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Analytical Method Development and Validation of Pyrimethamine and Sulphadoxine in Pharmaceutical Dosage Forms by RP-HPLC

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Abstract
A simple, accurate reverse phase high performance liquid chromatographic method has been developed for the simultaneous estimation of Pyrimethamine and Sulphadoxine in bulk and Pharmaceutical dosage forms. The analytical method development was carried on Agilent made HPLC instrument using RP - C18 column. The mobile phase employed for the estimation is Phosphate Buffer**:** Acetonitrile which was pumped at a flow rate of 0.9 mL min^{-1} in the ratio of 70:30*v/ v* and the eluents were monitored at 224 nm. Linearity was obtained in the concentration range of 5-37.5 µg/ml for Pyrimethamine and 100-750 µg/ml for Sulphadoxine. The correlation coefficient and % curve fitting for Pyrimethamine and Sulphadoxine was found to be 0.9997 and 0.9999 respectively. The mean percentage recovery for Pyrimethamine and Sulphadoxine was found to be between 99.51-100.53% and 99.44-100.46% respectively. The method was statistically validated and RSD was found to be less than 2% indicating high degree of accuracy and precision of the proposed HPLC method. Due to its simplicity, rapidness, high precision and accuracy, the proposed HPLC method can be applied for determining Pyrimethamine and Sulphadoxine in bulk and in pharmaceutical dosage form.

Keywords
Pyrimethamine and Sulphadoxine, Method development, Validation.

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INTRODUCTION
Pyrimethamine is an anti-malarial drug used in the treatment of protozoa infection and it Becomes White, Odorless and Crystalline powder. Practically insoluble in water; slightly soluble in acetone, in alcohol, and in chloroform. Side effects include hypersensitivity reactions, Megaloblastic anemia, Leucopenia, Thrombocytopenia, Pancytopenia, Atrophic Glossitis, Hematuria, cardiac arrhythmias, pulmonary eosinophilia (rare), hyperphenylalaninemia. IUPAC Name is 5-(4-chlorophenyl)-6-ethyl-2, 4-pyrimidinediamine.

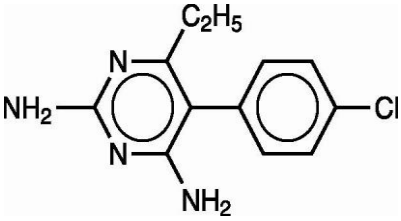

Figure 1: The chemical structure of Pyrimethamine

Sulphadoxine also known as Sulfomethoxine. It is a synthetic origin and belongs to Sulphonamide. It belongs to Dihydropteroate synthetase inhibitor pharmacological group on the basis of mechanism of action. IUPAC Name is 4-amino-N-(5, 6-dimethoxy-4-pyrimidinyl) benzene sulfonamide.

Figure 2: The chemical structures of Sulphadoxine

It's White or creamy white, crystalline powder, odorless and slightly soluble in water, ethanol (96%) and methanol, insoluble in ether, soluble in dilute sulphuric acid. The symptomatic adverse reactions produced by Sulfadoxine are more or less tolerable and if they become severe, they can be treated symptomatically, these include Headache, Fatigue, Fever, Rashes, Stomatitis, Pruritus, Photosensitivity, Nausea and vomiting, Orthostatic hypotension, Feeling of fullness, Hepatic granulomata, Polyneuritis.

MATERIALS AND METHODS

Methanol HPLC Grade, Acetonitrile HPLC Grade and Water HPLC Grade were commercially procured from Merck chemical division, Mumbai. Orthophosphoric acid, Potassium dihydrogen Orthophosphate and Triethylamine and all other solvents were of AR grade and obtained from S.D. Fine chem. Limited, Mumbai.

METHOD DEVELOPMENT

Based on drug solubility and pK_a values, following conditions were used to develop the method for simultaneous estimation of Pyrimethamine and Sulphadoxine.

Preparation of Solutions:

Buffer:

Accurately weighed 2.72gm of Potassium dihydrogen orthophosphate taken in a 1000ml volumetric flask. About 900ml of milli-Q water was added and sonicated to dissolve and finally made up the volume with water. Then added 0.5ml of Triethylamine. Then p^H was adjusted to 3.0 with dil. Orthophosphoric acid solution.

Standard stock solution:

Accurately weighed and transferred 500mg of Sulphadoxine and 25mg of Pyrimethamine into a 50 ml clean, dry volumetric flask. Then 30ml of diluents was added, sonicated for 5 minutes, filtered and made up the final volume with diluents.

From this stock solution, 0.5ml was taken into a 10ml clean, dry volumetric flask and made up the volume with diluents to get a solution containing 25µg/ml of Pyrimethamine and 500µg/ml of Sulphadoxine.

Sample Preparation:

5 tablets were weighed and average weight was determined and powdered. An amount of powder equivalent to average weight of 5 tablets (equivalent to 25mg of Pyrimethamine and 500mg of Sulphadoxine) was weighed and transferred into a 100 ml volumetric flask. 60ml of diluents was added and sonicated for 25 min to ensure complete dissolution. Then the solution was filtered and further the volume was made up with diluents. From this solution, 0.2ml was taken into a 10 ml volumetric flask and made up the volume with diluents.

Chromatographic conditions:

Column : Waters X Bridge, C_{18}, 150×4.6mm, 5µm or equivalent

Mobile phase: Phosphate Buffer**:** Acetonitrile (70**:**30)

Flow rate: 0.9 l

Detector wavelength: 224nm

Runtime: 8minutes

Diluent: Methanol

Temperature: 300C

Injection Volume: 10µL

VALIDATION OF DEVELOPED HPLCMETHOD:

A HPLC method was developed for the simultaneous estimation of Pyrimethamine and Sulphadoxine in tablet dosage form using Waters X Bridge (150 x 4.6 mm, 5□.) Mobile phase Buffer and Acetonitrile (70:30), detection wavelength at 224 nm, at flow rate of 0.9 ml/min with retention time of 2.89 min for Pyrimethamine, 3.47 min for Sulphadoxine.

Since the HPLC method was developed, validation of the method by using various parameters was performed to ensure that the performance characteristics of the method meet the requirements for intended analytical applications.

PRECISION:

The precision of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Precision should be performed at three different levels: repeatability, intermediate precision and reproducibility.

A. Method precision

5 tablets were weighed and average weight was determined and powdered. An amount of powder equivalent to average weight of 5 tablets (equivalent to 25mg of Pyrimethamine and 500mg of Sulphadoxine) was weighed and transferred into a 100 ml volumetric flask. 60ml of diluent was added and sonicated for 25 min to ensure complete dissolution. Then the solution was filtered and further the volume was made up with diluent. From this solution, 0.2ml was taken into a 10 ml volumetric flask and made up the volume with diluent. The values were represented in the table 1 given below.

Table 1: Method Precision parameters

Sample No	Sample (Pyrimethamine)	%Assay	Sample (Sulphadoxine)	%Assay
1.	729016	100.88	3153905	101.33
2.	722032	99.91	3151224	101.24
3	721890	99.89	3151735	101.26
4	721402	99.82	3138290	100.82
5	724051	100.19	3139110	100.85
6	720344	99.68	3127897	100.49
AVG	723123	100.062	3143694	101.00
S.D	3130.0	0.4331	10250.7	0.3293
% RSD	0.43	0.43	0.33	0.33

The % assay of Pyrimethamine and Sulphadoxine were found to be 100.06% and 101% respectively. The %RSD for both drugs is less than 2%.

B. Intermediate Precision Results

Accurately weighed and transferred 500mg of Sulphadoxine and 25mg of Pyrimethamine into a 50 ml clean, dry volumetric flask. Then 30ml of diluent was added, sonicated for 5 minutes, filtered and made up the final volume with diluent. From this stock solution, 0.5ml was taken into a 10ml clean, dry volumetric flask and made up the volume with diluent to get a solution containing 25µg/ml of Pyrimethamine and 500µg/ml of Sulphadoxine. Intermediate Precision Results were given in the table 2.

Table 2: Intermediate Precision Results

Injections (n)	Pyrimethamine			Sulphadoxine		
	Peak area	Retention time	Tailing factor	Peak Area	Retention Time	Tailing Factor
1	1078936	2.963	1.36	4694010	3.515	1.23
2	1078927	2.954	1.4	4691388	3.508	1.25
3	1079968	2.946	1.37	4694073	3.499	1.24
4	1087035	2.862	1.42	4716215	3.424	1.25
5	1084503	2.9	1.43	4706324	3.456	1.25
6	1082001	2.923	1.41	4703315	3.481	1.24
Mean	1081895			4700888		
SD	3301.8			9532.7		
%RSD	0.31			0.20		

Acceptance Criteria: % RSD Should not be is more than 2.0%.

ACCURACY:

Accuracy may often the expressed as percent recovery by the assay of known added amounts of analyte.

Recovery study for Pyrimethamine and Sulphadoxine at 3 levels:

Standard stock solution of Pyrimethamine (500µg/ml):

Accurately weighed 25mg of Pyrimethamine was taken in a clean, dry 50ml volumetric flask, added with sufficient volume of diluent and sonicated for 5min. Then the solution was filtered and volume was made up to 50ml with the diluent.

Standard stock solution of Sulphadoxine (10000µg/ml):

Accurately weighed 500 mg of Sulphadoxine was taken in a clean, dry 50 ml volumetric flask, added with sufficient volume of diluent and sonicated for 5min. Then the solution was filtered and volume was made up to 50ml with the diluent. Suitable dilutions were made for further stock solutions. The accuracy results were given in the table 3 a & 3 b for pyrimethamine and sulphadoxine respectively.

Table 3 a: Accuracy results of Pyrimethamine

Level (%)	S. No	Peak Area	Recovery (%)	Mean Recovery (%)	SD	RSD (%)
50 %	1	364238	100.80	100.53	0.5623	0.56
	2	364609	100.91			
	3	360919	99.89			
100 %	1	1132080	99.92	100.21	0.4198	0.42
	2	1132813	100.02			
	3	1137663	100.69			
150 %	1	1729008	99.54	99.51	0.0341	0.03
	2	1728321	99.48			
	3	1758903	99.53			

Table 3 b: Accuracy results of Sulphadoxine

Level (%)	S.No	Peak Area	Recovery (%)	Mean Recovery (%)	SD	RSD (%)
50 %	1	1541057	99.02	99.44	0.6781	0.68
	2	1542142	99.09			
	3	1559854	100.23			
100 %	1	4917393	100.15	100.46	0.3985	0.40
	2	4929643	100.35			
	3	4941311	100.92			
150 %	1	7463988	99.89	99.77	0.1710	0.17
	2	7462128	99.85			
	3	7619325	99.58			

Acceptance criteria: The % recovery should be within the limits of 98% to 102%.

LIMIT OFDETECTION

LOD parameter was performed to know the the lowest concentration of the analyte in a sample that can be detected, though not necessarily quantitated. The values were shown in the table 4.

Table 4: LOD report of Pyrimethamine and Sulphadoxine

Drug	Limit of Detection (µg/ml)
Sulphadoxine.	2.63 µg/ml
Pyrimethamine.	0.49 µg/ml

The S/N ratio for both Pyrimethamine and Sulphadoxine was 3.1. Limit of detection of Sulphadoxine and Pyrimethamine were found to be 2.63µg/ml and 0.49µg/ml respectively.

LIMIT OF QUANTITATION:

LOQ parameter was performed to know the lowest amount of analyte in sample which can be determined and quantified with precision and accuracy. The values were shown in the table 5.

Table 5: LOQ report of Pyrimethamine and Sulphadoxine

Drug	Limit of Quantification (µg/ml)
Sulphadoxine	7.9 µg/ml
Pyrimethamine	1.51 µg/ml

The S/N ratio of Pyrimethamine and Sulphadoxine was found to be 9.2 and 9.1 respectively. Limit of quantitation of Sulphadoxine and Pyrimethamine were found to be 7.9µg/ml and 1.51 µg/ml respectively.

LINEARITY AND RANGE:

The linearity parameter was performed to ensure that the test results are directly proportional to the concentration of analyte sample. Solutions of various percentages were prepared and injected into HPLC. The peak area and concentration were plotted to get a standard calibration curve. The correlation coefficient and regression coefficient was calculated. The results obtained were tabulated in table 6a and 6b.

Table 6 a: Linearity of Pyrimethamine

S. No	Concentration (µg/ml)	Area
1	5	226874
2	12.5	544892
3	17.5	762650
4	25	1108842
5	30	1307580
6	37.5	1619089

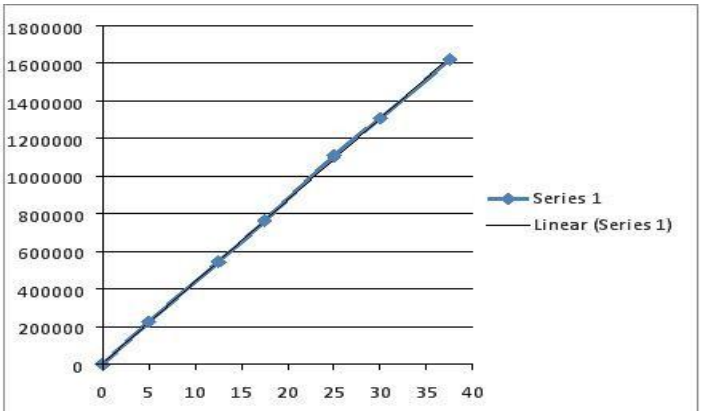

Figure 3: Linearity curve for Pyrimethamine

Table 6 a: Linearity of Sulphadoxine

Sl. No	Concentration (µg/ml)	Area
1	100	948862
2	250	2330482
3	350	3240604
4	500	4602627
5	600	5568667
6	750	6999686

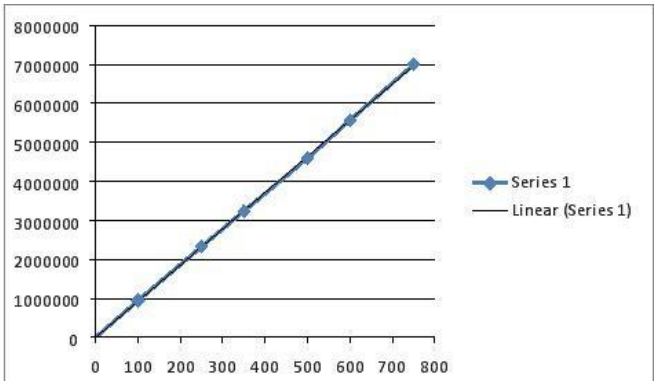

Figure 4: Linearity curve for Sulphadoxine Analytical method validation:

The developed method was validated as per the ICH guidelines in terms of accuracy, precision, linearity, specificity, sensitivity and robustness and and the results were summarized below in table 7.

Table 7: Method validation report

Parameters	Acceptance criteria	Pyrimethamine	Sulphadoxine
Method Precision	% RSD not more than 2	0.43	0.33
Intermediate Precision	% RSD not more than 2	0.31	0.20
Accuracy	% Recovery should be within limits of 98%- 102%	99.51-100.53%	99.44-100.46%
Linearity Range	Correlation coefficient not less than 0.999	0.9997 5-37.5µg/ml	0.9999 100-750µg/ml
LOD	S/N ratio shall be 3	3.1	3.1
LOQ	S/N ratio shall be 10	9.2	9.1
Specificity	No peaks at the retention time of analytes.	No interference	No interference
System Precision	% RSD not more than 2	0.34	0.46

SUMMARY AND CONCLUSION:

The proposed RP-HPLC method was validated as per ICH guidelines and applied for the determination of Pyrimethamine and Sulphadoxine in bulk and pharmaceutical formulations. The method was found to be simple, selective, reproducible, sensitive, robust, specific and accurate with good precision. At the same time the chromatographic elution step is undertaken in a short time (< 4 min). No interference was seen from any components of pharmaceutical dosage form. In conclusion, the high sensitivity, good selectivity, accuracy and precision of the proposed method is suitable for simultaneous determination of Pyrimethamine and Sulphadoxine in bulk and pharmaceutical formulations.

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