INTERNATIONAL JOURNAL OF PHARMACY AND ANALYTICAL RESEARCH

ISSN: 2320-2831

IJPAR |Vol.9 | Issue 3 | Jul - Sep - 2020
Journal Home page: www.ijpar.com

Research article ***Open Access***

A novel stability indicating method development and validation for the quantification of related substances in pyrimethamine tablet dosage form by RP-HPLC

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ABSTRACT

A new stability indicating RP-HPLC method was developed to analyse the related substances in Pyrimethamine tablets. The projected method shows with accurate, precise, linear, robust and rugged. The developed method validated as per ICH guidelines by using high performance liquid chromatography. Column configuration - Agilent zorbax SB-C8, 150 x 4.6 mm 3.5µm column. Mobile phase contains buffer pH 4.0: methanol in the ratio of 55:45 %v/v with the flow rate of 1.2 ml/min. Detection fixed at 280 nm. The retention time of the pyrimethamine is 5.007 min. Runtime 12 minutes for standard and 40 minutes for blank and sample. The validation parameters such as precision, accuracy, linearity, robustness, ruggedness, forced degradation study and filter study were evaluated. Linearity range covered from LOQ level. Correlation coefficient square found not less than 0.98.Recovery % occurs between 99% to 102 % for impurities.

Keywords: RP- HPLC, Related substance, Pyrimethamine, Method development, Method Validation

INTRODUCTION

Pyrimethamine can play vital role as folic acid antagonists and also it possess anti-malarial activity. Pyrimethamine restricts with the regeneration of tetrahydrofolic acid from dihydrofolate by inhibiting the enzyme dihydrofolate reductase.[1] Tetrahydrofolic acid is key factor for DNA and RNA synthesis in many species, which includes including protozoa. IUPAC name of pyrimethamine is 5,4-chlorophenyl-6-ethyl-2,4-pyrimidinediamine.[2,3]

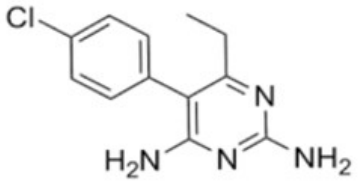

Fig 1: Structure of pyrimethamine

Related substances are structurally interrelated to a drug substance. These substances may be identified or unidentified degradation products or impurities arising from a manufacturing process or during storage of a material. The existence of impurities, particularly the impurities from API, degradation and interaction-related impurities even in trace level also may impact the efficacy and safety of pharmaceutical products[4]. Impurities are categorised as Organic impurities, Inorganic impurities and residual solvents according to ICH guidelines The regulatory requirements and different management approaches are required to be established and complied sources of impurities shall be carefully categorized prior to proceed consecutive steps such as development of analytical methods and acceptance criteria[5,6].

Literature survey reveals that few HPLC[7,8] UV[9] and LC-MS[10,11,12] methods for the estimation of pyrimethamine along with its combination dosage form have been reported. Based on the literature survey, aim of the current study is to develop a precise, accurate, linear, robust and rugged related substance method by using RP-HPLC, for the analysis of pyrimethamine in tablet dosage forms as per ICH guide lines Q2 R1[13,14, 15]

MATERIALS AND METHODS

Drugs and chemicals

Pyrimethamine Reference standard procured from USP. Impurity 258U53, Impurity 42W75 and Impurity 25U52 obtained from Dalton and wuxi app. HPLC grade sodium acetate trihydrate, triethylamine, glacial acetic acid and methanol and Milli-Q water obtained from Merck.

Instrumentation

A waters HPLC instrument controlled with Empower -3 software. Column manufacturer is Agilent zorbax SB-C8, 150 x 4.6 mm 3.5µm Part No: 863953-906.

Mobile Phase preparation

Buffer preparation: Accurately weighed 6.8g of sodium acetate trihydrate dissolved into a 1000 ml of water contains 2.0 ml of triethylamine. Sonicated and mixed well. Glacial acetic acid was used to adjust the pH 4.0 ± 0.05. Filtered the mobile phase through with 0.45 µm nylon membrane. Buffer pH 4.0 combined with methanol in the ratio of 55:45 % v/v.

Diluent Preparation

Mixed methanol and 1% glacial acetic acid in the ratio of 1:1 % v/v and mixed well.

Standard preparation

Accurately weighed and transferred about 50 mg of Pyrimethamine RS into a 100 ml volumetric flask. Added 70 ml of diluent sonicated to dissolve and made up to the volume with diluent and mixed well. Further pipetted out 5.0 ml of above stock solution into a 25 ml volumetric flask. Made up to the volume with diluent and mixed well

Sample preparation

Randomly selected 20 tablets. Determined the average tablet weight. Crushed the required number of tablets and mixed then powder uniformly. Weighed the powder equivalent to 50 mg of Pyrimethamine into 100 ml volumetric flask. Added 70 ml of diluent and sonicated for 20 minutes with intermittent shaking. Made up to the volume with diluent and mixed well. Filtered through 0.45µ nylon filter by discarding first 4 ml of filterate.

Chromatographic conditions

Agilent zorbax SB-C8, 150 x 4.6 mm 3.5µm, Part No: 863953-906. Mobile phase composition Buffer pH 4.0: Methanol (55:45), at flow rate of 1.2 ml/min. Run time 10 minutes for standard, 40 minutes for blank and sample with detection wavelength at 280 nm. Injection volume 20 µl, Column oven temperature 30°C, Sampler temperature 10°C.

Method validation

The developed related substance method was validated as per the ICH guidelines.

System precision

To demonstrate the system precision, standard solution was prepared and injected as per the procedure. The % Relative standard deviation for peak area responses from six replicate injections of the standard solution, USP tailing factor and The % relative standard deviation of six replicate standard injections reported in Table 1. Acceptance criteria for USP tailing factor should be not more than 2.0 and the % related standard deviation of six replicate injections should not be more than 6.0%.(Figure 1)

Method precision

Precision of the method was determined by injecting six individual sample solutions of Pyrimethamine tablets spiked with known impurities at the level of 0.27%.The samples were prepared as per the method (Figure 1-Figure 5). The percentage relative standard deviation from six individual sample preparations should be not more than 15.0%.Calculated the % Relative standard deviation if percent impurity observed equal or more than 0.05% (Table 2)

Linearity

Solutions of Pyrimethamine and all impurities were

prepared as follows Pyrimethamine –LOQ to 162 %, Impurity 258U53 –LOQ to 295%, Impurity 42W75 – LOQ to 330%, Impurity 25U52 – LOQ to 337%.Each of the above upper limit refers to the % of the specification level 0.15% for unknown and 0.1% for known impurities with respect to the sample concentration. Each were prepared and injected into the HPLC system. The linearity graph plotted form QL to appropriate level. Correlation co efficient should be not less than 0.98. (Table 3 – Table 6)

Precision at LOQ level

Precision at LOQ level was determined by preparing and injecting Pyrimethamine its impurities solution at LOQ level. Signal to noise ratio for LOQ solution should be more than 10 and the % Relative standard deviation from the six replicate injections of LOQ solution should be not more than 25%.(Table 7 – Table 8)

Accuracy

Accuracy of the method demonstrated by placebo and taken different amounts of Pyrimethamine and impurities representing about LOQ to 550% of the specification level for known impurities 0.1% and about LOQ to 260 % of the specification level for unknown 0.15% were added to the flask.The spiked samples were prepared as per the method in triplicate and injected. The average % recovery should be between 70%-130% for LOQ level and should be between 80%-120% for other levels. The % relative standard deviation should be not more than 25% for LOQ level and not more than 15% for other levels. he overall average percentage recovery should be between 98.0%-102.0% and the overall % RSD should be not more than 3.0% (Table 8 - Table 12).

Relative retention time (RRT) and relative response factor (RRF)

RRT of know impurities were determined from spiked sample containing Pyrimethamine and its impurities. (Table 13)

Specificity

A forced degradation study is performed in order to show that the method is stability indicating. Pyrimethamine tablets were stressed under Acid, base, peroxide, Uv light and heat. The acceptance criteria for specificity study, any secondary peak arising from forced degradation study should not interfere with Pyrimethamine peak retention time (Figure 6-Figure-12). No interference should be observed from diluent, placebo and all the known impurities at the retention time of Pyrimethamine peak. In peak purity analysis purity threshold for stress samples should be higher than the purity angle (Table 14 –Table 15).

Robustness

In robustness study of the method established by injecting standard solution as per method and the same standard solution was injected by varying the method parameters. System suitability data calculated for standards injected as per method and altered method conditions. (Table 16)

Filter study

The spiked sample solution of Pyrimethamine tablets was filtered by discarding 2ml, 4ml, 6ml and 8ml of the filtrate by using 0.45 µm nylon filter and the sample were injected. Unfiltered centrifuged solution was also prepared and injected. Compared the results of filtered sample with that of centrifuged sample results. The % difference between in % impurity between the centrifuged sample and filtered sample should be not more than 25 %.(Table 17 – Table 18)

LOD (Limit of detection) Level

LOD was determined by preparing and injecting pyrimethamine and its impurities solutions as LOD level. The signal to noise ratio for LOD solution should be more than 3. (Table 19)

Ruggedness

The ruggedness of the related substance method was established by injecting six individual sample solutions of Pyrimethamine tablets spiked with known impurities at the levels of 0.27% by second analyst using different day system and different column on a different day. The % relative standard deviation of impurities from six individual sample preparations should be NMT 15%.Calculated % relative standard deviation if percent impurity observed equal or more than 0.05%. The % difference in % impurity between method precision and intermediate precision results should be Not more than 25%. (Table 20)

RESULTS AND DISCUSSION

Table 1: System precision data results

S.no	Name	Retention time	Area	USP Tailing
1	Standard-1	5.007	260517	1.2
2	Standard-2	5.010	259123	1.2
3	Standard-3	5.011	258697	1.2

4	Standard-4	5.008	258492	1.2
5	Standard-5	5.011	259317	1.2
6	Standard-6	5.016	258795	
Mean			259157	
% RSD			0.3	

Table 2: Method precision results

S.no	Name	Imp 258U53	Imp 42W75	Imp 25U52
1	Sample-1	0.276	0.294	0.266
2	Sample-2	0.275	0.285	0.271
3	Sample-3	0.275	0.279	0.271
4	Sample-4	0.273	0.280	0.275
5	Sample-5	0.274	0.291	0.287
6	Sample-6	0.273	0.293	0.275
Mean		0.274	0.287	0.274
% RSD		0.4	2.3	2.5

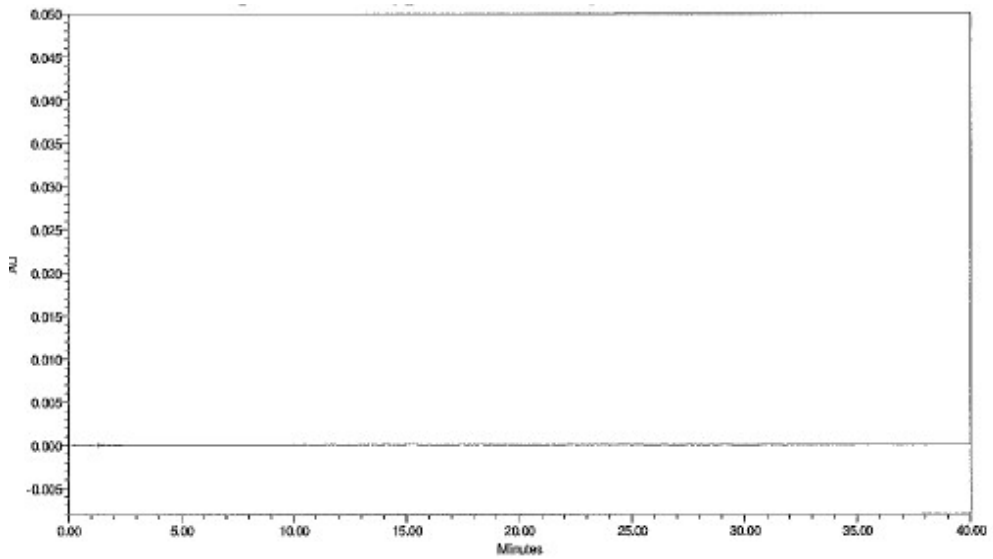

Fig 1: Typical chromatogram of blank

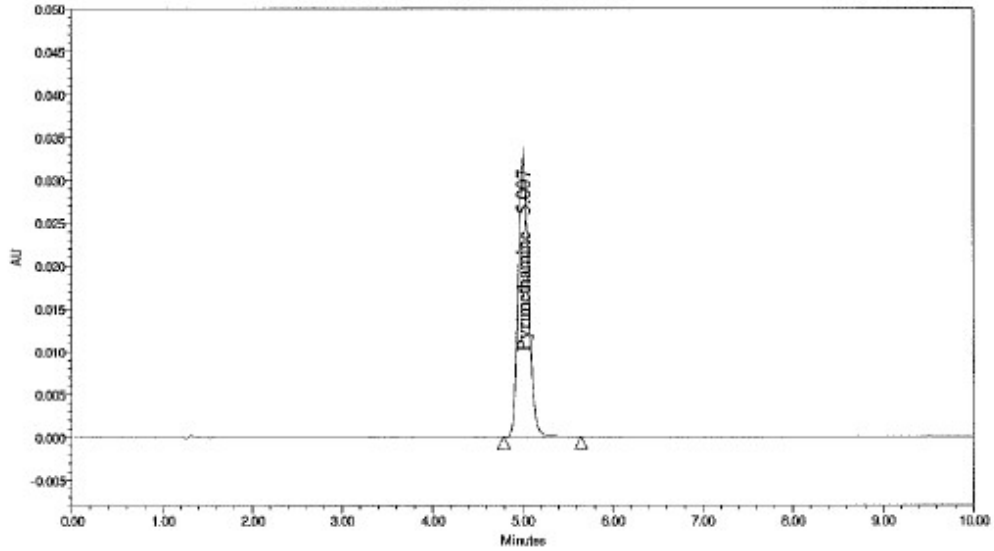

Fig 2: Typical chromatogram of standard

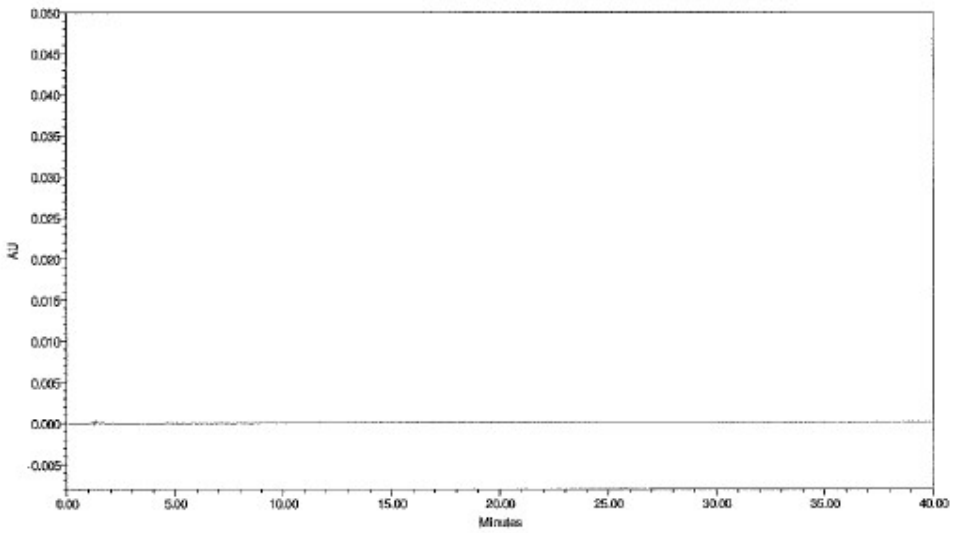

Fig 3: Typical chromatogram of placebo

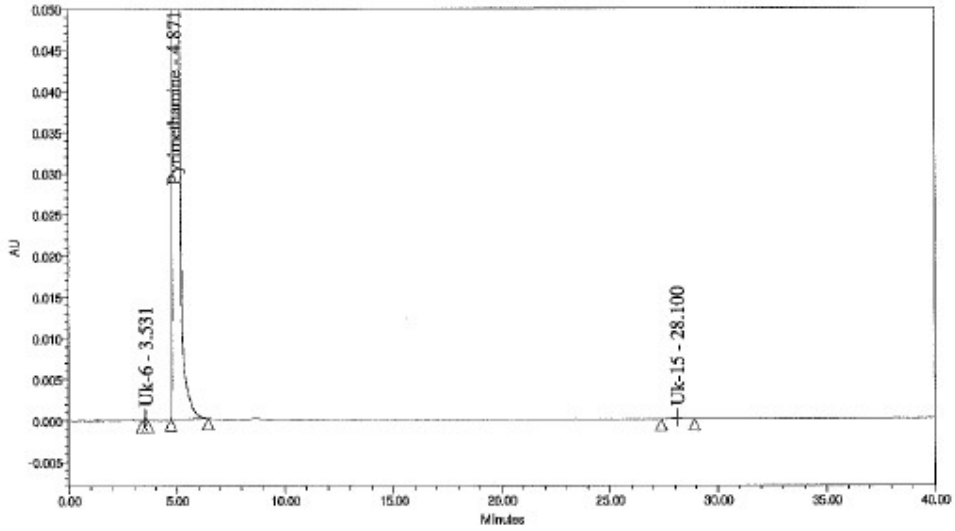

Fig 4: Typical chromatogram of control sample

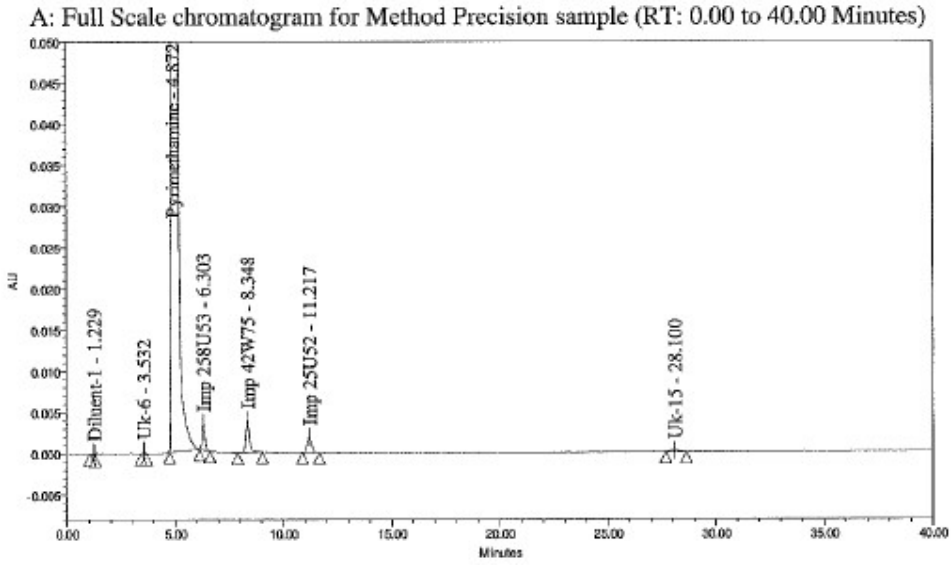

Fig 5: Typical chromatogram of method precision sample

Table 3: Linearity data for Pyrimethamine

S.no	Name	Area
1	Linearity - 34% (LOQ)	7203
2	Linearity - 54%	11761
3	Linearity - 81%	17290
4	Linearity - 108%	23150
5	Linearity - 135%	28624
6	Linearity - 162%	34744
Correlation coefficient square = 1.000		

Table 4: Linearity data for impurity 258U53

S.no	Name	Area
1	Linearity - 49% (LOQ)	5610
2	Linearity - 74%	8612
3	Linearity - 147%	17117
4	Linearity – 221%	25464
5	Linearity - 295%	34249
Correlation coefficient square = 1.000		

Table 5: Linearity data for impurity 42W75

S.no	Name	Area
1	Linearity - 55% (LOQ)	9382
2	Linearity - 82%	14220
3	Linearity - 165%	28081
4	Linearity – 247%	42089
5	Linearity - 330%	56292
Correlation coefficient square = 1.000		

Table 6: Linearity data for IMPURITY 25U52

S.no	Name	Area
1	Linearity - 56% (LOQ)	6112
2	Linearity - 84%	9500
3	Linearity - 168%	18756
4	Linearity – 253%	28250
5	Linearity - 337%	37812
Correlation coefficient square = 1.000		

Table 7: LOQ Precision for pyrimethamine and impurities

S.no	Sample name	Pyrimethamine	IMP 258U53	IMP 42W75	IMP 25U52
1	LOQ Precision-1	6668	5589	9608	6276
2	LOQ Precision-2	6657	5774	9567	6047
3	LOQ Precision-3	6641	5717	9351	6060
4	LOQ Precision-4	6713	5639	9456	6184
5	LOQ Precision-5	6633	5603	9425	6014
6	LOQ Precision-6	6764	5737	9499	6136
Mean		6679	5676	9468	6119
%RSD		1	1	1	2

Table 8: Precision at LOQ Level for pyrimethamine

S.no	Sample name	LOQ Concentration	Linearity level With relative to sample concentration	USP S/N
1	Pyrimethamine	0.2399	0.048	136
2	IMP 258U53	0.2456	0.049	101
3	IMP 258U53	0.2748	0.055	148
4	IMP 258U53	0.2806	0.056	76

Table 9: Accuracy data for Pyrimethamine

S.no	Name	Area	Amount added µg/ml	Amount found µg/ml	% Recovery	Mean	% RSD
1	LOQ preparation-1	6613	0.2493	0.2507	101	102	1
2	LOQ preparation-2	6685	0.2493	0.2534	102		
3	LOQ preparation-3	6736	0.2493	0.2553	102		
1	Recovery 133% -1	26876	0.9973	1.0187	102	100	2
2	Recovery 133% -2	26131	0.9973	0.9905	99		
3	Recovery 133% -3	25940	0.9973	0.9832	99		
1	Recovery 266% -1	52707	1.9946	1.9978	100	99	1
2	Recovery 266% -2	52738	1.9946	1.9978	100		
3	Recovery 266% -3	51454	1.9946	1.9978	98		

Table 10: Accuracy data for Pyrimethamine impurity – 258U53

S.no	Name	Area	Amount added µg/ml	Amount found µg/ml	% Recovery	Mean	% RSD
1	LOQ preparation-1	6119	0.282	0.2825	100	99	3
2	LOQ preparation-2	6142	0.282	0.2836	101		
3	LOQ preparation-3	5848	0.282	0.2700	96		
1	Recovery 133% -1	30338	1.4102	1.4007	99	100	1
2	Recovery 133% -2	30652	1.4102	1.4152	100		
3	Recovery 133% -3	30557	1.4102	1.4108	100		
1	Recovery 266% -1	62780	2.8204	2.8986	103	102	1
2	Recovery 266% -2	61885	2.8204	2.8573	101		
3	Recovery 266% -3	62351	2.8204	2.8788	102		

Table 11: Accuracy data for Pyrimethamine impurity – 25U52

S.no	Name	Area	Amount added µg/ml	Amount found µg/ml	% Recovery	Mean	% RSD
1	LOQ preparation-1	5768	0.2768	0.2799	101	99	2
2	LOQ preparation-2	5581	0.2768	0.2708	98		
3	LOQ preparation-3	5644	0.2768	0.2739	99		
1	Recovery 277% -1	28141	1.3842	1.3654	99	100	1
2	Recovery 277% -2	28816	1.3842	1.3982	101		
3	Recovery 277% -3	28839	1.3842	1.3993	101		
1	Recovery 554% -1	57787	2.7685	2.8039	101	101	0
2	Recovery 554% -2	57930	2.7685	2.8108	101		
3	Recovery 554% -3	57954	2.7685	2.8120	102		

Table 12: Accuracy data for Pyrimethamine impurity – 42W75

S.no	Name	Area	Amount added µg/ml	Amount found µg/ml	% Recovery	Mean	% RSD
1	LOQ preparation-1	9326	0.2681	0.2791	104	99	5
2	LOQ preparation-2	8918	0.2681	0.2669	100		
3	LOQ preparation-3	8462	0.2681	0.2533	94		
1	Recovery 268% -1	43094	1.3404	1.2898	96	97	1
2	Recovery 268% -2	43575	1.3404	1.3042	97		
3	Recovery 268% -3	44109	1.3404	1.3202	98		
1	Recovery 536% -1	88131	2.6808	2.6378	98	99	1
2	Recovery 536% -2	88858	2.6808	2.6596	99		
3	Recovery 536% -3	87785	2.6808	2.6295	98		

Table 13: RRT and RRF for pyrimethamine Impurities

S.no	Sample name	RRT	RRF
1	Pyrimethamine	1.00	1.00
2	IMP 258U53	1.29	0.83
3	IMP 258U53	1.71	1.23
4	IMP 258U53	2.30	0.81

Table 14: Forced degradation – peak purity results

S.no	Name	Purity angle	Purity threshold
1	Control sample	0.118	0.264
2	Acid degradation sample (5N HCl/80°C/5 hrs)	0.648	1.114
3	Base degradation sample (1N NaOH/80°C/4 hrs)	0.097	0.266
4	Peroxide degradation sample (3% H2O2/80C/4 hrs)	0.105	0.263
5	Heat degradation (80°C/16 hrs)	0.126	0.266
6	Uv light degradation sample (Uv light/16 hrs)	0.139	0.267
7	Spiked sample	0.147	0.267

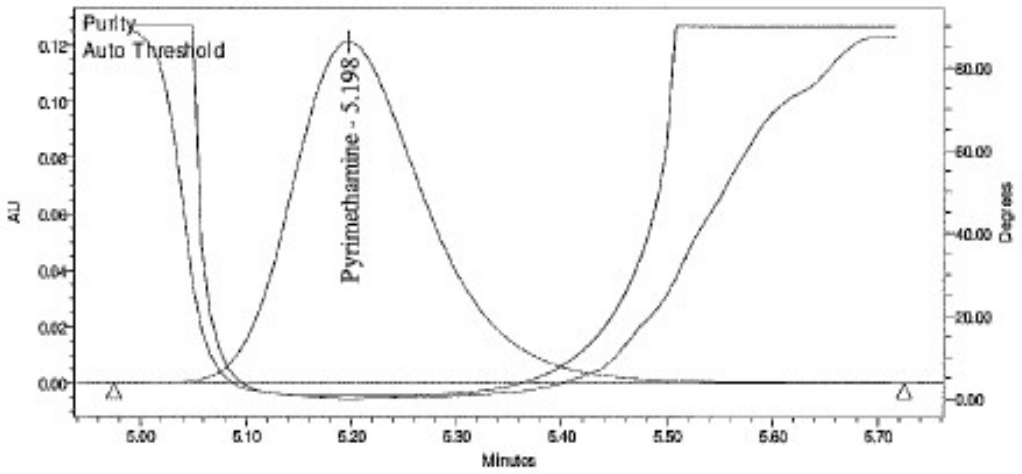

Fig 6: Peak purity plot for acid stress sample

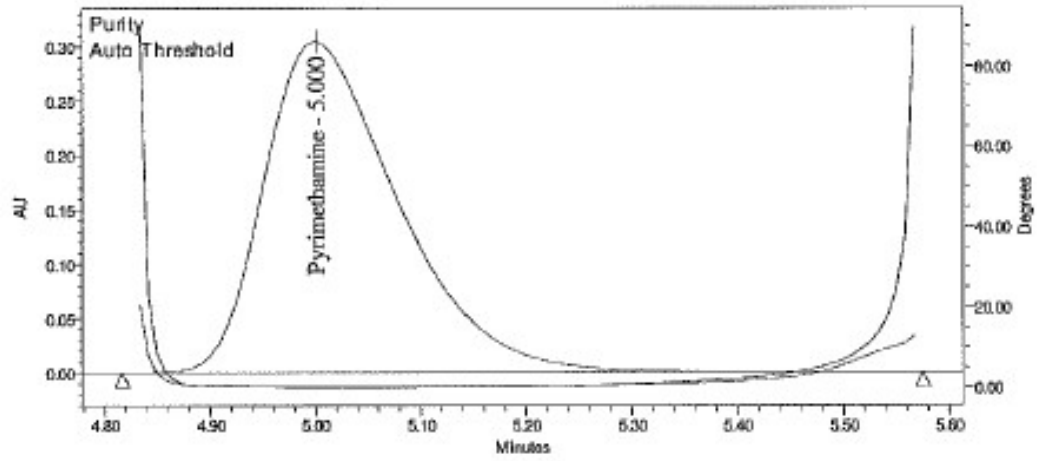

Fig 7: Peak purity plot for base stress sample

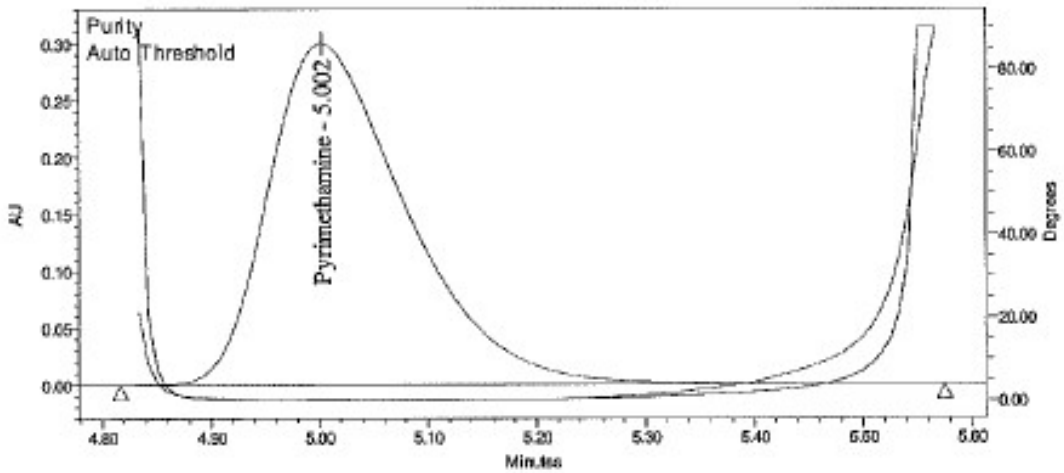

Fig 8: Peak purity plot for peroxide stress sample

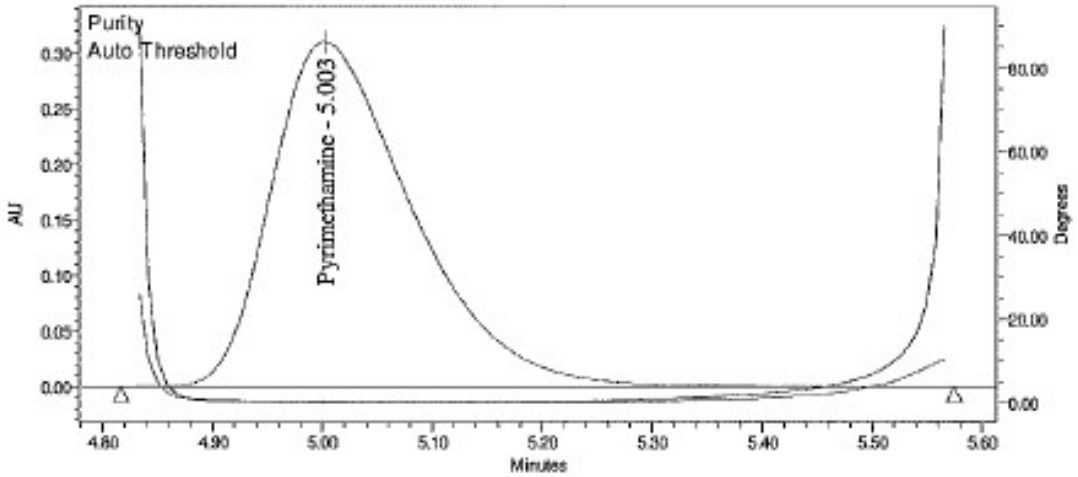

Fig 9: Peak purity plot for heat stress sample

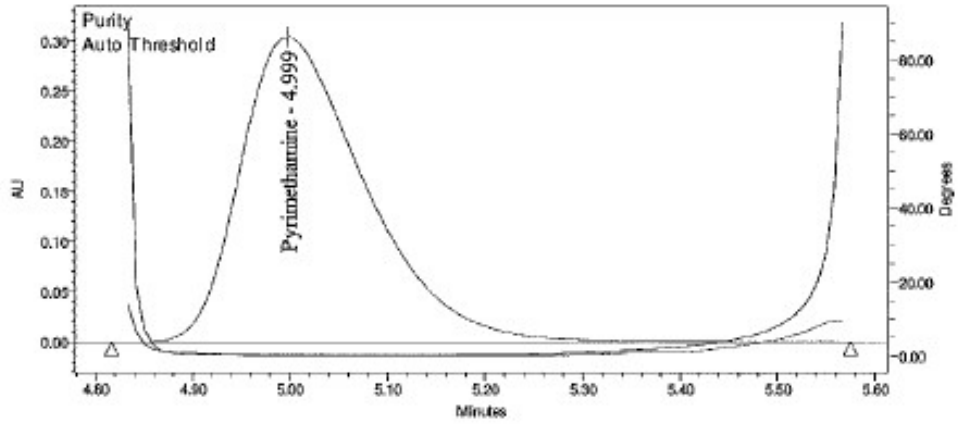

Fig 10: Peak purity plot for UV light stress sample

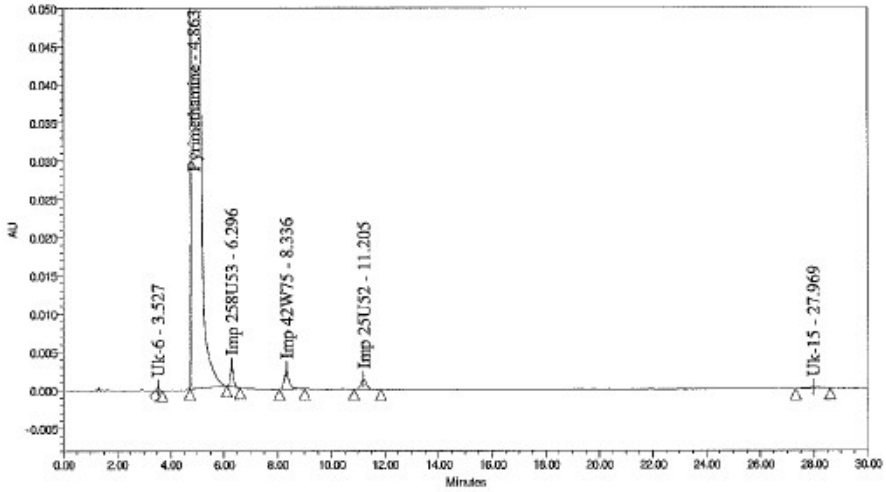

Fig 11: Typical chromatogram of spiked sample

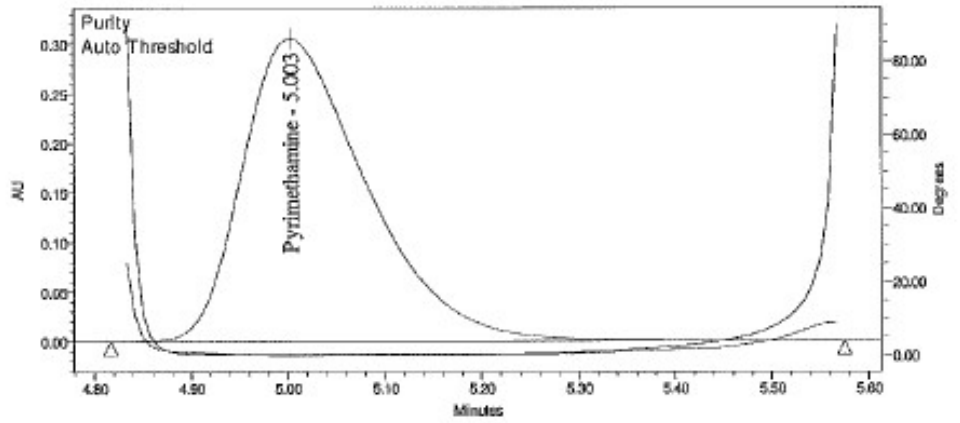

Fig 12: Peak purity plot for spiked sample

Table 15: Spiked sample RT and RRT

S.no	Sample Name	Name	RT	RT Ratio
1	Spiked sample	Pyrimethamine	4.863	1.00
2	Spiked sample	Imp 258U53	6.296	1.29
3	Spiked sample	Imp 42W75	8.336	1.71
4	Spiked sample	Imp 25U52	11.205	2.30

Table 16: Robustness data for Pyrimethamine

Parameter	% RSD
Column temperature plus	0.10
Column temperature minus	0.30
Flow rate plus	0.10
Flow rate minus	0.20
pH plus	0.10
pH minus	0.10

Table 17: Filter study

S.no	Sample name	Imp 258U53	Imp 42W75	Imp 25U52
1	Centrifuged sample	0.275	0.299	0.277
2	Filter study_2ml discarded	0.277	0.266	0.279
3	Filter study_4ml discarded	0.274	0.291	0.277
4	Filter study_6ml discarded	0.275	0.269	0.279
5	Filter study_8ml discarded	0.277	0.280	0.277

Table 18: Filter study % difference in % impurity

S.no	Sample name	Imp 258U53	Imp 42W75	Imp 25U52
1	Centrifuged sample	NA	NA	NA
2	Filter study_2ml discarded	0.7	0.7	11.0
3	Filter study_4ml discarded	0.4	0.0	2.7
4	Filter study_6ml discarded	0.0	0.7	10.0
5	Filter study_8ml discarded	0.7	0.0	6.4

Table 19: LOD for pyrimethamine and its impurities

S.no	Sample name	LOD Concentration µg/ml	USP S/N
1	Pyrimethamine	0.0256	9
2	Imp 258U53	0.0257	5
3	Imp 42W75	0.0254	7
4	Imp 25U52	0.0256	3

Table 20: Ruggedness data

Impurity name	% Impurity		% Difference
	Analyst-1	Analyst-2	
Impurity 258U53	0.274	0.284	4
Impurity 42W75	0.287	0.279	3
Impurity 25U52	0.274	0.268	2

CONCLUSION

The developed RP-HPLC method for the related substance analysis in Pyrimethamine tablets was found to be Accurate, precise, Specific, linear, robust and rugged. Forced degradation study demonstrated the stability of the method and its suitable to analyse the product from different stability conditions. Filter study shows the absence of interference. All the results were complies with the acceptance criterions as per the ICH guidelines. Hence this method is appropriate to analyse the impurities present in the Pyrimethamine tablet dosage form.

ACKNOWLEDGEMENT

The authors are thankful to Department of Chemistry, Jaipur National University, Rajashthan. India for providing necessary facilities to carry out the research work

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