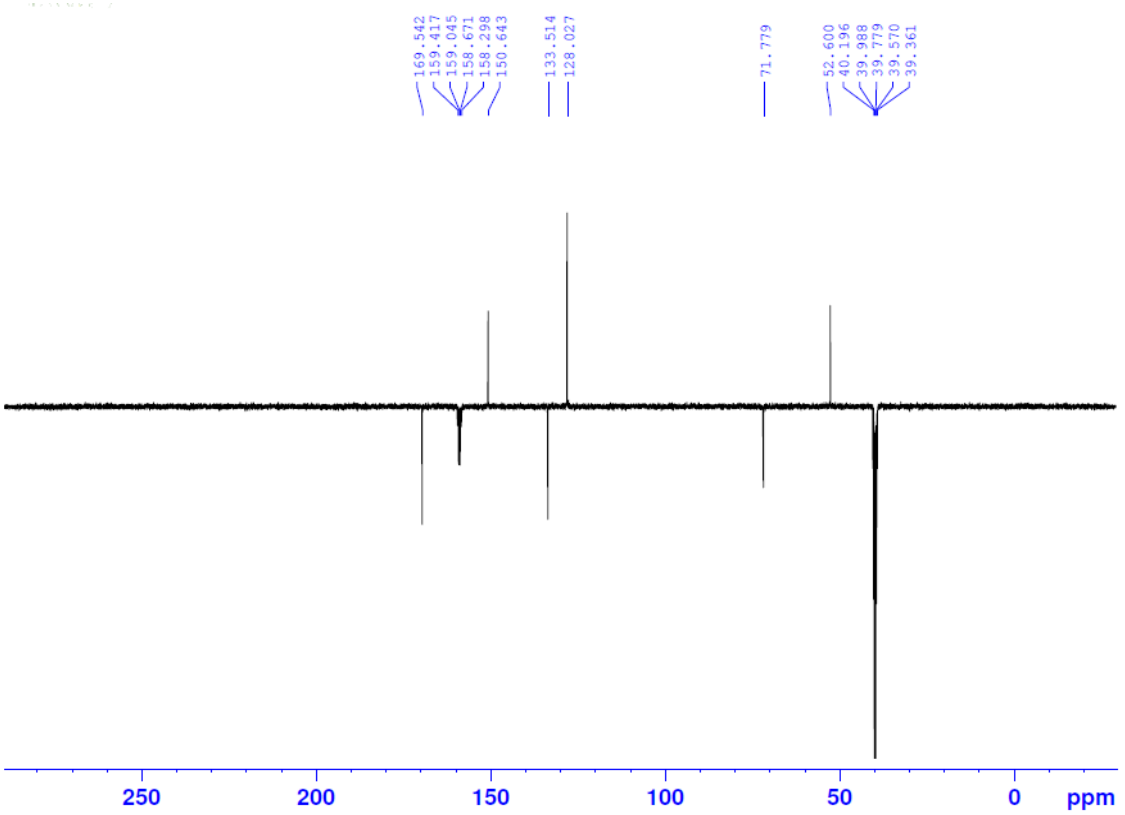
4.04
4.02
2.11
0.80

B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ppm

C

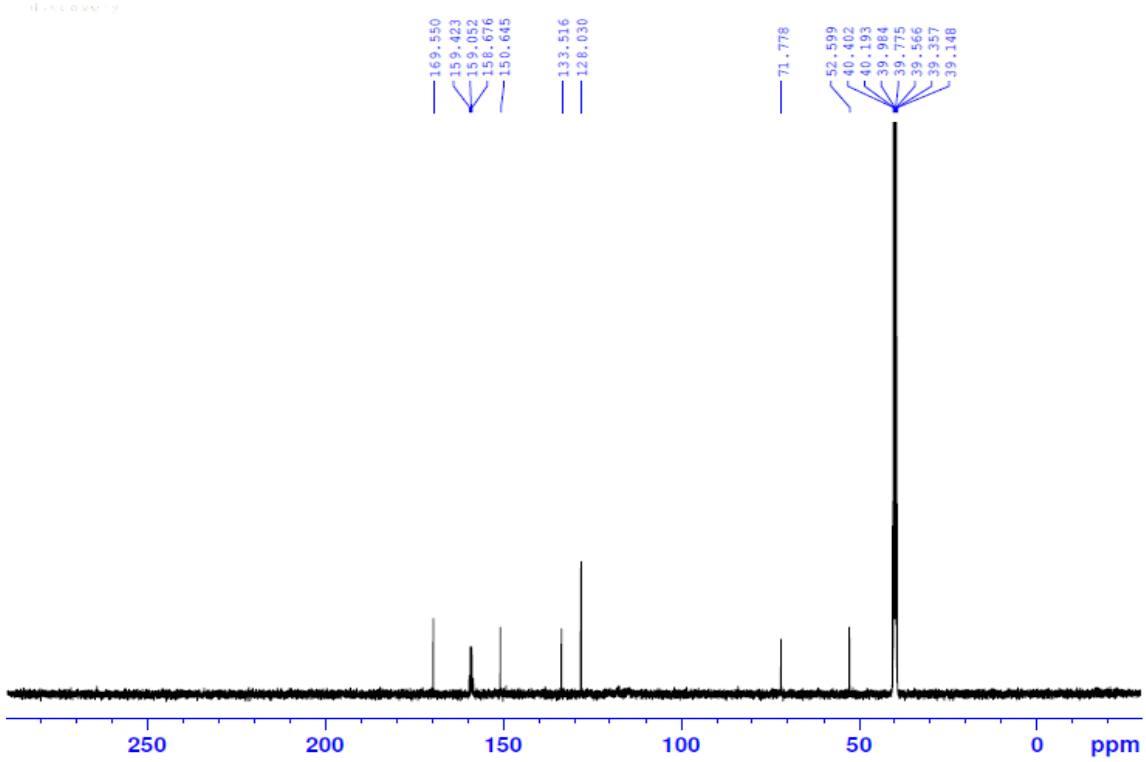


Fig.3.30 A) Proton NMR B) APT NMR C) C^{13} NMR for neutral DP-N3

The analysis of NMR is shown in Table 3.26.

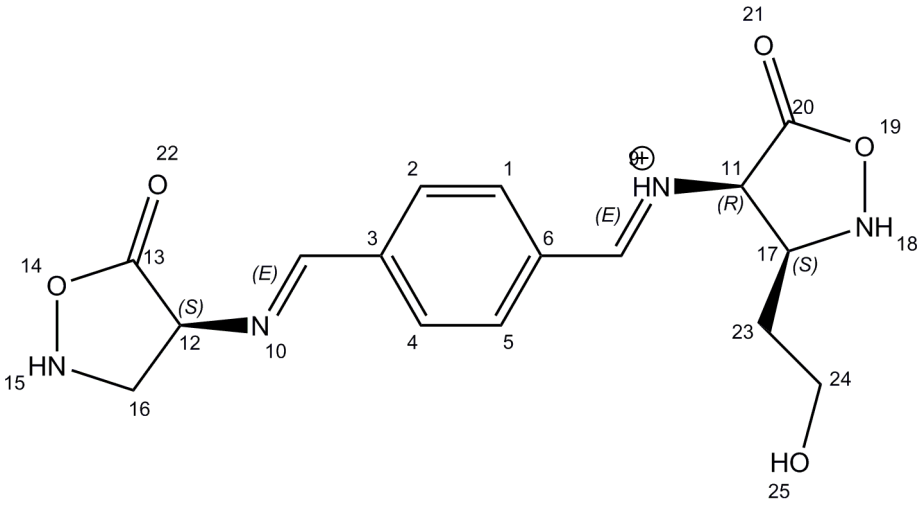
Chemical shift (∂, ppm)(Multiplicity)				
α-position	**TRZ API**	**DP-N3**		
		^{1}H	**^{13}C**	**APT**
1	7.88*(s)*	7.6*(s)*	128.0	-CH(*uf*)
2	7.88*(s)*	7.6*(s)*	128.0	-CH(*uf*)
3	C	C	133.5	-C(*lf*)
4	7.88*(s)*	7.8*(s)*	128.0	-CH(*uf*)
5	7.88*(s)*	7.6*(s)*	128.0	-CH(*uf*)
6	C	C	133.5	-C(*lf*)
7	8.5*(s)*	8.4*(s)*	150.4	-CH(*uf*)
8	8.5*(s)*	8.4*(s)*	150.4	-CH(*uf*)
9	N	N	-	-
10	N	N	-	-
11	4.6*(m)*	4.5*(m)*	52.6	-CH(*uf*)
12	4.6*(m)*	4.5*(m)*	52.6	-CH(*uf*)

13	C	-	169.5	-C(*lf*)
14	O	-	-	-
15	N	-	-	-
16	4.4,4.5 *(t)*	4.5*(t)*	39.3	-CH(*lf*)
17	4.4, 4.5*(t)*	*2.4*(m)*	39.3	-CH(*lf*)
18	N	-	-	-
19	O	-	-	-
20	C	-	159.5	-C(*lf*)
21	O	-	-	-
22	O	-	-	-
23	-	-	71.2	-2CH*(lf)*
24	-	*4.4(*m*)	71.2	-2CH(*lf*)
25	-	*4.3 (*s*)	-	OH

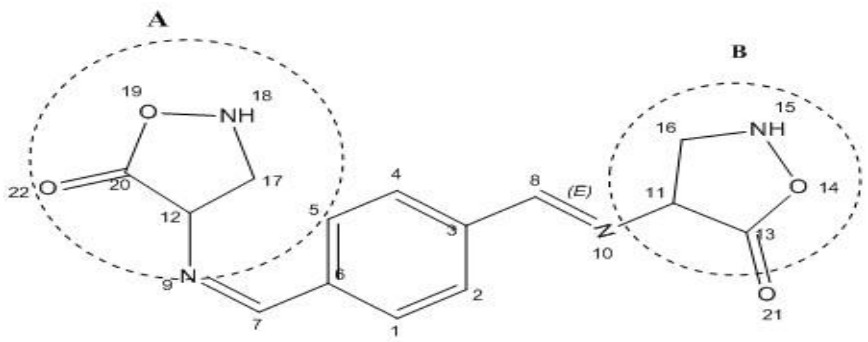
*Changes observed in NMR spectrum

Table 3.26 NMR analysis of neutral DP-N3

The structure elucidated for DP-N3 of neutral degradation of TRZ is shown in Fig 3.31.



A



B

Fig.3.31 Chemical structure for A) DP-N3 and B) TRZ bulk drug

IR analysis

The IR spectrum obtained for DP-N3 of neutral condition for TRZ is shown in Fig.3.32 and analysis for IR spectrum is shown in Table 3.26.

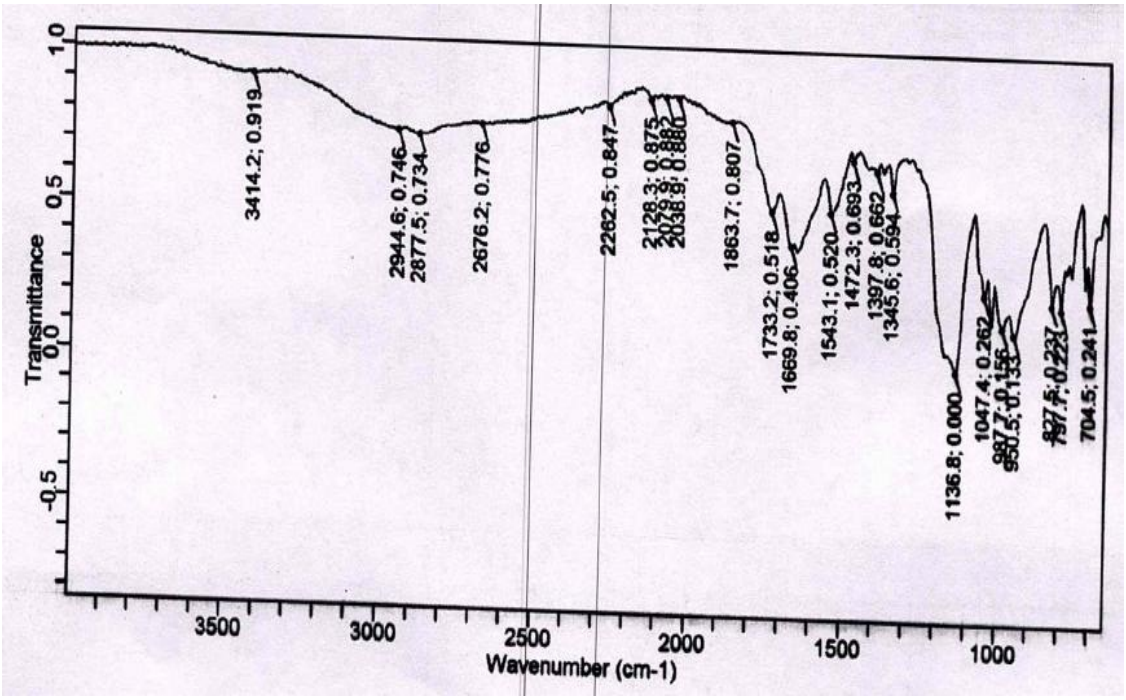


Fig.3.32 IR spectrum for isolated DP-N3 of neutral condition for TRZ

The analysis of IR spectrum is shown in Table 3.27.

Groups Present	Obtained Wave number	Assigned wave number
Benzene derivative	704.5	700±20
C-H stretching	797.5	780±20
C-H bending	827.5	810±20
C=C bending	950.5	980-960
	987.7	995-985
C-O stretching	1136.8	1159-1085
C-N stretching	1345.6	1345-1266
C-H bending	1472.3	1465
N-O stretching	1543.1	1500-1550
C=C stretching	1669.8	1675-1665
C=O stretching	1733.2	1740-1720
O-H stretching	2877.5	3200-2700
N-H stretching	2944.6	3000-2800
O-H stretching	3414.2	3550-3200

Table 3.27 IR spectrum analysis for DP-N3 of neutral degradation of TRZ

The IR spectrum confirms the groups present in isolated DP-N3 of neutral condition, the groups are matching with elucidated structure for DP-N3.

3.7.2.4. Oxidative degradation impurity (DP-O7)

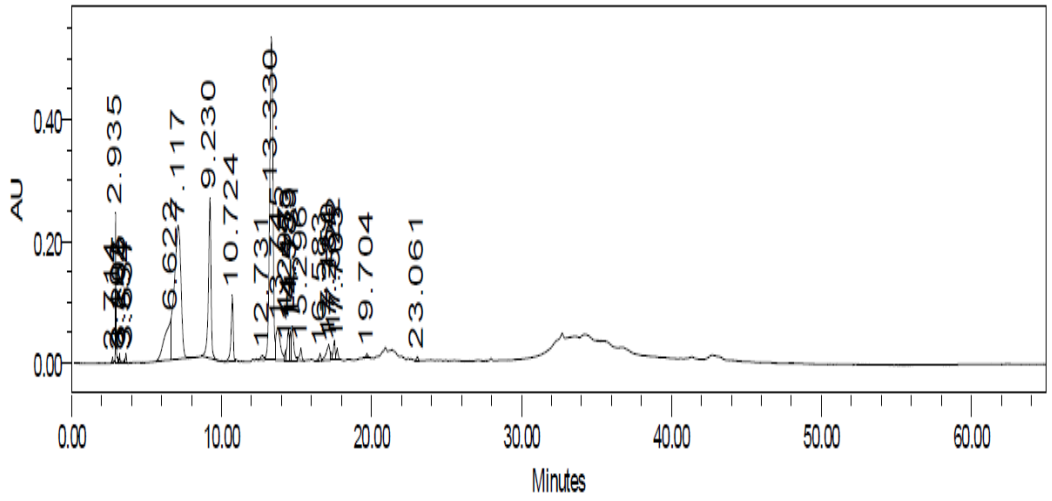
Isolation and purification of DP

The isolation and purification method is described in section 3.7.2.1. The major DP in oxidative condition was observed at Rt 13.30 min with major peak area (46.75%) and named as O7 where 'O' stands for oxidative condition.

Confirmation of DP-O7

The confirmation of isolated DP-O7 as desired DP was done by HPLC, UPLC, MS/MS spectra of isolated DP. The isolated DP chromatogram and mixture of DPs chromatogram were compared to confirm the desired DP was isolated (Fig. 3.33).

A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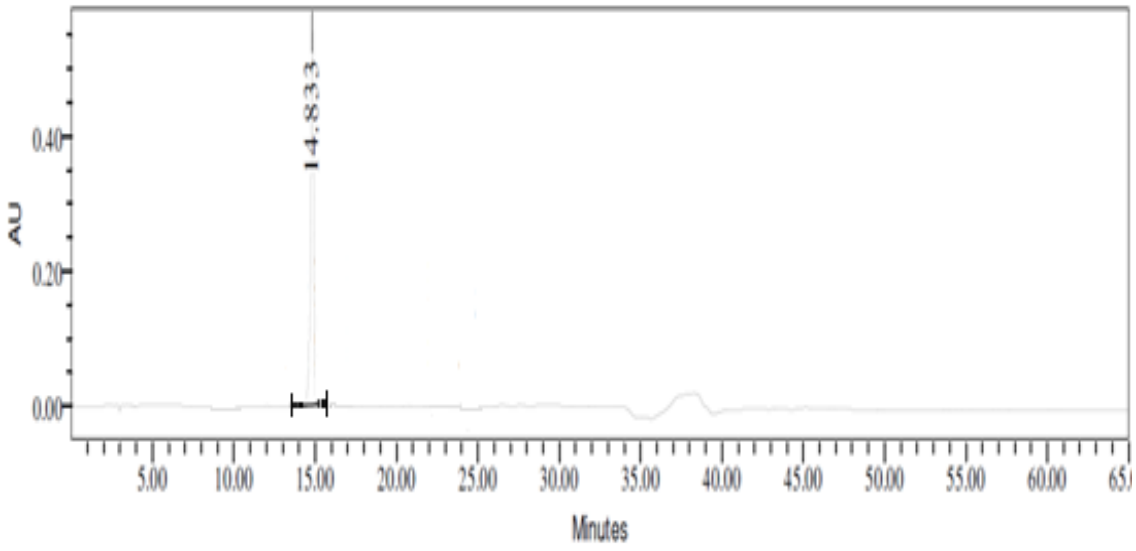
Minutes

B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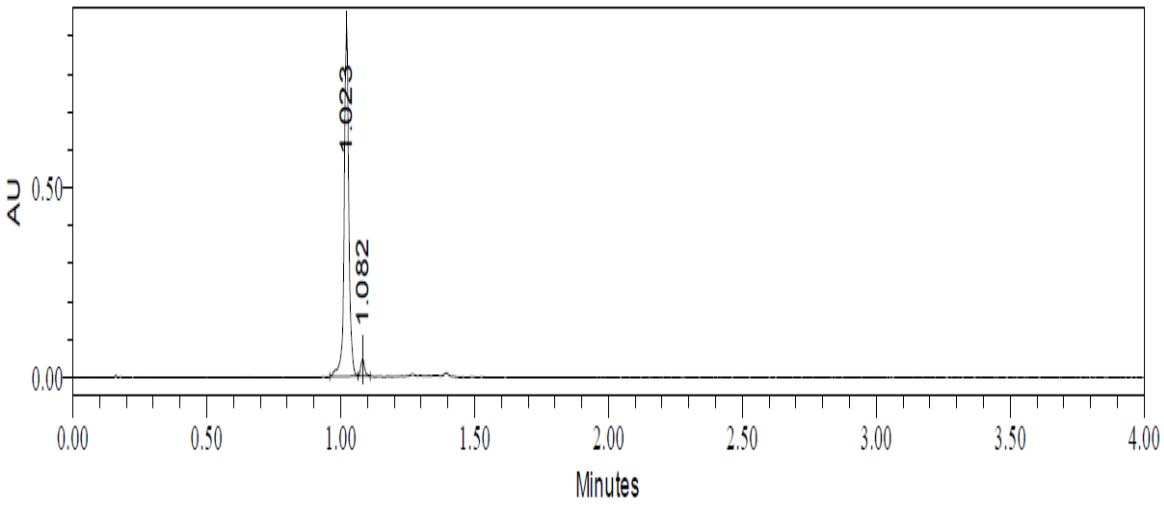
45.00
50.00
55.00
60.00
Minutes

C



D

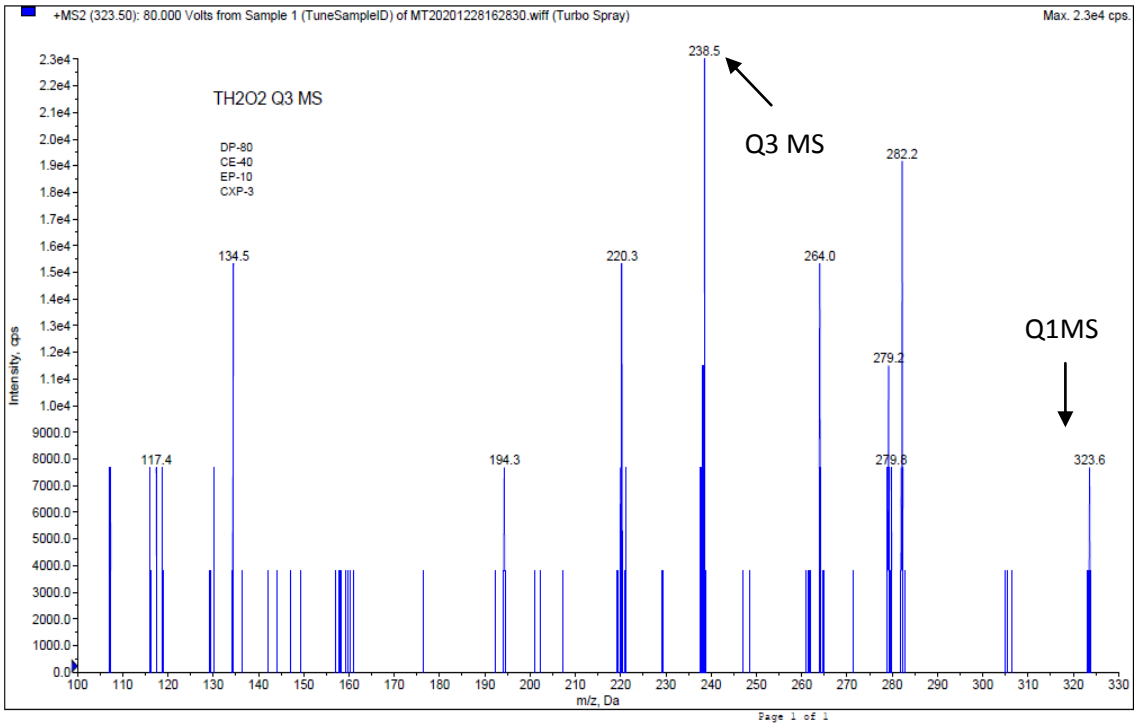


Fig.3.33 A) HPLC chromatogram for peroxide degraded TRZ sample B) HPLC chromatogram for isolated DP C) UPLC chromatogram for isolated DP D) MS/MS spectra of DP-O7

The HPLC chromatogram showed that desired DP was isolated; UPLC chromatogram also showed that the desired DP of peroxide degradation of TRZ was isolated. The MS/MS spectra showed *m/z* ion of 323.6 and fragmentation at 238.5 *m/z.* The *m/z* of isolated DP is 21

amu higher suggest that group or atoms are attached to the TRZ. Chemical structure of DP-O7 was derived using proton NMR and fragmentation pathway is shown in Fig 3.34.

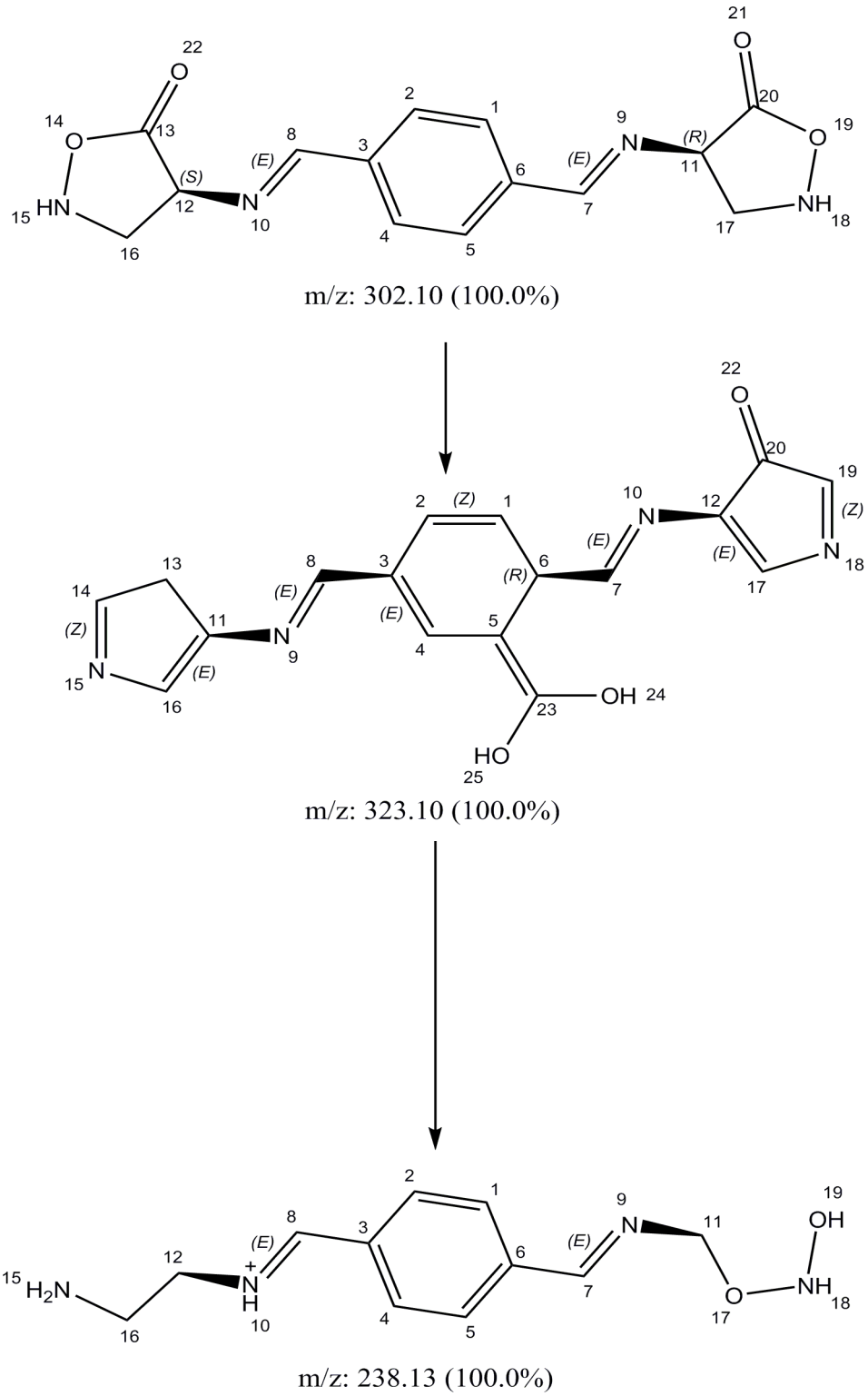


Fig. 3.34 Fragmentation pathway for peroxide DP-O7 of TRZ

The Tandem MS study showed that precursor DP-O7 fragmented into 238 *m/z* ion, the possible fragmentation is shown in Fig 3.34.

Characterization of DP-O7

Characterization of oxidized DP of TRZ was done by proton NMR study. The proton NMR for DP-O7 is shown in Fig 3.35.

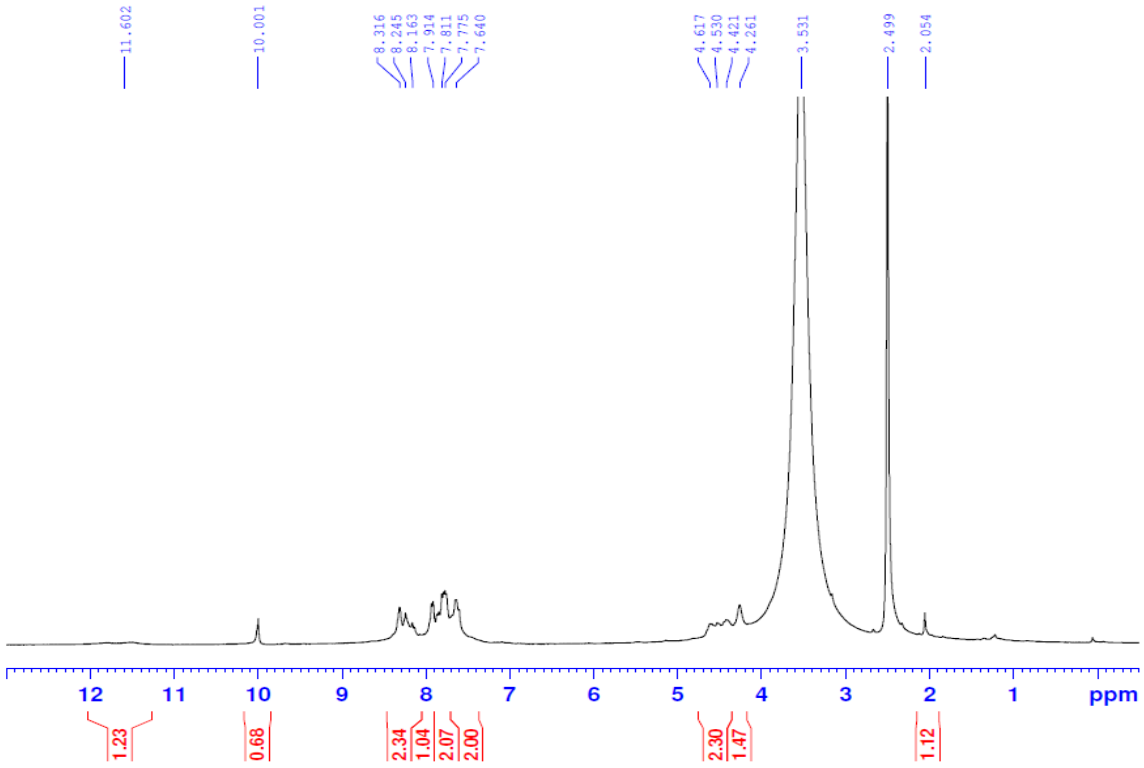


Fig. 3.35 Proton NMR of peroxide DP-O7

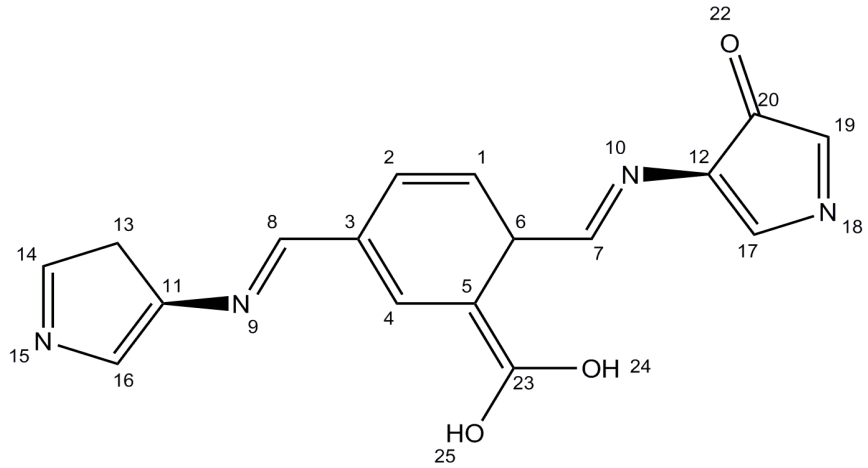
The assignment of NMR is shown in Table 3.28 with α-position and assigned NMR chemical shift (∂) in ppm.

	Chemical shift (∂) ppm	
A-position	**TRZ API**	**DP-O7**
1	7.88*(s)*	*7.64 *(m)*
2	7.88*(s)*	*7.64 *(m)*
3	C	-
4	7.88*(s)*	7.64 *(m)*
5	7.88*(s)*	-
6	C	*4.26*(brs)*
7	8.5*(s)*	8.31 *(m)*
8	8.5*(s)*	8.31 *(m)*
9	N	N
10	N	N
11	4.6*(m)*	*-
12	4.6*(m)*	*-
13	C	*2.05(s)
14	O	*4.4 *(brs)*
15	N	N
16	4.4,4.5 *(t)*	*4.4 *(brs)*
17	4.4, 4.5*(t)*	*4.4 *(brs)*
18	N	N
19	O	*7.7 *(m)*
20	C	C
21	O	-
22	O	O
23	-	C
24	-	11.60 *(s)* (OH)
25	-	11.60 *(s)*(OH)

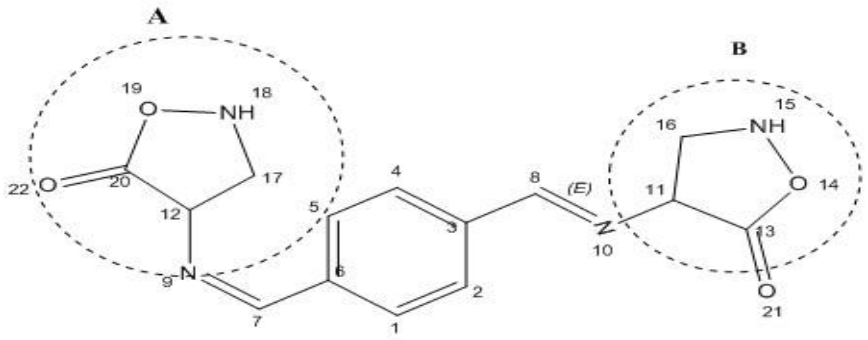
*Changes observed in NMR spectrum

Table 3.28 NMR assignment for DP-2 of oxide degradation of TRZ

The NMR assignment and MS/MS spectra suggested structure is shown in Fig 3.36.



A



B

Fig. 3.36 suggested chemical structure for A) DP-O7 B) TRZ API

The suggested structure show that TRZ was oxidized in presence of oxidizing agent, the –OH group of H_2O_2 was attached to TRZ chemical structure via C=C bond at position C-5.

IR analysis

The isolated DP was analyzed for groups present in it by IR analysis; the IR spectrum of isolated DP-O7 is shown in Fig.3.37.

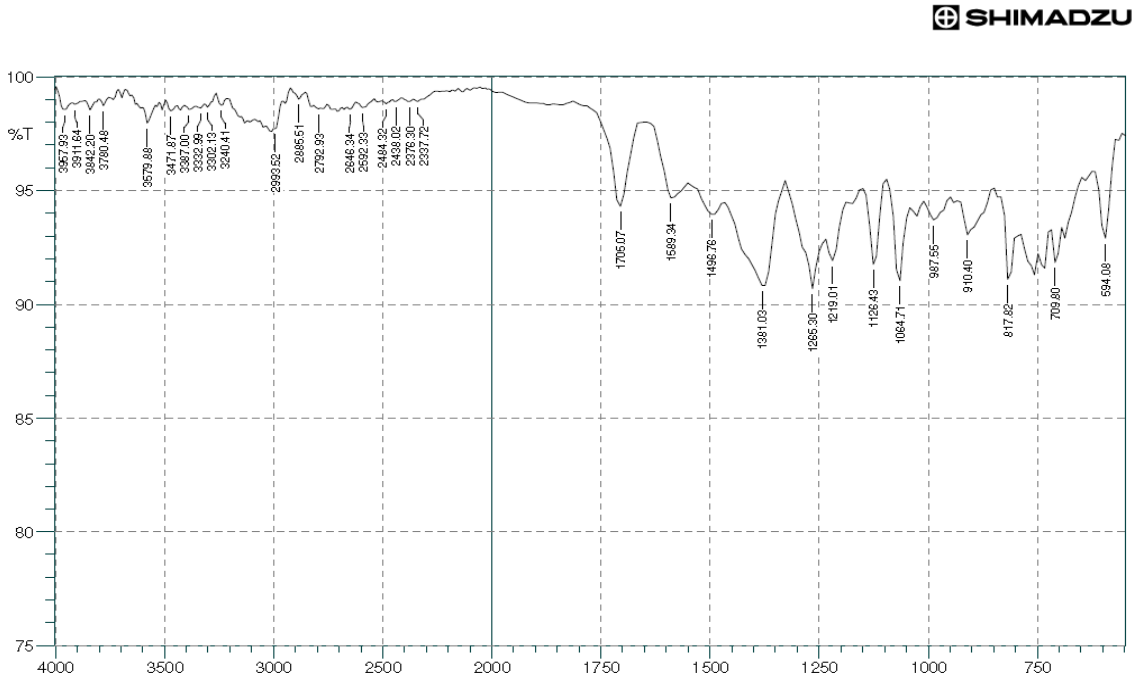


Fig. 3.37 IR spectrum for isolated DP-O7 of oxidizing condition for TRZ

The analysis of IR spectrum is shown in Table 3.29;

Observed groups	Obtained wave number	Assigned wave number
C=C bending	694.8,709.8	730-665
	817.8	840-790
	987.5	995-985
C-N stretching	1064.7	1250-1020
C-O stretching	1126.4	1205-1124
C-H bending	1381.0	1385-1380
C=C bending	1599.3	1650-1566
C=O stretching	1705.0	1725-1705
O-H stretching	2592.3, 2646.3, 2792.9, 2885.9, 2993.5 3240.4	3300-2500
O-H stretching	3471.8	3550-3200
	3579.8, 3780.4	3700-3500

Table 3.29 IR spectrum analysis for DP-O7 of TRZ in oxidative medium

The analysis of IR spectrum showed all the present group in DP-O7 of TRZ in oxidative medium, the present group is shown in structure elucidated for DP-O7.

PART-D

3.8. IMPURITY PROFILING OF TERIZIDONE

The process related impurities and degradation products (DPs) of TRZ were analyzed using sophisticated instruments. The isolation of major DPs were carried out using preparative HPLC, identification of DPs were carried out using UPLC-Tandem MS and characterization of major DPs were completed using NMR (proton).

3.8.1. Experimental

3.8.1.1. Chemicals and reagents

The chemicals and reagents are same as described as in section 3.5.1.1.

3.8.1.2. Equipments and chromatographic conditions

The equipments for stress degradation studies were same as described in section 3.5.1.2. UPLC-Tandem MS and NMR instrument and chromatographic conditions are described in section 3.7.1.2.

3.8.1.3. Sample Preparations

Analytical sample preparation, stress degradation sample and buffer preparations are same as described in section 3.7.1.3. Sample preparation for isolation is described in section 3.7.2.

Enrichment of DPs for identification

UPLC/Tandem MS analysis : From the stock solution (described in section 3.7.1.3) 1ml aliquot (neutralized in case of acid sample) was diluted with methanol to produce 10ml (10mg/ml), from this solution 0.5ml aliquot was again diluted with methanol to produce 10ml (0.5mg/ml). The solution was filtered through 0.45µ Pall syringe filter prior to injection in chromatographic condition described for UPLC/MS for identification purpose.

3.8.2. Results and Discussion

The process related impurity was noticed in TRZ bulk drug during analysis; it was identified by UPLC/ESI-MS.

The DPs were not identified for alkaline medium as TRZ structure was broken down and formed small fragments that appeared as clusters of *m/z* peaks in mass spectrum. There was no major or identifiable peak.

The DPs formed under acid pH media, in water and in presence of oxidizing agent were identified and structures were proposed for DPs. The major degradation products from acid,

neutral and oxidative conditions were isolated and characterized for structure elucidation and confirmation by NMR.

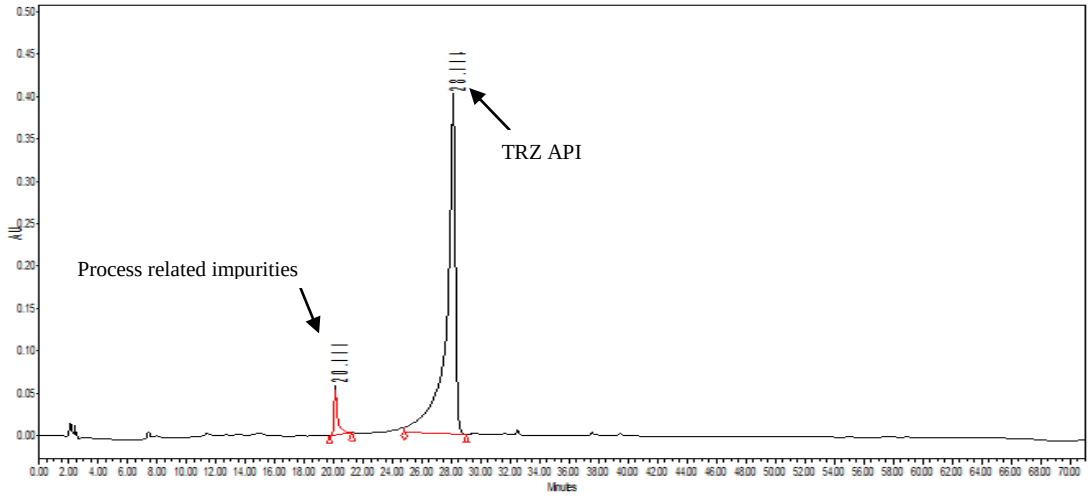
3.8.2.1.Analysis of TRZ bulk drug and process related impurities

TRZ bulk drug was analyzed using LC/ESI-MS and NMR. The IR spectrum analysis is shown in section 3.4.1.

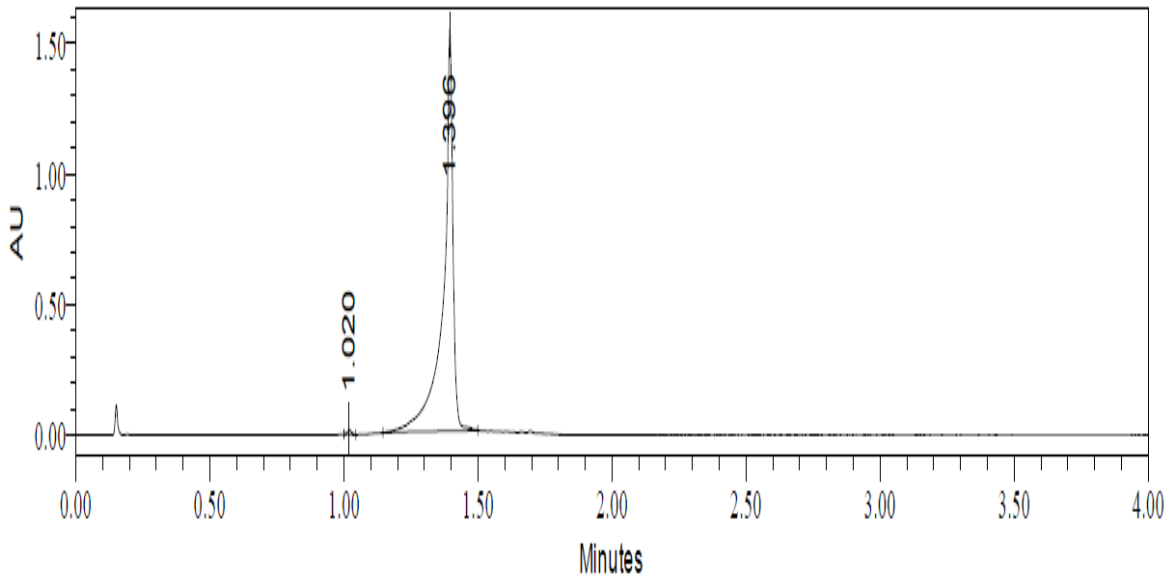
ESI-MS Spectrum analysis

The ESI-MS and proton NMR was performed for TRZ bulk drug, the data was utilized to study about process related impurity and to compare degradation impurity data (NMR) with TRZ bulk drug data (NMR). The chromatographic separation was done using method and equipments described in section 3.5.1.2. The chromatogram (HPLC and UPLC) for TRZ bulk drug for process related impurities and ESI-MS spectrum is shown in Fig. 3.38.

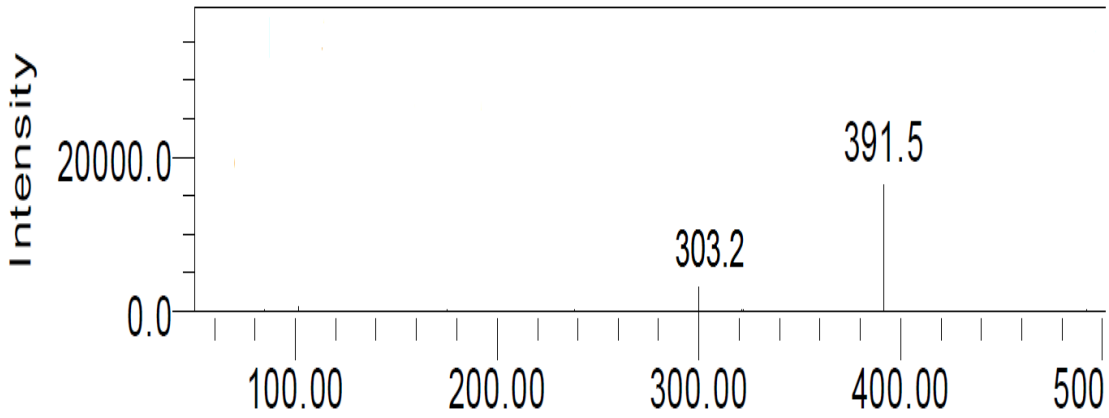
A



B


1.50
1.00
0.50
0.00
AU
1.020
1.396
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
Minutes

C


Intensity
20000.0
0.0
391.5
303.2
100.00
200.00
300.00
400.00
500

D

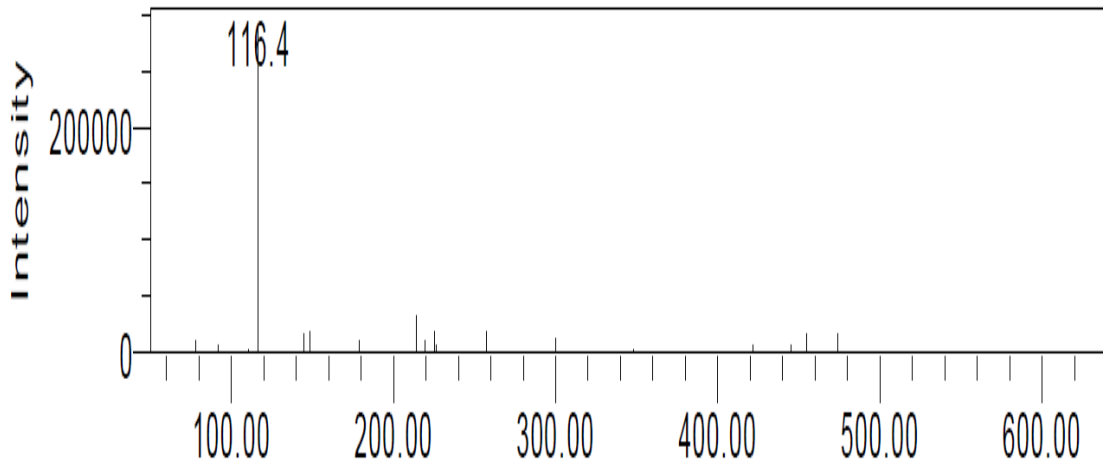


Fig.3.38 TRZ bulk drug HPLC identification A) HPLC Chromatogram B) UPLC chromatogram for TRZ and process related impurity C) ESI/MS spectrum for TRZ process related impurity D) ESI/MS spectrum for process related impurity

The chromatogram in Fig 3.38 (A) showed extra peak other than TRZ bulk drug peak indicates the presence of process related impurity (*m/z* 116.4). While the ESI/MS spectrum of TRZ showed base peak of 391.5 *m/z* that indicates that adduct was formed with formate ion that suggest the pseudo impurity formed due to mobile phase used for the analysis [M+ Formic acid (88) + H^+]. The area covered by process related impurity and TRZ with % mass balance in chromatogram is shown in Table 3.30.

	Name	Retention Time	Purity angle	Purity Threshold	Area	% Area
1	Impurity-1	20.111	0.168	0.565	931253	5.30
2	API	28.114	0.117	0.246	16630701	94.70

Table 3.30 Analysis of HPLC chromatogram of TRZ bulk drug

The chromatogram of TRZ bulk drug showed process related impurity which covered 5.30% area of total area. The *m/z* for process related impurity was 116.4 amu (Fig 3.33).The proposed structure for process related impurity is shown in Fig 3.39.

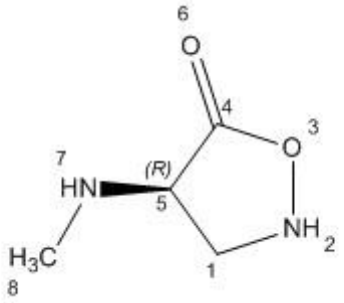


Fig.3.39 Process related impurity of TRZ

The bond between Enantiomeric carbon and benzene ring is broken to form process related impurity. The impurity might be due to incomplete reaction or the by-product of TRZ synthesis reaction. The *m/z* of impurity-1 (116 amu) is matching with the chemical formula $C_4H_8N_2O_2$ and the suggested chemical name is *(R)*-4-(methylamino) isoxazolidin-5-one.

NMR identification of TRZ bulk drug

The proton NMR study was performed for TRZ sample characterization. Instrumental details are provided in section 3.7.1.2. The NMR spectrum is shown in Fig. 3.23 and analysis of spectrum is shown in Table 3.23.

3.8.2.2. Acid degraded Impurities

The acid degradation impurity of TRZ was identified by UPLC/ESI-MS of acid degraded sample of TRZ. The major degradation product DP-A4 was isolated and characterized by preparative HPLC and NMR (Proton, C-13 and APT), respectively.

Degradation behavior of TRZ in acid media

The stress degradation study of TRZ is discussed in section 3.5.2.3. TRZ showed one major DP and other minor DPs in acidic pH. The peak purity study of acid degraded sample chromatogram showed that one peak at Rt 8.01 minute was having co-elution of peaks and other peaks were pure. Table 3.31 shows the %area covered by each DPs and % mass balance in chromatogram.

	Name	Retention Time	Purity Angle	Purity Threshold	Area	% Area
1	Mix. DPs	3.083	12.532	0.375	238496	1.61
2	Mix. DPs	3.136	27.740	0.501	199755	1.35
3	Mix. DPs	8.124	1.415	0.281	630355	4.25
4	Peak-6	15.351	0.133	0.248	6933381	46.79
5	Peak-7	15.900	0.241	0.349	168754	1.14
6	API	26.450	3.584	0.248	6648030	44.86

Table 3.31 acid degradation product behavior

It can be seen from Table 3.30 that area covered by mixture of DPs are in low % compared to peak % area of major DP and API. The total area covered by mixture of DPs are 8.35; the reporting threshold for impurity is 0.5% (1000mg daily dose- <2g/day dose) and identification threshold is 1% of TRZ daily dose. [19]

Identification of degradation impurities

The identification of DP was done by ESI-LC-MS; instrument and chromatographic condition is described in section 3.7.1.2. For analysis using ESI/MS high concentration sample was used to reduce S/N (signal to noise) ratio and better signals. The mixture UPLC chromatogram is shown in Fig. 3.23. The peaks are showing broadening and tailing as high concentration sample was used for analysis to detect the minute responses in ESI/MS. The ESI-MS spectrum showed seven DPs among which one was major DP and other DPs were eluted at Rt 0.198, 0.669, 0.981, 1.204, 1.278 1.495 and 1.863 (Fig 3.40).

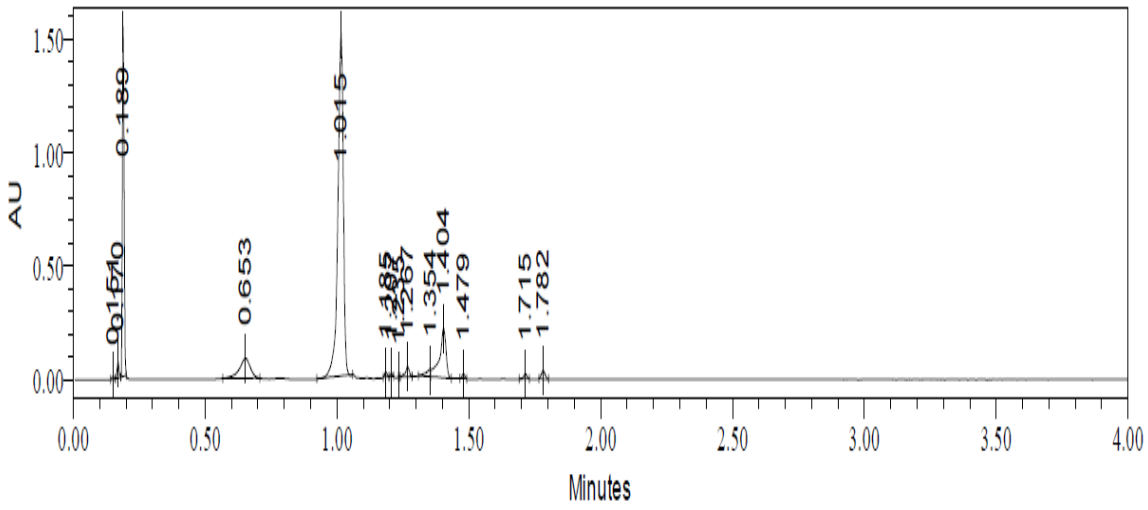
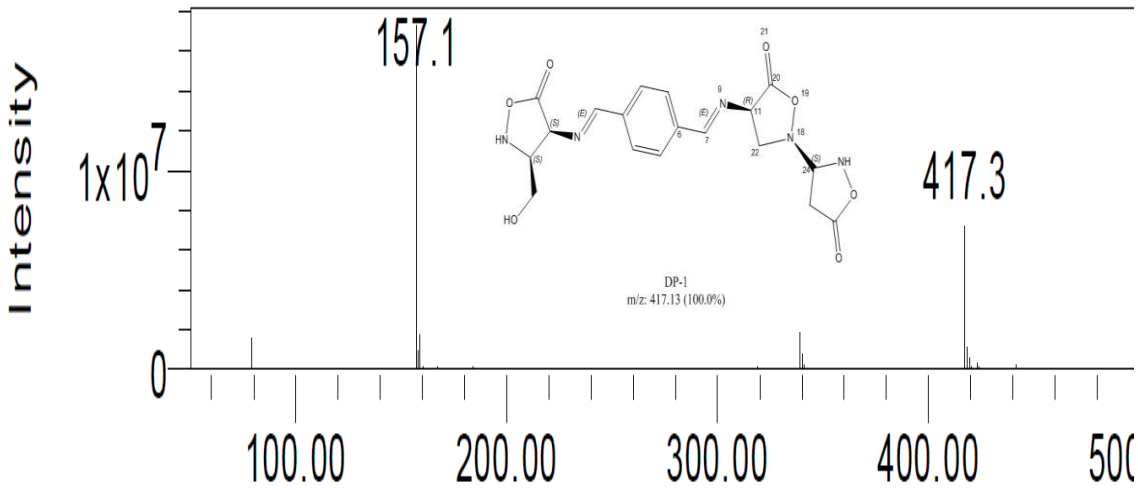


Fig. 3.40 UPLC chromatogram of acid degraded sample

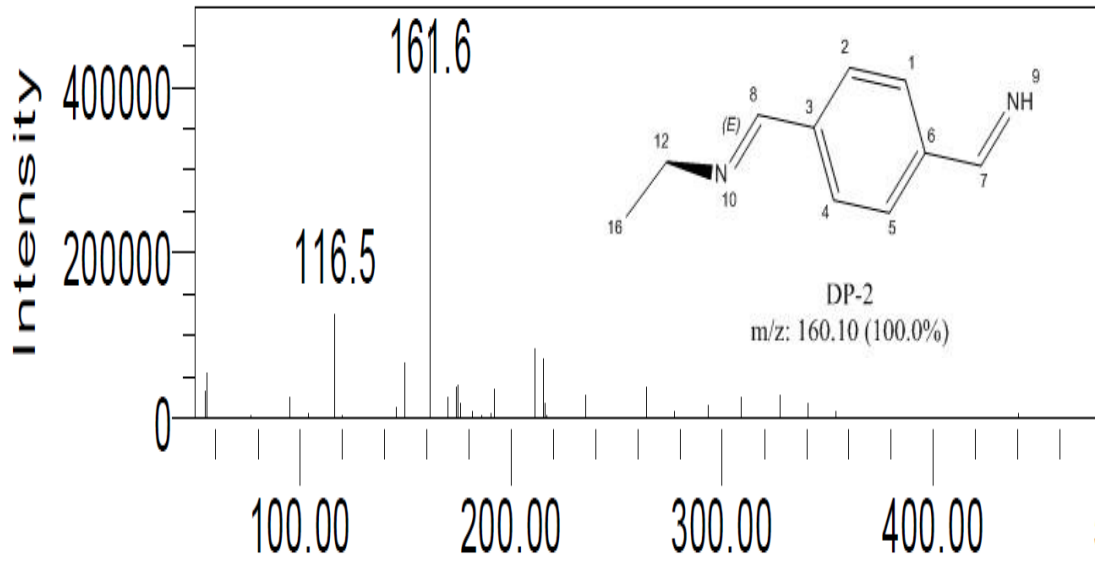
The name of the DPs are mentioned in the increasing order of *m/z* for DPs; DP-A1(*m/z 417* Rt, 0.198*)*, DP-A2 *(m/z 161* co-elution with *m/z 116* Impurity-1, Rt, 1.863*)*, DP-A3*(m/z 170* Rt,0.664*)*, DP-A4 *(m/z 237.2*,Rt,0.956,0.981, 1.029*)*, DP-A5*(m/z 279*, Rt, 1.204*)*, DP-A6 *(m/z 283*, Rt, 1.278*)* and DP-A7 *(m/z 455*, Rt, 1.495*)*. Here 'A' stands for acid DPs.

The data is gathered in Table 3.31 and shown in Fig 3.41. DP-4 was major DP; it was isolated, identified and characterized by preparative HPLC, tandem-MS and proton NMR.

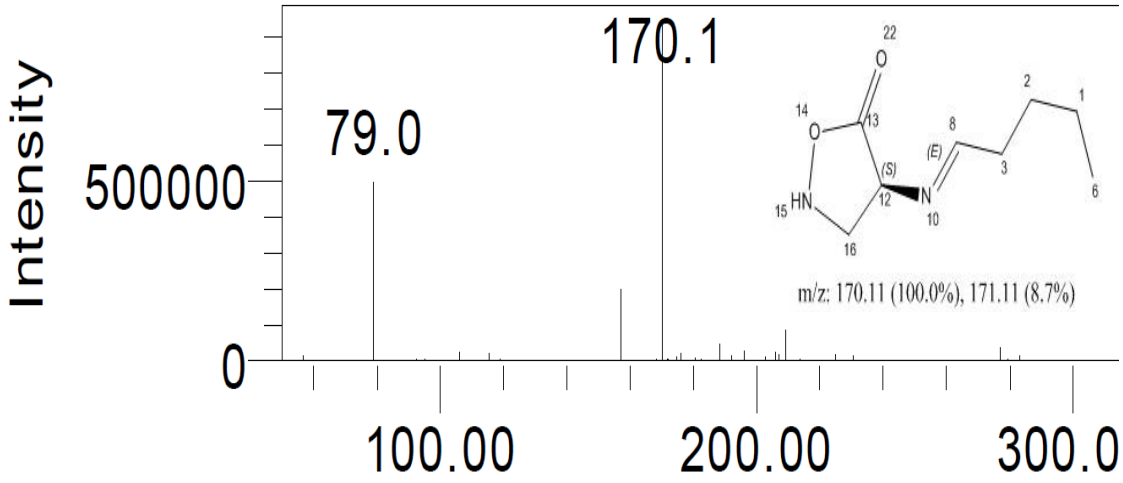
A



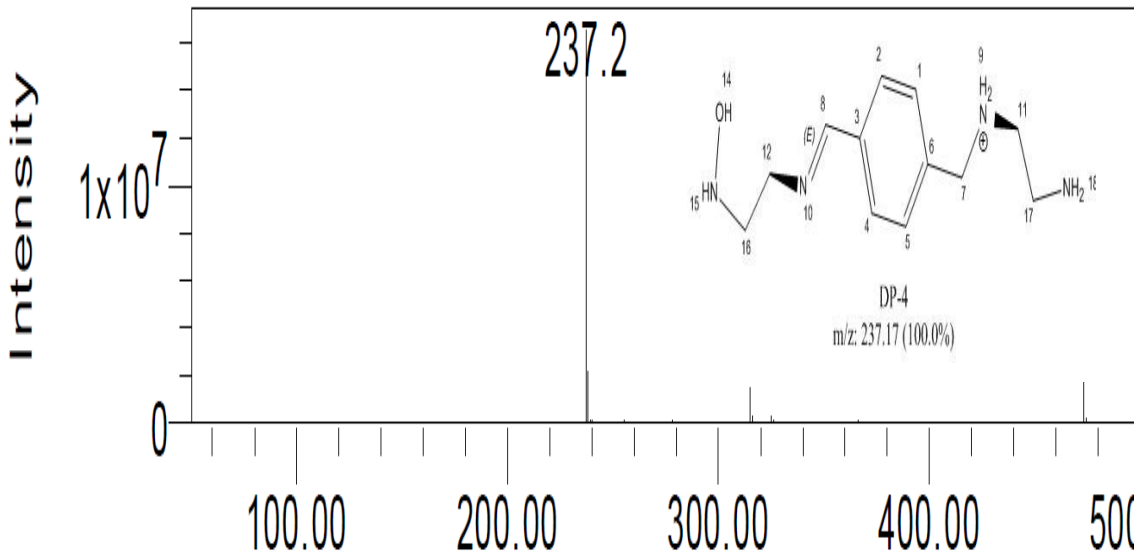
B


161.6
116.5
Intensity
400000
200000
0
100.00
200.00
300.00
400.00
DP-2
m/z: 160.10 (100.0%)

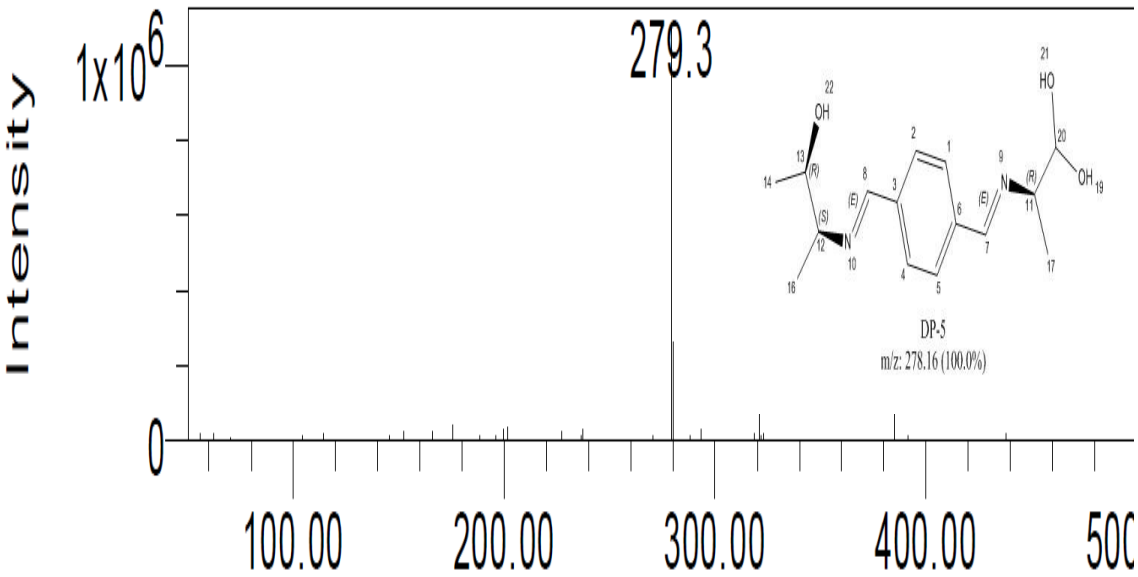
C


170.1
79.0
Intensity
500000
0
100.00
200.00
300.0
m/z: 170.11 (100.0%), 171.11 (8.7%)

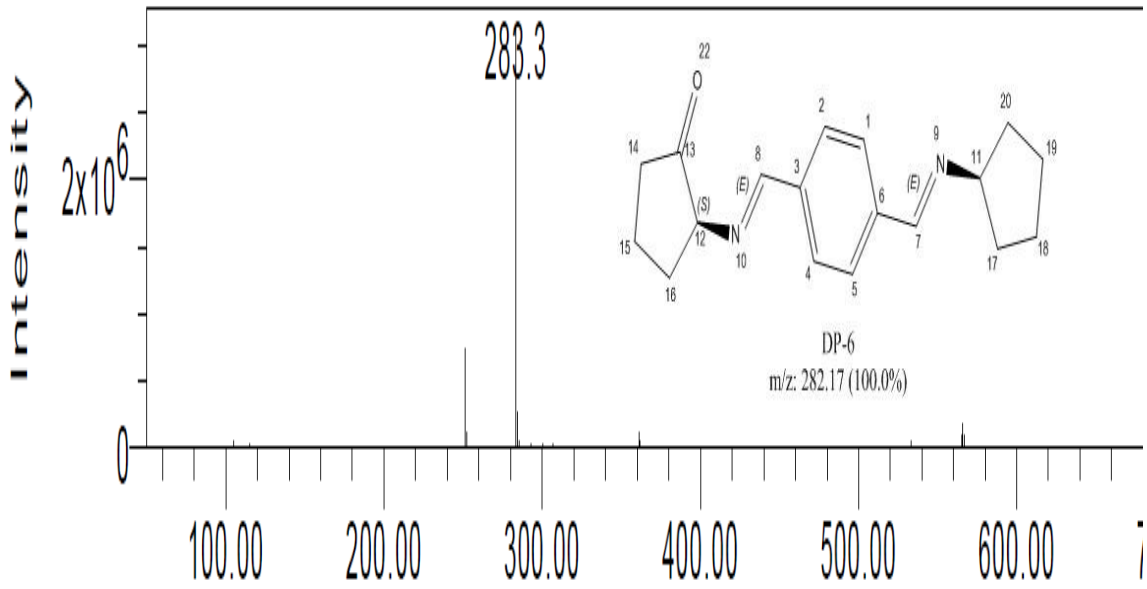
D


237.2
Intensity
1x10^7
0
100.00
200.00
300.00
400.00
DP-4
m/z: 237.17 (100.0%)

E


279.3
Intensity
1x10^6
0
100.00
200.00
300.00
400.00
DP-5
m/z: 278.16 (100.0%)

F



G

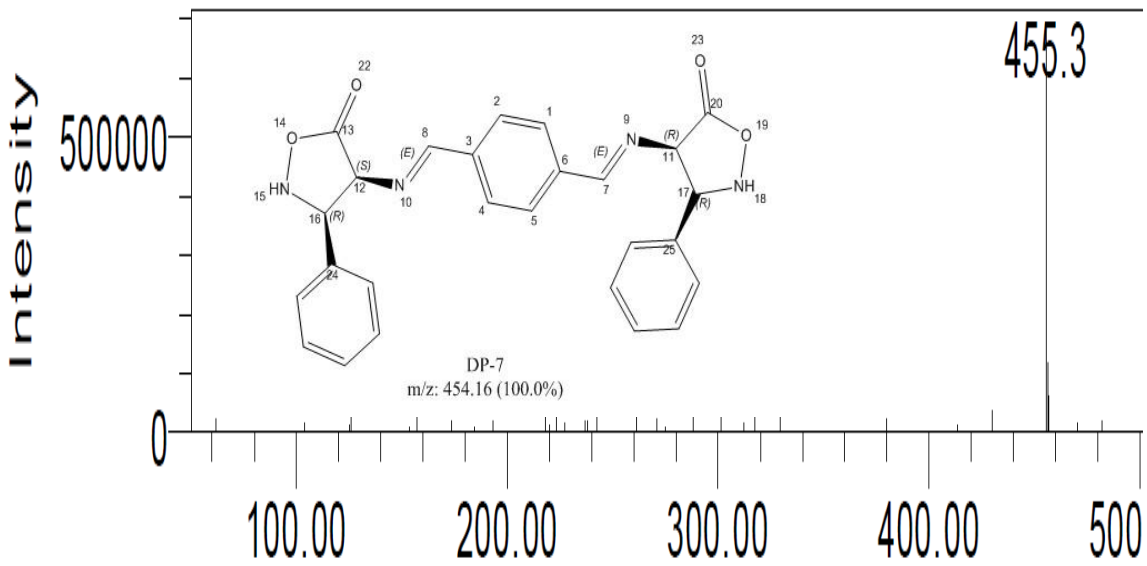
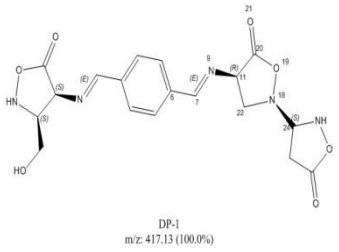
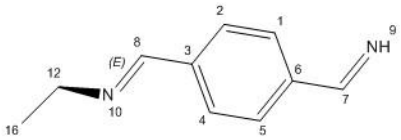
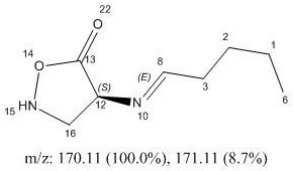
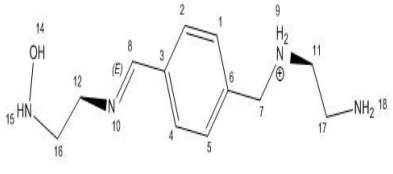
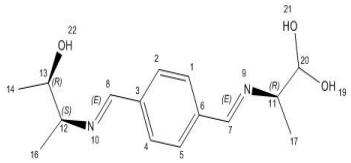
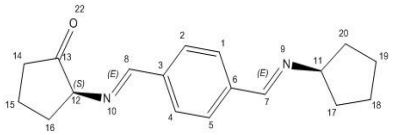
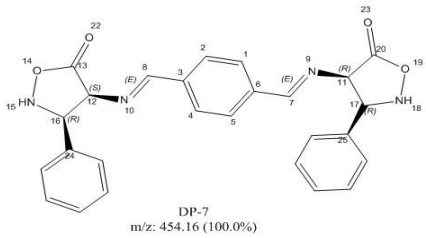


Fig. 3.41 ESI/MS spectra for A) DP-A1 B) DP-A2 C) DP-A3 D) DP-A4 E) DP-A5 F) DP-A6 and G) DP-A7

The ESI/MS spectra and proposed structure are shown in Fig 3.41 for HCl degradation products of TRZ. The possible mechanism for formation of DP is discussed in next section. The analysis of LC/ESI-MS spectra obtained for TRZ in acid media is shown in Table 3.32.

DP with Rt	*m/z*	Chemical structure	Chemical formula
DP-A1 (0.198)	*417*	 DP-1 m/z: 417.13 (100.0%) (*3's, 4R)*-4-(((E)-(((3S, 4S)-3-(hydroxymethyl)-5-oxoisoxazolidin-4-yl) imino) methyl) benzylidene) amino)-[2, 3'-biisoxazolidine]-5,5'-dione	$C_{18}H_{19}N_5O_7$
*DP-A2 (1.863)	*161*	 DP-2 m/z: 160.10 (100.0%) (*E)-N*-ethyl-1-(-4(iminomethyl) phenyl)methaimine	$C_{10}H_{12}N_2$
DP-A3 (0.664)	*170.1*	 m/z: 170.11 (100.0%), 171.11 (8.7%) *(S ,E)*-4-(pentylideneamino) isooxazolidine-5-one	$C_8H_{14}N_2O_2$
DP-A4 (0.956,0.981, 1.029)	*237.2*	 DP-4 m/z: 237.17 (100.0%)	$C_{12}H_{21}N_4O$

DP-A5 (1.204)	*279.3*	 DP-5 m/z: 278.16 (100.0%) *(R)*-2-(((*(E)*-4-*((E)*-(((*2S, 3R)-3*-hydroxubutan-2-yl)imino)methyl)benzylidene)amino)propane-1,1-diol	$C_{15}H_{22}N_2O_3$
DP-A6 (1.278)	*283.3*	 DP-6 m/z: 282.17 (100.0%) *(S)*-2-*(((E)*-4-*((E)*-(cyclopentylimino)methyl)benzylidine)amino) cyclopentan-1-one	$C_{18}H_{22}N_2O$
DP-A7(1.495)	*455.3*	 DP-7 m/z: 454.16 (100.0%) *(3R, 2S)*-4-((*(E)*-4-((E), (((3R, 4R)-5-oxo-3-phenylisooxazolidin-4-yl)imino)methyl)benzylidene)amino)-3-phenylisoxazolidin-5-one	$C_{26}H_{22}N_4O_4$

*Reported in literature [12]

Table 3.32 ESI/MS spectrum data analysis for TRZ in acid medium

Mechanism for formation of DPs

The seven DPs were formed in acid degradation study of TRZ; the probable mechanism of formation of DP in acid media is described below;

DP-A1

The *m/z* base value in DP-A1 is 157.1 which is common background ion found in MS study. The *m/z* 157.1 belongs to the DMSO $[2M+H]^+$ while DP-A1 *m/z* is 417.3; the DP can be formed by conjugation between TRZ API and other Enantiomeric fragment of TRZ API with amine group of five member ring-A while on another side $–O-CH_2$ group attached with amine group of five member ring-B. The attachment sight for TRZ is position $C^{15 \text{ and } 17}$ and $N^{14 \text{ and } 18.}$ The DP-A1 is formed due to addition of groups. The suggested chemical name for DP-A1 is (*3's, 4R)*-4-(((E)-(((3S, 4S)-3-(hydroxymethyl)-5-oxoisoxazolidin-4-yl) imino) methyl) benzylidene) amino)-[2, 3'-biisoxazolidine]-5,5'-dione and chemical formula is $C_{18}H_{19}N_5O_7$.

DP-A2

DP-A2 is formed due to degradation of five member ring attached to Enantiomeric carbon at α – position 7 and 8. Another MS peak shown along with DP-A2 is of process related impurity which is identified in previous section 3.8.2.1. The cleavage between C-8 and N-10 and between N-9 and C-11 formed DP-2. The suggested chemical name for DP-A2 is *(E)-N*-ethyl-1-(-4(iminomethyl) phenyl)methaimine and chemical formula is $C_{10}H_{12}N_{2.}$

DP-A3

The *m/z* for DP-A3 is 170.1 which suggest the degradation of benzene ring and five member ring in TRZ structure. Another MS peak of 79 is because of solvent DMSO $[M+H]^+$. The suggested chemical name for DP-A3 is *(S ,E)*-4-(pentylideneamino) isooxazolidine-5-one and chemical formula is $C_8H_{14}N_2O_{2.}$

DP-A4

DP-A4 is a major DP with *m/z* of 237.0, identification and characterization is described in details in section 3.7.2.1. The DP-A4 is formed due to cleavage of five member rings. Amine groups of five member ring remains attached while alcohol and ketone group is removed due to acid effect. The alcohol group remains attached to the amine in one of the side and it was confirmed by proton NMR spectrum analysis. The suggested chemical name for DP-A4 is *(E)*-2-amino-*N*-(4-(((2-(hydroxyamino)ethyl)imino)methyl)benzyl)ethan-1-aminium with chemical formula $C_{12}H_{21}N_4O$.

DP-A5

DP-A5 is formed due to degradation of five member ring; the alcohol group remains attached on both side and one more alcohol group remains attached to one of the side while other groups are removed due to acid. The suggested chemical name forDP-A5 is *(R)*-2-(((*E)*-4-*((E)*-(((*2S, 3R)-3*-hydroxubutan-2-yl)imino)methyl)benzylidene)amino)propane -1,1-diol with chemical formula $C_{15}H_{22}N_2O_{3.}$

DP-A6

The DP-A6 is formed due to removal of alcohol and amine and ketone group from five member rings of TRZ chemical structure. The ketone group in one of the side remains attached with five member ring. The suggested chemical name for DP-A6 is *(S)*-2-*(((E)*-4-*((E)*-(cyclopentylimino)methyl)benzylidine)amino)cyclopentan-1-one with chemical formula $C_{18}H_{22}N_2O$.

DP-A7

The DP-A7 is formed due to attachment of benzene rings at attachment sites of TRZ chemical structure. The benzene ring is the fraction of TRZ chemical structure which is ruptured in acid media. The chemical name for DP-A7 is *(3R, 2S)*-4-((*E)*-4-((E), (((3R, 4R)-5-oxo-3-phenylisooxazolidin-4-yl)imino)methyl)benzylidene)amino)-3-phenylisoxazolidin-5-one with chemical formula C_{26} H_{22} N_4 O_4.

The possible degradation pathway for TRZ in acid media is shown in Fig. 3.42.

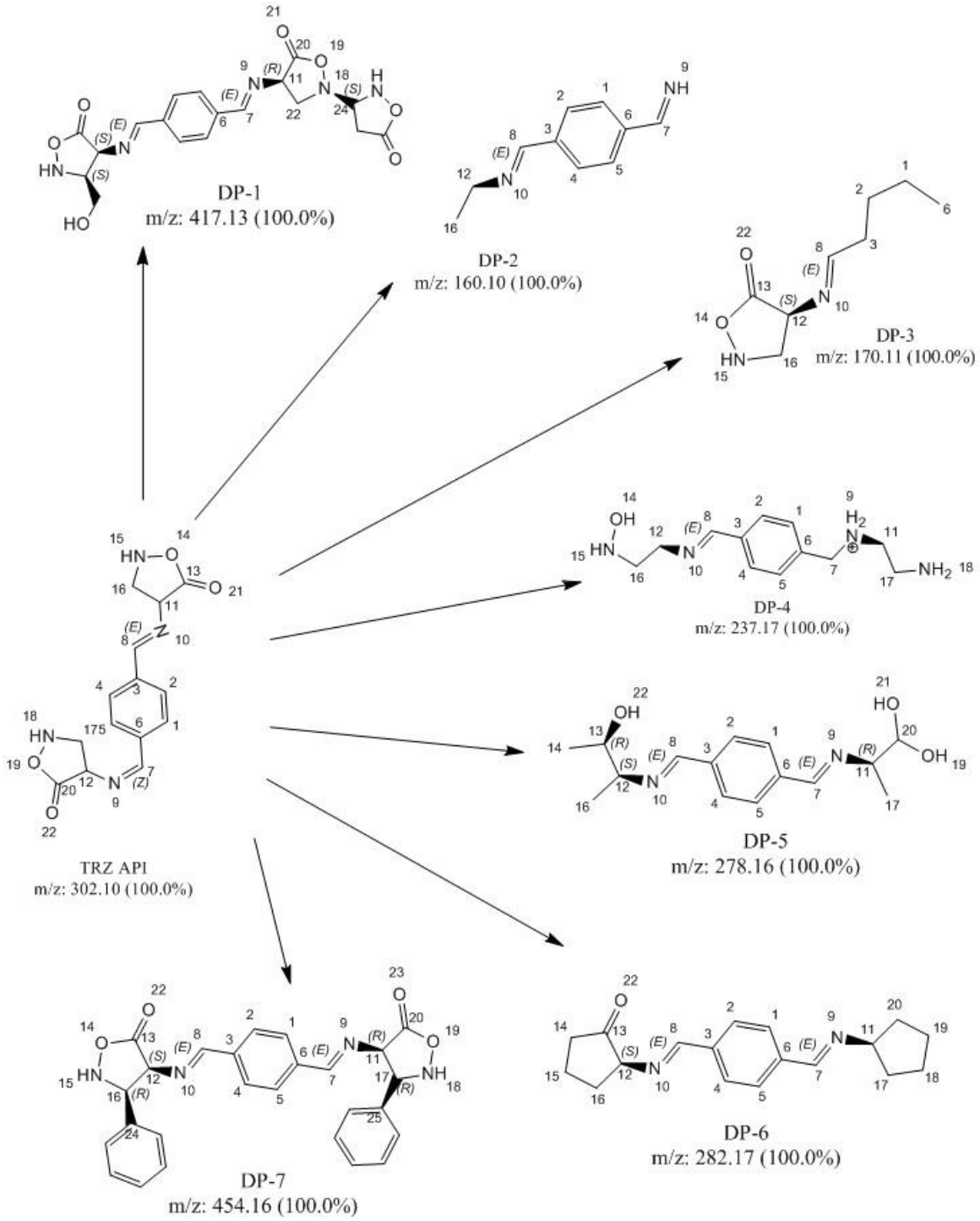


Fig. 3.42 Degradation pathway for TRZ in acid medium

3.8.2.3. Neutral degraded impurities

In neutral medium (water), TRZ formed five new peaks in HPLC chromatogram among which one major DP was formed at Rt 16.2minutes named as DP-N3. The major DP was isolated using preparative HPLC and identified using ESI-MS and Tandem MS; characterization was done using Tandem MS.

Degradation behavior of TRZ in neutral media

The TRZ bulk drug was degraded in water at RT in dark and it exhibited five new peaks in TRZ chromatogram among which one major DP was formed at Rt16.2minutes while one DP formed at Rt 24.02minutes showed co-elution of substance with it. The area covered by each peak and mass balance (100%) in HPLC chromatogram is shown in Table 3.33.

	Name	Retention Time	Purity1 Angle	Purity1 Threshold	Area	% Area
1	Peak-1	10.963	0.241	0.373	329832	2.40
2	Peak-2	16.248	0.104	0.241	4532948	32.99
3	Peak-3	24.150	1.868	1.711	59604	0.43
4	API	28.681	0.188	0.274	8634786	62.85
5	Peak-4	35.920	1.155	1.300	66380	0.48
6	Peak-5	37.053	0.310	0.947	114786	0.84

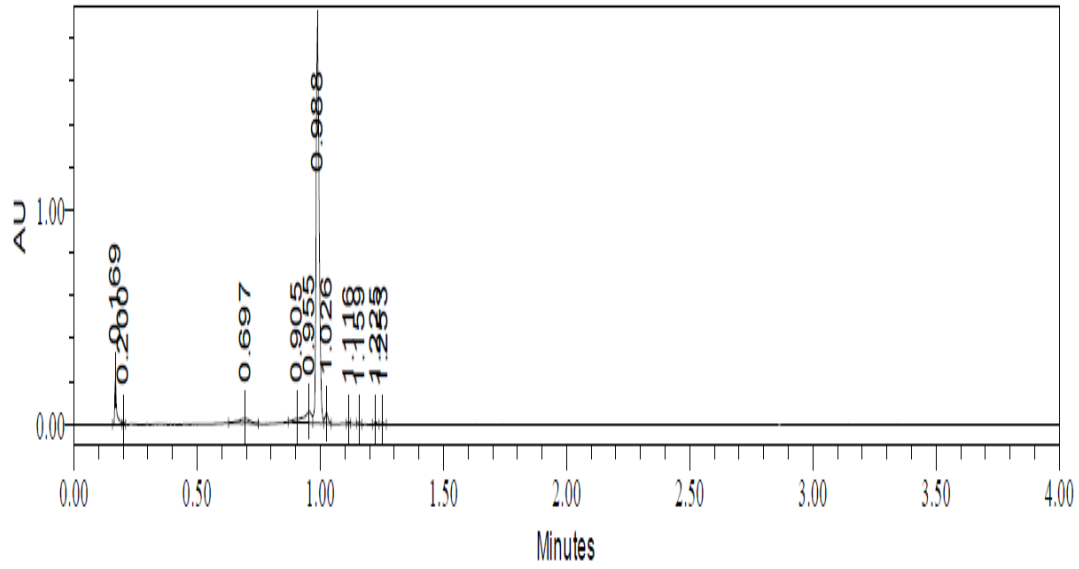
Table 3.33 TRZ neutral degraded sample chromatogram behavior

TRZ daily dose is <2gm therefore reporting impurity threshold is 0.1% of dose (1mg for 1000mg dose) and identification threshold is 0.10% of dose (1mg for 1000mg daily dose). The DPs formed in neutral degradation study were less than the identification and reporting threshold except DP-N1 and DP-N2. For that reason in UPLC-ESI/MS data the mass peak without co-elution was identified and other mass clusters were not identified as area covered by cluster is too low to identified therefore it is reported by *m/z* of DPs.

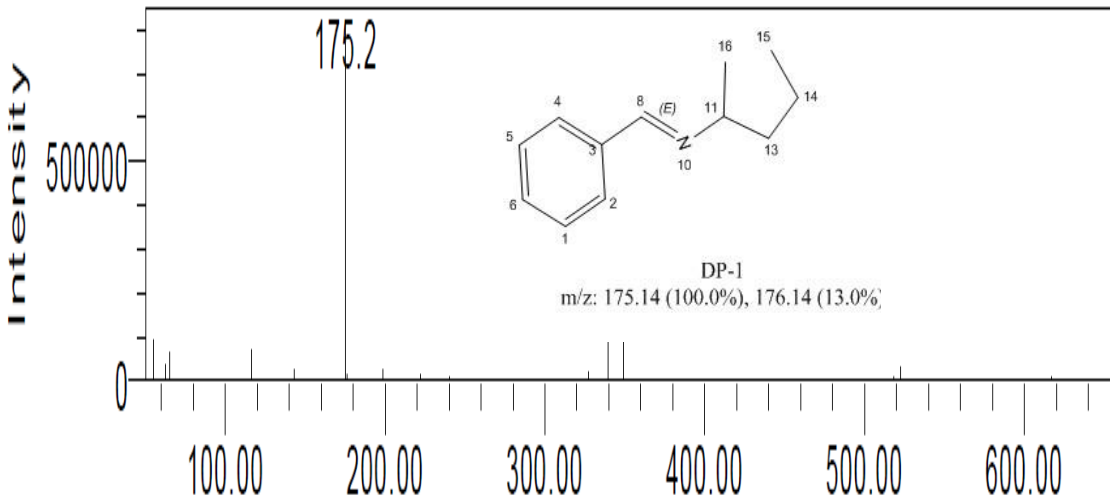
Identification of degradation impurities

The identification of DP was carried out using ESI/MS spectrum; the major DP identification and characterization is discussed in section 3.8.1.2. The UPLC chromatogram for TRZ neutrally degraded sample is shown in Fig 3.43 with mass peak.

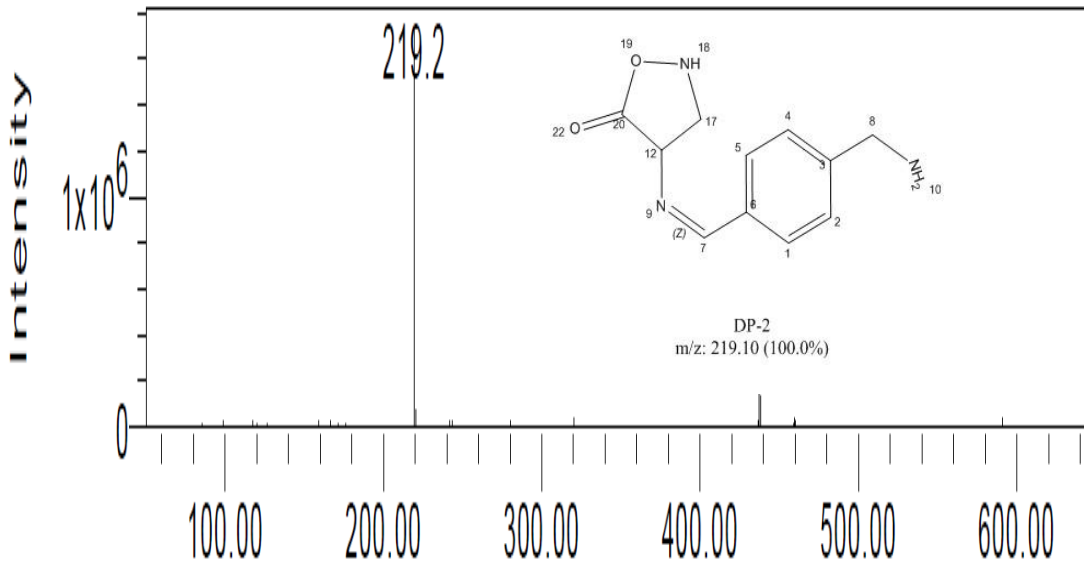
A


AU
1.00
0.00
0.988
0.697
0.905
0.955
1.026
1.116
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
Minutes

B


Intensity
500000
0
175.2
DP-1
m/z: 175.14 (100.0%), 176.14 (13.0%)
100.00
200.00
300.00
400.00
500.00
600.00

C



D

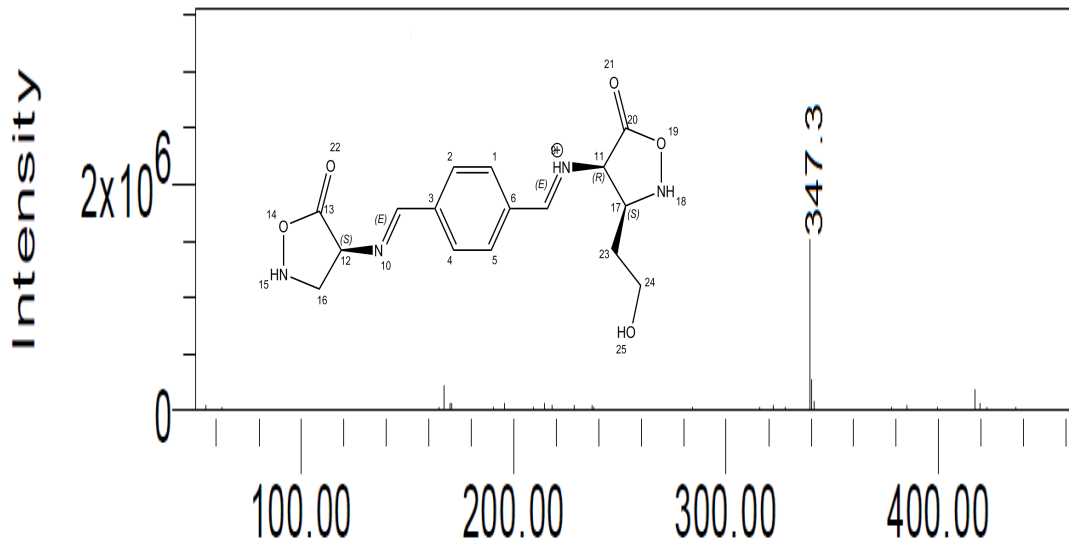


Fig. 3.43 ESI/MS spectrum analysis for TRZ degraded in neutral media

The UPLC chromatogram of TRZ neutral degraded sample showed a number of DPs and clusters of mass peaks were observed. The peaks at Rt 0.697, 0.905and 0.988 were single eluted peaks and identified. DP at 0.988 was a major DP and it was isolated by preparative HPLC, identified by UPLC, ESI-MS and Tandem MS. The clusters of mass peaks are reported in degradation pathway. The analysis of ESI/MS spectrum is shown in Table 3.34.

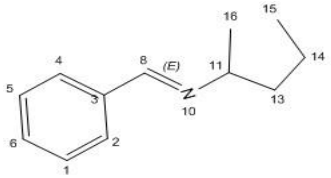
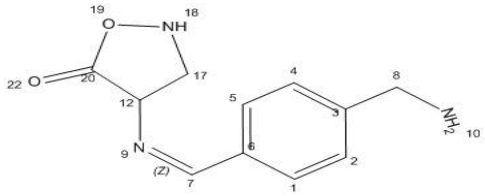
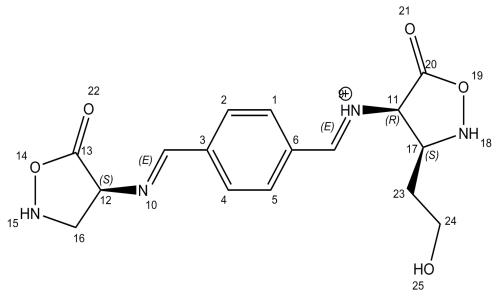
DP and Rt	*m/z*	Chemical structure	Chemical formula
DP-N1 (0.905)	*175.2*	 DP-1 m/z: 175.14 (100.0%), 176.14 (13.0%) *(E)-N*-(pentan-2-yl)-1-pheneylmethanimine	$C_{12}H_{17}N$
DP-N2(0.697)	*219*	 DP-2 m/z: 219.10 (100.0%) *(Z)*-4-((4-(aminomethyl)benzylidene)amino)isoxazolidin-5-one	$C_{11}H_{13}N_3O_2$
DP-N3(0.988)	*347.3*	 *(3S, 4R)* -3-(2-hydroxyethyl)-5-oxo-*N- (((E)*-4-(((*S*)-5-oxoisoxazolidin-4-yl) imino) methyl) benzylidene) isoxazolidin-4-aminium	$C_{16}H_{19}N_4O_5$

Table 3.34 ESI/MS data analysis of TRZ neutral degraded sample

Mechanism for formation of DPs

The ESI-MS spectrum showed nine very small peaks, the *m/z* of those peaks were reported while two major peaks were observed for which structures were elucidated based on *m/z* and one major DP was isolated and characterized by NMR and IR data.

Mechanisms for formation of DPs are described below;

DP-N1 (*m/z 175.1)*

The DP-N1 is formed due to removal of five member ring from one side and on another side removal of ketone (=O), alcohol (-O) and amine ($-NH_2$) group. The suggested chemical name for DP-N1 is *(E)-N*-(pentan-2-yl)-1-pheneylmethanimine and chemical formula is $C_{12}H_{17}N$.

DP-N2 *(m/z 219)*

The DP-N2 showed *m/z* 219 which suggest the removal of five member - ring attached to amine group. The cleavage of bond between amine (-NH) and carbon of five member ring formed the DP-N2. The suggested chemical name for DP-N2 is *(Z)*-4-((4-(aminomethyl)benzylidene)amino)isoxazolidin-5-one and chemical formula is $C_{11}H_{13}N_3O_2$.

DP-N3 *(m/z 347.3)*

The attachment of -ethyl group at C-17 resulted in formation of DP-N3. TRZ was not stable in proteolytic solution and formed a number of DPs; the attachment may be result of constant conformational change that stabilized in the form of DP-N3 in neutral medium. The suggested chemical name for DP-N3 is *(3S, 4R)* -3-(2-hydroxyethyl)-5-oxo-*N*- *(((E)*-4-(((*S*)-5-oxoisoxazolidin-4-yl) imino) methyl) benzylidene) isoxazolidin-4-aminium, and chemical formula is $C_{16}H_{19}N_4O_5$.

The proposed degradation pathway is shown in Fig 3.44.

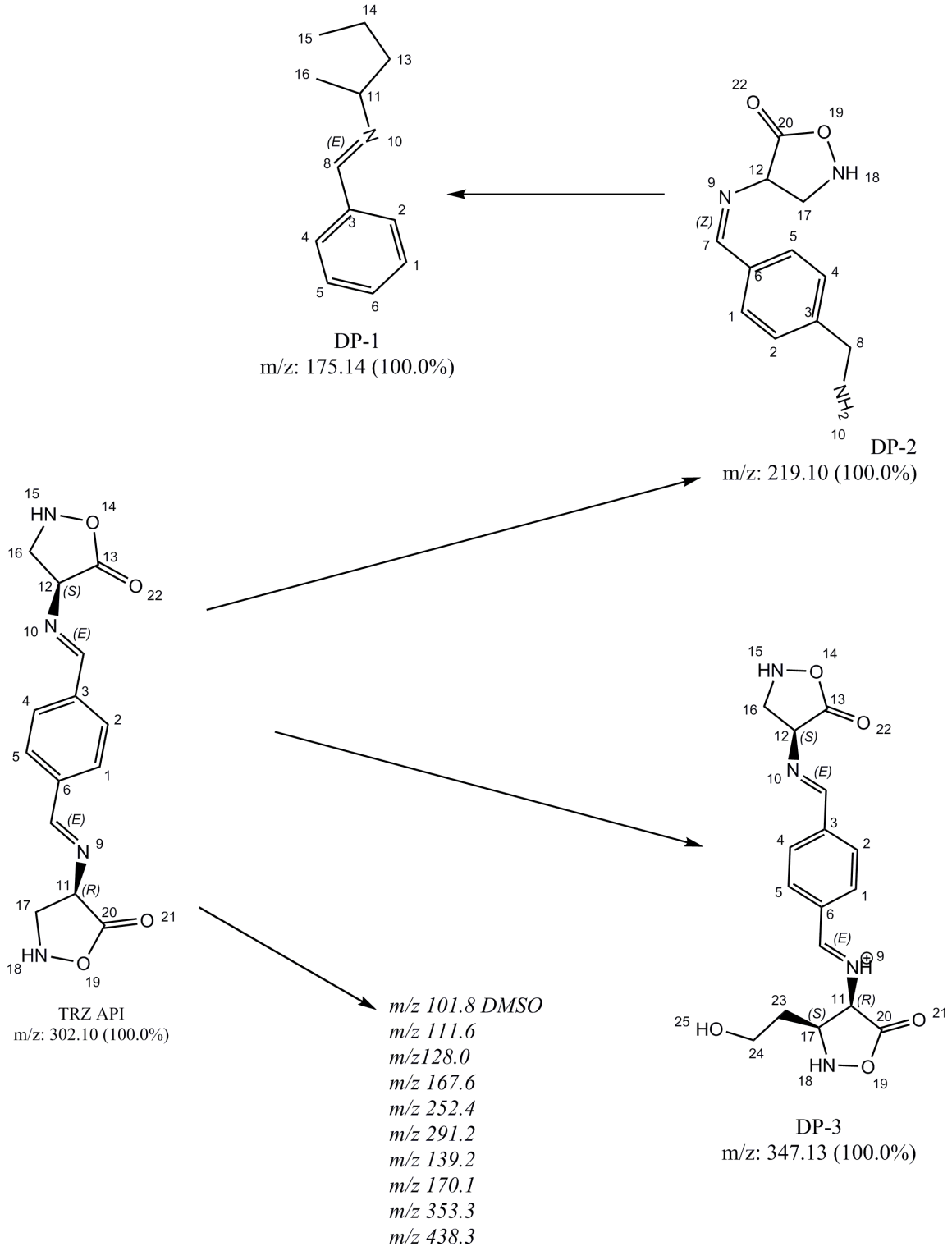


Fig.3.44 proposed degradation pathway for TRZ in neutral media

3.8.2.4. TRZ in alkaline medium

The stress degradation of TRZ in 0.5N NaOH alkaline medium showed that TRZ chemical structure broken down into fractions after a certain time point (60 minutes) before that there was no DP peak appeared. The lower concentration of alkali was used to find out if any major or identifiable peak appears (during kinetic study 0.1N NaOH) but the same degradation pattern was observed although the time point for disappearance of TRZ peak and appearance of fractions peak was extended.

The ESI/MS study showed several DPs which are not in the concentration that it could be identified or reported but it confirms that the TRZ is highly susceptible for alkaline condition (even for 0.01N NaOH) generating several degradation products in small quantities those are difficult to isolate and characterize.

3.8.2.5. TRZ in oxidative media

TRZ was kept in presence of oxidative agent to notice any change in TRZ chemical structure. The stress degradation study is discussed in section 3.5.2.3.

Degradation behavior of TRZ in oxidative media

Degradation behavior of TRZ in oxidative medium is shown in HPLC chromatogram (Fig. 3.5 (F)); the chromatogram showed two additional peaks other than TRZ and hydrogen peroxide peak. The peak-1 was co-eluting peak while major peak-2 was passed in purity test and was isolated by preparative HPLC, identified by ESI/MS and Tandem MS, characterized by proton NMR. The co-eluting peaks in peak-1 were identified by ESI/MS. The analysis of peroxide degraded sample and mass balance (100%) of TRZ chromatogram is shown in Table 3.35.

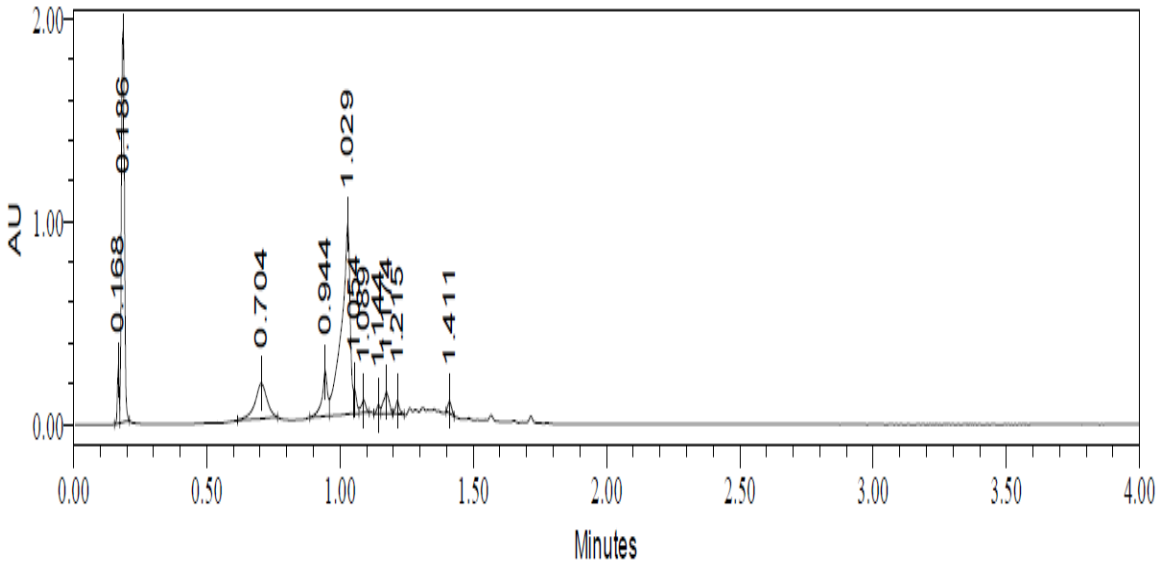
	Name	Retention Time	Purity1 Angle	Purity1 Threshold	Area	% Area
1	H_2O_2	2.657	1.177	3.919	2011321	26.62
2	1 (Mix. DP)	8.027	9.945	1.115	1443756	19.11
3	DP-O7	15.262	0.248	0.237	3539411	46.85
4	API	26.168	0.244	0.248	560435	7.42

Table 3.35 TRZ behavior in oxidative media

Identification of degradation impurities

Identification of DP was carried out by ESI/MS. The UPLC chromatogram and ESI/MS spectrum is shown in Fig 3.45.

A



B

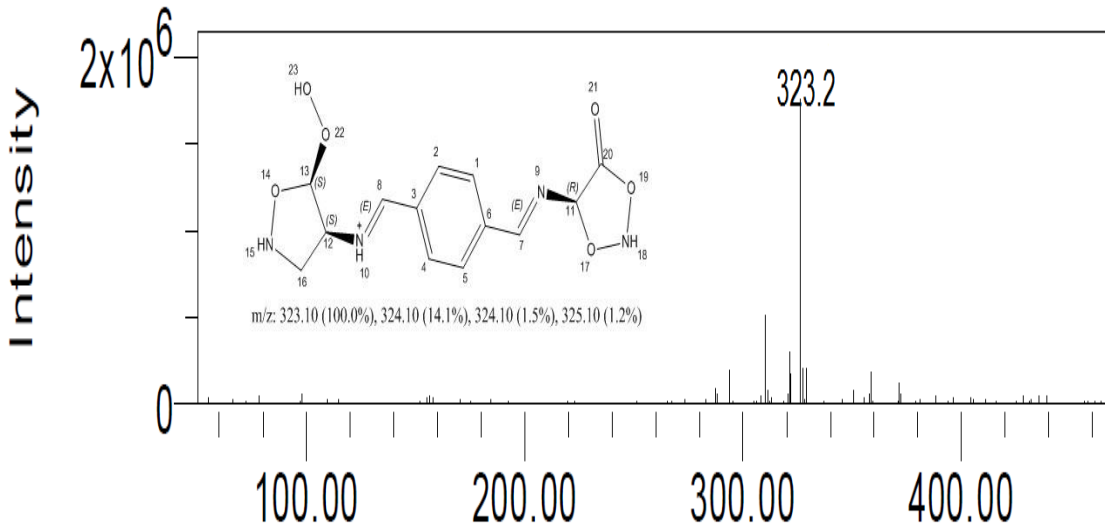


Fig.3.45 A) UPLC chromatogram of oxide degraded sample of TRZ B) ESI/MS spectrum of major DP

The UPLC chromatogram showed 11 mass peaks among which one was major DP which has *m/z* 323.3 (named as DP-O7). The ten DPs were eluted in HPLC chromatogram at Rt 8.027minutes therefore DPs with *m/z* 170, 417 and 237 are identified in oxidative medium while other DPs are not identified as the ESI-MS peaks appeared as mixture of DPs as cluster of peaks. The *m/z* is shown in proposed degradation pathway. (Fig 3.46)

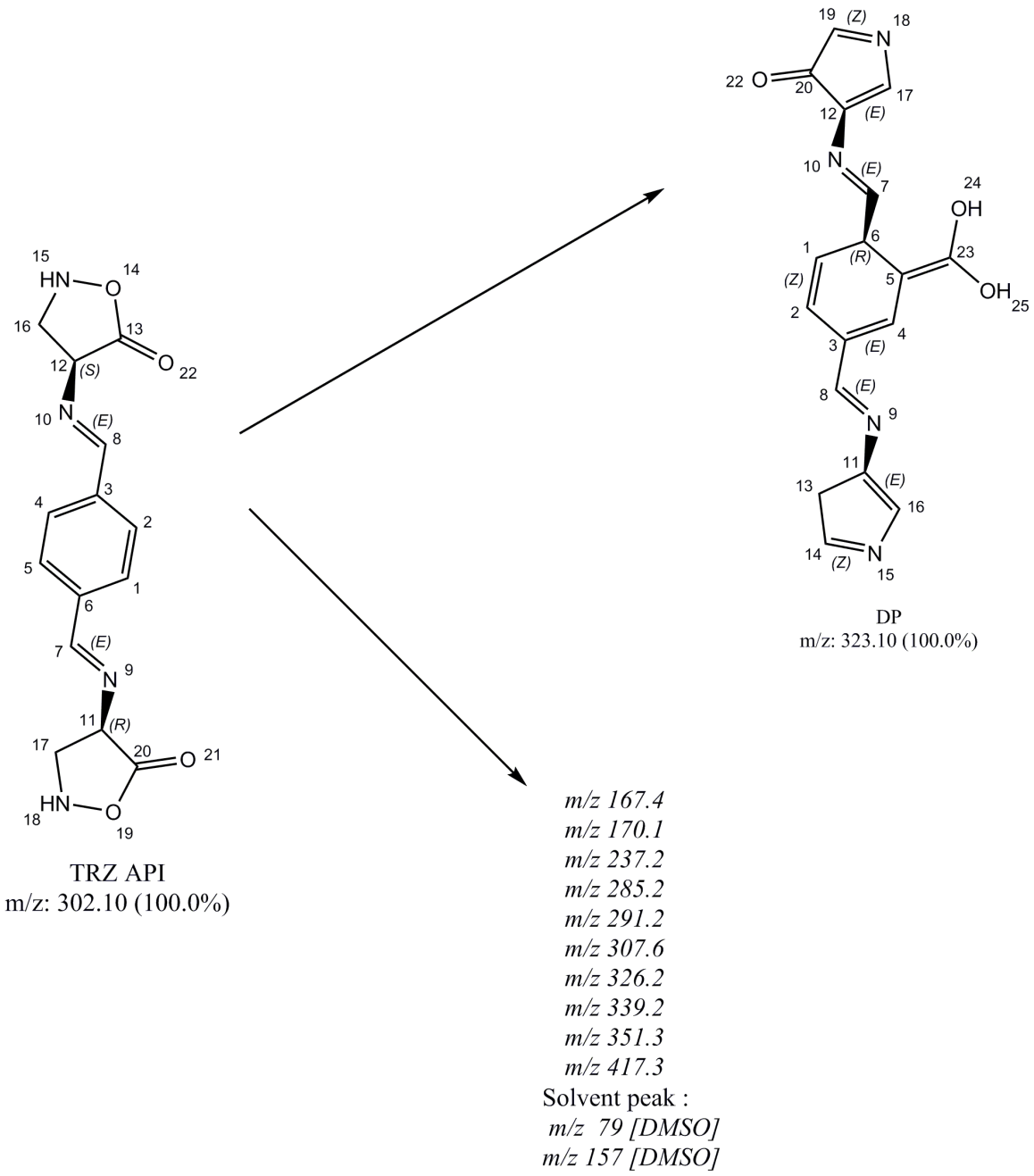


Fig.3.46 Proposed degradation pathway for TRZ in oxidative medium

Mechanism for formation of DPs

The cluster of 11 DPs were observed in ESI/MS spectrum of TRZ sample in oxidative medium, among which four DPs (*m/z 157, 170.1, 237.3and 417.3)* are identified in section 3.8.2.2. The major DP with *m/z* 323.1 suggests the oxidation of TRZ. The oxidation in presence of oxidizing agent was occurred at position 5 in TRZ chemical structure. The conjugation or/and fragmentation of TRZ formed other minor DPs. The Oxidative DP-O7 is named as 4-(((*E*)-(4-((*E*)-((3*H*-Pyrrol-4-yl)imino)methyl)-6-(dihydroxymethelene)cyclohexa-

2,4-dien-1-yl)methylene)amino)-*3H*-pyrrol-3-one that matches with chemical formula $C_{17}H_{14}N_4O_3$.

- **Conclusion:**

A new RP-HPLC method was developed for stability study of TRZ in different conditions (hydrolytic, oxidative, photolytic and thermal). The study results showed that TRZ is prone to degradation in hydrolytic solutions (acidic, alkaline and neutral condition) and oxidized in presence of oxidative agent while it was stable under thermal and photolytic conditions for more than 28days. TRZ is highly sensitive for alkaline medium. These results suggest that during formulation preparation or storage, acidic and/or alkaline and/or water and/or oxidative conditions as well as excipient or solvent should be avoided to stop degradation of TRZ bulk drug. If possible the formulation should be coated with suitable coating agent or should be transferred in capsule formulation to avoid contact with environmental or other stressor that can degrade TRZ in formulation.

The degradation kinetic study of TRZ was completed in acid, neutral and oxidative condition and TRZ followed first order kinetics (hydrolytic and oxidative conditions) under the effect of stressor concentration. Degradation kinetic parameters (rate constant, half life, shelf life, activation energy, enthalpy of reaction and entropy of reaction) were also evaluated for the degradation kinetic study. The reactions were endothermic and used environmental energy to degrade TRZ.

The impurity profile was created for TRZ by reporting, identifying and characterizing the process related impurity and degradation impurity. The DP reported in literature is marked with asterisk in the respective section. The major DP of respective condition (acidic, neutral and oxidative condition) was identified by RP-HPLC, UPLC/ESI-MS and Tandem MS while characterized by either proton NMR and/or C-13 and attached proton test NMR. The IR test was performed to confirm the presence of groups in DP structure.

The highlight of the study was DPs identification and characterization in different conditions; In acidic condition 7 DPs were identified (one DP *m/z 161* is reported in literature) and structure was elucidated based on UPLC/ESI-MS data among which one major DP was isolated and characterized using sophisticated instruments.

In neutral media total 11 DPs were reported among which only two DPs were in high concentration to identify and structure elucidation while one major DP was isolated and characterized using sophisticated instruments.

In oxidative condition total 11 DPs were reported as these DPs were in very small amount, the structure was not elucidated for them but one major DP was isolated and characterized using sophisticated instruments.

The process related impurity and other degradation impurities are reported for the first time. This process related impurity and degradation impurity can be helpful further for toxicity study and/or pharmacological study of DPs. This TRZ impurity profile is helpful in suggesting storage condition for formulation and bulk drug of TRZ. This study would be helpful in taking precautions to avoid certain conditions, excipient, media and solvent during formulation preparation.

References:

1. TBCAB (Global TB Community Advisory Board) (Internet), TBonline, Terizidone, Available on: http://www.tbonline.info/posts/2011/8/24/terizidone/

2. WHO (World Health Organization) (Internet), essential medicines list, Available on:https://www.who.int/selection_medicines/comMittees/expert/20/applications/terizidone/en/

3. Central drug standard control organization (Internet), Drugs, Available on : https://cdscoonline.gov.in/CDSCO/Drugs

4. Vora A., Drug Review: Terizidone, *JAPI*, 2010; 58, 267.

5. National library for medicines, National center for biotechnology information (Internet),Pubchem, Terizidone, Available on: https://pubchem.ncbi.nlm.nih.gov/compound/Terizidone

6. European Chemicals agency (ECHA) (Internet), Terizidone, Available on: https://echa.europa.eu/substance-information/-/substanceinfo/100.042.882

7. Khairnar S. K., Nagras M. A and Sonawane M. A., Development and Validation of UV Spectrophotometric Method for the Estimation of Terizidone in Bulk and Pharmaceutical Dosage Form, *Inventi Rapid: Pharm Analysis & Quality Assurance*, 2016; 3, 1.

8. Jain H. K. and Mane R. R., Estimation of Terizidone in bulk and capsule dosage form by Area under curve and first order derivative spectrophotometry, *Int J Pharma Tech Res*, 2016; 9(9), 457.

9. Mulubva M. and Mugabo P., Analysis of Terizidone in plasma using HPLC-UV method and its application in a pharmacokinetic study of patients with drug resistant tuberculosis, *Biomed Chromatogr*, 2018; 32(11), 4325.

10. Gandhi S. V., Shevale V. P. and Choudhari G. B, Development and validation of a stability indicating RP-HPLC method for the determination of Terizidone, *Indo Am J PSci*, 2018; 5(3), 1353.

11. Bhole R. P. and Phadke S., Development and validation of thin layer chromatography and MS-MS method for estimation of Terizidone in pharmaceutical dosage form, *TJPS*, 2018; 42(4), 196.

12. Patil M.P.,Alalaiwe A., Two new degradation products of Terizidone: An application of forced degradation study and hyphenated chromatographic techniques, *Int J Pharma Bio Sci*, 2019; 10(2),58-70.

13. Mulubwa, M, Mugabo, P. Steady-state population pharmacokinetics of Terizidone and its metabolite Cycloserine in patients with drug-resistant tuberculosis. *Br J Clin Pharmacol.*, 2019; 85: 1946– 1956.

14. Shirkhedkar A A, Khan M M G, Chaudhari P, Pawara P I, Analytical techniques for Pirfenidone and Terizidone: A review. *Int J Pharm Chem Anal*, 2019;6(1):1-5.

15. Vanavi P J. and Rajput S. J.,. Impurity profiling of first line anti-tb drug-terizidone using chromatographic and related techniques. *International journal of pharmacy and pharmaceutical sciences*, 2021; 13(5), 83-95.

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

17. National library for medicines, National center for biotechnology information (Internet),Pubchem, Terizidone, Available on: https://pubchem.ncbi.nlm.nih.gov/compound/Terizidone

18. ICH (Internet), Quality Guideline, Available on: https://www.ich.org/

19. ICH (Internet), Stability Guidelines, Available on: https://www.ich.org/