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Development Of Novel Stability Indicating RP-HPLC Method For Quantification Of Trientine Hydrochloride And Characterization Of Its Degradation Product By Spectroscopic Techniques.

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ABSTRACT

The present work reports the development of a novel stability indicating RP-HPLC method for the quantification of Trientine hydrochloride. The stability of Trientine hydrochloride was studied under different stress conditions recommended by ICH guidelines. Trientine contains amino group which makes it vulnerable to oxidative degradation. The chromatographic separation of the drug and its degradation product was achieved in a Waters Reliant C_8 column (250mm x 4.0mm ID, 4µm particle size) employing gradient elution using Acetronitrile and Ammonium formate buffer at pH 5.3 ± 0.05 (90:10) v/v, at a flow rate of 0.8mL/min and detection performed at 220 nm. The develop method has been validated as per ICH guidelines. Structural characterisation and identification of the degradation product was done by LC-MS, ^{1}H NMR, ^{13}C NMR and IR spectroscopic studies. Toxicity prediction for the degradation product was achieved using the Software OSIRIS.

Keywords: Trientine, Stability indicating RP-HPLC, Validation, Characterisation, Toxicity prediction.

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INTRODUCTION

Trientine hydrochloride (TNT) is chemically N'-(2-(2-aminoethylamino)ethyl)ethane-1,2-diaminehydrochloride.[1] (Figure 1) It is a metal chelating agent used to bind and remove excess copper in the body to treat Wilson's disease. It also possesses potent anti-angiogenic activity. In addition, trientine may inhibit copper-induced secretion of interleukin-8(IL-8). Literature review revealed spectroscopic method[2], fluorimetric methods,[3,4] RP-ion pairing HPLC and conductivity detection[5] for estimation of TNT and simultaneous estimation of TNT and its two major metabolites by HPLC.[6] In the present work, stability indicating RP-HPLC method using acetronitrile : ammonium formate as mobile phase in gradient elution technique has been reported for the quantification of Trientine in bulk and pharmaceutical dosage form.

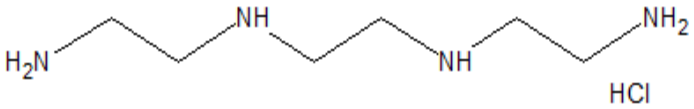

Figure 1. Structure of Trientine hydrochloride

EXPERIMENTAL

Instrumentation

The Water HPLC 2996 system equipped with photo diode array detector was used for the study.

Software used for this system is Empower 3. HPLC was performed using Waters Reliant C_8 column (250 mm x 4.0 mm ID, 4µm particle size).

Reagents and chemicals

TNT ≥99.0% was obtained from Saimirra Innopharm Pvt Ltd. All the chemicals and reagents used were of HPLC grade.

Mobile phase

The mobile phase used in the present work was Acetronitrile: Ammonium formate buffer pH 5.3 (90:10) v/v in gradient elution technique. The flow rate was set to 0.8mL/min.

Preparation of standard solution

About 200mg of TNT standard was accurately weighed and transferred into a 100ml volumetric flask, dissolved and made upto the volume with diluent.10ml of standard stock solution was pipetted out into a 100ml volumetric flask, 40ml of diluent, 1ml of 1N sodium hydroxide and 1.5ml of ammonium hydroxide solution were added and shaken well. The flask was kept in an ice-bath to allow the solution to reach the temperature 2 to 8ºC and the same temperature was maintained for about 15 min. 2ml of benzoyl chloride was added to the flask and kept in ice-bath for 2 min. The flask was kept at room temperature for about 30 min and made upto volume with diluent. 20µL of the above solution was injected and the chromatogram was recorded. The retention time of TNT was found to be 12.497 mins.

Quantification of TNT formulation

For analysis of the capsule dosage form, twenty capsules (Saimirra Pvt.Ltd) containing 250 mg of TNT was weighed and the average weight was determined.The powder equivalent to the weight of 200 mg of TNT was transferred to a 100ml volumetric flask, dissolved and made upto the volume with diluent. 10ml of the solution was pipetted out into a 100ml volumetric flask, 40ml of diluent, 1ml of 1N sodium hydroxide and 1.5ml of ammonium hydroxide solution were added and shaken well. The flask was kept in an ice-bath to allow the solution to reach the temperature of 2 to 8ºC, the same temperature was maintained for about 15 min. 2ml of benzoyl chloride was added to the flask and kept in an ice-bath for 2 min. The flask was kept at room temperature for about 30 min and made upto volume with diluent. 10ml of the above solution was pipetted out into a 25ml

volumetric flask and made upto volume with diluent to get the final concentration of 80µg/mL.20µL of solution was then injected for quantitative analysis.

METHOD VALIDATION

The developed method was validated[7-13] as per ICH guidelines Q2(R1)[4].

Linearity:

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in sample. Linearity test solution for the assay method was prepared by diluting the stock solution to the required concentrations. Five different concentration levels of the solutions were prepared in the range of 40 to 120µg/mL. RSD value for the slope and Y-intercept of the calibration curve was calculated (Table 1). Peak area under the curve (average peak area of five observations) was plotted against the respective concentration level. Straight lines were obtained and the calibration equation obtained from regression analysis was used to calculate the corresponding predicted responses.

Accuracy:

Accuracy of an analytical procedure expresses the closeness of agreement between a value which is accepted either as a conventional true value or an accepted reference value and the value found. The accuracy of the assay method was evaluated in triplicates at three different concentration levels, 50%, 100%, and 150% i.e. 40, 80, 120 µg/mL in the bulk drug sample. Percentage recoveries were calculated from the slope and Y-intercept of the calibration curve developed for the drug. Percentage recoveries for the drug was within the range 99.87–100.08% (Table 2).

Precision:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability (same day), intermediate precision (Interday-Three different day, Intraday- different time interval on the same day), and reproducibility (different lab). Precision was carried out by injecting six replicates at the 100% level. The RSD of the peak areas was calculated (Table 3).

Limit of detection (LOD) and Limit of Quantification (LOQ):

The LOD is defined as the lowest amount of analyte in a sample which can be detected, but not necessarily quantify as an exact value and the LOQ was defined as the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The LOD and LOQ for trientine was determined as S/N ratio of 3:1 and 10:1 respectively by injecting a series of dilute solution with known concentration. The values of LOD and LOQ obtained are shown in Table 4.

Robustness:

To determine the robustness, deliberate changes in the following parameters were made: different columns, pH, wavelength and column temperature (Table 5).

Solution stability:

Stability of sample solution was established by storage of the sample solution at 6°C for 48 hrs. Trientine hydrochloride was reanalysed after 24 and 48 hrs time intervals and assay value was determined and compared against fresh sample. Sample solution does not show any appreciable change in assay value when stored at 6°C for 48 hrs.

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FORCED DEGRADATION STUDY

Forced degradation study was carried out on Trientine hydrochloride capsules under various stress conditions like acid and base hydrolysis, oxidation, thermal and photolytic degradation.

Acid hydrolysis :

The study was performed by adding 10ml of 1M HCl to 50mg of TNT in a 50mL volumetric flask. The mixture was kept at 70°c for 1 hr and the mixture was neutralised with 10ml of 1M NaOH and the chromatogram was recorded.

Base hydrolysis :

10ml of 1M NaOH was added to 50mg of TNT in a 50mL volumetric flask. The mixture was kept at 70°c for 1 hr and the mixture was neutralised with 10ml of 1M HCl and the chromatogram was recorded.

Oxidative degradation :

Oxidative degradation was performed by adding 5ml of 3% H_2O_2 to 50mg of TNT in a 50mL volumetric flask and the mixture was kept at 70°c for 1 hr and the chromatogram was recorded.

Thermal degradation :

Thermal degradation was done by treating 50mg of TNT at 105°c for 5 hrs in an oven, after which it was cooled to room temperature and the chromatogram was recorded.

Photolytic degradation :

Photolytic degradation was done by subjecting TNT at 350nm in UV-visible light for 5 hrs and the chromatogram was recorded.

CHARACTERIZATION OF OXIDATIVE DEGRADATION PRODUCT OF TNT:

The degradation product obtained in oxidative degradation study was targeted for its structural characterization. The oxidative degradation product was analysed by LC-MS. The mass spectrometer was run in positive ionization mode and negative ionization mode with turbo ion spray interface and mass to charge (m/z) ratio was recorded. Further structure elucidation of the oxidative degradation product was done by ^{1}H NMR, ^{13}C NMR and IR spectroscopic studies.

TOXICITY PREDICTION:

Toxicity Prediction was done by OSIRIS Property explorer, the online software of Thomas Sander Actelion Pharmaceuticals Ltd, Switzerland.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

Column chemistry, solvent type, solvent strength, detection wavelength and flow rate were varied to determine the chromatographic conditions for best separation. The mobile phase conditions were optimised so that the components were free from the interference of solvent and excipients. Mobile phase and the flow rate selection was based on the peak parameters like height, area, tailing, theoretical plates and the run time. The best result was obtained by use of gradient mobile phase A and mobile phase B (Programmed). Under the optimum chromatographic conditions, the retention time obtained for TNT was 12.497mins.The values obtained for the validation parameters show that the chromatographic conditions are appropriate for separation and determination of the selected drug.

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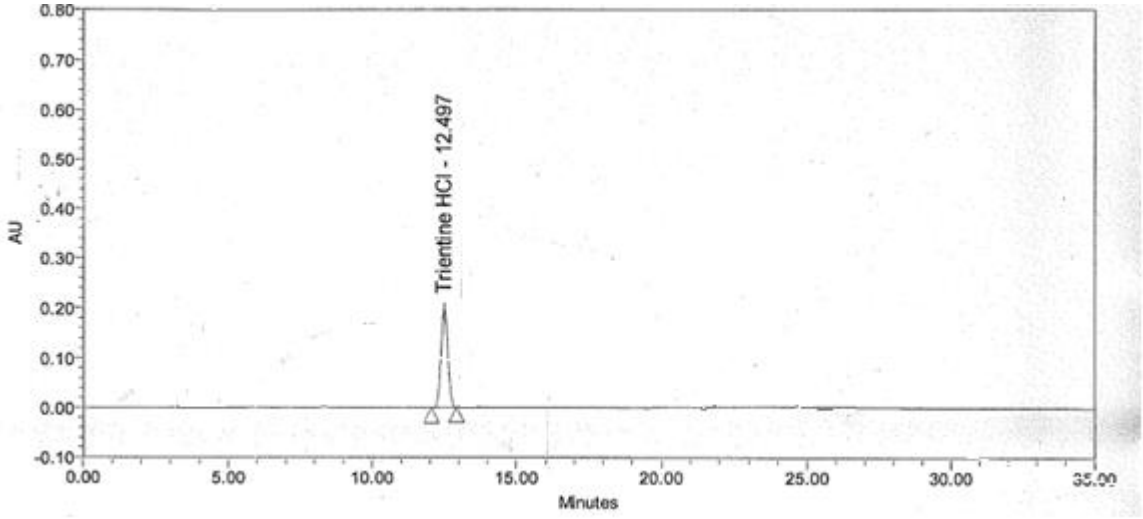

Figure 2. Chromatogram of Trientine hydrochloride

Report of Validation

Table 1. Linear regression data for the Calibration curve*

Parameter	TNT
Linearity range	40-120 µg/mL
r^2	0.9983
Slope	39824
Intercept	22147
y Intercept	0.68%

*n=5

Table 2. Results of Accuracy study*

Amount added (%)	Theoretical content(mg/caps)	Measured conc.±SD	Recovery (%)	%R.S.D
50	125.15	124.98	99.87	0.62
100	250.23	250.21	99.99	0.58
150	375.44	375.73	100.08	0.15

*n=9.

Table 3. Results of Precision study*

Drug conc. (µg/mL)	Repeatability Found conc.±S.D.	% R.S.D	Intermediate precision Found conc.±S.D.	% R.S.D
40	40.20±0.17	0.42%	39.90±0.04	0.09%
80	80.04±0.34	0.43%	80.39±0.43	0.53%
120	120.50±0.81	0.67%	120.15±0.56	0.47%

*n=9.

Table 4. Limit of dectection (LOD) and Limit of quantitation(LOQ)*

Parameter	TNT
LOD	1.785µg/mL
LOQ	5.409µg/mL

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Table 5. Robustness evaluation of the method*

Factor	Level	Retention time(R_t)	Asymmetry(T)
A. Columns from different manufactures			
Waters C_8 column		12.521	1.02
Phenomenex C_8 column		12.315	1.05
B. Column Temperature			
29°C	-1	12.502	1.04
30°C	0	12.499	1.02
31°C	+1	12.501	1.01
C. Wavelength			
218nm	2	12.511	1.11
220nm	0	12.502	1.15
222nm	+2	12.503	1.09
D. Buffer pH			
5.2	1	12.498	1.03
5.3	0	12.497	1.05
5.4	+1	12.502	1.07

***n=6**

Table 6. Assay of the marketed formulation by the proposed method*

Drug	Amount taken,µg/mL	Drug content(%)±S.D	% RSD
TNT	80 µg/mL	250.11mg ± 1.07	0.43

*n=6.

The amount of drug obtained by the proposed method was well within the label claim, thus proving that the developed method can be utilised for the quantification of TNT in its pharmaceutical dosage form.

In any chromatographic method the system suitability parameters have to be checked to prove that the developed chromatographic conditions are adequate for the analysis. The results shown in table 7 proves that the developed chromatographic conditions are suitable for the analysis of TNT.

Table 7. System suitability parameters*

Parameter	TNT	Reference values
Theoretical plates(N)	12765	NLT 2000
Tailing Factor	1.04	NMT 2.0

Stress Degradation study of TNT:

TNT was found to be stable in acid, alkali, thermal, and photolytic conditions. A degradation product was formed in oxidative stress conditions at the R_t of 28.355 mins. The chromatogram of TNT under various stress conditions are shown in figures 3 to 7.

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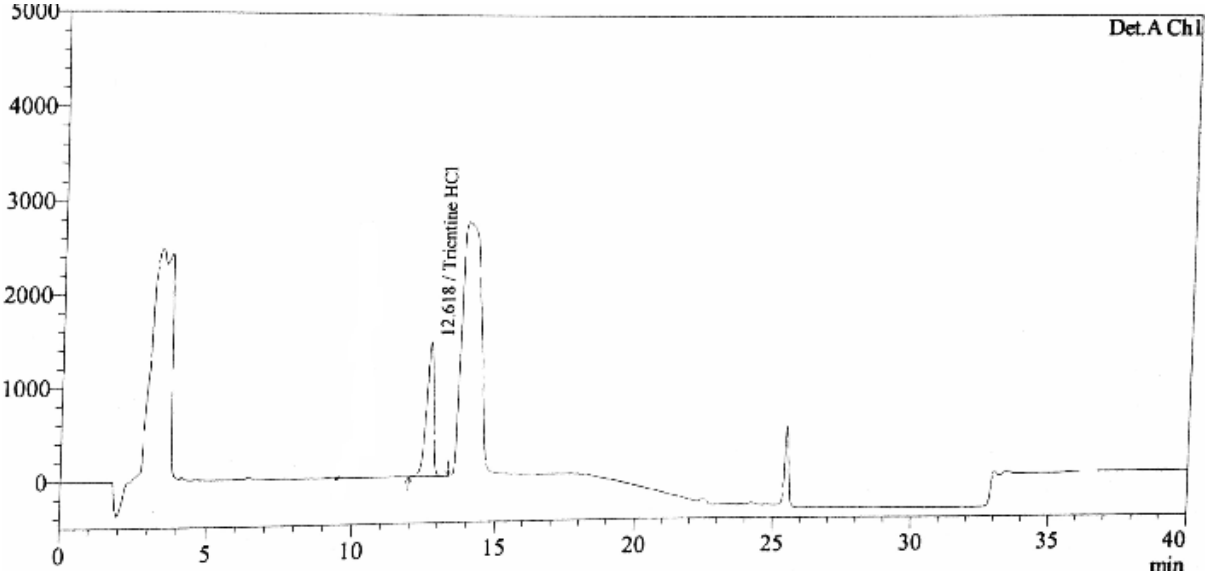

Figure 3. Acid degradation of TNT

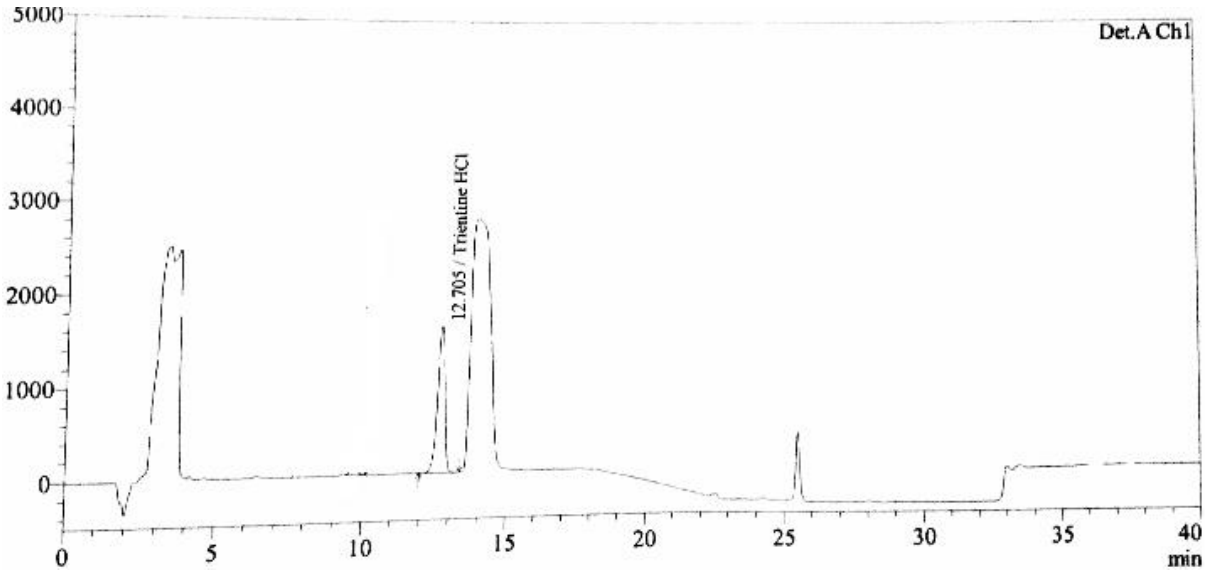

Figure 4. Base degradation chromatogram of TNT

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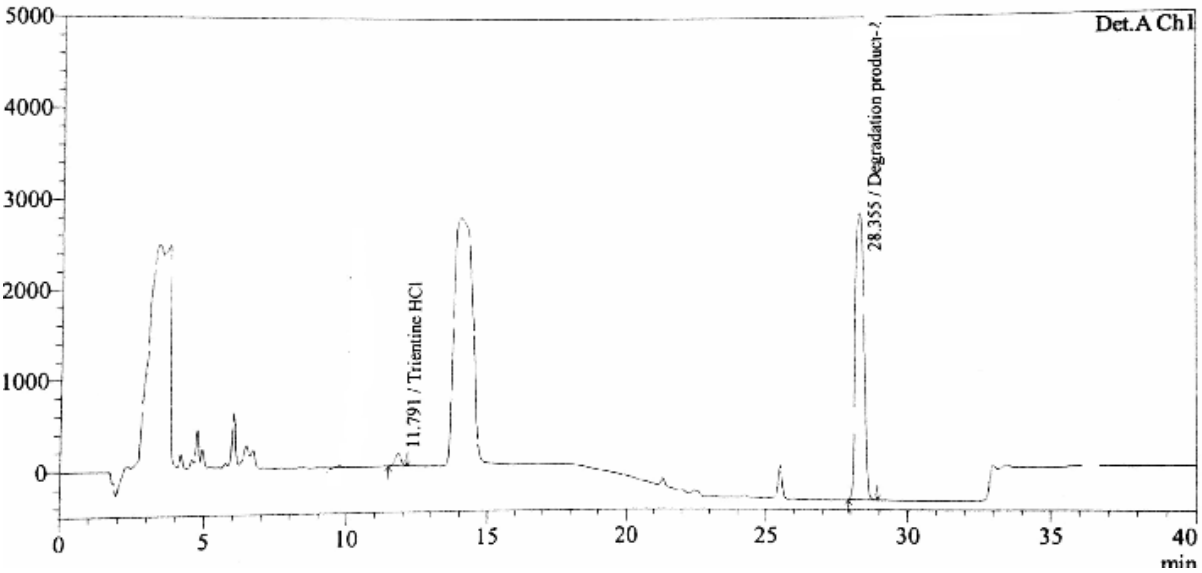

Figure 5. Oxidative degradation chromatogram of TNT

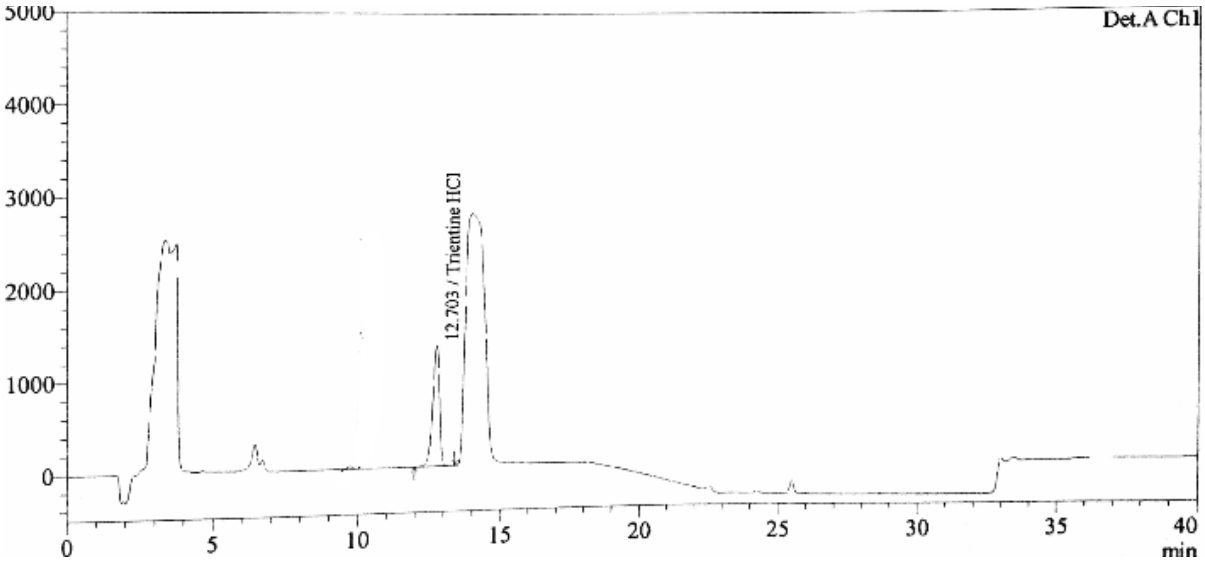

Figure 6. Photolytic degradation chromatogram of TNT

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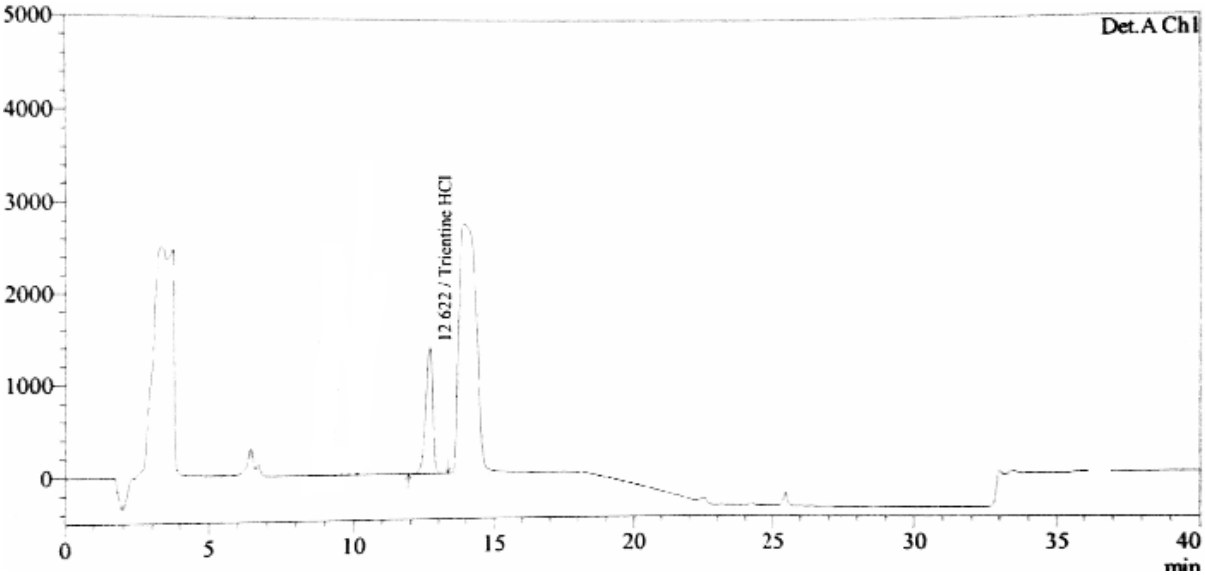

Figure 7. Thermal degradation chromatogram of TNT

Characterisation of Degradation Product

The degradation product obtained in oxidative stress condition was further subjected to LC-MS study for characterization and structural elucidation. LC-MS spectrum thus obtained is shown in Figure 8

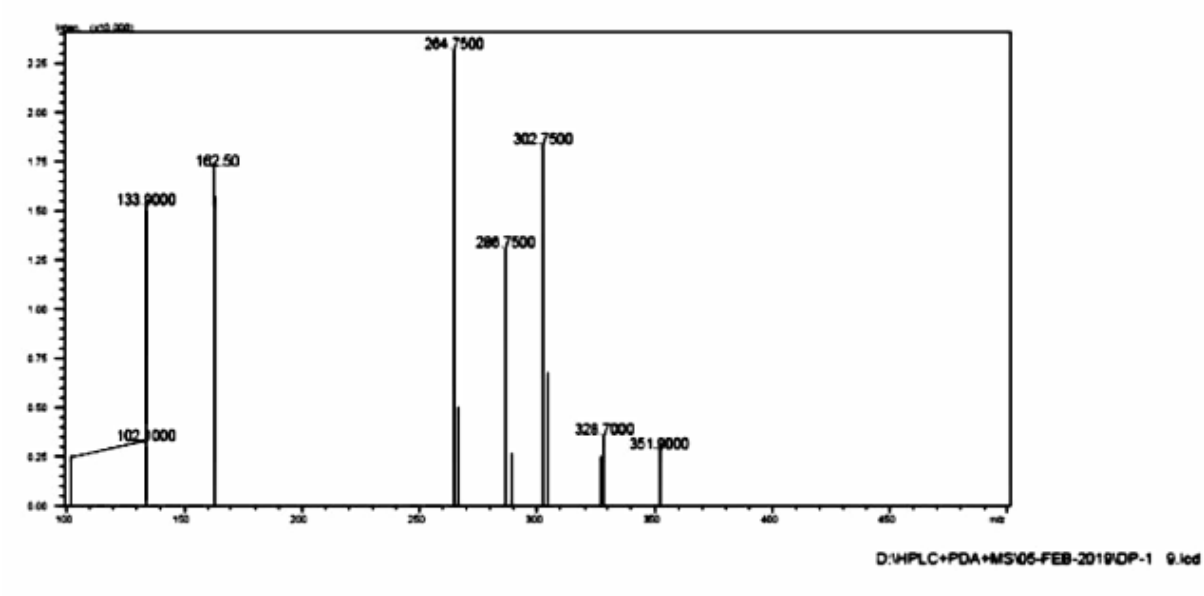

Figure 8. LC-MS Spectrum of degradation product

The m/z value of 162.50 in the spectrum is indicative of the formation of N-Oxide during oxidative degradation.

^{1}H NMR and ^{13}C NMR.

^{1}H NMR and ^{13}C NMR spectra were recorded for the degradation product as shown in Figure 9 and Figure 10 respectively.

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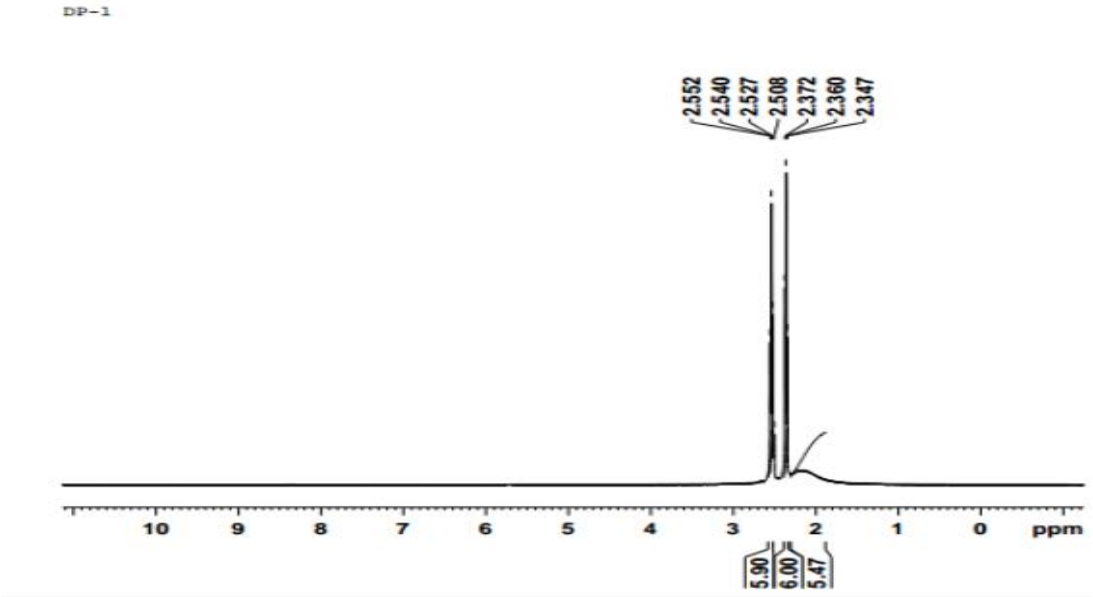

Figure 9. ^{1}H NMR Spectrum of degraded product

^{1}H NMR (400MHz, DMSO, δ in ppm)

2.54 (t, 6H CH_2)– 1,4,7) ; 2.35 (t, 6H CH_2 – 2,5,8) ; 2.24 (t, 6H NH_2 – 3,6,9)

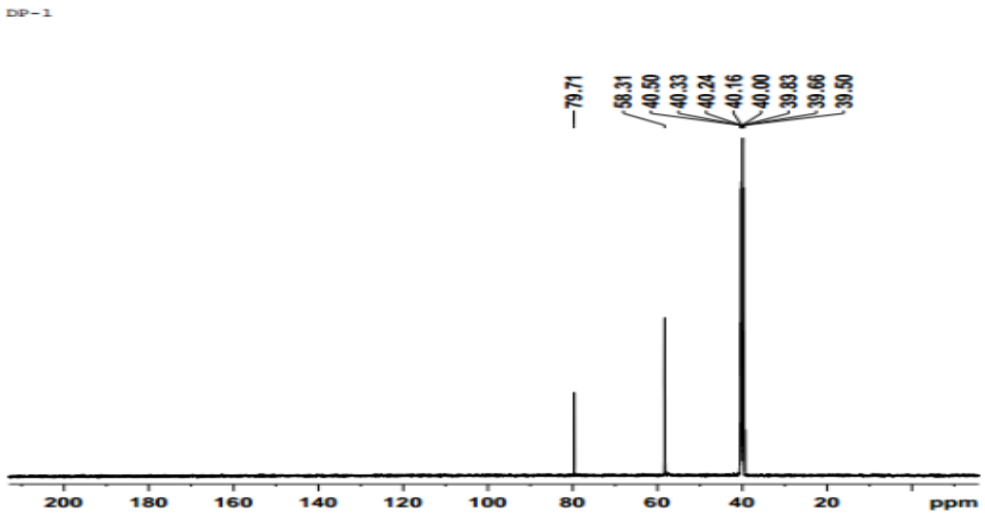

Figure 10. ^{13}C NMR Spectrum of degraded product

^{13}C NMR (100MHz, DMSO, δ in ppm)
C_1- 58.31 ; C_2 – 39.50 ; C_3 – 40.50 ; C_4 – 39.66 ; C_5 – 40.16 ; C_6 – 39.83.

Spectral characterisation of Oxidative degradation product :

IR : The IR spectrum was recorded for the oxidative degradation product and was compared with that of the parent drug.

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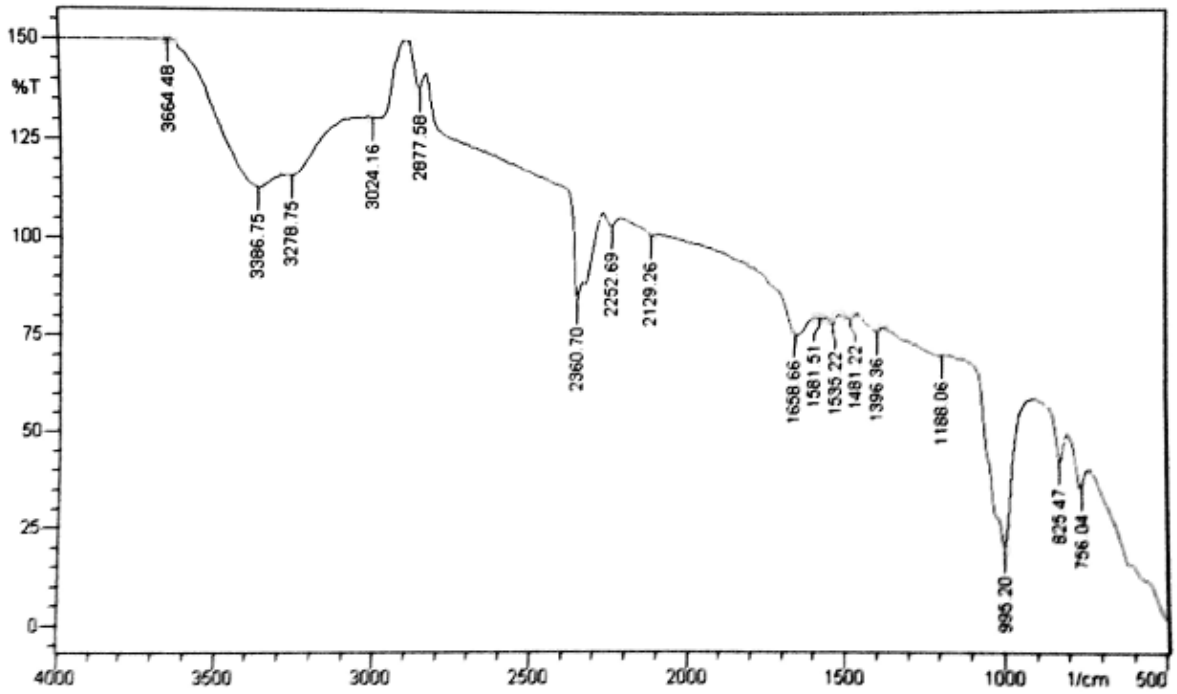

Figure 11. IR Spectrum of TNT Degradation product

Interpretation of the IR Spectrum

Wave number cm^{-1}	Types of stretching	Remarks
3278.75, 3024.16	N-H Stretching	Presence of primary amine
1581.51, 1658.66	N-H Bending	Presence of primary amine
995.20	N-O	Presence of amine oxide

The results of LC-MS and various spectroscopic studies indicate the formation of N-Oxide of TNT as the degradation product in oxidative conditions.

The degradation product has 16 amu more than Trientine showing the addition of one oxygen atom in Trientine moiety. Thus the structure of degradation product (N-Oxide) may be as shown in Figure 12.

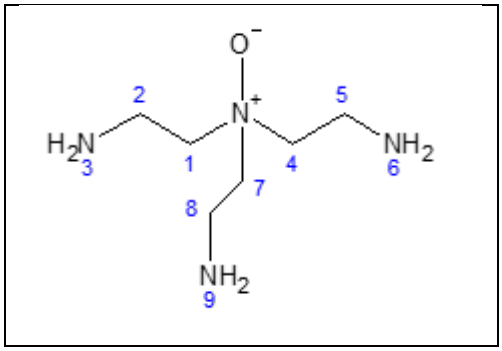

Figure 12. Proposed structure of TNT Degradation product

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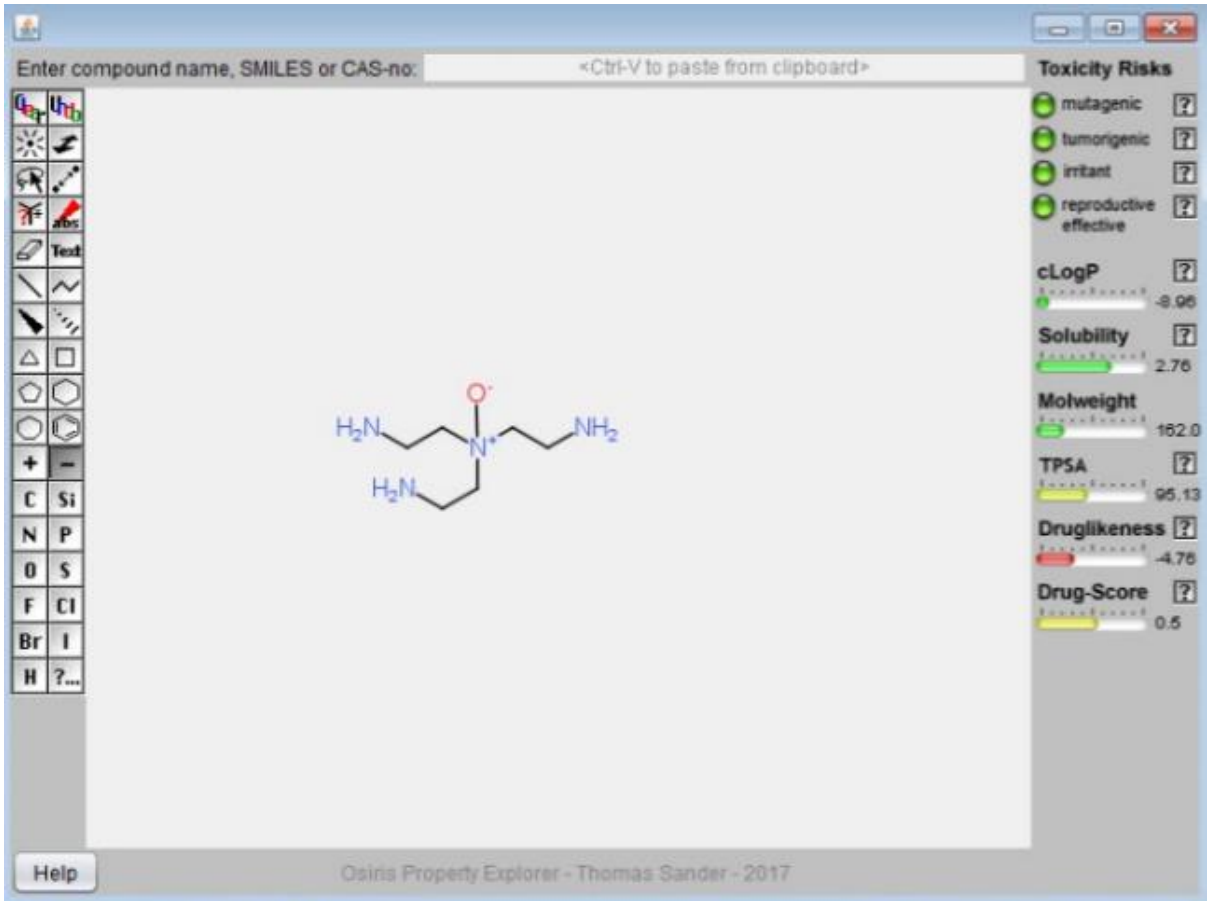
TOXICITY PREDICTION

The chemical structure was drawn in OSIRIS property explorer to show the biological properties of the compound. Properties like high risks of undesired effects like mutagenicity, tumorigenicity and reproductive effect are shown in red. Green colour indicates the drug conform behaviour. It was found that the degraded product is non mutagenic, non tumorigenic and has no irritability and reproductive effects.



CONCLUSION

The developed RP-HPLC method is specific, sensitive and robust. Since the developed method can be used to determine TNT in presence of its degradation product, the method is Stability- indicating.

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