ORIGINAL ARTICLE

Development and Validation of Stability Indicating RP-HPLC Method for Pyrimethamine, Sulfadoxine, and Artesunate Estimation in Bulk and Its Formulation Applying DOE

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Abstract

Introduction: Artesunate (ART) is a prodrug used as an antimalarial agent that acts by malarial protein damage through alkylation. Pyrimethamine (PYR) is an antiprotozoal agent that acts by interfering with the synthesis of tetrahydrofolic acid. Sulfadoxine is a dihydropteroate synthetase inhibiter which makes difficulty in parasite reproduction. A stability-indicating high-performance liquid chromatography (HPLC) technique has been established for the quantification of PYR, sulfadoxin, and ART applying design of experiment. **Material and Methods:** The phosphate buffer (pH 3.0):acetonitrile (80: 20) was used as the mobile phase, and hypersil BDS C18 (250 × 4.6 mm; 4 m) column at a flow rate of 1 mL/min was used for the chromatographic separation. The detection wavelength was set at 237 nm. The mobile phase was optimized using 3^2 full factorial design. **Results and Discussion:** The optimized method contains the retention times of PYR, sulfadoxin, and ART at 3.653, 4.920, and 8.310 min, respectively. The method shows a good linearity in the concentration range of 0.5–2.5 µg/mL for PYR, 10–50 µg/mL for sulfadoxin, and 4–20 µg/mL for ART. The stability study of the drugs was performed by acid, alkali, oxidation, thermal, and photolytic degradation. **Conclusion:** The proposed stability indicating HPLC method was found to be simple, specific, practical, accurate, quick, and affordable. It was also suitable for the routine analysis, quality control, and percentage degradation of pharmaceutical preparations containing these drugs either individually or in combination.

Key words: Artesunate, design of experiment, high-performance liquid chromatography, pyrimethamine, stability, sulfadoxine

INTRODUCTION

The plasmodium genus of protozoans is responsible for malaria, a parasitic disease. It spreads from one person to another through the bite of an infected female anopheles mosquito.[1] A stability-indicating chromatographic technique is an analytical procedure used to separate, identify, assay of component, and measure the decrease in the amount of the active pharmaceutical ingredient (API) in drug product as a result of degradation. Analytical quality by design (QbD) procedures reveals the fundamental information about the variables that were considered in the method.[2-4]

Artesunate (ART) [Figure 1a] is a prodrug that is rapidly hydrolyzed to its active form dihydroartemisinin (DHA) in the presence of plasma esterase enzyme. The DHA increases oxidative stress and causes malarial protein damage through alkylation. It is chemically 4-oxo-4-[[(1R,4S,5R,8S,9R,10S,12R,13R)-1,5,9-trimethyl-11,14,15,16tetraoxatetracyclo[10.3.1.$0^{4,13}$0^{8,13}] exadecane-10-yl]oxy] butanoic acid.[5,6] Pyrimethamine (PYR) [Figure 1b] acts by interferes with the synthesis of tetrahydrofolic acid by inhibiting dihydrofolate reductase, which is necessary

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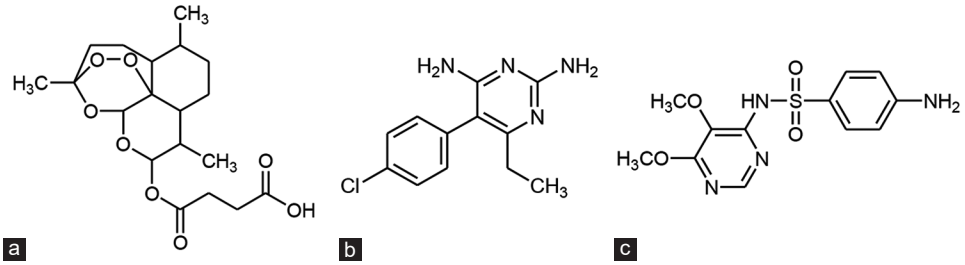

Figure 1: Molecular structure of (a) Artesunate (b) Pyrimethamine (c) Sulfadoxine

for DNA and RNA synthesis in many species, including protozoa. Chemically, it is 2,4-diamine-5-(P-chlorophenyl)-6-ethylpyrimidine.[7-9] Chemically, sulfadoxine (SPD) [Figure 1c] is 4-amino-N-(5, 6-dimethoxy-4-pyrimidinyl) benzene sulfonamide, dihydropteroate synthetase inhibiter which is an enzyme necessary in the conversion of PABA to folic acid. Folic acid plays a vital role in the synthesis, repair, and methylation of DNA. By the lacking of this vital nutrient, the parasite has difficulty in reproduction.[10-12] The quantification of ART, PYR, and sulfadoxin alone and in combination with other medications was reported using some analytical methods from the review of the literature, such as high-performance liquid chromatography (HPLC), high-performance thin-layer chromatography, and spectrophotometric methods. Stability-indicating reversed-phase HPLC (RP-HPLC) and spectrophotometric methods were reported for the forced degradation study of ART, PYR, and sulfadoxin in single and in combination with other drugs, moreover one LC-MS method was developed for the estimation of ART, PYR, and sulfadoxin in human plasma in combination, but no method was reported on stability indicating assay of ART, PYR, and sulfadoxin in combination by QbD approach. Therefore, it was thought to develop a stability-indicating RP-HPLC method for ART, PYR, and sulfadoxin in combination by the novel approach of QbD.[13-26]

MATERIALS AND METHODS

Material

Reference standard of PYR, SPD, and ART was obtained as gift samples from Bharat Parentrals Limited, Vadodara, Gujarat. The pharmaceutical formulation containing 200 mg ART, 25 mg PYR, and 500 mg SPD was procured from the local pharmacy. The necessary solvents and chemicals used in this method were of analytical grade.

Instrumentation

The separation was performed using a Shimadzu prominence isocratic HPLC system with an LC 20 AT pump, SPD-20A detector, Spinchrom CFR software, and a Hypersil BDS C18 (250 4.6 mm; 4 m) column. Shimadzu UV-visible spectrophotometer was used for the spectral analysis. Adventurer Electronic balance, Systronic digital pH meter, and Whatman filter papers are used throughout analytical procedure. Design Expert 11 software was used Quality-by-Design approach.

Preparation of standard stock solution

Standard stock solutions of PYR, SPD, and ART were obtained by dissolving 2.5 mg of PYR, 50 mg SPD, and 20 mg of ART in 50 mL methanol, respectively. The concentrations of resulted solutions were 50 µg/mL for PYR, 1000 µg/mL for SPD, and 400 µg/mL for ART, respectively.

Preparation of working stock solution

1 mL each from the above prepared standard stock solutions was taken in a 10 mL of volumetric flask. The solution is diluted up to mark with mobile phase (mobile phase which used for trials) to get PYR 5 µg/mL, SPD 100 µg/mL, and ART 40 µg/mL.

Method optimization

Various mobile phases such as water: methanol, water: aceronitrile, buffer: acetonitrile, buffer: methanol in different ratios and different pH of buffer solution, i.e., pH 2.5, 3, 3.5 at a flow rate of 0.75 mL/min, 1 mL/min, 1.25 mL/min were tried. The mobile phase containing phosphate buffer (pH 3):aceotonitrile (80:20, v/v) at a flow rate of 1 mL/min was ultimately chosen to carry out additional optimization using factorial design. Various chromatographic responses, such as retention time, area, and resolution, were assessed.

Software-aided method optimization

A 3^2 factorial design was used to optimize the chromatographic conditions. Three levels and two independent variables are included in this 3^2 full factorial design, involving only 13 iterations for optimization. The two independent variables chosen were A (organic phase ratio on mobile phase) and B, and the three levels were low (−1), medium (0), and high (+1) (flow rate). Resolution 1 (Y1) and Resolution 2 were the chromatographic responses noted in the experiment (Y2). For producing a response surface and creating various models with Design Expert, a 3^2 complete factorial design

was appropriate (Version 11.0.4.0). Analysis of variance (ANOVA) was used to analyze the responses and determine significance of the model. 3D response surface plots and perturbation plots were created to visualize the impact of the independent variable and their interactions on the responses. Each response's relationship to the optimization design was demonstrated by the generation of regression equations.

Chromatographic conditions

In this method, the separation was achieved by chromatographic conditions such as phosphate buffer (pH-3.0):acetonitrile (80:20) as mobile phase, Hypersil BDS C18 (250 × 4.6 mm; 4 µm) with flow rate of 1 mL/min using UV detection at 237 nm. The chromatographic conditions such as organic phase ratio in the mobile phase and flow rate were optimized by applying design of experiment (DOE). This mobile phase resolves the peaks with retention time 3.56 min for PYR, 4.9 min for SPD, and 8.34 for ART.

Preparation of calibration curve

From the standard stock, solution of PYR, SPD, and ART mixed working standard solution comprising of PYR (0.5–2.5 µg/mL), SPD (10–50 µg/mL), and ART (4–20 µg/mL) was prepared and analyzed the solutions 5 times under the pre-determined HPLC conditions. To generate the calibration curve, peak regions were plotted against the corresponding concentration.

Estimation of PYR, SPD, and ART in formulation

20 Tablets of PYR, SPD, and ART were taken and weighed accurately. Crushed the tablets and powder equivalent to 25 mg of PYR, 500 mg of SPD, and 200 mg of ART was transferred to a 100 mL volumetric flask. 60 mL methanol was added to it and sonicated for 15 min. Volume made up to mark with methanol and filtered.0.5 mL from this solution diluted up to 50 mL with mobile phase. The areas of resulting peak were measured at 237 nm.

Method validation

The method was validated in accordance with the official requirements for the validation of analytical techniques provided by the "International Conference on Harmonization."[27,28]

Specificity

It was investigated how precisely and particularly the medications under investigation are appraised in the presence of anticipated formulation components. To determine whether excipients and drug peaks interfered with one another in the proposed RP-HPLC method, placebo, mixed working standard, and sample solutions were simultaneously injected and their peak positions were compared to those of the standard drug.

Linearity and range

In the concentration ranges of 0.5–2.5 g/mL for PYR, 10–50 g/mL for SPD, and 4–20 g/mL for ART, the linearity of response was analyzed. For PYR, SPD, and ART, the calibration curve was plotted using peak areas versus concentrations to provide the correlation coefficient and regression line equation. In terms of the correlation coefficient of a linear regression line, linearity is expressed.

Precision

Repeatability

Repeatability of the method was evaluated by applying six injections of mix working standard solution containing 1.5 µg/mL of PYR, 30 µg/mL of SPD, and 12 µg/mL of ART and determining %RSD.

Intraday precision

Combined mixed solutions containing 0.5 µg/mL, 1.5 µg/mL, 2.5 µg/mL of PYR, 10 µg/mL, 30 µg/mL, 50 µg/mL of SPD and 4 µg/mL, 12 µg/mL, 20 µg/mL were analyzed 3 times on the same day. The % RSD for the PYR, SPD, and ART was calculated.

Interday precision

Combined mixed solutions containing 0.5 µg/mL, 1.5 µg/mL, 2.5 µg/mL of PYR, 10 µg/mL, 30 µg/mL, 50 µg/mL of SPD and 4 µg/mL, 12 µg/mL, 20 µg/mL were analyzed 3 times on the three different days. The % RSD for the PYR, SPD, and ART was calculated.

Accuracy

Analyte recovery was estimated to assess the accuracy of the projected method by adding standard analytes to pre-analyzed sample solution at different % levels (80, 100, and 120%). The resulting combined solutions were reanalyzed for the calculation of % recovery.

1 mL of sample solutions containing 10 µg/mL of PYR, 200 µg/mL of SPD, and 80 µg/mL of ART were taken in three separate flask label A, B, and C. 80%, 100%, and 120% of standard solutions were spiked and diluted to 10 mL. The peak area of each solution was measured at 237 nm.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ of the drug were derived using the following equations.

$$LOD = 3.3 \times (SD/Slope)$$
$$LOQ = 10 \times (SD/Slope)$$

Robustness

If a procedure is robust, it is unaffected by minor adjustments to the operational environment. It is done by analyzing a variety of factors, such as the pH of buffer solutions, flow rate, and ratio of organic solvent in the mobile phase.

Stability study[2,29-33]

Acid degradation

Acid degradation for sample was carried out by taking 1 mL of sample stock solution in 10 mL volumetric flask. 2 mL of 0.1 N HCl was added and kept for 4 h in room temperature. To stop the further degradation, the solution was neutralized with 2 mL of 0.1 M NaOH solution. Volume made up with mobile phase.

Alkali degradation

Alkali degradation for sample was carried out by taking 1 mL of sample stock solution in 10 mL volumetric flask. 2 mL of 0.1 N NaOH was added and kept for 3.5 h in room temperature. To stop the further degradation, the solution was neutralized with 2 mL of 0.1 N HCl solution. Volume made up with mobile phase.

Oxidative degradation

Oxidative degradation for sample was carried out by taking 1 mL of sample stock solution in 10 mL volumetric flask. 2 mL of 3% H_2O_2 was added and kept for 4 h in room temperature. Volume made up to mark with mobile phase.

Photolytic degradation

It was carried out for sample by exposing the thin layer of tablet powder PYR, SPD, and ART in a Petri dish to UV light for 8 h. Sample stock solution was prepared. 1 mL from this solution was taken in 10 mL volumetric flask and volume made up to mark with mobile phase.

Thermal degradation

It was carried out for sample by exposing the thin layer of tablet powder PYR, SPD, and ART in a Petri dish in oven at 105°C for 3 h. Sample stock solutions were prepared. 1 mL from this solution taken in 10 mL volumetric flask and volume made up to mark with mobile phase.

Table 1: Dependent and independent variables selected for the design of experiment (DOE)

Independent variable			
Factor	**Level used**		
	Low (−1)	**Medium (0)**	**High (+1)**
A. Organic phase ratio	10	20	30
B. Flow rate (mL/min)	0.75	1	1.25
Dependent variable			
Chromatographic response	Value		
Y1=Resolution 1	3.021<Y1<7.123		
Y1=Resolution 2	8.825<Y2<11.671		

RESULTS AND DISCUSSION

Development and optimization of HPLC method

A 3^2 full factorial design using 13 experimental runs was carried to optimize the proposed method. Table 1 lists the independent and dependent variables chosen for the DOE. Independent and dependent factors were used in the experiment's design (DOE).

The 13 experimental results were carried out for PYR, SPD, and ART with the chromatographic conditions and observed responses. The best-fitted model for Resolution 1 and Resolution 2 was found to be quadratic model [Table 2]. For both responses, the difference between projected R^2 and adjusted R^2 was <0.2, indicating that there was a satisfactory

Table 2: Summary of statistical analysis on various chromatographic responses

Response	Model	R^2	Adjusted R^2	Predicted R^2	Standard deviation	Adequate precision
Resolution 1	Quadratic	0.9987	0.9981	0.9962	0.0567	118.2206
Resolution 2	Quadratic	0.9978	0.9967	0.9947	0.0494	93.5777

Table 3: Regression equations for various chromatographic responses

Response	Model	Regression equation
Resolution-1	Full quadratic	Y_1=5.45−1.82 A−0.1437 B−0.0180 AB−0.0886 A^2−0.3811 B^2
	Reduced quadratic	Y_1=5.45−1.82 A−0.1437 B−0.0886 A^2−0.3811 B^2
Resolution-2	Full quadratic	Y_2=10.37+1.18 A−0.1255 B−0.0135 AB+0.0961 A^2−0.3794 B^2
	Reduced quadratic	Y_2=10.37+1.18 A−0.1255 B+0.0961 A^2−0.3794 B^2

degree of agreement between them. There was adequate signal for all responses indicated by the adequate precision >4.0. Table 3 provides the regression equations for various chromatographic reactions determined by ANOVA analysis. The element that favors the optimization was represented by a positive value in the equation, while an inverse link between the independent variable and the response was represented by a negative value. 3D surface plots were obtained to determine the relationship between the variables and the chromatographic responses.

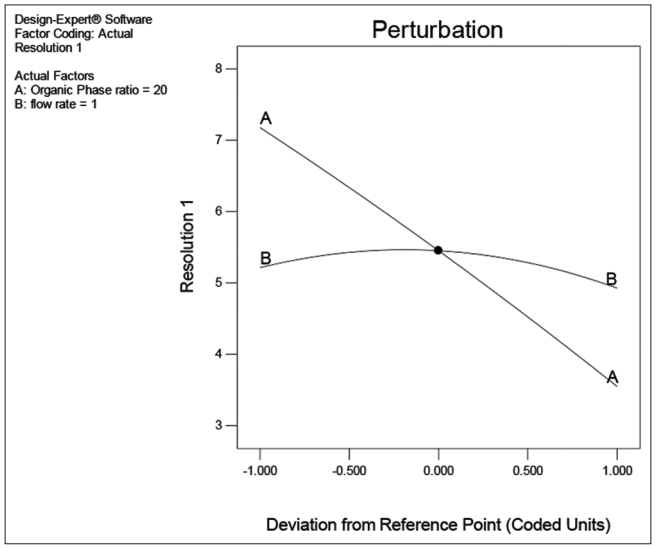

Figure 2: The perturbation plot for Resolution 1

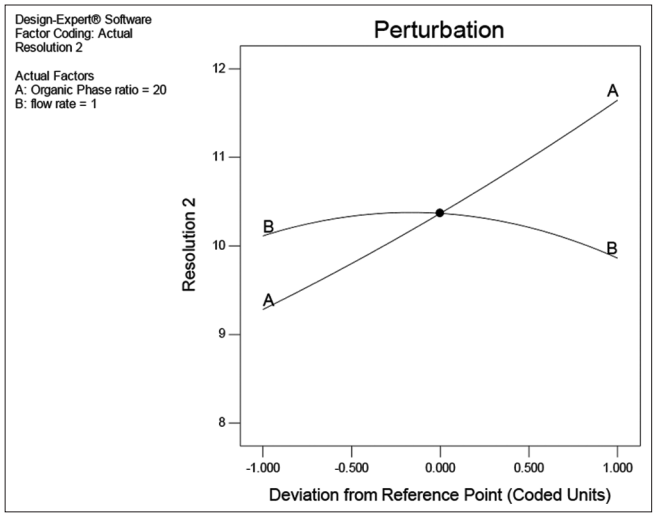

Figure 3: The perturbation plot for Resolution 2

The perturbation plot aids in comparing the effects of all the variables at a certain location in the design space. The response is plotted by changing only one variable over its range while keeping all the other factors constant.

Perturbation plot for Resolution 1 is shown in Figure 2. The plot indicates the effect of both the factors at a center point in design space. The relatively flat line of flow rate shows lower effect of this factor on the Resolution 1 in the design space. It can be seen from Eq. (Y_1= 5.45−1.82 A−0.1437 B−0.0886 A^2−0.3811 B^2). The steep slope line for organic phase ratio demonstrates negative effect of factor on Resolution 1.

Perturbation plot for Resolution 2 is shown in Figure 3. The plot indicates the effect of both the factors at a center point in design space. The relatively flat line of flow rate shows lower effect of this factor on Resolution 2 in the design space. It can be seen from Eq. (Y_2 = 10.37+1.18 A−0.1255 B+0.0961 A^2−0.3794 B^2). The steep slope line for organic phase ratio demonstrates positive effect of factor on Resolution 2.

Contour and 3D surface plots indicate that with increase in organic phase ratio from 10 to 30 mL, Resolution 1 was found to be significantly decreased which is evident that lower organic phase ratio is more effective for higher resolution.

Figure 4 indicates that with the increase in flow rate from 0.75 mL/min, a slower increase in Resolution 1 was found but flow rate above 1 mL/min and decrease in Resolution 1 was observed.

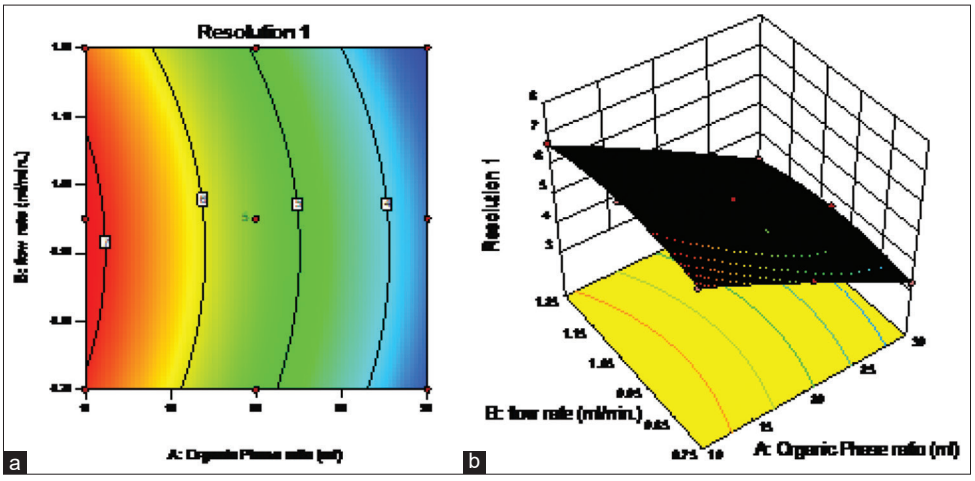

Figure 4: (a) Contour plot (b) 3D surface plots for Resolution 1

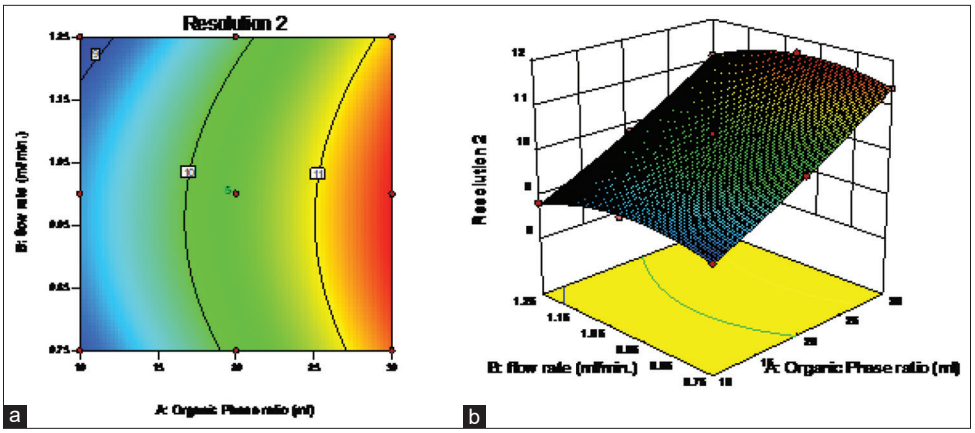

Figure 5: (a) Contour plot (b) 3D surface plots for Resolution 2

With increase in organic phase ratio, increase in resolution 2 was observed [Figure 5] which indicates the positive impact of independent variable on selected response. Figure 5 indicates that the initial increase in flow rate increases resolution 2 but after it was found to be reduced above 1 mL/min.

Linearity and range

Linearity was observed in the concentration range of 0.5–2.5 µg/mL for PYR, 10–50 µg/mL for SPD, and 4–20 µg/mL for ART. Figure 6 shows the linearity chromatograms of PYR, SPD, and ART. Calibration curves for PYR, sulfadoxine, and ART are shown in Figure 7.

Precision

The proposed approach's repeatability, intraday, and interday precisions were all assessed. The findings revealed that for PYR, SPD, and ART, the % RSD is <2% at each level [Table 4].

Accuracy

Accuracy of the analytical method was confirmed by percentage recovery study. Values of recovery within the acceptable range show that the suggested procedure is reliable for drug analysis [Table 4].

LOD and LOQ

In this proposed method, the LOD and LOQ were determined to be 0.069 µg/mL and 0.21 µg/mL for PYR, 0.014 µg/mL and 0.043 µg/mL for SPD, and 0.017 µg/mL and 0.051 µg/mL for ART, respectively [Table 4].

Robustness

The robustness of this analytical procedure measures of its capacity to remain unaffected by small but deliberate variations in method parameters such as flow rate, mobile phase composition, and pH of the buffer solution in the mobile phase [Table 5].

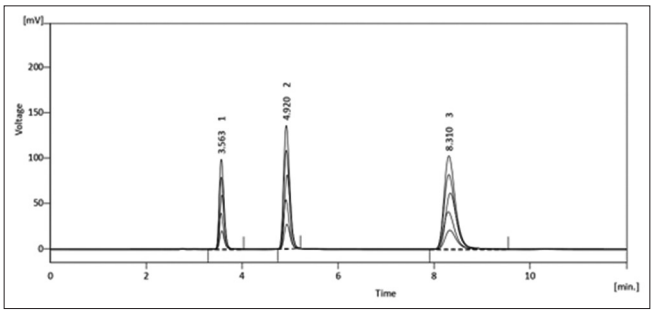

Figure 6: Linearity chromatograms for pyrimethamine, sulfadoxine, and artesunate

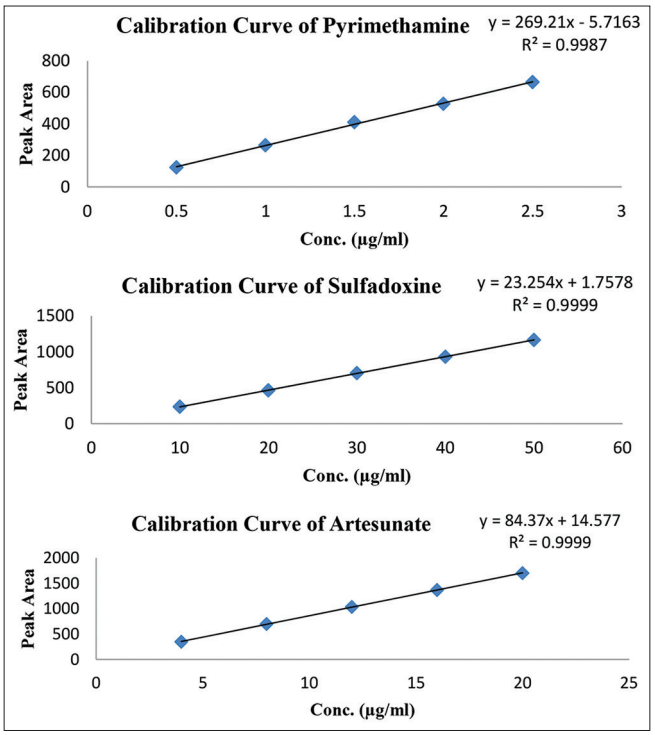

Figure 7: Calibration curves of pyrimethamine, sulfadoxine, and artesunate

Table 4: Summary of validation parameters

Parameters	PYR	SPD	ART
Linearity range (µg/mL)	0.5–2.5	10–50	4–20
Correlation coefficient	0.998	0.999	0.999
Regression equation	y=269.2×–5.716	y=23.25× + 1.757	y=84.37× + 14.57
LOD (µg/mL)	0.069	0.014	0.017
LOQ (µg/mL)	0.21	0.043	0.051
Precision (%RSD)			
Repeatability (*n*=6)	0.972	0.699	1.213
Intraday (*n*=3)	1.071	0.642	1.188
Interday (*n*=3)	1.091	0.986	1.119
Accuracy			
80% (*n*=3)	98.58	98.50	98.37
100% (*n*=3)	98.04	99.08	98.67
120% (*n*=3)	97.59	98.08	97.35

LOD: Limit of detection, LOQ: Limit of quantification, PYR: Pyrimethamine, SPD: Sulfadoxine, ART: Artesunate

Table 5: Results of robustness

Parameters	% RSD		
	PYR	SPD	ART
Change in flow rate			
0.8 mL/min	1.075	1.229	1.073
1.2 mL/min	0.898	1.129	1.311
Change in organic phase ratio in mobile phase			
+2%	1.373	1.401	1.046
–2%	1.229	1.156	1.332
Change in pH of buffer in mobile phase			
pH 3.2	1.057	1.107	1.294
pH 2.8	1.210	0.968	1.040

PYR: Pyrimethamine, SPD: Sulfadoxine, ART: Artesunate

Table 6: Assay of marketed formulation

Drugs	Labeled amount (mg)	Amount found (mg)	Amount found (%)*	% RSD
PYR	25	24.89	99.56±1.67	1.68
SPD	500	493.50	98.70±1.66	1.68
ART	200	199.24	99.62±1.39	1.39

*mean±SD, PYR: Pyrimethamine, SPD: Sulfadoxine, ART: Artesunate

Estimation of drug content

The assay results of the pharmaceutical formulation showed that the drug content of the formulation is within the range and percentage of RSD was below 2, indicating that this method is suitable for drug content assay [Table 6].

The results of acid, alkali, oxidative, photolytic, and thermal degradation for PYR, SPD, and ART are shown in Tables 7-9.

Forced degradation was carried out to evaluate stability-indicating properties of the method, by exposing samples of the drug substance and drug product to stress conditions of hydrolysis, oxidation, photodegradation, and thermal degradation.

From the forced degradation study, it was established that no degradant was found to interfere with the retention time of PYR, SPD, and ART and its impurities. The results interpret the percentage degradation of PYR, SPD, and ART in each

Table 7: Results of stress degradation studies of pyrimethamine

S. No.	Stress types	Stress conditions	% assay	% degradation
1	Acid degradation	0.1 N HCl, 4 h	91.26	8.74
2	Alkali degradation	0.1 N NaOH, 3.5 h	88.65	11.35
3	Oxidative degradation	3% H_2O_2, 4 h	81.83	18.17
4	Photolytic degradation	UV light, 8 h	89.13	10.87
5	Thermal degradation	105°C, 3 h	88.59	11.41

Table 8: Results of stress degradation studies of sulfadoxine

S. No.	Stress types	Stress conditions	% assay	% degradation
1	Acid degradation	0.1 N HCl, 4 h	87.09	12.91
2	Alkali degradation	0.1 N NaOH, 3.5 h	86.14	13.86
3	Oxidative degradation	3% H_2O_2, 4 h	82.58	17.42
4	Photolytic degradation	UV light, 8 h	93.28	6.72
5	Thermal degradation	105°C, 3 h	92.94	7.06

Table 9: Results of stress degradation studies of artesunate

S. No.	Stress types	Stress conditions	% assay	% degradation
1	Acid degradation	0.1 N HCl, 4 h	84.02	15.98
2	Alkali degradation	0.1 N NaOH, 3.5 h	96.74	3.26
3	Oxidative degradation	3% H_2O_2, 4 h	89.29	10.71
4	Photolytic degradation	UV light, 8 h	86.64	13.36
5	Thermal degradation	105°C, 3 h	89.17	10.83

condition. From the forced degradation study, it was found that PYR degraded maximum in the presence of H_2O_2 and least in acidic degradation, SPD degraded maximum by oxidation and least under photolytic and thermal degradation, and ART degraded maximum by acid hydrolysis and least by alkali hydrolysis.

CONCLUSION

For the simultaneous estimation of PYR, sulfadoxine, and ART in bulk and pharmaceutical dosage form, the proposed stability indicating HPLC method was found to be simple, specific, practical, accurate, quick, and affordable. It was also suitable for the routine analysis, quality control, and percentage degradation of pharmaceutical preparations containing these drugs either individually or in combination.

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