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RP-HPLC Method Development for the Determination of Azathioprine in Bulk drug and Pharmaceutical Dosage Forms

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ABSTRACT: A RP-HPLC method has been developed for the quantitative estimation of Azathioprine in pure drug and its formulations. The quantification was carried out using RP C_{18} stainless steel column in Isocratic mode with mobile phase containing acetonitrile,water,methanol in the ratio of 25:70:05 and pH adjusted to 4±0.1 using glacial acetic acid at a flow rate of 1.0 ml/minute, and the detection wavelength was set at 280 nm and the linearity was found to be in the range of 30-90 µg/ml.The proposed method was found to be simple,precise,accurate,reproducible for the estimation of Azathioprine in pure drugs and its formulations .
Key words : HPLC, Azathioprine pure drug and tablets.

INTRODUCTION

Azathioprine[1-6] is chemically 6-[(1-Methyl-4-Nitro-1*H* imidAzathioprinel-5yl) sulfanyl]-7*H*-purine, having immunosuppressive action given either orally or intravenously and can be used to prevent rejection in organ transplantation. It is official in U.S.P, B.P and European Pharmacopoeia . A few analytical methods LC-MS, GC-MS were reported in literature and estimated the drug levels in blood plasma[7,8]. Hence this HPLC method was developed which is simple, accurate, precise for the determination of bulk drug and its formulation.

MATERIALS AND METHODS

Experimental Instrumentation:

An isocratic high performance liquid chromatograph (Knauer HPLC) with Wellchrom HPLC-pump K501 and UV-Visible detector K2501 (Knauer) with software C2000 version 1.7, column C_{18}, 250 x 4 mm i.d:, particle size of 5µm and packing material eurosphere-100 was used.

Chemicals and Reagents:

Azathioprine was obtained as gift sample from RPG-Life Sciences-Mumbai. Acetonitrile, water, methanol, ammonium hydroxide and glacial acetic acid were of HPLC grade from Spectrochem Pvt.Ltd, Mumbai-India.

Chromatographic conditions:

The chromatographic column used was a 250x4mm i.d: stainless steel with 5 µm particle size and packing material eurosphere-100.The HPLC instrument operated at ambient temperature. The flow rate of the mobile phase was maintained at 1ml/min.Detection was carried out at 280 nm and the injection volume was 20 µltr.

Working standard of Drug Solution:

Accurately weighed quantity of 50 mg of Azathioprine was transferred into a 100ml volumetric flask. Then added 30 ml of methanol and 1ml of ammonia solution to the flask swirled and sonicated for 5 minutes. Diluted upto the volume with Methanol and mixed

well. 10 ml of this solution was transferred to a 50 ml volumetric flask, diluted with water to volume and mixed well. This solution has given a concentration of 100 µg/ml.

Preparation of stock solution for the commercially obtained tablets:

20 tablets each containing 50 mg of Azathioprine were taken and crushed to get fine fine powder. Then weight equivalent to 50 mg of Azathioprine transferred into a 100 ml volumetric flask. Added about 30 ml of methanol and 1 ml ammonia solution, swirled and sonicated for 5 mins. Diluted upto volume with Methanol and mixed well. 10 ml of this solution was transferred to 50 ml volumetric flask, diluted with water to volume and mixed well. This has given a stock solution of 100 µgm/ml.

Assay procedure:

Working solutions of 10-100 µg/ml were prepared by appropriate dilution of the stock solution with the diluent solution of mobile phase.

Composition and flow rate of the mobile phase was programmed from mother pump and the mobile phase using Acetonitrile, water, methanol (25:70:05) was prepared and the pH adjusted to 4±0.1 with glacial acetic acid and then filtered through 0.45 µ membrane filter. Then it was delivered at 1.0 ml/min for column stabilization.

During this period the baseline was continuosly monitored. The wavelength selected for the detection 280 nm .The prepared dilution containing concentrations of Azathioprine in the range of 10-100 µg/ml were injected.The peak areas were recorded for all the chromatograms.Calibration curve was constructed by plotting peak area vs concentration and the linear relationship was evaluated by calculation of regression line by method of least squares.

RESULTS AND DISCUSSION

The development of an analytical method for the determination of drugs by HPLC has received considerable attention in recent years because of their importance in quality control of drugs and drug products. The objective of this study was to develop a rapid and sensitive HPLC method for the analysis of Azathioprine in pure drug and its formulations using the most commonly employed RP C_{18}- column with UV detection.

The run time was set at 10 min and Azathioprine appeared as chromatogram at Retention time of 3.23 min as shown in **Fig.1**, when the sample was injected 6 times, the retention time of the drug was same .The average of 6 such determinations of peak areas are shown in **Table-1**.

When the concentration of Azathioprine and its respective peak areas were subjected to regression analysis by least square method, a high correlation coefficient was observed (r^2 =0.9999) in the range of 30-90 µg/ml .The only regression of Azathioprine concentration over its peak area ratio was found to be Y=7.0328 X -2.3088 where Y is peak area and X is the concentration of Azathioprine .The regression equation was used to estimate the amount of Azathioprine in the formulation and in validation study (precision and accuracy).

The proposed RP-HPLC method was validated for intraday and interday precision. When the solutions containing 30 and 40 µg/ml of Azathioprine as shown in **Table-2** were repeatedly injected on the same day, the coefficient of variance (CV) in the peak area of the drug for six replicate injections was found to be less than 0.1%. Also the interday variation (3 days and six injections) was found to be less than 0.1 %. Thus, the results showed that the proposed RP-HPLC method is highly reproducible.

When the known amount of drug solution (10 and 20 µg) was added to a known concentration of drug solution (30 µg/ml) there was a high recovery (100.98±0.6%) of Azathioprine as shown in **Table-3** indicating that the proposed method is highly accurate.

The HPLC method developed in the present study has been used to quantify Azathioprine in parenteral dosage forms also. Azathioprine in tablets (each containing 50 mg of the drug) was analyzed as per the procedure described above .The average drug content was found to be 49.99±0.05% of the labeled amount as shown in **Table-4**. No interfering peaks were found in the chromatogram indicating that excipients used in the formulation didn't interfere the estimation of the drug by the proposed RP-HPLC method.

CONCLUSION

The proposed HPLC method was found to be highly accurate, sensitive and precise; therefore this method can be applied for the routine quality control and analysis of Azathioprine in its tablets and dosage forms.

Table 1 :Calibration of the HPLC method for the estimation of Azathioprine

Concentration of Azathioprine (µg/ml)	Peak area *	C.V (%)
0.00	0	0
30	207.38	0.09904
40	275.32	0.07474
70	491.48	0.04187
90	631.41	0.03259

*Mean of six determinations
Regression Equation: $Y = 7.0328X - 2.3088$ ($r^2 = 0.9999$)

Table 2:Intra and Inter day precision for the Azathioprine assay in Pharmaceutical Dosage forms by the proposed HPLC method

Concentration of Azathioprine (µg/ml)	Concentration of Azathioprine found on			
	Intraday		Inter-day	
	Mean (n=6)	C.V. (%)	Mean (n=6)	C.V. (%)
30	30.06	0.09902	30.09	0.09990
40	40.04	0.07473	40.01	0.07471

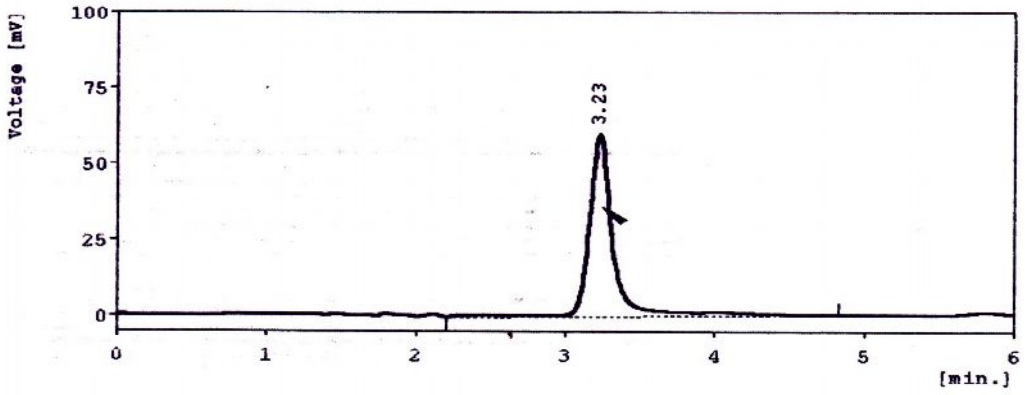
Table 3: Recovery of Azathioprine using the proposed HPLC method

Amount of drug added (µg)	Amount found (µg) Mean (± S.D) (n = 6)	% recovery Mean (± S.D) (n = 6)
10	9.98±0.04	99.96±0.5
20	20.02±0.06	100.98±0.6

Table 4 : Mean (± S.D) amount of Azathioprine in tablet dosage forms by proposed HPLC method

Formulation (tablets)	Labelled amount of drug (mg)	Amount found (µg) Mean (± S.D) (n = 6)	% Purity Mean (± S.D)
T_1	50	49.99±0.05	100.02±0.06

Fig .1:Chromatogram of Azathioprine showing Retention time of 3.23 mins



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