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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPTLC METHOD FOR ANALYSIS OF NOVEL NITROIMIDAZOOXAZINE ANTIMYCOBACTERIAL DRUG PRETOMANID

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Keywords:

Pretomanid, Stability indicating analytical method, HPTLC, Method Development, Validation, Degradation products

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ABSTRACT: The medication pretomanid effectively treats drug-resistant tuberculosis (MDR TB). For the analysis of pretomanid, a simple, focused, and accurate HPTLC method has been created and approved. The samples were applied as bands on a silica gel-coated TLC plate made of aluminium. Pretomanid was completely separated using ethyl acetate and n-hexane as the mobile phase; the RF value was 0.68 0.046. Densitometry was used for detection, namely in absorbance mode at 330 nm. The peaks produced by this approach were discovered to be crisp, symmetrical, and highly resolved. A linear relationship was found across the concentration range of 200-1200ng/spot with coefficients of determination of 0.987. According to the International Conference on Harmonization's criteria, the method's accuracy, recovery, repeatability, and robustness were all confirmed. The limit of quantitation was determined to be 439.36 ng/spot, while the lowest detectable level was found to be 144.9 ng/spot. According to statistical analysis of the data, the procedure is exact, accurate, repeatable, and selective for the examination of Pretomanid. The suggested single approach permitted analysis of Pretomanid in the presence of their degradation products created under various stress conditions, according to the findings of the validation research. The established method might be used to assess Pretomanid's stability in commercial pharmaceutical dosage form. Regarding the HPTLC-based Pretomanid degrading behaviour, no prior technique has been documented. Pretomanid was successfully estimated and quantified using the technique in a commercially available preparation.

INTRODUCTION: A nitroimidazooxazine antimycobacterial agent called protomanid is used with other antituberculosis medications to treat multidrug-resistant TB. Pretomanid **Fig. 1** is (6S) - 2 - nitro - 6- [4-(trifluoromethoxy) phenyl] methoxy] in its chemical form. **Fig. 1** illustrates - 6,7-dihydro-5H-imidazo [2,1-b] [1,3] oxazine [5].

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In August 2019, the US Food and Drug Administration authorized protomanid. To treat individuals with pulmonary extensive drug-resistant tuberculosis, the combination therapy of bed aquiline, pretomanid, and linezolid (BPaL regimen) has been authorised (TB).

Mycolic acid production is inhibited by proto-manid. This results in improper cell wall synthesis, eventually resulting in bacterial cell death. Both replicating and non-replicating M. tuberculosis are susceptible to it. It cannot be analyzed using spectroscopic or HPTLC techniques. Therefore it is necessary to build Pretomanid analytical techniques. The current study's objective was to

provide a precise, specific, repeatable HPTLC approach for the evaluation of pretomanid. The developed method was validated as per ICH guideline using linearity, accuracy, precision, robustness, ruggedness, LOD, LOQ [1, 2].

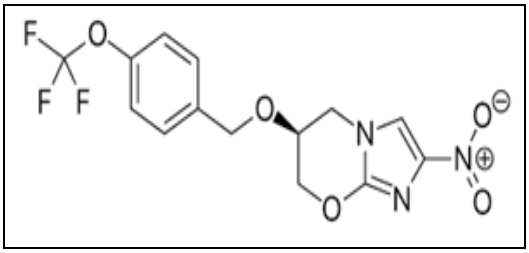

FIG. 1: STRUCTURE OF PRETOMANID

Thin-layer chromatography is a type of chromatography that uses an inert backing and a thin stationary phase to separate and analyze mixtures. The use of this method in qualitative and quantitative analysis of pharmaceutical, environmental, toxicological, food, and agricultural samples has steadily increased since the mid-1970s with the introduction of modern methodological and instrumental advancements made in TLC (known as high-performance TLC) (Sherma, 2010; Sherma, 2000, 2009; Le Roux et al., 1992; Rakesh *et al.,* 2013). This pattern persisted to the point where quantitative HPTLC is now often utilized in some quality control laboratories (Ferenczi-Fodor, Renger, Veigh, 2010).

The following are the main benefits of HPTLC: ease of use, minimal pre-treatment, effectiveness with small amounts of sample, parallel analysis of samples (up to 72 samples can be analysed concurrently and under identical conditions on a 2010 cm plate), numerous nondestructive detection methods (such as visualization and scan in visible or UV light at different wavelengths), a wide range of developing solvent options, and low consumption of s. (The expenses associated with providing solvents and maintenance are much lower than HPLC.) These benefits and the method's dependability, sensitivity, and repeatability make it a viable option to other chromatographic processes like HPLC. For instance, the outcomes of a study showed that HPTLC was more cost-effective than HPLC, even though the precision and accuracy of the HPLC and HPTLC techniques of phospholipids might be equivalent. In the current work, a straightforward and practical analytical procedure using the HPTLC-densitometric method is described for identifying and quantitatively measuring protomanid in bulk drugs. ICH guidelines were used to create and validate the suggested technique.

MATERIALS AND METHODS: Pretomanid pure drug was obtained as a gift sample from Mylan, Hyderabad. HPLC grade Ethyl acetate, methanol and n-hexane were purchased from Qualigen(India) Ltd., Mumbai, India. All other chemicals are of analytical grade from S.D. Fine Chemical Ltd., Worli, India. and the volumetric glassware of class A grade were used all over the experimental work.

Instrumentation: An automatic sample applicator (CamagLinomat) equipped with a Linomat 100-µL syringe 695.0014 was employed for sample application on the HPTLC plate. Chromatographic separations were performed on 20×20 cm aluminium packed plates precoated with 0.2 mm layers of silica. A Camag twin-trough chamber for 20×20 cm plates, with a stainless-steel lid, was used for ascending development of the plates. Densitometric scanning was performed on Camag TLC scanner III, operated with WINCAT software, while the source of radiation was a D2lamp.

HPTLC Analysis:

Pre-conditioning: After selecting the chromatographic layer, plates were prewashed with methanol and activated at 70 °C for 60 min.

Sample Application: Using an automatic applicator, the samples were spotted with a constant application rate of 5 s/µL in the form of bands of 6 mm in width. The space between bands was 9 mm. The distance from the left edge and the bottom of the plate was kept at 30 and 20 mm, respectively. Samples were applied under a continuous drying stream of nitrogen gas at a constant application rate of 150 nL/s

Selection of Suitable Mobile Phase: The following solvent mixtures were selected as candidates for the method development: Ethyl acetate: n-hexane

Chromatographic Development: The tank was saturated for 20 min before the insertion of the spotted plate. Plates were developed with 10 mL of mobile phase in strict light-protected conditions.

The development distance was approximately 80 mm. The chromatography was run at 25 °C± 3 and at relative humidity of 33%± 3.

Detection and Scanning: After development, the plate was dried with dryer for 1min. Densitometric scanning was then performed in the absorbance mode using the D2 light source at 330 nm (λmax for the compounds). Monochromator bandwidth was kept at 6mm and the dimension of slit was set at 6*0.30 mm.

Preparation of Stock and Working Solutions: A stock solution was prepared (1000 µg/mL) by dissolving and diluting 10mg of Pretomanid in 10 mL with methanol. The solution was sonicated for 10 minutes.

Calibration Curve: The concentrations 0.2µl, 0.4µl, 0.6 µl,0.8 µl,1 µl,1.2 µl were applied in three replicates on the TLC plate. The spotted plates were developed and scanned as described above. The calibration curve was constructed by plotting average peak areas versus the corresponding amounts, and the regression equation was calculated for Pretomanid.

RESULTS AND DISCUSSION:

Method Development and Optimization: Various chromatographic conditions were investigated to attain satisfactory Pretomanid qualitative and quantitative analysis results.

Developing the mobile phase individually on glass and aluminum TLC plates and comparing results indicated that aluminum backing plates produce well-defined spots with better resolution. Hence, this work selected aluminium sheet plates precoated with silica gel as the stationary phase.

Method Validation: HPTLC method was carried out as per the International Conference on Harmonization (ICH) guidelines Q2 (R1) linearity, accuracy, precision, robustness, limit of detection (LOD) and limit of quantitation (LOQ) [10].

Linearity: The linearity of the method was evaluated by constructing calibration curves at six concentration levels. The calibration curve was plotted over a concentration range of 200–1200 ng/spot **Table 1** Aliquots of standard working solution of Pretomanid were applied to the plate (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 µL/spot). The calibration curves were developed by plotting peak area versus concentrations (n = 6) with the help of the win CATS software **Fig. 2**. 2D Densitogram of Pretomanid Standard is obtained at Rf 0.68 **Fig. 3**. A typical 3D Densitotogram of standard Pretomanid is shown in **Fig. 4.**

TABLE 1: LINEARITY OF PRETOMAID

Sr. no.	Conc.(ng/spot)	Area
1	200	4006.41
2	400	4515.85
3	600	5769.33
4	800	6708.03
5	1000	8191.96
6	1200	8993.40

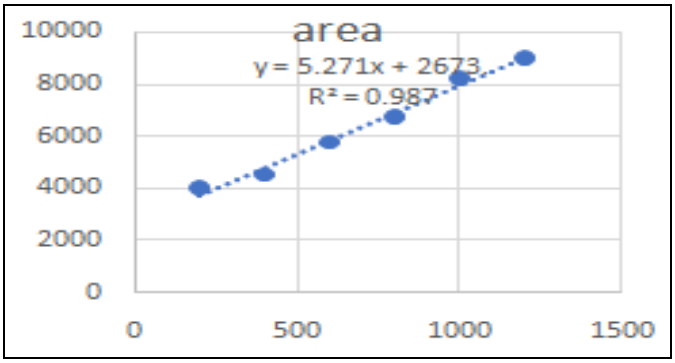

FIG. 2: CALIBRATION CURVE FOR PRETOMAID

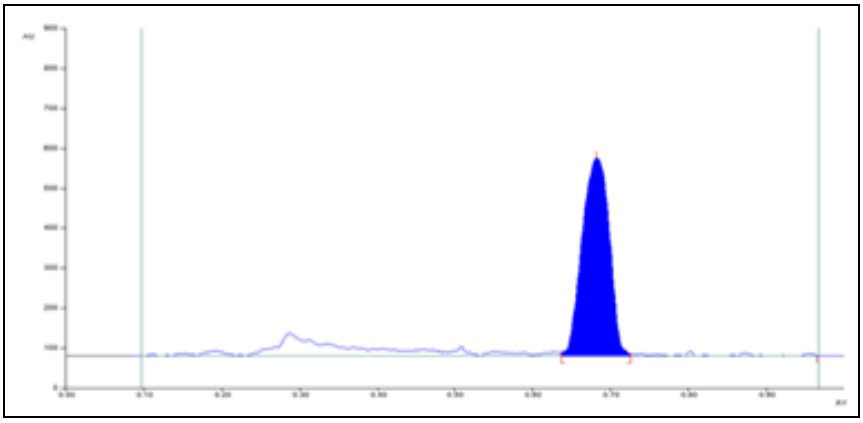

FIG. 3: 2D DENSITOGRAM OF PRETOMANID STANDARD (RF 0.68 ± 0.046)

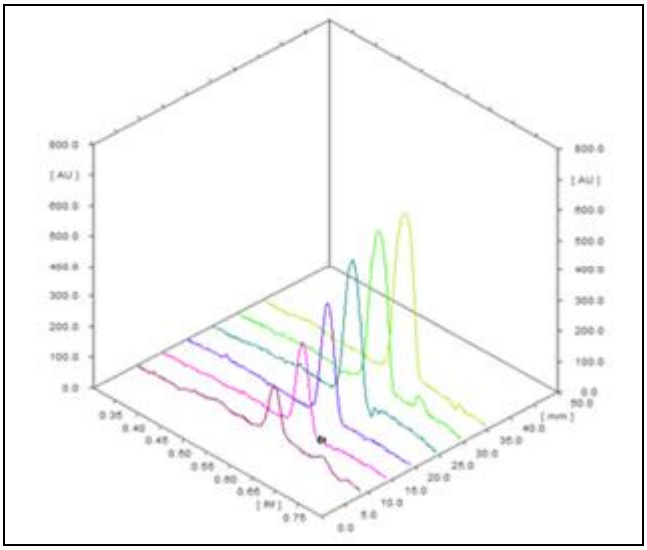

FIG. 4: A TYPICAL 3DDENSITOTOGRAM OF STANDARD PRETOMANID

Precision: To assess three sources of variance, the accuracy was measured at three distinct concentration levels of 0.6, 0.8, and 1 L/spot. When a densitometer measures the same location three times, accuracy was initially evaluated. Third, overall repeatability was assessed when the study was performed under the same conditions by the same analyst on two separate days. Second, the same answer is found three times. The results are shown in **Tables 2** and **3** as relative standard deviation percentages (%RSD). RSD values were under 3%, confirming both the appropriate intermediate accuracy and the strong repeatability of sample application and peak area measurement. RSD values between 1% and 5% in TLC densitometry are considered acceptable.

Intraday Precision:

TABLE 2: INTRADAY PRECISION

Sr. no.	Conc. (ng/spot)	Area	Mean	S.D.	%RSD
1	600	6442.9	6518.266	85.2220	1.3
2	600	6474.5			
3	600	6637.4			
4	800	8576.1	8579.7	112.133	1.3
5	800	8444.2			
6	800	8718.8			
7	1000	10265.8	10196.56	57.590	0.56
8	1000	10199.1			
9.	1000	10124.8			

Interday Precision:

TABLE 3: INTERDAY PRECISION

Sr. no.	Conc.(ng/spot)	Area	Mean	S.D.	%RSD
1	600	6386.7	6383.6	20.00	0.3
2	600	6406.4			
3	600	6357.7			
4	800	8351.7	8465.7	84.17	0.9
5	800	8461.0			
6	800	8589.4			
7	1000	9754.5	9623.56	101.7	1.05
8	1000	6506.3			
9.	1000	9609.9			

Accuracy (Recovery Studies): Recovery studies were carried out to assess the accuracy of the method. These studies were carried out at three levels. The percentage recovery was found to be within the limits and shown in **Table 4.**

TABLE 4: RECOVERY STUDIES

%Recovery	Standard API	Admixture	Area	Mean	%Recovery.
50	0.4µl(400ng)	0.2 µl(200 ng)	5973.2	6015.43	105
50	0.4µl(400ng)	0.2 µl(200 ng)	6012.5		
50	0.4µl(400ng)	0.2 µl(200 ng)	6060.6		
100	0.4µl(400ng)	0.4 µl(200 ng)	7192.0	7145.5	106
100	0.4µl(400ng)	0.4 µl(200 ng)	7013.2		
100	0.4µl(400ng)	0.4 µl(200 ng)	7231.5		

150	0.4µl(400ng)	0.6 µl(200 ng)	8120.5	8166.1	104.1
150	0.4µl(400ng)	0.6 µl(200 ng)	8104.3		
150	0.4µl(400ng)	0.6 µl(200 ng)	8273.5		

Robustness: Robustness was determined by altering chromatographic conditions like mobile phase composition. The low value of RSD indicates the robustness of the method. The results were shown in the **Table 5.**

TABLE 5: ROBUSTNESS

Conc. (µL)	Area	Mean	S.D.	R.S.D.
0.6 µL	6211.2	6329	100.7	1.65
0.6 µL	6457.4			
0.6 µL	6318.4			
0.8µL	8257.4	8188.3	55.8	0.6
0.8µL	8187.1			
0.8µL	8120.5			
1.0 µL	9311.0	9146.0	121.3	1.32
1.0 µL	9104.7			
1.0 µL	9022.4			

LOD and LOQ: The sensitivity of the proposed method was estimated in terms of Limit of Detection (LOD) and Limit of Quantitation. The ICH indicates that LOD (which they call DL, the detection limit) can be calculated as LOD = 3.3σ / S, and the limit of quantification (which they call QL, the quantitation limit) LOQ = 10σ / S. Here σ is the standard deviation of the response and S is the slope of the calibration curve. S is estimated from the slope of the calibration curve for the analyte **Table 6.**

TABLE 6: LOD AND LOQ

Drug	LOD	LOQ
Pretomanid	144.9ng	439.36ng

Stress Degradation Studies of Bulk Drug: Stability studies were carried out to provide evidence on how the quality of drug varies under the influence of various environmental conditions like hydrolysis, oxidation, temperature, *etc*. Dry heat and photolytic degradation were carried out in the solid state [11, 13].

Acid Hydrolysis: To 1 ml standard stock solution of drug (1000 µg/ml), 1 ml of 2 N HCl was added and volume made to 10 ml with methanol to get 100 µg/ml solution. The solution was kept for 24 hr in dark, neutralized with 2 N NaOH and 10 µl of this solution was applied on TLC plate (1000 ng/band concentration).

Acid degradation blank is prepared in the same way without using an analyte. Under acid hydrolysis, percent recovery obtained for Pretomanid was 98.87 % with no degradant peak. The representative chromatogram is shown in **Fig. 5.**

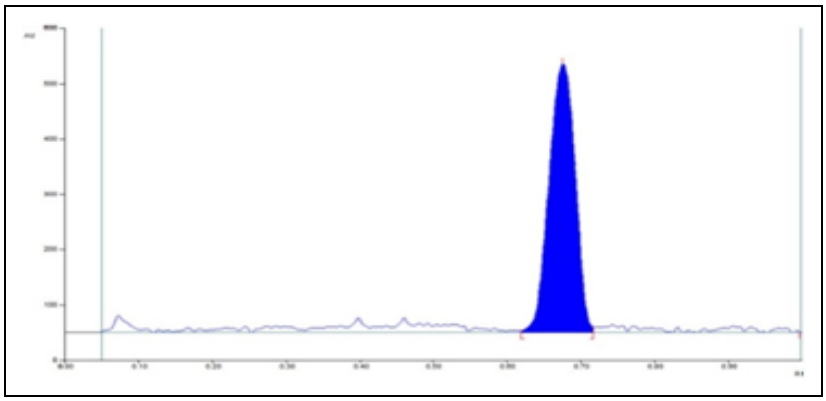

FIG. 5: DENSITOGRAM OF PRETOMANID AFTER ACID DEGRADATION

Alkaline Hydrolysis: To 1 ml standard stock solution of drug (1000 µg/ml), 1 ml of 1N NaOH was added and volume made to 10 ml with methanol to get 100 µg/ml solution. The solution

was kept for 24 hr in the dark, neutralized with 1 N HCl and 2 ml diluted to 10 ml with methanol to get 20 µg/ml solution. Alkali degradation blank is prepared in the same way without using analyte. Under alkaline hydrolysis, the percent recovery obtained for Pretomanid was 59.38 % with two degradant peaks. The representative chromatogram is shown in **Fig. 6.**

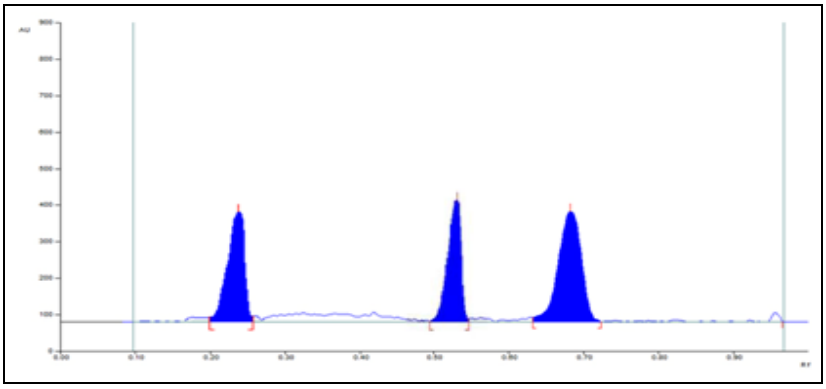

FIG. 6: DENSITOGRAM OF PRETOMANID AFTER ALKALINE DEGRADATION

Degradation under Oxidative Condition: To 1 ml standard stock solution of the drug (1000 µg/ml), 1 ml of 30% H_2O_2 was added and volume made to 10 ml with methanol to get 100 µg/ml solution. The solution was kept for 24 hr in dark and 2 ml diluted to 10 ml with methanol to get 20 µg/ml solution. The blank is prepared in the same way without using an analyte. Under oxidative degradation, the percent recovery obtained for Pretomanid was 97.22 % with no degradant peak. The representative chromatogram is shown in **Fig. 7.**

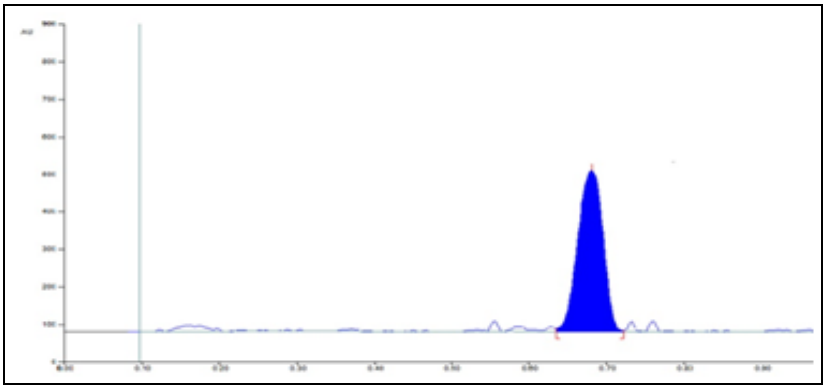

FIG. 7: DENSITOGRAM OF PRETOMANID AFTER OXIDATION WITH 30% V/V H_2O_2

Degradation under Dry Heat: Dry heat study was performed by keeping the drug sample in an oven (100° C) for a period of 2 hour. A sample was withdrawn 10 mg was dissolved in methanol to get a solution of 1000 µg/ml and further diluted with methanol to get 20 µg/ml as final concentration and injected. Under dry heat degradation conditions, the percent recovery obtained for Pretomanid was 99.26 % with no degradant peak. The representative chromatogram is shown in **Fig. 8.**

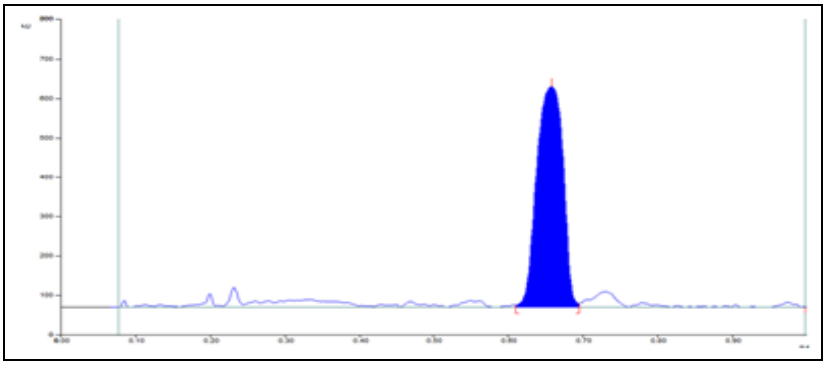

FIG. 8: DENSITOGRAM OF PRETOMANID AFTER DRY HEAT DEGRADATION

Photodegradation Studies: The photodegradation stability study of the drug was studied by exposing the drug to UV light providing illumination of NLT 200 watt hr/m^2 and exposure to cool white fluorescence light of NLT 1.2 million Lux-Hr. After exposure and accurately weighed 10 mg of drug was transferred to 10 ml of volumetric flask; the volume was made up with methanol. Further dilution was made with methanol to get 20 µg/ml as final concentration and injected. An average 83.74 % of Pretomanid was recovered with a peak of degradant at RT 2.238 after exposure to UV light. An average 98.62 % of Pretomanid was recovered with no degradant peak after exposure to fluorescence light. The representative chromatogram is shown in **Fig. 9** and **Fig. 10,** respectively [12].

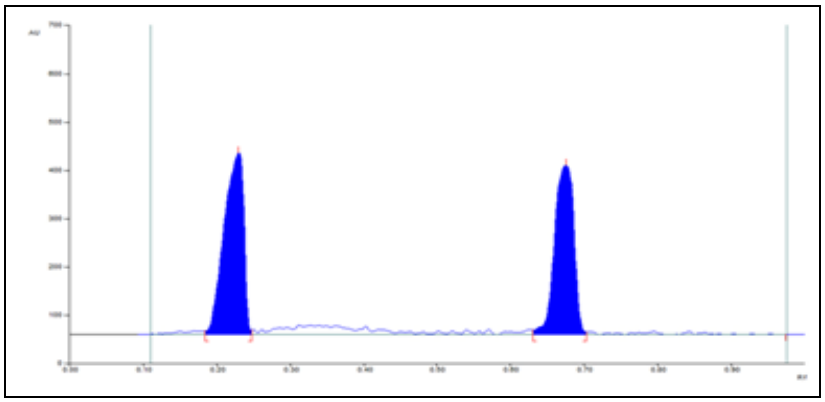

FIG. 9: DENSITOGRAM OF PRETOMANID AFTER UV ILLUMINATION EXPOSURE

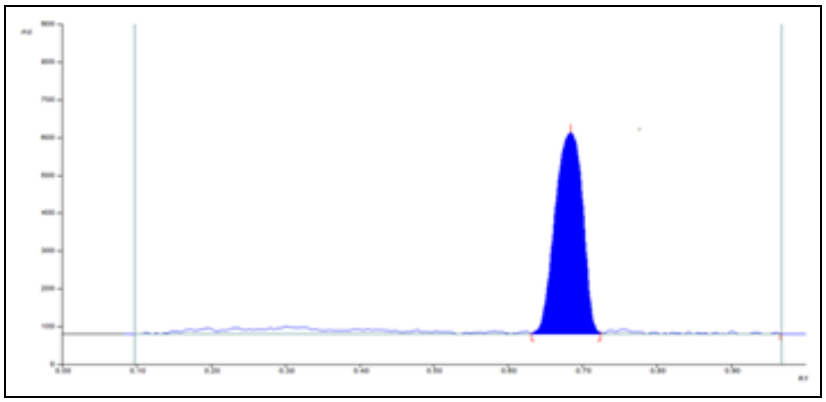

FIG. 10: DENSITOGRAM OF PRETOMANID AFTER FLUROSCENT LIGHT EXPOSURE

TABLE 7: SUMMARY OF DEGRADATION PARAMETERS

Stress condition/Duration	% Recovery of Analyte	RT of degraded products
Acidic/ 2 N HCl for 24 hours	98.87 %	-
Alkaline/ 1 N NaOH for 24 hours	59.38 %	Degradant at Rf – 0.23 and Rf – 0.52
Oxidative/ 30 % H_2O_2 at room temperature 24 hours	97.22 %	-
Dry heat / 100ºC/ 24 hours	99.26 %	-
UV illumination NLT 200 watt hours/square meter	83.74 %	Degradant at Rf 0.22
Fluroscent light NLT 1.2 X 10^6 Lux hr	98.62 %	-

CONCLUSION: For the investigation of Pretomanid, a novel HPTLC densitometric technique was created, verified by ICH criteria, and found to be repeatable, precise, accurate, and robust. In compared to other analytical techniques, the process is quick, easy, and affordable. It is also sufficiently feasible enough to be recommended for the regular analysis of Pretomanid. For the safety of patients, the quality of pharmaceutical goods is crucial. The presence of degradation chemicals or contaminants may impact the effectiveness and safety of medications. Pretomanid's forced degradation research was conducted under the ICH-required circumstances to examine its degradation profile and clarify the structures of the degradation products. An environmentally friendly, sensitive, exact, precise, affordable, time- and cost-efficient, and repeatable stability-indicating HPTLC method

is developed. The validation experiments demonstrated that the methods were linear, precise, accurate, specific, and selective to the medication in the presence of degradation products. The drug has been shown to be susceptible to photolytic and alkali hydrolytic degradation at room temperature. Research on stability indications was conducted in compliance with ICH Q1A (R2) guidelines.

The method has been applied to evaluate tablet items that are currently on the market. The presented method may be used frequently in quality control laboratories to analyse protomanid for pharmaceutical tablet dosage.

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CONFLICTS OF INTEREST: Authors declare that there is no conflict of interest

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