Asian Journal of Pharmaceutical Technology & Innovation
ISSN: 2347-8810

Research Article

RP-HPLC Method Development And Validation For Simultaneous Estimation of Artemether and Lumefantrine in Pharmaceutical Dosage Form

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Received on: 22-07-2016
Accepted on: 28-07-2016
Published on: 07-08-2016

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ABSTRACT

The importance of Antimalarial drugs are increasing day by day & used for method development by reverse phase HPLC. In literature survey few analytical methods have been reported for determination of drug out of which some are time consuming and costly. So this research work is dedicated to develop simple, efficient, Economical, fast, reliable and new method for estimation of Selected drug.

RP-HPLC analysis was performed on the Grace C-18 (250 mm × 4.6 mm), 5 µm particle size and using **Acetonitrile: Potassium Phosphate buffer (60:40*v/v*)** as mobile phase**;** flow rate was adjusted to **1 ml/min**. The detection was carried out at **215 nm**. The average retention times of Artemether and Lumefantrine was observed **8.11 min** and **10.25 min** at **215 nm** wavelength respectively. Linearity was observed in the concentration ranges of **2–10µg/ml** (**r^2 = 0.999**) for ART and **12 - 60 µg/ml** (**r^2 = 0.999**) for LUME. The method has been successively applied for the determination of ARTEMETHER and LUMAFANTRINE in tablet formulation. There was no interference from the excipients commonly present in the tablet. Accuracy of the method was studied by the recovery studies at three different levels 80 %, 100 % and 120 % level. The % recovery was found to be within the limits of the acceptance criteria with average recovery of **97-105%**. The method was found to be accurate, precise and specific for estimation of Artemether and Lumefantrine drugs in pharmaceutical dosage forms.

Key-words: RP-HPLC Method Development, Artemether and Lumefantrine, method validation etc.

Cite this article as:
Ganesh Mali,Vilas Arsul, Anup Puranpole, Yogesh Nalte,RP-HPLC Method Development And Validation For Simultaneous Estimation of Artemether and Lumefantrine in Pharmaceutical Dosage Form, Asian Journal of Pharmaceutical Technology & Innovation, 04 (19); 85-94, 2016. www.asianpharmtech.com

INTRODUCTION:-

For hydrocarbon type or non-polar stationary phase, we need to choose a polar mobile phase. This mode of partition chromatography is called as Reverse phase chromatography. Here the most polar component elutes first. Increasing mobile phase polarity leads to decrease in elution time. Common solvents used in this mode include Methanol/Acetonitrile/Isopropanol etc. Control of pH is another way to control resolution. In the case of an aromatic acid, a low pH buffer will suppress the ionization, and will increase the capacity factor, while with a high pH buffer, the capacity factor will decrease. Small amounts of modifiers like Sodium phosphate/ Sodium acetate reduce peak tailing, and increase the separation efficiencies. Mostly used for separation of ionic and polar substances.

Both Artemether and Lumefantrine act as blood schizontocides. Artemether is concentrated in the food vacuole. It then splits its endoperoxide bridge as it interacts with haem, blocking conversion to haemozoin, destroying existing haemozoin and releasing haem and a cluster of free radicals into the parasite.

Lumefantrine is thought to interfere with the haem polymerisation process,a critical detoxifying pathway for the malaria parasite. Both Artemether and Lumefantrine have a secondary action involving inhibition of nucleic acid and protein synthesis within the malarial parasite. An 8 - amino-quinoline derivative such as primaquine should be given sequentially after the combination in cases of mixed infections of P. falciparum and P. vivax to achieve hypnozoites eradication. The combination isalso associated with rapid gametocyte clearance.

The importances of Antimalarial drugs are increasing day by day & used for method development by reverse phase HPLC. In literature survey few analytical methods have been reported for determination of drug out of which some are time consuming and costly. So this research work is dedicated to develop simple, efficient, Economical, fast, reliable and new method for estimation of Selected drug.

The aim of present work was to develop and validate RP-HPLC method for simulataneous estimation of ART and LUMA in pharmaceutical dosage form.

Material and Equipment:-

Table 1 : List of Chemicals

Sr.No.	Drug	Company
1	Methanol	AR grade Rankem
2	Acetonitrile	AR grade Rankem
3	potassium phosphate	AR grade Spectrochem
4	Distilled Water	AR grade
5	Orthophosphoric Acid	AR grade Rankem
6	Artemether	Analytical grade
7	Lumefantrine	Analytical grade

Table 2 : List of Instruments and Equipment's

Sr.No.	Name of Instrument	Make
1	HPLC	Younglings Autocgro 3000
2	UV Spectrophotometer	Jasco 530
3	pH Meter	Electrolab
4	Melting Point	Labtronics

METHODOLOGY:

The Reverse phase HPLC was selected for separation because nature of drug it is convenient and rugged than other forms of the liquid chromatography and is more likely to result in a satisfactory final separation.

Selection of HPLC column: The Colum selection is important step in method development by HPLC system. Generally, C-18 Colum is selected for reverse phase high performance liquid chromatography.

Selection of Mobile phase: The standards and sample solutions of Artemether and Lumefantrine were prepared in mobile phase. Different pure solvents of varying polarity in different proportions were tried as mobile phase for development of the chromatogram.

Optimization of Mobile Phase Strength: The mobile phase was chosen after several trials with methanol and water with phosphate buffer in various proportions. A mobile phase consisted of Acetonitrile and phosphate buffer (0.1 % OPA for pH 2.5) (60:40 *v/v*) was selected to achieve symmetrical peak. The effects of flow rates in the ranges of 0.5 to 1.1 ml/min were examined. A flow rate of 1 ml/min gave good results, system suitability parameter and reasonable retention time.

Selection of detector and detection wavelength: UV- detector was selected, as it is reliable and easy to set at the correct wavelength. By appropriate dilution of each standard stock solution with mobile phase, varying concentrations of ART and LUMA were prepared separately. The solutions were scanned using double beam UV visible spectrophotometer in the spectrum mode between the range of 400 nm to 200 nm and their overlain spectra were obtained.

Preparation of Standard Stock Solution: Standard stock solution was prepared by dissolving 10 mg of Artemether and 60 mg of Lumefantrine in 25ml of Methanol that gives concentration of 400µg/ml and 2400 µg/ml for Artemether and Lumefantrine respectively.

Linearity studies: From stock standard solution, 0.25ml were taken in 10 ml volumetric flasks and diluted up to the mark with Mobile phase such that to obtained concentration of Artemether and Lumefantrine in the range 2-10µg/ml and 12-60 µg/ml respectively. Volume of 20 µL of each sample was injected with the help of Hamilton syringe. All measurements were repeated two times for each concentration and calibration curve was constructed by plotting the peak area *versus* the drug concentration.

Analysis of Physical Laboratory Mixture: In order to see the feasibility of the method in the marketed formulation, it was first tried in physical laboratory mixture. Accurately weighted quantity of 10 mg (ART) and 60 mg (LUME) were transferred to 50 ml volumetric flask containing Methanol and volume was adjusted up to the mark. It was further diluted to get concentration of 10 µg/ml of ART and 60µg/ml of LUME. Constant volume 20 µl was injected into column and peak was recorded. The concentration of both the drugs was determined from curve linearity equation. The concentration of both the drugs was determined from curve linearity equation,

$$y = mX + C$$

Where,Y= y axis data X= x axis data m= Slope of graph and c= Intercept

Analysis of tablets: To determine the contents of drugs in tablets (Label claim 4 mg of ART and 24 mg of LUME per tablet); the twenty tablets (LUMEFANTRINE – LUMERAX-20DT (IPCA LAB)) were weighted, average weight determined and then finally powdered. Powder of Lumefantrine 543 mg was transferred to 25 ml of Methanol. The resulting solution was sonicated for 15 min and diluted with Methanol. The solution was filtered, using 0.45 µm of filter (Milifilter, Milford, MA). The solution was further diluted to get concentration of 10 µg/ml of ART (60 µg/ml of LUME). The aliquots were subjected to proposed method and amount of ART and LUME was determined.

Brand Name- LUMEFANTRINE – LUMERAX-20DT (IPCA LAB)

Validation of proposed method: The proposed method was validated as per ICH guidelines. The drug solutions were prepared as per the earlier adopted procedure given in the experiment.

Accuracy: The accuracy of the method was studied by recovery study using standard addition method at 80%, 100% and 120% level. To the sample solution (4µg/ml of ART; 24µg/ml of LUME) a known quantity of standard ART (2 µg/ml, 4µg/ml and 6µg/ml) and LUME (12µg/ml, 24µg/ml and 36µg/ml) were added and analyzed by the proposed RP-HPLC method.

Precision: Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions. It was verified by repeatability and intermediate precision studies. Intra-day precision was studied by analysing 4, 6 and 8 µg/ml of ART and 24, 36 and 48 µg/ml of LUME for three times on the same day. Inter day precision was checked analysing the same concentration for three

different days over a period of weak.[51]

Repeatability: It is measured by multiple injections of a homogenous sample of 10 µg/ml of ART and 60 µg/ml of LUME that indicates the performance of the HPLC instrument under chromatographic conditions.

Robustness: Robustness of the method was studied by making deliberate changes in few parameters viz; flow rate, wavelength and mobile phase combination. The effects on the results were studied by injecting 8µg/ml of ART and 48 µg/ml of LUME; one factor was changed at the original flow was 1 ml/min and the robustness study were carried out by taking at 0.9 ml/min and 1.1 ml/min respectively. The original wavelength was taken as 215 nm and robustness study were carried out by taking at 214 nm and 216 nm.

Ruggedness: In addition to intra and inter day precision reproducibility study was also carried out and it was checked by determining precision on the same instrument, but by a different analyst. From stock solution, sample solution of 20 µg/ml of ART and 50 µg/ml of LUME were prepared and analysed by two different analysts using similar operational and environmental conditions.

Sensitivity: The Quantitation limit is a parameter of quantitative assay for low levels of compounds in sample matrices, and is used particularly for determination of impurities and/or degradation products. The limit of detection (LOD) and the limit of Quantitation (LOQ) were determined using following formulae,

$$LOD = 3.3 \times I / S \qquad LOQ = 10 \times I/S$$

Where I = Intercept of the graph, (S) = the slope of calibration curve.

Specificity and selectivity: The analytes should have no interference from other extraneous compounds and be well resolved from them. Specificity is procedure to detect quantitatively the analyte in presence of component that may be expected as being present in the sample matrix, while selectivity is the procedure to detect qualitatively the analyte in presence of component that may be expected as being to be present in the sample matrix. The method is quite selective. There was no other interfering peak around the retention time of both the drugs; also the base line did not show any significant noise. The specificity of the HPLC method was determined by complete separation of ART and LUME along with other parameter like retention time (t_R), tailing factor etc.

System suitability test: System suitability testing is essential for the assurance of the quality performance of the chromatographic system. Earlier prepared solutions for chromatographic conditions were tested for system suitability testing. System Suitability Requirements are the Relative Standard Deviation for the peak area response of standard preparation is not more than 2.0 %.

RESULTS AND DISCUSSION:-

Table 3 : Chromatographic conditions

HPLC	Youngling (S.K) Gradient System UV Detector
Software	Autochrome -3000
Column	4.6 x 250 mm
Particle size packing	5 µm
Stationary phase	C-18 (Grace)
Mobile Phase	Acetonitrile : Phosphate buffer (60 : 40 V/V)
Detection Wavelength	215 nm (Isobastic Point)
Flow rate	1 ml/min
Temperature	Ambient
Sample size	20 µl

Selection of detector and detection wavelength: By appropriate dilution of each standard stock solution with mobile phase, varying concentrations of ART and LUMA were prepared separately. The solutions were scanned using double beam UV visible spectrophotometer in the spectrum mode between the range of 400 nm

to 200 nm and their overlain spectra were obtained. The isobastic point was found to be at 215nm.

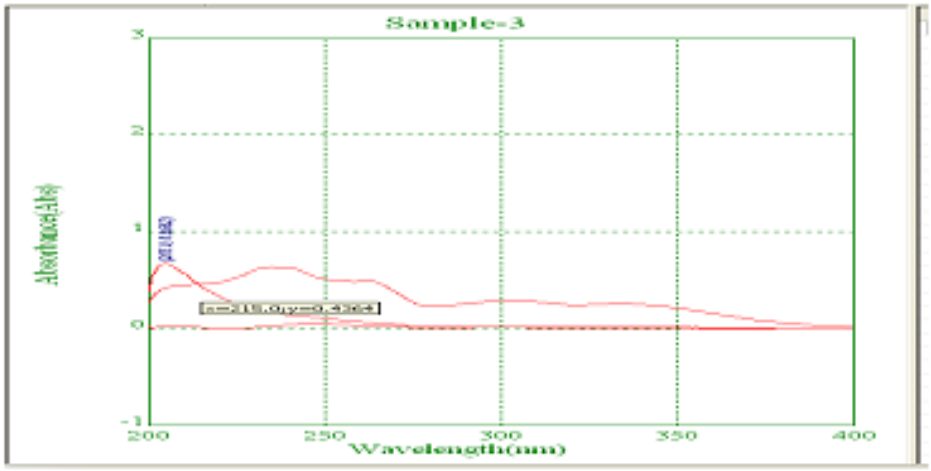

Figure 1: An overlay spectra of ART and LUME

Linearity study:

The linearity of an analytical procedure is its ability to elicit test results that are proportional to the concentration of analyte in the sample. From stock standard solution, aliquots of 2,4,6,8,and 10 ml were taken in 10 ml volumetric flasks and diluted up to the mark with Acetonitrile: Potassium Phosphate Buffer such that to obtained concentration of ART and LUME in the range 2-10 µg/ml and 12-60 µg/ml respectively. The data obtained for linearity study for ART and LUME are shown in Table 4 and Table 5. The calibration curve are plotted by using concentration verses area for ART and LUME are shown in Figure 2.

Table 4: Data showing Linearity study of ARTEMETHER

Sr.No.	Concentration of Art [µg/ml]	Mean peak area	%RSD (n=2)
1	02	436.28	1.85
2	04	854.69	0.22
3	06	1276.94	1.33
4	08	1686.11	0.33
5	10	2091.89	0.29

Table 5: Data showing Linearity study of LUMEFANTRINE

Sr.No.	Concentration of Lume [µg/ml]	Mean peak area	%RSD (n=2)
1	12	681.65	0.48
2	24	1383.07	0.73
3	36	1956.72	0.44
4	48	2569.36	0.09
5	60	3214.28	0.39

From the calibration curves the coefficient of correlation of ART and LUME were found tobe 0.999 and 0.999 respectively.

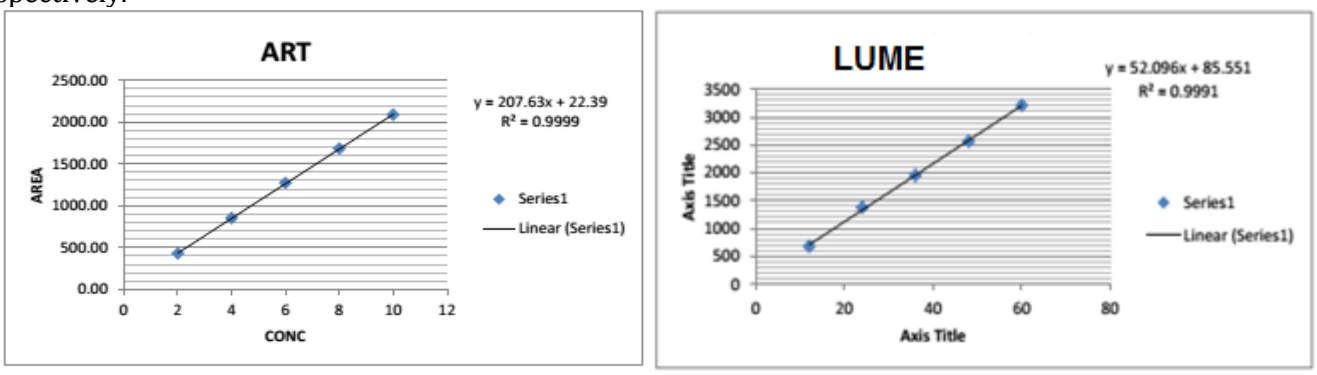

Figure 2: Calibration curve for ARTEMETHER & LUMEFANTRINE

Linearity and range of method was ranging from 2 µg/ml-10 µg/ml for ART and 12µg/ml-60µg/ml for LUME.

Graphs were plotted with concentrations on X axis and mean peak areas on Y-axis. The R^2 value is found to be 0.999 and 0.999 for ART and LUMA, respectively.

The linearity study were performed by taking 2-10 µg/ml for ART and 12-60µg/ml for LUMA and the respective chromatograms are shown in Figure 3.

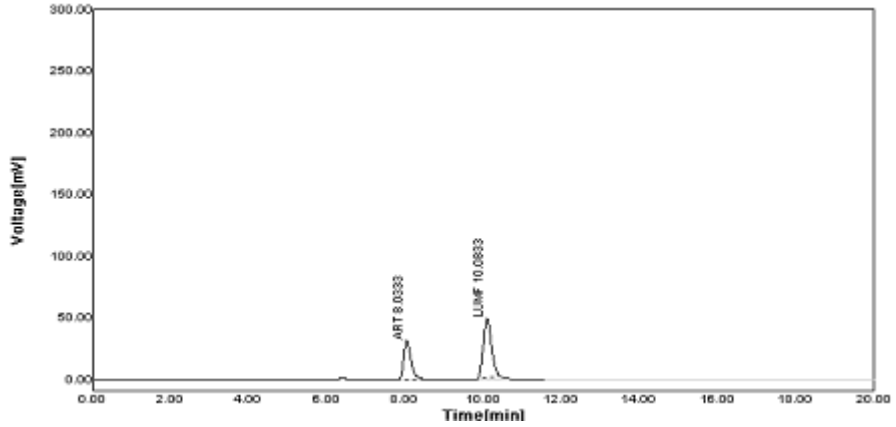

Figure 3: Chromatogram for linearity

No.	RT[min]	Area[mV*s]	Area%	TP	TF	Resolution
1	8.0333	436.9230	37.46	10918.3	1.3570	0.0000
2	10.0833	679.3584	62.54	13765.7	1.2064	6.2952
Sum		1086.2815				

Analysis of Physical Laboratory Mixture: The analysis of physical laboratory mixture was carried out. The chromatograms obtained areshown in Figure 4 and the results are shown in Table 6.

Table 6: Analysis of Physical Laboratory Mixture

Sr. no.	Component	Amounttaken (µg)	Amount found	% Amount found	% RSD
1	ART	4.00	4.06±0.12	101.51	0.45
2	LUME	24.00	24.83±0.36	103.46	0.18

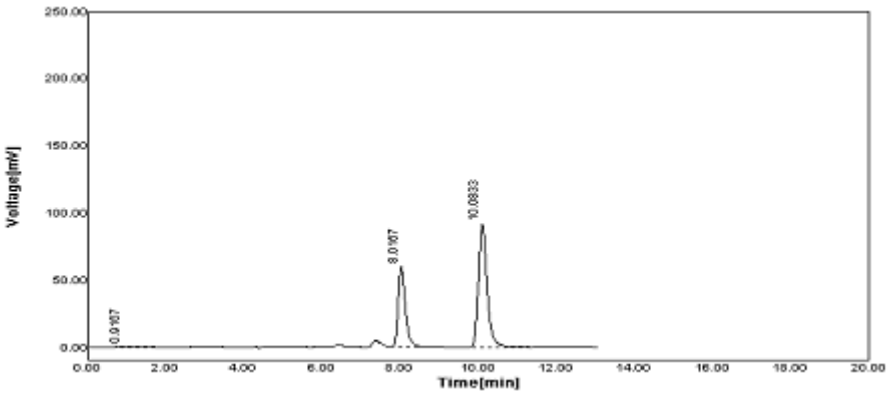

Figure 4: Chromatogram for standard Laboratory mixture of ART and LUME Acetonitrile: Potassium Phosphate buffer (60:40 v/v)

No.		RT[min]	Area[mV*s]	Area%	TP	TF	Resolution
1		0.9167	17.9176	0.83	5.2	1.9810	0.0000
2		8.0167	865.4078	35.39	9385.8	1.3747	7.3210
3		10.0833	1379.1598	63.78	15473.1	1.4085	6.3081
Sum			2262.4851				

Analysis of tablets: The tablet was analysed to determine the concentration of 10 µg/ml of ART and 60 µg/ml of LUMA. The chromatogram for tablets assay is shown in Figure 5 and result are mentioned in Table 7.

Brand Name- LUMEFANTRINE – LUMERAX-20DT (IPCA LAB)

Table 7: Analysis of tablets formulations

Sr. No.	Component	Label claim (mg)	Amount found (mg) ±SD	% Label claim	% RSD
1	Artemether	4	4.06	101.51	0.45
2	Lumefantrine	24	24.83	103.46	0.18

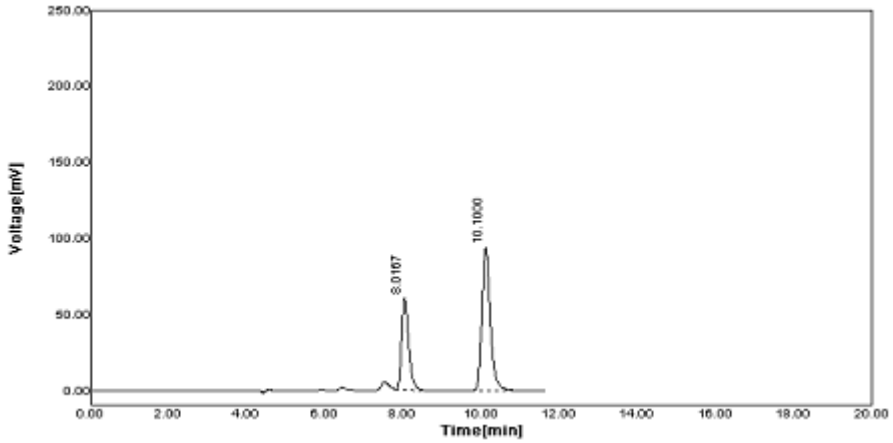

Figure 5: Chromatogram for tablets Assay

No.	Name	RT[min]	Area[mV*s]	Area%	TP	TF	Resolution
1	ART	8.0167	859.8685	35.47	11513.6	1.4680	0.0000
2	LUME	10.1000	1382.5997	64.53	13591.1	1.3275	6.4561
Sum			2242.4683				

The results shows that the % Label Claim for ART 101.51% and for LUMA 103.46 %. The %RSD were found to be within the limits (NMT 2%).

Validation of proposed method:

The proposed method was validated as per ICH guidelines. The results were found as follows,

Accuracy: Accuracy of an analytical method is the closeness of the test results obtained by that of the true value. The results for recovery study are shown in Table 8.The chromatograms obtained at standard 80 % addition, 100 % addition, 120 % addition.

Table 8 : Data Showing Results of Recovery Studies

Drugs	Initial amount(µg/ml)	Excess drug added (%)	Amount recovered± SD (µg/ml)	Recovery (%)
ART	4	80	3.20±0.12	100.27
	4	100	4.00±0.20	100.00
	4	120	8.87±0.23	101.49
LUME	24	80	19.21±0.30	100.07
	24	100	23.64±0.31	98.51
	24	120	28.60±0.35	99.32

Precision: Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions. The results for Precision are shown in table 9.

Table 9: Data Showing Results of Precision Studies (Intermediate Precision)

		Intra-day		Inter day	
Drugs	**Conc. (µg/ml)**	**Amount found (%) (n=3)**		**Amount found (%) (n=3)**	
		Mean	**% RSD**	**Mean**	**% RSD**
	4	101.17	0.47	101.43	0.56
ART	6	99.85	0.19	99.85	0.26
	8	99.88	0.90	99.75	0.72
	24	103.61	0.18	102.13	1.06
LUME	36	100.45	0.21	99.92	0.22
	48	98.40	0.08	99.15	0.47

The results shows that the the proposed method is precise for the given concentration range as % RSD was less than 2%. Hence, this method might be used precisely for the analysis of ART and LUMA.

Repeatability: Results for repeatability are shown in Table 10.

Table 10: Data Showing Results of Repeatability Studies

Drugs	**Concentration[µg/ml]**	**Area Mean ± SD (n=6)**	**% RSD(n=6)**
ART	10	2070.43±4.80	0.64
LUME	60	3242.02±51.02	0.09

The results for repeatability shows that the method gives repeated results for given concentration range as %RSD was found to be less than 2%.

LOD and LOQ: The LOD and LOQ were found to be **0.16µg** and **0.50µg** for ART and **0.42µg** and **1.29µg** for LUME, respectively.

Robustness: The original mobile phase combination used was ACN: PHOSPHATE BUFFER (60: 40 % v/v) and robustness study were carried out by taking ACN: phosphate buffer (59: 41 % v/v) and ACN: phosphate buffer (61: 39% v/v) .

Table 11: Data for robustness studies

Parameter	% RSD	
	ART	LUME
Flow rate (±0.1 ml) (Original- 1.0 ml)	1.37	1.32
Wavelength (±1 nm) (Original- 215 nm)	0.80	1.74
Mobile phase combination(±1) (Original-ACN: Phosphate buffer (60:40 v/v)	1.57	1.26

Ruggedness: The Peak area measured for same concentration solutions for six times; the results are shown in table12.

Table 12 : Data for ruggedness studies

Drugs	**Conc. Taken**	**% Amount found (n=6)**		**% RSD (n=6)**	
	(µg)	**Analyst I**	**Analyst II**	**Analyst I**	**Analyst II**
ART	4	99.83	100.29	1.65	1.81
LUME	24	98.46	98.10	1.37	1.58

The results for ruggedness shows that method is found to be rugged as the %RSD was found to be less than 2%,

System suitability test: System suitability testing is essential for the assurance of the quality performance of the chromatographic system. Earlier prepared solutions for chromatographic conditions were tested for system suitability testing; results are shown in Table 13.

Table 13: Data Showing System Suitability Test

System suitability parameters	Proposed method	
	ARTEMETHER	LUMEFANRINE
Retention time (tR)	7.41	9.36
Theoretical plate (N)	6316.5	7548.9
Tailing factor (T)	1.3672	1.3683
Resolution	0.0000	4.8477

The data for system suitability test confirmed that the method is suitable for that analysis of ART and LUMA.

CONCLUSION:-

RP-HPLC analysis was performed on the Grace C-18 (250 mm × 4.6 mm), 5 µm particle size and using **Acetonitrile: Potassium Phosphate buffer (60:40*v/v*)** as mobile phase**;** flow rate was adjusted to **1 ml/min**. The detection was carried out at **215 nm**. The average retention times of Artemether and Lumefantrine was observed **8.11 min** and **10.25 min** at **215 nm** wavelength respectively. Linearity was observed in the concentration ranges of **2–10µg/ml** (**r^2 = 0.999**) for ART and **12 - 60 µg/ml** (**r^2 = 0.999**) for LUME. The method has been successively applied for the determination of ARTEMETHER and LUMAFANTRINE in tablet formulation. There was no interference from the excipients commonly present in the tablet. Accuracy of the method was studied by the recovery studies at three different levels 80 %, 100 % and 120 % level. The % recovery was found to be within the limits of the acceptance criteria with average recovery of **97-105%**. The% RSD below 2 showed the high precision of proposed method. According to USP**,** system suitability tests are an integral part of chromatographic methods. They are used to verify the reproducibility of the chromatographic system. To ascertain its effectiveness, system suitability tests were carried out on freshly prepared stock solution. The retention time, theoretical plate and tailing factor for ART were found to **7.41** min, **6316.5** and **1.3672**, respectively and retention time, theoretical plate and tailing factor for LUME were found to be **9.3167** min, **7548.9** and **4.8477**, respectively. The method was found to be accurate, precise and specific for estimation of Artemether and Lumefantrine drugs in pharmaceutical dosage forms.

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