HPLC Method Development and Validation of Artesunate and Amodiaquine in Tablet Dosage Form

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Abstract: *An accurate and precise HPLC method was developed and validated for simultaneous estimation of Amodiaquine and Artesunate in tablet dosage form. The column was used was C18 column (150 x 4.6mm, 5µm) column and a mobile phase composed of Potassium Dihydrogen o-Phosphate Buffer (pH-5): Acetonitrile (50:50 v/v). The flow rate was kept 1 ml/min and the detection wavelength was 280nm. The retention time for Amodiaquine and Artesunate was found to be 3.1 min and 5.3 min respectively. The linearity range was found to be 12.5-37.5 µg/ml for Artesunate with corelation coefficient 0.999 and 32.5-97.5 µg/ml for Amodiaquine corelation coefficient 0.9999 for Artesunate and Amodiaquine respectively. The LOD and LOQ for Artesunate 0.32 and 0.97 µg/ml was found to be and for Amodiaquine was found to be 0.50 and 0.82 respectively. The repeatability, intraday and interday precision was found to be less than 2%. The proposed methods for estimation of Amodiaquine and Artesunate were found to be selective, precise and accurate. The developed validated method is applicable for the simultaneous determination of Amodiaquine and Artesunate in tablet dosage form.*

***Key Words*:** HPLC, Amodiaquine, Artesunate

Received Date: 18/07/2023 Revised Date: 22/09/2023 Acceptance Date: 27/09/2023

1. INTRODUCTION

Malaria is a life-threatening disease caused by parasites of the Plasmodium genus. Malaria is caused by 4 species of the protozoa parasite. Plasmodium is endemic. In the most parts of India four species of plasmodium cause human malaria: Plasmodium falciparum, Plasmodium Vivax, Plasmodium Malariae and Plasmodium Ovale. Malaria parasites are transmitted from one person to another by the female anopheles' mosquitoes. Artemisinin based combination therapy is based on the use of two drugs with different modes of action. Artemisinin-derivative that causes rapid and effective reduction of parasite biomass and gametocyte carriage and second drug that has a longer duration of action.

1.1. Artesunate

Artesunate is an antimalarial agent of chemical class a hemisuccinate derivative of dihydroartemisinin and is chemically known as 4-oxo-4-{[(1R,4(3R,5aS,6R,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, which is itself obtained by the reduction of artemisinin, a sesquiterpene lactone endoperoxide. Amodiaquine is class of synthetic amino 4-quinoline and chemically known as chloroquinolin-4-yl)amino]-2-[(diethyl amino)methyl]phenol. Its activity is characterized by a schizonticidal action on all Plasmodium species. So it is used to treat acute illnesses by destroying intra erythrocytic forms.2

The mechanism of action of Artesunate has been widely studied and appears well established. The Artesunate endoperoxyde's bridge is split by heme within the infected erythrocyte, generating singlet oxygen. Parasite proteins, particularly in membranous structures, are thus alkylated, leading to parasite death.3,4 The amodiaquine, penetrate the infected red blood cells in a specific way and prevent the parasite from polymerizing heme into an insoluble product called hemozoin, leading to parasite death.

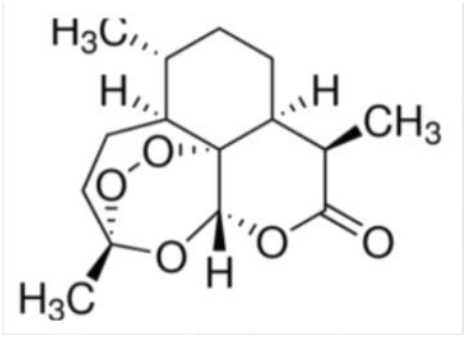

Fig - 1: Chemical structure of Artesunate

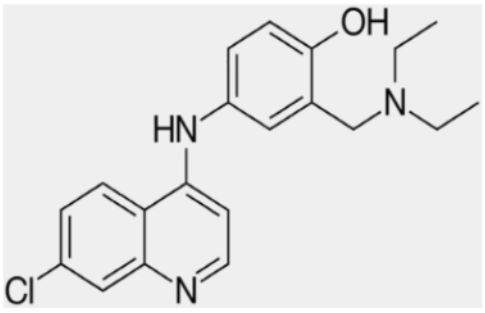

Fig - 2: Chemical structure of Amodiaquine

For Artesunate is official HPLC methods are reported in IP 5 and USP 6. Literature survey reveals that, Spectrophotometric method for Artesunate with curcumin in liposomal formulation 7, HPLC method for the simultaneous determination of Artesunate and mefloquine hydrochloride in fixed dose combination Tablets 8 and simultaneous estimation of Artesunate and Mefloquine hydrochloride in bulk and marketed formulation by UV spectroscopic 9, Stability Indicating Method Development and Validation for Simultaneous Estimation of Mefloquine and Artesunate in Tablet Dosage Form 10 were reported.

Compendial methods for estimation amodiaquine are not available. The literature survey reveals that Stability Indicating HPLC Method for the Determination of Amodiaquine Hydrochloride 11 and UV Spectrophotometric and HPLC Methods for Quantitative Determination of Chloroquine and Amodiaquine in Pharmaceutical Formulations 12 were reported in research articles.

Very few methods reported for the determination of Artesunate and Amodiaquine in combined dosage form by different instrumental techniques are Simultaneous Determination of Artesunate and Amodiaquine in Human Plasma Using LC-MS/MS 13, RP-HPLC Method for Simultaneous Estimation of Artesunate and Amodiaquine in Combined Tablet Dosage Form 14, RP-HPLC Method for Simultaneous Estimation of Artesunate and Amodiaquine HCL in their Combined Pharmaceutical Dosage Form 15 Spectrophotometric method determination of Artesunate and Amodiaquine in combined dosage form16 were reported.

The aim of present work was to develop and validate an accurate, cost effective and precise HPLC method.

2. MATERIAL AND METHODS

Instruments and Apparatus

The instruments were used; HPLC System- Liquid Chromatograph: LC-2010 CHT (Shimadzu), Detector-UV VIS Detector (UV-2487) – Dual Absorbance Detector, YMC C-18 column (150 x 4.6mm, 5µm), UV-Visible Spectrophotometer: Shimadzu UV double beam spectrophotometer 1800, Electronic analytical balance (ME204) and Digital melting Point Apparatus. The calibrated volumetric apparatus were used for preparation and dilutions.

Reagents and Chemicals

API was provided as a gift sample from IPCA Laboratory ltd, Athal, Silvassa. All Chemicals and reagents used were of Analytical Grade and HPLC Grade. Marketed Formulation used for assay was by IPCA LABORATORIES LIMITED having Label Claim 25mg of Artesunate and 67.5mg Amodiaquine.

Standard stock solution of Artesunate and Amodiaquine

Weigh an accurately about 25 mg Artesunate in 100 ml volumetric flask and dissolved with 100 ml of methanol. (250 µg/ml). Weigh an accurately about 65 mg Artesunate in 100 ml volumetric flask and dissolved with 100ml of methanol. (650 µg/ml).

Working standard preparation (Combine standard preparation)

Pipette out 1 ml from Artesunate standard stock solution and 1ml from Amodiaquine standard stock solution in 10 ml with Mobile phase (Potassium Dihydrogen o-Phosphate Buffer (pH-5): Acetonitrile (50:50 v/v)) (ART-25 µg/ml), (AMO-65 µg/ml).

System Suitability Studies

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 analyzed constitute an integral system that can be evaluated as such. The system suitability study was evaluated from the standard chromatogram by three replicate injections of Artesunate and Amodiaquine. The %RSD, theoretical plate was calculated for standard solution (ART-25 µg/ml -25µg/ml, AMO -65 µg/ml).

Method validation

The analytical method validation was performed as per ICH guidelines ICH Q2 (R1): Validation of Analytical Procedures: Text and Methodology (2005)

3. LINEARITY AND RANGE

Preparation of calibration curve for Artesunate

The standard stock solution of 250 µg/ml was prepared by dissolving 25 mg of Artesunate in 100 ml methanol. The standard sub-stock solution of concentrations 12.5, 18.75, 25, 31.25 and 37.5 µg/ml were prepared from above standard solution with methanol.

Preparation of calibration curve for Amodiaquine

The standard stock solution of 650 µg/ml was prepared by dissolving 65 mg of Amodiaquine in 100 ml methanol. The standard sub-stock solution of concentrations 32.5, 48.75, 65, 81.25 and 97.5 µg/ml were prepared from above standard solution with methanol.

Each sample injected in triplicate for each concentration level and calibration curve was constructed by plotting the peak area versus the drug concentration.

The HPLC chromatogram and area were shown in Figure 3 and Table 1 respectively.

Table -1: Calibration Data of Artesunate and Amodiaquine

Sr no.	Conc. (µg/ml)		Peak area ± SD (n=3)		%RSD	
	ART	AMO	ART	AMO	ART	AMO
1	12.5	32.5	174.759 ± 1.39	1808.692 ± 14.32	0.79	0.79
2	18.75	48.75	266.284 ± 5.23	2752.823 ± 32.56	1.96	1.18
3	25	65	357.079 ± 6.69	3698.559 ± 25.88	1.87	0.69
4	31.25	81.25	444.168 ± 3.25	4600.067 ± 45.56	0.73	0.99
5	37.5	97.5	534.26 ± 5.20	5533.136 ± 56.17	0.97	1.01

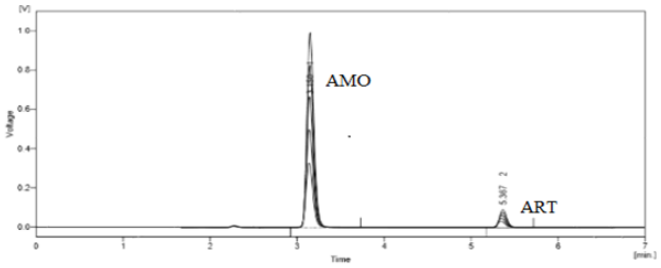

Fig - 3: Chromatogram of Linearity of Artesunate (12.5-37.5 µg/ml) and Amodiaquine (32.5-97.5µg/ml)

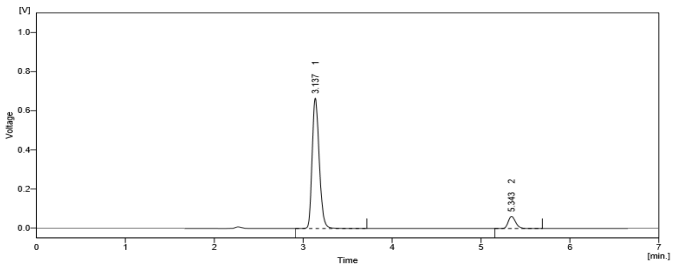

Fig – 4: Chromatogram for Repeatability 1 (100 µg/ml ART and AMO)

Precision

The injection system precision was determined by performing 6 replicate injection for Repeatability and 3 replicate injections for Intra-day and Inter-day Precision. The precision expressed as standard deviation or relative standard deviation.

Repeatability.

The data for repeatability of peak area measurement for Artesunate and Amodiaquine based on six times measurement of same concentration (100µg/ml). The % RSD was found to be 0.19 and 0.12 for Artesunate and Amodiaquine. The HPLC chromatogram was shown in Figure 4.

Intraday Precision

Artesunate (12.5, 25 and 37.5 µg/ml) and Amodiaquine (32.5, 65, 97.5 µg/ml) were taken in a ratio was analyzed at three levels of mention concentration for three times in a day.

Interday precision

The Artesunate (12.5, 25 and 37.5 µg/ml) and Amodiaquine (32.5, 65, 97.5 µg/ml) were taken in a ratio was analyzed at three levels of mention concentration on three different consecutive days.

The Results of Intraday Precision and Interday Precision for Artesunate and Amodiaquine were shown in Table 2.

Table - 2: Results of Intraday Precision and Interday Precision

		Intraday Precision			Interday Precision		
Name of drug	Conc. (µg/ml)	Area (n=3)	SD (n=3)	%RSD	Area (n=3)	SD (n=3)	%RSD
ART	12.5	179.641	1.93	0.10	173.86	0.35	0.20
	25	354.96	0.69	0.19	355.31	0.71	0.25
	37.5	531.09	1.05	0.20	531.61	1.07	0.20
AMO	32.5	1796.41	1.94	0.11	1797.56	1.86	0.10
	65	3673.61	4.078	0.11	3677.63	4.32	0.11

	97.5	5496.23	6.45	0.12	5501.79	6.42	0.12

Accuracy

Accuracy was done by % Recovery Study. The assay sample solutions were prepared by spiking the API at 3 levels i.e. 80%, 100% and 120%. The Percent Recovery data obtained by the proposed RP-HPLC method. The results were shown in Table 3.

Table -3: Result of Accuracy (%Recovery)

Sr no.	Tablet content Taken eq. to (mg)		Standard Added (mg)		Total Drug Recovered mg		%Recovery of Standard Added	
	ART	**AMO**	**ART**	**AMO**	**ART**	**AMO**	**ART**	**AMO**
Blank	25	67.5	-	-	24.96	67.35	-	-
	25	67.5	-	-	25.00	67.50	-	-
	25	67.5	-	-	24.60	66.90	-	-
	Mean ± SD				24.85 ± 0.22	67.25 ± 0.31		
80%	25	67.5	20	54	44.94	121.50	99.84	99.78
	25	67.5	20	54	49.90	119.56	100	100
	25	67.5	20	54	45.00	120.56	94.4	99.11
	Mean ± SD				35.61 ± 0.21	88.45 ± 0.31	98.93 ± 0.59	98.28 ± 0.34
100%	25	67.5	25	67.5	50.10	131.23	100.86	99.38
	25	67.5	25	67.5	49.96	134.86	99.92	98.92
	25	67.5	25	67.5	51.23	132.66	101.25	102.46
	Mean ± SD				39.67 ± 0.44	101.52 ± 1.21	99.17 ± 1.10	101.52 ±1.21
120%	25	67.5	30	81	54.96	147.44	99.92	99.28
	25	67.5	30	81	55.00	148.97	100.00	100.31
	25	67.5	30	81	55.06	149.02	100.01	100.35
	Mean ± SD				44.81 ± 0.21	108.35 ± 0.70	101.83 ± 0.48	98.50 ± 0.63

Table - 4: Results of Robustness

Sr. No	Parameter	Mean area (n=3)		SD (n=3)		%RSD	
	Potassium dihydrogen o-phosphate buffer: Acetonitrile	**ART**	**AMO**	**ART**	**AMO**	**ART**	**AMO**
1	48:52v/v	349.22	3614.34	2.13	18.32	0.61	0.51
2	52:48 v/v	370.69	3836.70	2.17	18.26	0.58	0.48
	Flow rate						
1	1.2 ml/min	349.22	3614.34	2.13	18.32	0.61	0.51
2	0.8 ml/min	370.69	3836.70	2.17	18.26	0.58	0.48
	pH						
1	5.2	341.40	3533.37	2.12	18.67	0.62	0.53

2	4.8	366.61	3797.60	2.34	17.83	0.64	0.47

Robustness

The Robustness of the method was evaluated by the change in following parameters such as by changing the flow rate: ± 0.2 ml/min, by changing the Mobile Phase: ± 2.0% solvent in Mobile Phase, by changing the pH: ± 0.2. The results were shown in Table 4.

4. LOD AND LOQ

LOD:

Analyte must reliably differentiate from background noise. It is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified.

LOQ:

The Limit of Quantitation is the minimum injected amount that gives precise measurements in chromatography typically requiring peak height 10 times higher than the baseline noise. The results of LOD and LOQ for Artesunate and Amodiaquine was shown in Table 5.

Table - 5: Results of LOD and LOQ

Parameter	Artesunate	Amodiaquine
LOD (µg/ml)	0.32	0.50
LOQ (µg/ml)	0.97	1.51

System Suitability Studies

A standard solution for Artesunate and Amodiaquine was prepared as per the test method and was injected six times into HPLC System.

The system suitability parameters were evaluated from standard chromatogram by calculating %RSD from six replicate injections for Artesunate and Amodiaquine.

Table - 6: System suitability test parameters

Parameter	Artesunate	Amodiaquine
Number of Theoretical Plates (N)	7443	18339
Retention Time	5.3	3.1
Asymmetry	1.318	1.333
Resolution	13.933	0.00

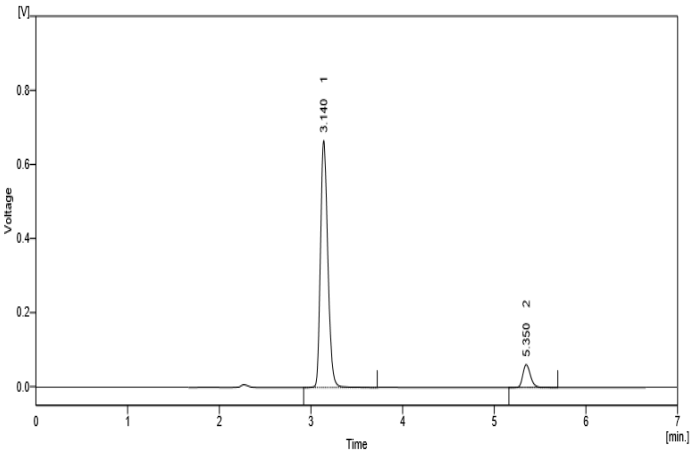

Fig - 5: Chromatogram of Artesunate (25 µg/ml) and Amodiaquine (65 µg/ml) Standard Solution

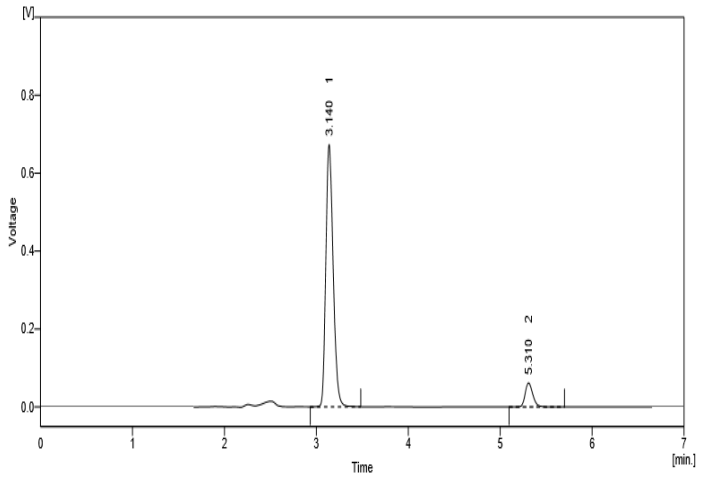

Fig -6: Chromatogram of Artesunate (25 µg/ml) and Amodiaquine (65 µg/ml) Tablet solution

Table - 7: Assay of Artesunate and Amodiaquine

Sr. no	Drug	Label Claim (mg)	Amount found (mg)	Area of samples	%Assay	%ASSAY ± SD	%RSD of assay
1	ART	25	24.46	350.38	98.22	98.42 ± 0.19	0.20
2		25	24.60	351.07	98.42		

3		25	24.65	351.76	98.61		
		Average			98.42		
1	AMO	67.5	67.87	3715.18	100.55	100.70 ± 0.13	0.13
2		67.5	68.01	3722.67	100.75		
3		67.5	68.03	3723.87	100.79		
		Average			100.70		

5. RESULTS AND DISCUSSION

The RP-HPLC method was validated in terms of linearity and range, precision, robustness, LOD, LOQ, assay and accuracy. The develop RP- HPLC method was found to be linear for the range from 12.5-37.5 µg/ml for Artesunate and 32.5-97.5 µg/ml for Amodiaquine. The precision of the method was studied as an intra-day, inter-day variations and repeatability. The % RSD value was found to be less than 2 indicates that the method was precise. The limits of detection were found to be 0.32 and 0.82 µg/ml for Artesunate and Amodiaquine respectively. Limit of Quantitation were found to be 0.50 and 1.51 µg/ml for Artesunate and Amodiaquine respectively. The % accuracy was found to be 99.41± 0.88 % - 100.86±1.39 % and 99.62±0.46 % - 100.86±.1.39% for Artesunate and Amodiaquine respectively. The developed method was found to be selective and specific as the assay results indicate there is no interference of excipients.

6. CONCLUSIONS

The proposed HPLC method was developed and validated according to ICH Guidelines and was found to be precise, accurate and reproducible for the determination of Artesunate and Amodiaquine. The recovery studies revealed excellent accuracy and high precision than previous reported method. The developed selective and validated method can be applied for quality control and routine analysis.

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