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Development and Validation of Rapid RP- HPLC Method for the Determination of Azathioprine in Bulk and Pharmaceutical Dosage Form

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ABSTRACT

The core aim of present work was to develop a simple, precise, rapid and reproducible isocratic reverse phase high performance liquid chromatographic (RP-HPLC) method for the estimation of Azathioprine in pure and in tablet dosage form. An isocratic RP-HPLC was performed by utilizing Welchrom C_{18} column, (250 mm × 4.6 mm i.d., particle size 5 µm) maintained at ambient temperature and mobile phase composed of a mixture of acetonitrile: water (50:50 v/v) with apparent pH of 3.3 adjusted with o-phosphoric acid. The flow rate was adjusted 1.0 mL/min and UV detection was performed at 276 nm. The developed method was statistically validated for its linearity, precision, accuracy, specificity, Robustness and ruggedness. The retention time of Azathioprine peak was found at 3.080 minutes. The developed method was linear in the range of 1-5 µg/mL with correlation coefficient of 0.999. The method was found to be specific and accurate with the overall mean % recovery of 99.74%. The % RSD of intra and inter-day precision was found to be 0.833 and 0.877 respectively. The developed method was highly sensitive with LOD of 0.0480 µg/mL and LOQ of 0.1456 µg/mL. Assay content of Azathioprine was determined and the mean % found for Azathioprine was in good agreement with the label claim. The proposed method was found to be simple, highly sensitive, precise, accurate and rapid and can be employed for quantification of Azathioprine in bulk and tablet dosage form.

Keywords: RP - HPLC, Azathioprine, Validation, ICH guidelines.

INTRODUCTION

Azathioprine [1-6] is chemically 6-[(1-Methyl-4-Nitro-1*H* imidAzathioprinel-5yl) sulfanyl]-7*H*-purine, containing immunosuppressive action which is given either orally or intravenously and can be utilized to stop rejection in organ transplantation. It is used in autoimmune disease such as rheumatoid arthritis or inflammatory bowel disease or Crohn's disease and acts by preventing DNA synthesis. Infact it is official in U.S.P, B.P and European Pharmacopoeia. Azathioprine, also known as "Imuran", a "pro-drug", that gets converted or metabolized into the required substance, for Mercaptopurine (6MP). Azathioprine as well as its metabolite 6-mercaptopurine have been discovered by G. Ellion more than 45 years ago. A thorough literature survey revealed that few methods have been described for determination of Azathioprine such as spectrophotometry [7], HPLC [8], ^{1}H NMR [9], Chemiluminescence (CL) [10] HPTLC [11] have been used for the determination of Azathioprine. Most of the available reported methods for the estimation of Azathioprine are in human serum and plasma. As a matter of fact the available RP-HPLC method has got disadvantages like peak tailing, long run time, less sensitivity, selectivity and expensive. Keeping in view of this, an attempt has been made to develop a novel RP-HPLC method with

simple, precise, accurate, economical method for the estimation of Azathioprine in tablet dosage form. The proposed method was validated as per the International Conference on Harmonization (ICH) guidelines [12]. Figure 1 shows the structure of Azathioprine.

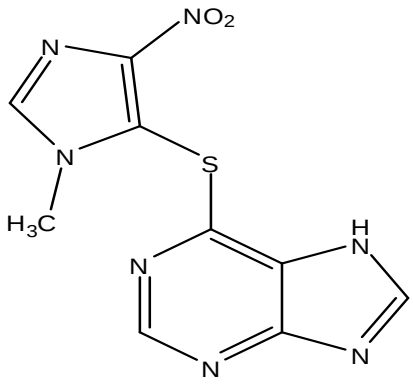

Figure 1. Chemical structure of Azathioprine.

MATERIALS AND METHODS

EXPERIMENTAL:

Materials used

An analytically pure sample of Azathioprine standard was gifted from Hetero Labs Ltd., Hyderabad India. All the chemicals were analytical grade. HPLC grade acetonitrile and triethylamine were obtained from Merck pharmaceuticals private Ltd., Mumbai, India. Methanol and water utilized were of HPLC grade and purchased from Merck specialties private Ltd., Mumbai, India. Commercial tablets of Azathioprine formulation was procured from local pharmacy. IMURAN tablets containing Azathioprine with labeled amount of 50 mg per tablet are manufactured by Glaxo Smithkline Pharmaceuticals Ltd. India.

Instruments used

The HPLC analysis was carried out on Shimadzu LC-20AT Prominence Liquid Chromatograph comprising a LC-20AT VP pump, Shimadzu SPD-20A, variable wavelength programmable UV/VIS detector SPD-20AVP and Welchrom C_{18} column (4.6 mm i.d. X 250 mm, 5 micron particle size). A manually operating sample Rheodyne injector with 20 µL fixed sample loop was equipped with the HPLC system. The HPLC system was equipped with "Spinchrom" data acquisition software.

Preparation of Reagents and Standards

Mobile phase

Mobile phase preparation was done by mixing potassium dihydrogen phosphate (1.48 gms) and dipotassium hydrogen phosphate (0.288 gms) to 500 mL of water, and make up to 1000 mL with 500 mL of acetonitrile pH of the buffer should be 3.3. (Note: water mixture with buffer should not be added to acetonitrile may it cause precipitation of the entire mobile phase).

Preparation of standard stock and working standard of Drug Solution

10 mg of Azathioprine was accurately weighed and transferred to a 10 mL clean, dry volumetric flask with the addition of mobile phase, upto the mark, sonicate the solution to dissolve if necessary. This is primary stock standard solution of Azathioprine 1000 µg/mL concentration. Secondary stock solution having the concentration range of 100 µg/mL made by taking 1mL from primary and make up with buffer to 10 mL. From this solution prepared working range concentration. Linearity range solutions containing 1 µg/mL,2 µg/mL, 3 µg/mL, 4µg/mL and 5 µg/mL of Azathioprine were prepared.

Preparation of stock solution for the commercially obtained tablets

Twenty tablets each containing 50 mg of Azathioprine were taken and crushed to get fine powder. Then a quantity of the powder equivalent to 10 mg of Azathioprine was extracted with 20 mL of mobile phase, followed by another 2 extractions each with 10 mL of mobile phase. It was filtered through whattman filter paper no. 42 to remove insoluble materials. The volume of filtrate was diluted to 100 mL with mobile phase. This was stock solution of 100

µg/mL. An aliquot of 1.0 mL of this solution was transferred to 10 mL volumetric flask and adjusted up to the mark. From this solution 1 to 5 mL were transferred to 10 mL different flasks to get the final concentrations of 1,2,3,4,5 µg/mL. The content of the tablets was calculated either from the formerly plotted calibration graphs or by means of regression equations.

Optimization of mobile phase and method development

Optimization of mobile phase was performed based on trial and error method. A series of trials were conducted in order to get proper optimized HPLC conditions. In the first instance several mobile phase trials were done such as methanol: water, acetonitrile: HPLC grade water, methanol: acetonitrile: water in different ratio without adjusting pH, infact there are different problems such as high tailing factor, good symmetry and proper chromatographic peak were not obtained. Finally after reviewing the results, a mobile phase comprising of phosphate buffer mixture duly adjusted to pH 3.3, acetonitrile in the proportion of 50:50 v/v which full fill all the criteria of system suitability and also obtained sharp, well- gaussian shape peak. This mobile phase was also selected as the diluent because the drug is freely soluble in the mobile phase. The stationary phase made up of Welchrom C_{18} column with 4.6 X 250 mm, 5 µm were observed and they are found to be utmost suitable for Azathioprine. The ultra violet spectrum of diluted solutions of various concentrations of Azathioprine in mobile phase was recorded by utilizing UV spectrophotometer ie., systronic double beam SL 2203. An absorption maximum was found to be 276 nm. This wavelength was optimum for the detection of Azathioprine. Figure 2 shows the absorption maxima of Azathioprine. The developed method gave symmetric peak at retention time of 3.080 minutes and satisfied all the peak properties as pursuance of ICH guidelines.

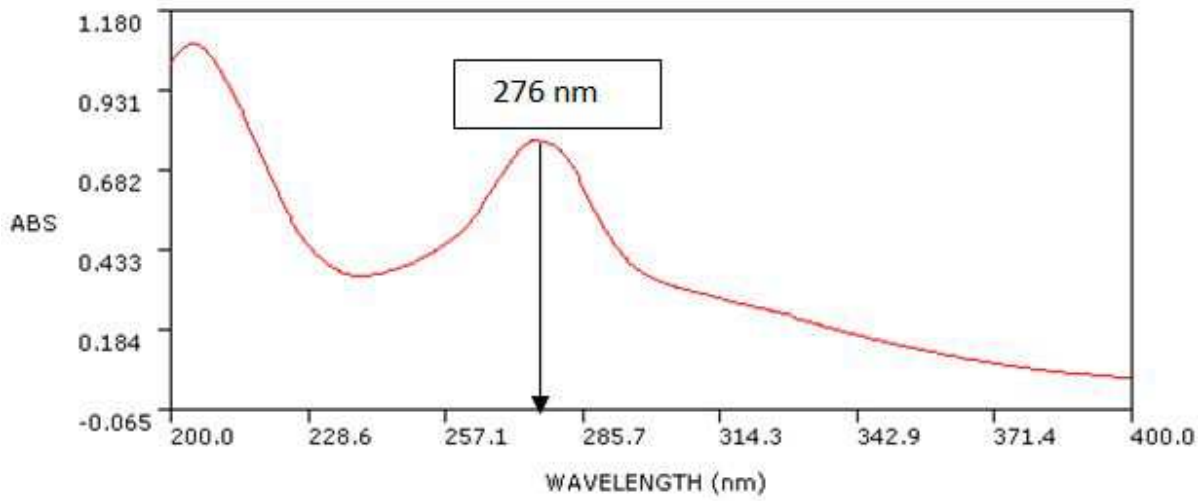

Figure 2. Absorption maxima for Azathioprine.

Solution stability

In order to check the stability of the both standard and sample solutions throughout the analysis, both solutions were analyzed over a period of seventy-two hours at an intermission twenty four hours at room temperature. The results showed for solutions, the retention time and peak area of Azathioprine remained unchanged and no degradation occur within indicated period, which indicates that both solutions were stable for seventy-two hours.

Validation of analytical method

The proposed RP-HPLC method of analysis was validated in pursuance of ICH Q2 (R1) for the parameters like system suitability, specificity, linearity, precision, accuracy, robustness, limit of detection (LOD) and limit of quantitation (LOQ).

System suitability

The chromatographic systems used for analysis must pass system suitability limits before sample analysis can commence. Set up the chromatographic system, allow the HPLC system to stabilize for 40 minutes. Inject blank preparation (single injection) and standard preparation (six replicates) and record the chromatograms to evaluate the

system suitability parameters like resolution (NLT 2.0), tailing factor (NMT 1.5), theoretical plate count (NLT 3000) and % RSD for peak area of six replicate injections of Azathioprine standard (% RSD NMT 2.0). The system suitability data and the optimum chromatographic conditions are reported in Table 1.

Table 1. Optimum chromatographic conditions and system suitability data.

Parameter	Chromatographic conditions
Instrument	SHIMADZU LC-20AT prominence liquid chromatograph
Column	WELCHROM C_{18} Column (4.6 mm i.d. X 250 mm, 5 µm particle size)
Detector	SHIMADZU SPD-20A prominence UV-Vis detector
Diluents	Acetonitrile : water (50 : 50) pH : 3.3 using o-phosphoric acid)
Mobile phase	Acetonitrile : water (50 : 50) pH : 3.3 using o-phosphoric acid)
Column modifier	Triethylamine (0.5 mL)
Flow rate	1 mL/min.
Detection wave length	UV at 276 nm.
Run time	4 minutes
Column back pressure	91 kgf
Temperature	Ambient temperature (25°C)
Volume of injection loop	20 µL
Retention time (t_R)	3.080 min
Theoretical plates [th.pl] (Efficiency)	8212
Theoretical plates per meter [t.p/m]	164,240
Tailing factor (asymmetry)	1.069

Linearity

Linearity was checked by preparing standard solutions at 5 different concentration levels of each of Azathioprine. Azathioprine standard solutions (1, 2, 3, 4, 5 µg/mL) were injected into the HPLC system to get the chromatograms. The average peak area and retention time were recorded. The calibration curve was constructed between concentrations versus peak area by the prepared concentration of 1-5 µg/mL of stock solution. The linearity range was found to be 1-5 µg/mL and the results are presented in Table 2. The standard chromatograms of Azathioprine calibration standards are depicted in Figure 3 to Figure 7. Results show that a phenomenal correlation exists between peak area and concentration of drug within the linearity range. The calibration graph of Azathioprine is presented in Figure 8. The data of regression analysis is presented in Table 3.

Table 2. Calibration data of the proposed method for the estimation of Azathioprine.

S.	Concentration, µg/m	Retention time, (t_R) m	Peak area, mV
1	0	-	0
2	1	3.080	43.545
3	2	3.080	86.352
4	3	3.080	131.16
5	4	3.080	174.49
6	5	3.080	216.773

Table 3. Linear regression data of the proposed method of Azathioprine.

Parameter	Method
Detection wavelength (λ_{max})	UV at 276 nm
Linearity range (µg/mL)	1-5 µg/mL
Regression equation (Y = a + bX)	Y = 43.472x + 0.0409
Slope (b)	43.472
Intercept (a)	0.0409
Standard error of slope (S_b)	0.1515
Standard error of intercept (S_a)	0.4586
Standard error of estimation (S_e)	0.633
Regression coefficient (r^2)	0.9999
Percentage range of errors* (Confidence limits) 0.05 significance level 0.01 significance level	 0.4024 0.5289

**Average of 6 determinations; Acceptance criteria < 2.0.*

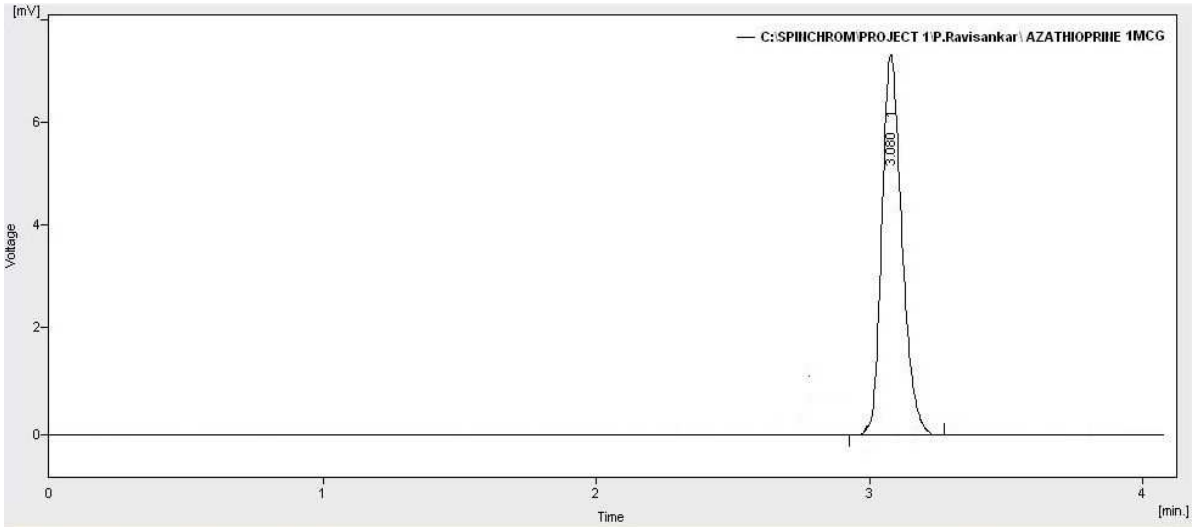

Figure 3. Standard chromatogram of Azathioprine (1 µg/mL).

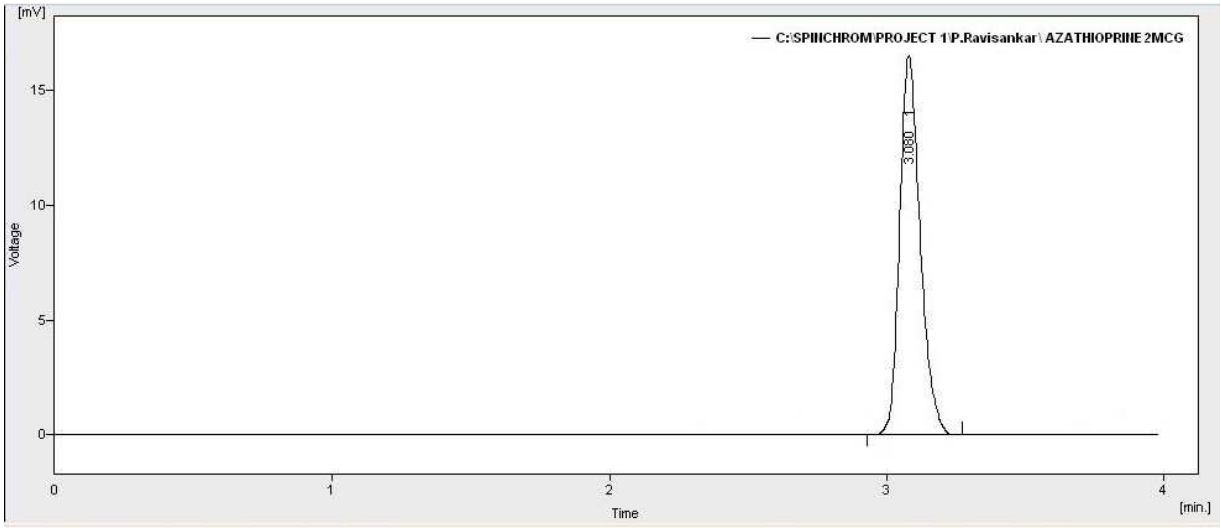

Figure 4. Standard chromatogram of Azathioprine (2 µg/mL).

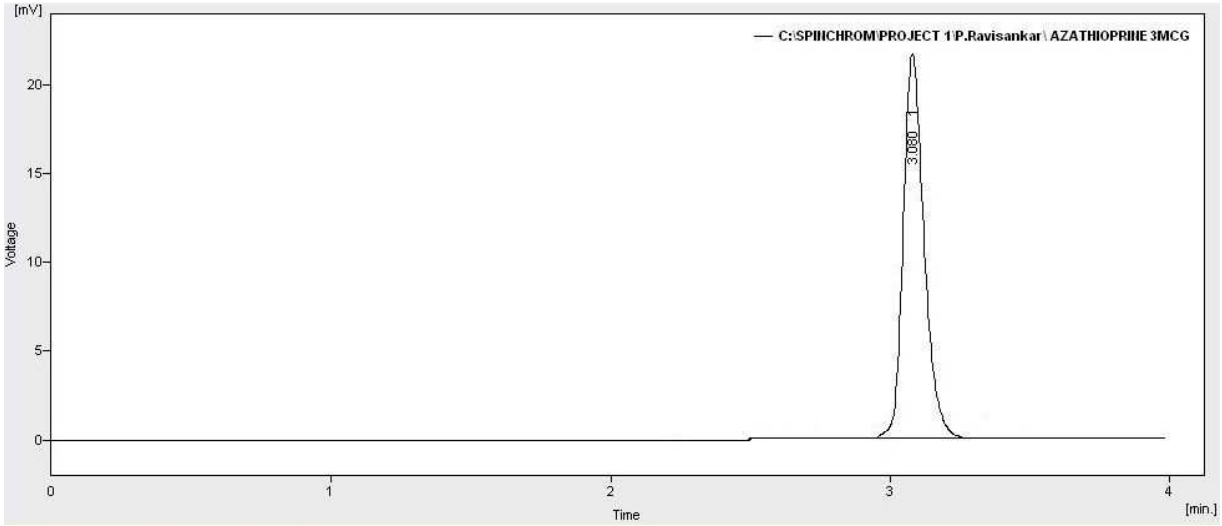

Figure 5. Standard chromatogram of Azathioprine (3µg/mL).

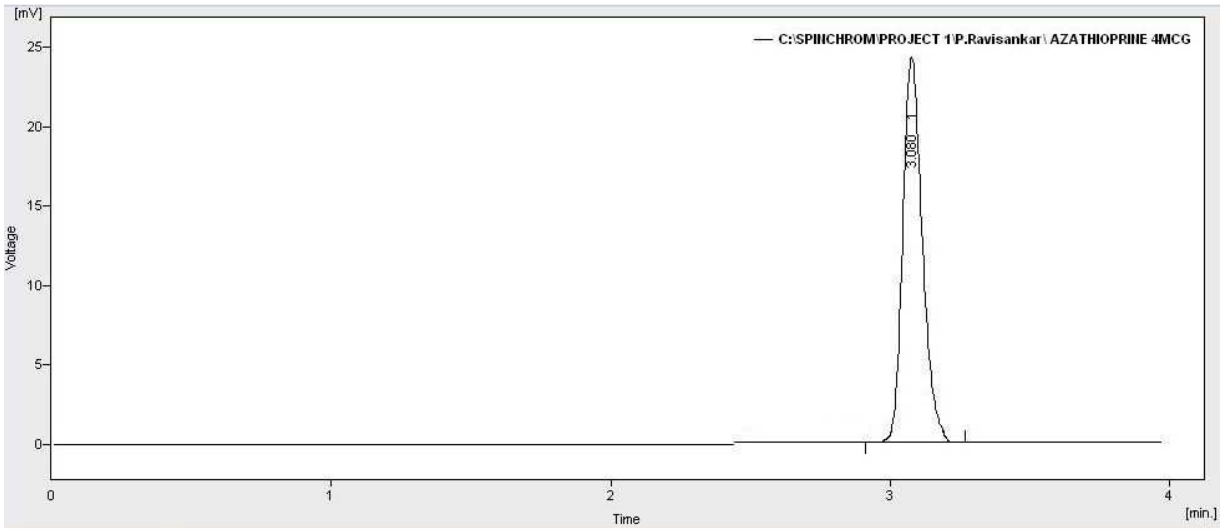

Figure 6. Standard chromatogram of Azathioprine (4µg/mL).

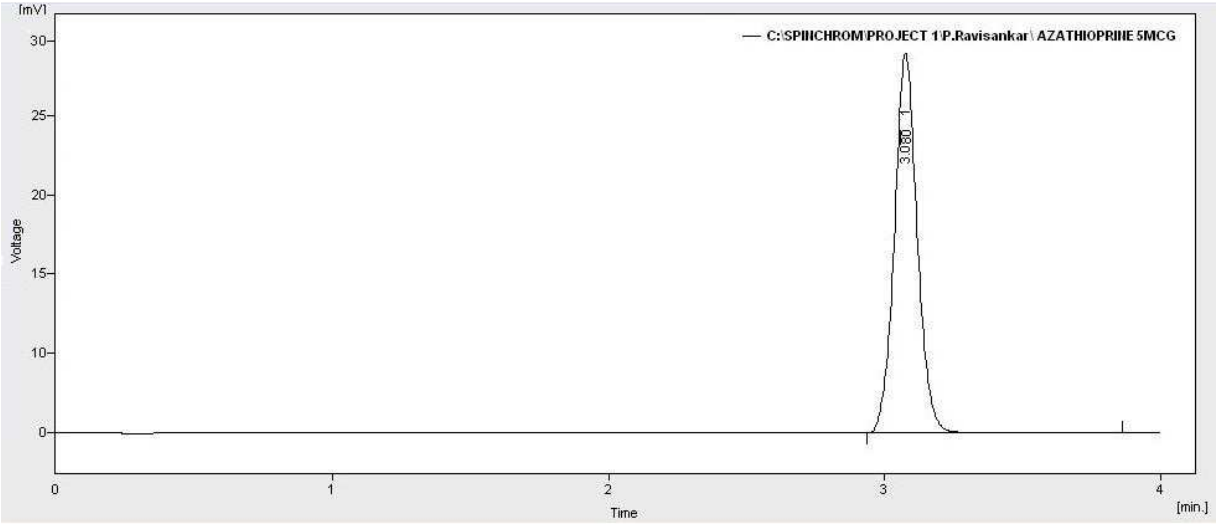

Figure 7. Standard chromatogram of Azathioprine (5µg/mL).

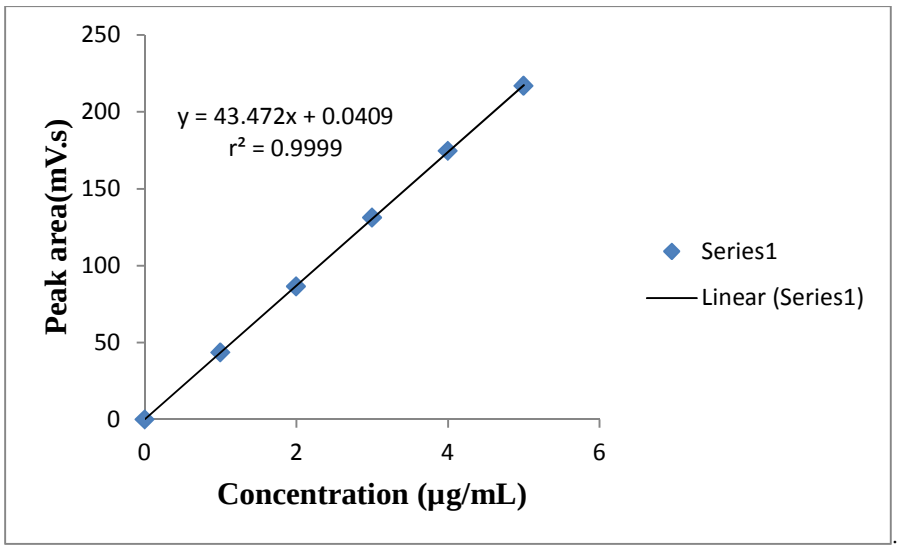

Figure 8. Calibration plot of Azathioprine.

Specificity

The specificity of the method was determined by the prepared standard, sample solutions and the blank solution were injected and check any other excipients interference occurs or not. It was shown that the excipients present in pharmaceutical tablets of Azathioprine did not show any interference with Azathioprine peak because no excipients peaks appear in the chromatogram of the prepared tablet. Furthermore the well-shaped peaks also indicate the specificity of the method. The results for specificity are tabulated in Table 4. The chromatogram for placebo indicating the specificity of developed method is presented in Figure 9.

Table 4. Specificity study for Azathioprine

Name of the solution	Retention time, (t_R) min.
Mobile phase	No peaks
Placebo	No peaks
Azathioprine, 10 µg/mL	3.080 min.

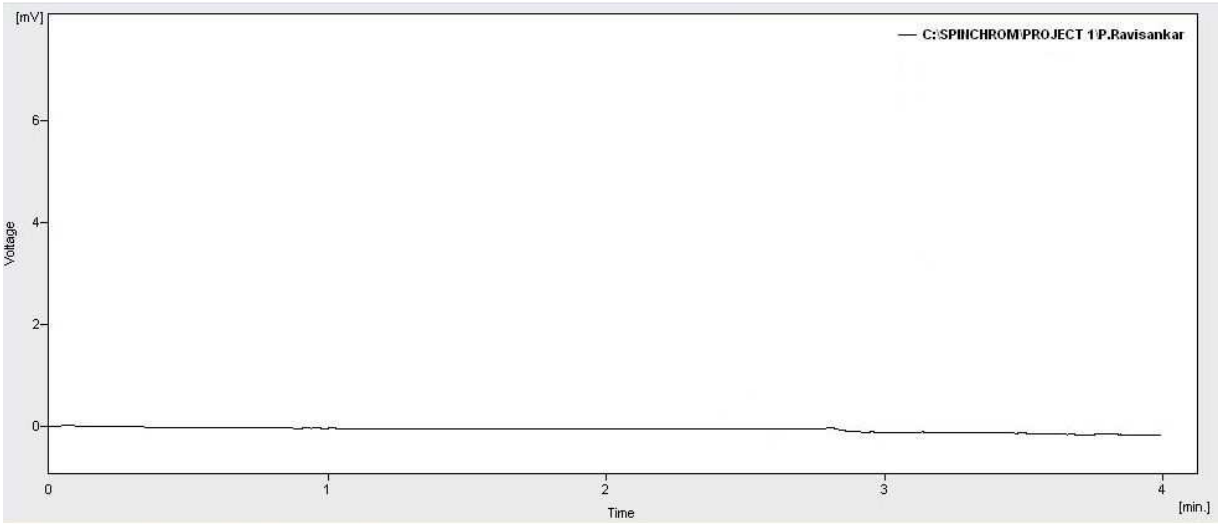

Figure 9. Chromatogram of placebo.

Precision

Intra - day precision was investigated by replicate applications and measurements of peak area for Azathioprine for six times on the same day under similar conditions. Inter - day precision was obtained from % RSD values obtained by repeating the assay six times on two different days. The percent relative standard deviation (% RSD) was calculated which is within the acceptable criteria of not more than 2.0. The intra-day and inter-day precision results were shown in Table 5 and Table 6 respectively.

Table 5. Results of precision study (intra-day) for Azathioprine.

Sample	Concentration (µg/mL)	Injection no.	Peak area (mV.s)	% RSD[#]
Azathioprine	3	1	131.16	0.833
		2	133.986	
		3	132.709	
		4	133.809	
		5	131.98	
		6	132.098	

[#] *Acceptance criteria < 2.0.*

Table 6. Results of precision study (inter-day) for Azathioprine.

Sample	Concentration (µg/mL)	Injection no.	Peak area (mV.s)	% RSD[#]
Azathioprine	3	1	131.16	0.877
		2	132.09	
		3	131.78	
		4	133.908	
		5	133.945	
		6	132.053	

[#] *Acceptance criteria < 2.0.*

Accuracy/Recovery

The accuracy of the method was find out by standard addition method. The recovery experiment was carried out the previously analyzed sample (5 µg/mL), a known amount of standard drug was added at 50%, 100% and 150% level. The concentrations were re-analyzed with the above described procedure. The percent recovery of the triplicate solutions was determined and average of the percent recovery was calculated. The results are presented in Table 7.

Table 7. Recovery data for Azathioprine.

Type of recovery in % level	Sample conc. (µg/mL)	Amount of the standard added (µg/mL)	Total conc. (µg/mL)	Found conc. (µg/mL)	% recovery*	% RSD
50	5	2.5	7.5	7.47	99.66	0.26
100	5	5	10	9.992	99.92	0.17
150	5	7.5	12.5	12.48	99.88	0.060

*RSD: relative standard deviation. *mean of three determinations*

Robustness

Robustness is the ability to provide accurate and precise results under a variety of conditions. To evaluate the robustness of HPLC method, a few parameters were deliberately changed. The parameters such as the slight variation in acetonitrile percentage composition in the mobile phase, flow rate, wavelength detection. The results of robustness study is shown in Table 8 indicated that the small change in the conditions did not significantly affect the determination of Azathioprine.

Table 8. Robustness results of Azathioprine.

S. no	Parameter	Optimized	Used	Retention time (t_R), min	Plate count$^{\$}$	Peak asymmetry$^{\#}$	Remark
1.	Flow rate (±0.2 mL/min)	1.0 mL/min	0.8 mL/min	3.089	8220	1.080	*Robust
			1.0 mL/min	3.080	8212	1.089	*Robust
			1.2 mL/min	3.070	8206	1.023	*Robust
2.	Detection wavelength (±5 nm)	276 nm	271nm	3.080	8212	1.080	*Robust
			276 nm	3.080	8212	1.080	Robust
			281 nm	3.080	8212	1.080	Robust
3.	Mobile phase composition (Acetonitrile : Water)	50:50 v/v	55:45v/v	3.074	8209	1.079	Robust
			50:50 v/v	3.080	8212	1.080	*Robust
			45:55v/v	3.076	8219	1.086	*Robust

*Acceptance criteria (Limits): $^{\#}$Peak Asymmetry < 1.5, $^{\$}$ Plate count > 3000, * Significant change in Retention time.*

Ruggedness

Ruggedness is the degree of reproducibility of the results obtained under variety of conditions. Form the stock solution containing 10 µg/mL solution of Azathioprine was prepared and analyzed by 2 different analysts and two different instruments using the same operational conditions in different experimental period. The % recoveries of the replicates were calculated. It was observed that the obtained results are reproducible under differences in analysts. The results of ruggedness are represented in Table 9.

Table 9. Ruggedness results

S.No	Analyst 1	Analyst 2	Instrument 1	Instrument 2
% Recovery	99.9 %	99.8 %	100.1	99.9 %
% Deviation	0.1 %		0.2 %	

LOD and LOQ

Limit of Detection is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. The limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. Limit of Detection and Limit of Quantitation were calculated using following formula LOD = 3.3 σ/S and LOQ = 10 σ/S, where SD = standard deviation of response (peak area) and S = slope of the calibration curve. The LOD and LOQ values are presented in Table 10. The results of LOD and LOQ supported the sensitivity of the developed method.

Table 10. Limit of detection and Limit of quantitation

Limit of Detection(LOD)	0.0480 µg/mL
Limit of Quantitation(LOQ)	0.1456 µg/mL

Application to commercial tablet

Using the developed RP-HPLC chromatographic method, assay of Azathioprine in tablet was carried out as mention in the experimental section. Six replicate determinations were made. Satisfactory results were obtained and were good agreement with the label claim and assay results were shown in Table 11. The results were very close to the

labeled value of commercial tablets. The representative sample chromatogram of Azathioprine is shown in Figure 10.

Table 11. Assay results of Azathioprine formulation

S. No	Formulations	Labelled claim	Amount found (mg) (mean±SD) (n=6)	Assay ± % RSD
1	IMURAN tablets (Glaxo Smithkline Pharmaceuticals Ltd. India).	50 mg/tablet	49.821±0.20 mg/tablet	99.642 ±0.40

SD: standard deviation. RSD: relative standard deviation.

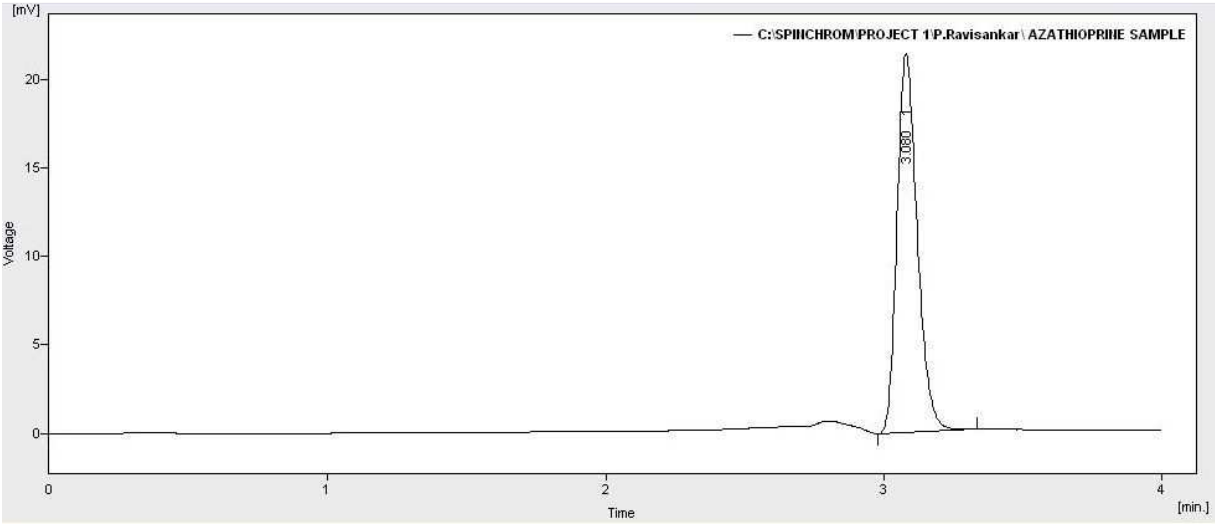

Figure 10. Chromatogram of marketed formulation IMURAN.

RESULTS AND DISCUSSION

The present study was aimed at developing a precise, sensitive, rapid and accurate HPLC method for the analysis of Azathioprine in bulk drug and in pharmaceutical dosage forms. In order to achieve phenomenal retention time and peak asymmetry, C_{18} stationary phase column (250 mm X 4.6 mm i.d, 5 µm particle size) and mobile phase composed of water, acetonitrile (50:50) v/v with pH adjusted to 3.3 using ortho phosphoric acid and triethylamine as column modifier at a flow rate of 1mL/min was selected. The retention time for Azathioprine was found to be 3.080 minutes. UV spectra of Azathioprine showed that the drug absorbed maximum at 276 nm, so this wavelength was selected as the detection wavelength. The correlation coefficient (0.9999) of regression was found almost equal to one in the range of 1-5 µg/mL which states that the method was good linear to the concentration versus peak area responses. The comparison of chromatograms of placebo, standard and sample, there was no interference observed from the peaks of placebo, standard and sample. It shows that the method is specific. The precision studies were performed and the % RSD of the determinations was found to be 0.833 for intra-day precision and 0.877 for inter-day precision which are within the limits which indicates that the proposed method was found to be precise. The accuracy of the method was found to be good with the overall % RSD for recovery at 50 %, 100 % and 150 % levels were all within the limits which indicate that the proposed method was found to be accurate. Method validation following ICH guidelines indicated that the developed method had high sensitivity with LOD of 0.0480 µg/mL and LOQ of 0.1456 µg/mL. The method was found to be robust even though on slight deliberate variation in the method conditions did have a tiny effect on the peak asymmetry, plate count and retention time and all are within the limits which indicated that the method is robust. Regarding the assay results satisfactory results were obtained and were in a good agreement with the label claim.

CONCLUSION

The developed RP-HPLC method provides a convenient and efficient method for the estimation of Azathioprine in dosage form. This method has various advantages like less retention time, low solvent consumption, outstanding peak symmetry, and phenomenal linearity, highly sensitive, precise, accurate and robust. The mobile phase can be easily prepared and diluents are economical and readily available and it does not need sample preparation with sophisticated techniques. The drug solutions employed in the study were stable up to 48 hours. These attribute the high quality of the method. There was no interference from the excipients used in the tablet formulation. The proposed method can be used for the routine analysis of Azathioprine in pharmaceutical dosage forms for routine application in quality control laboratories.

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