DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR DETERMINATION OF MERCAPTOPURINE-AN ANTI CANCER DRUG IN PHARMACEUTICAL FORMULATIONS

Pallapati Suman[1,2], Tirukkovalluri Siva Rao[1*], Dr. S. Radha Krishna, Kallam Venkata Siva Rama Krishna Reddy[2]

[1]Department of Inorganic & Analytical Chemistry, Andhra University, Visakhapatnam- 530003, Andhra Pradesh, India.
[2]Laurus Labs Limited, Visakhapatnam- 531021, Andhra Pradesh, India.

*Corresponding Author: Tirukkovalluri Siva Rao

Department of Inorganic & Analytical Chemistry, Andhra University, Visakhapatnam- 530003, Andhra Pradesh, India.

ABSTRACT

A quantitative determination of Mercaptopurine in pharmaceutical formulation employing a very simple and rapid chromatographically technique was validated and developed. The analysis was performed at temperature (25 ± 2 °C) by employing a reverse phase Kromasil C18 (250mm x 4.6mm, 5µm) column and a mobile phase made up of methyl alcohol, acetonitrile, and water in the ratio of 50:15:35 (v/v/v) at rate of flow of 0.9 mL/min and analyte was detected at 217nm by employing a UV-detector. During this method, Mercaptopurine has separated altogether the formulations within 6.1min. The Linear calibration curves obtained the concentration range of 2-12µg/mL and the correlation coefficient is 0.9993 for Mercaptopurine. Stress testing was accomplished to reveal the specificity of the optimized chromatographic HPLC method. This proposed method was suitable for the separation Mercaptopurine and its degradative products in pharmaceutical formulations.

KEYWORDS: Mercaptopurine, RP-HPLC, Stability-indicating method, Method Validation, ICH, MERCAPTO©.

1. INTRODUCTION

3, 7-dihydropurine-6-thione (6-MP), is effective both as an anticancer & an immunosuppressive agent used to the treatment of acute lymphocytic leukemia (ALL), chronic myeloid leukemia (CML), Crohn's disease, and ulcerative colitis.[1,2] Nobel Prize laureates Gertrude B. Elion & George H. Hitchings, were discovered this 6-MP.[3] 6-MP was approved in 1953 for therapeutic uses[1] in the United States (USP). WHO had published a list of essential, efficacious, safe and cost-effective medicines needed for a health system and It is in the list.[4]
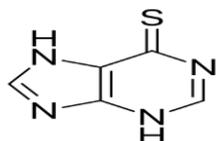
Figure 1: chemical structure of Mercaptopurine.

The drug works by interfering the nucleic acid synthesis by inhibiting purine metabolism and is employed, generally in combination with other drugs, in the treatment of or in maintenance therapy of acute lymphatic leukemia (ALL) & remission induction. For all, it is generally used with methotrexate. The drug is available in tablet dosage form.[1] Common side effects incorporate dizziness, swelling, liver toxicity, bone marrow suppression, vomiting, inflammation of the pancreas, loss of appetite & trouble in breathing. Additional adverse effects embody an enhanced risk of future cancer; pancreatitis and use in gestation might danger the baby.[1] 6-MP is an ant metabolite family & the thiopurine of medications.[2,3] A very few analytical methods were reported in the literature for the estimation of Mercaptopurine in plasma and bioequivalence studies in presence of its metabolites in single.[5-15] and in combination with other drugs.[16, 17] Simultaneous determination of 6-MP and its oxidative metabolites in human plasma & synthetic solutions employing spectrophotometric multivariate calibration methods.[18] The study was conducted on the effect of the basic solvent by using UV-spectrophotometer for Mercaptopurine (6-MP).[19] No analytical method was reported previously for the quantification of Mercaptopurine in pharmaceutical formulations and

stability studies. Hence the aim of this research work to develop a simple, rapid & robust stability indicating a method for the estimation of 6MP in pharmaceutical formulations by RP-HPLC.

2. MATERIALS AND METHODS
2.1. Materials
Mercaptopurine was gifted by GlaxoSmithKline Pharmaceuticals Ltd, India, and used as reference standard without further purification. HPLC grade methanol (CH_3OH), water (H_2O) and Acetonitrile (CH_3CN) were purchased from Merck Specialties, Pvt. Ltd. Mumbai, India. All solvents & solutions degassed before utilizing by ultrasonication (Ultrasonicator, PCI, and Mumbai, India). The pharmaceutical dosage form with brand name MERCAPTO$^©$ (Chandra Bhagat Pharma Pvt Ltd, Mumbai) was purchased from a local pharmacy.

2.2 Instrumentation
Chromatography was performed using PEAK LC 7000 isocratic HPLC with PEAK 7000 delivery system, Analytical column Kromasil C18 (250mm x 4.6mm, 5μm) column, Electronic balance-(Make: DENVER) (Model No: SI234), Rheodyne manual sample injector with switch (77251), manual Rheodyne injector with a 20 μL loop was utilized for the injection of sample. PEAK LC software was employed. UV 2301 Spectrophotometer was used to determine the wavelength of maximum absorbance.

2.3 Preparation of standard stock solution
A standard stock solution of the drug was prepared by dissolving the pure drug in the mobile phase, i.e. 10mg Mercaptopurine in 100 mL methanol. The stock solution was sonicated and filtered through Whatman filter paper. The resulting solution was further diluted with the mobile phase [CH_3OH, CH_3CN, and H_2O (50:15:35 v/v/v)] to a concentration of 100μg/mL. 25mL from 100μg/mL was further diluted to 50mL to get working standard solution concentration of 50μg/mL. This solution was used for the preparation of further dilutions for constructing a calibration curve.

2.4 Preparation of sample solution
Ten formulation tablets of Mercaptopurine (MERCAPTO$^©$ – 50mg) were powdered to get uniform sample powder. From the sample solution, an amount equivalent to the 10mg standard drug was weighed accurately and was dissolved in 10mL in a volumetric flask containing little amount of mobile phase and was shaken to dissolve the drug of Mercaptopurine thoroughly and the volume made up to 10mL with sample mobile phase. Then the sample solution obtained was filtered through Nylon membrane filter paper. Sample solution having Mercaptopurine concentration of 1000μg/mL was obtained was further diluted to 6μg/mL the solution was used for the formulation assay.

2.5 Degradation studies
All stress decomposition studies were carried out at a working standard drug concentration of 6μg/mL in methanol. Acid hydrolysis was carried out in 0.1 N HCl for 24h. An alkaline stress study was achieved in 0.1 N NaOH for 24 h. The studies under the oxidative conditions were accomplished at room temperature with 3.0% hydrogen peroxide for 24 h. For the studies of photo-degradation, their solutions were exposed in a chamber with 200–400 nm for 1 h, ultra-violet light for 24h. Appropriate controls were reserved in the dark. Samples were withdrawn & analyzed by using HPLC later suitable dilution. Mass balance & peak purity and were observed for every degradation condition.

2.6 Validation of the method
2.6.1 Specificity and selectivity
The specificity of the method for the drug was established by determining interference (if any) from solvent or degradation products, with a resolution of the Mercaptopurine drug peak from the adjacent resolving peak. On the whole, selectivity was fixed by determining the peak purity.

2.6.2. Linearity and range
The stock solution of Mercaptopurine was diluted with mobile phase in order to prepare solutions containing the concentration 2–12μg/mL. Each one concentration was injected 6 times into the analytical HPLC column, and the peak areas were recorded and plotted against the corresponding concentration to attain a calibration curve.

2.6.3 Precision
Injected 6μg/mL concentration solution 6 times onto the RP-HPLC column, were given on the same day, and the % RSD (6μg/mL solution of Mercaptopurine) was calculated to determine intra-day precision. Same concentration was repeatedly analyzed in three days for inter-day precision. The %RSD was calculated.

2.6.4 Accuracy
To determine the accuracy of the method carried out studies on recovery by the standard addition method with three concentrations (50%, 100%, and 150%) of the spiked drug.

2.6.5 Robustness
The effects of change in one condition to another condition of the mobile phase composition, mobile phase pH and wavelength were studied in robustness parameter.

2.6.6 Limits of detection and quantification
LOD: The lowest amount of an analyte (smallest concentration of an analyte) that can be detected in a sample is known as the limit of detection.

LOQ: The lowest amount of an analyte (smallest concentration of an analyte) in a sample that can be quantitated with allowable accuracy & precision under

the declared operational conditions is the limit of quantification. According to the ICH guidelines detection limit (LOD) and quantification limit (LOQ) were calculated.

2.6.7 Analysis of Mercaptopurine in dosage form

The formulation solution prepared using MERCAPTO© (50mg) was analyzed in the optimized conditions and the peak area obtained was compared with standard and the %assay was calculated.

3. RESULTS AND DISCUSSION

In order to validate an effective method for analysis of drug (Mercaptopurine) in a pharmaceutical product, preliminary tests were performed with the target to pick out adequate and optimum conditions. Parameters, like an ideal mobile phase and their proportions, wavelength detection, standard

concentration solutions & optimized pH were thoroughly studied and therefore the rate of flow of 1 mL/min was chosen after preliminary tests. Developed a stability indicating assay method was optimized for the quantitative determination of Mercaptopurine (6-MP) in pharmaceutical formulations. Pure drug solution of 6-MP and also the degraded products were injected by employing rheodyne manual sample injector into the HPLC system and run in several solvent systems. at first methyl alcohol, acetonitrile and water in varying ratios were tried on Kromasil C18 (250mm x 4.6mm, 5μm) column. Finally, the mobile phase made up of Methanol: Acetonitrile: water in the ratio of 50:15:35 (v/v/v) at a rate of flow of 0.9 mL/min. UV detection at a wavelength of 217nm provided a sharp, obvious and well-defined symmetrical peak at Rt = 6.13min as shown in figure 2.
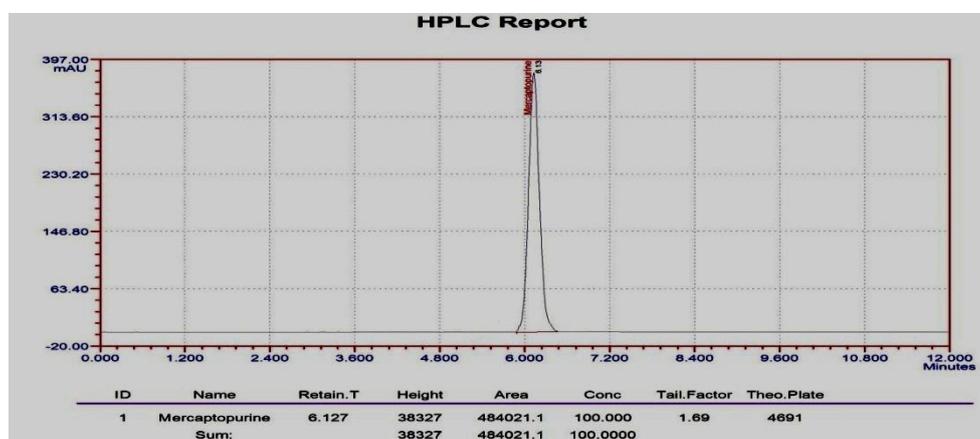

Figure 2: Standard chromatogram of Mercaptopurine

ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1	Mercaptopurine	6.127	38327	484021.1	100.000	1.69	4691
	Sum:		38327	484021.1	100.0000		

The linear regression data of the proposed method for the calibration curves (n = 3) of Mercaptopurine at 217nm (Table 1) explain a good linear relationship over a concentration range of 2–12μg/mL with respect to the peak area. No important variation was discerned in the slopes of standard curves. The observed equation of the straight line was found as; $40040x + 24469$ with R^2-value above 0.9993 (Figure 3). Linearity results showed over the concentration range of Mercaptopurine and these linearity results suggested the intensity of the detector response is proportional to the concentration of the analyzed substance (6-MP).

Table 1: Linearity results for Mercaptopurine.

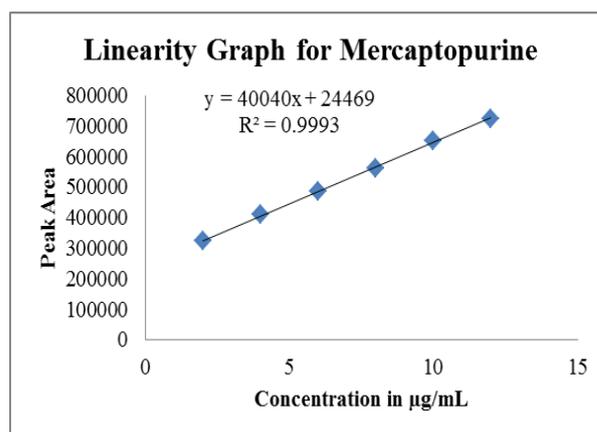| Level	Concentration in μg/mL	Peak Area
1	2	322336
2	4	408679
3	6	484021
4	8	561810
5	10	651181
6	12	721835
Slope: 40040		
Intercept: + 24469		
Correlation Coefficient: 0.9993		

Figure 3: Linear calibration curve for Mercaptopurine.

Mercaptopurine peak area in intraday precision and Interday precision studies shown low values of %RSD 0.478 and 0.517 for intra-day precision & inter-day precisions indicates an outstanding precision of the proposed HPLC method. When mobile phase components were purposely changed as much as ±5mL each, practically no important difference was observed in

the area & retention time (Rt) and no effect was observed in the chromatogram. There was no important distinction in the retention time but a little increase in the area of the chromatogram was identified when pH of the mobile phase was decreased in order 0.1 units and a slight increase in the area of the chromatogram was observed when pH of the mobile phase was increased in 0.1. The

change in detector response doesn't influence the elution time and peak area response was noticed. The low values of % change (table 2) along with almost unchanged Rt values of Mercaptopurine obtained after introducing small deliberate changes as mentioned above in the method indicated the robustness of the developed HPLC method.

Table 2: Robustness results.

S. No.	Parameters changes	Change	Peak Area	% Change
1.	Optimized	--	484021	--
2.	WL-change-1	214nm	481171	0.589
3.	WL-change-2	220nm	483843	0.037
4.	MP-change-1	Methanol: Acetonitrile: water 45:34:20 (v/v/v)	480990	0.626
5.	MP-change-2	Methanol: Acetonitrile: water 35:45:20 (v/v/v)	486705	0.554
6.	MP pH Change-1	6.0	485711	0.349
7.	MP pH Change-2	6.2	484979	0.198

The accuracy of the proposed HPLC assay method was assessed in triplicate at 3 concentration levels 6, 8 and 10 μg/mL, i.e. (50, 100 and 150%). The percentage of RSD & recoveries percentage was calculated from the slope and y-intercept of the calibration curve obtained. The

recovery values attained were between the range of 98.361% - 100.776% (Table 3). The %RSD was found to be 0.524, 0.750 and 0.447 for 50, 100 and 150% spiked levels ascertaining the accuracy of the developed HPLC method respectively.

Table 3: Recovery results.

Level	Target In μg/mL	Amount of spiked (μg/mL)	Total in μg/mL	Amount of Recovered (μg/mL)	% Recovery	RSD of recovery
50 %	4	2	6	5.985	99.754	**0.524**
	4	2	6	5.955	99.247	
	4	2	6	6.018	100.293	
100%	4	4	8	7.944	99.298	**0.750**
	4	4	8	8.021	100.263	
	4	4	8	8.062	100.776	
150%	4	6	10	9.919	99.192	**0.447**
	4	6	10	9.903	99.034	
	4	6	10	9.836	98.361	

LOD and LOQ and were observed to be 0.006 μg/mL and 0.020 μg/mL, respectively, which indicated the appropriate sensitivity of the HPLC method. The prepared 6 μg/mL pharmaceutical formulation solution was analyzed in the developed method. 5.977μg/mL was

recovered in the formulation solution. The %assay in formulation analysis was observed to be 99.625%. Therefore the proposed HPLC method was established to be appropriate for the estimation of Mercaptopurine in pharmaceutical formulations.

Table 4: Formulation analysis result.

S. No.	Brand Name	Form	Dosage	Amount Prepared	Amount Found	% Assay
1.	MERCAPTO©	Tablet	50mg	6μg/mL	5.977μg/mL	99.625

The chromatograms of the samples treated with acid (0.1 N HCl), base (0.1 N NaOH), H_2O_2, dry heat (80°C), and UV-light (254 nm), illustrated well-separated peaks of pure Mercaptopurine, in addition, some additional peaks at totally different Rt-values. Degradants identification relied on the comparison of UV-spectra of "stressed samples" with that of the "standard solution". Peaks of the degradation products have been properly separated from the Mercaptopurine drug peak and also the number of degradation products with their Rt values (Fig.3). The

number of degradation products with their Rt-values, the content of Mercaptopurine remaining, and calculated percentage recovery are listed in Table 5. Summary of validation results was presented in table 6.

Table 5: Forced Degradation results.

S. No.	Condition	No of additional peaks observed	Peak Area	% Obtained	% degradation
1.	Standard [6μg/mL]	--	484021	100	--
2.	Acid	3	457347	94.489	5.511
3.	Base	1	467913	96.672	3.328
4.	Light	2	464872	96.044	3.956
5.	Peroxide	1	475857	98.313	1.687
6.	Thermal	2	470358	97.177	2.823
7.	UV	2	421976	87.181	12.819

Drug recovery at the level of 94.489% and 96.672% from the acid (Figure 4) and base (Figure 5) stressed samples, respectively, implies an important degradation of the drug in acidic and basic conditions (hydrolysis condition). The degradation products peaks were well separated from the Mercaptopurine drug peak. The chromatogram of the sample treated with 3.0% (v/v) H_2O_2 showed only one additional peak at Rt, 5.65min. The area of the H_2O_2 degradation product peak was significant and accountable for 98.313% recovery of Mercaptopurine from the H_2O_2-stressed sample (Figure 6). The chromatogram of the dry-heat stressed sample (Figure 7) shows the appearance of 2 additional peaks at Rt 5.64 and 7.50min and showed 97.177% recovery of Mercaptopurine. Drug recovery at the level of 87.181% and 96.044 % were observed for the UV light and light-exposed sample (Figure 8) and (Figure 9) respectively, shows that the drug is unstable more toward the UV light and light stressed degradation.
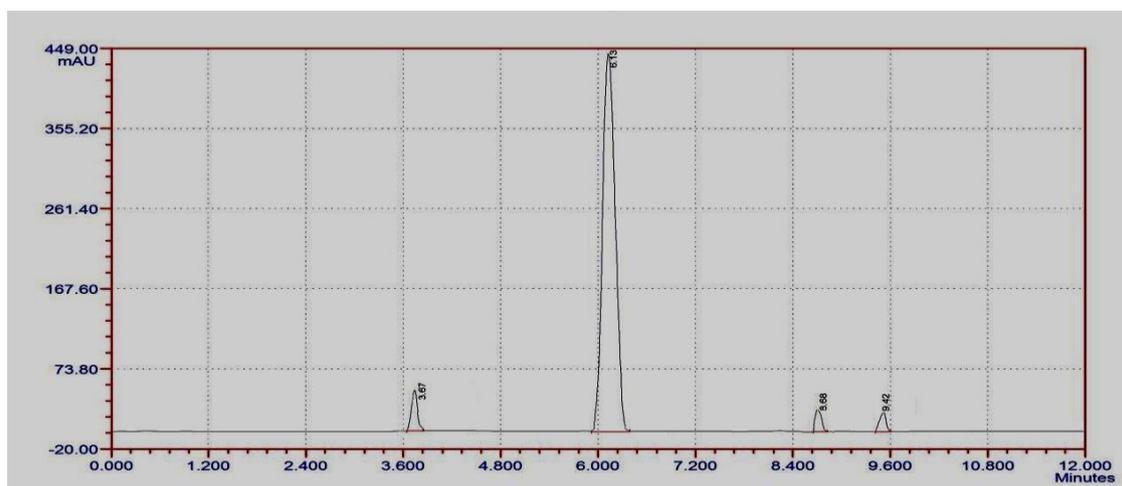
Fig 4: Acid stress degradation chromatogram.
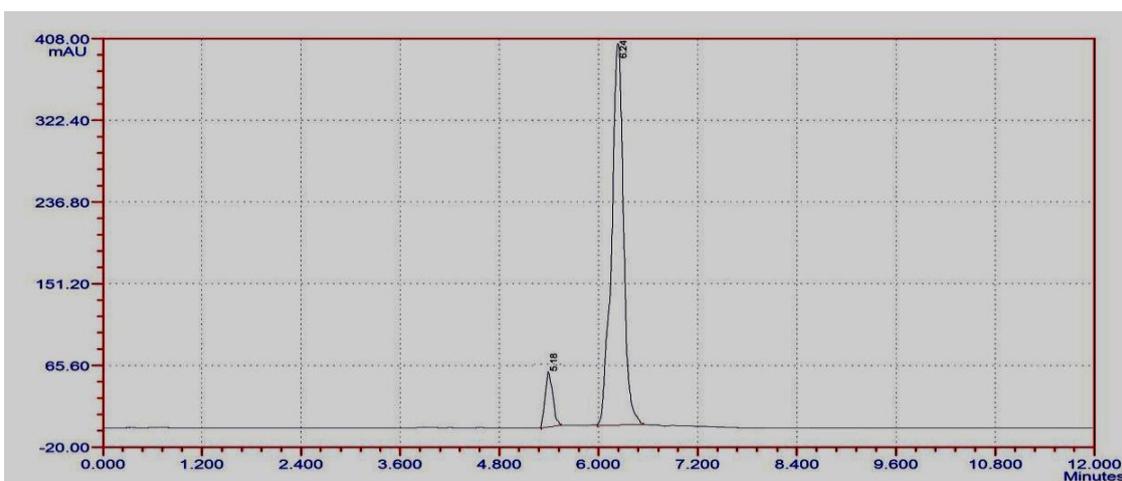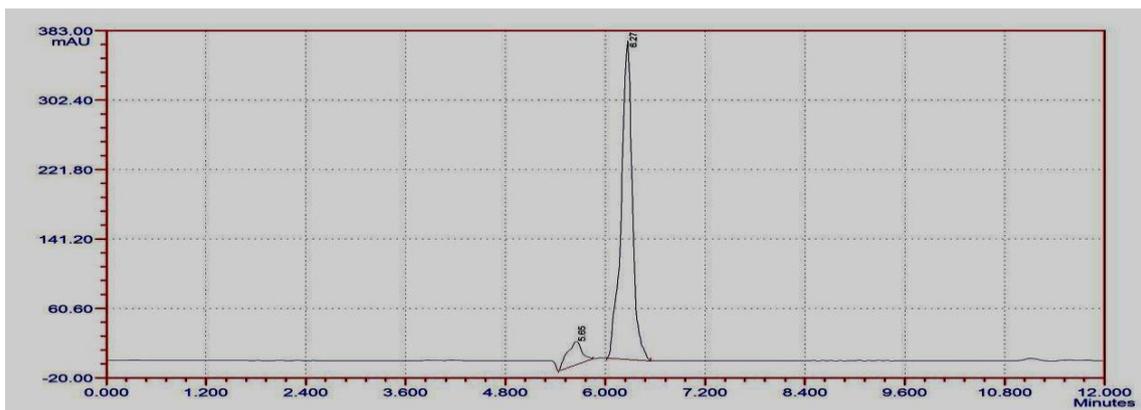

Fig 5: Base stress degradation chromatogram.

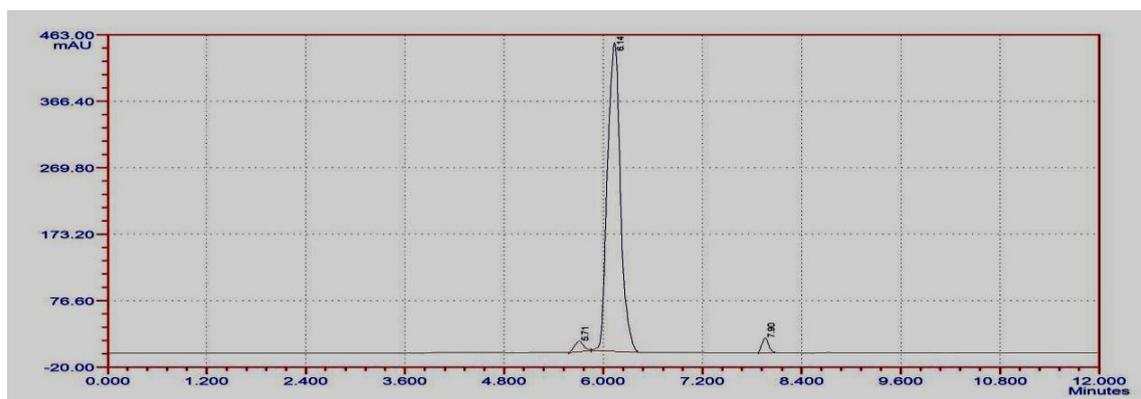**Fig 6: Peroxide stress degradation chromatogram.**

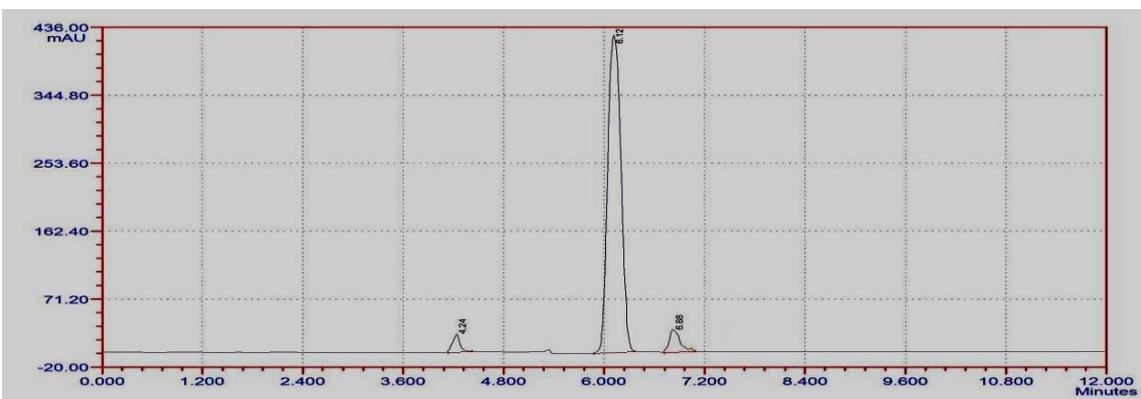**Fig 7: Thermal stress degradation chromatogram.**

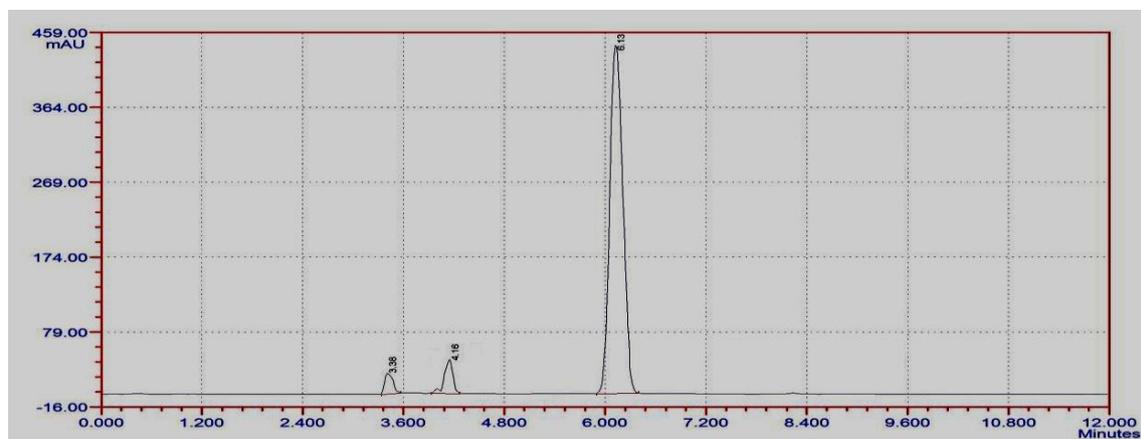**Fig 8: UV light stress degradation chromatogram.**

Fig 9: Light stress degradation chromatogram.

Table 6: Summary of validation.

S. No	Validation Parameter	Result
1.	Tailing factor	1.69
2.	Theoretical plates	4691
3.	Linearity	2-12µg/mL
4.	Intraday precision (%RSD)	0.478
5.	Interday precision (%RSD)	0.517
6.	Ruggedness (%RSD)	0.792
7.	LOD	0.006µg/mL
8.	LOQ	0.020µg/mL

4. CONCLUSION

The developed HPLC method (proposed HPLC method) was validated in respect of accuracy, precision and robustness. An excellent linear relationship was ascertained for Mercaptopurine in the concentration ranges of 2–12µg/mL. The correlation coefficient (R^2) was found to be 0.9993. The intra-day precision (0.478) & inter-day precision (0.517) results were adequate to elucidate that the developed HPLC method was precise and reproducible. Preparation of samples was easy and efficient. UV detection at 217 nm was found to be appropriate. The assay experiment illustrated that the contents of Mercaptopurine (6-MP) determined in the tablet dosage form free from the interference of excipients. This proven that the proposed HPLC method was simple, fast as indicated through short retention time, sensitive, accurate, precise, efficient & could be appropriate for the regular routine quality control (Q.C) analysis of Mercaptopurine from its bulk drug & pharmaceutical dosage forms. The forced degradation studies results exhibit that the developed HPLC method is stability indicating.

5. ACKNOWLEDGMENT

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