# UV-Visible Spectrophotometric Determination of Azathioprine in Pharmaceutical Formulations Based on Oxidative coupling reaction with MBTH

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## Abstract
A simple, sensitive, selective precise and accurate spectrophotometric method has been developed for the determination azathioprine in pure form, pharmaceutical formulations and blood sample by MBTH reagent. Spectrophotometric method was developed for determination of azathioprine at $P^H$–4.0 which is extractable at 620 nm. Beer's law is obeyed in the concentration ranges 10-60 µg $ml^{-1}$ for formulations and 4-24 µg $ml^{-1}$ for blood sample. %R.S.D was found to be 0.1933% & 0.2901 and Recovery 99.65% & 99.16% respectively. The result obtained in this method would have a great value when applied in quality control studies in laboratories. Any ingredients and excipients were not observed during the study. The developed method was validated with respect to accuracy, precision, and Specificity and Selectivity.

**Keywords:** UV-Visible Spectrophotometry, Azathioprine, Blood Sample & MBTH / $FeCl_3$.

## Introduction
Azathioprine is chemically 6-(1-methyl-4-nitroimidazole-5-yl) (in fig-1). Azathioprine is an immunosuppressive agent. It is used in the chemotherapy of acute leukemia, for immunosuppressant after solid-organ transplantation, and increasingly for immunomodulation in autoimmune disease. Currently, azathioprine (AZA) is used for treating patients suffering from chronically active inflammatory bowel disease (IBD). It inhibits lymphocyte activation, lymphocyte differentiation, in vitro lymphocyte stimulation, and in vitromixed lymphocyte reaction. It reduces the activity of natural killer lymphocytes.[1,2] It is also used in systemic anti-inflammatory states, such as rheumatoid arthritis, lupusery thematosus, colitis ulcerosa, auto immunological hepatitis and Crohn's disease.[3] In addition it acts as an immunosuppressive agent, and is given orally or by the intravenous (IV) route.[4] Impurities in azathioprine bulk drug are determined by using UPLC method.[5] United State Pharmacopoeia monograph of azathioprine API, thin layer chromatographic test is described for 6-mercaptopurine.[6,7]

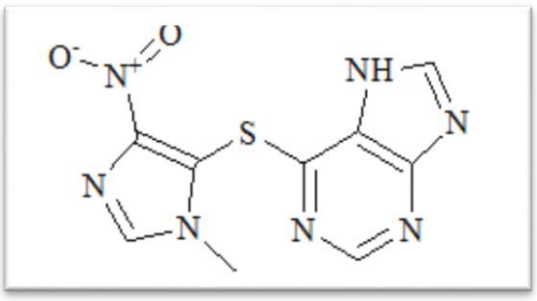


**Fig: 1 Chemical structure of Azathioprine**

Administration of azathioprine in transplant patients leads rapidly to severe my-elosuppression when the homozygote allele for thiopurine methyltransferase (TPMT) deficiency is expressed.[8] High 6-TGN concentrations in RBCs could be correlated with low erythrocyte TPMT activity, which exhibits a favorable clinical out-come in child with acute lymphoblastic leukemia treated by6-MP.[9] However, the relation between high6-TGNconcentrations in RBCs and myelosuppression induced by azathioprine remains controversial.[10] More recently, Me6-TIMP was shown to strongly in-hibit purine de novo synthesis in a concentration dependent manner, leading to cytotoxicity in Molt F4 cells. This suggests that methyl 6-mercaptopurine nucleotides (Me6-MPNs) could explain part of the pharmacologic activity of azathioprine[11] A few analytical methods LC-MS, GC-MS were reported in literature and these were used to estimate the drug levels in blood plasma.[12,13,14] 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.[15] Very few analytical HPLC and liquid chromatography tandem mass spectrometry methods[16,17] have been developed for the quantification in biological samples of plasma.[18,19,20] 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.

There is, however, no reported UV- Visible spectrophotometric method for the analysis of azathioprine in its technical grade, formulations and Blood Sample. In the present study therefore UV-

Visible spectrophotometric method for the quantitative determination of azathioprine. Functional group used for color development of azathioprine was primary amine group. The results obtained in this method were based on the Oxidative coupling reaction with 3-Methyl-2-benzothiazolinone hydrazone (MBTH)/ Ferric chloride.

## Materials & Methods

### Chemicals and Instruments

The pure gift samples was collected from Bio-Leo Analytical Labs INDIA pvt ltd, Plot No 135, Oasis Towers 9, IDA, Kukatpally, Prashantinagar, Hyderabad – 500 072. 3-methyl-2-benzothiazolone hydrazone hydrochloride, Bromophenol Blue and acetonitrile all chemicals are analytical grade were purchased in SSR Enterprises, Tirupati-517 501 A.P, India. UV-Vis spectrophotometer (UV-1800 Shimandzu, North America) connected to computer loaded with spectra manager software vision light was employed with spectral bandwidth of 1 nm and wavelength accuracy of ± 0.3 nm with a pair of 10 mm matched quartz cells. For scanning, the wavelength range selected was 300 nm to 1000 nm with medium scanning speed. All weights were taken using electronic balance (Denver, Germany). All experiments were performed at room temperature (25 ± 1) °C.

### Preparation of standard stock solution

Accurately weighed 100 mg of azathioprine was dissolved in 40 ml of acetonitrile in a 100 ml volumetric flask and it was made up to the mark with acetonitrile. (Stock solution A) 10 ml of the solution was pipetted out into a 100 ml volumetric flask and the same was made up to the mark with acetonitrile to obtain a final concentration of 100 µg $ml^{-1}$ (Stock solution B).

### Preparation of Calibration curve

Fresh aliquots of azathioprine ranging from 1 to 6 ml were transferred into a series of 10 ml volumetric flasks to obtain final concentration range of 10 to 60 µg $ml^{-1}$. 1ml of (0.5%) MBTH, 1ml of (0.7%) Ferric chloride solutions were added to each flask and resulting solution was heated for 15 min and finally 1ml (0.5N) Hydrochloric acid solution was added. The solutions were cooled to room temperature and made up to mark with acetonitrile. The absorbance of green colored chromogen was measured at 620 nm against the reagent blank. The color species was stable for 32 h. The amount of azathioprine present in the sample solution was computed from its calibration curve.

### Procedure for formulations

Twenty tablets containing azathioprine were weighed and finely powdered. An accurately weighed portion of the powder equivalent to 100 mg of azathioprine was dissolved in a 100 ml of acetonitrile and mixed for about 5 min and then filtered. The acetonitrile was evaporated to get a dry powder. The dry powder was diluted in a 100 ml volumetric flask with acetonitrile to get the stock solution A. 10 ml of aliquots were pipetted out into 100 ml volumetric flasks and the volume was made up to the mark with acetonitrile to obtain a final concentration of 1000µg $ml^{-1}$ (Stock solution B). Subsequent dilutions of this solution were made with acetonitrile to get a concentration range 10 to 60 µg $ml^{-1}$. These were analyzed at the selected wavelength, 620 nm, and the results were statistically validated.

### Procedure for Blood sample

Blood samples collected were centrifuged. To isolate azathioprine from plasma, acetonitrile was used for protein precipitation. Liquid- Liquid extraction was performed with plasma by alkalinization with 1M NaOH (sodium hydroxide), using by extraction with 30% dichloromethane in hexane. The upper organic layer was evaporated to dryness. The dry residue of 100 mg was dissolved in 100 ml of acetonitrile (1000 µg $ml^{-1}$). 10 ml of aliquot was taken into a 100 ml of volumetric flask and made up to the mark with acetonitrile. (100 µg $ml^{-1}$). Samples of aforesaid solutions ranging from 0.4-2.4 ml (4-24 µg $ml^{-1}$) were transferred in to 10 ml volumetric flasks. 1ml of (0.5%) MBTH solution was added followed by 1ml of (0.7%) Ferric chloride solution and then to each flask made up to the mark with acetonitrile. The resulting solutions were heated and finally 1ml (0.5N) hydrochloric acid solution was added. The solutions were cooled to room temperature and made up to the mark with acetonitrile. The color species was stable for 32 h. The absorbance of green colored chromogen was measured at 620 nm against the reagent blank. The amount of azathioprine present in the sample solution was computed from its calibration curve.

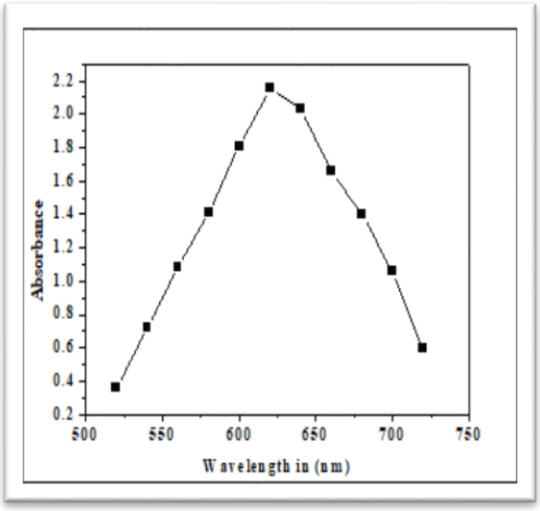


**Fig. 2: Absorption spectrum of Azathioprine with MBTH /$FeCl_3$**

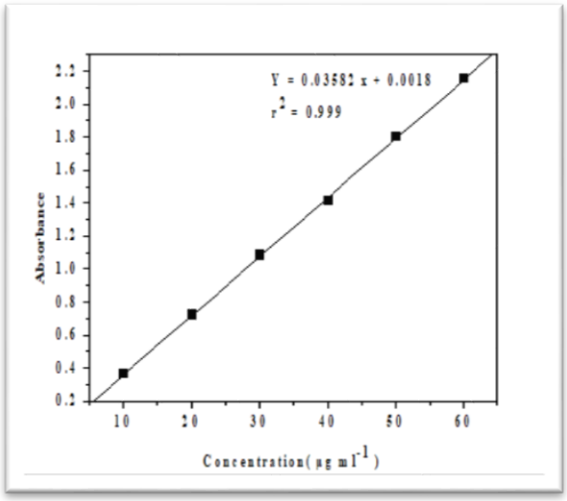


**Fig. 3: Beer's law plot of Azathioprine with MBTH/$FeCl_3$**

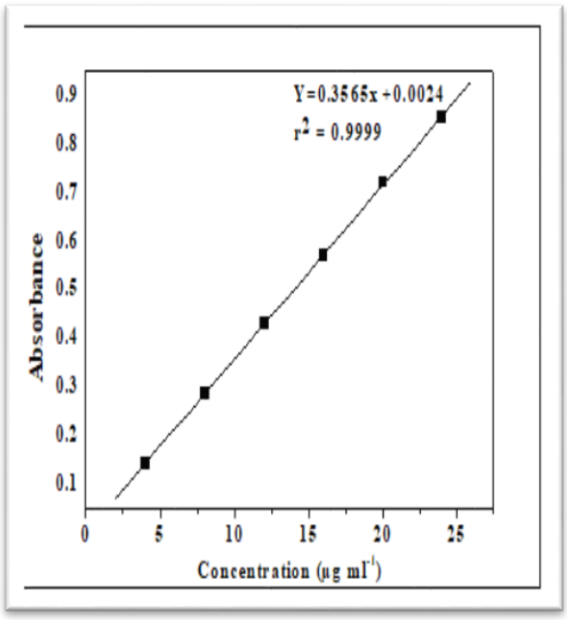


**Fig. 4: Beer's law plot of Azathioprine with MBTH in Blood sample**

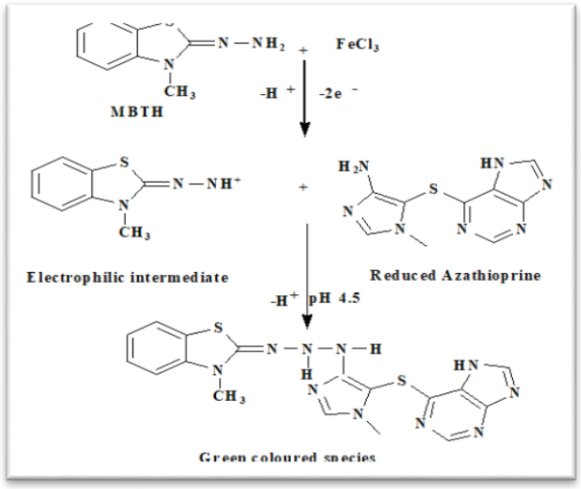


**Fig. 5 Scheme 1: Reaction Mechanism of Azathioprine with MBTH**

**Table 1: Optical characteristics and precision by MBTH**

| Parameter | MBTH method |
|---|---|
| Color | Green |
| Absorption maxima (nm) | 620 |
| Beer's law limits (µg $ml^{-1}$) | 10-60 |
| Molar absorptivity (l $mol^{-1}cm^{-1}$) | $0.1358x10^4$ |
| Sandell's Sensitivity (µg $cm^{-2}$) | 0.4646 |
| Regression equation (Y*) | Y=bc+a |
| Slope (b) | 0.03582 |
| Intercept(a) | 0.0018 |
| Standard deviation(SD) | 0.01278 |
| Correlation coefficient ($r^2$) | 0.999 |
| %RSD (Relative Standard deviation) | 0.2785 |
| Limits of detection (LOD)(µg $ml^{-1}$) | 0.0837 |
| Limits of quantification (LOQ) (µg $ml^{-1}$) | 0.2791 |

% RSD of six independent determinations

**Table 2: Assay results of Azathioprine in formulations by visible Method**

| Name of the Formulation | Formulation in (mg) | Amount found by the proposed method (mg) | Amount found by the reference method[36-38] (mg) | % Recovery |
|---|---|---|---|---|
| AZORAN | 250 | 249.53<br>t=0.002967<br>F=6.38481 | 248.23 | 99.53 |
| AZOPRINE | 250 | 249.25<br>t=0.00296<br>F=6.38481 | 248.75 | 99.25 |

- T and F- values refer to comparison of the proposed method with reference method.
- Theoretical values at 95% confidence limits t= 0.00297 and F= 5.9177

**Table 3: Determination of accuracy of Azathioprine**

| Amount of AZA in formulation (mg) | Amount of Standard AZA added (mg) | Total amount found (mg) | % Recovery |
|---|---|---|---|
| 249.53 | 200 | 449.15 | 99.15 |
| 249.62 | 200 | 449.31 | 99.31 |
| 249.70 | 200 | 449.46 | 99.46 |
| 249.53 | 250 | 499.06 | 99.06 |
| 249.62 | 250 | 499.24 | 99.24 |
| 249.67 | 250 | 499.34 | 99.34 |
| 249.53 | 300 | 548.96 | 98.96 |
| 249.59 | 300 | 549.09 | 99.09 |
| 249.64 | 300 | 549.20 | 99.20 |

**Table 4: Statistical data for accuracy determination**

| Total amount found (mean) | Standard deviation | % RSD |
|---|---|---|
| 449.31 | 0.15503 | 0.0345 |
| 499.21 | 0.14189 | 0.0284 |
| 549.09 | 0.12014 | 0.0218 |

The results are the mean of three readings at each level of recovery.

**Table 5: Repeatability data for Azathioprine at 620 nm**

| Conc. (µg ml$^{-1}$) | Abs 1 | Abs2 | Abs3 | Mean | Std. deviation | (%)RSD |
|---|---|---|---|---|---|---|
| 10 | 0.360 | 0.359 | 0.358 | 0.359 | 0.001 | 0.2785 |
| 20 | 0.721 | 0.720 | 0.719 | 0.72 | 0.001 | 0.1388 |
| 30 | 1.082 | 1.081 | 1.081 | 1.0813 | 0.00058 | 0.0536 |
| 40 | 1.412 | 1.411 | 1.412 | 1.4116 | 0.00058 | 0.0410 |
| 50 | 1.803 | 1.801 | 1.800 | 1.8013 | 0.00153 | 0.0849 |
| 60 | 2.152 | 2.152 | 2.153 | 2.1523 | 0.00058 | 0.0269 |

Average of six determinations.

**Table 6: Assay results of Azathioprine in Blood sample**

| Name of the Formulation | Formulation in (mg) | Amount found by the proposed method in (mg) | Amount found by the reference method[36 to38] (mg) | % of Recovery |
|---|---|---|---|---|
| AZORAN | 2 | 1.99<br>t=0.00296<br>F= 6.2307 | 1.86 | 99.99 |
| AZOPRINE | 2 | 1.94<br>t=0.0029<br>F=6.2868 | 1.88 | 99.94 |

- T and F values used for the comparison of the proposed method with reference method.
- Theoretical values at 95% confidence limits t=0.00276 and F=.5.6981

**Table 7: Determination of accuracy of Azathioprine**

| Name of the Formulation in (mg) | Amount of Drug in Blood sample (mg) | Amount of Standard Drug added in (mg) | Total amount found (mg) | % Recovery |
|---|---|---|---|---|
| AZORAN (2mg) | 1.99 | 2 | 3.98 | 99.98 |
| AZOPRINE(2mg) | 1.94 | 2 | 3.88 | 99.88 |

The results are the mean of two readings at each level of recovery.

**Table 8: Repeatability data for Azathioprine at 620nm**

| Concentration in µg ml$^{-1}$ | Abs1 | Abs2 | Abs3 | Mean | Std. Deviation | (%) RSD |
|---|---|---|---|---|---|---|
| 4 | 0.144 | 0.142 | 0.143 | 0.143 | 0.001 | 0.69930 |
| 8 | 0.288 | 0.286 | 0.287 | 0.287 | 0.001 | 0.34843 |
| 12 | 0.432 | 0.432 | 0.431 | 0.4316 | 0.00058 | 0.13438 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 16 | 0.569 | 0.570 | 0.569 | 0.5693 | 0.00058 | 0.10187 |
| 20 | 0.721 | 0.720 | 0.721 | 0.7206 | 0.00058 | 0.08048 |
| 24 | 0.855 | 0.854 | 0.855 | 0.85467 | 0.00058 | 0.06786 |

Average of six determinations

## Results and Discussions

### Optical parameters

Absorption ($\lambda_{max}$) formed in the proposed UV-Visible spectrophotometric method and colored species formed in each two visible spectrophotometric methods, specified amount of azathioprine in solution which contained 10 µg $ml^{-1}$ was taken and the colors were developed following the procedure described earlier. The absorption spectra were scanned on spectrophotometer in the wavelength regions of 350-800 nm against corresponding reagent blank. The regent blank absorption spectrum of each method was also recorded against distilled water / acetonitrile. The results are graphically represented in (fig-2).

### Parameters fixation

In developing these methods, a systematic study of the effects of various relevant parameters encountered in these methods concerned were under taken by verifying one parameter at a time and controlling all other parameters to get the maximum color development for this method, reproducibility and reasonable period of stability of colored species. The following studies were conducted. The results obtained in this method were based on oxidation followed by coupling reaction of azathioprine with MBTH, Ferric chloride and HCl to get a green colored chromogen that exhibited maximum absorption at 620 nm against the corresponding reagent blank. The functional group used for the color development in this method was primary amino group present in azathioprine. A schematic reaction mechanism of azathioprine with MBTH reagent is shown in (Scheme-1). The effect of various parameters like concentration and volume of MBTH and strength of acid were studied by means of control experiments varying one parameter at a time.

### Optical Characteristics

The reference method which follows by beer's law was studied at appropriate wave length of a set of solutions containing different amounts of azathioprine using specified amounts of reagents. In order to test whether the colored species formed in this method (discussed earlier) adhere to beer's law, the absorbance at appropriate wavelength of a set of solutions containing different amounts of azathioprine and specified amount of reagents (as described in the recommended procedure) were noted against appropriate reagent blanks. The beers law plots of the system are illustrated graphically in (figs-3&4). Least square regression analysis was carried out for the slope; intercept and correlation coefficient. Beer's law limit, molar absorptivity, Sandell's sensitivity for azathioprine with each of mentioned reagents were calculated. The optical characteristics are presented in the table -1.

### Precision

The precision of each one amongst the three proposed spectrophotometric methods were ascertained separately from the absorbance values obtained by actual determination of a fixed amount of azathioprine 10 µg $ml^{-1}$ in final solution. The percent relative standard deviations were calculated for the proposed methods and are given table -1.

### Analysis of formulations

Commercial formulations of azathioprine were successfully analyzed by using the aforesaid methods. The values obtained from the proposed and reference methods were compared statistically by the T and F tests and were found that the proposed methods are more suitable than those reported in literature. The results are given in the table-2.This method was also applied for biological samples (Blood). Recoveries values are given in table-6.

### Accuracy

Recovery studies were carried by applying the standard method to drugs samples present in formulations to which known amount of azathioprine. Applying the aforesaid method to biological sample (Blood) to which known amount of azathioprine correspond to 2 mg formulations taken by the patient. By following of standard addition method, 2 mg of label claim was added. And the contents were transferred to 100 ml volumetric flask and dissolved in acetonitrile. Finally the volume was made up to the mark with solvent. The solution was filtered through Whitman No. 41filter paper. The mixed sample solutions were analyzed and their absorbance values were determined. At each level of recovery five determinations were performed and these are presented in Tables-3 &7. The results were compared with expected results and were statistically validated in Table-4.

### Linearity and Range

The linearity of analytical method is its ability to elicit test results that are directly proportional to the concentration of analyze in a sample within a given range.

### Specificity and Selectivity

Specificity and Selectivity is a procedures to used to detect quantitatively an analyze in the presence of components that may be expected to be present in the sample matrix. The results of the present study show that they follow rules of Specificity and Selectivity.

### Repeatability

Standard solutions of azathioprine were prepared and absorbance was measured against the solvent as the blank. The observance of the same concentration solution was measure a six times and standard deviation was calculated and presented in tables-5&8.

## Conclusion

The developed uv-visible sectrophometric method was found to be simple, economical and sensitive. Statistical parameters and recovery study data clearly indicate the reproducibility and accuracy of the method. Analysis of blood samples and formulation containing azathioprine showed no interference from common excipients. Hence this method could be considered for the determination of azathioprine in quality control laboratories.

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