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DEVELOPMENT AND VALIDATION OF A STABILITY INDICATING ASSAY METHOD OF PYRIMETHAINE BY USING DIFFERENT STRESS DEGRADATION CONDITIONS

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ABSTRACT

The aim of the present work is to develop simple accurate, precise and cost effective UV-Vis spectrophotometric method for the estimation of pyrimethamine in bulk and pharmaceutical dosage form and determination of percent degradation by using different stress condition. The solvent used was methanol and distilled water (40:60) and λmax of the drug was found to be 272.5nm. A linear response was observed in the range 2-20 µg/ml with a regression coefficient 0.999. The method was then validated for different parameters as per the ICH guidelines. This method can be used for the determination of pyrimethamine in quality control of formulation without interference of the excipients. Pyrimethamine was subjected to stress degradation under different conditions recommended by ICH. The samples generated were used for degradation studies using the developed method.

INTRODUCTION: Pyrimethamine is chemically a 5-(4-chlorophenyl)-6-ethyl-2, 4-pyrimidinediamine.

Pyrimethamine is an antiparasitic compound commonly used as an adjunct in the treatment of uncomplicated, chloroquine resistant, Plasmodium falciparum malaria. Pyrimethamine is a dihydrofolate reductase inhibitor. It has been demonstrated that the drug binds the parasite enzyme with much greater affinity than it binds the corresponding enzyme from the host [1-13].

The aim of this work is to develop and validate an analytical method by using UV-Vis spectrophotometry for the estimation of pyrimethamine in bulk and pharmaceutical dosage forms and also perform degradation studies on the drug as per ICH guidelines using the proposed method [14-17].

MATERIAL AND METHODS: Pyrimethamine sample was obtained from GlaxoSmithKline, Mumbai. The instrument used for the present study was Jasco V-530 UV-Vis double beam, high speed scanning spectrophotometer. The solvent used was distilled water, methanol (AR grade), NaOH (AR grade), HCl (AR grade) and H_2O_2 (AR grade). These chemicals were purchased from Merck Chemicals (Mumbai, India).

Preparation of stock solution: Standard stock solution of pyrimethamine was prepared by dissolving 10 mg of pyrimethamine in 100 ml of methanol and distilled water (40:60) which gives 100µg/ml solution.

Preparation of working solution: From the above stock solution 1 ml was transferred into 10 ml volumetric flask and volume make up with methanol to give 10µg/ml. Then sample was scanned with UV-Vis

spectrophotometer in the range 200-400 nm and the wavelength corresponding to maximum absorbance was noted (λ_{max} 272.5 nm) (**Figure 1**).

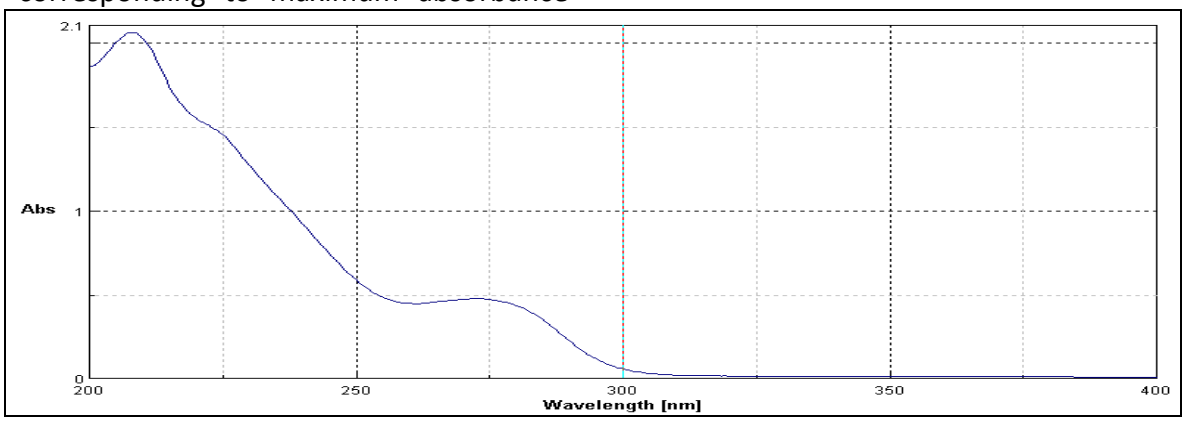

FIGURE 1: λ_{max} OF PYRIMETHAMINE SHOWING AT 272.5 nm

Preparation of calibration curve: 0.2-2ml of 100µg/ml solutions were taken and diluted up to 10 ml using methanol and distilled water to produce 2-20µg/ml solutions respectively. Then graph was plotted taking concentration on x-axis and absorbance on y-axis which shows a straight line (**Figure 2**).

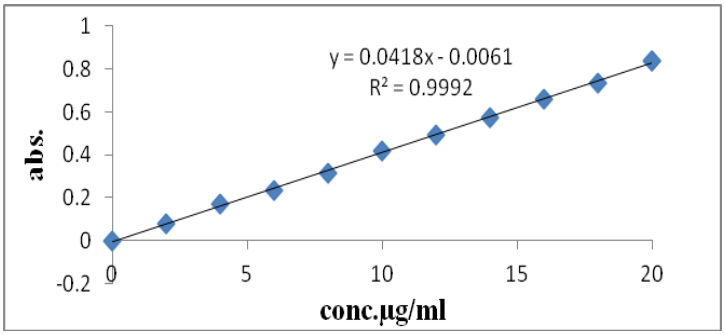

FIGURE 2: CALIBRATION CURVE OF PYRIMETHAMINE

Method Validation:

Linearity: Various aliquots were prepared from the stock solution (100µg/ml) ranging from 2-20µg/ml. The samples were scanned in UV-Vis Spectrophotometer against methanol and distilled water (40:60) as blank. It was found that the selected drug shows linearity between the ranges of 2-20µg/ml (**Figure 2 & Table 1**).

TABLE 1: OPTICAL CHARACTERISTICS

Beer's law limit (µg/ml)	2-20µg/ml
Correlation coefficient	0.999
Regression equation (Y*)	0.041x - 0.006
Slope (a)	0.041x
Intercept (b)	0.006

Precision: Precision of the method was demonstrated by intraday and interday variation studies. In intraday variation study six different solutions of same concentration 10µg/ml were analyzed three times in a day and the absorbance was noted. In the interday variation studies, solution of same concentration 10µg/ml were analyzed three times for the three consecutive days and the absorbance result mean, standard deviation and % RSD was calculated and given in **table 2 and 3**.

TABLE 2: INTRA-ASSAY PRECISION

Concentration (µg/ml)	Absorbance 1	Absorbance 2	Absorbance 3	Average % RSD
10	0.4192	0.4194	0.4192	
10	0.4192	0.4191	0.4194	
10	0.4192	0.4192	0.4192	
10	0.4194	0.4192	0.4192	
10	0.4196	0.419	0.4196	
10	0.4192	0.4192	0.4192	
% RSD	0.039	0.031	0.039	0.036%

TABLE 3: INTER-ASSAY PRECISION

Concentration (µg/ml)	%RSD			Average %RSD
	Day 1	Day 2	Day 3	
10	0.028	0.036	0.038	0.034%

Accuracy: Solutions were prepared in triplicate at levels 80%, 100%, and 120% of test concentration using pyrimethamine working standard as per the method and taken absorbance of each solution in triplicate (**Table 4 and 6**).

Specificity: 10 mg of pyrimethamine was spiked with 50% (5 mg), 100% (10 mg), and 150% (15 mg) sample was analyzed for % recovery of pyrimethamine (Table 6).

Robustness: Robustness of the method was determined by carrying out the analysis under different temperature condition. The respective absorbance of 10µg/ml was noted and the result was indicated as % RSD (**Table 5**).

Ruggedness: Ruggedness of the method was determined by carrying out the analysis by different analyst. The respective absorbance of 10µg/ml was noted then result indicated as % RSD (Table 5).

TABLE 4: ACCURACY READING OF PYRIMETHAMINE

No. of Preparations	Concentration (µg/ml)		% Recovery	Statistical Results		
	Formulation	Pure drug		Mean	SD	%RSD
S_1:80%	10	8	99.12			
S_2:80%	10	8	99.98	99.74	0.54	0.54
S_3:80%	10	8	100.12			
S_4:100%	10	10	99.96			
S_5:100%	10	10	100.18	100.58	0.89	0.89
S_6:100%	10	10	101.6			
S_7:120%	10	12	99.36			
S_8:120%	10	12	100.12	99.61	0.43	0.43
S_9:120%	10	12	99.36			

TABLE 5: RESULT SHOWING ROBUSTNESS & RUGGEDNESS OF PYRIMETHAMINE

Analyst-1		
Concentration (µg/ml)	Absorbance	Statistical Analysis
10	0.4192	Mean=0.41925
10	0.4191	
10	0.4194	SD= 0.000122
10	0.4192	
10	0.4194	
10	0.4192	%RSD=0.029

Analyst-2		
Concentration (µg/ml)	Absorbance	Statistical Analysis
10	0.4192	Mean=0.4194
10	0.4194	
10	0.4194	SD= 0.000126
10	0.4194	
10	0.4196	
10	0.4194	%RSD=0.030

Room Temperature		
Concentration (µg/ml)	Absorbance	Statistical Analysis
10	0.4194	Mean=0.41923333
10	0.4192	
10	0.4192	SD= 0.00013744
10	0.4192	
10	0.4194	
10	0.419	%RSD=0.032

Temp. 18°C		
concentration(µg/ml)	Absorbance	Statistical Analysis
10	0.4192	Mean=0.4192
10	0.4193	
10	0.4192	SD= 0.000133
10	0.4194	
10	0.419	
10	0.4192	%RSD=0.031

Limit of Detection (LOD): The limit of detection (LOD) was determined from solutions of different concentrations ranging from 0.1-0.5µg/ml. The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantification as an exact value (**Table 6**).

Limit of Quantification (LOQ): The LOQ is the concentration that can be quantification reliably with a specified level of accuracy and precision. The LOQ was calculated using formula (Table 6).

Assay of Pyrimethamine: A quantity of powder equivalent to 25mg of Pyrimethamine was taken in a 100ml volumetric flask and it was dissolved and diluted upto the mark with methanol and water. Then solution was ultrasonicated for 5 minutes and then filtered using Whatmann filter paper no. 40. From the filtrate, appropriate dilutions were made in methanol and water (40:60) to obtain the desired concentration (25µg/ml). This solution was then analyzed in UV and the result was indicated by % recovery given in table 6.

Degradation studies:

1. **Acidic degradation:** 1 ml of stock solution of Pyrimethamine, and 5 ml of 5 N HCl was added in 10 ml of volumetric flask and the volumetric flask was kept at normal condition for 3 hour. After 3 hours, solution neutralized and diluted with methanol and water (40:60) up to 10 ml and the dilution was done to achieve the appropriate concentration (10µg/ml) (**Figure 3 & Table 7**)

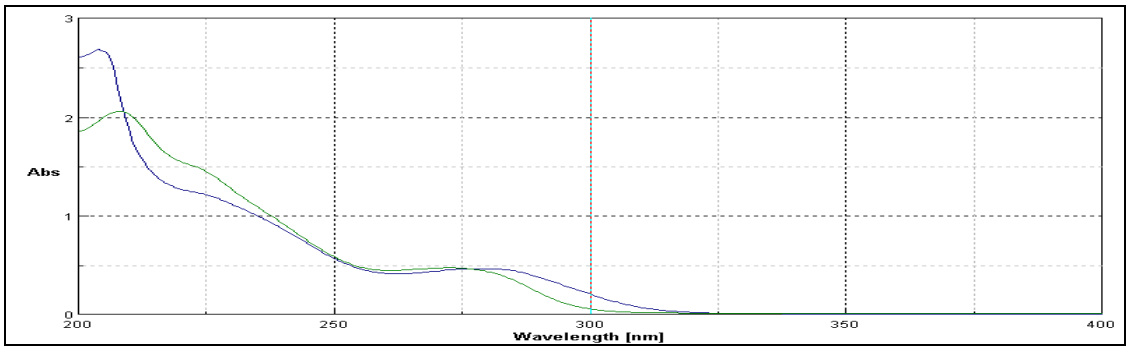

FIGURE 3: COMPARISON BETWEEN STANDARD PYRIMETHAMINE (10µg/ml) AND ACID DEGRADED SAMPLE OF PYRIMETHAMINE (10 µg/ml). Drug got degraded and its λ_{max} shifted

Alkali degradation: 1 ml of stock solution of Pyrimethamine and 5 ml of 0.1 N NaOH was added in 10 ml of volumetric flask and the volumetric flask was kept at normal condition for 3 hour. After 3 hours, solution neutralized and diluted with methanol and water (40:60) up to 10 ml and the dilution was done to achieve the appropriate concentration (10µg/ml) (**Figure 4 & Table 7**).

Oxidative degradation: 1 ml of the stock solution of Pyrimethamine and 5 ml of 3 % w/v of hydrogen peroxide added in 10 ml of volumetric flask and volumetric flask was kept at normal condition for 3 hour. After 3 hours, solution diluted with methanol and water (40:60) up to 10 ml and the dilution was done to achieve the appropriate concentration (10µg/ml) (**Figure 5 & Table 7**).

Dry heat induced degradation: Pyrimethamine sample was taken in a petriplate and exposed to a temperature of 72°C for 48 hours in an oven. After 48 hours, 10 mg of the sample was diluted with methanol and water (40:60) up to 10 ml. From this solution, dilution was done to achieve the appropriate concentration (10µg/ml) (**Figure 6 & Table 7**).

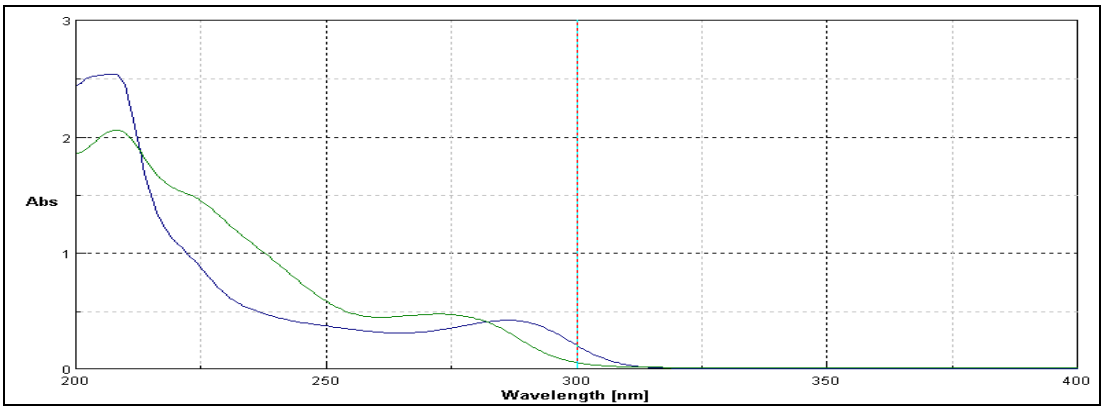

FIGURE 4: COMPARISON BETWEEN STANDARD PYRIMETHAMINE (10µg/ml) AND ALKALI DEGRADED SAMPLE OF PYRIMETHAMINE (10 µg/ml). Drug got degraded and its λ_{max} shifted**.**

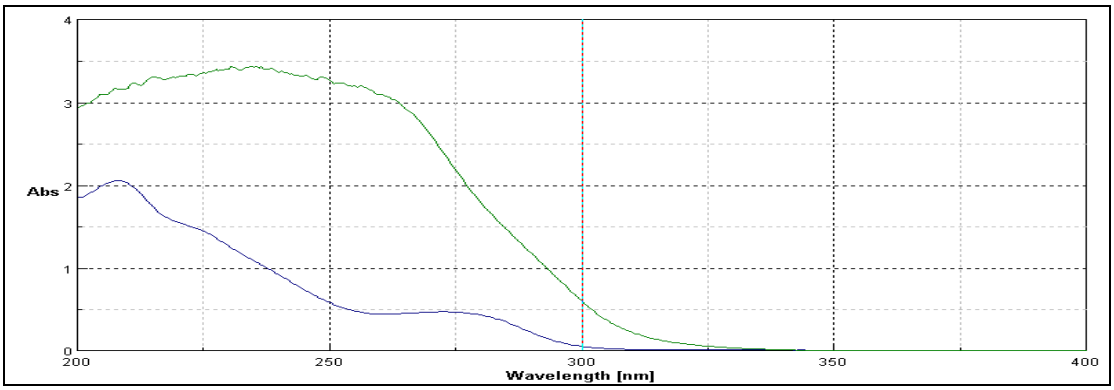

FIGURE 5: COMPARISON BETWEEN STANDARD PYRIMETHAMINE (10µg/ml) AND OXIDISED SAMPLE OF PYRIMETHAMINE (10 µg/ml). Drug got degraded and its λ_{max} shifted**.**

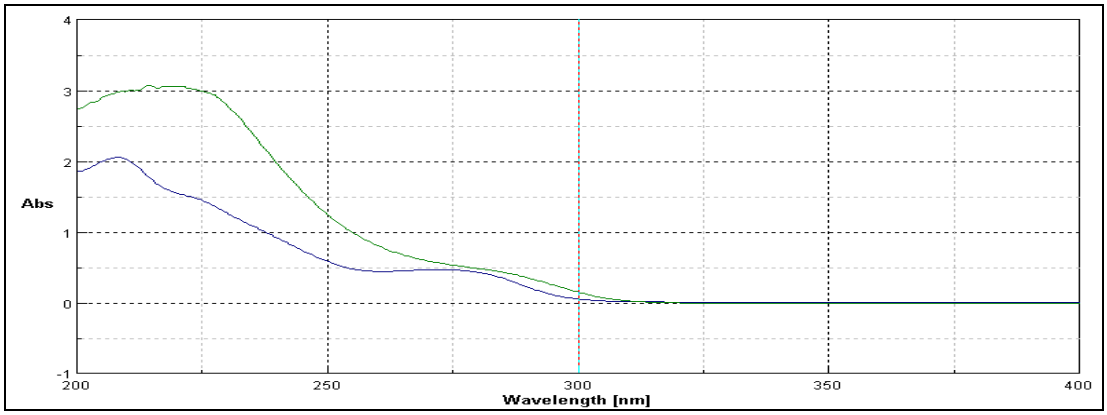

FIGURE 6: COMPARISON BETWEEN STANDARD PYRIMETHAMINE (10µg/ml) AND TEMPERATURE DEGRADED SAMPLE OF PYRIMETHAMINE (10 µg/ml). Drug got degraded and its λ_{max} shifted.

RESULTS AND DISCUSSION: The developed method was found to be precise as the %RSD values for intraday and inter-day were found to be less than 2%, recoveries (99.97% to 101.4%) of the drug, indicating that the method was accurate.

The method was also found to be specific. The LOD and LOQ were found to be in sub-microgram level indicating the sensitivity of the method. The method was also found to be robust and rugged as indicated by the % RSD values which are less than 2 %.

The results of assay show that the amount of drug was in good agreement with the label claim of the formulation as indicated by % recovery (99.98%). Summary of validation parameters of proposed spectrophotometric method is shown in table 6.

The stress degradation studies showed that Pyrimethamine undergoes degradation in acidic, oxidation, alkaline and dry heat (15.47%, 14.24% 12.77%, 16.70% respectively). Results are shown in table 7.

TABLE 6: SUMMARY OF VALIDATION PARAMETERS

Parameter	Result
Linearity indicated by correlation coefficient	0.999
Precision indicated by % RSD	0.035%
Accuracy indicated by % recovery	99.9766%
Specificity indicated by % recovery	100%
Limit of Detection	0.28µg/ml
Limit of Quantification	0.853µg/ml
Range	2-20µg/ml
Linear regression equation	0.041x - 0.006
Robustness indicated by % RSD	0.0305%

TABLE 7: RESULT OF STRESS DEGRADATION STUDIES

Stress condition	Time	Observation	% Degradation
Acidic degradation	3 hours	λ_{max} shifted	15.47%
Alkali Degradation	3 hours	λ_{max} shifted	14.24%
Oxidative Degradation	3 hours	λ_{max} shifted	12.77,
Dry Heat	48hours	λ_{max} shifted	16.70 %

CONCLUSION: All the above factors lead to the conclusion that the proposed method as accurate, precise, simple, sensitive, robust and cost effective and can be applied successfully for the estimation of Pyrimethamine in bulk and pharmaceutical formulation and percentage degradation. The proposed method is also useful for determination of Pyrimethamine stability in sample of pharmaceutical dosage forms.

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