DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR AZATHIOPRINE IN PHARMACEUTICAL DOSAGE FORM.

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ABSTRACT
The method developed used for assay of azathioprine in pharmaceutical dosage form. A simple, rapid and reproducible high performance reverse phase liquid chromatographic method has been developed for quantitative estimation of Azathioprine in tablets form using a, Phenomenex 250 mm x 4.9 mm C18, 5 um, inertsil and UV detection at 240nm. The isocratic elution was used to quantify the analyte and the mobile phase was acetate buffer: Acetonitrile (30: 70) was pumped at 1.0 ml/min. The method was linear between 50-300 µg/ml, statistically validated for its linearity, precision and accuracy. The intra-and - inter day variation was found to be less than 1% showing high precision of the assay method. The method is selective and sensitive which can be used for the estimation of azathioprine in presence of excipients.

KEYWORDS: HPLC, Azathioprine Validation ICH guidelines.

INTRODUCTION

![Structure of Azathioprine](image)

Its IUPAC name: 6-[(1-methyl-4-nitro-1H imidazol-5yl) sulfanyl]-7H-purine. It is having marked effect on T-lymphocytes and immunosuppressive action which is given orally or by I.V route. It is co administered with cyclosporine and corticoids to prevent rejection after transplantation. It is also used in systemic anti-inflammatory states, such as rheumatoid arthritis, lupus erythematosus, colitis ulcerosa, auto immunological hepatitis and Crohn’s disease.

Several methods have been described for determination of Azathioprine in pharmaceutical preparations including HPTLC, HPLC and NMR had been used for the determination of Azathioprine. The proposed method was selective and sensitive for assay of azathioprine in pharmaceutical dosage form with sensitivity of LOQ level.

2. EXPERIMENTAL

2.1 Materials
All chemicals and reagents used were of analytical grade and purchased from Qualigens Fine Chemicals, Mumbai, India.

Acetonitrile HPLC grade, Water HPLC grade, Triethylamine HPLC grade, Sodium acetate AR grade and Azathioprine Standard.

Preparation of solutions
Sodium acetate Buffer solution
0.05M acetate buffer: 2.7 gm of acetic acid and 0.41 gm of sodium acetate in 1000 ml beaker, dissolve in HPLC grade water and dilute to the mark with the same HPLC grade water. pH adjusted to 5.0 with triethylamine Filtered through 0.45 micron membrane filter.

2.2 Preparation of standard stock solution
Standard Solution: 150ppm of Azathioprine (Prepared standard in duplicate for similarity)
Accurately weigh and transfer 50 mg of Azathioprine standard into a 50 ml volumetric flask. Add 1.5 ml acetonitrile and sonicate to dissolve and dilute up to mark with same medium and mix it well.

Dilute 1.5 ml of this solution to 10 ml with ACN solution.
2.3 Selection of wavelength for analysis of Azathioprine
Accurately weigh and transfer 50 mg of Azathioprine standard into a 50 ml volumetric flask. Add 3 ml acetonitrile and sonicate to dissolve and dilute up to mark with same medium and mix it well.

Dilute 1.5 ml of this solution to 10 ml with ACN solution.

The resulting solution was scanned in UV range (200nm–400nm). In spectrum Azathioprine showed absorbance maximum at 240 nm.

Table: 1

<table>
<thead>
<tr>
<th>Conc. µg/ml</th>
<th>Avg. Area</th>
<th>SD</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>85199</td>
<td>1.51</td>
<td>0.001%</td>
</tr>
<tr>
<td>100</td>
<td>975900</td>
<td>70.7</td>
<td>0.007%</td>
</tr>
<tr>
<td>150</td>
<td>1854964</td>
<td>0.54</td>
<td>0.002%</td>
</tr>
<tr>
<td>200</td>
<td>2732600</td>
<td>1.87</td>
<td>0.002%</td>
</tr>
<tr>
<td>300</td>
<td>4308900</td>
<td>1.50</td>
<td>0.003%</td>
</tr>
<tr>
<td>---</td>
<td>1991513</td>
<td>---</td>
<td>----</td>
</tr>
<tr>
<td>---</td>
<td>1.00</td>
<td>---</td>
<td>----</td>
</tr>
</tbody>
</table>

2.4 Validation of the method
The method was validated in terms of linearity, accuracy, precision and ruggedness.

2.4.1 Linearity study
Different aliquots of Azathioprine in range 50 - 300 µg/ml were transferred into series of 10 ml volumetric flasks and the volume was made up to the mark with acetonitrile to get concentrations, respectively.

Figure 1: Linearity plot

Figure 2: Overlay Linearity of Azathioprine 50 to 300 (µg/ml)
Table: 2

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Parameters</th>
<th>Azathioprine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Linearity range</td>
<td>50 - 300µg/ml</td>
</tr>
<tr>
<td>2</td>
<td>Correlation coefficient ($r^2$)</td>
<td>1.00</td>
</tr>
<tr>
<td>3</td>
<td>Intercept</td>
<td>189567</td>
</tr>
<tr>
<td>4</td>
<td>Slope</td>
<td>65984</td>
</tr>
<tr>
<td>5</td>
<td>Precision</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intraday Average % RSD (n = 5)</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>Interday Average % RSD (n = 5)</td>
<td>1.06</td>
</tr>
<tr>
<td></td>
<td>Reproducibility of measurements %RSD</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>% Recovery</td>
<td>99.61 -102.24</td>
</tr>
<tr>
<td></td>
<td>Reproducibility of measurements %RSD</td>
<td>0.33</td>
</tr>
<tr>
<td>6</td>
<td>Limit of detection (µg/ml)</td>
<td>0.0067</td>
</tr>
<tr>
<td>7</td>
<td>Limit of quantification (µg/ml)</td>
<td>0.022</td>
</tr>
</tbody>
</table>

2.4.2 Accuracy
To the preanalysed sample solutions, a known amount of standard stock solution was added at different levels i.e. 80%, 100% and 120%. The solutions were reanalyzed by proposed method.

2.4.3 Precision
Precision of the method was studied as intra-day and inter-day variations. Method Intra-day precision was determined by analyzing the 150ppm solution of Azathioprine for method precision and system precision have been. Inter-day precision was determined by analyzing the 150 ppm solution of Azathioprine daily for three days over the period of week.

2.4.4 Sensitivity
The sensitivity of measurements of Azathioprine by the use of the proposed method was estimated in terms of the Limit of Quantification (LOQ) and Limit of Detection (LOD). The LOQ and LOD were calculated using equation LOD = 3.3 x N/B and LOQ = 10 x N/B, where, ‘N’ is standard deviation of the peak areas of the drugs (n = 3), taken as a measure of noise and ‘B’ is the slope of the corresponding calibration curve.

2.4.5 Repeatability
Repeatability was determined by analyzing 150 ppm concentration of Azathioprine solution for six times.

2.4.6 ruggedness
Ruggedness of the proposed method is determined for 0.1 mg/ml concentration of Azathioprine by analysis of aliquots from homogenous slot by two analysts using same operational and environmental conditions

2.5 Determination of Azathioprine in bulk
Test solution: 150 ppm of Azathioprine
Take 20 tablets, find out its average weight and weigh powder equivalent to about 50 mg of Azathioprine in 50 ml of volumetric flask add some amount of acetonitrile and shaken well. The solution is sonicated for approx. 15 minutes. Following cooling to room temperature, the solution is filled up to volume up to the 50ml with the same acetonitrile diluent only. Mixed well and then filtered through a 0.45µm nylon membrane. The first 5 ml of filtrate are discarded. Transfer the 1.5ml of above stock solution in to 10 ml volumetric flask and make up to the 10 ml with the same diluent acetonitrile and mixed well.

2.6 Application of proposed method for pharmaceutical formulation
3. RESULTS AND DISCUSSION
3.1 Method Validation
The proposed method was validated as per ICH guidelines. The solutions of the drugs were prepared as per the earlier adopted procedure given in the experiment.

3.1.1 Linearity studies
The linear regression data for the calibration curves showed good linear relationship over the concentration range 50-300 µg/ml for Azathioprine. Linear regression equation was found to be $y = 10268x$ $R^2 = 0.999$

3.1.2 Accuracy
The solutions were reanalyzed by proposed method; results of recovery studies are reported, which showed that the % amount found was between 98.54% to 99.98% with %R.S.D. >2.

3.1.3 Precision
The precision of the developed method was expressed in terms of % relative standard deviation (% RSD). These result shows reproducibility of the assay. The % R.S.D. values found to be less than 2, so that indicate this method precise for the determination of both the drugs in formulation.

3.1.4 Sensitivity
The linearity equation was found to be $Y =16921x -71583$ The LOQ and LOD for Azathioprine were found to be 0.0067 and 0.022, respectively.

3.1.5 Repeatability
Repeatability was determined by analyzing 1.2 µg/ml concentration of Azathioprine solution for six times and
the % amount found was between 98% to 102% with % R.S.D. less than 2.

3.1.6 Ruggedness
Peak area was measured for same concentration solutions, six times. The results are in the acceptable range for both the drugs. The results are given in Table 5. The result showed that the % R.S.D. was less than 2%.

Determination of Azathioprine in bulk
The concentrations of the drug were calculated from linear regression equations. The % amount found was between 99.17% to 100.43%.

Application of proposed method for pharmaceutical formulation:
The spectrum was recorded at 240 nm. The concentrations of the drug were calculated from linear regression equation. The % amount was found between 98.36% to 101.31%.

CONCLUSION
The HPLC method has been developed for quantification of Azathioprine tablet formulation. The validation procedure confirms that this is an appropriate method for their quantification in the API and formulation. It is also used in routine quality control of the raw materials as well as formulations containing this entire compound. As per study the future research proposed in impurities and azathioprine in same method.

ACKNOWLEDGEMENTS
The authors are thankful to the Principal and management, R.C. Patel Institute of Pharmaceutical Education and Research, Shirpur (M.S.), India for providing the required facilities to carry out this research work.

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