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A Stability Indicating RP-High-Performance Liquid Chromatographic Method for the Determination of Azathioprine (AZA) in Tablet

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Abstract

A simple, selective, rapid, precise and economical RP-HPLC stability-indicating method has been developed and validated for the quantitative estimation of AZA in tablet dosage form. An isocratic separation was completed using a C18, 250×4.6 mm, 5 µm particle size columns with a flow rate of 1 ml/minute and using a PDA detector to monitor the elute at 284 nm. The mobile phase comprised of Water:Acetonitrile (80:20v/v). The drug candidate was subjected to oxidation, hydrolysis, photolysis and thermal degradation. AZA was found to degenerate in acidic, basic, and oxidative, Photolytic condition, under the neutral condition and also dry heat. Complete separation of degenerated products gained from the parent drug candidate. All degradation products in an overall analytical run time of approximately 10 minutes with the parent compound AZA eluting at around 6.44 minutes. The scheme was linear over the concentration range of 10-90 µg/ml (r^2= 0.999) with a limit of detection and limit of quantitation of 0.21369 µg/ml and 0.647546 µg/ml respectively. The method has the requisite accuracy, selectivity, sensitivity, precision and to assay AZA in Tablet. Degradation products as a result of the stress studies did not get in the way with the detection of AZA, and the assay is thus stability-indicating.

Keywords: Stability-indicating, RP-HPLC, PDA, oxidation, hydrolysis, photolysis

1. INTRODUCTION

According to ICH (International Conference on Harmonization) Q1A (R2) drug stability tests guidelines the stability-indicating assay method must employed for the analysis of stability samples.[1, 2] The stress study involves the degradation behavior of drug under hydrolytic, oxidation, photolysis and thermal conditions. It is important that the degradation products that which are formed under a variety of environmental conditions should be identified and degradation pathways established.[3] In order to detect accurately and to quantify drug-related impurities, a stability indicating analytical method is needed. Ideally, such a method should resolve all drug-related impurities from the parent and from each other[4-6]

Chemically AZA is 6-[(1-methyl-4-nitro-1H imidazol-5yl) sulfanyl]-7H-purine[7-9] (see fig 1). It is having a notable response on T-lymphocytes and immunosuppressive action which is given orally or by I.V route. It is co-administered with corticoids and cyclosporine to forbid rejection after organ transplantation. It is also used in systemic anti-inflammatory states, such as lupus erythematosus, rheumatoid arthritis, autoimmunological hepatitis, Crohn's disease and colitis ulcerosa. Several techniques were reported for the determination of AZA in pharmaceutical preparations including HPLC[10], HPTLC[11], ^{1}H NMR[12], UV-spectrophotometry[13], spectrophotometric and atomic absorption spectrometric[14] and capillary zone electrophoresis[15] had been used for the determination of AZA. The International Conference on Harmonization (ICH) guideline entitled “Stability testing of new drug substances and products” requires that stress testing is carried out to elucidate the inherent stability characteristics of the active substance.[1] The proposed method turn into validated in line with ICH guidelines as indicated by International Conference on Harmonization (ICH).[16-17] Regression analysis is to be performed on the linearity curve.[18] After thorough literature survey, only one HPLC method was found to be reported and the reported method was found to be more time taking and more solvent consuming as it shows the prolonged retention time for the pure drug. The proposed validated method is precise, accurate, specific more cost effective for the quantitative determination of AZA in pharmaceutical dosage form.

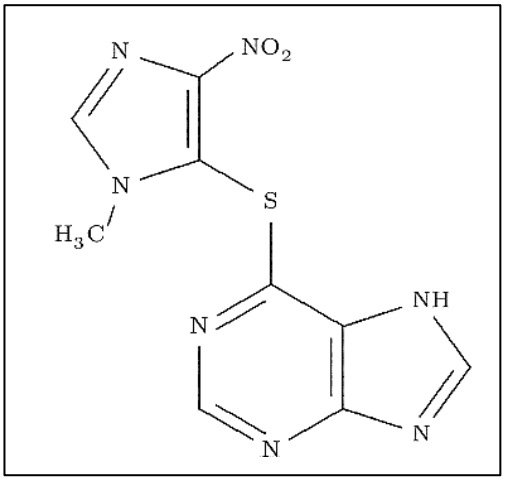

Fig 1: Structure of Azathioprine (AZA)

2. MATERIALS AND METHODS

2.1. **Chemicals and reagents** AZA pure compound was kindly supplied by Aarti Drugs, Boisar, India and was used without further purification. AZA Tablet, (Azoran 50 mg, RPG Life Sciences Ltd.) containing 50 mg AZA as per label claim was acquired from a local pharmacy. All the chemicals utilized were of HPLC grade. Purified HPLC grade water was achieved by double distillation and filtered by means of a filter (Millipore, Milford, MA) and

was used to make all the solutions. The mobile phase was vacuum filtered via 0.22 mm polytetrafluoroethylene (PTFE) filter membrane and degassed using a sonicator to remove the dissolved gasses prior to use.

2.2. **HPLC instrumentation and conditions** The HPLC system includes a pump (Jasco PU-2080, intelligent HPLC pump) with injecting facility programmed at 20 µl capacity per injection was used. The detector comprised of a PDA (Jasco PDA 2075) operated at a wavelength of 284 nm. The software used was Jasco CHROMPASS. The most common separation variables include solvent type, mobile phase PH, column type, and temperature. The chromatographic separation was performed using a HiQsil, C18 5µm, 250×4.6 mm column. Separation was done using a mobile phase comprising of Water-acetonitrile (80:20 v/v) solution at a flow rate of 1 ml/minute. The eluent was observed using PDA at a wavelength of 284 nm. The column was perpetuated at ambient temperature and injection volume of 20µl was used.

2.3. **Preparation of solutions**

2.3.1. **Selection and Preparation of mobile phase**

Various mobile phases containing water, acetonitrile, methanol, in different ratios were tried with different flow rates. Good symmetrical peak was found with the mobile phase comprising acetonitrile and water in the ratio 80:20 (v/v). The mobile phase was prepared by mixing 800 mL of HPLC grade acetonitrile with 200 mL of MilliQ water. The mobile phase made was sonicated for 10 min and filtered through the 0.45 µm membrane filter.

2.3.2. **Preparation of Standard Solutions:**

Accurately measured and transferred 10 mg of AZA to 10ml volumetric flask. 2 ml of Acetonitrile was added to dissolve AZA completely it is sonicated. And the volume was made up to the mark with Acetonitrile, to obtain a solution of 1000g/ml, the resultant solution was sonicated for 5 minutes and filtered through a 0.45µ membrane filter.

2.3.3. **Preparation of Sample Solution (Tablets)**

To determine the content of AZA in tablets (label claim: 50 mg AZA) 20 tablet of AZA was weighed and finely powdered. A quantity equivalent to 10mg was transferred into 10 ml volumetric flask, and 2 ml of Acetonitrile was added. The above solution was sonicated, and the volume was made with Acetonitrile and filtered through a 0.45µ membrane filter to obtain a concentration of 1000 µg/ml. 1 ml of this solution was pipetted out and transferred to a 10 ml volumetric flask and made the volume up to mark with dilution solution to get a concentration of 100µg/ml. 1 ml of 100 µg/ml solution was pipetted out and transferred to a 10 ml volumetric flask and made the volume up to mark with dilution solution to get a final concentration of 10µg/ml. 20 µl of the sample and standard solutions were injected in HPLC in triplicate and chromatograms were recorded.

2.4. **FORCED DEGRADATION STUDIES**

Stress testing is defined as the stability testing of drug substances and drug products under conditions exceeding those used for accelerated testing.[19] Although it is an intrinsic part of the information provided to regulatory authorities in registration application dossiers.[1] Forced degradation studies should be performed whenever a stability indicating method is required.[20] Stability testing is a vital part of the process of drug product development.[21, 22] The motive of stability testing is to endow proof of how the quality of a drug substance or drug product changes with time under a different environmental conditions, (for example temperature, humidity, and light) and allow recommendation of storage conditions, retest durations, and shelf life and to be established and to reveal the degradation mechanisms such as hydrolysis, oxidation, thermos-analysis or photolysis of the pure drug and drug product.[1, 23] The two most important aspects of the pharmaceutical product that plays a vital role in the shelf-life determination are assay of the active pharmaceutical ingredient and the degradation products produced during forced degradation studies.[19]

2.4.1. **Preparation of stock solution** Accurately weighed and transferred 10mg of AZA to 10ml volumetric flask, added 2 ml of Acetonitrile and sonicated to dissolve it completely and the volume was made up to the mark with Acetonitrile, to obtain a solution of 1000µg/ml, resultant solution was sonicated for 5 minutes and filtered through 0.45µ membrane filter. 5 ml of this solution was pipetted out and transferred to a 50 ml volumetric flask and made the volume up to mark with dilution solution to get a final concentration of 100µg/ml.

2.4.2. **Stress testing under acidic conditions** In Round bottomed flask (RBF) 5 ml of AZA stock was taken, and to it 5 ml of 1 N solution of HCL was added, it was then heated under reflux on a heating mantle at 80°C for 3 hrs. After cooling at room temperature acid was neutralized with 1 N NaOH.

2.4.3. **Stress testing under Basic Conditions** In Round bottomed flask (RBF) 5 ml of AZA stock was taken, and to it, 5 ml of 1 N solution of NaOH was added, it was then heated under reflux on a heating mantle at 80°C for 3hrs. After cooling at room temperature base was neutralized with 1 N HCl.

2.4.4. **Stress testing under Neutral Conditions**

In Round bottomed flask (RBF) 5 ml of AZA stock was taken, and to it, 5 ml of Water was added, it was then heated under reflux on a heating mantle at 80°C for 3 hrs.

2.4.5. **Oxidative stress testing**

5 ml of AZA stock was taken in a 10 ml stoppered volumetric flask, and to it, 5 ml 3% V/V solution of H_2O_2 was added, it was solicited for 2 minutes. To ensure even mixing of the drug in solution and then kept in the dark condition at room temperature for 24 hours.

2.4.6. **Stress testing under dry heat conditions**

AZA was kept in hot air oven at 80°Cor 3 hours. From the above stressed sample, 10 mg weighted accurately and diluted with acetonitrile to make up the volume up to 10 ml and sonicated for 10 minutes.

2.4.7. **Stress testing under Photolytic conditions**

AZA was directly exposed to daylight for 8 hours. From the above stressed sample, 10 mg weight accurately and diluted with acetonitrile to make up the volume to10 ml and sonicated for 10 minutes.

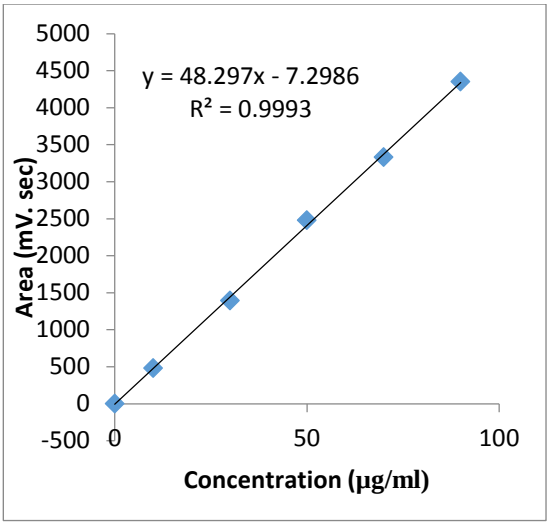

Fig 2: Linearity of Azathioprine

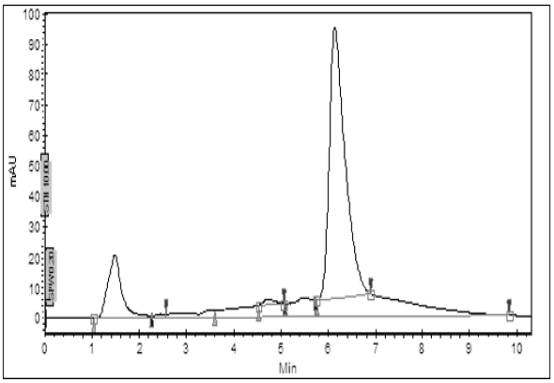

Fig 3: Chromatogram for Acid Degradation

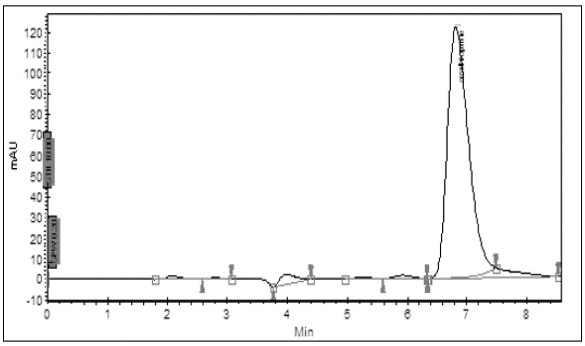

Fig 4: Chromatogram for Base Degradation

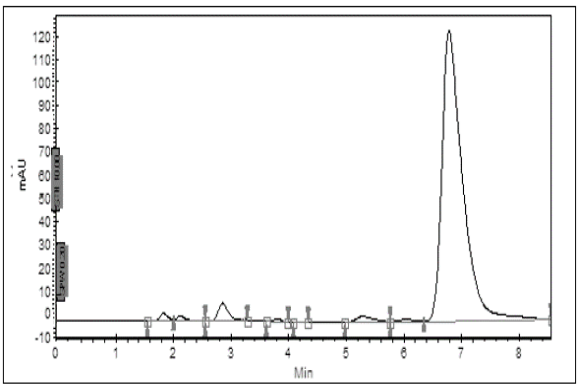

Fig 5: Chromatogram for Neutral Degradation

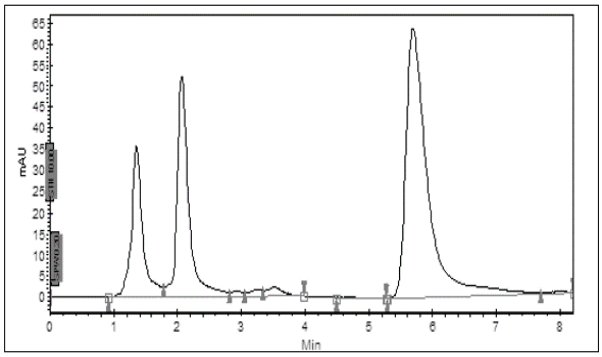

Fig 6: Chromatogram of Oxidative Degradation

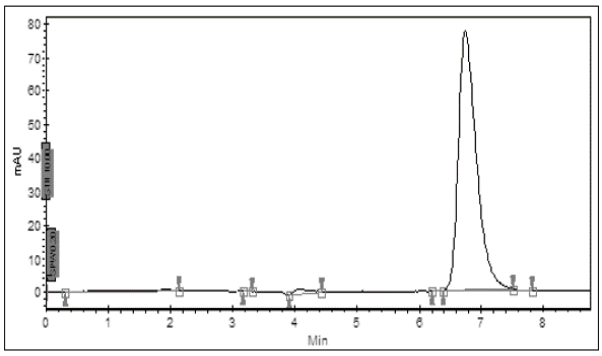

Fig 7: Chromatogram of Photolytic Degradation

3. RESULTS AND DISCUSSIONS

The HPLC procedure was enhanced with a purpose to develop a stability indicating assay method. Pure drug candidate together with its degenerated products was injected and run in different solvent systems. Initially, acetonitrile and water in particular ratios had been tried. It was observed that water and acetonitrile system shows good results, water:acetonitrile in the ratio of 50:50 was not able to give good peak symmetry with adequate retention time. An attempt to enhance peak symmetry was done by increasing the volume of acetonitrile to the mobile phase. The presence of more acetonitrile in mobile phase resulted in excellent overall chromatography with appropriate peak symmetry and complete baseline resolution. Finally, the mobile phase consisting of Water and acetonitrile (80:20 v/v) was taken for validation and stability studies. The method was validated with respect to parameters including linearity, limit detection (LOD), limit of quantitation (LOQ), system suitability, recovery, precision, accuracy, and selectivity and a summary of validation parameters were presented in Table 1.

Parameters	Inference
Linearity range	10-90 µg/ml
Correleation coefficient	0.999
LOD	0.21369 µg/ml
LOQ	0.647546 µg/ml
Recovery	99-101%

Table 1: Summary of Validation Parameters

AZA showed linearity in the concentration range of 10-90 µg/ml (r2 = 0.999) for HPLC.

Linearity was analyzed by determining ten standard

working solutions containing 10-90 µg/ml thrice in triplicate. Peak areas of AZA were plotted versus AZA concentration and linear regression analysis performed on the resultant curve. For HPLC method the linearity of calibration graphs and adherence to the system to Beer's law was validated by the G value of correlation coefficient and the standard deviation for intercept value was less than 2%.

Sr. No.	Conc (µg/ml)	Area mV. Sec
1	10	480
2	30	1392
3	50	2478.3
4	70	3330.6
5	90	4349.5

Table 2: Linearity Data

Sr. No.	RT (min)	Theoretical plates	Tailing factor
1	6.987	5319	1.21
2	6.986	5387	1.23
3	6.989	5290	1.21
4	6.989	5302	1.2
5	6.989	5365	1.2
Average	6.988	5332.6	1.21
SD	0.001265	37.26983	0.010954
% RSD	0.018101	0.698905	0.905327

Table 3: System suitability test parameters for Azathioprine 8

The Limit of Detection and Limit of Quantitation were determined based on signal-to-noise ratio and were based on analytical responses of 3 and 10 times the background noise, respectively. The LOD was found to be 0.21 9 µg/ml. The LOQ was determined to be 0.647546µg/ml

Proposed method when used for extraction and subsequent estimation of AZA from the pharmaceutical dosage form after spiking with an additional drug, afforded recovery of 99-101% and mean recovery of AZA from the marketed formulation are listed in Table 4.

The precision of assay was determined with respect to each repeatability and reproducibility. An amount of the product powder identical to 100% of the label claim of AZA was competently weighed and assayed. Method repeatability was determined by 6 replicate applications and 6 times folds of a sample solution at the analytical concentration. The repeatability of sample application and measurement of peak area for active compound have been expressed in terms of % RSD (relative standard deviation). Method repeatability was obtained from RSD value by repeating the assay two times in a same day for intra-day precision. Inter-day precision was assessed by the assay of three sample sets on different days (inter-day precision). The intra-day and inter-day variation for determination of AZA was carried out at three different concentration levels 8, 40, 80 µg/ml (Table 5, 6 and 7).

The efficiency of the assay was determined by interpolation of replicates (n=3) peak areas of accuracy standards (8, 40, 80µg/ml) from a calibration curve prepared as previously described.

Accuracy level	Amount added in	Area mV. Sec	Amount recovered in µg	Average % Recovery	Average %RE
90	50	4349.5	50.0283	100.057	0.03145
90	50	4336.8			
90	50	4356.1			
100	50	4839.9	50.1408	100.282	0.14082
100	50	4831.4			
100	50	4836.1			
110	50	5308.3	50.02	100.04	0.0182
110	50	5312.5			
110	50	5317.8			

Table 4: Recovery of Azathioprine

Conc. (µg/ml)		1	2	3	Average	SD	% RSD
8	Area mV. Sec	346	339.6	330.9	329.7	14.2095	4.309828
	Conc. µg/ml	8.440671	8.284543	8.072307	8.043033	0.346641	4.309828
40	Area mV. Sec	1636.9	1691.3	1622.8	1640.733	33.25242	2.02668
	Conc. µg/ml	39.93218	41.25927	39.58821	40.0257	0.811193	2.02668
80	Area mV. Sec	3263.1	3298.9	3265.3	3304.9	19.04022	0.576121
	Conc. µg/ml	79.60334	80.47668	79.65701	80.62305	0.464486	0.576121

Table 5: Determination of Inter-day Precision

Conc. (µg/ml)		1	2	3	average	SD	% RSD
8	Area mV. Sec	344.2	329.1	315.8	329.7	14.2095	4.309828
	Conc. µg/ml	8.39676	8.028396	7.703942	8.043033	0.346641	4.309828
40	Area mV. Sec	1629.7	1678.1	1614.4	1640.73	33.25242	2.02668
	Conc. µg/ml	39.75654	40.93726	39.38329	40.0257	0.811193	2.02668
80	Area mV. Sec	3283.8	3310.1	3320.8	3304.9	19.04022	0.576121
	Conc. µg/ml	80.10831	80.7499	81.01093	80.62305	0.464486	0.576121

Table 6: Determination of intra-day precision

Conc. (µg/ml)		1	2	3	Average	SD	% RSD
8	Area mV. Sec	320.5	326.7	332.9	331.4	8.254696	2.490856
	Conc. µg/ml	7.818599	7.969848	8.121097	7.969848	0.151249	1.897766
40	Area mV. Sec	1631.2	1653.7	1636.8	1648.267	18.64067	1.130926
	Conc. µg/ml	39.79313	40.34202	39.92974	40.02163	0.285748	0.713984
80	Area mV. Sec	3282.1	3311.8	3293.9	3295.15	11.35266	0.344526
	Conc. µg/ml	80.06684	80.79137	80.3547	80.40431	0.364804	0.453712

Table 7: Determination of intra-day precision

Degradation	**Area mV. sec**	**% Degradation**
Acid	2271.2	9.53%
Base	2075.5	17.32%
Oxidation	1889.8	24.72%
Neutral (Wet)	2128.2	15.22%
Photostability	1356.4	10.22%

Table 8: Stressed study data of Azathioprine

Sr. No	Amount per tablet	Area mV.sec	Conc.(µg/ml)	% Assay	Average	S.D	%RSD
1	100	421.5	10.28249	102.8249	101.1986	1.445883	1.428758
2	100	415.9	10.14588	101.4588			
3	100	407.1	9.931206	99.31206			

Table 9: Determination of Azathioprine in tablet dosage form

The results of stress testing studies indicated a high degree of selectivity of this method for AZA. Typical chromatograms obtained from the assay of the pure drug sample and stressed samples are shown in figures. The mean retention time for AZA was found to be 6.44 minutes. The peaks obtained were sharp and have clear baseline separation. The results of stress testing studies indicated a high degree of selectivity of this method for AZA. Typical chromatograms obtained from the assay of the pure drug sample and stressed samples are shown in figures. The mean retention time for AZA was found to be 6.44 minutes. The peaks obtained were sharp and have clear baseline separation.

AZA is characterized by an anthraquinone moiety with a side chain of acetoxy group and carboxylic acid group, which was prone to hydrolysis. All the main degradation products were separated from the parent compound. AZA was found to be stable under dry heat conditions. The drug was unstable under exposure of solid drug powder to light, which was kept in daylight for 8hrs. The drug was unstable under basic stress conditions when kept for 3hrs under reflux at 80ºC temperature. The drug was degraded approximately to 17.32%.

Also, it was unstable under acidic conditions when kept for 3hrs under reflux at 80ºC temperature. The drug was degraded approximately to 9.53%. When kept under oxidative stress conditions with 3% H_2O_2 for 24hrs in the dark at room temperature, the drug was degraded to around 24.72%. In neutral conditions when the drug was refluxed with water for 3hrs, around 15.22% degradation was shown. The stability of stock solution was determined by quantitation of AZA and comparison to a freshly prepared standard. No significant change was observed in the stock solution response, relatively to freshly prepared standard (Table 8).

The proposed method was applied to the determination of AZA in Azoran Tablet. The result of these assay yielded 98.09 of label claim of the tablet. The results of the assay indicate that the method is selective for the determination of AZA without interference from the excipients used in these tablets.

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