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Sensitive and spectrophotometric methods for the determination of Azathioprine in tablets using indigo carmine and methyl orange

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Abstract

A simple, economical, precise and fast visible spectrophotometric method has been developed for the determination of Azathioprine in tablet dosage form. Developed method is based on the formation of extractable colored complex of drug with colouring agent Indigo Carmine and Methyl orange. A wavelength maximum was found to be 683.7 nm and 566 nm. The concentration range of 15-50 μg mL^{-1} with of percentage recovery 100.05-101.31 %, while the linear regression, LOD and LOQ were 99.42-99.08 %, 0.38 and 0.84 μg mL^{-1} and 0.74 and 0.18 μg mL^{-1}, respectively. Two new sensitive spectrophotometric methods are proposed for the determination of Azathioprine using bromate-bromide mixture and two dyes, methyl orange and indigo carmine, as reagents. The methods entail the addition of a known excess of bromate-bromide mixture to Azathioprine, in acetic acid medium followed by determination of residual bromine by reacting with a fixed amount of either indigo carmine and measuring the Absorbance at 683.6 nm (Method A) or methyl orange measuring the absorbance at 566 nm (Method B).

Keywords: Azathioprine; Spectrophotometric; Indigo Carmine; Methyl orange.

1. Introduction

Azathioprine [1-8] is chemically 6-[(1-methyl-4-nitro-1*H* imidazol-5yl) sulfanyl]-7*H*-purine (Fig. 1). It is having immunosuppressive action which is given orally or by I.V route. It has marked effect on T-lymphocytes and suppresses cell mediated immunity.

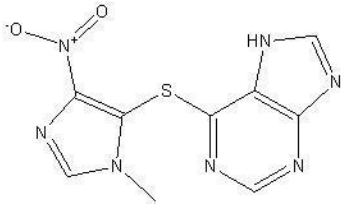

Fig. 1. Chemical structure of Azathioprine.

It is mainly used to prevent rejection in organ transplantation and also useful in a variety of auto-immune disorders. It is converted in the body to the anti-metabolite Mercaptopurine. It is

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official in U.S.P, European Pharmacopoeia and in British Pharmacopoeia. In literature no simple analytical methods were reported for its quantitative estimation in bulk drug as well as its formulations .The present work deals with the development of two simple and sensitive colorimetric methods for the quantitative estimation in bulk drug as well as its formulations. Author of the article and his research team has developed a UV Method development different pharmaceutical dosage form [9-17].The proposed method was validated as per ICH guidelines According to International Conference on Harmonization (ICH), the objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose [18, 19] and its updated international convention [20].

2. Experimental

2.1. Material and methods

UV Visible spectrophotometer (Shimadzu Model 1601) was employed with spectral bandwidth of 1 nm and wavelength accuracy of 0.3 nm (with automatic wavelength correction with a pair of 1 cm matched quartz cells). Single component tablet formulations of Azathioprine (2 mg) (formulation A Eurepa, manufactured by Torrent Pharma. Ltd., Ahmadabad) were purchased from local market. All chemicals and reagents used were of AR/HPLC grade, Chloroform, ammonia (SD'S) and methanol were used for mobile phase preparation and as solvent. All chemicals used in this study were analytical grade and used without further purification.

2.2. Preparation of calibration curve

Standard drug solution (200 μg mL^{-1}) was prepared in double distilled water and was diluted with same, so as to give several dilutions in concentration range 10-50 mL of drug. To 10 mL of each dilution taken in separating funnel, 10 mL of Indigo Carmine solution was added and shaken gently. Then 5 ml of chloroform was added reaction mixture was shaken gently and allowed to stand so as to separate aqueous and chloroform layer. The chloroform layer was separated out and transferred to 50 mL of volumetric flask. Reaction mixture was extracted further with 3 mL and 2 mL of fresh chloroform and combined it with previously extracted chloroform layer containing complex. Absorbance of this final extracted chloroform layer was measured at wavelength maxima 438.6 nm against blank. Calibration curve was plotted between concentration of drug and measured absorbance.

2.3. Standard drug solution:

A stock standard solution containing 2 mg mL^{-1} Azathioprine was prepared by dissolving accurately weighed 100 mg of pure drug in water and diluting to the mark in a 250 mL calibrated flask. This solution was used for titrimetric work, and for spectrophotometric work, the same was diluted appropriately with water to get a working concentration of 5 μg mL^{-1} for method A, and 20 and 40 μg mL^{-1}.

2.4. Sample solution

Twenty Azathioprine tablets were powdered and an accurately weighed quantity powder equivalent to 10 mg of Azathioprine from each brands were dissolved in methanol. The excipients were separated by filtration using Whatmann filter paper (No. 41) and the filter paper washed three times with distilled water for effective liberation of drug from the core. Filtrate and washings of the tablet samples were transferred into 100 ml flask and diluted to the mark with absolute ethanol, and the spectrophotometric procedure was followed.

2.5. Reagent Preparation

0.1 mg of methyl orange was weighed and transferred into a 100 mL standard flask and the volume was made up to the mark to get the required concentration (0.1 %w/v). 50 mg of Indigo Carmine was weighed and transferred into a 100 mL standard flask and the volume was made up to the mark to get the required concentration (0.1%w/v).

2.6. Method using indigo carmine (method A)

Aliquots of pure Azathioprine solution (50 µg mL^{-1}) were transferred into a series of 10 mL calibrated flasks and the total volume was adjusted to 4.0 mL with water. To each flask were added 5 mL of 1 mol L^{-1} Acetic acid followed by 1 mL of bromate-bromide mixture (10 µg mL^{-1} in KBrO3). The content was mixed well and the flasks were set aside for 10 min with occasional shaking. Finally, 1 mL of 50 µg mL^{-1} indigo carmine solution was added to each flask, diluted to the mark with water and the absorbance of solution was measured at 683.7 nm against reagent blank after 10 min.

2.7. Method using methyl orange (method B)

Varying aliquots (0.5-3.0 µg mL^{-1}) of standard 35 µg mL^{-1}Azathioprine solutions were measured accurately and delivered into a series of 10 mL calibrated flasks and the total volume was brought to 5.0 mL with ether. To each flask were added 1 mL of 0.5 M acetic acid and 1.0 mL of bromate-bromide mixture (30 µg mL^{-1} in $KBrO_3$) by means of micro burette; the flasks were let stand for 15 min with occasional shaking. Then, 1 mL of 50 µg mL^{-1} methyl orange solution was added to each flask, the volume was adjusted to the mark with water and mixed well. The absorbance of each solution was measured at 566.0 nm against a reagent blank after 10 min. In either method, the concentration of the unknown was read from the calibration graph or computed from the regression equation derived from the Beer's law data.

2.8. Analysis for tablets

An amount of finely ground tablet powder equivalent to 100 mg of Azathioprine was accurately weighed into a beaker; 10 mL of glacial acetic acid was added and stirred for 20 min, and warmed. Then, the content was transferred to a 100 mL calibrated flask, the beaker was washed with water and the washings were also transferred to the flask, and the volume was diluted with water to the mark, mixed well, and filtered using a Whatmann No 40 filter paper. First 10 mL portion of the filtrate was discarded and a suitable aliquot of the subsequent portion (200 µg mL^{-1} Azathioprine) was diluted appropriately to get 15 and 40 µg mL^{-1} concentrations for analysis by method A and method B, respectively.

2.9. Optimization of the method variables method validation

2.9.1. Effects of reagent concentration

The effect of Indigo Carmine and methyl orange concentration on the reaction was checked out at room temperature and away from direct sunlight. The reaction of Azathioprine was dependent on the concentration of dye used. A concentration of 0.5% (w/v) was selected as the optimum reagent concentration. Higher concentrations caused a distinct decrease in the absorbance.

2.9.2. Effect of time

The absorbance of the solution was measured after 20 minutes after adding reagent, and up to 3.5 hrs. The reactions was slow and the formed colour was stable up to 6 hrs

2.9.3. Validation of the Developed Methods

The developed methods for simultaneous estimation of Azathioprine were validated as per ICH guidelines.

2.9.4. Accuracy

To check the accuracy of the developed methods and to study the interference of formulation additives, analytical recovery experiments was carried out by standard addition method. From that total amount of drug found and percentage recovery was calculated (Table 1).

Table 1
Accuracy of the proposed method.

Sample	Label Claim	Estimated amount (mg/tab)	Spike Level (%)	Amount of Drug Added	Amount of Drug recovered	% Recovery	RSD (% n=5)
Method A	50	50.10	50	20	50.03	100.00	0.04
			100	40	49.92	99.92	0.27
			150	60	49.99	99.99	0.93
Method B	50	50.04	50	20	49.98	99.94	0.17
			100	40	50.01	100.06	0.62
			150	60	50.05	100.05	0.39

2.9.5. Repeatability

To check the degree of repeatability of the methods, suitable statistical evaluation was carried out. Six samples of the tablet formulations were analyzed for the repeatability study. The standard deviation, coefficient of variance and standard error was calculated.

2.9.6. Intermediate Precision (inter-day and intra-day precision)

The intra and inter-day precision was calculated by assay of the sample solution on the same day and on different days at different time intervals respectively. The results are presented in Table 2. A linear correlation was found between absorbance at λ max and concentration of STV in the ranges given in Table 1. The graphs showed negligible intercept as described by the regression equation:

$$Y = a + bX$$

(where Y = absorbance of 1-cm layer of solution; a = intercept; b = slope and X = concentration in μg mL^{-1}). Regression analysis of the Beer's law data using the method of least squares was made to evaluate the slope (b), intercept (a) and correlation coefficient(r) for each system and the values are presented in Table 3. The optical characteristics such as Beer's law limits, molar absorptivity and Sandell sensitivity values of both methods are also given in Table 3. The limits of detection (LOD) and quantitation (LOQ) calculated according to ICH guidelines are also presented in Table. 1 and reveal the very high sensitivity of the methods.

Table 2
Intraday, Interdays, data of tablet formulation.

sample	Intra day precision %COV (n=6)	Interday precision %COV		
		Day 1[a]	Day 2[a]	Day 3[a]
Method A	0.852	0.428	0.145	0.662
Method B	0.620	0.27	0.90	0.75

COV: Coefficient of variance

Table 3
Analytical and regression parameters of the proposed methods.

Parameter	Indigo Carmine	methyl orange
Beer's law limits	15-50	15-50
λ_{max}, nm	683.7 nm	566 nm
Molar absorptivity	3.6×10^3	1.4×10^3
Sandell sensitivity	0.0281	0.193
Limit of detection	0.093	0.036
Limit of quantification	0.73	0.41
Regression equation *		
(a) Intercept	0.0131	0.249
(b) Slope	0.032	0.004
Correlation coefficient, (r)	0.9989	0.9996

2.9.7. Recovery studies

The method was validated by recovery study were carried out by the addition of different amount of drugs to pre analyze solution (10 µg mL^{-1}). From the stock solution of 100 µg mL^{-1} of each drug 1 mL solution was taken in each of four volumetric flask (10ml), then 1.2, 0.8, 0.4 mL of mixed standard stock solution (200 µg mL^{-1} of Azathioprine) added in three flasks so that remaining one flask contains no added solution. These solutions were scanned at 298 nm. Percentage recovery was found in the range of 100 % to 105%.

2.9.8. Robustness and Ruggedness

Robustness and Ruggedness of the method were also studied by altering wavelength of estimation and changing the dye's concentration which were also within the acceptable limit with respect to % RSD (Table. 4). In case of ruggedness difference in the estimation was studied by means of analyzing the samples in two different days by following same procedure and the results were summarized in (Table 4)

3. Results and Discussion

The proposed spectrophotometric methods are indirect and are based on the determination of the residual bromine (*in situ* generated) after allowing the reaction between Azathioprine and a measured amount of bromine to be complete. The bromine was determined by reacting it with a fixed amount of methyl orange, indigo carmine or thymol blue dye. The methods make use of bleaching action of bromine on the dyes, the decolouration being caused by the oxidative destruction of the dyes. Azathioprine when added in increasing amounts to a fixed amount of *insitu* generated bromine consumes the latter proportionately and there occurs a concomitant

falls in the amount of bromine. When a fixed amount of dye is added to decreasing amounts of bromine, a concomitant increase in the concentration of dye results. Consequently, a proportional increase in the absorbance at the respective λ_{max} is observed with increasing concentration of Azathioprine, as shown by the correlation coefficients of 0.9989 and 0.9996 for method A and method B, respectively. The reaction between *insitu* bromine and Azathioprine, and bleaching of dye by bromine, acetic acid medium was found to be ideal. The reaction was complete in 10 and 15 min in method, and the same quantity of acid was employed for the estimation of the dye. Contact time of 15 or 25 min is not critical and any delay up to 55 min in either method had no effect on the absorbance.

Table 4
Robustness and day to day variation of the method.

Parameters Studied	**Recovery (%±RSD**
Indigo Carmine Concentration(%,w/v)	
0.05	99.95±0.16
0.15	99.97±0.04
Wave length	
533 nm	72.40±0.29
589 nm	89.04±0.04
683.6 nm	99.99±0.15
Ruggedness(day-to-day variation)	
Day1	95.74±0.01
Day 2	98.89 ±0.13
Day 3	99.94±0.07
Methyl orange Concentration(%,w/v)	
0.05	101.11±0.63
0.15	99.87±0.27
Wave length	
473 nm	83.54±0.01
526 nm	90.12±0.8
566 nm	99.96±0.2
Ruggedness(day-to-day variation)	
Day1	99.01±0.43
Day 2	99.12±0.25
Day 3	100.1±0.28

The absorbance of the measured colour was constant for several days even in the presence of the reaction product. In present research work a UV Spectrometric method has been developed for determination of Azathioprine from its tablet formulations. The developed method was based on formation of absolute ethanol extractable complex of drug with Indigo Carmine in and methyl orange double distilled water. Wavelength maxima of Azathioprine were found to be at 683.7 nm and 566.0 Indigo Carmine and methyl orange respectively. Linearity was observed in concentration range of 15-50 μg mL^{-1}. Percentage label claim estimated for tablet formulation was found to be in the range of 100.05-101.31 % and respective values of standard deviation were found in the range of 0.5981-0.9371 for different colouring agents tablet formulations of Azathioprine (Table 1). To fix the linearity a calibration curve was constructed by plotting the absorbance as a function of the corresponding concentrations. The regression equation for the results was:

$$A=0.5486 * -0.8731 (r = 0. 0.9989)$$

where *A* is the absorbance at 683.7 nm, *C* the concentration of Azathioprine in μg mL^{-1} in the range of 15-50 μg mL^{-1} and *r* is the correlation coefficient. The molar absorptivity (*å*) was found to be

$$0.3802* 0.1093 \text{ lit mol } cm^{-1}.$$
$$A=0.3713 * -0.3015 (r = 0.\ 0.9996)$$

where *A* is the absorbance at 566.0 nm, *C* the concentration of Azathioprine in μg mL^{-1} in the range of 15-50 μg mL^{-1} and *r* is the correlation coefficient. The molar absorptivity (*å*) was found to be

$$2.552 * 0.6271 \text{ lit mol } cm^{-1}$$

The limit of detection (LOD) and limit of quantitation (LOQ) were determined using the formula: LOD or LOQ = κ S.D.*a*/*b*, where κ = 3 for LOD and 10 for LOQ, S.D.*a* is the standard deviation of the intercept and *b* is the slope. The LOD and LOQ were 0.38 and 0.84 μg mL^{-1}, respectively. The detection and quantitation limits determined were 0.74 and 0.18 μg mL^{-1} respectively. These low values indicated the high sensitivity of the purposed method. Recovery studies were carried out by adding a known quantity of pure drug to a pre-analyzed formulations and the proposed method was followed. From the amount of drug found, percentage recovery was calculated. The results of analysis and recovery studies are given in Table.1. Inter-day precision was calculated from results from the same samples analyzed on five consecutive days. The results obtained are listed in Table 3. The low relative standard deviation (RSD 0.428 0.145, 0.662 at three different level (intra-day precision), 0.27, 0.90, 1.05 at three different level (inter-day precision) showed the good precision of the method.

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