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Development and Validation of Stability Indicating HPLC Method for Estimation of Novel Nitroimidazooxazine Antitubercular Drug Pretomanid Followed by LC-MS

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KEYWORDS

Pretomanid, Stability indicating analytical method, HPLC, Degradation products, LC/MS

ABSTRACT:

Introduction: A reliable and fast method using reversed phase high performance liquid chromatography (RP-HPLC) has been developed and validated to determine the concentration of pretomanid in the presence of its degradation products. The drug was subjected to various stress tests according to international guidelines, and the results showed that pretomanid is susceptible to degradation under certain conditions such as alkali hydrolysis and photolysis at room temperature.

Objective: To develop a simple, precise, accurate, and economic stability indicating analytical methods for determination of Pretomanid by HPLC .To validate developed methods as per ICH guidelines. To study forced degradation study of selected drugs at recommended conditions in guidelines which are thermal, photolytic, oxidation and acid/ alkaline hydrolysis and identify the degradation products by LC-MS

Method: The RP-HPLC method was effective in separating the drug from its degradation products using a HiQ Sil C18 column and a mobile phase composed of 10 mM ammonium acetate solution pH3: methanol (65:35 v/v). The validation study demonstrated that the method was accurate, precise, and robust. LC-MS was used to identify the degradation products and to suggest possible fragmentation.

Result: Developed method was validated and found to be linear with R^2= 0.9993 for concentration in the range 5-30 µg/ml, accurate, precise, robust and Limit of detection (0.817 µg/ml) and limit of quantitation (2.476 µg/ml)

Conclusion: This method can be useful for assessing the stability of pretomanid in commercial pharmaceutical dosage forms. No previous method has been documented for the degradation behavior of pretomanid, and chemical analysis through mass spectrometry

1. Introduction:

Pretomanid (*6,7-dihydro-5H-imidazo[2,1-b] [1,3]oxazine*) is an antimycobacterial drug used in combination with other drugs to treat multidrug resistant tuberculosis. It works by inhibiting the synthesis of mycolic acid, which is essential for bacterial cell wall formation, ultimately leading to bacterial cell death. It is effective against both replicating and non-replicating Mycobacterium tuberculosis. The drug was approved by the United States Food and Drug Administration in August 2019 and is part of a combination regimen called BPaL, used to treat pulmonary extensive drug resistant tuberculosis in adults. This study aimed to develop and validate a stability-indicating analytical method to quantify pretomanid in tablets and identify degradation products, proposing a degradation pathway. The method was validated following the guidelines of the

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International Conference on Harmonization for linearity, accuracy, precision, robustness, ruggedness, LOD, and LOQ [3].

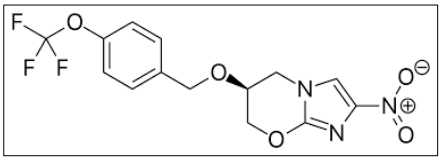

Fig 1: Structure of Pretomanid

2. Materials and methods:

The pure pretomanid drug used in this study was obtained as a gift sample from Mylan in Hyderabad. HPLC grade water and methanol were purchased from Qualigen (India) Ltd. in Mumbai, India, while all other chemicals used were of analytical grade from S.D. Fine Chemical Ltd. in Worli, India. Class A grade volumetric glassware was used throughout the experimental work. Millipore membrane filters with a pore size of 0.45 µ were used to filter the mobile phase and working solution.

2.1 Instrumentation:

HPLC instrumentation consisting of Model PU-2080 Intelligent plus pump (JASCO, Tokyo, Japan), with Rheodyne sample injection port and HiQ Sil C18 column (250*4.6mm,5μ) were used. Detection was carried out using PU 2010 Plus UV detector (JASCO, Tokyo, Japan). Data acquisition was done by Borwin chromatography software version 1.5.

2.1.1 Preparation of standard stock solution

Standard stock solution of drug was prepared by dissolving 10 mg of drug in 10 ml of methanol to get concentration of 1000 µg mL^{-1}. From this solution further dilutions were made in methanol.

2.1.2 Selection of detection wavelength

From the standard stock solution (1000 µg mL^{-1}) further dilutions were made using methanol and scanned over the range of 200-400 nm and the spectra was obtained. It was observed that the drug showed linear, stable and considerable absorbance at 320 nm.

2.1.3 Selection of mobile phase:

In this study, a standard solution of pretomanid with a concentration of 10 µg/ml was injected into the HPLC system and run using different solvent systems. Various mobile phases were tested, including methanol and distilled water, as well as varying proportions of mobile phase components like ACN and distilled water, and different pH conditions were attempted in order to obtain suitable system parameters for the analysis of pretomanid. After several trials, a mobile phase consisting of 10 mM ammonium acetate (pH 3 was maintained using acetic acid) and MeOH in a ratio of 65:35 (v/v) was selected, as it gave acceptable peak parameters.

2.1.4 Preparation of 10 mM ammonium acetate buffer (pH 3) and mobile phase

To prepare the 10 mM ammonium acetate buffer (pH 3), 770 mg of ammonium acetate was dissolved in 400 ml of HPLC grade water. The solution was then filtered using a 0.45µm HPLC certified nylon filter. Next, 950 ml of HPLC grade water was added, and the pH of the solution was checked and adjusted to 3 using acetic acid. The volume was then made up to 1000 mL using HPLC grade water. The mobile phase was prepared by mixing the ammonium acetate buffer and methanol in a ratio of 65:35 (v/v). The mixture was then filtered and sonicated for 10 minutes.

2.1.5 Preparation of sample solution-

Tablets with label claim 200 mg of pretomanid (pretomanid Tablets 200 mg, Mylan) were weighed and powdered. A quantity of powder equivalent to 10 mg of pretomanid was transferred to a 10 ml volumetric flask containing 5 ml of methanol. The mixture was ultra-sonicated for 10 min and the resulting sample stock solution was filtered with Whatman filter paper 41 and the volume was made up with the methanol to get concentration of 1000 µg/ml. Further dilution was done to get concentration 10 µg/ml which was injected on system.

2.1.6 Chromatogram and system suitability parameter of drug

The column was equilibrated with the mobile phase (indicated by constant back pressure at desired flow rate). Working standard solution of pretomanid (10 µg mL^{-1}) was injected on system. The retention time for the drug was found be 4.213 min (Table 1). Chromatogram of pretomanid is shown in Fig 2.

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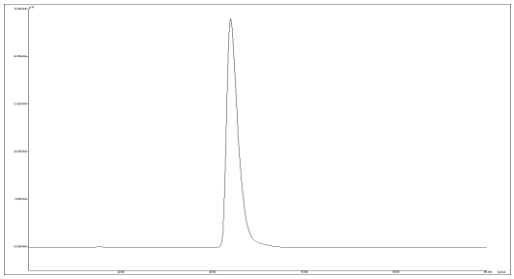

Fig 2: Chromatogram of Pretomanid (10 µg/ml)

System suitability parameters for Pretomanid
Concentration(µg/ml)- 10
RT (Min)- 4.213 ± 0.034
Area-446486
Plates-3951
Asymmetry-1.33

Summary of Chromatographic parameters
Column- HiQ Sil C18 (250 x 4.6 mm, 5 µm)
Mobile phase-10mM ammonium acetate solution pH3: methanol (65:35 v/v)
Flow rate-1 mL min^{-1}
Detection Wavelength-320nm
Sample injector-50 µl loop
Column temperature- Ambient

3. Results and Discussion

3.1. VALIDATION OF ANALYTICAL METHOD:[3]

3.1.2 Linearity

A solution of pretomanid was prepared in methanol with a concentration of 100 µg ml^{-1}, which was then used to prepare six different solutions of varying concentrations. The linearity between the peak area and concentration was established by analyzing these solutions over a range of 5-30 µg mL^{-1}. The resulting data was used to generate a calibration curve with an equation of y = 40875 x + 48415, which is shown in Figure 3. Table I displays the results obtained for pretomanid.

Table I: Linearity study of Pretomanid

Conc (µg/ml)	Avg AREA	SD	% RSD
5	250784.000	4589.964	1.830
10	454057.500	7171.421	1.579
15	676102.167	11791.613	1.744
20	851205.833	13820.863	1.624
25	1078480.000	11412.610	1.058
30	1271733.167	23786.981	1.870

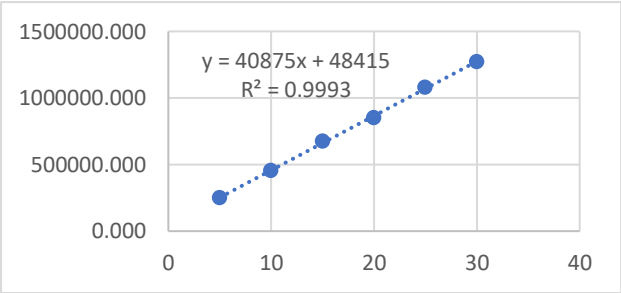

Fig. 3: Linearity curve of Pretomanid (5-30 µg/ml)

3.1.3 Precision

The precision of the method was demonstrated by intra-day and inter-day variation studies. In the Intra-day studies, 3 replicates of 3 different concentrations were analysed in a day and percentage RSD was calculated. For the inter day variation studies, 3 different concentrations were analyzed on 3 consecutive days and percentage RSD was calculated. The results obtained for intraday and inter day variations are shown in Table II and III respectively.

Table II: Intra-day precision study of Pretomanid

SN	Therotical conc	Area	Practical Conc	% x	AVG	SD	%RSD
1	10	459309	10.052	100.525	100.251	0.237	0.237
2	10	457694	10.013	100.129			
3	10	457570	10.010	100.099			
4	15	664974	15.084	100.560	100.117	0.426	0.425
5	15	659766	14.957	99.711			

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6	15	662033	15.012	100.080			
7	20	859822	19.851	99.255			
8	20	865069	19.979	99.897	99.774	0.471	0.472
9	20	867319	20.034	100.172			

Table III: Inter-day precision of Pretomanid

SN	Therotical conc	Area	Practical Conc	% x	AVG	SD	%RSD
1	10	455635	9.963	99.626			
2	10	454032	9.923	99.234	99.762	0.608	0.610
3	10	458909	10.043	100.427			
4	15	663382	15.045	100.300			
5	15	664248	15.066	100.442	100.302	0.140	0.139
6	15	662537	15.024	100.163			
7	20	868142	20.054	100.272			
8	20	866799	20.022	100.108	100.047	0.262	0.261
9	20	863954	19.952	99.760			

3.1.4 Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ are calculated from the formula: -

$$LOD = \frac{3.3\ \sigma}{S} \qquad LOQ = \frac{10\ \sigma}{S}$$

Where,

σ = S.D of the response at lowest concentration or standard deviation of Y intercept;

S = Average of slope of the calibration curve (Table IV)

Table IV: LOD and LOQ of Pretomanid

Method	**Avg slope**	**S.D**	**LOQ (µg/ml)**	**LOD (µg/ml)**
1. Using S.D of the response at lowest concentration	40874.954	4589.964	0.371	1.123
2. Using S.D of y-intercept	40874.954	10119.234	0.817	2.476

3.1.5 Specificity

The specificity of the method was ascertained by peak purity profiling studies. The peak purity values were found to be more than 998, indicating the no interference of any other peak of degradation product, impurity or matrix. The purity tail and purity front of pretomanid were found to be 999.12 and 998.54

3.1.6 Assay

Pretomanid Tablets formulation analysis was carried out as mentioned under section preparation of sample solution. Procedure was repeated for six times. Sample solution was injected and area was recorded. Concentration and % recovery was determined from linear equation. The results obtained are shown in Table V.

Table V: Assay of marketed formulation

Sr. No.	**Peak Area**	**Amount Recovered (µg/ml)**	**%Recovery**	**Mean ± % RSD**
1	458953	10.044	100.437	

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2	452825	9.894	98.938	100.294 ± 0.858
3	455820	9.967	99.671	
4	462751	10.137	101.367	
5	460273	10.076	100.760	
6	459567	10.059	100.588	

3.1.7 Accuracy

To check accuracy of the method, recovery studies were carried by spiking the standard drug to the tablet sample solution, at three different levels around 50, 100 and 150 %. Basic concentration of sample solution chosen was 10 µg mL^{-1} % recovery was determined from linearity equation. The results obtained are shown in Table VI.

Table VI: Accuracy of Pretomanid

Level	Conc. of Sample solution (µg/ml)	Conc. of Standard solution spiked (µg/ml)	Area	Amount recovered (µg/ml)	% Recovery	% Recovery (Mean ± %RSD)
50 %	10	5	658549	14.927	99.512	100.005 ± 0.524
			661221	14.992	99.948	
			664944	15.083	100.555	
100 %	10	10	864512	19.966	99.828	100.095 ± 0.462
			871058	20.126	100.629	
			864512	19.966	99.828	
150 %	10	15	1079933	25.236	100.944	100.348 ± 0.709
			1065790	24.890	99.560	
			1075813	25.135	100.540	

3.1.8 Robustness-

Robustness of the method was checked by carrying out the analysis under conditions during which mobile phase composition (± 2 ml Composition), detection wavelength (± 1 nm), flow rate (± 0.05 mL/min) were altered and the effect on the area were noted. Robustness of the method checked after deliberate alterations of the analytical parameters showed that areas of peaks of interest remained unaffected by small changes of the operational parameters indicating that the method is robust (Table VII)

Table VII: Robustness study

% RSD Found for Robustness Study (Peak Area)								
MP COMPOSITION (± 2 ml Composition)			**DETECTION WAVELENGTH (± 1 nm)**			**FLOW RATE (± 0.05 ml/min)**		
63:37	65:35	67:33	319	320	321	0.95	1	1.05
0.362	0.354	0.144	0.453	0.672	0.144	0.843	0.956	0.812

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Summary of validation Parameters:

Table VIII: Summary of Validation Parameters by HPLC

Sr. No.	Validation parameters	Pretomanid Results
1.	Linearity equation R^2 Range	y = 40875 x + 48415 R^2= 0.9993 5-30 µg/ml
2.	Precision	(%RSD)
	Intraday	0.237-0.472
	Interday	0.139-0.610
3.	Assay	100.294 ± 0.858
4.	Accuracy	Mean ± %RSD
	50	100.005 ± 0.524
	100	100.095 ± 0.462
	150	100.348 ± 0.709
5.	Limit of detection	0.817 µg mL^{-1}
6.	Limit of quantitation	2.476 µg ML^{-1}
7.	Specificity	Specific
8.	Robustness	Robust

4. STRESS DEGRADATION STUDIES:

Stability studies were carried out to provide evidence on how the quality of drug varies under the influence of a variety of environmental conditions like hydrolysis, oxidation, temperature, etc. Dry heat and photolytic degradation were carried out in the solid state. [9]

4.1 Acid hydrolysis

A 1 ml sample of a standard stock solution of a drug (with a concentration of 1000 µg/mL) was mixed with 1 ml of 2N HCl. The resulting solution was diluted to 10 mL with methanol to obtain a solution with a concentration of 100 µg mL^{-1}. The solution was then kept in the dark for 24 hours, and then neutralized with 2 N NaOH. Next, 2 mL of the solution was further diluted to 10 mL with methanol to obtain a solution with a concentration of 20 µg mL^{-1}. A blank solution without the drug was prepared using the same procedure for acid degradation testing. The drug was subjected to acid hydrolysis, and it was found that the recovery percentage of the drug (Pretomanid) was 99.32% with no degradation peak detected. The representative chromatogram is shown in Fig. 4(a). [7]

4.2 Alkaline hydrolysis

A 1 ml sample of a standard stock solution of a drug (with a concentration of 1000 µg/mL) was mixed with 1 ml of 1 N NaOH. The resulting solution was diluted to 10 ml with methanol to obtain a solution with a concentration of 100 µg mL^{-1}. The solution was then kept in the dark for 24 hours, and then neutralized with 1 N HCl. Next, 2 ml of the solution was further diluted to 10 mL with methanol to obtain a solution with a concentration of 20 µg mL^{-1}. A blank solution without the drug was prepared using the same procedure for alkali degradation testing. The drug was subjected to alkaline hydrolysis, and it was found that the recovery percentage of the drug (Pretomanid) was 62.65% with two degradation peaks detected. The representative chromatogram is shown in Fig. 4(b).[7]

4.3 Degradation under oxidative condition

A standard stock solution of a drug (with a concentration of 1000 µg/mL) was mixed with 1 ml of 30% H2O2 to obtain a solution with a concentration of 100 µg mL^{-1}.. The resulting solution was then diluted to 10 mL with methanol and kept in the dark for 24 hours. Next, 2 mL of the solution was further diluted to 10 mL with methanol to obtain a solution with a concentration of 20 µg mL^{-1}. A blank solution without the drug was prepared using the same procedure for oxidative degradation testing. It was found that the recovery percentage of the drug (Pretomanid) was 98.85% with no degradation peak detected under oxidative degradation.The representative chromatogram is shown in Fig.4(c).

4.4 Degradation under dry heat

In a dry heat study, a drug sample was kept in an oven at 1000°C for 2 hours. A sample was withdrawn, and 10 mg of it was dissolved in methanol to obtain a solution with a concentration of 1000 µg mL^{-1}. The resulting solution was further diluted with methanol to obtain a final concentration of 20 µg/ml, which was then injected. It was found that the recovery percentage of the drug (pretomanid) was 99.63% with no degradation peak detected under the dry heat degradation condition. The representative chromatogram is shown in Fig. 4(d)[7]

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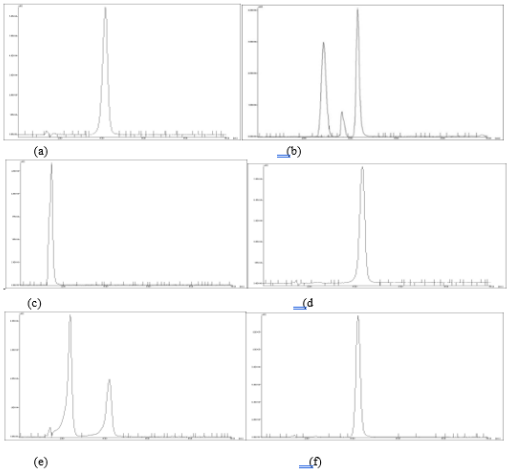

Fig. 4: Chromatogram of Pretomanid after (a) acid degradation (b) alkaline degradation (c) oxidation with 30% v/v H_2O_2 (d) under dry heat (e) UV illumination exposure(f) florescent light exposure

4.5 Photo-degradation studies:

The drug's photo degradation stability was studied by exposing it to UV light with an illumination of at least 200 watt hr/m2 and cool white fluorescence light with at least 1.2 million Lux-Hr. After exposure, 10 mg of the drug was accurately weighed and transferred to a 10 ml volumetric flask, and the volume was made up with methanol. The resulting solution was further diluted with methanol to obtain a final concentration of 20 µg ml^{-1}, which was then injected. It was found that after exposure to UV light, an average of 78.49% of pretomanid was recovered with a degradation peak at RT 2.238. However, after exposure to fluorescence light, an average of 99.13% of pretomanid was recovered with no degradation peak detected. The representative chromatogram is shown in Fig.4(e) and Fig 4(f)respectively. [8]

4.6. Summary of degradation parameters:

The degradation was found maximum in alkaline condition (37.05%) and photolytic condition (21.51%) and stable in acidic, oxidative and thermal conditions. The percent assay of active substance and their RT values of degradation products are given in Table XII.

Table IX: Summary of Degradation

Stress condition/Duration	**% Recovery of Analyte**	**RT of degraded products**
Acidic/ 2 N HCl for 24 hours	99.32 %	-
Alkaline/ 1 N NaOH for 24 hours	62.95%	Degradant at RT – 2.916 min and RT – 3.758 min
Oxidative/ 30 % H_2O_2 at room temperature 24 hours	98.85%	-
Dry heat / 100ºC/ 24 hours	99.63%	-

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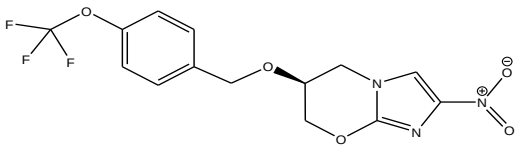
UV illumination NLT 200 watt hours/square meter	78.49 %	Degradant at RT 2.238 min
Fluorescent light NLT 1.2 X 10^6 Lux hr	99.13%	-

5. Identification of degraded products by LC-MS

LC-MS

Instrument: Agilent 6540 UHD Accurate-Mass Q-TOF MS connected to Agilent 1260 Infinity II HPLC
Ion Source: Dual AJS ESI
Mass Range (*m/z*): 50 to 1700
Polarity: Positive
Ion Source Parameters:
Gas Temp: 300 degree
Gas Flow: 8 l/min
Nebulizer Gas: 35 psig
Sheath Gas Temperature: 350 degree
Sheath Gas Flow: 11 l/min
Capillary Voltage: 3500 V
Nozzle Voltage: 1000 V
Column: Agilent HPH-C18, 4.6X100 mm, 2.7 micron
Column Temp: RT
Mobile Phase A: 0.1% Formic acid in water
Mobile Phase B: 0.1% Formic acid in acetonitrile
Flow rate: 0.5 mL/min
Gradient:
Time (min): Mobile Phase B (%): 0 min: 20% B, 1 min: 20% B, 13 min: 100% B, 14 min: 100% B, 15 min: 20% B
Post run: 20 % B for 4 min



Structure of Pretomanid

$C_{14}H_{12}F_3N_3O_5$
Exact Mass: 359.0729
Mol. Wt.: 359.2574
m/e: 359.0729 (100.0%), 360.0763 (15.1%), 360.0699 (1.1%), 361.0796 (1.1%), 361.0772 (1.0%)
C, 46.80; H, 3.37; F, 15.86; N, 11.70; O, 22.27

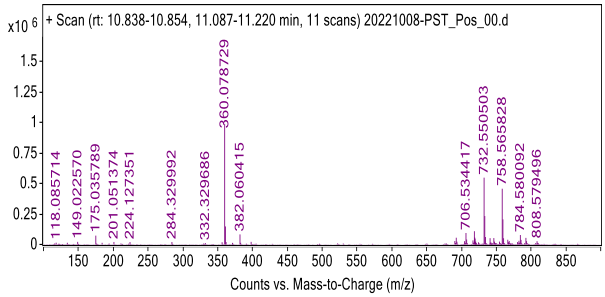


Fig 5: LC-MS mass spectrum of standard Pretomanid (RT – 10.921 min)

The MS spectra of stadardard pretomanid (Retention time-RT-10.921min) display product ions at m/z 360.07 due to loss of electron forming m+ ion.

5.1. Alkaline hydrolysis

After alkaline hydrolysis, Pretomanid shown 62.65% recovery i.e.37.05% degradation with two peaks of degradant at 9.162 ad 8.473

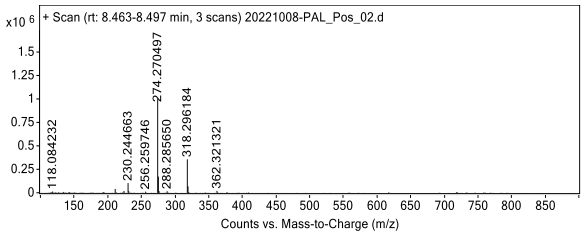
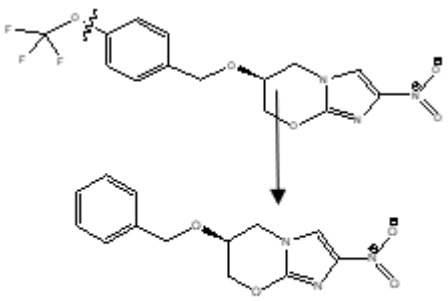


Fig 6: LC-MS Spectrum of degradant at RT- 8.473 under alkali treatment



m/z = 274

Fig 7: Major peak at m/z 274 observed for degradant at RT – 8.473 under alkali treatment

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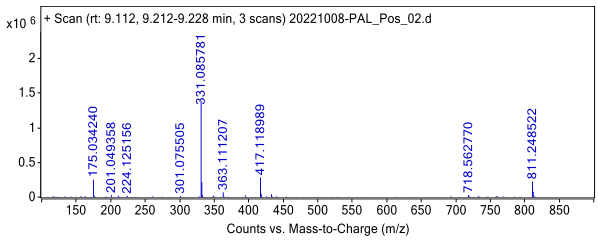
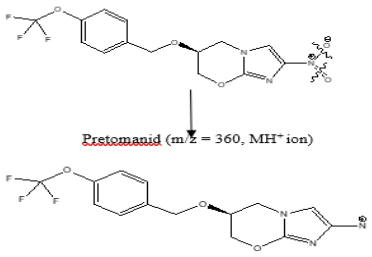


Fig 8: LC-MS Spectrum of degradant at RT- 9.162 under alkali treatment



m/z = 331

Fig 9: Major degradant peak at m/z 331 observed for degradant under alkali treatment

5.2. Photo degradation studies

78.49% of pretomanid was recovered with peak of degradant at RT 2.238 after exposure to UV light and average 99.13% of pretomanid was recovered with no peak of degradation after exposure to fluorescence light.

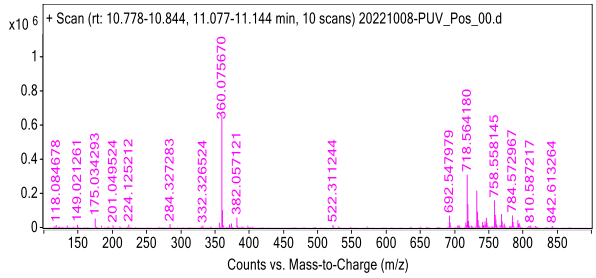


Fig 10: LC-MS mass spectrum of UV light treated Pretomanid (RT – 10.927)

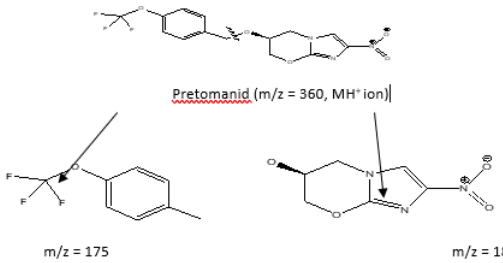


Fig 11: Major degradant peak at m/z 175 observed apart from quasimolecular ion peak of Pretomanid at m/z = 360 for standard Pretomanid analyte

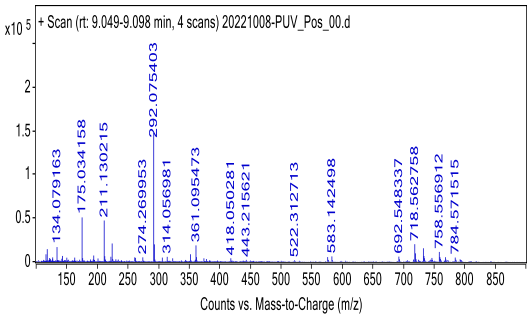
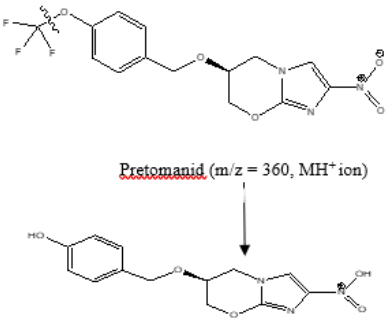


Fig 12: LC-MS Spectrum of degradant at RT- 9.067 under UV light exposure



m/z = 292

Fig 13: Major degradant peak at m/z 292 observed for degradant under UV light exposure (RT – 9.067 min)

Conclusion

The quality of pharmaceutical products is crucial for ensuring the safety of patients. Any degradation compounds or impurities in the product can have an impact on its efficacy and safety. This study focuses on the forced degradation of pretomanid under ICH prescribed conditions to analyze its degradation profile and identify its degradation product structures. The study developed HPLC- and LC-MS-MS methods that are green, sensitive, accurate, precise, cost-effective, and reproducible. These methods were validated to be linear, precise, accurate, specific, and selective for the drug in the presence of degradation products.The study found that pretomanid is susceptible to alkali hydrolytic and photolytic degradation at room temperature. The degradation products were characterized and identified by MS/MS methods. The suggested method can be applied in testing commercially available tablets and used for routine analysis of pretomanid in quality control laboratories in the pharmaceutical tablet dosage. The study was performed according to the ICH Q1A (R2) guidelines to ensure the stability-indicating study's reliability.

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Conflict of Interests

Authors declare that there is no conflict of interest

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