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A VALIDATED STABILITY-INDICATING RP-LC METHOD FOR PROPYLTHIOURACIL WITH LC-MS STUDIES OF FORCED DEGRADATION PRODUCTS AND SIMULTANEOUS ESTIMATION OF ITS IMPURITY

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Keywords:

Propylthiouracil, Thiourea, Impurity, Stability Indicating, LC-MS

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ABSTRACT: A simple, precise, accurate, sensitive and robust stability-indicating HPLC method for simultaneous estimation of propylthiouracil and its impurity thiourea has been proposed. The separation was achieved on a C_{18} column (4.6 mm × 150 mm, particle size 5.0 µm) maintained at 45 ºC with a mobile phase composed of water: methanol: acetonitrile (50:35:15 v/v/v) with 0.1% acetic acid and detection wavelength was 241 nm. In statistical analysis, the linear response in the range of 30 - 300 µg/ml for propylthiouracil and 0.3 - 30 µg/ml for thiourea with a correlation coefficient greater than 0.99 was obtained. In forced degradation studies, PTU was found to degrade under basic hydrolysis, oxidative and photo stress while found resistant to acid/neutral hydrolysis and thermal degradation. The probable structures of six major degradants generated under stress conditions were identified by LC-MS studies and the most likely degradation pathway was proposed from mass spectral data. The information presented herein could be very useful for the impurity profiling of drugs as well as can be employed to check the drug product quality during stability studies.

INTRODUCTION: Propylthiouracil (PTU) belongs to anti-thyroid drugs class called thion-amides, commonly used to treat hyperthyroidism, thyrotoxicosis and hyperthyroidism associated with pregnancy. It is a potent inhibitor of thyroid peroxidase enzyme and impairs the oxidation and organic binding of thyroid iodide thus blocks thyroid hormone synthesis [1]. PTU is cited in various Pharmacopoeia to have contaminated by impurity; thiourea (TU). Therefore, it was thought worth determining this impurity to ensure safety, efficacy and quality of the final formulation [2, 3].

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Detailed literature indicated different methods *viz*; HPLC [3], titrimetry [2], potentiometry [2, 3] are available for quantification of PTU in bulk and formulation. Simultaneous estimation methods *viz*; voltammetry [4] and UPLC-MS/MS [5] with other anti-thyroid drugs are also reported in the literature.

Official TLC method to detect impurity; TU is a semi-quantitative method and lacks stability-indicating potential [2]. Two stability-indicating HPLC methods have been reported in the literature; one is applicable to bulk drug [6] and other is to tablet assay [7]. The reported stability-indicating method is applicable for assay but is not applicable to its impurity; TU. These methods do not involve the identification of degradation products and are not suitable for LC-MS studies. Other reported methods include the study of the effect of temperature on stability of extemporaneously

prepared oral suspensions by HPLC [8], bioanalytical HPLC methods, for estimation in plasma, serum, [9, 10] urine [11] and metabolism studies by LC-MS/MS [12].

From the preceding details of literature, it became apparent that no report on the identification of the degradation products is available. Hence, there was a need to develop a validated stability-indicating method for simultaneous estimation of PTU and TU along with the characterization of degradation products. Thus, the ultimate objective of the present research work was the degradation of PTU under stress conditions, resolution of the degradation products, and selected impurity; TU by HPLC using MS suitable mobile phase and validation of the developed method. Finally, the characterization of the major degradation products by LC-MS.

MATERIALS AND METHODS:

Reagents and Chemicals: PTU (Purity 100.25%) was provided as gratis sample by Macleods Pharmaceutical Ltd., Daman, while TU (Purity 99.51%) was procured from Aarti Drugs Ltd., Thane and were used without further purification.

HPLC grade methanol, acetonitrile was purchased from E. Merck (India) Ltd., Mumbai. AR grade HCl, NaOH and H_2O_2 were purchased from Research Lab Fine Chem. Ind., Mumbai. Double distilled water filtered through 0.2 µm filter before use was used throughout the experiment. Marketed formulation, PTU tablet (Macleods Pharmaceuticals Ltd.) containing 50 mg of propylthiouracil was procured from market and used for analysis.

Chromatographic Condition: Resolution of a drug from its Impurity; TU and the degradation products was achieved on Waters HPLC system consisted of a binary pump (Waters 515 HPLC pump), autosampler (717 plus), column oven (Waters CHM) and PDA detector (Waters 2998). The separation was carried out on Cosmocil C_{18} (4.6 mm × 150 mm, 5.0 µm) column maintained at 45 ºC. The mobile phase composed of water: methanol: acetonitrile (50:35:15 v/v/v) and 0.1% acetic acid, filtered through 0.45 µ membrane filter and sonicated for 15 min before use. The flow rate was 0.80 ml/min. The wavelength of detection was 241 nm; λ_{max} of TU at which PTU also bears considerable absorbance **Fig. 1**.

Data were integrated using Empower version 2 software. Bruker (Impact II HD MS) equipped with an electrospray ionization source was used for LC-MS studies. The samples were infused into the mass spectrometer from the HPLC system through the ESI interface. The Brucker Compass Data Analysis 4.2 software was used for data acquisition and processing.

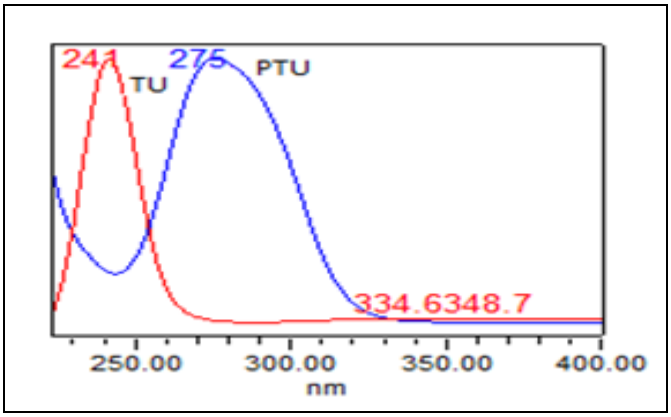


FIG. 1: ONLINE OVERLAIN UV SPECTRA OF PTU AND TU

Standard Stock Solution: Accurately weighed 100 mg of PTU and 10 mg TU was dissolved separately in methanol and volume was made up to 100 ml to obtain a standard stock solution of 1000 µg/ml of PTU and 100 µg/ml of TU. A series of solutions in the range of 30 - 300 µg/ml of PTU and 0.3 - 30 µg/ml of TU were prepared using a stock solution.

Formulation Assay and System Suitability Test (SST): Twenty tablets were weighed and crushed to a fine powder. An accurately weighed tablet triturate equivalent to 100 mg of PTU was transferred to a 100 ml volumetric flask and volume was made up to 50 ml with methanol. The solution was sonicated for about 10 min to ensure complete extraction of PTU and volume was made with methanol (1000 µg/ml). The resultant solution was filtered through Whatman filter paper no. 41.

The sample solution was prepared by diluting 1.5 ml of the above stock sample solution to 100 ml with methanol (150 µg/ml) and 10 µl was injected after filtration through a syringe filter (0.45 µ PALL Life Sciences) into stabilized chromate-graphic conditions and chromatogram was recorded. Drug concentration in the formulation was calculated using a calibration curve.

Before the sample analysis system suitability test was performed by injecting standard PTU solution spiked with standard impurity TU (150 µg/ml PTU spiked with 15 µg/ml of TU) into the stabilized chromatographic conditions five times. The following parameters were measured *viz*; a number of theoretical plates, AUC, resolution, peak purity, tailing and capacity factor.

Forced Degradation Studies: The aim was to achieve partial degradation of the drug, preferably in 10 - 20% range. The degradation was performed in two sets at two different temperatures; one set was kept at room temperature up to 4 h while another set of solutions was refluxed at 80 ºC for 40 min using 1000 µg/ml of PTU. The degradation samples were diluted with the mobile phase to achieve the nominal concentration of 100 µg/ml of PTU. The degradation was carried out under hydrolytic, oxidative condition using stresser; 0.1 N HCl (acid hydrolysis), 0.1N NaOH (base hydrolysis), HPLC grade water (neutral hydrolysis) and 6% hydrogen peroxide (oxidation). The working standard of 50 mg was spread as a thin film in two separate petri dishes (5 cm diameter) and exposed to 100 ºC in a hot air oven for 5 h; cooled to room temperature and 10 mg of powder was diluted appropriately to obtain a solution that contains 1000 µg/ml of an analyte with methanol. For photolytic stress, drug substance in the solid-state was irradiated with near UV radiation (366 nm) and daylight for 26 h. All samples were then diluted suitably and injected into stabilized chromatographic conditions.

Method Validation: The method was validated as per ICH guidelines for linearity, accuracy, precision, robustness *etc*. [13]

Specificity: Specificity was established by peak purity profiling to demonstrate the chromatographic peak is attributed to one component. Thus, chromatograms were observed for separation and resolution between analytes (PTU and TU) and separation and resolution between the analyte and its degradation products.

Linearity and Range: The series of solutions in the range of 30 to 300 µg/ml and 0.3 - 30 µg/ml of PTU and TU, respectively. The analytes were resolved under optimized chromatographic conditions. A standard calibration graph of peak area *vs*. concentration was plotted, the entire procedure was executed thrice, starting from the weighing of analytes. The linearity of the method was evaluated by linear regression analysis using the least square method. The slope and intercept were calculated. To further confirm linearity, F test and residual plot of relative response against concentration were plotted, and the pattern was observed; were performed [14]. LOD and LOQ were calculated using the regression equation.

Accuracy (Recovery Studies): The standard addition method in triplicate at 80%, 100% and 120% concentration level was applied to demonstrate the accuracy of the developed method. The standard addition method was used to demonstrate the recovery of PTU and TU from tablet triturate. For PTU recovery studies from tablet formulation, the amount of tablet triturate containing 75 mg of PTU was spiked with 80%, 100%, and 120% of standard PTU separately. Similarly, to check impurity recovery from formulation, the amount of tablet triturate equivalent to 300 mg of PTU was spiked with 0.05%, 0.1%, and 1% of impurity separately. The sample preparation was executed, starting from weighing thrice and was analysed. The percent recovery and % RSD was calculated.

Precision: The precision of the proposed analytical method was demonstrated by repeatability and intermediate precision studies. Analysis of sample solution containing 300 µg/ml of PTU spiked with 0.3 µg/ml of TU was performed thrice on the same day and on different days by different analysts to demonstrate intermediate precision. For this, tablet blend equivalent to 300 mg of PTU was spiked with 3 mg of TU and extraction was executed as per the assay procedure.

Each time the sample solution was prepared starting from weighing. Similarly, six independent assays of sample containing 150 µg/ml PTU was also considered. The % RSD and % mean recovery was checked.

Robustness: To assess robustness, deliberate changes in the flow rate, column temperature and use of different brand columns were considered. Solution stability studies were also executed by

using a sample solution containing 150 µg/ml of PTU spiked with 15 µg/ml of TU. The effect on resolution, % assay and % RSD was considered to establish the robustness.

RESULTS AND DISCUSSION:

Method Validation:

Specificity: Optimum resolution was achieved with elution of PTU at 3.99 min and TU at 2.75 min as shown in **Fig. 2**. The average result for SST parameters is summarized in **Table 1**. The SST parameters were found acceptable. The resolution was greater than 3.9 and peak tailing was less than 1.5. The formulation chromatogram is shown in **Fig. 3** and the % mean assay was found 99.79 with 0.55 % RSD. The peak purity angle values were always found less than purity threshold values for analyte as indicated in **Table 1** and **Table 5**.

This proves the homogeneous peak of a single component and absence of co-elution of inter-ferents and thus demonstrate the specificity of the developed method.

TABLE 1: SYSTEM SUITABILITY AND PEAK PURITY DATA (n=5)

Parameter↓ / Analytes →	PTU	TU
Peak Area	2437098	1216726
(% RSD)	(0.89)	(1.03)
No. of theoretical plates (% RSD)	3933 (3.07)	3150 (3.19)
USP Tailing Factor (±SD)	1.41 (0.08)	1.46 (0.083)
USP resolution (R) (±SD)	--	3.96 (0.73)
Capacity Factor (k) (±SD)	3.717 (0.62)	2.709 (1.07)
Typical Peak Purity data		
Peak Angle	0.137	0.146
Peak threshold	0.185	0.201
Formulation assay results (n=6)		
Mean AUC	2427066	
Mean % assay	99.79	
%RSD	0.55	

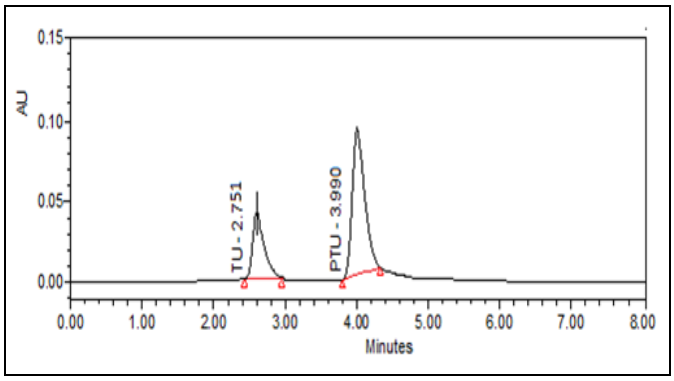


FIG. 2: REPRESENTATIVE CHROMATOGRAM ACQUIRED WITH PTU SPIKED WITH TU

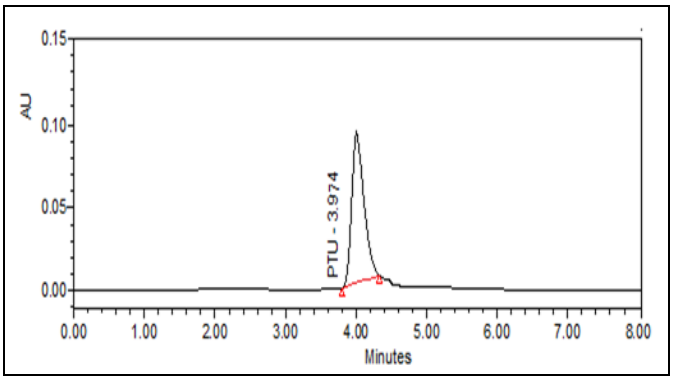


FIG. 3: REPRESENTATIVE CHROMATOGRAM ACQUIRED WITH FORMULATION

Linearity and Range: It was demonstrated by regression analysis, F-test and residual plots. The least square regression analysis showed correlation coefficient greater than 0.999 while experimental F value found less than critical tabulated F value at 95% confidence level as represented in **Table 2**. The residual plot of both the analytes was without any trend as seen in **Fig. 4.**

Thus, demonstrate the excellent correlation between peak area and concentration of the analytes within the selected range. LOD and LOQ values were found satisfactory as seen in **Table 2.** Thus, developed method can be utilised successfully to detect and quantify the impurity up to 0.05% level in formulation and bulk.

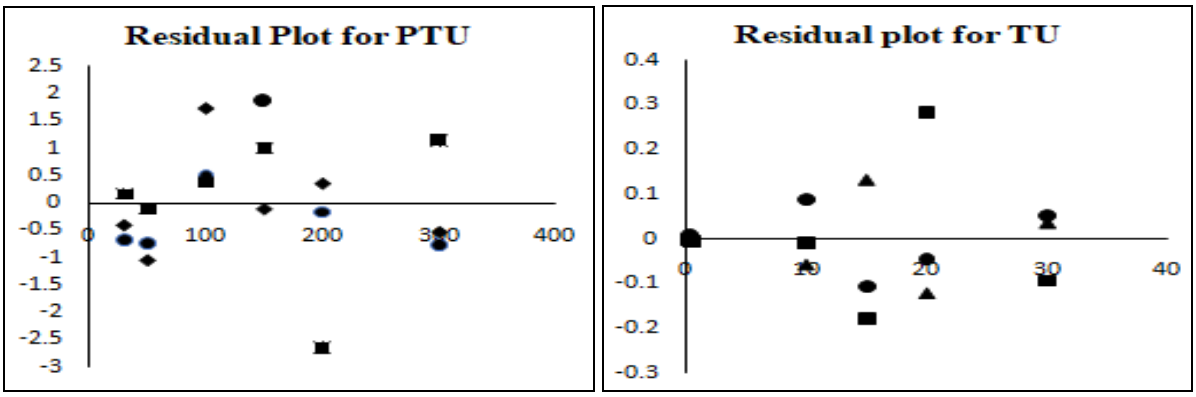


FIG. 4: RESIDUAL PLOT FOR PTU AND TU

TABLE 2: REGRESSION CHARACTERISTICS, METHOD SENSITIVITY AND PRECISION STUDIES OF PROPOSED RP-HPLC METHOD

Parameter↓ / Analytes →	PTU	TU
Wavelength for detection	241 nm	
Concentration range (µg/ml)	30 – 300	0.3 - 30
Retention time (t_R) (min)	3.959	2.790
Regression equation (Y= b × Concentration ± a)		
Intercept (± SD)	-13776.5 (± 797)	692.2 (±149)
Slope (± SD)	16305.9 (± 41)	81065.9 (± 339)
Correlation coefficient (r^2)	0.999	0.999
F Test (12 and 4 degrees of freedom)		
Experimental Fischer variance ratio	2.015	2.161
Tabulated Fischer variance ratio	3.259	
Method sensitivity (µg/ml)		
Limit of Detection	1.20	0.018
Limit of Quantitation	3.63	0.056
Intermediate precision (n=3)		
Intra-day, Assay (%RSD)	100.04 (1.05)	99.76 (1.32)
Inter-day, Assay (%RSD)	100.28 (1.24)	98.79 (1.53)

Accuracy: The % mean recovery values were found in the acceptable range; 100 ± 2.0% and % RSD value was less than 2 as summarized in **Table 3**. Consequently, the proposed method is found accurate to estimate PTU as well as its impurity TU

TABLE 3: RESULT OF RECOVERY STUDIES (ACCURACY) (n=3)

Sample spiked	PTU Recovery			Impurity Recovery		
	Level of Recovery (PTU)			Level of Recovery (TU)		
	80%	100%	120%	0.05%	0.1%	1%
Mean AUC (n=3)	2166635	2458020	2668203	12716	24912	246387
% Mean recovery	99.03	101.03	99.65	98.88	99.59	101.02
% RSD	0.71	0.88	0.97	1.98	1.75	1.55

Precision: In independent sample analysis, the mean % recovery was 99.76 % with % RSD 0.55 demonstrate the repeatability of the developed method. During intermediate precision, the % RSD was found less than 2%, while the mean recovery was 100 ± 2% as indicated in **Table 2**; establish the precision parameter.

Robustness: There was no substantial impact of small modifications in the HPLC method on the resolution, % recovery and % RSD of the analytes as indicated in **Table 4.** This revealed that the developed method is robust to these changes.

TABLE 4: RESULT OF ROBUSTNESS STUDIES (n=3)

Parameter (Limit)	Level	Resolution	PTU % Assay, % RSD	TU % Amount, % RSD
Flow Rate	(–) 0.75	3.92	99.26, 0.52	98.24,0.87
(± 0.02 ml/min)	(+) 0.85	3.86	100.07,0.74	100.45,0.92
Column Temp.	(–) 43	3.96	99.08.0.67	99.08, 0.81
(± 2 ºC)	(+) 47	3.84	98.12.0.83	101.46,1.02
Column (C18)	Cosmocil	4.01	101.12,0.56	100.25,0.97
	Symmetry	3.95	100.41, 0.75	98.26, 1.26

Characterization of Forced Degradation Products: To gain high-intensity signal and higher sensitivity to characterize the degradation products; ESI source conditions were optimized. The parameters *viz*; drying gas flow, its temperature, nebulizing gas flow, voltage were optimized. The ionization of the drug and its degradation products was carried out on negative mode due to the presence of two strong electronegative atoms, namely; sulphur and oxygen, to form negative ions rapidly. Standard PTU was introduced to validate the output of the mass spectrometer. Then the drug under stressed condition was introduced into the column. The $[M-H]^-$ value observed for PTU was

169.04, which matched with its exact mass (170.05). The column and mobile phase as described above were used for the characterization of degradation products by LC-MS. The analysis was carried out by using electrospray ionization as a source of ionization. Mass spectra were acquired using Bruker (Impact II HD-MS) mass spectrometer. Optimized electron spray ionization parameters were: capillary voltage 3000 V, source temperature 200 ºC, sheath gas/nebulizing gas was nitrogen at pressure 0.3 bar, declustering/entrance potential was -10 V and scan range: m/z 50 to 600. All the samples were prepared in methanol. Mass spectra were acquired and processed using Brucker Compass Data Analysis 4.2 software. The mass spectrum acquired for PTU and LC-MS chromatogram is shown in **Fig. 5**.

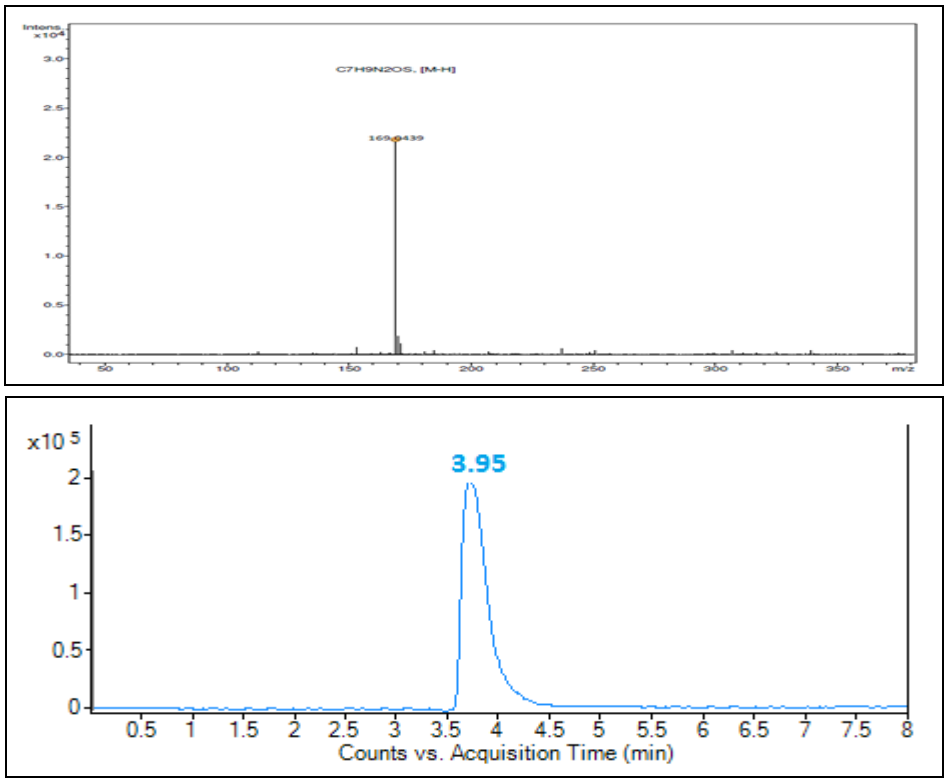


FIG. 5: A) LC-MS SPECTRUM OF PTU M/Z= 169.04 B) LC-MS CHROMATOGRAM OF PTU

Hydrolytic Degradation Studies: The comparison of degraded sample chromatograms obtained by acid and neutral hydrolysis under reflux and pure sample revealed the absence of additional peaks in the degraded samples indicating stability towards these conditions. In base induced degradation studies, additional degradation peaks were observed in chromatogram acquired with base induced degraded samples. The comparison of degraded sample chromatograms revealed the presence of two additional peaks, A and B in **Fig. 6.**

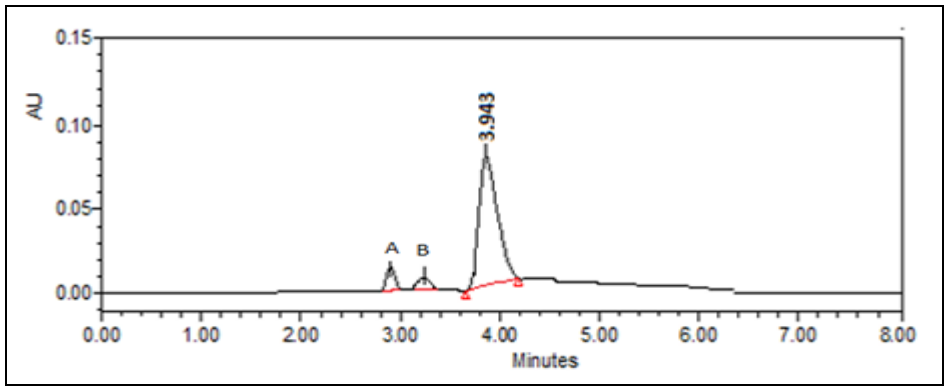


FIG. 6: CHROMATOGRAM OF BASE HYDROLYSIS

The LC-MS spectrum of base induced degradation product B is shown in **Fig. 7** while the probable reaction mechanism and the reaction product is depicted in **Fig. 8**. Since, the degradant, A formed neutral molecule, it was not detected in MS studies but was eluted at the same t_R as that of TU. This confirms the formation of TU under base hydrolysis.

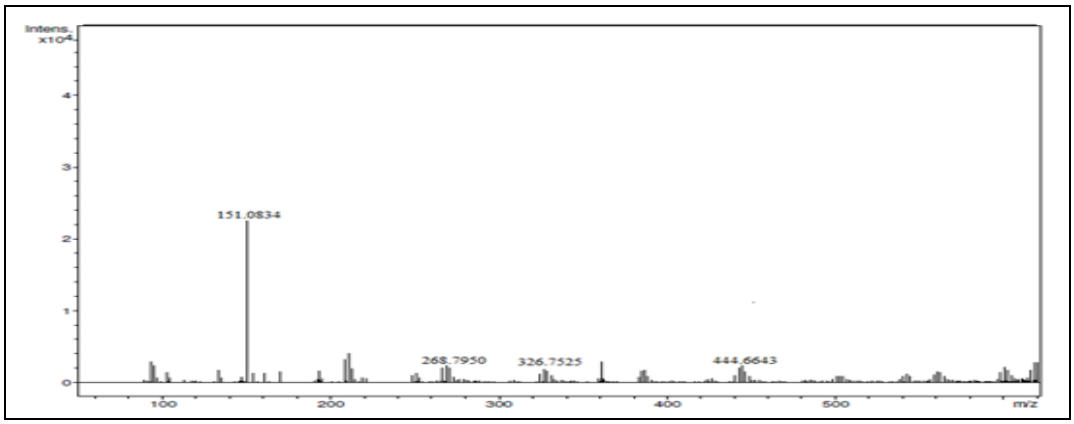


FIG. 7: NEGATIVE ION ESI LC–MS SPECTRUM OF BASE HYDROLYSIS PRODUCT B (M/Z = 151)

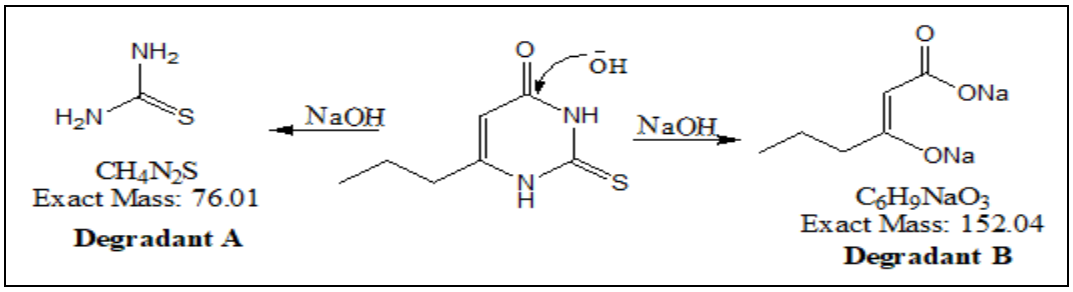


FIG. 8: BASE HYDROLYSIS OF PTU

Oxidative Degradation Studies: PTU in oxidative degradation produced two degradants upon refluxing, depicted as C and D in **Fig. 9**. The LC-MS spectrum is shown in **Fig. 11** and **12** revealed the formation of degradant with m/z ratio 217 and 201, respectively. The probable degradation products have been deduced from MS data and the oxidative degradation reaction is depicted in **Fig. 10**.

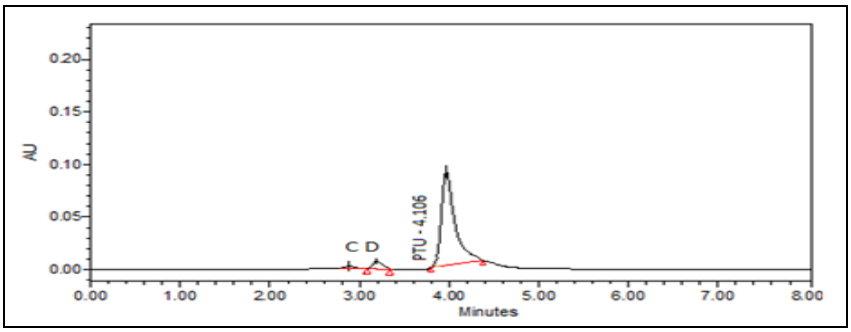


FIG. 9: CHROMATOGRAM OF OXIDATIVE DEGRADATION PRODUCT

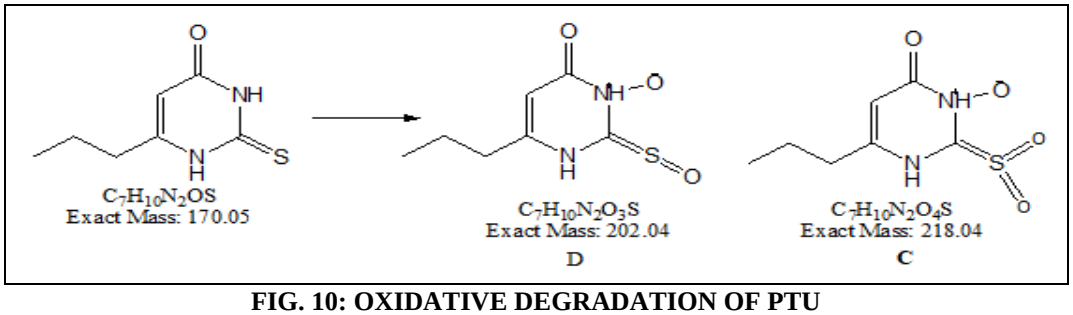


FIG. 10: OXIDATIVE DEGRADATION OF PTU

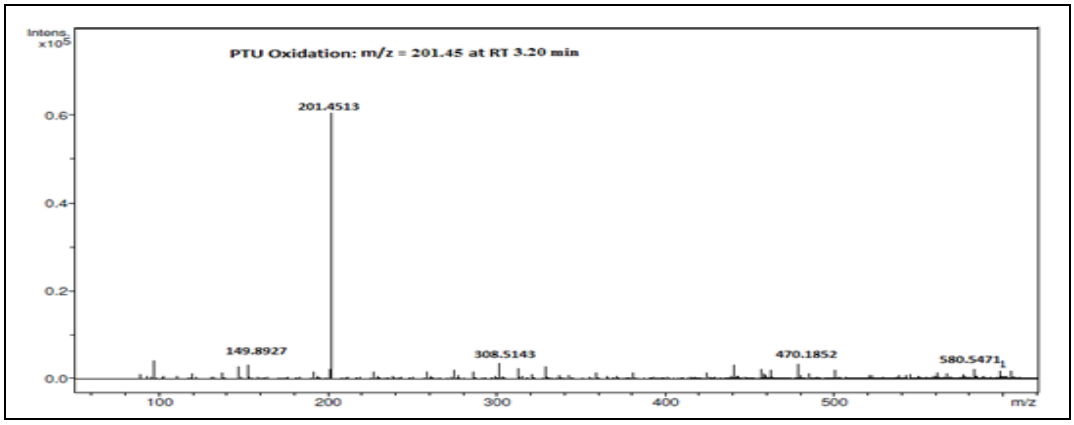


FIG. 11: NEGATIVE ION ESI LC-MS SPECTRUM OF OXIDATION DEGRADATION PRODUCT D (M/Z = 201)

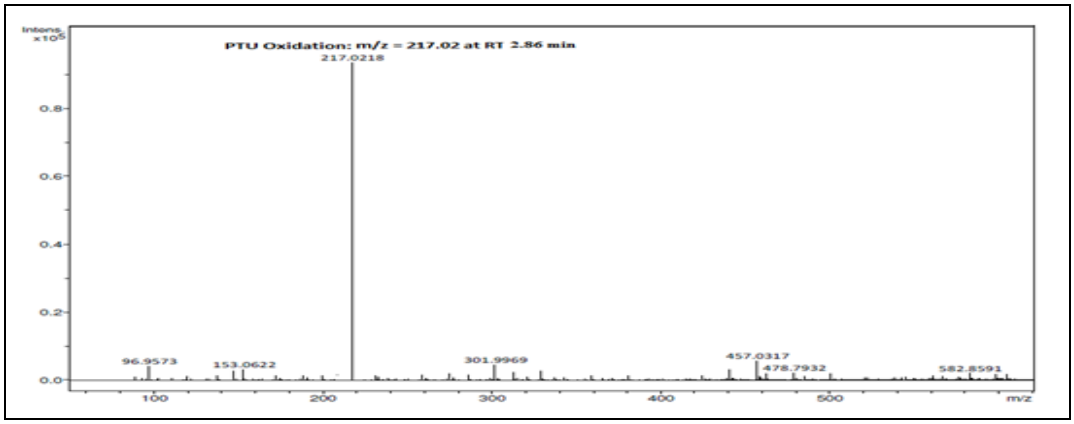


FIG. 12: NEGATIVE ION ESI LC-MS SPECTRUM OF OXIDATION DEGRADATION PRODUCT C (M/Z = 217)

Photo Degradation: PTU was found sensitive towards photo stress and formed two degradants E and F as shown in **Fig. 13.** The ESI-LC-MS spectra are shown in **Fig. 15** and **Fig. 16**. In LC-MS studies, the degradant with m/z ratio 362 (E) and 339.09 (F) were revealed. The probable reaction products and the reaction is deduced in **Fig. 16**. PTU formed disulphide linkage upon exposure to photo stress lead to the formation of the dimer. There was no degradation while an additional peak observed in the sample subjected to thermal stress. Thus, it indicates resistance to thermal stress.

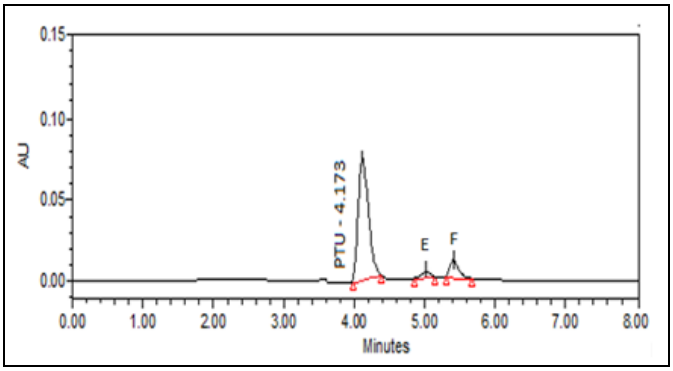


FIG. 13: CHROMATOGRAM OF PHOTO DEGRADATION PRODUCT

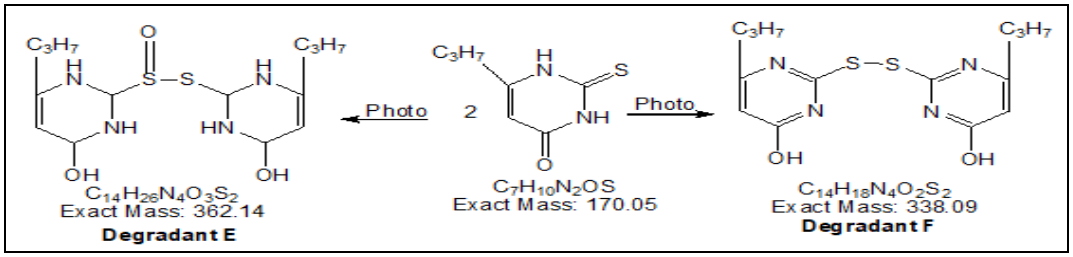


FIG. 14: PHOTO STRESS DEGRADATION OF PTU

Thus, in forced degradation studies, PTU found sensitive towards basic hydrolysis, oxidative and photo stress and was stable towards neutral/acid hydrolytic and thermal conditions. The degradation products of PTU along with the probable structures of degradation products is represented in **Table 6**. The results of forced degradation studies are summarised in **Table 5**.

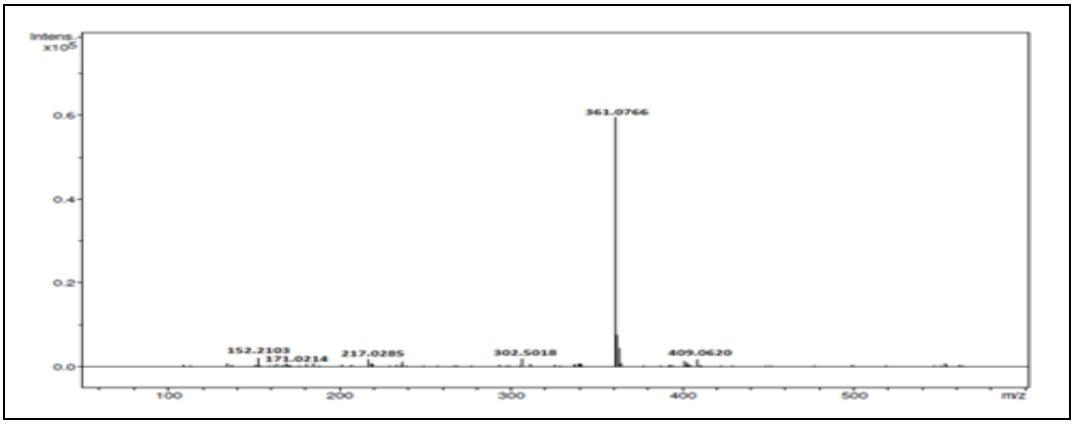


FIG. 15: NEGATIVE ION ESI LC-MS SPECTRUM OF PHOTO DEGRADATION PRODUCT E (M/Z = 361.07)

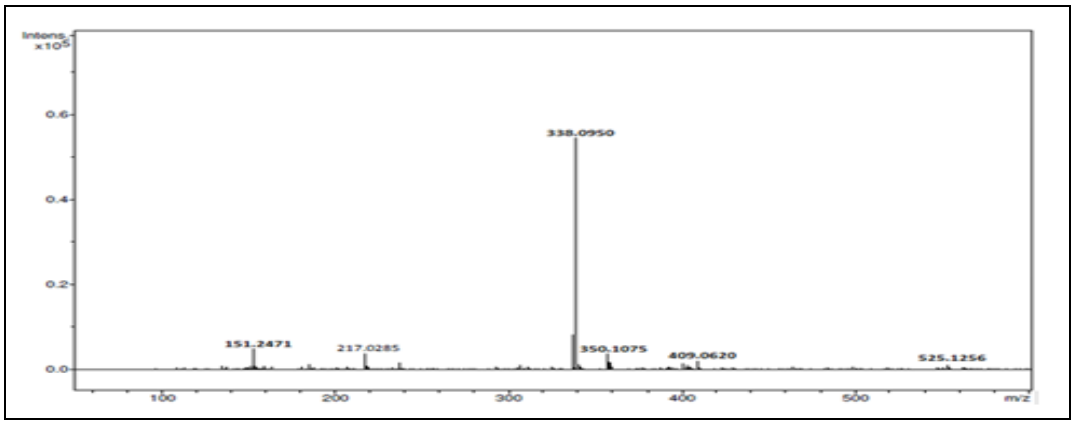
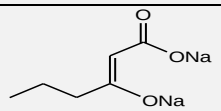
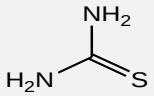
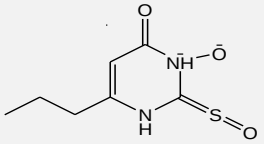


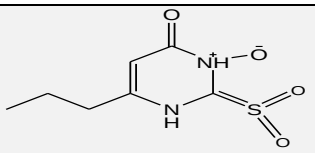
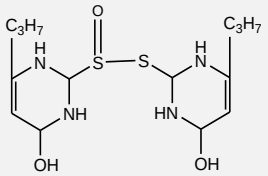
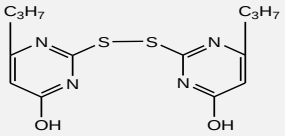
FIG. 16: NEGATIVE ION ESI LC-MS SPECTRUM OF PHOTO DEGRADATION PRODUCT F (M/Z = 338.09)

TABLE 5: SUMMARY OF FORCED DEGRADATION STUDY OF PTU

Analyte→ Stress condition ↓	Experimental Mass	t_R of Degraded Product	% Recovery	Peak angle, Threshold
Base (1 N NaOH, 1 h 80 ºC)	151.08	3.20	85.61	0.175,0.241
	76.04	2.88		0.275,0.310
Oxidation Hydrogen peroxide 6%	217.02	2.86	89.68	0.146,0.188
(1 h 80 ºC)	201.45	3.20		0.235, 0.289
Photo stress	361.07	5.02	90.25	0.211,0.285
	338.09	5.41		0.187,0.209

TABLE 6: SUMMARY OF DEGRADATION PRODUCTS OF PTU ALONG WITH THE PROBABLE STRUCTURES OF DEGRADATION PRODUCTS

Molecular Formula	Theoretical Mass (Experimental Mass)	Generation of peak	Probable structure
$C_6H_7Na_2O_3^-$	151.08 (152.04)	Base Hydrolysis	 3-Hydroxy-hex-2-enoic acid disodium
CH_4N_2S	76.04		 Thiourea
$C_7H_9N_2O_3S^-$	202.04 (201.45)	Oxidative stress	 3-methyl-6-propyl-2-thioxo-2,3-dihydro-1H-pyrimidin-4-one

$C_7H_9N_2O_4S^-$	218.04 (217.02)		 3-methyl-6-propyl-4-on-2,3-dihydro-1H-pyrimidin-3-ol-2-sulfone
$C_{14}H_{25}N_4O_3S_2^-$	362.14 (361.07)	Photo stress	 6-propyl-2-methylsulfoxyl-pyrimidin-4-ol; compound with 6-propyl-2-mercapto-pyrimidin-4-ol
$C_{14}H_{18}N_4O_2S_2$	338.09 (338.09)		 6-propyl-2-methylsulfoxyl-pyrimidin-4-ol; compound with 6-propyl-2-mercapto-pyrimidin-4-ol

CONCLUSION: The proposed RP-HPLC method is simple, specific, precise, accurate, sensitive and robust, as evidenced from the validation data for both; assay and impurity determination in bulk and in tablet formulation. In the proposed work, efforts have been taken to develop LC/MS suitable methods to identify and characterize the major degradation products generated under stress conditions. The structures of six major degradants generated under base, oxidative and photo stress have been proposed on the basis of their mass-to-charge ratio and known reactivity. The proposed method can be used in the routine analysis of production samples and the quality monitoring of bulk samples. The information presented herein could be very useful for the impurity profiling of drugs as well as can be employed to check the drug product quality during stability studies.

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