

A NOVEL HPLC-PDA METHOD FOR QUANTITATIVE DETERMINATION OF RELATED IMPURITIES IN AZATHIOPRINE ACTIVE PHARMACEUTICAL INGREDIENT

M. Sucheta¹, Y. Prashanthi^{2,✉}, and Tentu Nageswara Rao³

¹Department of Chemistry, MVSR Engineering College, Nadargul-501510, Telangana, India

²Department of Chemistry, Mahatma Gandhi University, Nalgonda, Telangana-508254, India

³Department of Analytical Chemistry, Isosphere Biosciences Pvt Ltd., Hyderabad, Telangana-501301, India

✉Corresponding author: dryprasanthi@gmail.com

ABSTRACT

An innovative and cost-effective method has been devised using high-performance liquid chromatography with PDA detection for the assessment of Azathioprine and its related impurities (1-methyl-4-nitro-1H-imidazol-5-amine and 7H-purine-6-thiol). Validation was performed in accordance with ICH guidelines, confirming that the method is linear, accurate, precise, and robust. The established method is suitable for routine quality control analysis of Azathioprine in pharmaceutical formulations (SDG-3). The chromatographic separations were conducted on a (150×4.6 mm), 3.5 µm Waters Symmetry C18 column, utilizing a mobile phase of 0.1% orthophosphoric acid buffer and Acetonitrile in a 30:70 ratio at a flow rate of 1.0 mL/min. Azathioprine and its two impurities were eluted in less than 10 minutes. The column oven temperature was maintained at 30° C, the sample temperature was kept at 20° C, and a detector wavelength of 235 nm was used. The method was validated successfully by establishing System Suitability, Specificity, Linearity, Accuracy, Limit of Detection, and Limit of Quantification. Specificity was validated by assessing the separation of impurities and confirming the absence of interference from the placebo. Linearity was established by analysing a series of standard solutions at various concentration levels, with the correlation coefficient determined to be greater than 0.999. Accuracy was determined through recovery studies conducted at three concentration levels, with the percentage recovery remaining within the acceptable range of 80-120%. The limit of detection (LOD) and limit of quantification (LOQ) were determined using the signal-to-noise ratio method.

Keywords: Azathioprine, Related Impurities, HPLC-PDA, Method Validation, Linearity, LOQ, LOD.

RASAYAN J. Chem., Vol. 19, No.4, 2026

INTRODUCTION

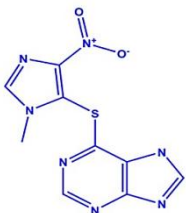
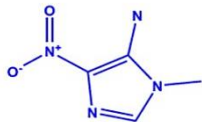
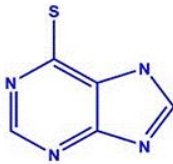
Azathioprine is an oral immunosuppressant prescribed for organ transplants and various autoimmune diseases, acting as a prodrug of 6-mercaptopurine.¹ Its mechanism of action involves the suppression of lymphocyte proliferation through its conversion to 6-thioguanine nucleotides, which inhibits the synthesis of DNA and RNA.² Its primary uses include maintenance immunosuppression after renal or liver transplants and treatment for rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus, and other autoimmune disorders.³ Before starting treatment, patients should have TPMT/NUDT15 testing due to the risk of severe myelosuppression, which requires regular blood monitoring. Common side effects include hematologic issues (such as leukopenia and thrombocytopenia) and gastrointestinal symptoms (like nausea).⁴ Long-term use increases the risk of lymphoma and skin cancers. The usual dosing is individualised at 1-3 mg/kg/day, with necessary adjustments for interactions, particularly with allopurinol, which raises toxicity risks. Azathioprine regulatory submissions require a thorough Chemistry, Manufacturing, and Controls (CMC) package, a risk-based pharmacovigilance strategy, and a designated bioequivalence (BE) study-single-dose, two-way crossover on 50 mg tablets for the US, in compliance with FDA regulations.⁵ For submissions in the EU, please consult Jayempi EPAR dossiers, and for India, adhere to CDSCO guidelines.⁶ The high-level requirements vary by authority, emphasising the need to define the regulatory pathway, the content of the CMC dossier, the prerequisites for the BE study, and the local submission requirements.⁷ Essential components include stability data,

impurity profiles, and safety considerations for labelling. Risks involve insufficient CMC/stability documentation and the requirements for patient studies related to BE, especially concerning interactions with allopurinol.⁸ The actionable next steps consist of confirming the target markets and identifying the type of product for customised submissions.⁹ Azathioprine is produced on a global scale, mainly by manufacturers in India and China, with regulatory compliance requiring the oversight of key impurities, such as 6-mercaptopurine, in line with ICH Q3A(R2).¹⁰ Considerations for regulatory submissions include the sources of API, the status of GMP audits, impurity profiles, and stability data. Generic pharmaceutical companies in India, Europe, and North America are engaged in the production of finished formulations.¹¹ The impurity analysis of azathioprine requires the focused identification of 6-mercaptopurine and nitroimidazole-related degradation products, along with forced-degradation profiling via LC-MS/MS/IDMS. Manufacturers must adhere to ICH Q3A(R2) impurity thresholds in the CMC dossier and provide validated LC-MS methods in conjunction with stability data.¹² Key impurities identified include 6-mercaptopurine, nitroimidazole derivatives, and oxidation by-products. Recommended analytical methods include LC-HRMS for the detection of unknown degradation products, LC-MS/MS for the quantification of known impurities, and IDMS for regulatory submissions. It is essential to strictly follow ICH Q3A(R2) thresholds, avoiding the assumption of fixed limits, and the CMC section must include forced-degradation data, impurity structures, and validation certificates.¹³ An effective workflow involves the collection of reference standards, conducting forced-degradation studies, developing validated assays, and establishing specification limits based on ICH Q3A calculations while managing risks associated with degradation. Robust documentation is crucial to prevent regulatory complications.

EXPERIMENTAL

Chemicals

The reference standards of Azathioprine (Purity 99.7%), 1-methyl-4-nitro-1H-imidazol-5-amine (Purity 97.22%), and 7H-purine-6-thiol (Purity 99.96%) were acquired from Dr. Ehrenstorfer. Azathioprine API sample (Purity 98.56%) was purchased from the India mart. HPLC grade acetonitrile (Purity 99.9%) was obtained from Rankem, Chennai, India. Potassium dihydrogen orthophosphate (Purity 99.6%) AR grade was supplied by M/s. Merck India Ltd., Mumbai. The details of Azathioprine and its impurities (1-methyl-4-nitro-1H-imidazol-5-amine and 7H-purine-6-thiol) are presented below:

API/Impurity Name	Structure	Molecular Mass	Molecular Formula
Azathioprine		277.26 g·mol ⁻¹	C ₉ H ₇ N ₇ O ₂ S
1-methyl-4-nitro-1H-imidazol-5-amine (Impurity A)		142.12 g·mol ⁻¹	C ₄ H ₆ N ₄ O ₂
7H-purine-6-thiol (Impurity B)		152.17 g·mol ⁻¹	C ₅ H ₄ N ₄ S

Preparation of Mobile Phase A

A precise amount of 2.0004 g of Potassium dihydrogen orthophosphate was measured and placed into a 1000 mL volumetric flask. Following this, 500 ml of HPLC-grade water was introduced, mixed thoroughly, sonicated, and the solution was brought up to the mark with additional HPLC-grade water.

Preparation of Mobile Phase B

Methanol of HPLC grade was used as Mobile Phase B.

Preparation of Standard Stock Solutions**Azathioprine Standard Stock Solution**

A precise amount of 50.16 mg of the reference item (99.7%) was weighed into a clean and dry 10 mL volumetric flask, dissolved in 2 mL of diluent and made up to the mark with the same diluent. The concentration of this prepared solution is equivalent to 5000.95 µg/mL.

Impurity -A Standard Stock Solution

A precise amount of 50.98 mg of the reference item (97.22%) was weighed into a clean and dry 10 mL volumetric flask, dissolved in 2 mL of diluent and made up to the mark with the same diluent. The concentration of this prepared solution is equivalent to 4956.28 µg/mL.

Impurity -B Standard Stock Solution

A precise amount of 50.11 mg of the reference item (99.96%) was weighed into a clean and dry 10 mL volumetric flask, dissolved in 2 mL of diluent and made up to the mark with the same diluent. The concentration of this prepared solution is equivalent to 5009.00 µg/mL.

Preparation of Mixed Stock Solution

The standard stock solutions mentioned above, consisting of 4.999 mL of Azathioprine, 5.044 mL of Impurity -A, and 4.991 mL of Impurity -B, were transferred into a 20 mL volumetric flask and diluted to the mark with acetonitrile as the diluent. The concentration of the resulting solution is 1250 µg/mL.

HPLC Method Conditions

Azathioprine, 1-methyl-4-nitro-1H-imidazol-5-amine, and 7H-purine-6-thiol were quantified by HPLC using a Waters system with a PDA detector (230 nm). The mobile phase was 10:90% v/v 0.2% potassium dihydrogen orthophosphate and acetonitrile. A Waters Symmetry column (150 mm × 4.6 mm × 3.5 µm) was used at a flow rate of 1.0 mL/min, with a 10 µL injection volume. The run time was 7.5 minutes, with retention times of 4.2, 3.0, and 3.5 minutes for Azathioprine, 1-methyl-4-nitro-1H-imidazol-5-amine, and 7H-purine-6-thiol, respectively.

Method Validation**Specificity**

PLC assessed specificity by injecting aliquots of mobile phase solvents, diluent, standard, and sample solutions.

Preparation of sample solution

Precision solution (P1) was used.

Preparation of reference item solution

Linearity solution (Linearity-4) was used.

Limit of Quantification (LOQ)

Limit of quantification was determined at the lowest concentration of analyte to determine with acceptable % RSD. The LOQ was established by S/N ratio.

Limit of Detection (LOD)

The limit of detection (LOD) indicates the smallest concentration of an analyte that can be recognised, yet it cannot be accurately quantified. The determination of LOD is based on the S/N ratio, which is expected to be approximately 3.

Linearity

Various concentrations of standards (0.03, 0.1, 1.0, 10.0, 50.0, and 100.0 µg/mL) were created in individual 10 mL volumetric flasks through the dilution of the standard stock mixed solution. Information regarding the serial dilution and the corresponding responses can be found in Table-1.

Table-1: Preparation of Serial Dilutions for Linearity Standard Solutions

Standard Code	Standard Stock (µg/mL)	Volume taken (mL)	Final volume (mL)	Final Concentration (µg/mL)
Linearity-1	1.00	0.300	10	0.030
Linearity-2	10.00	0.100	10	0.10
Linearity-3	100.00	0.100	10	1.00
Linearity-4	100.00	1.000	10	10.00
Linearity-5	1250.00	0.400	10	50.00
Linearity-6	1250.00	0.800	10	100.00

Method Precision

System Precision

Mixed standard Linearity solution (50.0 µg/mL) was used for system precision.

Recovery

The analytical method was validated in terms of recovery of the standard at two fortification levels.

Preparation of Standard Solution

The Linearity Standard Solution (Linearity-4) was used for recovery determination.

Preparation of Blank Sample Solution

An amount of 16.01 mg of test item was accurately weighed and was taken into a clean and dry 100 mL volumetric flask, dissolved and made up to the mark with the acetonitrile. The concentration of the sample solution was equivalent to 16.00 mg/L. This prepared solution was used for recovery determination.

Lower-Level Fortification

An aliquot of 0.5 mL Linearity Standard Solution (Linearity-4) of standard solutions was fortified into a 5 mL volumetric flask and was diluted with blank sample solution and made up to the mark with blank sample solution. The concentration of this solution was equivalent to 1.00 µg/µL for Azathioprine, Impurity A and B.

Higher Level Fortification

An aliquot of 2.5 mL Linearity Standard Solution (Linearity-4) was fortified into a 5 mL volumetric flask, diluted with blank sample solution and was made up to the mark with blank sample solution. The concentration of this solution was equivalent to 5.00 µg/µL for Azathioprine, Impurity A and B.

$$\begin{aligned} \text{Fortified area} &= \text{Detected area} - \text{Average blank sample area} \\ \text{Recovered Conc. (mg/L)} &= [\text{Standard conc. (µg/mL)} / \text{Average standard area}] \times \text{Fortified area} \\ \text{Recovery (\%)} &= [\text{Recovered conc. (µg/mL)} / \text{Fortified standard conc. (µg/mL)}] \times 100 \end{aligned}$$

RESULTS AND DISCUSSION**Method Validation****Specificity**

The analytical method demonstrated specificity. There was no significant interference observed at the metconazole peak retention time. The interference at retention time in the diluent was 0.0%, considerably lower than the threshold of <3.0%.

Linearity

Standard solutions of azathioprine, 1-methyl-4-nitro-1H-imidazol-5-amine, and 7H-purine-6-thiol were prepared at concentrations of 0.03, 0.1, 1.0, 10.0, 50.0, and 100.0 µg/mL by diluting a 1250 ng/mL stock solution. These were analysed by HPLC (chromatogram in Fig.-1), and the responses are presented in Table-2. Calibration curves (Fig.-2) were generated by plotting peak area (Y) against concentration (X). The regression equations were $Y = 8311.91X + 52.71$ for azathioprine, $Y = 8764.11X + 11.07$ for 1-methyl-4-nitro-1H-imidazol-5-amine, and $Y = 7342.75X + 13.32$ for 7H-purine-6-thiol. High correlation coefficients ($R^2 \geq 0.9997$) demonstrate excellent linearity, ensuring quantitative accuracy and regulatory

compliance. The document also discusses determining concentration from peak area and the impact of a diminished slope on method sensitivity.

Table-2: Detector Linearity Solutions Response

Final volume (mL)	Final Concentration (µg/mL)	Azathioprine Standard Area	Impurity A Standard Area	Impurity B Standard Area
Linearity-1	0.03	289	305	238
Linearity-2	0.1	802	852	703
Linearity-3	1	8026	8296	7125
Linearity-4	10	79965	81963	70895
Linearity-5	50	422281	449415	372271
Linearity-6	100	828252	871397	731985
Slope		8311.91	8764.11	7342.75
Intercept		52.71	11.07	13.32
Correlation coefficient (R)		0.9999	0.9999	1.0000
Coefficient of determination (R ²)		0.9999	0.9997	0.9999

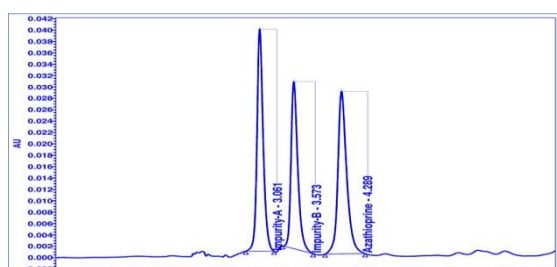


Fig.-1: Representative Linearity Chromatogram (100 µg/mL) of Azathioprine and its Impurities

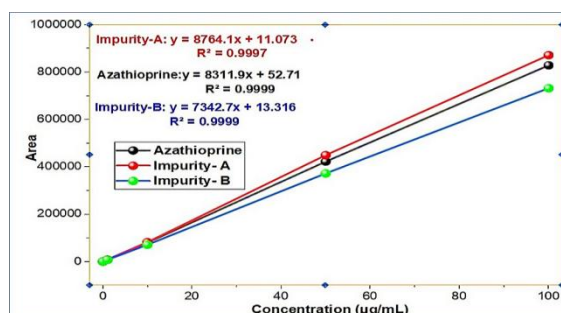


Fig.-2: Calibration Curve of Azathioprine and its Impurities

Limit of Quantification (LOQ)

The quantification limit was defined as the minimum concentration of the analyte that could be accurately assessed while maintaining an acceptable % RSD. The LOQ was derived from the signal-to-noise (S/N) ratio. Initially, the S/N ratio was calculated from the LOQ blank and impurity standards in a 0.1 µg/µL solution. Noise height was taken from the blank, and signal height was taken from the standard (0.1 µg/µL).

Noise height in the blank (h)- 139

Signal height in 0.1 µg/µL Azathioprine and its Impurities A and B (H)- 697, 713 and 701

S/N Ratio = 2(H)/h = 2(697)/139 = 10.03, 2(713)/139 = 10.26 and 2(701)/139 = 10.09.

Based on the S/N ratio obtained at the 0.1 µg/µL concentration level as LOQ. The representative chromatogram is shown in Fig.-3.

Limit of Detection (LOD)

The limit of detection (LOD) is the lowest analyte concentration that can be detected, but cannot be exactly quantified. LOD was determined with an S/N ratio. It should have a S/N of about 3.

Noise height in the blank (h)- 139

Signal height in 0.1 µg/µL impurity 2 standard solution (H)- 209, 214 and 211.

$S/N \text{ Ratio} = 2(H)/h = 2(209)/139 = 3.01$, $2(214)/139 = 3.08$, $2(211)/139 = 3.04$
Based on the S/N ratio obtained at 0.03 $\mu\text{g/mL}$ concentration level as LOD.

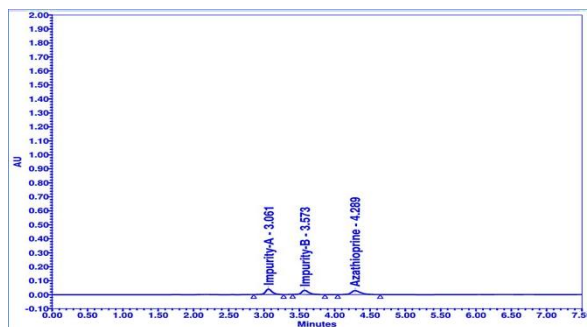


Fig.-3: Representative LOQ Chromatogram (0.1 $\mu\text{g/mL}$) of Azathioprine and its Impurities

System Precision

Injected 10 μl of metconazole standard solution (50.0 $\mu\text{g/mL}$) six times. Recorded the peak area of metconazole. Calculated the % RSD of peak area. The resulting data are presented in Table-3. The representative Chromatogram presented in Fig.-4.

Table-3: Precision Details

Concentration in $\mu\text{g/mL}$	Azathioprine Standard Area	Impurity A Standard Area	Impurity B Standard Area
50	423696	450217	373069
	420965	449854	372854
	422985	452336	371759
	424087	449859	371187
	421195	451235	372859
	420874	450824	373053
Mean	422300.33	450720.83	372463.50
Standard deviation	1459.32	962.47	793.58
% Relative standard deviation	0.35	0.21	0.21

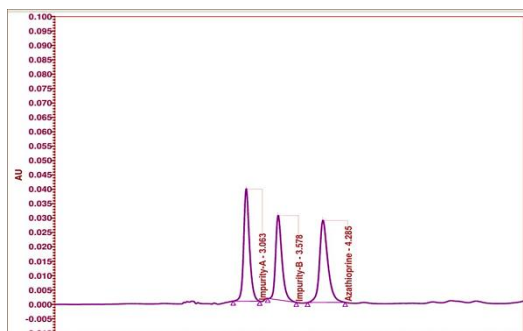


Fig.-4: Representative Precision Chromatogram (50 $\mu\text{g/mL}$) of Azathioprine and its Impurities

Recovery

The recovery assessment was performed at two levels. At the lower level of 1.0 $\mu\text{g/mL}$, the recovery percentages were recorded as 94.18% for Azathioprine, 91.47% for Impurity A, and 93.36% for Impurity B. At the higher level of 5.0 $\mu\text{g/mL}$, the recovery percentages were noted to be 98.29% for Azathioprine, 95.76% for Impurity A, and 96.17% for Impurity B. The recovery findings were established to be well within the specified limits of 80.0% to 120.0%. The results of the recovery are illustrated in Table-4.

CONCLUSION

The analytical approach adopted was carefully customised and optimised for the specific analyte of interest. This specificity ensured that the approach accurately and reliably measured the target compound without interference from other substances. The linearity of the method was rigorously assessed and confirmed to be acceptable, with a correlation coefficient exceeding 0.99, indicating a strong linear

relationship between the analyte concentration and the instrument response. Precision, which is a measure of the method's repeatability, was thoroughly evaluated and expressed as the per cent relative standard deviation (%RSD) of both the peak area and the per cent recovery.

Table-4: The Recovery Details of Azathioprine and its Impurities

Statistical parameters	Lower-Level Fortification		
	Azathioprine	1-methyl-4-nitro-1H-imidazol-5-amine (Impurity A)	7H-purine-6-thiol (Impurity B)
Mean	94.18	91.47	93.36
SD	0.67	1.28	1.36
% RSD	0.59	0.97	1.15
	Higher Level Fortification		
	Mean	98.29	95.76
	SD	0.41	1.11
	% RSD	0.38	0.94

The observed %RSD values were all found to be within pre-defined and acceptable limits, demonstrating the method's ability to generate consistent and reproducible results. Comprehensive method validation studies were conducted to confirm the overall suitability of the analytical methods for their intended purpose in this study. These validation studies provided documented evidence that the methods are accurate, precise, reliable, and robust, thereby ensuring the integrity and validity of the data generated.

AKNOWLEDGEMENTS

The authors would like to extend their heartfelt thanks to the Department of Chemistry at Mahatma Gandhi University, Nalgonda, Telangana-508 254, which has been instrumental in the successful completion of this research work.

CONFLICT OF INTERESTS

All authors confirm that there are no conflicts of interest or financial interests related to this research work.

AUTHOR CONTRIBUTIONS

This study is associated with *SDG-3: Good Health and Well-being*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

M. Sucheta  <https://orcid.org/0000-0003-3447-0716>

Y. Prashanthi  <https://orcid.org/0009-0004-5397-050X>

Tentu Nageswara Rao  <http://orchid.org/0000-0001-7623-3343>

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Cite this article as: M. Sucheta *et al.*, *Rasayan J. Chem.*, 19(4), 2241-2248(2026), <https://doi.org/10.31788/RJC.2026.1949941>

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