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Development And Validation Of A Stability-Indicating Rp-Hplc Method For The Estimation Of Mercaptopurine In Pharmaceutical Formulation

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ABSTRACT

A stability-indicating reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed to assay Mercaptopurine and assess its stability in pharmaceutical formulations. Chromatographic separation was achieved using a YMC ODS AQ C18 (100 mm x 4.6 mm, 3µm) at 35 °C using a gradient mobile phase composed of 1 mL formic acid in 1000 mL water (A) and Methanol (B), with detection at 325 nm, and with a 5 µL injection volume. Mercaptopurine and its degradation products were detected at 260 nm. The method was validated as per ICH guidelines and demonstrated linearity in the range of 5 – 150 µg/mL (correlation coefficient: 0.9999) with good accuracy (101.4 – 101.6 % recovery) and precision (% RSD < 2.0 %). Forced degradation studies showed significant degradation on exposure to alkaline, photolytic, and peroxide oxidative stress, but no degradation was observed on exposure to acidic and thermal stress. Thus, the method was sensitive, specific, and suitable for stability testing of Mercaptopurine in pharmaceutical formulations.

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1. INTRODUCTION:

Mercaptopurine is a crucial medication for the treatment of immune system-related illnesses as well as tumours including leukaemia and lymphoma. It works by preventing purine production in cancer cells, but its usage necessitates close observation for adverse effects and individual differences in drug metabolism. Mercaptopurine belongs to a group of drugs known as purine antagonists.

Mercaptopurine is a chemotherapeutic medication that is mostly used to treat leukaemia and other cancers. It is categorized as an antimetabolite, which means that it disrupts the regular metabolic functions of cells, particularly those that divide quickly, like cancer cells. Inhibiting the synthesis of purines, which are necessary building blocks for DNA and RNA, is how Mercaptopurine functions. In the end, this activity stops cancer cells from growing and proliferating, which kills them.

Although Mercaptopurine is typically taken orally as pills, it can occasionally be given intravenously. The type of cancer or illness being treated, as well as the patient's reaction to the medication, determines the dosage.

In an attempt to create leukaemia treatments that worked, Mercaptopurine was initially produced in the 1950s. It was among the first chemotherapeutic substances designed to interfere with the creation of DNA and RNA in cells. Additionally, it was

among the first medications to be categorized as an antimetabolite, opening the door for other medications in the similar family. Mercaptopurine has since emerged as a crucial element in the management of leukaemia and other malignancies. [1-4] Figure 1 shows the chemical structures of Mercaptopurine.

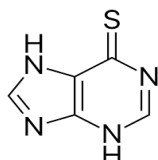


Figure 1. Chemical Structure of Mercaptopurine

A literature survey revealed that few chromatographic methods are reported for the determination 6-Mercaptopurine in presence of its metabolites. RP-HPLC [5] method for the estimation of 6-mercaptopurine and its metabolites in bulk and pharmaceutical formulations, LC-MS/MS [6] assay for the simultaneous determination of methotrexate, 6-mercaptopurine and its active metabolite 6-thioguanine in plasma of children with acute lymphoblastic leukaemia, RP-HPLC [7] method for the estimation of 6-mercaptopurine in spiked human plasma and pharmaceutical formulations, RP-HPLC [8] method for the estimation of 6-mercaptopurine in rat plasma and various tissue homogenates, HPLC [9] method for the rapid and simultaneous determination of 6-mercaptopurine and four of its metabolites in plasma and red blood cells.

To the best of our knowledge, no HPLC technique has been created for the determination of the Mercaptopurine. Therefore, it was decided to create an estimation of Mercaptopurine.

2. MATERIALS AND METHODS:

2.1 Chemical and reagents:

Mercaptopurine API was received by Fermion. Merck provided the methanol. Sigma-Aldrich provided the HPLC-grade formic acid. The Milli-Q system supplied the HPLC-grade water that was used.

2.2 Instrumentation:

The HPLC system (Waters Instrument) with a PDA detector and a data station running Lab Solution software. UV spectra were recorded using UV – Vis Spectrophotometer (Shimadzu, UV-1900I). A water purification system (Merck, Milli-Q DQ 5) was used to obtain Type-I water. Semi-micro balance (Sartorius, Cubis MCA3.6P20-IN-M) was used for weighing samples. A digital ultrasonicator (Labman LMUC-6) facilitated the sonication of the sample.

2.3 Chromatographic conditions:

The Separation was achieved on YMC ODS AQ C18, 100 mm x 4.6 mm, 3µm. The mobile phase consisted of 1 mL formic acid diluted in 1000 mL Milli-Q water (mobile phase A) and Methanol (mobile phase B) at a flow rate of 1.0 mL/min. The Gradient program is presented in Table 1. The Injection volume was 5 µL, detection was performed at 325 nm, and the column temperature was maintained at 35°C.

Table 1. Gradient elution program for separation of Mercaptopurine

Time (Min)	Mobile Phase A (%)	Mobile Phase B (%)
0.00	98	2
10.00	98	2
15.00	25	75
20.00	25	75
20.01	98	2
30.00	98	2

2.4 Preparation of Calibration Standard:

250 mg of Mercaptopurine was accurately weighed, and transferred to 250 mL volumetric flask. Add 5 ml of Dimethyl sulfoxide sonicated to dissolve. Add 15 mL of Methanol and mix well. Further, make up to the mark with Mobile Phase A and mix (Stock Solution: 1000 µg/mL) and appropriately diluted to obtain calibration standards having a concentration of 5, 50, 80, 100, 115, and 150 µg/mL of Mercaptopurine.

2.5 Forced degradation studies:

Forced degradation studies (stress testing) were performed to evaluate Mercaptopurine susceptibility to degradation and demonstrate the specificity of the developed analytical method. [10, 11] Mercaptopurine was subjected to acid, alkali, and oxidative stress by adding 5 N HCl, 5 N NaOH at 65°C for 4 hours, and 3 % H₂O₂, 65°C for 4 hours, respectively. Additionally, in the thermal degradation study, the volumetric flask was kept at 65°C for 4 hr. Photo stress degradation was assessed by exposing the sample under a photo stability chamber for 1 cycle.

2.6 Method validation:

The ICH Q2 (R1) recommendations were followed in the validation of the established RP-HPLC technique. [12]

2.6.1 System suitability:

To evaluate system suitability, five replicate injections of the Mercaptopurine solution was examined using RP-HPLC. System suitability was deemed acceptable if the relative standard deviation was not more than 2.0 percent, the tailing factor was below 2.0, and the number of theoretical plates for the Mercaptopurine was at least 2000.

2.6.2 Specificity:

The specificity of the method was determined by comparing the chromatograms obtained by injecting the diluent (blank), Mercaptopurine standard, forced degradation samples, as well as the placebo and Mercaptopurine formulation samples. Mercaptopurine peak purity in forced degradation samples was evaluated using a photodiode array detector between 200 – 400 nm.

2.6.3 Linearity:

Linearity was evaluated using six calibration standard concentrations ranging from 5 to 150 µg/mL, prepared in triplicate. Calibration curves were obtained by plotting the Mercaptopurine average area versus the concentration expressed as µg/mL. The Correlation coefficient was calculated to determine the linearity of the method.

2.6.4 Precision:

The precision, defined as the closeness of agreement (degree of scatter) between a series of measurements (measured values) obtained from multiple sampling of the same homogeneous sample under the prescribed conditions, was evaluated at three levels: repeatability (intra-day precision), intermediate precision (inter-day precision) and reproducibility (inter-laboratory precision) [23,27]. Intra-day and inter-day precision were calculated as the relative standard deviation (% RSD) between three replicates of Mercaptopurine quality control (QC) standards containing 100 µg/mL of Mercaptopurine within the same day and on three consecutive days, respectively.

2.6.5 Solution stability:

Sample solution of Mercaptopurine and its known impurities were prepared and stored at room temperature (25°C). The sample solution was analysed over a chromatographic time interval of 48 hr.

2.6.6 Accuracy:

The accuracy, defined as the closeness of agreement between the reference or theoretical concentration (true value) and the measured concentration (value obtained) [23], was determined by spiking Mercaptopurine at three concentrations (50, 100 and 150 µg/mL). Each sample was prepared in triplicate and analyzed for drug recovery.

2.6.7 Robustness:

The Method's robustness was investigated by varying the experimental conditions and evaluating their impact on the chromatographic separation and relative retention times of the analytes. Small

deliberate adjustments were made to different chromatographic conditions, including column temperature ($\pm 2^\circ\text{C}$), flow rate ($\pm 0.1\text{ mL/min}$), and Wavelength ($\pm 1\text{ nm}$).

3. RESULT AND DISCUSSION**3.1 Method Development**

To determine the appropriate wavelength for the analysis of Mercaptopurine, the solutions of Mercaptopurine was scanned in the wavelength range of 200–400 nm. The detection wavelength of 325 nm was chosen based on the overlapping UV spectra from these solutions. (Figure 2).

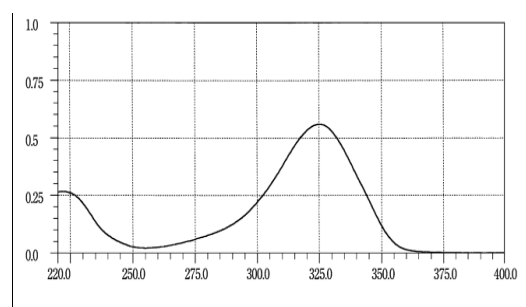


Figure 2. UV spectra of Mercaptopurine

The chromatographic conditions were also optimized, specifically the diluent, and mobile phase composition, to achieve proper peak shape for Mercaptopurine. Preliminary attempts at baseline separation of Mercaptopurine by isocratic elution using various mobile phases consisting of water with methanol were unsuccessful. To elute Mercaptopurine, the pH of the mobile phase was adjusted using formic acid, and a gradient elution procedure was used. Various mobile phase compositions were used to elute Mercaptopurine. Mobile phase A was prepared by dissolving 1 ml of formic acid into 1000 ml of Milli-Q water while mobile phase B consisted of methanol. The combination yielded sharp and resolved peaks of Mercaptopurine, with the retention time for Mercaptopurine around 5.3 min. The optimised chromatographic conditions for Mercaptopurine are depicted in Figure 3, with a summary of the system suitability results provided in Table 2.

Table 2: System suitability data

Parameters	Mercaptopurine	USP Standard
Relative standard Deviation	0.1%	Not more than 2 %.
Theoretical plates (N)	7075	Not less than 3000
Tailing factor	1.4	Not more than 2

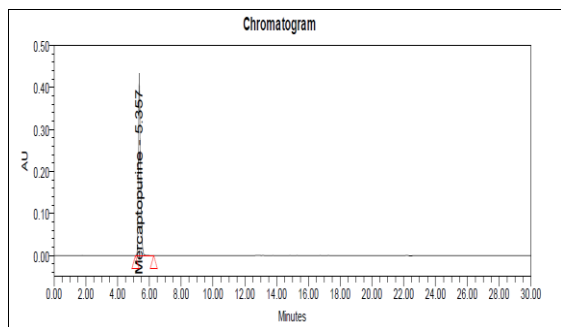


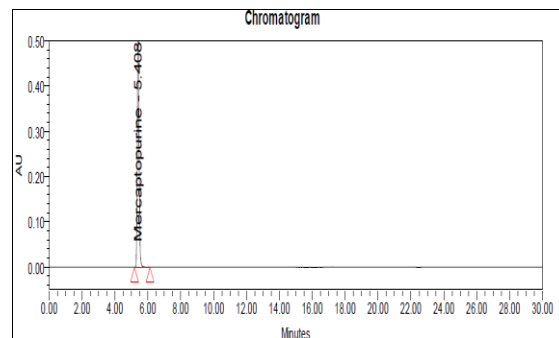
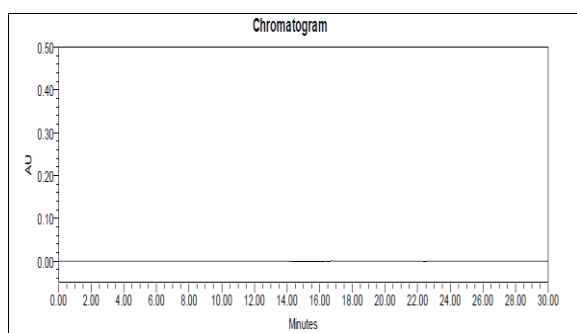
Figure 3. Optimized chromatogram of Mercaptopurine

3.2 Forced degradation studies

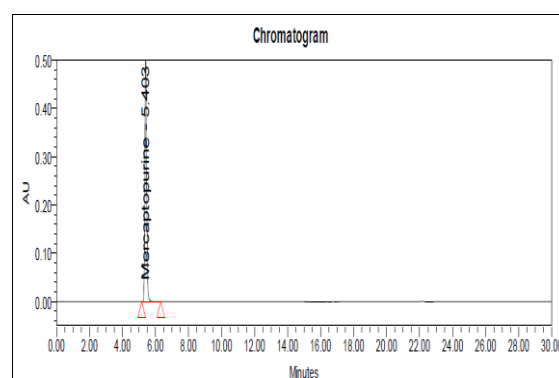
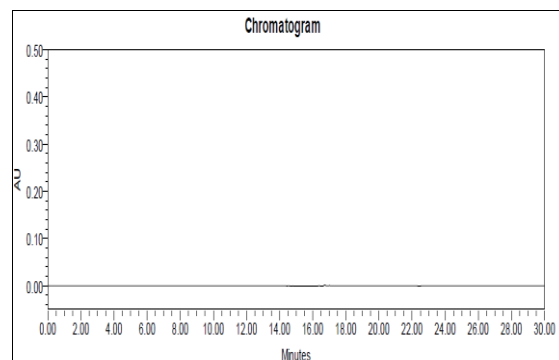
Forced degradation was evaluated on Mercaptopurine to demonstrate the specificity of the method, and the results provided in Table 3. The results indicated that Mercaptopurine remained stable under acid and thermal stress conditions with no observed potential degradation products or impurity interferences. However, during Basic, Peroxide, and Photolytic degradation, Mercaptopurine decomposed by 1.0, 2.0, 4.1, 6.1, and 10.2%, respectively.

Table 3: Stress degradation study data

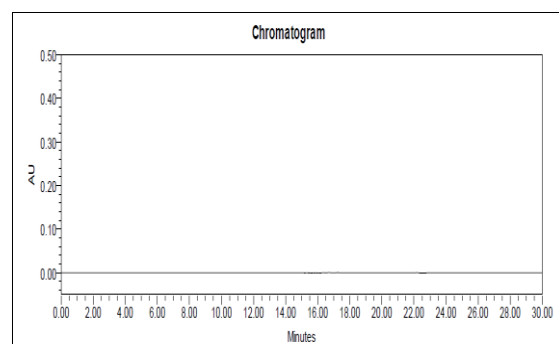
Stress Type	% Assay	Purity angle of Mercaptopurine Peak	Purity Threshold of Mercaptopurine Peak
As Such	99.2	0.033	0.233
Acid Hydrolysis	100.2	0.022	0.243
Alkali Hydrolysis	94.9	0.030	0.242
Peroxide Oxidation	93.1	0.048	0.272
Thermal Degradation	101.7	0.030	0.242
Photolytic Degradation	92.6	0.042	0.280

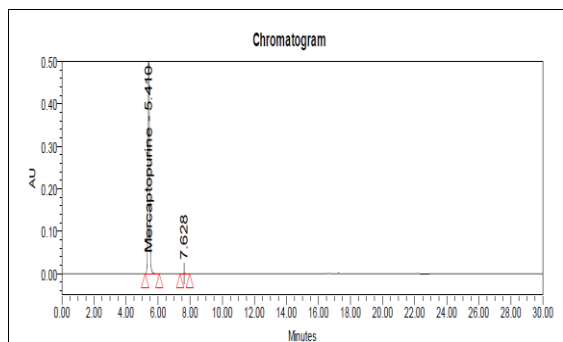


(a)

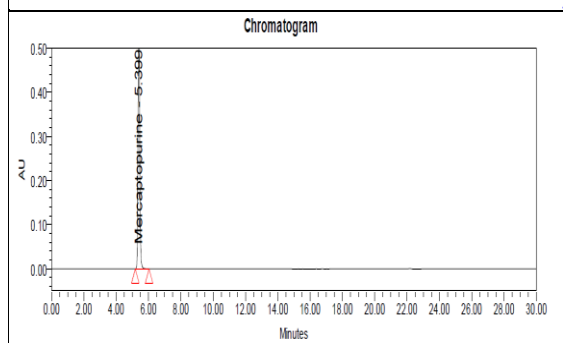
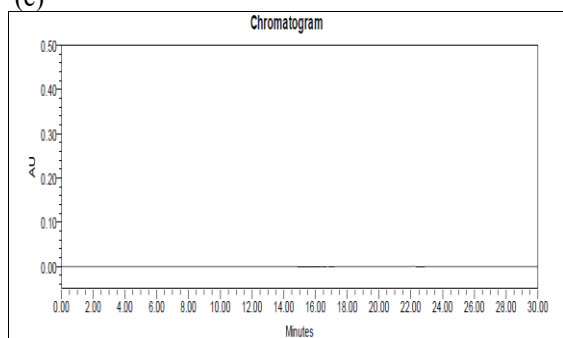


(b)

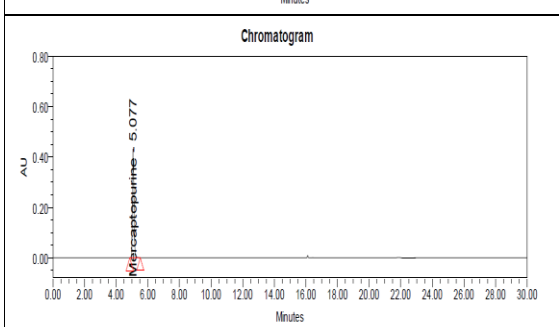
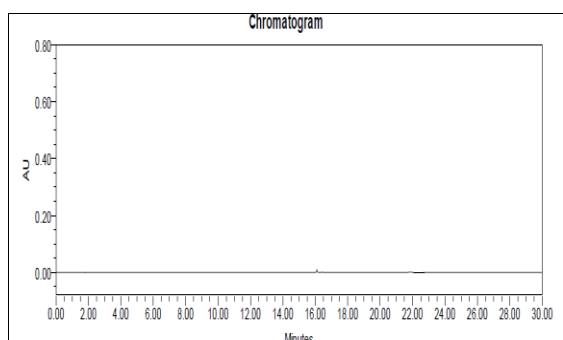




(c)



(d)



(e)

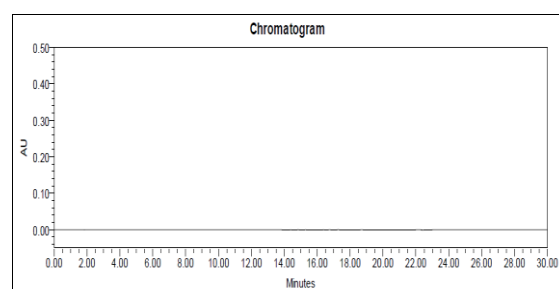
Figure 4: Forced Degradation Chromatograms of Placebo

and Sample (a) Acid degradation (b) Alkali Degradation (c) Peroxide Degradation (d) Thermal Degradation (e) Photolytic degradation

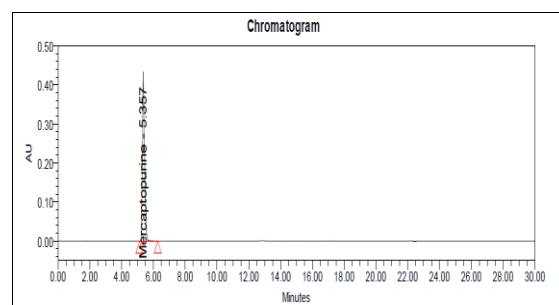
3.3 Method Validation

3.3.1 Specificity

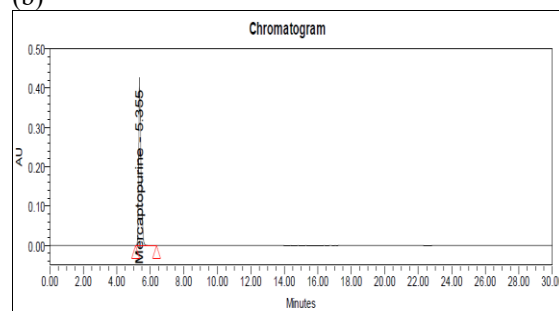
The chromatograms in Figure 5 demonstrate that Mercaptopurine peak was effectively separated. The results of the blank solvent interference experiment indicate that the Mercaptopurine peak is free from diluent interference. No interferences were observed with degradation products, and the main peak of Mercaptopurine.



(a)



(b)



(c)

Figure 5. Chromatograms of blank solution (a), standard solution (b) and sample solution (c)

3.3.2 Linearity

As presented in Table 4 and 5, the determination coefficients obtained from linear regression analysis exceeded 0.999 for each calibration curve, indicating excellent correlation and strong linearity for the method. Figures 6 and 7 display the acquired results.

Table 4: Linearity data for Mercaptopurine

Concentration (µg/mL)	Mean Area ± SD (n=3)	% RSD
5	202029 ± 557.2001	0.27
50	2050953 ± 6859.643	0.33
85	3421452 ± 16196.28	0.47
100	4030072 ± 4193.85	0.10
120	4875142 ± 30044.97	0.61
150	6014253 ± 14770.75	0.24

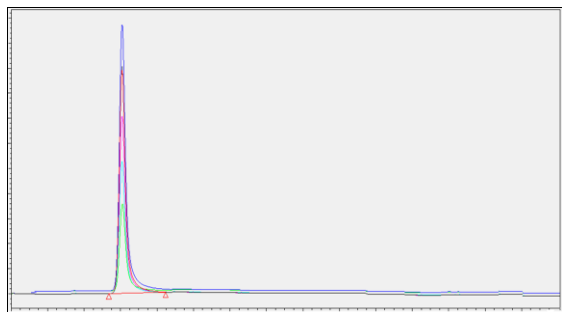


Figure 6. Overlain chromatogram of Mercaptopurine

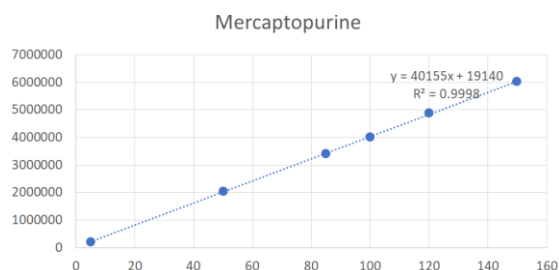


Figure 7. Calibration curve of Mercaptopurine

Table 5: Calibration curve analysis using linear regression data

Parameters	Mercaptopurine
Linearity range	5-150 µg/mL
Slope	40155
Intercept	19140
Correlation coefficient(r ²)	0.9998

3.3.3 Precision

The overall %RSD for both Repeatability and intermediate precision of the proposed method was less than 2% (see Table 6 and 7).

Table 6: Repeatability data for Mercaptopurine

Conc. (µg/mL)	Mean Peak Area (n=6) ± SD	% RSD
100	4042392 ± 17423.11	0.43

Table 7. Intermediate precision data for Mercaptopurine

Concentration (µg/mL)	Intraday Precision	Inter day Precision
	Mean±S.D (n=3), %RSD	Mean±S.D (n=3), %RSD
5	201832 ± 278.6001, 0.13	202771 ± 1050.054, 0.51
100	2048528 ± 3429.468, 0.16	2048095 ± 4042.529, 0.19
150	6019476 ± 7385.73, 0.12	6023334 ± 12841.77, 0.21

3.3.4 Solution Stability

The Stability of the impurities was evaluated using system suitability solutions and the spike solution at room temperature (25°C) for 48 h. The stability of suitability solutions was analysed at 0, 2, 8, 12, 24, and 48 h. No significant changes were observed in peak area with the %RSD of less than 2.0%. The result indicates that it is feasible to analyse samples within a 48 h timeframe.

3.3.5 Accuracy

The accuracy of this method was measured through recovery experiments and is expressed as the “recovery rate.” At three concentration levels (50%, 100% and 150%) for Mercaptopurine, the recovery rates were determined to be within an acceptable range. (Table 8).

Table 8: Accuracy Results

Sr. No.	Name of Product	50% level (n=3)	100% level (n=3)	150% level (n=3)
1	Mercaptopurine	101.4	101.5	101.6

3.3.6 Robustness

The robustness of the method was verified by intentionally changing chromatographic parameters, including column temperature (± 2°C), flow rate (± 0.1 mL/min), and Wavelength (± 1 nm). Based on these results (Table 9), the method exhibits excellent durability.

Table 9: Robustness data for Mercaptopurine

Drug name	Parameters	Optimized condition	Used condition	Mean Area (n=3)	Rt (min) (n=3)	% Assay (n=3)
Mercaptopurine (µg/mL)	Flow rate (±0.1mL/min)	1 mL/min	0.9 mL/min	4039867	5.348	98.7
			1.0 mL/min	4044389	5.402	99.0
			1.1 mL/min	4050404	5.439	98.4
			Avg	4044887	5.396	98.7
			SD	5286.099	0.04	0.3
			%RSD	0.13	0.84	0.3
	Wavelength (± 1 nm)	325	324	4033193	5.408	98.7
			325	4044389	5.402	99.0
			326	4049361	5.382	98.8
			Avg	4042314	5.390	98.8
			SD	8281.258	0.01	0.15

	Column oven temperature ($\pm 2^\circ\text{C}$)	35	%RSD	0.20	0.25	0.15
			33	4045443	5.41	99.1
			35	4044389	5.402	99.0
			37	4035625	5.382	98.8
			Avg	4041819	5.398	98.9
			SD	5389.987	0.01	0.15
			%RSD	0.13	0.26	0.15

Table 9: Summary of validation parameters

Parameters	Mercaptopurine
Linearity range (n=3)	5-150 $\mu\text{g/mL}$
$y = mx + c$	$y = 40155x + 19140$
Correlation coefficient(r^2)	0.9998
Precision (%RSD)	Repeatability
	Intraday
	Inter day
Accuracy (%Recovery)	101.4-101.6
Specificity	Specific
Robustness	Robust

4.0 APPLICATION OF THE METHOD

The developed RP-HPLC method was utilized to analyse the Mercaptopurine Oral suspension formulation. This method is suitable for both qualitative and quantitative determination of Mercaptopurine in actual commercial samples.

5. CONCLUSION

In summary, a gradient liquid chromatography method was developed to examine Mercaptopurine. The method's selectivity, precision, accuracy, Linearity, and robustness were confirmed by validation tests. Forced Degradation studies were conducted to determine the specificity of the degrading impurities. It was observed that the formation of decomposition products, indicating its instability and susceptibility to degradation in acidic, Basic, Peroxide, and Photolytic degradation. This chromatographic method can be employed for analysing Mercaptopurine in Mercaptopurine oral suspension. Furthermore, the optimized HPLC method can also be conveniently utilized for stability analysis.

6.0 ACKNOWLEDGEMENT

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