International Journal of Pharmacy and Analytical Research (IJPAR)

IJPAR |Vol.14 | Issue 4 | Oct - Dec -2025

www.ijpar.com

ISSN: 2320-2831

DOI : https://doi.org/10.61096/ijpar.v14.iss4.2025.1169 -1185

Research

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ARTESUNATE AND MEFLOQUINE

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Published on:

Published by:
Futuristic Publications

Abstract

A simple, precise, rapid and accurate reversed-phase high performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of Artesunate and Mefloquine was developed and validated as per ICH Guidelines. Chromatography was carried out by isocratic technique on a Develosil C18 (4.6mm×150mm, 5µm) Column with mobile phase mixture of Acetonitrile: Phosphate buffer pH-5.2 (25:75v/v) was used as a mobile phase and the pH was adjusted into 5.2 by using with ortho-phosphoric acid, at a flow rate of 1.0 ml/min. The UV range was detected at 248 nm for Artesunate and Mefloquine respectively. The different analytical performance parameters such as linearity, precision, accuracy, and specificity, limit of detection (LOD) and limit of quantification (LOQ) were determined according to International Conference on Harmonization ICH Q2B guidelines. The linearity of the calibration curves for each analyte in the desired concentration range is good (r2 >0.9). The recovery of the method was between 99.95% and 100.10% for Artesunate and Mefloquine respectively. Hence the proposed method is highly sensitive, precise and accurate and it successfully applied for the reliable quantification of API content in the commercial formulations of Artesunate and Mefloquine.

Keywords: Artesunate and Mefloquine, RP-HPLC, ICH Guidelines, Accuracy, Precision.

1. INDTRODUCTION

1.0 Introduction to HPLC:

High Performance Liquid Chromatography (HPLC) was derived from the classical column chromatography and, is one of the most important tools of analytical chemistry today.1In the modern pharmaceutical industry, high-performance liquid chromatography (HPLC) is the major and integral analytical tool applied in all stages of drug

discovery, development, and production.2 HPLC is the method of choice for checking peak purity of new chemical entities, monitoring reaction changes is in synthetic procedures or scale up, evaluating new formulations and carrying out quality control / assurance of the final drug products.3

The Goal of HPLC method is to try & separate, quantify the main drug, any reaction impurities, all available synthetic intermediates and any degradants. 4High Performance Liquid Chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantify the compounds that are present in any sample that can be dissolved in a liquid. HPLC is the most accurate analytical methods widely used for the quantitative as well as qualitative analysis of drug product and used for determining drug product stability. 5 HPLC principle is the solution of sample is injected into a column of porous material (stationary phase) and liquid phase (mobile phase) is pumped at higher pressure through the column. The principle of separation followed is the adsorption of solute on stationary phase based on its affinity towards stationary phase. (Figure-1) The technique of HPLC has following features.6

- High resolution
- Small diameter, Stainless steel, Glass column
- Rapid analysis
- Relatively higher mobile phase pressure
- Controlled flow rate of mobile phase

2.0 HPLC Method Development:

Methods are developed for new products when no official methods are available. Alternate methods for existing (Non-Pharmacopoeial) products are to reduce the cost and time for better precision and ruggedness. When alternate method proposed is intended to replace the existing procedure comparative laboratory data including merit/demerits are made available. The goal of the HPLC-method is to try & separate, quantify the main active drug, any reaction impurities, all available synthetic inter-mediates and any degradants.7

Steps involved in Method development are. 6,7

- Understanding the Physicochemical properties of drug molecule.
- Selection of chromatographic conditions.
- Developing the approach of analysis.
- Sample preparation
- Method optimization
- Method validation (figure-2)

2.1 Understanding the physicochemical properties of drug molecules:

Physicochemical properties of a drug molecule play an important role in method development. For Method development one has to study the physical properties like solubility, polarity, pKa and pH of the drug molecule. Polarity is a physical property of a compound. It helps an analyst, to decide the solvent and composition of the mobile phase. 6 The solubility of molecules can be explained on the basis of the polarity of molecules. Polar, e.g. water, and nonpolar, e.g. benzene, solvents do not mix. In general, like dissolves like i.e., materials with similar polarity are soluble in each other. Selection of diluents is based on the solubility of analyte. The acidity or basicity of a substance is defined most typically by the pH value. Selecting a proper pH for ionizable analytes often leads to symmetrical and sharp peaks in HPLC.7

2.2 Selection of chromatographic conditions

During initial method development, a set of initial conditions (detector, column, mobile phase) is selected to obtain the first "scouting" chromatograms of the sample. In most cases, these are based on reversed-phase separations on a C18 column with UV detection. A decision on developing either an isocratic or a gradient method should be made at this point.

2.2.1 Selection of Column:

A column is of course, the starting and central piece of a chromatograph. A appropriately selected column can produce a good chromatographic separation which provides an accurate and reliable analysis. An improperly used column can often generate confusion, inadequate, and poor separations which can lead to results that are invalid or complex to interpret.9The heart of a HPLC system is the column. Changing a column will have the greatest effect on the resolution of analytes during method development. Choosing the best column for application requires consideration of stationary phase chemistry, retention capacity, particle size, and column dimensions. The three main components of an HPLC column are the hardware, the matrix, and the stationary phase.

Normal phase chromatography utilizes a polar stationary phase and a non-polar mobile phase. Generally, more polar compounds elute later than non-polar compounds. Commonly used reverse phase columns and their uses are listed below. Propyl (C3), Butyl (C4), and Pentyl (C5) phases are useful for ion-pairing chromatography (C4) and peptides with hydrophobic residues, and other large molecules. C3–C5 columns generally retain non-polar solutes more poorly when compared to C8 or C18 phases. Examples include Zorbax SB-C3, YMC-Pack C4, and Luna C5. These columns are generally less stable to hydrolysis than columns with longer alkyl chains. Octyl (C8, MOS) phases have wide applicability. This phase is less retentive than the C18 phases, but is still quite useful for pharmaceuticals, nucleosides, and steroids.10Selection of the stationary phase/column is the first and the most important step in method development. The development of a rugged and reproducible method is impossible without the availability of a stable, high performance column. To avoid problems from irreproducible sample retention during method development, it is important that columns be stable and reproducible. The separation selectivity for certain components vary between the columns of different manufacturer as well as between column production batches from the same manufacturer. Column dimensions, silica substrate properties and bonded stationary phase characteristics are the main ones. The use of silica-based packing is favored in most of the present HPLC columns due to several physical characteristics.6

INSTRUMENTS USED

HPLC WATERS, software:	Empower 2, Alliance 2695 separation module. 996 PDA detector.
pH meter	Lab India
Weighing machine	Sartorius
Volumetric flasks	Borosil
Pipettes and Burettes	Borosil
Beakers	Borosil
Digital ultra sonicator	Labman

CHEMICALS USED:

Artesunate	Sura labs
Mefloquine	Sura labs
Water and Methanol for HPLC	LICHROSOLV (MERCK)
Acetonitrile for HPLC	Merck

RESULTS AND DISCUSSION

Optimized Chromatogram (Standard)

Mobile phase	: Acetonitrile: Phosphate buffer pH-5.2 (25:75v/v)
Column	: Develosil C18 (4.6mm×150mm, 5µm) Column
Flow rate	: 1 ml/min
Wavelength	: 248 nm
pH	: 5.2
Column temp	: 36ºC
Injection Volume	: 20 µl
Run time	: 7 minutes

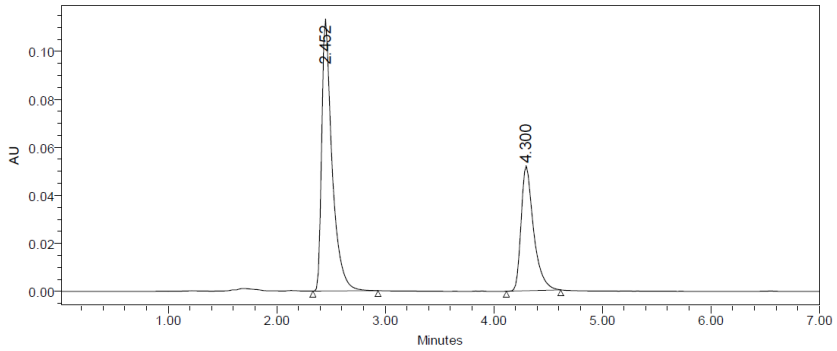

Fig-: Optimized Chromatogram

Table-: Peak Results for Optimized Chromatogram

S. No.	Peak name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Artesunate	2.452	8567524	452685		1.56	6358
2	Mefloquine	4.300	526586	36586	5.64	1.43	5425

Observation: From the above chromatogram it was observed that the Artesunate and Mefloquine peaks are well separated and they shows proper retention time, resolution, peak tail and plate count. So it's optimized trial.

Optimized Chromatogram (Sample)

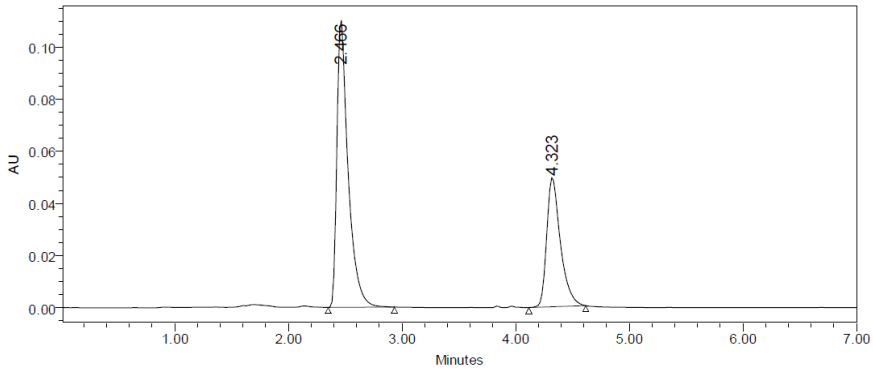

Figure-: Optimized Chromatogram (Sample)

Table: Optimized Chromatogram (Sample)

S. No.	Peak name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Artesunate	2.466	8659825	468545		1.57	6485
2	Mefloquine	4.323	536584	37856	5.65	1.44	5596

Acceptance Criteria:

- Resolution between two drugs must be not less than 2.
- Theoretical plates must be not less than 2000.
- Tailing factor must be not less than 0.9 and not more than 2.

- It was found from above data that all the system suitability parameters for developed method were within the limit.

METHOD VALIDATION

Blank:

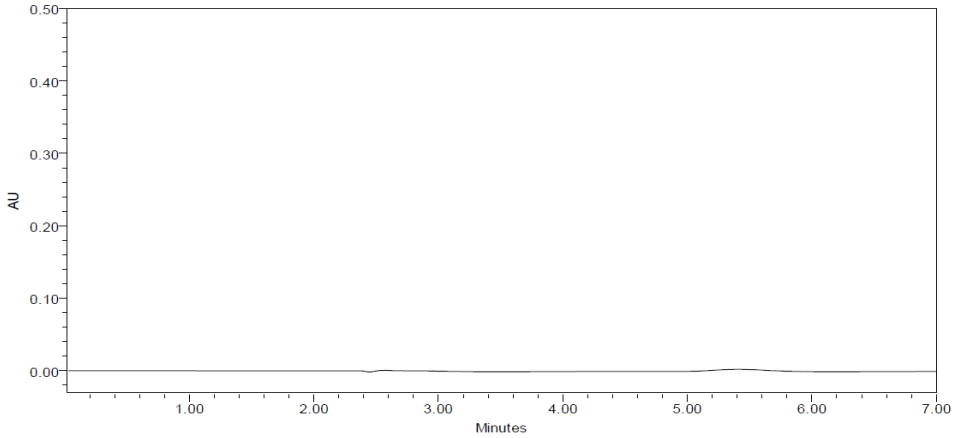

Fig-: Chromatogram showing blank (mobile phase preparation)

System Suitability:

Table-: Results of system suitability for Artesunate

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Artesunate	2.459	856983	458695	6452	1.57
2	Artesunate	2.466	854787	468562	6369	1.58
3	Artesunate	2.472	856964	458798	6485	1.56
4	Artesunate	2.452	859625	465258	6385	1.58
5	Artesunate	2.450	865242	458263	6482	1.58
Mean			858720.2			
Std. Dev			4028.793			
% RSD			0.469163			

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is suitable.

Table-: Results of system suitability for Mefloquine

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution

1	Mefloquine	4.322	526985	36985	5486	1.43	5.65
2	Mefloquine	4.323	528526	37582	5525	1.44	5.64
3	Mefloquine	4.342	524685	36547	5478	1.43	5.66
4	Mefloquine	4.300	524986	37856	5529	1.44	5.66
5	Mefloquine	4.295	526425	36854	5486	1.43	5.65
Mean			526321.4				
Std. Dev			1563.046				
% RSD			0.296976				

Acceptance criteria:

- %RSD for sample should be NMT 2.
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

SPECIFICITY

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components.

Analytical method was tested for specificity to measure accurately quantitate Artesunate and Mefloquine in drug product.

Assay (Standard):

Table-: Peak results for assay standard

S.No.	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Artesunate	2.456	8569853	458698		1.56	6358	1
2	Mefloquine	4.312	526585	36586	5.68	1.44	5485	1
3	Artesunate	2.457	8598524	458986		1.57	6385	2
4	Mefloquine	4.308	526954	36985	5.69	1.45	5479	2
5	Artesunate	2.456	8542655	4587522		1.58	6328	3
6	Mefloquine	4.312	532432	36896	5.68	1.45	5496	3

Assay (Sample):

Table-: Peak results for Assay sample

S. No.	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Artesunate	2.465	8658542	468598		1.57	6485	1
2	Mefloquine	4.337	536985	37854	5.68	1.45	5596	1
3	Artesunate	2.474	8657547	462587		1.58	6429	2
4	Mefloquine	4.356	532645	38652	5.68	1.46	5587	2
5	Artesunate	2.465	8625985	469365		1.57	6552	3
6	Mefloquine	4.337	536985	37856	5.69	1.45	5589	3

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

The % purity of Artesunate and Mefloquine in pharmaceutical dosage form was found to be 100.258%.

LINEARITY

CHROMATOGRAPHIC DATA FOR LINEARITY STUDY:

Artesunate:

Concentration µg/ml	Average Peak Area
40	5758685
50	7096524
60	8549852
70	9856584
80	11135872

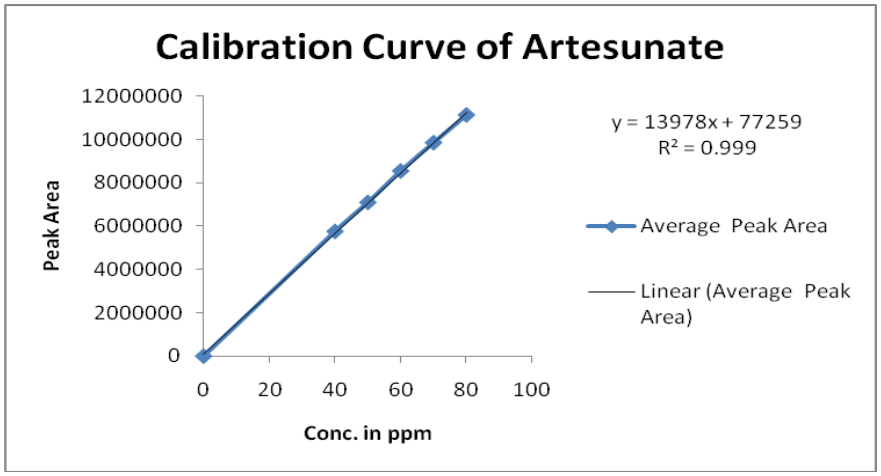

Figure 6.3.4 Calibration graph for Artesunate

LINEARITY PLOT:

The plot of Concentration (x) versus the Average Peak Area (y) data of Artesunate is a straight line.

Y = mx + c

Slope (m) = 13978

Intercept (c) = 77259

Correlation Coefficient (r) = 0.999

VALIDATION CRITERIA: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

CONCLUSION: Correlation Coefficient (r) is 0.99, and the intercept is 77259. These values meet the validation criteria.

Mefloquine

Concentration µg/ml	**Average Peak Area**
60	372685
80	498542
100	618528
120	747575
140	864598

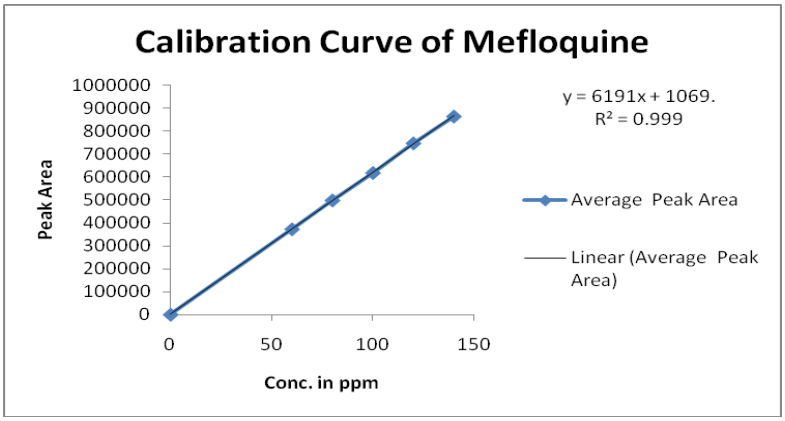

Figure 6.3.4 Calibration graph for Mefloquine

LINEARITY PLOT:

The plot of Concentration (x) versus the Average Peak Area (y) data of Mefloquine is a straight line.

Y = mx + c

Slope (m) = 6191

Intercept (c) = 1069

Correlation Coefficient (r) = 0.999

VALIDATION CRITERIA: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

CONCLUSION: Correlation Coefficient (r) is 0.99, and the intercept is 1069. These values meet the validation criteria.

PRECISION:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

REPEATABILITY

Obtained Five (5) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD.

Table-: Results of Repeatability for Artesunate:

S. No.	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Artesunate	2.453	8566257	458652	6358	1.56
2	Artesunate	2.455	8547556	465325	6342	1.57
3	Artesunate	2.453	8569897	458264	6398	1.56
4	Artesunate	2.452	8564875	465823	6357	1.57
5	Artesunate	2.450	8569854	459858	6359	1.56
Mean			8563688			
Std. Dev			9284.684			

% RSD			0.108419			

Acceptance criteria:

- %RSD for sample should be NMT 2.
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Table-: Results of method precision for Mefloquine:

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Mefloquine	4.289	526583	36585	5486	1.43	5.65
2	Mefloquine	4.309	532422	37562	5482	1.44	5.65
3	Mefloquine	4.306	528687	36534	5496	1.43	5.64
4	Mefloquine	4.300	523658	36598	5548	1.43	5.66
5	Mefloquine	4.295	532654	36584	5489	1.44	5.64
Mean			528800.8				
Std. Dev			3851.63				
% RSD			0.728371				

Acceptance criteria:

- %RSD for sample should be NMT 2.
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Intermediate precision:

Day 1:

Table-: Results of Intermediate precision for Artesunate

S. No.	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Artesunate	2.465	8659856	468542	6485	1.57
2	Artesunate	2.472	8659854	469587	6492	1.58
3	Artesunate	2.467	8757458	465875	6475	1.58
4	Artesunate	2.466	8659852	478541	6483	1.59
5	Artesunate	2.472	8654754	475826	6528	1.57
6	Artesunate	3.424	8759898	465985	6592	1.59
Mean			8691945.333			

Std. Dev			51734.38066			
% RSD			0.595199103			

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2.

Table-: Results of Intermediate precision for Mefloquine

S. No.	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Mefloquine	4.323	536985	37256	5589	1.45	5.65
2	Mefloquine	4.343	548723	37458	5587	1.46	5.66
3	Mefloquine	4.324	536587	38562	5569	1.48	5.65
4	Mefloquine	4.323	542154	37452	5592	1.46	5.66
5	Mefloquine	4.342	536852	38659	5574	1.48	5.65
6	Mefloquine	4.323	548576	37489	5692	1.45	5.65
Mean			541646.2				
Std. Dev			5808.059				
% RSD			1.072298				

Acceptance criteria:

- %RSD of six different sample solutions should not more than 2.
- The %RSD obtained is within the limit, hence the method is rugged.

Day 2:

Table-: Results of Intermediate precision Day 2 for Artesunate

S. No.	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Artesunate	2.456	8457852	448568	6235	1.54
2	Artesunate	2.457	8496587	447524	6248	1.53
3	Artesunate	2.456	8457265	436898	6199	1.54
4	Artesunate	2.459	8398986	447584	6258	1.54
5	Artesunate	2.467	8457245	445823	6247	1.53

6	Artesunate	2.459	8459852	436985	6265	1.53
Mean			8454631			
Std. Dev			31330.74			
% RSD			0.370575			

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2.

Table-: Results of Intermediate precision for Mefloquine

S. No.	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Mefloquine	4.312	512867	35685	5386	1.42	5.62
2	Mefloquine	4.308	513645	35784	5397	1.41	5.61
3	Mefloquine	4.312	514285	34698	5299	1.42	5.61
4	Mefloquine	4.322	518569	35865	5368	1.42	5.62
5	Mefloquine	4.324	517548	34999	5347	1.41	5.61
6	Mefloquine	4.322	516985	34758	5326	1.42	5.62
Mean			515649.8				
Std. Dev			2346.637				
% RSD			0.455083				

Acceptance Criteria:

- %RSD of six different sample solutions should not more than 2.
- The %RSD obtained is within the limit, hence the method is rugged.

ACCURACY:

Accuracy at different concentrations (50%, 100%, and 150%) was prepared and the % recovery was calculated.

Table-: The accuracy results for Artesunate

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery

50%	496704.3	30	30.007	100.023%	99.95%
100%	914386	60	59.888	99.813%	
150%	1335442	90	90.011	100.012%	

Table-: The accuracy results for Mefloquine

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	312042	50	50.229	100.458%	100.10%
100%	619652	100	99.916	99.916%	
150%	929157	150	149.909	99.939%	

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

LIMIT OF DETECTION

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

$$\mathbf{LOD = 3.3 \times \sigma / s}$$

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Result:

Artesunate:

= 1.98µg/ml

Mefloquine:

= 2.36µg/ml

LIMIT OF QUANTITATION

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

LOQ=10×σ/S

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Result:

Artesunate:

= 5.94µg/ml

Mefloquine:

= 7.08µg/ml

ROBUSTNESS

Table-: Results for Robustness

Artesunate:

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	8567524	2.452	6358	1.56
Less Flow rate of 0.9 mL/min	9125478	2.741	6825	1.62
More Flow rate of 1.1 mL/min	8425685	2.270	6145	1.54
Less organic phase	8362514	3.266	6296	1.53
More organic phase	8287546	2.147	6486	1.53

Acceptance Criteria:

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

Mefloquine:

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	526586	4.300	5425	1.43
Less Flow rate of 0.9 mL/min	584265	4.830	5952	1.48
More Flow rate of 1.1 mL/min	516524	3.979	5326	1.42
Less organic phase	506985	3.266	5184	1.43
More organic phase	524576	2.147	5298	1.45

Acceptance Criteria:

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

SUMMARY

The analytical method was developed by studying different parameters.

First of all, maximum absorbance was found to be at 248nm and the peak purity was excellent.

Injection volume was selected to be 20µl which gave a good peak area.

The column used for study was Develosil C18 (4.6mm×150mm, 5µm) Column because it was giving good peak.

36ºC temperatures was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area and satisfactory retention time.

Mobile phase is Acetonitrile: Phosphate buffer pH-5.2 (25:75v/v) was fixed due to good symmetrical peak. So this mobile phase was used for the proposed study.

Run time was selected to be 7min because analyze gave peak around 2.452, 4.300 ±0.02min respectively and also to reduce the total run time.

The percent recovery was found to be 98.0-102 was linear and precise over the same range. Both system and method precision was found to be accurate and well within range.

The analytical method was found linearity over the range 40-80mg/ml of Artesunate and 60-140mg/ml of Mefloquine of the target concentration.

The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

CONCLUSION

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of Artesunate and Mefloquine in bulk drug and pharmaceutical dosage forms.

This method was simple, since diluted samples are directly used without any preliminary chemical derivatisation or purification steps.

Artesunate was found to be soluble in organic solvents such as ethanol, DMSO, and dimethyl formamide and also soluble in methanol, Acetonitrile, ethanol, isopropanol, and PG), soluble in sodium bicarbonate solution. Mefloquine was found to be very slightly soluble in water; freely soluble in methanol R; soluble in ethanol (~750 g/l) TS; sparingly soluble in dichloromethane, ethyl acetate.

Acetonitrile: Phosphate buffer pH-5.2 (25:75v/v) was chosen as the mobile phase. The solvent system used in this method was economical.

The %RSD values were within 2 and the method was found to be precise.

The results expressed in Tables for RP-HPLC method was promising. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods.

This method can be used for the routine determination of Artesunate and Mefloquine in bulk drug and in Pharmaceutical dosage forms.

ACKNOWLEDGEMENT

The Authors are thankful to the Management and Principal, Princeton College of Pharmacy, Narapally, Ghatkesar, Telangana, for extending support to carry out the research work. Finally, the authors express their gratitude to the Sura Pharma Labs, Dilsukhnagar, Hyderabad, for providing research equipment and facilities.

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