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Original Article

VALIDATED STABILITY-INDICATING RP-HPLC ASSAY METHOD FOR AZATHIOPRINE IN PHARMACEUTICAL DOSAGE FORM ACCORDING TO ICH GUIDELINES

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ABSTRACT

Objective: A simple, sensitive, precise and accurate stability-indicating HPLC method has been developed and validated for determination of Azathioprine in bulk drug and in pharmaceutical dosage form in the presence of degradation products.

Methods: An isocratic, reversed phase HPLC method was developed to separate the drug from the degradation products, using a Hypersil C_8, 150 X 4.6 mm, 5µ, Thermostat column compartment connected with Waters (alliance) Empower software. Mobile phase consists mixture of Potassium dihydrogen phosphate(A) and Acetonitrile(B) in the ratio (pH 6.50±0.1, ratio 60:40 v/v) at a flow rate of 1.0 mL /min. The linear regression analysis data for the calibration curve showed a good linear relationship with regression coefficient 1. The detection was carried out at a wavelength of 254 nm.

Results: The linearity of the method were excellent over the range 2.5-15 µg/ml, the linear regression equation was Y=76621×+12515. The Azathioprine was subjected to stress conditions of hydrolysis (acid, base), photolysis and thermal degradation. Degradation was observed for Azathioprine in acid, base, heat and UV. The degradation products were well resolved from the main peak. The percentage recovery of Azathioprine was from (98.0 to 102.0 %.) in the pharmaceutical dosage form.

Conclusion: The results demonstrated that the method would have a great value when applied in quality control and stability studies. The developed method was validated with respect to linearity, accuracy (recovery), precision, system suitability, specificity and robustness. The forced degradation studies prove the stability indicating power of the method.

Keywords: Azathioprine, Assay HPLC, Validation, Stability-indicating, Degradation profile.

INTRODUCTION

Azathioprine is an immunosuppressive drug used in organ transplantation and autoimmune diseases and belongs to the chemical class of purine analogues.[1] Synthesized originally as a cancer drug and a prodrug formercaptopurine in 1957, it has been widely used as an immunosuppressant for more than 50 years.[2] Azathioprine acts as a pro drug for mercaptopurine, inhibiting an enzyme that is required for the synthesis of DNA. Thus it most strongly affects proliferating cells, such as the T cells and B cells of the immune system.[3]Azathioprine, is chemically 6-(1-methyl-4-nitroimidazole-5-yl) thiopurine (Figure- 1), is an immunosuppressive agent. They are used in the chemotherapy of acute leukemia, for immunosuppressant after solid-organ transplantation, and increasingly for immunomodulation in autoimmune disease. Currently, AZA is standard treatment in patients with chronically active inflammatory bowel disease (IBD). Azathioprine acts on several modes in cellular immunity processes. It inhibits lymphocyte activation, lymphocyte differentiation, in vitro lymphocyte stimulation, and in vitromixed lymphocyte reaction and it reduces the activity of natural killer lymphocytes.[4, 5] It is also used in systemic anti-inflammatory states, such as rheumatoid arthritis, lupuserythematosus, colitis ulcerosa, auto immunological hepatitis and Crohn's disease [6]. And immunosuppressive action, and is given orally or by intravenous (IV) route [7]. A validated UPLC method for the determination of process-related impurities in Azathioprine bulk drug [8]. In the United State Pharmacopoeia monograph of Azathioprine API, thin layer chromatographic test is described for 6-mercaptopurine [9].

Administration of Azathioprine in transplant patients leads rapidly to severe my-elosuppression when the homozygote allele for TPMTdeficiency is expressed [15]. Then, high 6-TGN concentrations in RBCs could be correlated with low erythrocyteTPMT activity, which exhibits a favorable clinical out-come in children with acute lymphoblastic leukemia treated by6-MP [16]. However, the relation between high 6-TGN concentrations in RBCs and myelosuppressioninduced by Azathioprine remains controversial [17]. More recently, Me6-TIMP was shown to strongly in-hibit purine de novo synthesis in a concentration-depen-dent manner, leading to cytotoxicity in Molt F4 cells, suggesting that methyl 6-mercaptopurine nucleotides (Me6-MPNs) could explain part of the pharmacologic activity of azathioprine [18]. A few analytical methods LC-MS, GC-MS were reported in literature and estimated the drug levels in blood plasma [11, 12]. Azathioprine and the metabolite 6-MP are moderately bound to serum proteins (30%). Azathioprine and 6-MP are structurally very similar, differing only in that azathioprine has a methyl–nitro-imidazolyl group attached to the sulfur atom at the 6-position of the purine ring of 6-MP [30]. On the average, 47% of an orally administered dose of azathioprine is available to the systemic circulation. A number of analytical methods have been used to determine the azathioprine with 6-mercaptopurine. It involves mainly high performance liquid chromatography [31]. And very few liquid chromatography tandem mass spectrometry methods [32]. Have been developed for the quantification in biological samples of plasma [33, 34&35].

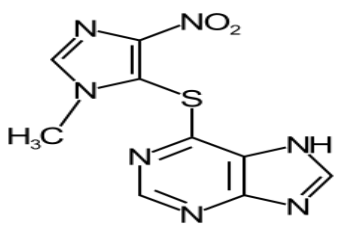

Fig. 1: Chemical structure of Azathioprine

The author has developed RP-HPLC method based on the use of symmetry column, without use of an internal standard. An attempt has been made to develop and validate all methods to ensure their

accuracy, precision, repeatability, reproducibility and other analytical method validation parameters as mentioned in the various guidelines. The Chemical Structure of Azathioprine in figure.1.

However, in the knowledge authors, no account has been reported for stability indicating assay method for determination of Azathioprine. According to current good manufacturing practices, all drugs must be tested with a stability-indicating assay method before release. Till date, no stability-indicating HPLC assay method for the determination of azathioprine is available in the literature. It was felt necessary to develop a stability indicating liquid chromatography (LC) method for the determination of Azathioprine as bulk drug and pharmaceutical dosage form and separate the drugs from the degradation products under the International Conference on Harmonization (ICH) suggested conditions (hydrolysis, photolysis and thermal stress) [36&37]. Therefore, the aim of the present study was to develop and validate a stability-indicating HPLC assay method for azathioprine as bulk drug and in pharmaceutical dosage form as per ICH guidelines [37].

MATERIALS AND METHODS

Material and reagents

Azathioprine bulk drug (purity 99.7) and tablet Azathioprine (100 mg) were obtained from Life Care labs, ameerpet (Hyderabad, India). Hydrochloric acid, sodium hydroxide pellets, dihydrogen phosphates and Acetonitrile from were obtained Bross Scientifics Laboratories Tirupati, AP India. All chemicals used are of HPLC grade. Milli-QWaterwas used throughout the experiment.

Instrumentation

Waters HPLC 2 2695 series consisting pump, Auto Sampler, UV-Vis detector, Thermostat column compartment connected with Waters(alliance) Empower software Hypersil C_8, 150 X 4.6 mm, 5µ, Thermostat column compartment connected with Waters(alliance) Empower software. At a flow rate of 1.0 mL/ min. The injection volume was 20 µL. The detection wavelength was 254 nm.

Chromatographic conditions

HPLC method development parameters	
Column	C_8, 150 X 4.6 mm, 5µ
Flow rate	1.0 mL / min
Wavelength	254 nm
Column temperature	30°C
Injection volume	20 µL
Run time	10 minutes
Diluents	Mobile phase
Elution	Isocratic

Preparation of solutions

Mobile phase preparation

2.72 gms of Potassium dihydrogen phosphate in 1 Ltr water, Adjust pH 6.50±0.1 with dilute potassium hydroxide solution: Acetonitrile in the ratio 60: 40. The mobile phase was premixed and filtered through a 0.45µ nylon filter and degassed.

Preparation of stock and standard solutions

All solutions were prepared on a weight basis and solution concentrations were also measured on weight basis to avoid the use of an internal standard. Standard solution of azathioprine was prepared by dissolving the drug in the diluents and diluting them to the desired concentration. Diluents were composed of Potassium dihydrogen phosphate and Acetonitrile in the ratio (60: 40 v/v). Approximately 50 mg of azathioprine was accurately weighed, transferred in a 100 mL volumetric flask, add 30 mL of diluents and sonicate to dissolve and dilute to volume with diluent. Transfer 10 mL of standard stock solution into 100 mL volumetric flask and dilute to volume with diluent. And an appropriate concentration of sample (20 μgmL^{-1} assay concentration) was prepared at the time of analysis.

Sample solution (tablets)

Crush to powder 20 tablets of azathioprine weigh and transfer the tablet powder equal to 50 mg of azathioprine into 100 mL volumetric flask add 30 mL of diluent, sonicate to dissolve for 10 minutes and dilute to volume with diluent. Further filtrate the solution through 0.45µ filter. And an appropriate concentration of sample (20 μgmL^{-1} assay concentration) was prepared at the time of analysis. Inject 20 µL of blank solution, placebo solution, three times of Standard solution, Disregard peaks due to blank and placebo.

Assay

Diluted to 10 ml of standard stock solution, into 100 mL and make up to volume with diluent.

Repeated same procedure for remaining three preparations

Sample Preparation

Crush to powder 20 tablets, weigh and transfer the tablet powder equal to 50 mg of

AZT into 100 mL volumetric flask added 30 mL of diluent, sonicate to dissolve for 10 minutes and dilute to volume with diluent. Further filtrate the solution through 0.45µ

Filter. Procedure**:** Inject 20 µL of blank solution, placebo solution, three times of Standard solution, Disregard peaks due to blank and placebo.

System suitability requirements from SST solution

a) Tailing factor: NMT 2.0

b) Theoretical Plates: NLT 2000

Procedure for forced degradation study

Stability testing is an important part of the process of drug product development. The purpose of stability testing is to provide evidence of how the quality of a drug substance or drug product varies with time under a variety of environmental conditions, for example temperature, humidity, and light and enables recommendation of storage conditions, retest periods, and shelf life to be established. The two main aspects of drug product that plays an important role in shelf-life determination are assay of the active drug and the degradation products generated during stability studies.

Acidic degradation

5 mg drug were dissolved in the diluents A&B. Forcibly degrade the sample by using 0.1 N HCl at room temperature. Collect 10 mL of the sample upto 48 hours.

Alkaline degradation

5 mg drug were dissolved in the diluents A&B. Forcibly degrade the sample by using 0.1 N NaOH at room temperature. Initially collect 10 mL of the sample upto 48 hours.

Thermal degradation

10 mg drug were forcibly degrading the sample exposed to heat under oven at 75°C of temperature. Then the working solution was prepared using diluents A&B. Collect the sample after 10^{th} day.

Photo degradation

10 mg of drug is exposed to the short wavelength (254 nm) UV light for 48 h. Then the working solution was prepared using diluents A&B forcibly degrades the sample under UV.

Method Validation

Specificity

Specificity is the ability of the method to assess unequivocally the analyte in the presence of components, which may be expected to be present. Typically, these might include degradation products, matrix the specificity of the developed HPLC method for azathioprine was carried out in the presence of its degradation products. Stress studies were performed for azathioprine in bulk drug to provide an

indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted to stress conditions exposing it with acid (01N hydrochloric acid), alkali (0.1N NaOH), heat (75^{0}C) and UV light (254 nm wavelength) to evaluate the ability of the proposed method to separate azathioprine from its degradation products. For light and heat study, the study period was 48 h whereas for acid and base 48 h. Peak purity test for azathioprine was by using PDA detector in stress samples.

Precision

Assay of method precision (intra-day precision) was evaluated by carrying out six independent assays of azathioprine test samples against reference standard; the percentage of RSD of six assay values obtained was calculated. The intermediate precision (inter-day precision) of the method was also evaluated using two different analysts, different HPLC systems and different days in the same laboratory the results were tabled in Table- 3.

Accuracy (recovery test)

Accuracy of the method was studied by recovery experiments. The recovery experiments were performed by adding known amounts of the drugs in the placebo. The recovery was performed at three levels, 50, 100 and 150% of the label claim of the tablet (50 mg of azathioprine). The recovery samples were prepared in the aforementioned procedure, and then 10 mL of azathioprine solution were transferred into a 50 mL volumetric flask and diluted to volume with diluent B. Three samples were prepared for each recovery level. The Solutions were then analyzed, and the percentage recoveries were calculated from the calibration curve. The recovery values for azathioprine ranged from 98.0 to 102.0%. The average recoveries of three levels for azathioprine was 99.86% the results were tabled in Table- 2.

Linearity

The linearity of the response of the drug was verified at six concentration levels, ranging from 25 to 150% of the targeted level (20 µg mL-1). Concentration standard solutions containing 2.5-15 µg mL-1 of azathioprine in each linearity level were prepared. Linearity solutions were injected in triplicate. The calibration graphs were obtained by plotting peak area versus the concentration data and were treated by least-squares linear regression analysis. The equation of the calibration curve for azathioprine obtained Y=76621×+12515, the Calibration graphs were found to be linear in the aforementioned concentrations. The correlation coefficient of determination is 1. The linearity calibration curve was showed in figure-8. The results were tabulated in table-4. The sample Chromatogram showed in Figure-3and the Overlaid Linearity Chromatogram showed in Figure- 2.

Limit of detection and limit of quantification (LOD and LOQ)

The limit of detection (LOD) and limit of quantification (LOQ) were determined by calibration curve method. Specific calibration curve was constructed using samples containing the analytes in the range of LOD and LOQ. The LOD and LOQ for azathioprine in LC were 1.95and 3.62 µg mL^{-1}, respectively. LOD and LOQ were calculated by using the following equations. LOD = 3.3Sa/b, LOQ = 10Sa/b. where Sa is the standard deviation of the calibration curve and b is the slope of the calibration curve. Precision at limit of quantification and limit of detection was checked by analyzing six test solutions prepared at LOQ and LOD levels and calculating the percentage RSD of area.

Robustness

To determine the robustness of the developed method experimental conditions were purposely altered and the resolution between azathioprine and acid degradation products were evaluated. The flow rate of the mobile phase was 1.0 mL min± 0.1 ml, and temp variation ± 5^{o}C to study the effect of flow rate on the resolution. The effect of percent organic strength on resolution was studied by varying. Acetonitrile from –10to+10%. The resolution in the robustness study was not less than <2.0 in all conditions. The stability of the standard solutions and the sample solutions was tested at intervals of 48hours and 10th day. The stability of solutions was determined by comparing results of the assay of the freshly prepared standard solutions. The RSD for the assay results determined up to 10th day for azathioprine was 0.53%. The assay values were within 1.5% after 10th day. The results indicate that the solutions were stable for 10th day at ambient temperature.

Ruggedness

The ruggedness of test method was demonstrated by carrying out precision study in six preparations of sample on a single batch sample by different analysts, the results of the intermediate precision study are tabulated as below table-3. The mean % RSD for both method precision and intermediate precision < 2.0.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

The primary target in developing this stability indicating HPLC method is to achieve the resolution between azathioprine and its degradation products. To achieve the separation of degradation products we used stationary phase C-8 and combination of mobile phase consist of Potassium dihydrogen phosphate: Acetonitrile in the ratio (60:40 v/v), The separation of the degradation product and azathioprine was achieved on Hypersil C_8 stationary phase and the combination of mobile phase consist of Potassium dihydrogen phosphate: Acetonitrile in the ratio (60:40 v/v), The tailing factor obtained was less than two and retention time was about 3.674 min for the main peak and less than 4 min for the degradation products, which would reduce the total run time and ultimately increase the productivity thus reducing the cost of analysis per sample. Forced degradation study showed the method is highly specific and entire degradation products were well resolved from the main peak. The developed method was found to be specific and method was validated as per ICH guidelines.

Result of forced degradation experiments

Degradation was observed for azathioprine samples during stress conditions like heat, UV light, base and acid. Azathioprine was degraded into acid (Figure 4), base (Figure5), Heat (Figure 6) and UV (Figure 7) forms polar impurities. In the acidic condition 7.5%, in the basic condition 4.78% after 48 h, in the heat condition 7.46 % and in the UV condition 7.93 % after 10th day. Degradation was observed for azathioprine. Peak purity results greater than 990 indicate that the azathioprine peak is homogeneous in all stress conditions tested. The unaffected assay of azathioprine in the tablets confirms the stability indicating power of the method the summary of forced degradation results were tabled in Table -1.

Determination of active ingredients in tablets

The contents of drug in tablets were determined by the proposed method using the calibration Curve.

Table 1: Summary of forced degradation results for Azathioprine

Stress conditions	Time / h	Peak Area	Assay of active	Degradation/ (%) Substance	Peak purity
Acid hydrolysis	48	3558178	92.5	7.5	0.999
Base hydrolysis	48	3659926	95.22	4.78	1.0
Heat	75^{o}C	3557060	92.54	7.46	0.999
UV	10th Day	3538833	92.07	7.93	1.0

Peak purity values in the range of 0.999 -1000 indicate the homogenous peak

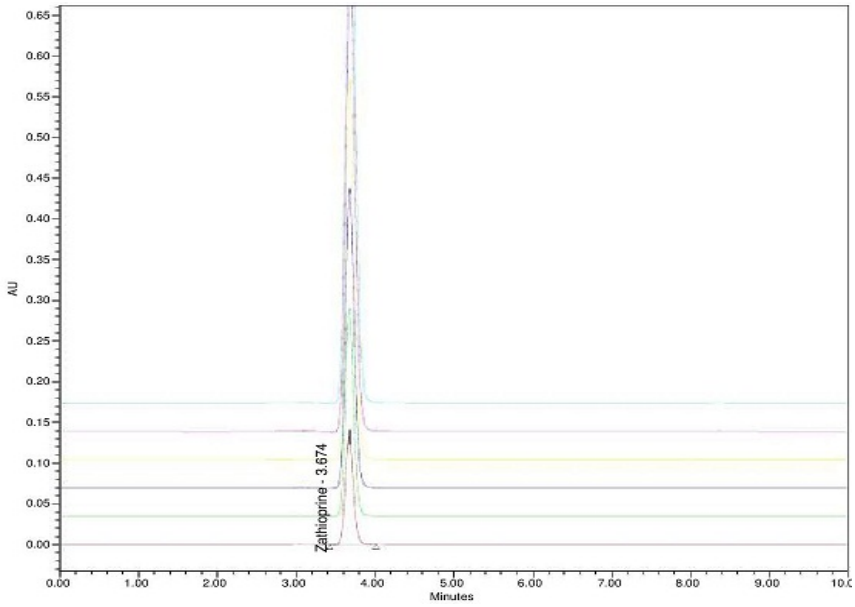

Fig. 2: Overlaid Linearity Chromatogram of Azathioprine

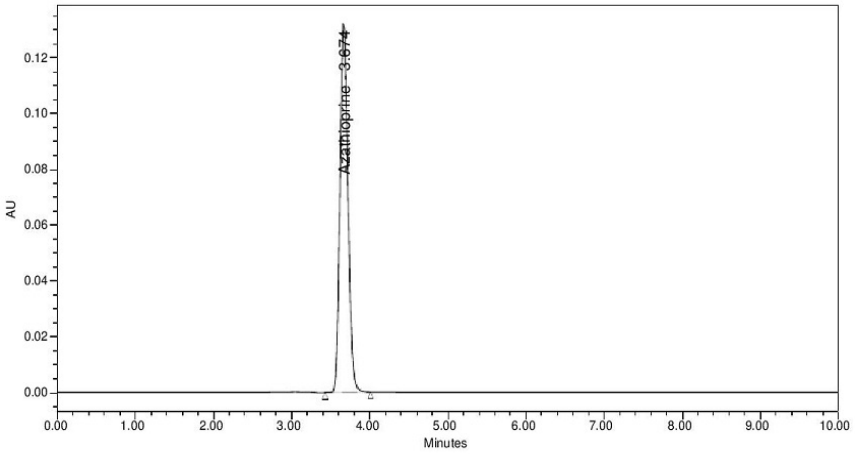

Fig. 3: Sample Chromatogram of 25 µg/mL of standard solution of Azathioprine

S. No.	Peak Name	RT	Area	Height	% Area	USP Tailing	USP Plate Count
1	Azathioprine	3.674	959197	132877	100.000	1.13	5924.98

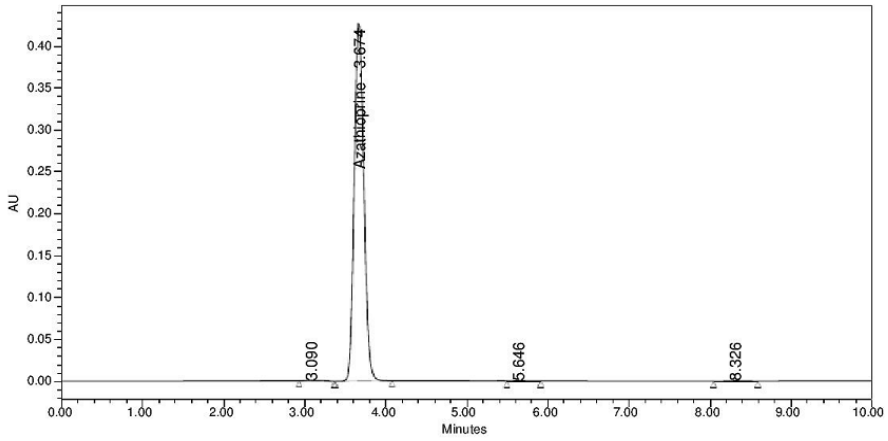

Fig. 4: Chromatogram of Azathioprine in acid degradation, in 0.1NHCl

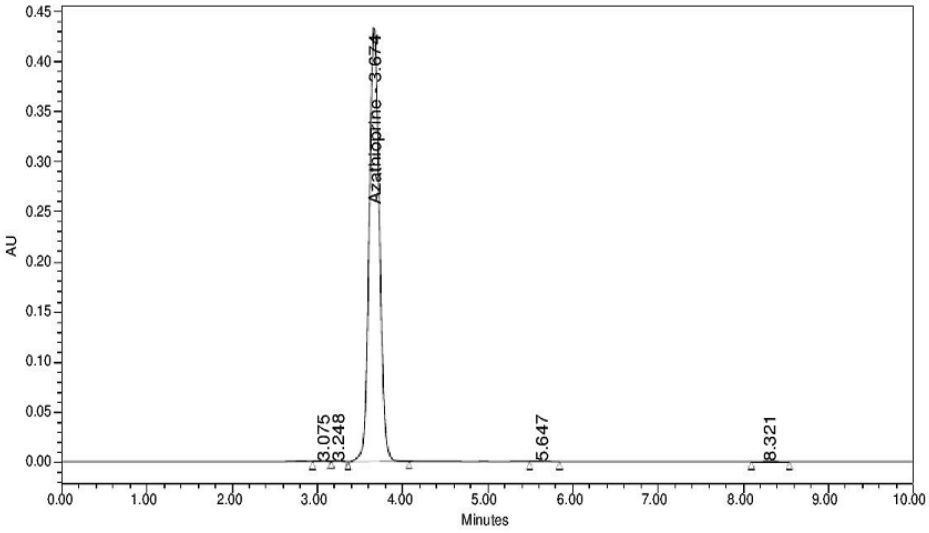

Fig. 5: Chromatogram of Azathioprine in Base degradation; in 0.1N NaOH

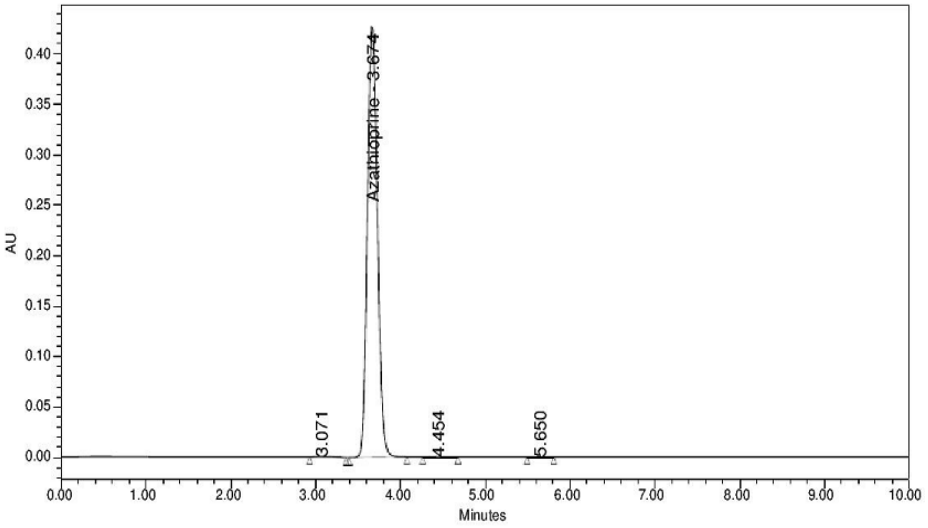

Fig. 6: Chromatogram of Azathioprine in Heat degradation at 75°C

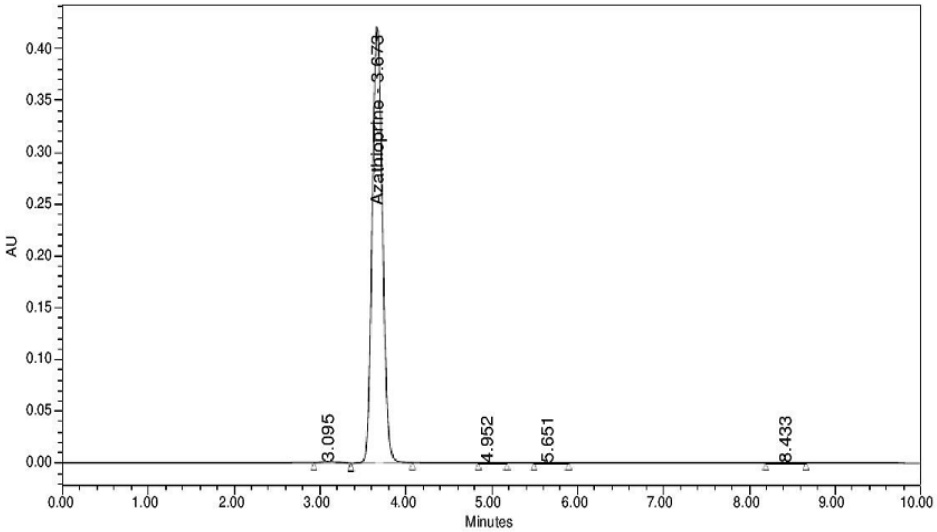

Fig. 7: Chromatogram of Azathioprine in UV degradation in 10th Day

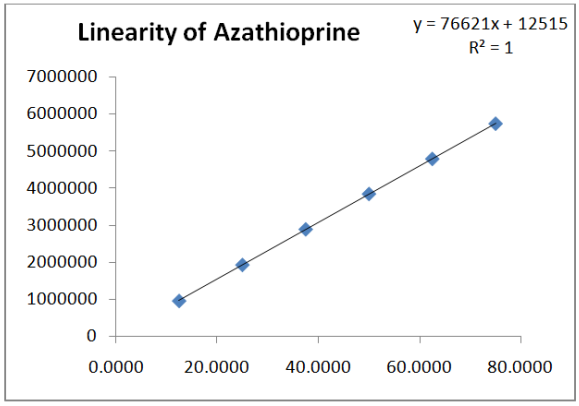

Fig. 8: Linearity of Azathioprine

Table 2: Results of recovery tests of Azathioprine

Level of addition / (%)	Amount added / µg	Recovery / (%)	Average recovery / (%)
50	25	100.25	99.86
100	50	99.87	
150	75	99.46	

Table 3: Result of precision of the Azathioprine test method

Analyst 1 (intra-day precision)				Analyst 2 (inter-day precision)		
S No	Name	RT	Area	Name	RT	Area
1	Injection-1	3.68	3784665	Injection-1	3.678	3840547
2	Injection-2	3.681	3788099	Injection-2	3.679	3843549
3	Injection-3	3.681	3794539	Injection-3	3.682	3848905
4	Injection-4	3.685	3820154	Injection-4	3.686	3855487
5	Injection-5	3.689	3833276	Injection-5	3.683	3852038
6	Injection-6	3.691	3840598	Injection-6	3.685	3859904
	AVG	3.685	3810222	AVG	3.682	3850072
	STDEV	0.005	24254.4	STDEV	0.003	7273.1
	%RSD	0.126	0.64	%RSD	0.087	0.189

Table 4: Linearity of Azathioprine

Linear solutions %	Conc.	Area
25	12.5000	959197
50	25.0000	1931934
75	37.5000	2894450
100	50.0000	3851429
125	62.5000	4799518
150	75.0000	5751594

CONCLUSION

The developed method is stability indicating and can be used for assessing the stability of azathioprine bulk drugs and pharmaceutical dosage form. The developed method is specific, selective, robust, and rugged and precise. This method can be conveniently used for assessing stability assay of selected substances and dissolution of tablets containing azathioprine in quality control laboratory. The study showed that the drug is moderately degraded in acid (7.5%), base (4.78%) heat (7.46%) conditions but highly degraded in the UV (7.93%).

CONFLICT OF INTERESTS

Declared None

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