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### A VALIDATED RP- HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ARTESUNATE, SULFADOXINE AND PYRIMETHAMINE IN BULK AND TABLETS FORMULATION

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#### ABSTRACT

A simple High Performance Liquid Chromatographic (HPLC) method for separation and quantitative analysis of Sulfadoxine (SDX), Pyrimethamine (PYR) and Artesunate (ART) in bulk and in tablets by RP – HPLC with PDA detector has been established and validated. The HPLC separation was carried out by reverse phase chromatography on ODS Hypersil (250 mm × 4.6 μm, 5μm) column, with mobile phase composed of 0.1M Potassium dihydrogen phosphate buffer (pH 2.2 – adjusted with orthophosphoric Acid) : Acetonitrile (55:45 v/v) in isocratic mode at a flow rate of 1.5 ml/min. The detection was monitored at 254 nm. The calibration curve for Artesunate, Pyrimethamine & Sulfadoxine was linear from 50 to 500 μg/ml, 10 to 60 μg/ml & 250 to 1500 μg/ml respectively. The intermediate precision was found within limits. The proposed method has adequate sensitivity, reproducibility and specificity for the determination of Artesunate, Sulfadoxine, & Pyrimethamine in bulk and its tablet dosage forms. LOD and LOQ for Artesunate were found to be 0.970μg/ml and 3.239μg/ml, for Sulfadoxine 0.221 and 0.735 and for Pyrimethamine were found to be 0.212 and 0.708. Accuracy and reproducibility were found to satisfactory. The suitability of this method for quantitative determination of these compounds was proved by validation in accordance with the requirements ICH guidelines. The method was used for routine analysis of these drugs in bulk and in formulation.

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## INTRODUCTION

Anti malarial chemotherapy has been the primary option in the fight against malaria and over the years many drugs have been developed and used in the treatment of this disease. However, the burden of this disease is still very heavy partly due to the development of multi-drug resistant *Plasmodium falciparum* strains.[1, 2] The rate of increase in the resistance of the malaria parasite – *Plasmodium falciparum* – to antimalarial drugs in many parts of the world is becoming more disturbing. Because of the resistance problems associated with Chloroquine which was considered first-line therapy globally for many years, WHO convened an Informal Consultation on the use of antimalarial Drugs. The potential value of malaria therapy using combinations of drugs was identified as a strategic and viable option in improving efficacy, and delaying development and selection of resistant parasites.[3-5]

Chemically Sulfadoxine is 4-Amino-N-(5, 6-dimethoxypyrimidin-4-yl) benzene-1-sulfonamide.[6] Sulfadoxine is a sulfa drug, often used in combination with Pyrimethamine to treat malaria. The sulfonamides are bacteriostatic antimicrobials that block the incorporation of p-aminobenzoic acid to form dihydropteroic acid. [7-10] (Figure 1) shows structure of Sulfadoxine.

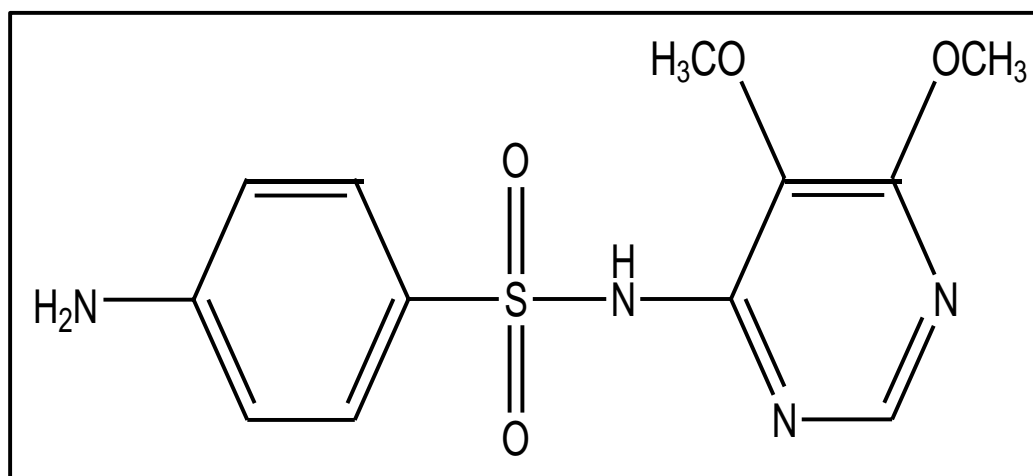


Figure 1: Structure of Sulfadoxine.

Chemically Pyrimethamine is a diaminopyrimidine derivative with the specific chemical name 5-(4-chlorophenyl)-6-ethyl-2,4-pyrimidinediamine. Pyrimethamine inhibits the dihydrofolate reductases of plasmodia and blocks the biosynthesis of purines and pyrimidines, which are essential for DNA synthesis and cell multiplication. This leads to failure of nuclear division at the time of schizont formation in erythrocytes and liver. (Figure 2) shows structure of Pyrimethamine.

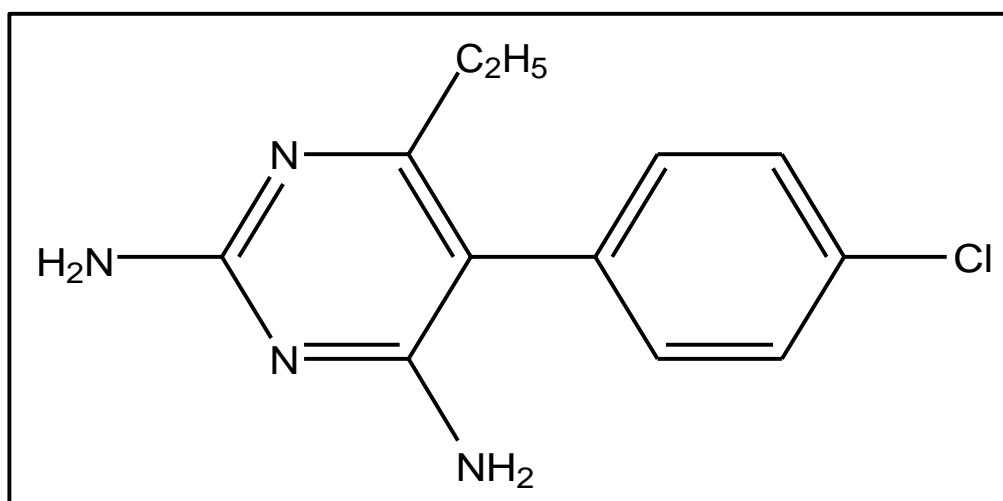


Figure 2: Structure of Pyrimethamine.

Chemically Artesunate is (3*R*,5*aS*,6*R*,8*aS*,9*R*,10*S*,12*R*,12*aR*)-Decahydro-3,6,9 trimethyl-3,12-epoxy-12*H*-pyrano[4,3-*j*]-1,2benzodioxepin-10-ol, hydrogen succinate. Artesunate and its active metabolite dihydroartemisinin are potent blood schizonticides, active against the ring stage of the parasite. Artesunate is ideal for the treatment of severe malaria, including cerebral malaria. It is also active against Chloroquine and Mefloquine resistant strains of *P. falciparum*. [11-14] (Figure 3) shows structure of Artesunate.

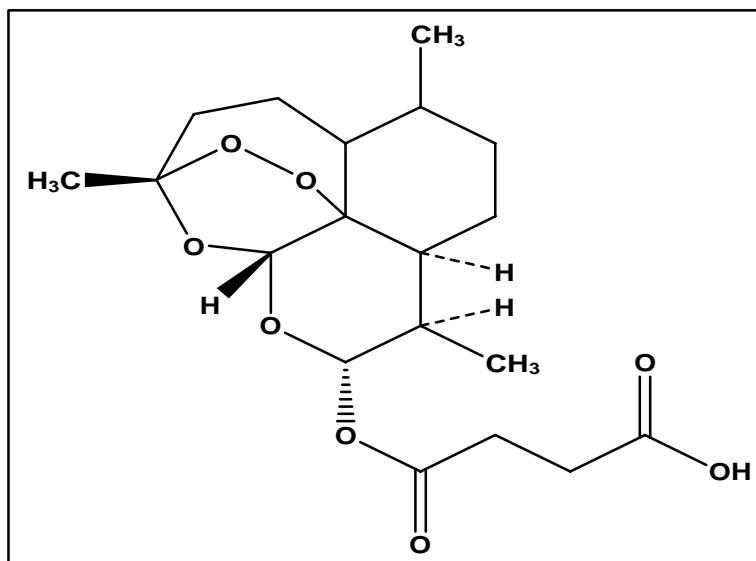


Figure 3: Structure of Artesunate.

Thus, for the purpose of study a validated RP- HPLC method was developed for simultaneous estimation of Artesunate, Sulfadoxine and Pyrimethamine in bulk and in tablets formulation.

## MATERIALS AND METHODS:

### Reagents and Samples

An analytically working standard of Artesunate was obtained from Cadila Pharmaceuticals Ltd. (Ahmadabad) and working standards of Sulfadoxine and Pyrimethamine were procured from Macleod Pharmaceutical Ltd, (Mumbai). Acetonitrile, Methanol, Water of HPLC grade and potassium dihydrogen phosphate and Phosphoric acid of analytical reagent grade were purchased from Rankem Pvt. Ltd. (Delhi).

### Mobile Phase Component

The mobile phase components were 55:45 (v/v) acetonitrile– 0.1M phosphate buffer, pH 2.2. Before use these solutions were filtered through a 0.45- $\mu$ m pore size Rankem filter and degassed in an ultrasonic bath.

### Stock Solutions

Sulfadoxine: 250 mg of Sulfadoxine standard was added to 100 mL volumetric flask and then, add Acetonitrile was added up to the mark. Then it was mixed and degassed by ultrasonication for 15 min. This will provide 100 mL solution of Sulfadoxine with 2500  $\mu$ g/mL concentration.

Pyrimethamine: 10 mg of Pyrimethamine standard was added to 100 mL volumetric flask then, Acetonitrile was added up to the mark. Then it was mixed and degassed by ultrasonication for 15 min. This will provide 100 mL solution of Pyrimethamine with concentration 100  $\mu$ g/mL.

Artesunate: 100 mg of Artesunate standard was added to 100 mL volumetric flask then, Acetonitrile was added up to the mark. Then it was mixed and degassed by ultrasonication for 15 min. This will provide 100mL solution of Artesunate with concentration 1000  $\mu$ g/mL.

### Assay Sample Preparation

Twenty tablets (*Falcigo-SP* Aurochem Pharmaceutical Ltd. India) of mixture containing Sulfadoxine, Pyrimethamine and Artesunate in combination were weighed; their average weight was determined and finally crushed to fine powder. Accurately weighed tablet powder containing equivalent amount of 750 mg of Sulfadoxine, 37.5 mg of Pyrimethamine & 200 mg of Artesunate was transferred to 100 mL volumetric flask & added Acetonitrile up to the mark. After sonication for 15 min, the solution was filtered through Whatman filter paper to give stock solution. Suitable aliquots of the solution were further diluted with Acetonitrile to obtain sample solution within the concentration range for all the three drugs.

### Chromatographic System and Condition

A HPLC method was performed in isocratic mode on HPLC system (Perkin Elmer series - 200) consisted of ODS HYPERSIL 5 $\mu$ m (250 mm $\times$ 4.6 mm i.d.) column. A mobile phase consisted of Acetonitrile: 0.1 M Phosphate buffer pH 2.2 (55:45 v/v), de-gassed and flow rate was kept at 1.5 ml/min. The run time was set to 10 min, and PDA detector was set to 254 nm.

## RESULTS AND DISCUSSION:

### Assay

The results of assay were shown in (Table 1).

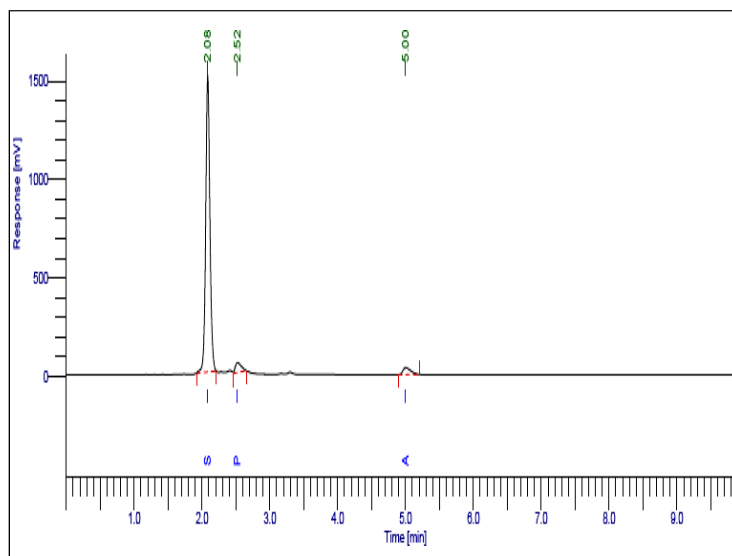
**Table 1: Statistical Validation of tablet analysis of SUL, PYR & ART.**

Component	%Mean*	S.D.	%R.S.D.
Sulfadoxine	99.97	0.8466	0.8468
Pyrimethamine	99.85	1.0010	1.0025
Artesunate	100.11	0.2361	0.2358

\*Mean of six determinations, S.D.- Standard Deviation, R.S.D.- Relative Standard Deviation

### RP-HPLC Chromatography

(Figure 3) shows a typical chromatogram of Sulfadoxine, Pyrimethamine and Artesunate after running sample through the system.



**Figure 4: Atypical chromatogram of Sulfadoxine, Pyrimethamine and Artesunate.**

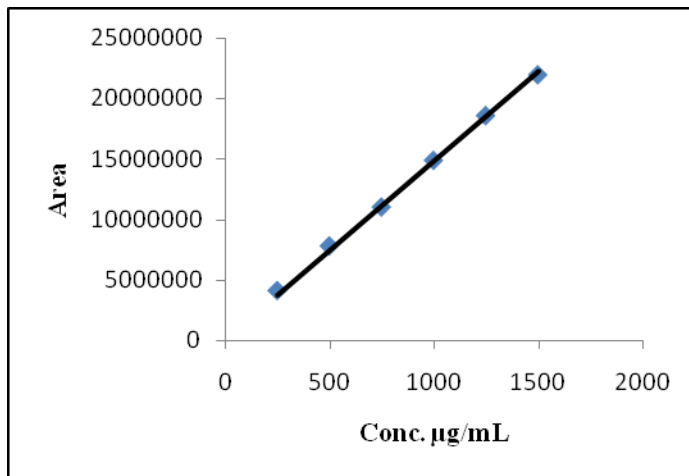
### Validation of the Method

The method was validated for accuracy, precision, sensitivity, recovery, linearity and robustness. The method validation was performed as per ICH guidelines.[15]

### Linearity

For each drug appropriate aliquots were pipette out from each standard stock solution into a series of 10 ml volumetric flasks. The volume was made up to the mark with mobile phase to get a set of solutions for having concentration range as, for SDX 250 - 1500 µg/mL, for PYR 10 - 60 µg/mL and for ART 50 - 500 µg/mL. Triplicate dilutions of each concentration of each drug were prepared separately. From these triplicate solutions, 20 µl injections of each concentration of each drug were injected into the HPLC system twice in number, separately and run under the conditions specified. Evaluation of all the three drugs was performed with PDA detector at 254 nm. Peak areas were recorded for all the peaks. Working calibration curves for SDX, PYR & ART were plotted separately with peak area Vs the respective concentration of SDX, PYR & ART.

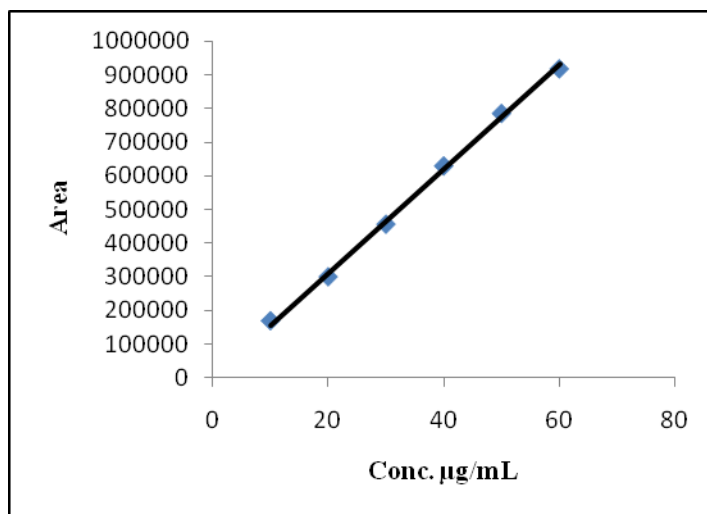
The calibration curve for Artesunate (Figure 5) (Table 4), Sulfadoxine (Figure 6) (Table 2) & Pyrimethamine (Figure 7) (Table 3) was found to be linear in the range of 10-60 µg/ml & 250-1500 µg/ml, 50-500 µg/ml, respectively. The correlation coefficients- $r^2$ , were 0.998 for SDX, 0.998 for PYR, and, 0.998 for ART. The average linear regression equations were  $y = 14815x + 2402$  for SDX,  $y = 15504x + 3210$  for PYR, and  $y = 3400x + 803$  for ART.



**Figure 5: Calibration curve for Sulfadoxine.**

**Table 2: Calibration table for Sulfadoxine.**

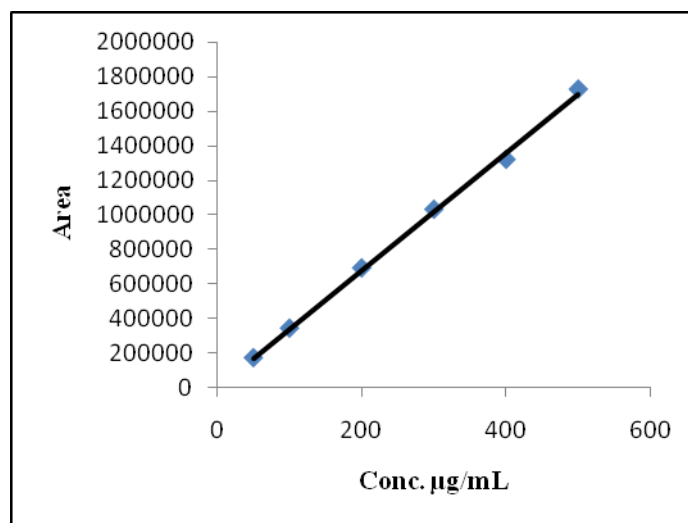
Sr. No.	Concentration of SDX (µg/ml)	Area
1.	250	4130122
2.	500	7817219
3.	750	11032810
4.	1000	14882014
5.	1250	18583585
6.	1500	21956372
Slope		14815
Y-intercept		2402
Correlation coefficient (r)		0.998



**Figure 6: Calibration curve for Pyrimethamine.**

**Table 3: Calibration table for Pyrimethamine.**

Sr. No.	Concentration of PYR (µg/ml)	Area
1.	10	169659
2.	20	300504
3.	30	456789
4.	40	630126
5.	50	786520
6.	60	919124
Slope		15504
Y-intercept		3210
Correlation coefficient(r)		0.998

**Figure 7: Calibration curve for Artesunate.****Table 4: Calibration table for Artesunate.**

Sr.No.	Concentration of ART (µg/ml)	Area
1.	50	170723
2.	100	341785
3.	200	689892
4.	300	1029338
5.	400	1317840
6.	500	1724230
Slope		3400
Y-intercept		803
Correlation coefficient(r)		0.998

### Sensitivity

The sensitivity of measurement of Artesunate, Sulfadoxine, and Pyrimethamine was estimated in terms of the limit of quantitation (LOQ). The smallest amounts detected under the chromatographic conditions used were estimated in terms of the limit of detection (LOD). LOQ and LOD were calculated by use of the equations:

$$\text{LOD} = (3.3 \times \sigma) \div S$$

$$\text{LOQ} = (10 \times \sigma) \div S$$

Where  $\sigma$  is the standard deviation of the peak areas of the drugs, taken as a measure of noise, and S is the slope of the corresponding calibration plot. LOD and LOQ for Artesunate were found to be 0.970 µg/ml and 3.239 µg/ml, for Sulfadoxine, 0.221 and 0.735 and for Pyrimethamine were found to be 0.212 and 0.708. (Table 5) shows the results of LOD and LOQ values.

**Table 5: Results of LOD and LOQ values.**

Parameter	SDX	PYR	ART
*L.O.D.(µg/mL)	0.221	0.212	0.970
*L.O.Q.(µg/mL)	0.735	0.708	3.239

LOD- Limit of Defection, LOQ- Limit of Quantification

**Precision:**

The precision of the method was investigated with respect to repeatability. For intraday precision, six concentrations of each compound were analyzed on the same day each concentration of sample was injected two times. For intraday samples are injected two times in a day, and for interday, samples are injected for one time once in a day & it is for two days. (Table 6) summarize the results of intermediate precision studies.

**Table 6: Intermediate Precision Data.**

Formulation	Parameter	Intra-day precision*	Inter-day precision*
SDX	Mean	99.89	100.1
	S.D.	0.8983	1.0010
	% R.S.D.	0.8993	1.0009
PYR	Mean	99.78	99.92
	S.D.	1.0144	1.0144
	% R.S.D.	1.0166	1.0155
ART	Mean	100.01	99.86
	S.D.	0.2367	0.2338
	% R.S.D.	0.2366	0.2341

\*Mean of six determinations, S.D.- Standard Deviation, R.S.D.- Relative Standard Deviation

**Accuracy (Recovery studies)**

The accuracy of proposed method was checked by recovery studies by carrying out at 80%, 100% and 120% of the test concentration as per ICH Guidelines [15]. As per label claim, the tablet consisted of 200 mg of ART, 750 mg of SDX and 37.5mg of PYR. For recovery studies different levels of the standard concentration according to 80%, 100% and 120% are made and % mean recoveries are calculated. (Table 7) shows the results of recovery studies.

**Table 7: Result of Recovery Study.**

Level of %Recovery	Amount present (mg/tab)			Amount of standard added (mg)			Total amount recovered (mg)			%Recovery*		
	SDX	PYR	ART	SDX	PYR	ART	SDX	PYR	ART	SDX	PYR	ART
80	750	37.5	200	600	30	160	1348.04	67.09	360.2	99.85	99.40	100.05
100	750	37.5	200	750	37.5	200	1497.15	74.64	399.48	99.81	99.53	99.87
120	750	37.5	200	900	45	240	1644.93	82.54	440.62	99.71	99.46	100.14
									Mean	99.79	99.46	100.02
									S.D.	0.0721	0.0650	0.1374
									% R.S.D.	0.0722	0.0653	0.1373

S.D.-Standard Deviation, R.S.D.-Relative Standard Deviation

**Specificity**

The specificity of the HPLC Method was determined by complete separation of Artesunate, Sulfadoxine & Pyrimethamine as shown in Figure 7 with parameters like retention time ( $t_R$ ), resolution ( $R_S$ ) and tailing factor ( $T_P$ ). Tailing factor for peaks of SDX, PYR & ART was less than 2 % and resolution was satisfactory. The average retention time  $\pm$  standard deviation (Av. RT Mean  $\pm$  S.D.) SDX, PYR & ART were found to be  $2.08 \pm 0.011$  for SDX,  $2.52 \pm 0.03$  for PYR &  $5.00 \pm 0.14$  for ART respectively for the six replicates. The peaks obtained for SDX, PYR & ART were sharp and have clear baseline separation.

### Robustness studies

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The evaluation of robustness should be considered during the development phase and depends upon the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in Method parameters. The parameters included flow rate, pH, composition of mobile phase ratio, and column temperature. The solution containing rationale amount of Artesunate, Sulfadoxine, and Pyrimethamine was injected into sample injector of HPLC three times using variation in the parameters like flow rate, percentage of Acetonitrile in the mobile phase, column temperature. The results obtained are given in the form of data in (Table 8) along with their statistical validation parameters.

**Table 8: Results of robustness testing.**

Factor	Level t <sub>R</sub>	T						Area Content			%		
Flow Rate		SDX	PYR	ART	SDX	PYR	ART	SDX	PYR	ART	SDX	PYR	ART
1.3	-2	2.23	2.93	5.21	1.18	1.21	1.28	4201887	223904	219896	99.44	98.07	99.48
1.5	0	2.08	2.52	4.97	1.12	1.19	1.15	4125907	213569	217384	99.99	99.85	99.77
1.7	2	2.01	2.49	5.18	1.15	1.37	1.19	4101278	213841	200436	99.38	99.55	99.26
<b>Mean</b>		<b>2.10</b>	<b>2.64</b>	<b>5.1</b>	<b>1.15</b>	<b>1.25</b>	<b>1.20</b>	<b>4143024</b>	<b>217104</b>	<b>212572</b>	<b>99.60</b>	<b>99.60</b>	<b>99.50</b>
<b>±S.D.</b>		<b>± 0.11</b>	<b>± 0.24</b>	<b>± 0.13</b>	<b>± 0.03</b>	<b>± 0.9</b>	<b>± 0.06</b>	<b>± 52443</b>	<b>± 5889</b>	<b>± 10584</b>	<b>± 0.33</b>	<b>± 0.95</b>	<b>± 0.25</b>
Mobile Phase		SDX	PYR	ART	SDX	PYR	ART	SDX	PYR	ART	SDX	PYR	ART
53:47	-2	2.15	2.72	5.19	1.01	1.32	1.27	4235238	222569	211602	100.1	99.65	99.57
55:45	0	2.08	2.52	4.97	1.12	1.19	1.15	4125907	213569	217384	99.99	99.85	99.77
57:43	2	2.02	2.38	4.69	1.17	1.21	1.19	4101790	215886	220693	99.67	99.56	99.83
<b>Mean</b>		<b>2.08</b>	<b>2.54</b>	<b>4.95</b>	<b>1.10</b>	<b>1.25</b>	<b>1.20</b>	<b>4154312</b>	<b>217341</b>	<b>216559</b>	<b>99.92</b>	<b>99.72</b>	<b>99.68</b>
<b>±S.D.</b>		<b>± 0.06</b>	<b>± 0.17</b>	<b>± 0.25</b>	<b>± 0.08</b>	<b>± 0.7</b>	<b>± 0.06</b>	<b>± 71114</b>	<b>± 4673</b>	<b>± 4601</b>	<b>± 0.22</b>	<b>± 0.13</b>	<b>± 0.14</b>
Temp.		SDX	PYR	ART	SDX	PYR	ART	SDX	PYR	ART	SDX	PYR	ART
28	-2	2.09	2.54	5.02	1.32	1.24	1.26	4198098	224008	220823	99.23	99.60	99.63
30	0	2.08	2.52	4.97	1.12	1.19	1.15	4125907	213569	217384	99.99	99.85	99.77
32	2	2.06	2.47	4.76	1.07	1.09	1.02	4011887	212904	209986	99.38	99.49	99.80
<b>Mean</b>		<b>2.07</b>	<b>2.51</b>	<b>4.91 ±</b>	<b>1.17</b>	<b>1.18</b>	<b>1.14</b>	<b>4111964</b>	<b>216827</b>	<b>216064</b>	<b>99.53</b>	<b>99.64</b>	<b>99.73</b>
<b>±S.D.</b>		<b>± 0.01</b>	<b>± 0.03</b>	<b>0.14 ±</b>	<b>± 0.13</b>	<b>± 0.7</b>	<b>± 0.12</b>	<b>± 93885</b>	<b>± 6227</b>	<b>± 5537</b>	<b>± 0.40</b>	<b>± 0.18</b>	<b>± 0.09</b>
pH		SDX	PYR	ART	SDX	PYR	ART	SDX	PYR	ART	SDX	PYR	ART
2.0	-	2.12	2.61	4.78	1.14	1.23	1.20	4017238	221369	222002	99.82	99.35	100.1
	0.2												
2.2	0	2.08	2.52	4.97	1.12	1.19	1.15	4125907	213569	217384	99.99	99.85	99.77
2.4	0.2	2.05	2.50	4.95	1.09	1.16	1.19	4163528	214157	211029	98.90	99.62	99.71
<b>Mean</b>		<b>2.08</b>	<b>2.54</b>	<b>4.90</b>	<b>1.11</b>	<b>1.19</b>	<b>1.18</b>	<b>4102224</b>	<b>216365</b>	<b>216805</b>	<b>99.57</b>	<b>99.60</b>	<b>99.86</b>
<b>±S.D.</b>		<b>± 0.03</b>	<b>± 0.06</b>	<b>± 0.10</b>	<b>± 0.0</b>	<b>± 0.3</b>	<b>± 0.02</b>	<b>± 75966</b>	<b>± 4343</b>	<b>± 5509</b>	<b>± 0.58</b>	<b>± 0.25</b>	<b>± 0.21</b>

S.D.-Standard Deviation

### Ruggedness

Degree of reproducibility of test results obtained by analysing the sample under variety of normal test conditions such as different analysts and days. Such experiments were performed by different analysts. The results of ruggedness are given in (Table 9).

**Table 9: Results of ruggedness.**

Formulation	Parameter	Different analysts*		Differen tday*	
		Analyst-I	Analyst-II	Day-I	Day-II
SDX	Mean	100.28	100.12	99.94	100.27
	S.D.	0.5058	0.7245	0.4176	0.4201
	% R.S.D.	0.5043	0.7236	0.4178	0.4189
PYR	Mean	99.66	99.84	99.79	99.61
	S.D.	0.3132	0.4005	0.1550	0.3510
	% R.S.D.	0.3142	0.4011	0.1553	0.3523
ART	Mean	99.19	100.11	99.20	99.43
	S.D.	0.5658	0.5819	0.5853	0.3493
	% R.S.D.	0.5704	0.5812	0.5900	0.3513

S.D.-Standard Deviation, R.S.D.-Relative Standard Deviation



**System suitability parameters:**

To ascertain resolution and reproducibility of the chromatographic system, system suitability parameters are studied and results are summarized in (Table 10).

**Table 10: System Suitability Studies.**

Parameters	Sulfadoxine	Pyrimethamine	Artesunate
Retention time(min.)	2.08	2.52	5.00
Tailing factor	1.12	1.19	1.15
Theoretical plates	5236	2652	7775
Resolution	3.01		11.47

**CONCLUSION**

The proposed RP-HPLC method enables simultaneous determination of ART, SDX & PYR with good separation and resolution of the chromatographic peaks. This is the first reported method for simultaneous quantitative analysis of ART, SDX & PYR, and is a significant advance in chromatographic analysis of such pharmaceutical mixtures. The method is suitable for qualitative and quantitative analysis of these pharmaceutical products. The results obtained are in a good agreement with the declared contents. Statistical analysis showed the method is accurate and precise. There was no interference from excipients in the tablets.

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**Conflict of Interest:**

The authors declare no conflict of interest.

**Abbreviations:**

SDX -Sulfadoxine  
PYR -Pyrimethamine  
ART -Artesunate  
LOD -LimitofDefection  
LOQ -LimitofQuantification  
S.D. -StandardDeviation  
R.S.D. -RelativeStandardDeviation

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