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Simultaneous Estimation of Pyrimethamine and Sulfamethoxypyrazine In a Marketed Formulation by using Chromatography

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Abstract: *The current research work explains the simultaneous estimation and validation of Pyrimethamine and sulfamethoxypyrazine present in a marketed formulation by using RP-HPLC. The chromatographic conditions for the separation was optimized on C_{18} inertsil column (250mm× 4.6mm i.d, 5µm particle size). The mobile phase used was potassium dihydrogen phosphate (10mmol) and Acetonitrile in the ratio of (70:30) with pH 3.5 using ortho phosphoric acid by isocratic method at a flow rate of 1ml/min, injection volume was10µl, column temperature was maintained at 25°c and pyrimethamine and sulfamethoxypyrazine were detected at 221 nm using an ultraviolet(UV) visible detector. The retention time was found to be 5.340 min and 9.260 min for Pyrimethamine and sulfamethoxypyrazine respectively. The developed method was validated as per ICH guidelines in terms of linearity, precision, accuracy, specificity, robustness and forced degradation studies.*

Key words: **Pyrimethamine, sulfamethoxypyrazine, Reverse phase high performance liquid chromatography, Validation, Forced degradation studies.**

INTRODUCTION

Pyrimethamine is 5-(4-chlorophenyl)-6-ethylpyrimidine-2,4-diamine [1]. It works by inhibiting the dihydrofolate reductase of plasmodia and there by blocking the biosynthesis of purines and pyrimidines, which are essential for DNA synthesis and cell multiplication, and gradually reduces the schizogony of malarial parasite in blood [2]. Chemical Formula: $C_{12}H_{13}ClN_4$ [3].

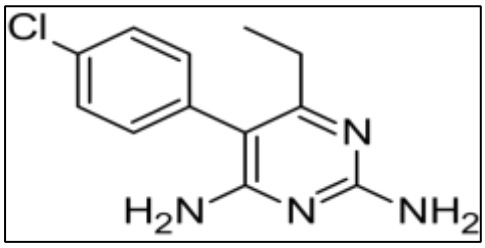

Fig. 1: Chemical structure of Pyrimethamine [2]

Sulfamethoxypyrazine is 4-amino-*N*-(3-methoxypyrazin-2-yl) benzenesulfonamide [4]. Sulfamethoxypyrazine is a competitive inhibitor of the bacterial enzyme dihydropteroate synthetase. Para-aminobenzoic acid (PABA), a substrate of the enzyme is prevented from binding. The inhibited reaction is necessary in these organisms for the synthesis of folic acid [5]. Chemical formula: $C_{11}H_{12}N_4O_3S$ [6].

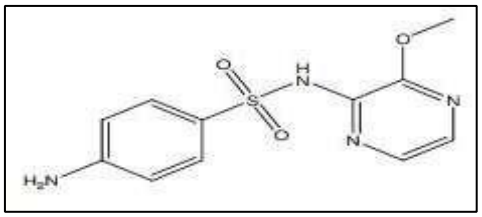

Fig. 2: Chemical structure of Sulfamethoxypyrazine [6]

Combination of pyrimethamine and sulfamethoxypyrazine (Lari 500) is used for Malaria, Bacterial infections, Irritation and redness in membrane covering the eye, Parasitic disease, Malaria infections, Urinary tract infections and other conditions [7].

The present work was carried to develop a new method according to ICH guidelines which was precise, accurate, rapid, and cost effective and to validate the method for simultaneous estimation of Pyrimethamine and sulfamethoxypyrazine.

Material and methods

Pyrimethamine and sulfamethoxypyrazine were procured as a gift sample from Piramal enterprises limited, Mumbai. LARI 500 (pyrimethamine-25 mg and sulfamtehoxypyrazine-500 mg) tablet purchased from local pharmacy. Acetonitrile (HPLC grade), Potassium dihydrogen phosphate (Merck), ortho phosphoric acid (Merck), Methanol (HPLC grade), Hydrogen Peroxide (Merck), Hydrochloric acid (Merck), Sodium hydroxide (Merck), and Milli Q water.

Instrumentation

Agilent 1260 infinity HPLC module, HPLC column C_{18} inertsil column (250mm× 4.6mm i.d, 5µm particle size) were used for the method development and validation, Kroma Tech (KL-1.5) sonicator was used for sonication, PH meter (LAB INDIA), UV 1800 (Schimadzu), Digital balance (Conitech).

Chromatographic condition

The separation of pyrimethamine and sulfamethoxypyrazine was carried out using Agilent 1260 infinity HPLC module system with C_{18} inertsil column (250mm× 4.6mm i.d, 5µm particle size). The mobile phase used was potassium dihydrogen phosphate (10 mmol) and Acetonitrile in the ratio of (70:30) with pH 3.5 using ortho phosphoric acid using isocratic method at a flow rate of 1ml/min, injection volume was 10µl, column temperature was maintained at 25°c, and pyrimethamine and sulfamethoxypyrazine were detected at 221 nm using an ultraviolet(UV) visible detector.

Selection of wavelength

Standard solution of pyrimethamine (10 ppm) and sulfamethoxypyrazine (10 ppm) were prepared and scanned by UV spectrophotometer separately, in the range of 200-400nm . The 221 nm wavelength was selected as detection wavelength for the separation of pyrimethamine and sulfamethoxypyrazine shown in Fig. 3.

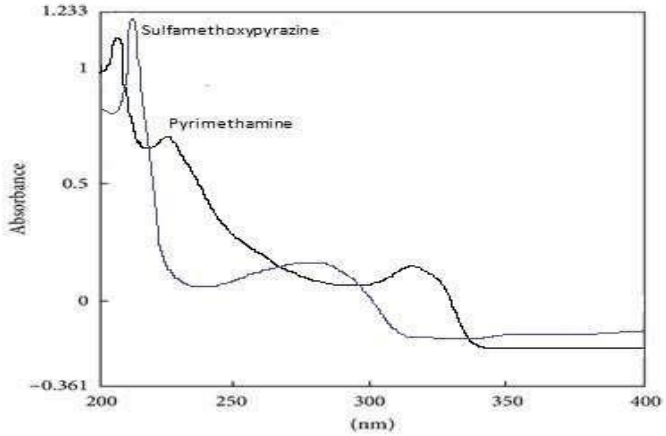

Fig. 3: UV Spectra of Pyrimethamine and sulfamethoxypyrazine

Preparation of standard stock solution

Standard stock solution was prepared by dissolving 50 mg of sulfamethoxypyrazine in a 50 ml volumetric flask about 30 ml of diluents was added, sonicated for 5 min and then volume was made up with the diluent.

10 ml of the above solution was further diluted into a 50 ml volumetric flask (giving 200 ppm of sulfamethoxypyrazine).

5 mg of pyrimethamine was dissolved into a 50 ml volumetric flask about 30 ml of diluents was added, sonicated for 5 min and then volume was made up with the diluent.

5 ml of the above solution was further diluted into the same 50 ml volumetric flask (giving 10 ppm of pyrimethamine).

Preparation of sample solution

5 tablets were weighed, crushed and the average weight of each tablet was calculated. The weight equivalent was transferred into a 50 ml volumetric flask about 30 ml of diluent was added, sonicated for 10 mins to dissolve and then the volume was made up with diluent filtered through 0.45µ filter.

Method validation

The developed method for pyrimethamine and sulfamethoxypyrazine was validated for parameter such as system suitability, specificity, linearity, precision, accuracy, robustness, and solution stability as per ICH guidelines [8-11].

Forced degradation studies

Forced degradation studies include the degradation of new drug substance and drug product at conditions more severe than accelerated conditions. These studies illustrate the chemical stability of the molecule which further facilitates the development of stable formulation with suitable storage conditions. ICH guidelines demonstrate certain degradation conditions like light, oxidation, dry heat, acidic, basic and hydrolysis etc [12].

For acid and alkali stress condition 2ml of 1N HCL and 2ml of 1N NaOH were added respectively to the sample solution and kept for 2hr, for oxidative degradation 2ml of 30% H_2O_2 was added and kept for 2hr for water hydrolysis 2ml of Milli q water was added kept for 2hr. Thermal degradation was performed by keeping the sample solution in a petridish and placing in an oven at 60°C for 2 hr.

Method development

A series of trials were carried out using different mobile phases such as acetonitrile: water (50:50), methanol: water (50:50), and buffer: methanol (60:40) using different column such as prontosil, inertsil ODS and shim pack C18 column to develop RP-HPLC method for simultaneous estimation of pyrimethamine and sulfamethoxypyrazine in a marketed tablet dosage form. Finally, a typical chromatogram was obtained using potassium dihydrogen phosphate (10mmol): Acetonitrile (70:30) (pH 3.5 by using orthophosphoric acid) as mobile phase on C_{18} inertsil column (250mm× 4.6mm i.d, 5µm particle size) with injection volume of 10□l at a flow rate of 1ml/min. The run time was 12 min and the column temperature was maintained at 25° C and detection was carried out at 221 nm. The retention time was 5.340 min and 9.260 min for pyrimethamine and sulfamethoxypyrazine respectively. Typical chromatograms of standard and sample solution of pyrimethamine and sulfamethoxypyrazine are shown in Fig. 4 and Fig. 5. The same developed method was applied for force degradation studies of pyrimethamine and sulfamethoxypyrazine marketed tablet dosage form. The optimized chromatographic conditions are tabulated in table1.

Table 1. Optimized chromatography condition for pyrimethamine and sulfamethoxypyrazine

Parameters	Optimized conditions
Mobile phase	potassium dihydrogen phosphate (10mmol): Acetonitrile (70:30), PH 3.5 with OPA
Diluent	Methanol (100%)
Column	C_{18} inertsil (250mm× 4.6mm i.d, 5µm particle size)
Column temperature	25℃
Wavelength	221 nm
Flow rate	1ml/min
Injection volume	10µl
Run time	12 mins
Retention time	5.340 min and 9.260 min

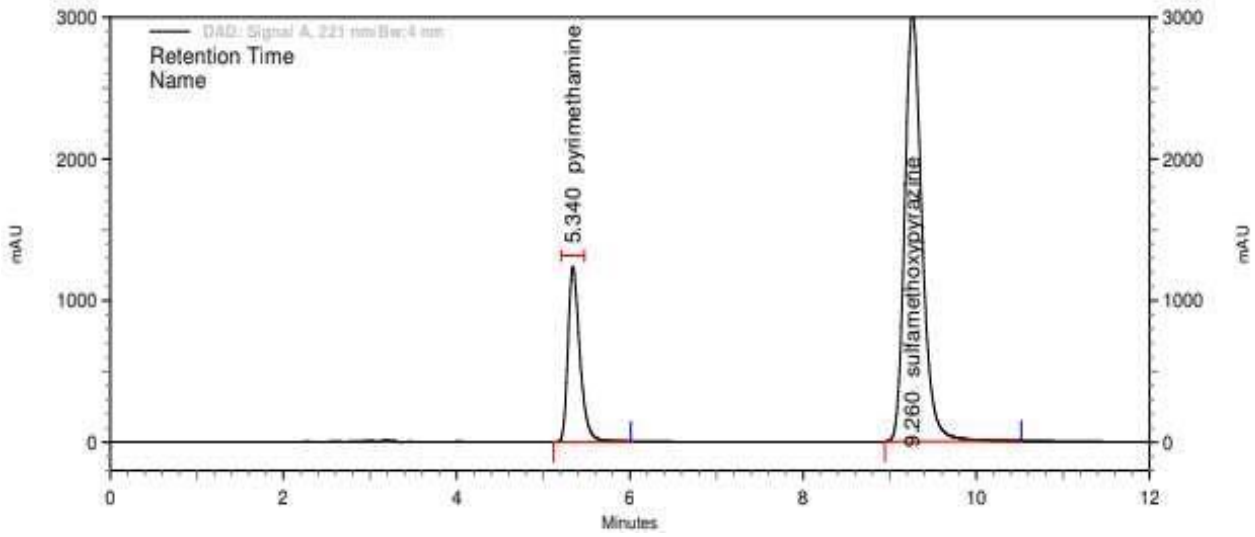

Fig. 4: Typical chromatogram of a standard mixture of pyrimethamine and sulfamethoxypyrazine

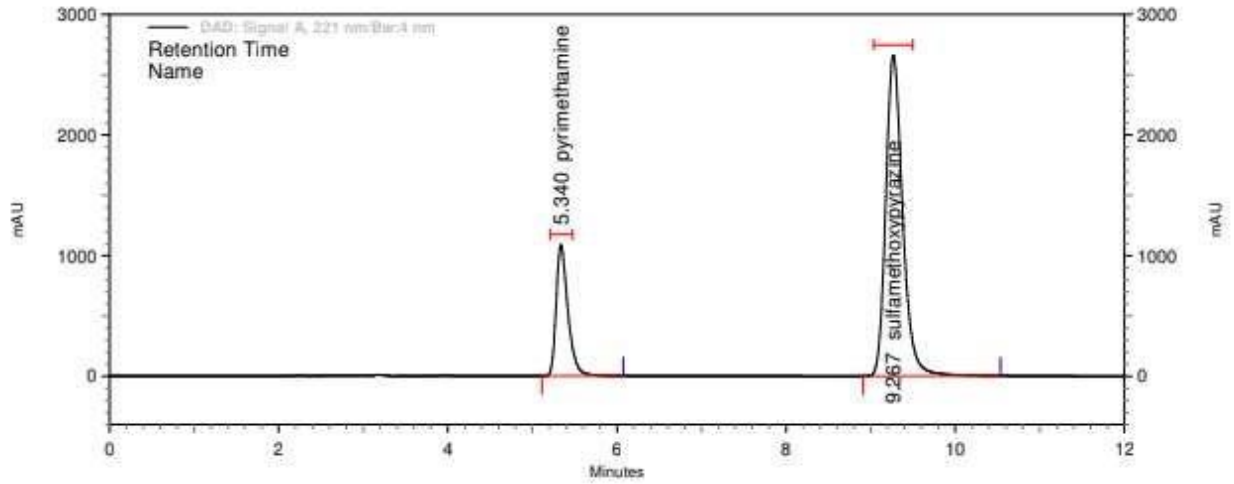

Fig. 5: Typical chromatogram of a sample of pyrimethamine and sulfamethoxypyrazine

System suitability

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics analytical operations and samples to be analysed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated [13].

System suitability was done by injecting six replicates injection of the standard solution and retention time, tailing factor, and number of theoretical plates were evaluated. The standard solution of pyrimethamine and sulfamethoxypyrazine were prepared as per the above method. system suitability results are tabulated in Table 2.

Table 2: System suitability results

Parameters	Pyrimethamine	Sulfamethoxypyrazine
Tailing factor	1.03	1.01
Retention time (mins)	5.34	9.26
Number of theoretical plates	5140	4213

Linearity

The linearity of a method is a check of how well a calibration plot of response vs. concentration estimate a straight line. Linearity can be determined by performing single measurements at several analyte concentrations [14].

The linearity of the developed method was determined at different concentration levels ranging from 80-120% for pyrimethamine and sulfamethoxypyrazine. The linearity curve was constructed by plotting peak area versus concentration and the regression coefficient (r^2) was found to be 0.999 for pyrimethamine and 0.999 for sulfamethoxypyrazine. From linearity results, it was found that the developed method is linear (Fig. 6 and 7).

Result are shown in table 3.

Table no 3: linearity results for pyrimethamine and sulfamethoxypyrazine

Sr.no	Concentration Level (%)	Peak areas	
		Pyrimethamine	sulfamethoxypyrazine
1	80	1702308	6170719
2	90	1921876	6924147
3	100	2120893	7654084
4	110	2340809	8375059
5	120	2550087	9172748
Correlation (r^2)		0.999	0.999

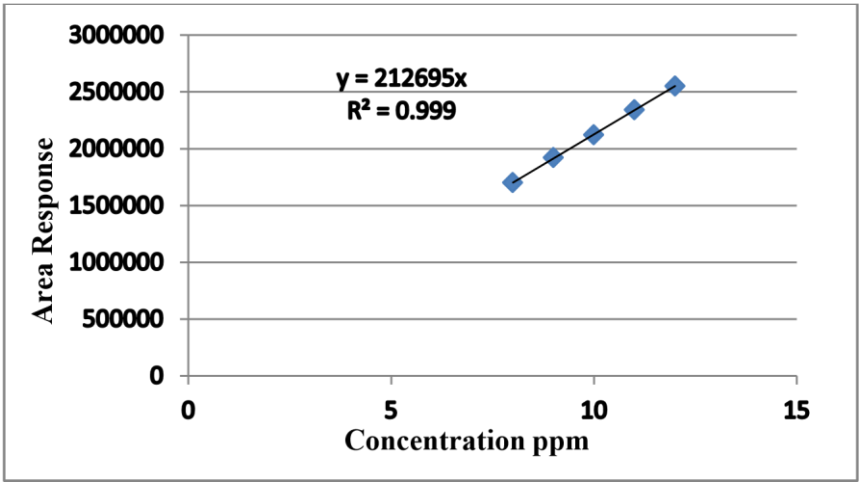

Fig. 6: Linearity graph of Pyrimethamine

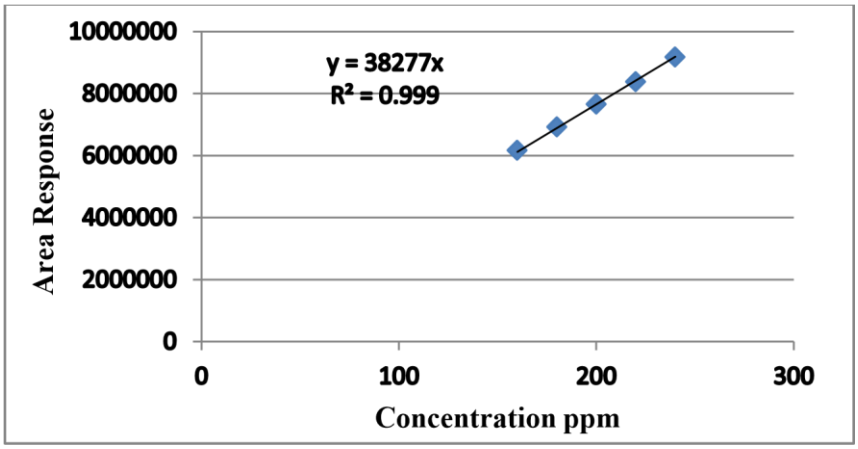

Fig. 7: Linearity graph of sulfamethoxypyrazine

Precision

The precision of an analytical procedure may be defined as the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings [15].

The system precision and method precision were performed by injecting six injections of pyrimethamine and sulfamethoxypyrazine standard and sample of the same concentration. The percentage relative standard deviation (%RSD) was calculated from the chromatogram area and it should be less than 2%. From precision result, it was found that the method is precise. The data of system and method precision are tabulated in Table 4.

Table 4: System precision results

Sr. No.	Peak area	
	Pyrimethamine	Sulfamethoxypyrazine
1	2145697	7734084
2	2155492	7756986
3	2144835	7732659
4	2146524	7745698
5	2143319	7789456
6	2146528	7712459
Average	2147066	7745224
SD	4301	26270
%RSD	0.20	0.34

Table 5: Method precision results

Sr. No.	% Assay	
	Pyrimethamine	Sulfamethoxypyrazine
1	99.00	99.50
2	99.80	100.50
3	99.90	99.20
4	99.70	99.80
5	99.90	99.20
6	99.00	99.20
Average	99.20	99.60
SD	0.42	0.52
%RSD	0.42	0.52

Accuracy

Accuracy shows the nearness of agreement between the value which is received either as a traditional true value or an accepted reference value and the value found [16].

The accuracy of pyrimethamine and sulfamethoxypyrazine was performed by calculating recovery studies of the test sample at three different concentration levels (80%, 100%, 120%). The mean percentage recovery for pyrimethamine and

sulfamethoxypyrazine was found to be within a limit of 98-102%, and from percentage recovery results, it was found that the developed method is accurate. The percentage recovery results are tabulated in Table 6 and 7.

Table 6. % recovery of pyrimethamine

Level	% recovery	Average	SD	%RSD
80%	99.60	99.20	0.57	0.57
	98.60			
	99.60			
100%	99.00	99.20	0.46	0.46
	99.80			
	99.00			
120%	98.90	98.50	0.34	0.34
	98.30			
	98.30			

Table 7. % recovery of sulfamet ıoxypyrazine

Level	% recovery	Average	SD	%RSD
80%	99.20	98.90	0.58	0.58
	98.20			
	99.20			
100%	99.20	99.60	0.53	0.53
	100.20			
	99.40			
120%	99.50	99.00	0.40	0.40
	98.80			
	98.80			

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present [18].

The specificity of the analytical method was determined by injecting the same concentration of standard solution and same concentration of sample solution. The chromatogram obtained were specific and separate from each other.

Assay

For assay of marketed formulation (Lari 500: Pyrimethamine 25 mg, sulfamethoxypyrazine 500 mg) 5 tablets were weighed, crushed and equivalent weight was transferred into a 50 ml volumetric flask about 30 ml of diluent was added, sonicated for 10 mins to dissolve and then the volume was made up with diluent filtered through 0.45µ filter.

5 ml of the above solution was further diluted into a 50 ml volumetric flask. The percentage assay for the marketed formulation was found to be 99.20% for Pyrimethamine and 99.70% for sulfamethoxypyrazine respectively shown in Table 8.

Table 8. %Assay of marketed formulation

Tablet	Drug	%Assay
Lari 500	Pyrimethamine	99.20
	Sulfamethoxypyrazine	99.70

Solution stability

Sample solution of Pyrimethamine and sulfamethoxypyrazine was injected at different time intervals and percentage assay was calculated. It was found that solution was stable over a period of 15 hr without any degradation of the solution.

Stability results are shown in Table 9.

Table 9. Solution stability results

Time interval	%Assay	
	Pyrimethamine	sulfamethoxypyrazine
Initial	100.30	99.90
10 hr	100.20	99.90
15 hr	100.30	100.20

Robustness

Robustness is the ability of the procedure to provide analytical results of acceptable accuracy and precision under variety of conditions [18].

The developed method was evaluated for robustness by small deliberate changes in optimized method parameters such as flow rate (±0.1 ml/min) and temperature (±2℃). It was found that none of the above parameters caused an alteration in the peak area and retention time. The %RSD was found to be within the limits, and the method was found to be robust. The robustness results are shown in table 10.

Table 10: Robustness results

Parameters	Proposed	Variation	Pyrimethamine	Sulfamethoxypyrazine
			%Assay	
Flow rate	1.0 ml/min	0.9 ml/min	99.30	99.80
		1.1 ml/min	99.00	99.50
Temperature	25°C	23°C	99.60	99.80
		27°C	99.50	99.40

Forced degradation studies

Forced degradation studies were carried out on pyrimethamine and sulfamethoxypyrazine marketed tablet formulation by treating the marketed formulation under stress conditions such as acidic, alkaline, hydrolysis, oxidative and thermal condition

to estimate the ability of the developed method to separate pyrimethamine and sulfamethoxypyrazine from its degradation product as shown in Fig. 8-12. The forced degradation results are within the limit and it is tabulated in table 11.

Acid degradation studies

In acid degradation, 2ml of 1N HCL was added into sample solution and kept for 2hr and the chromatogram was recorded to detect the stability of sample (Fig. 8). A degradant peak was seen at 2.260 min.

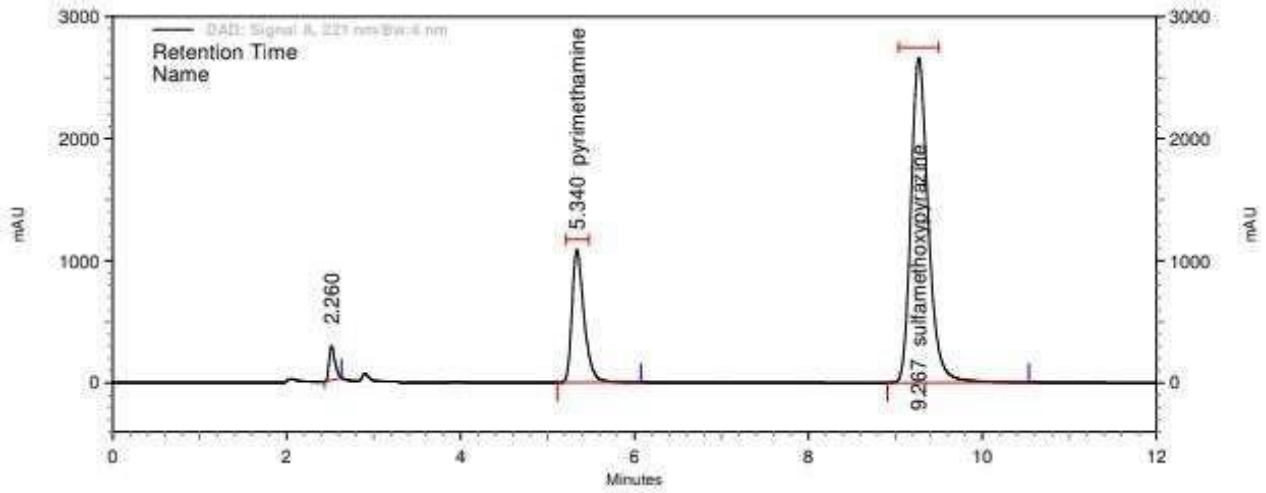

Fig. 8: Acid degradation chromatogram

Alkali degradation studies

In alkali degradation, 2ml of 1N NaOH was added into sample solution and kept for 2 hr. The chromatogram was recorded to detect the stability of sample (Fig. 9).

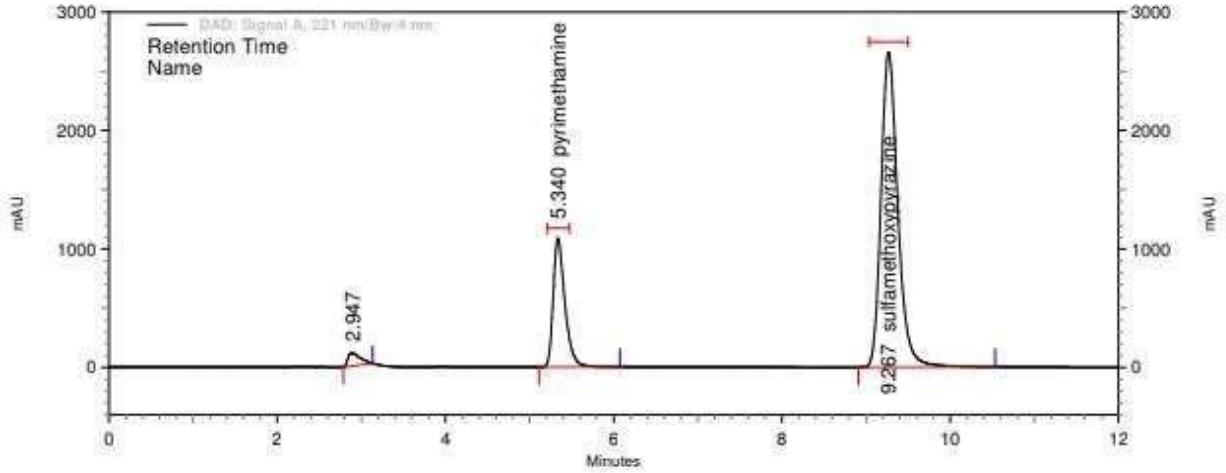

Fig. 9: Basic degradation chromatogram

Oxidative degradation

In oxidative degradation, 2ml of 30% H_2O_2 was added into sample solution and kept for 2hr. The chromatogram was recorded to detect the stability of sample (Fig.10).

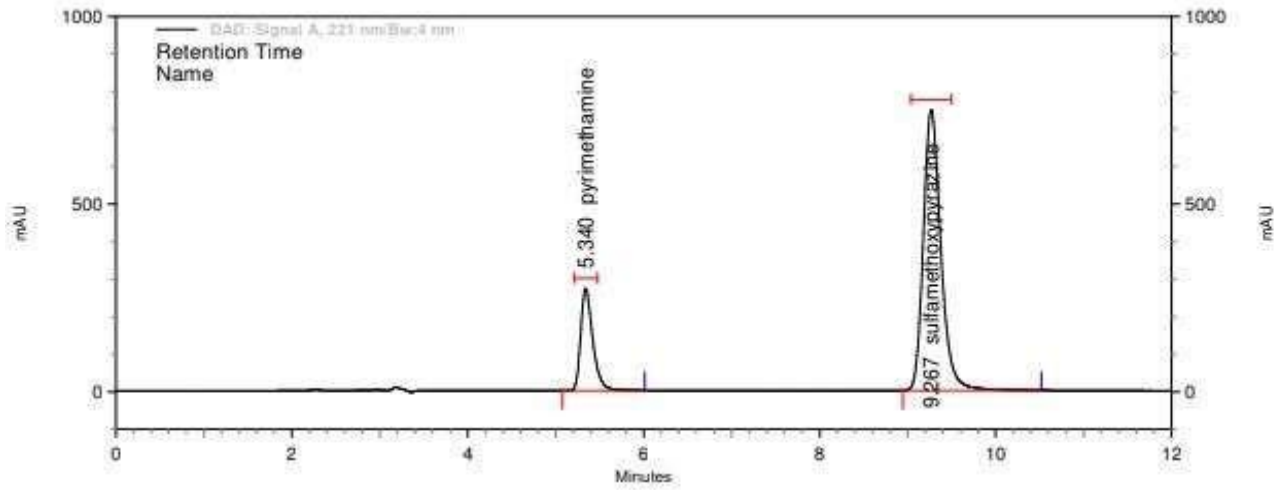

Fig. 10: Oxidative degradation chromatogram

Thermal degradation studies

In thermal degradation, sample solution was kept in a petri plate and placed in an oven at 60° C for 2 hr. The chromatogram was recorded to detect the stability of sample (Fig. 11).

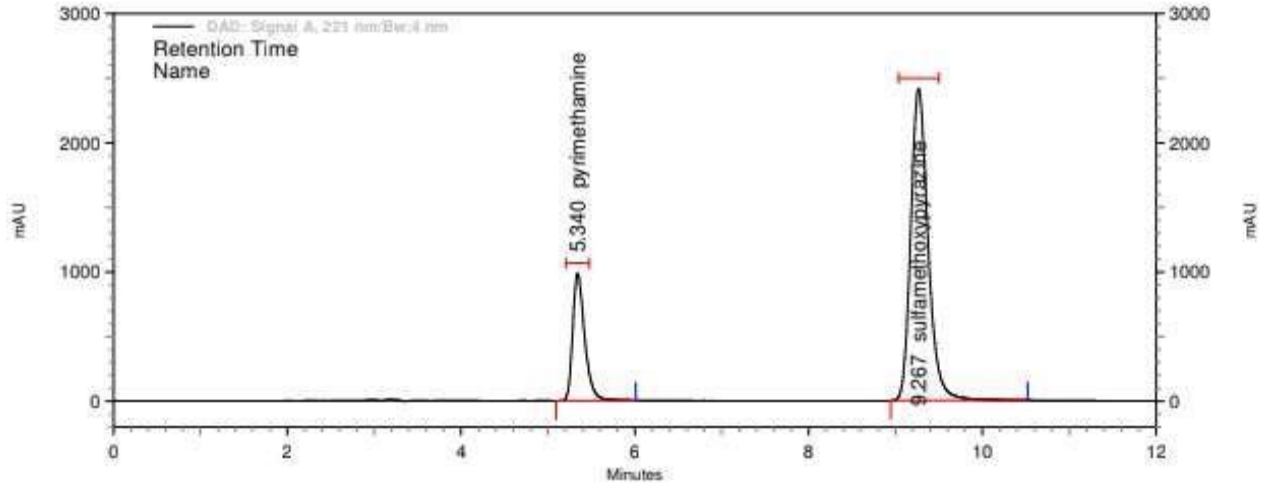

Fig. 11: Thermal degradation chromatogram

Hydrolysis degradation

In water hydrolysis, 2ml of Milli Q water was added into sample solution and kept for 2 hr. The chromatogram was recorded to detect the stability of sample (Fig. 12).

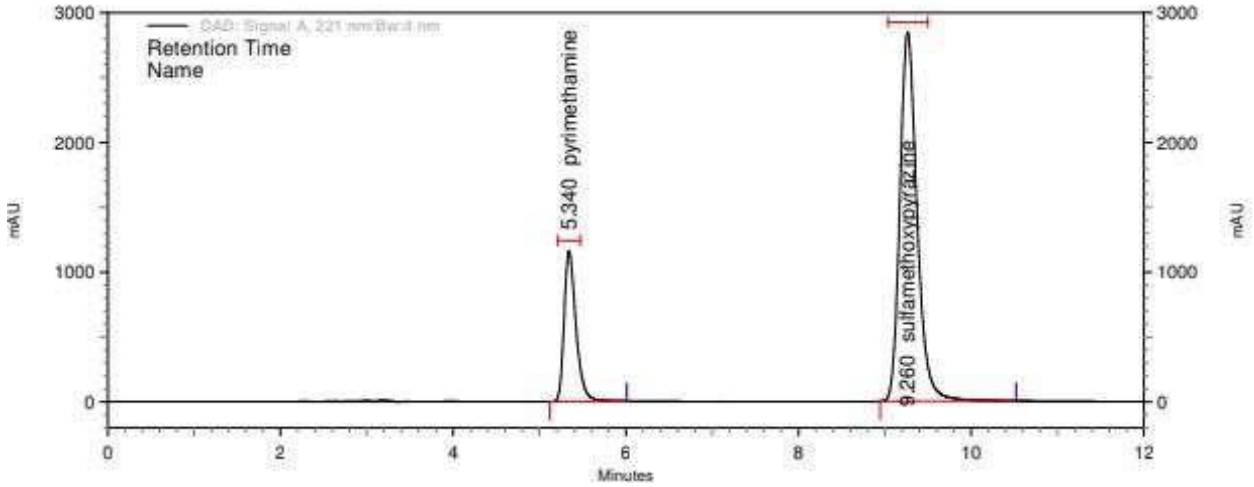

Fig. 12: Hydrolysis degradation chromatogram

Table 11: Forced degradation results

Conditions	% Assay	
	Pyrimethamine	Sulfamethoxypyrazine
Control sample	99.20	99.70
Acid treated sample	98.70	99.40
Base treated sample	98.90	99.30
Heat treated sample	99.30	99.50
Peroxide treated sample	99.10	99.65
Water treated sample	99.40	99.80

CONCLUSION

A new RP-HPLC method for simultaneous estimation has been developed and successfully validated for Pyrimethamine and sulfamethoxypyrazine in bulk and pharmaceutical dosage form. The above RP-HPLC method allows for novel, precise, accurate, robust and reliable estimation of drugs simultaneously in combined dosage form. Method was validated as per ICH guidelines and RSD for all parameters was found to be within limits, the developed method can be used for routine quantitative simultaneous estimation of Pyrimethamine and sulfamethoxypyrazine in pharmaceutical preparation.

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AUTHOR'S CONTRIBUTION

All the authors have contributed equally.

CONFLICTS OF INTEREST

The authors claim that there are no conflicts of interest regarding the publication of this article.

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