Development, Optimization, and Validation of a Novel HPLC Method for Simultaneous Quantification of Artesunate and Amodiaquine in Tablet Formulations

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Artemisinin-based combination therapy (ACTs) has become the primary first-line treatment for mild falciparum malaria in the majority of African countries. A fixed-dose combination of amodiaquine and artesunate is commonly employed to enhance treatment compliance and achieve successful malaria outcomes. In this study, a specific, accurate, linear, precise, and repeatable method was optimized, verified, and applied for the simultaneous estimation of artesunate and amodiaquine HCl in a commercially available artesunate-amodiaquine tablet with a dosage of 100 mg/270 mg. The optimization process involved two steps. Firstly, the top three were carefully selected out of seven characteristics influencing the separation. These key elements required fine-tuning, namely the column type, ion pair, and the residual amount of acetonitrile (ACN) remaining after elution. In the second step, a Box-Behnken experimental design, coupled with Derrenguer's desirability approach, was utilized to identify the ideal target conditions. The optimized method demonstrated excellent specificity, accuracy, linearity, precision, and repeatability, allowing for the reliable simultaneous estimation of artesunate and amodiaquine HCl in the artesunate-amodiaquine tablet. This method offers a valuable tool for quality control and dosage determination in the pharmaceutical industry. By employing advanced experimental techniques and focusing on critical parameters, the study contributes to analytical methodologies in malaria treatment.

Keywords: artesunate, amodiaquine, optimization, analytical validation, HPLC, tablet forms

Since the last decades, there has been a significant increase in political and financial support for attempts to control malaria, and the usage of artemisinin-based combination therapy (ACT) has become widely accepted as the first-line treatment for uncomplicated Plasmodium falciparum malaria has considerably lessened the impact of malaria [1-2] Chemically. Artesunate (AS) is (3R,5aS, 8aS,9R,10S,12R,12aR)-decahydro-3,6, 9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]- 1,2-benzodioxepin-10-ol hydrogen succinate, characterized by: $C_{19}H_{28}O_8$ is the chemical formula, and the molecular weight is 384.42 g/mol. It's a white crystalline powder with a melting point range of 132-1350 °C and is just marginally soluble in water. as well as amodiaquine (AD) hydrochloride is a known chemical under the name: 4-[(7-chloro-4-quinolyl) amino)-a-(diethylamino)-o-cresol; 4-[(7-chloro-4-quinolyl) amino-2-[(diethylamino) methyl] phenol, whose molecular weight is 355g/mol, and whose chemical formula is $C_{20}H_{22}C_lN_3O$. It is a yellow, crystalline powder that melts between 208 and 2090 °C and dissolves in water and alcohol. These two medicines are used to treat malaria [3]. Interestingly, the World Health Organization (WHO) presently recommends five artemisinin-based combinations [4], one of which is artesunate plus amodiaquine (ASAQ), which was first used as a first-line treatment in Ghana in 2005 [5]. When ASAQ was first introduced, 100% of children under five monitored for 28 days experienced therapeutic success [6]. Following that, cure rates ranging from 80 to 100 % were discovered in Ghanaian children aged six months to 10 years, with the maximum malaria incidence [7-8].

Additionally, combination therapy involves taking two or more blood schizonticidal medications at once that have distinct mechanisms of action and target various biochemical aspects of the parasite [9-10]. Thus, when drug components are combined in such a way to create a formulation, developing an analytical method to assess both the efficacy and the composition of the drug substance becomes an

alluring research problem. According to a literature review, some stabilities indicate methodologies are documented for the assessment of combination medication products that contain two or more active pharmacological ingredients [11-16].

In this situation, analytical approaches to identify AS and AD in fixed-dose combination tablets are required to evaluate their quality in endemic areas and to spot fake medications [17]. As a result, the Brazilian Pharmacopoeia provides instructions on DA determination in tablets, while the International Pharmacopoeia provides instructions on SA determination in tablets. Additionally, methods for the distinctive detection of AS or AD in tablets have been discovered in earlier scientific literature [18-19]. Furthermore, techniques for the concurrent HPLC measurement of AS and AD in plasma have been established [20]-21]. Before 2012, there was no reliable way to simultaneously determine both active components [22]. In order to simultaneously determine artesunate and amodiaquine in combination, Y. Le Vaillant et al. devised an RP-HPLC method with double UV detection [23].

This study aimed to improve the resolution (Rs) of fixed AS- and AD-based combination quality control by optimizing the RP-HPLC analytical method. To control chromatographic settings and select those that will best separate DA and its impurities, a two-stage design-of-experiments technique that (i) screens variables and (ii) optimizes separation may be used to address this issue. After being acquired, the new method must undergo the validation procedure utilizing the two approaches, SF-STP pharma and ICH Q2R1, to verify its effectiveness.

Materials and methods

Chemicals and reagents

Amodiaquine and artesunate were obtained from Supelco (Saint Quentin, France). HPLC-grade acetonitrile was purchased from Carlo Erba-Reagents S.A.S (Val de Reuil, France). Phosphoric acid was obtained from Carlo Erba-Reagents S.A.S (Val de Reuil, France), sodium 1-octanesulfonate from Panreac (Barcelona, Spain), and sodium 1-hexanesulfonate from Sigma-Aldrich (SaintnQuentin, France). Anhydrous potassium dihydrogen phosphate was purchased from Sigma-Aldrich (Germany), and ion pairs from Panreac (Barcelona, Spain). CoarsucamTM 100 mg/270 mg has been obtained from WHO. Potential DA impurities are Paracetamol (imp1) was supplied by Sigma-Aldrich (Saint Quentin, France), and 7-chloro-4-(4-hydroxyphenylamino) quinoline (imp2), 4-acetamido-2 diethylaminomethylphenole (imp3) and 4-7 dichloroquinoline, (imp4) were supplied by Sigma-Aldrich (Saint Quentin, France).

Instrumentation and chromatography

Chromatographic separation was performed by RT- HPLC system (Waters 2695) with 2998 diode array UV detector (reading wavelength: 300 nm for Amodiaquine and 210 nm for Artesunate). The mobile phase is a mixture of acetonitrile and phosphate buffer with sodium octane sulfonate acid added in 1L of distilled water at a pH of 3.0. Elution was carried out in gradient mode at a flow rate of 0.8 mL/min for 20min. The column used is of the C18 grafted silica type, 100 mm in length and 4.6 mm in internal diameter, with a particle size of 3 µm (Hypersil BDS model), conditioned at 22 C°.

Sample preparation

Preparation of sample dilution solvent

The dilution solvent we worked with was a mixture of 40% Acetonitrile and 60% a 1.36 g potassium phosphate dihydrate solution in 1000 mL distilled water with pH adjusted to 3 by phosphoric acid.

Preparation of active ingredients and reconstituted forms samples

For the 100% level, we decided to work with a concentration of 0.5 mg/mL of AD, translating to 50 mg of AD and 19 mg of AS, respectively. By combining the amounts of the two respective principles without and with 330 mg of placebo (for the matrix effect) and bringing the mixture to a final volume of 100 mL with the dilution solvent, we created three distinct series for each level. The samples spent 15 minutes in an ultrasound bath to completely dissolve them.

Method development and optimization

An initial basic investigation on AD-AS separation, using injection of a standard containing both AD and AS, was conducted in order to choose the target response for the method's development. The separation between AD and AS is good, and there is a high resolution (Rs) between the two peaks, which is the first conclusion we came to from this study. Therefore, there are no issues with separating these two molecules. The second point we made was that, unlike AD, AS's retention time (tR) does not change when HPLC parameters are changed, indicating that AS is essentially stable. A second preliminary study was conducted to visualize the resolution between the DA and all of its potential impurities. This was accomplished by injecting a standard containing the DA and its impurities: imp1; imp2; imp3; and imp4. According to the findings, we concluded that AD and imp3 are hard to distinguish because they have similar structures (assimilable UV spectra). Between the peaks of these two molecules, this causes a resolution issue. The resolution between AD and imp3 is the target response for method development.

We chose the set of factors already tested by the preliminary study above (Table 1): two qualitative factors, column and ion pair, and five quantitative factors, ion pair concentration, pH, flow rate, %ACN at the start, %ACN at the end. This allowed us to identify the factors that greatly influenced the target analytical response, resolution (Rs). For each aspect, we decided to work with three levels.

Resolution between AD and the impurity 7-Chloro-4-(4-HydroxyPhenylAmino) Quinoline is the target response in our study:

$$R_s = 1{,}18 \cdot \frac{t_R(AD) - t_R imp3}{\delta_{AD} + \delta_{imp3}}$$

A two-stage process was used to search for the best separation circumstances possible: (i) Factor screening using a factorial design: The top 7 factors that had the most significant impact on response were chosen (Table 2). To create the optimal chromatographic conditions for the best Rs, Box, and Behnken surface design optimization and application of the desirability concept were used (Table 3).

Multivariate analysis (MANOVA) was performed by Nemrod® with a critical error set at 5 %.

Table 1. The factors studied and their experimental levels.

Factors	Level 1	Level 2	Level 3
Column	ACE	ZORBAX	SYMMETRY
Ion pair	C5	C6	C7
Ion pair concentration (g/L)	6	8	10
pH	2.8	3	3.2
Flow rate (min/mL)	0.8	1	1.2
% ACN at the start	20	25	30
% ACN at the end	20	25	30

Table 2. Experimental matrix for screening factors.

Run	P.I*	pH	Conc P.I	Column	Flow rate	%ACN	%ACN	Rs
1	C5	3	8	Symmetry	1	30	30	6.32
2	C7	2.8	8	Zorbax	1.2	25	30	2.89
3	C7	3.2	6	Zorbax	1	30	25	2.71
4	C6	3.2	10	ACE	1	25	30	1.54
5	C7	3	10	Symmetry	0.8	25	25	0
6	C6	3.2	8	Symmetry	1.2	20	25	2.58
7	C6	3	10	Zorbax	1.2	30	20	6.29
8	C6	3	8	Zorbax	1	25	25	4.16
9	C7	3	8	ACE	1	20	20	1.7
10	C5	3.2	8	Zorbax	0.8	25	20	20.1
11	C5	2.8	10	Zorbax	1	20	25	4.82
12	C6	2.8	6	Symmetry	1	25	20	2.19
13	C5	3	6	ACE	1.2	25	25	9.08
14	C6	2.8	8	ACE	0.8	30	25	4.62
15	C6	3	6	Zorbax	0.8	20	30	4.96
16	C6	3	8	Zorbax	1	25	25	4.22

*C5: sodium pentane sulfonate; C6: sodium hexane sulfonate; C7: sodium heptane sulfonate.

Table 3. Experience matrix (Box-Behnken plane).

Run	P.I	Column	% ACN fin	Rs
1	C5	Symmetry	20	3.96
2	C7	Symmetry	20	1.27
3	C5	ACE	20	3.58
4	C7	ACE	20	1.77
5	C5	Zorbax	15	4.87
6	C7	Zorbax	15	3.44
7	C5	Zorbax	25	5.66
8	C7	Zorbax	25	2.98
9	C6	Symmetry	15	2.17
10	C6	ACE	15	2.89
11	C6	Symmetry	25	1.47
12	C6	ACE	25	2.08
13	C6	Zorbax	20	4.43

Method validation

Method validation was carried out in accordance with the 1992 SF-STP guide for linearity, precision, and accuracy in the concentration range of 50 %-140 % on Active ingredient samples alone (D1) and on reconstituted forms (D2) and with ICH guideline Q2(R1) for the specificity test. The chromatographic tracings of the placebo solution and the reconstituted form loaded with possible contaminants were recorded to evaluate the RT-HPLC approach's specificity.

Application of the method

The method was applied to a combination of the two molecules in a tablet dosed at 270 mg of AD and 100 mg of AS. For each compound, two standard solutions and adequately diluted tests were made. For AD, 50mg of Amodiaquine working standard (99.5%) was diluted in 100 ml of solvent. The test was prepared using a 50 mg of AD sample from a powder of 20 well-ground tablets. For AS, 19 mg of Artesuntaee working standard (99.2%) is diluted in 100 cc of solvent. A sample of 19 mg of AS from a powder of 20 tablets is ground to a fine powder. Three test preparations were made and determined the PA content of each tablet.

Results and discussions

Optimization of the method

Under the initial chromatographic settings, we injected a solution of AD and all of its related impurities at predetermined concentrations (0.1mg/mL). The acquired chromatogram shows that DA and imp3 are not separated (R3=0), therefore our goal is to optimize our internal method to better separate DA with all of its impurities, as well as the results obtained here are represented in the chromatogram and table below (Fig. 1 and Table 4).

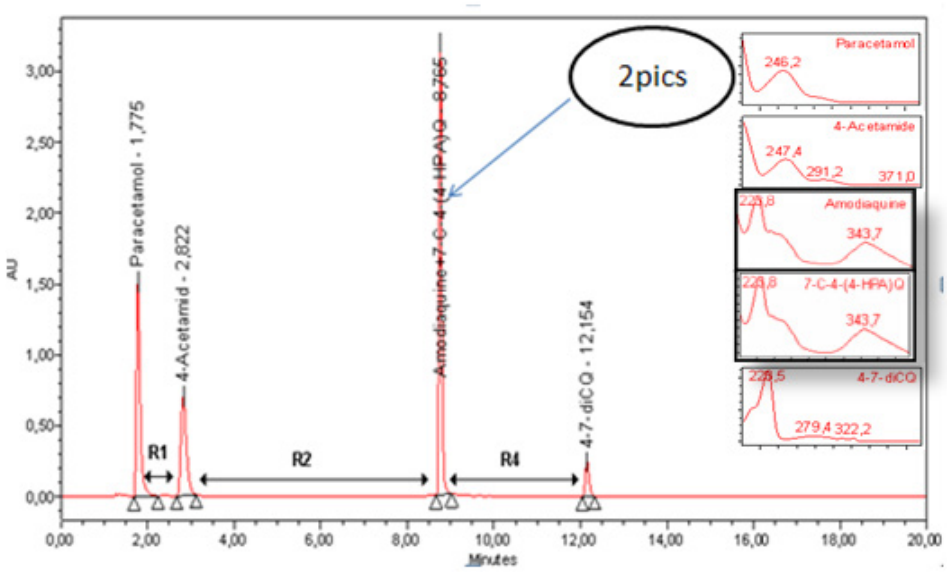

Figure 1. Chromatogram and UV spectra of a solution of DA with its impurities, according to the chromatographic starting conditions.

Table 4. Retention time, peak area, and resolution of AD and its impurities with starting chromatographic conditions.

Samples	Retention time (min)	Peak areas	Resolution*
Imp_1	1.775	9097892	-
Imp_2	2.822	6172620	R1=5.596703
Amodiaquine	8.765	14202095	R2=35.04263
Imp_3	8.765	14202095	R3=0
Imp_4	12.154	1456458	R4=25.17661

*R1: resolution between imp1and imp2, R2: resolution between imp2 and Amodiaquine, R3: resolution between Amodiaquine and imp3, R4: resolution between imp3 and imp4.

The experiment matrix is generated using the Nemrod software, and it defines the set of feasible trials for screening seven factors at three levels (Table 4). Based on the Pareto chart, it's clear that the ion pair is the most influential factor compared to the other factors studied (Figure 2). Based on these findings, the three factors listed in Table 3, the type of ion pair (X1), the column (X2), and the amount of ACN at the end of the gradient are those that have the most significant influence on the target response (Y = Rs). These three variables will be used in the box-Behnken experimental design to model and predict the best resolution based on the concept of attractiveness. One of the fundamental tenets of quality by design is using experimental design for optimization [24].

The present trend is toward the creation of eco-friendly practices [9].

Based on a detailed analysis of the results from the 13 trials, it can be inferred that the mathematical model explains 97.2 % of the observed response. Furthermore, the statistical test known as the Fisher Snedecor F1 yields a significant result of 11.58 (p=0.05), indicating that the model's variables have a substantial impact on the outcome. Additionally, the residual curves depicted in Figure 3 indicate that the model satisfies the assumption of homoscedasticity, meaning that the variability of the residuals remains constant across different levels of the predictors. These findings collectively provide strong support for the proposed mathematical model, which can be employed to predict the optimal conditions for achieving a more efficient separation between AD and its consequences.

After careful evaluation, only the relevant factors significantly influencing the response have been retained in the model equation. Specifically, the ion pair (b1) demonstrates a linear relationship with the response, while the column type (b22) exhibits a quadratic relationship. Consequently, the model equation can be represented as follows:

$$Y\hat{} = 0.163 + 0.558\,X1 + 0.634\,X22,$$

where X1 represents the ion pair, and X2 represents the column type.

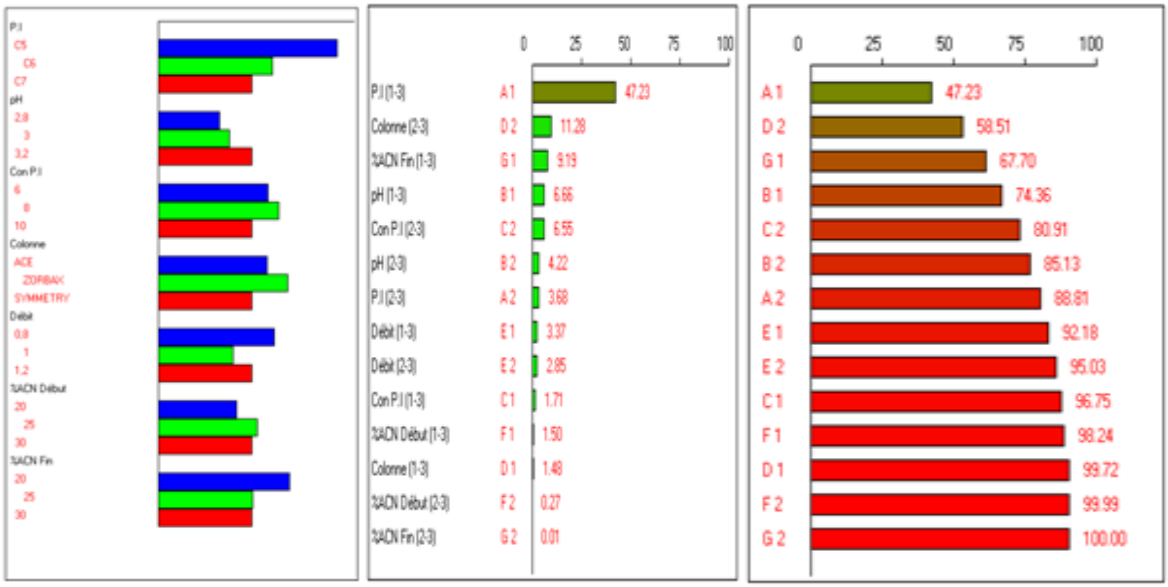

Figure 2. Total effects, Individual Pareto effect, and Accumulated Pareto effect, respectively.

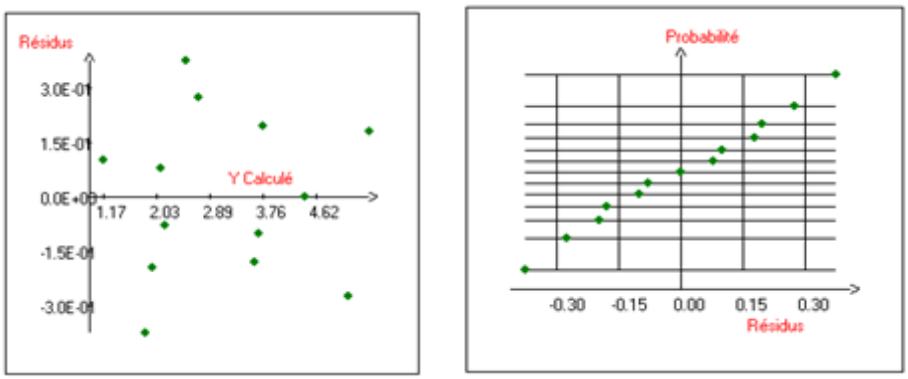

Figure 3. Residuals = F (Y calculated), Distribution of residuals on the Henry line.

Derringuer's desirability function optimization determined the ideal conditions for achieving optimal chromatographic separation. These conditions include the utilization of the sodium hexane sulfonate acid ion pair, the Zorbax type C18 150/4, 6 mm 3.5 m column, and a final ACN (acetonitrile) percentage of 25 mL. These optimized conditions resulted in the highest resolution, as depicted in Figures 4 and 5.

The revised chromatographic conditions were employed to validate the proposed model, and the resulting chromatogram is presented in Figure 6. The optimization of the separation conditions led to a significant improvement, with the transition from an initial R3 value of 0 to an enhanced value of R3 = 4.047560.

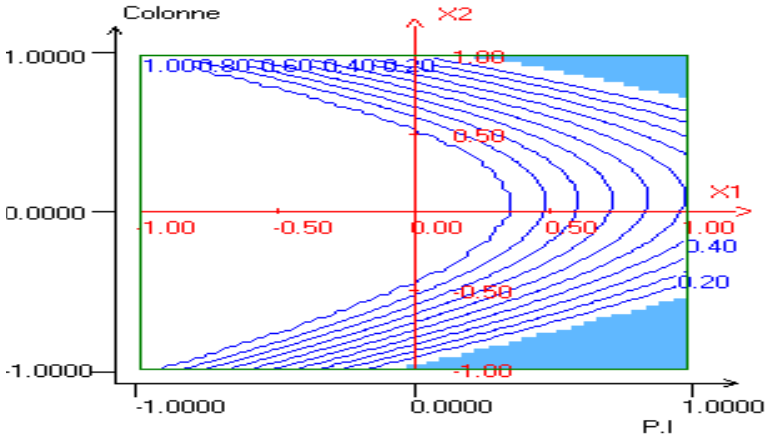

Figure 4. Desirability variation in the plane: P.I, Fixed factor column: % ACN fin = 25.

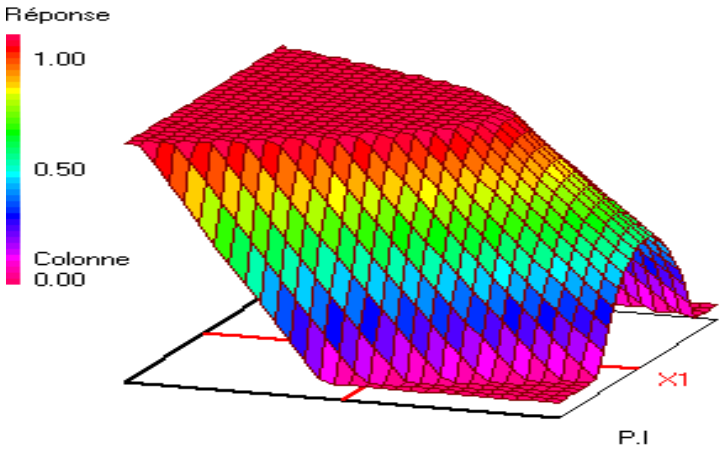

Figure 5. Desirability variation in space: P.I, Fixed factor column: % ACN fin = 25.

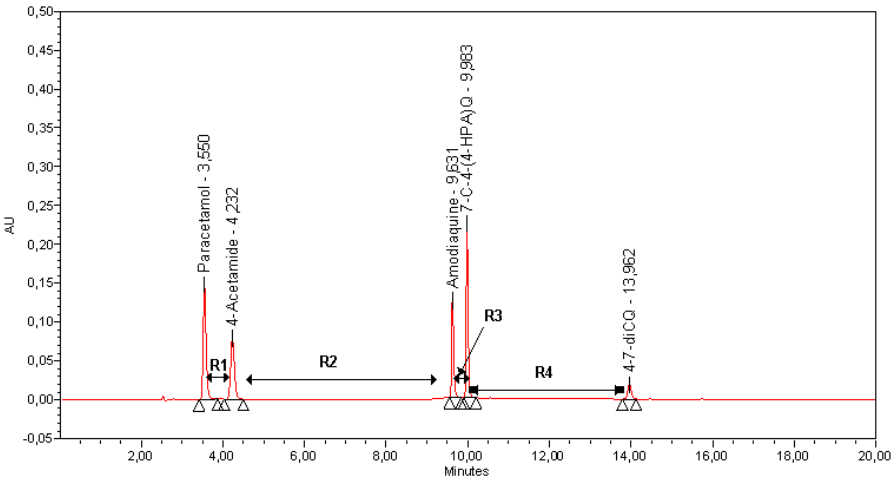

Figure 6. Chromatogram of AD solution with its impurities under optimized conditions.

Analytical validation of the method

Before proceeding with validation, several tests were conducted to ensure the stability and conformity of the system. Except for specificity, all criteria were validated using the SF-STP (Standard Flow-Standard Temperature-Pressure) method.

Specificity, which refers to the ability to accurately identify and quantify the target analytes in the presence of other components, was assessed through injections of a blank solution, a placebo solution, and a sample containing impurities. These injections aimed to determine if any interference occurred with the retention periods of the target analytes, namely AS (active substance) and AD (another active substance). The results indicated that there was no interference, confirming the specificity of the analytical method.

To evaluate the method's linearity, two forms of the samples were analyzed over a span of three days. The first form, referred to as D1, consisted of a solution containing only AS, while the second form, D2, represented the reconstituted pharmaceutical formulation containing both AS and AD. Analysis was performed at five different concentrations ranging from 0.25 to 0.7 mg/mL for AS and 0.096 to 0.266 mg/mL for AD base. The aim was to determine if the method's response exhibited a linear relationship within this concentration range. The regression equations for both the AS alone and the reconstituted form were statistically equivalent, indicating that the response was linear for both active components within 60 % to 140 % of the label claim (as shown in Table 5).

To assess the method's accuracy, three independently reconstituted forms of the sample were analyzed at three different concentrations, ranging from 50% to 140% of the assay concentration for each active molecule (AS and AD). The percentage recovery was determined by comparing the calculated concentrations of AS and AD with their known concentrations. Statistical analysis, involving the comparison of mean and standard deviation values at each concentration level, demonstrated the method's accuracy in simultaneously assessing these two active substances (as presented in Table 5).

Repeatability and intermediate fidelity were assessed by conducting two sets of experiments. In each set, six independent determinations were performed on the same sample of a pharmaceutical product and a combination of active molecules. These experiments were conducted on two different days by two other analysts using separate pieces of equipment and different batches of columns. The purpose of these evaluations was to investigate the consistency and reliability of the method under various conditions. Repeatability was evaluated to measure the precision when the same analyst performed multiple determinations on the same sample using identical equipment and column batches. Intermediate fidelity was examined to assess the method's robustness by considering variations introduced by different analysts, equipment, and column batches across multiple days. The results of these evaluations demonstrated acceptable levels of repeatability, indicating consistent and precise measurements. Furthermore, no discernible changes were observed between the two sets of experiments, indicating a stable and reliable method. The determination of the AS and AD contents obtained over the two days fell comfortably within the specified assay parameters of 90.0-110.0%. This range defines the acceptable content limits for AS and AD in the pharmaceutical product. Table 5 provides a clear and organized presentation of the results obtained from the repeatability and intermediate fidelity assessments.

Table 5. Final findings of method validation.

	Amodiaquine		**Artesunate**	
	AD alone	**AD in PF**	**AS alone**	**AS in PF**
Linearity (50-80-100-120-140%)				
- Regression equation	Y = 171593 x + 1577479	Y = 177568x + 9083395	Y = 4961 x - 2088	Y = 4844 x - 446
- Slope	171593.235	177568	4961.362	4844.458
- Order of origin	1577478.87	9083395	-2088.072	-445.896
- Correlation coefficient	0.9914	0.9961	0.999	0.999
Accuracy (50-80-100-120-140%)				
- Average recovery	100.38		99.74	
- Average recovery confidence interval	[98.85-101.9]		[99.28-100.19]	
Fidelity (repeatability)				
- CVr	1.76		0.44	
- Repeatability threshold	5.07		1.28	
Intermediate precision (IF)				
- CVFI	1.98		1.42	
- IF threshold	5.71		4.06	

Application

The validated approach was consistently employed to determine the content of the active molecules, AD and AS, in the final Coarsucam product. The results obtained from this method were highly satisfactory. The analysis revealed an Amodiaquine content of approximately 98.84 % in the finished product, with a confidence interval ranging from 98.03 % to 99.65 %. Additionally, the assessment indicated an Artesunate content of around 102.63 %, with a confidence interval between 101.96 % and 103.57 %.

These outcomes, obtained through the optimized chromatographic method, demonstrate the effectiveness of the new technology in accurately detecting both active components. Notably, this method can also be utilized for evaluating the presence of AD and AS in counterfeit medications, further emphasizing its potential for quality control purposes. This extends the method's applicability beyond standard quality control measures [25-27].

Conclusion

In conclusion, the suggested approach offers several advantages, including ease of use, accuracy, and repeatability. These qualities make it highly suitable for routine application in pharmaceutical formulations to measure the levels of artesunate and amodiaquine. Notably, the approach has undergone improvements that enhance its effectiveness in separating amodiaquine from impurities when utilizing the RT-HPLC method to simultaneously detect AS and AD in fixed-dose combination tablets.

The results of the statistical analysis demonstrate that the method exhibits high accuracy and good precision. The increased resolution achieved in the study can be primarily attributed to the specific column type and ion pair utilized. The 1992 SF-STP pharma methodology and the ICH Q2 (R1) guideline were employed to validate the improved method. The validation process confirmed the method's simplicity and feasibility for practical implementation.

Overall, the suggested approach represents a reliable and efficient solution for pharmaceutical laboratories seeking to assess the levels of artesunate and amodiaquine in their formulations. Its ease of use, accuracy, repeatability, and improved separation capabilities make it suitable for routine quality control and analysis use.

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