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A New Stability Indicating RP-HPLC Method for Simultaneous Determination of ARTRMRTHER and Lumifantrine in its Bulk and Pharmaceutical Dosage Forms

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ABSTRACT

The chromatographic conditions were successfully developed for the separation of by artemether and lumifantrine using symmetry C18 column (4.6×150mm) 5µ, A wavelength of 290 nm was selected and the mobile phase consists of triehtylamine buffer (PH 6 adjusted with ortho phosphoric acid) and methanol in 20:80 % v/v ratios at a flow rate of 0.8 ml/min were found to be optimum conditions for analysis. The peaks were well resolved with symmetry C18 column. System suitability studies were also carried out which includes theoretical plates, resolution and tailing factors etc. The accuracy studies were shown as % recovery for Artemether and Lumefantrine at 50 %, 100 % and 150 %. The limits of % recovered should be in the range of 98-102 %. The results obtained for Artemether and Lumefantrine were found to be within the limits. Hence the method was found to be accurate. The accuracy studies showing % recovery of Artemether were found to be 99.9 %, 99 % and 99.9. % respectively and the % recovery of Lumefantrine were found to be 99.9%, 99.9 % and 98.9 % respectively. In the System precision study, the % RSD was found to be less than 2 %. For Artemether 1.7 and Lumefantrine 1.2 which indicates that the system has good reproducibility. For ID precision studies six replicate injections of Artemether and Lumefantrine were performed. The % RSD was determined for peak areas of Artemether and Lumefantrine. The acceptance limit should not be more than 2 % and the results were found to be within the acceptance limits. Using the optimized chromatographic conditions, chromatograms of mixed standard solutions which contained Artemether and Lumefantrine were recorded. Retention times of Artemether and Lumefantrine were found to be 2.9 min and 4.6 min respectively. Calibration curves were obtained by using peak area vs. concentration. Artemether and Lumefantrine show linearity in the range of 0.1 – 0.5 µg/ml and 0.6 – 3.0 µ/ml. Calibration curve was plotted and correlation co-efficient for both the drugs Artemether and Lumefantrine were found to be 0.999 and 0.999 respectively

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INTRODUCTION

Pharmaceutical Analysis[21] is the branch of pharmacy that is responsible for developing sensitive, reliable and more accurate methods for the estimation of drugs in pharmaceutical dosage forms and in biological system. Pharmaceutical analysis may be defined as the application of analytical procedures used to determine the purity, safety and quality of drugs and chemicals. More recently it also deals with biological samples in support of biopharmaceutical and pharmacokinetic studies. Pharmaceutical analysis includes both qualitative and quantitative analysis. Quality assurance plays an important role in determining the safety and efficacy of medicines.

SCOPE AND SIGNIFICANCE OF PHARMACEUTICAL ANALYSIS

Pharmaceutical companies rely upon both qualitative and quantitative chemical analysis to ensure that the raw material used meet all the desired specifications, and also to check the quality of the final product. The examination of raw material is carried out to ensure that there is no unusual substance present which might deteriorate the manufacturing process or appear as a harmful impurity in the final product. The quantity of required ingredient in raw material is determined by a procedure known as Assay. The final manufactured product is subjected to quality control to ensure that desired components are present within a range and impurities do not exceed certain specified limits.

Analytical chemistry is a sub-discipline of chemistry that has the broad mission of understanding the chemical composition of all matter and developing the tools and experiments to make either qualitative or quantitative measurements. In a nutshell, analytical chemistry applies measurement of scientific principles along with an understanding of chemical systems to provide useful information; and it has significant overlap with other branches of chemistry through the measurement method that it provides.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY[9]

High Performance Liquid Chromatography (HPLC) is the fastest growing analytical technique for the analysis of drugs. Chromatographic separation in HPLC is the result of specific interaction between sample molecules with both the stationary and liquid mobile phases. HPLC is also being automated which involve automated sampling, separation, detection, recording, calculation and printing of results. Due to the high selectivity and sensitivity achieved by HPLC methods, it became the most selective method for the analysis in wide range of drugs. HPLC is one of the most versatile instruments used in the field of pharmaceutical analysis today with following advantages.

Normal Phase Chromatography[5]***:***

In normal phase chromatography, the stationary phase is a polar adsorbent and the mobile phase is generally a mixture of non-aqueous solvents.

The silica structure is saturated with silanol groups at the end. These OH groups are (very polar) in the stationary phase. This forms a weak type of bond with any molecule in the vicinity when any of the following interactions are present.

Reverse Phase Chromatography:

In 1960's chromatographers had started modifying the polar nature of silanol group by chemically reacting silica with organic silanes. The objective was to make less polar or non polar so that polar solvents can be used to separate water-soluble polar compounds. Since the ionic nature of the chemically modified silica is now reversed. Simple compounds are better retained by the reversed phase surface, the less water- soluble (i.e. the more non-polar) they are. As a general rule the retention increases with increasing contact area between sample molecule and stationary phase i.e. with increasing number of water molecules, which are released during the adsorption of a compound. Branched chain compounds are eluted more rapidly than their corresponding normal isomers.

In reversed phase systems the strong attractive forces between water molecules arising from the 3-dimensional inter molecular hydrogen bonded network, from a structure of water that must be distorted or disrupted when a solute is dissolved. Only higher polar or ionic solutes can interact with the water structure. Non-polar solutes are squeezed out of the mobile phase and are relatively insoluble in it but with the hydrocarbon moieties of the stationary phase.

Chemically bonded octadecyl silane (ODS) an alkaline with 18 carbon atoms it is the most popular stationary phase used in pharmaceutical industry. Since most pharmaceutical compounds are polar and water soluble, the majority of HPLC methods used for quality assurance, decomposition studies, quantitative analysis of both bulk drugs and their formulations use ODS HPLC columns. The solvent strength in reversed phase chromatography is reversed from that of adsorption chromatography (silica gel) as stated earlier. Water interacts strongly with silanol groups, so that, adsorption of sample molecules become highly restricted and they are rapidly eluted as a result. Exactly opposite applies in reversed phase system; water cannot wet the non-polar (hydrophobic) alkyl groups such as C_{18} of ODS phase and therefore does not interact with the bonded moiety. Hence water is the weakest solvent of all and gives slowest elution rate. The elution time (retention time) in reversed phase chromatography increases with increasing amount of water in the mobile phase.

HPLC INSTRUMENTATION

1. Solvent reservoir
2. Pump
3. Injector
4. Column
5. Detector
6. Data Acquisition and Control System

A. ***Solvent reservoir:***

It is used for storage of sufficient amount of HPLC solvents for continuous operation of the system. It could be equipped with an online degassing system and special filters to isolate the solvent from the influence of the environment.

B. ***Pump:***

The mobile-phase solvents are pulled from their reservoirs by the action of a pump. Most HPLC instruments use a reciprocating pump consisting of a piston whose back and forth movement is capable both of maintaining a constant flow rate up to several milliliters per minute and of obtaining the high output pressure needed to push the mobile phase through the chromatographic column.

a. ***Isocratic flow*** A separation in which the mobile phase composition remains constant throughout the procedure is termed isocratic flow. In isocratic elution, peak width increases with retention time linearly according to the equation for N, the number of theoretical plates.

b. ***Gradient elution*** A separation in which the mobile phase composition is changed during the separation process is called as a gradient elution. Gradient elution decreases the retention of the later-eluting components so that they elute faster, giving narrower (and taller) peaks for most components.

C. ***Injector :***

This allows an introduction (injection) of the analytes mixture into the stream of the mobile phase before it enters the column; most modern injectors are auto samplers, which allow programmed injections of different volumes of samples that are withdrawn from the vials in the auto sampler tray.

Column:

This is the heart of HPLC system; it actually produces a separation of the analytes in the

mixture. A column is the place where the mobile phase is in contact with the stationary phase, forming an interface with enormous surface. Most of the chromatography development in recent years went toward the design of many different ways to enhance this interfacial contact

D. ***Detector :***

This is a device for continuous registration of specific physical (sometimes chemical) properties of the column effluent. The most common detector used in pharmaceutical analysis is UV (ultraviolet),which allows monitoring and continuous registration of the UV absorbance at a selected wavelength or over a span of wavelengths(diode array detection). Appearance of the analyte in the detector flow-cell causes the change of the absorbance. If the analyte absorbs greater than the background (mobile phase), a positive signal is obtained.

Electrochemical Detectors

- These are another common group of HPLC detectors are those based on electrochemical measurements such as amperometry, voltametry, colometry, and conductivity

E. ***Data Acquisition and Control System:***

Computer-based system that controls all parameters of HPLC instrument (eluent composition (mixing of different solvents); temperature, injection sequence, etc.) and acquires data from the detector and monitors system performance.

MATERIALS AND METHODS

REAGENTS AMD CHEMICALS:

Table showing list of the chemicals used

S. No	Name	Grade	Manufacturer/ Supplier
1.	Artemether and Lumefantrine working standards	-	-
2.	Potassium di hydrogen ortho phosphate	HPLC	Merck
3.	Methanol	HPLC	Merck
4.	Triethylamine	HPLC	Merck
5.	Water	HPLC	Rankem
6.	Ortho phosphoric acid	Ar	Merck
7.	Sodium hydroxide		S D fine-chem limited
8.	Hydrocholoric acid	Ar	Finar chemical limited
9.	Hydrogen Peroxide	Lr	Alpha pharma limited

INSTRUMENTS:

Table showing list of the Instruments used

S. no	*Instruments*	*Make/Model*
1.	Analytical Balance	Afcos, ER-180A, Sartorius- M500P, Meter, AG 104
2.	Microbalance	Sartorius-M500P

3.	pH Meter	Thermo scientific
4.	HPLC	WATERS 2695 Separations module
5.	Detector	Photo diode Array Detector 996
6.	Software	Empower software

TRIAL 1

Preparation of standard solution:

Accurately weigh and transfer 10 mg equivalent weight of Artemether and 60 mg of Lumefantrine tablets powder into a 100 ml clean dry volumetric flask add about 70 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Pipette out 1.0 ml of the solution into a 10 ml volumetric flask and dilute up to the mark with diluents.

CHROMATOGRAPHIC CONDITIONS

Flow rate : **0.8** ml/min

Column : symmetry c_{18} (4.6 x 150 mm, 5 µm)

Detector wave length : 265 nm

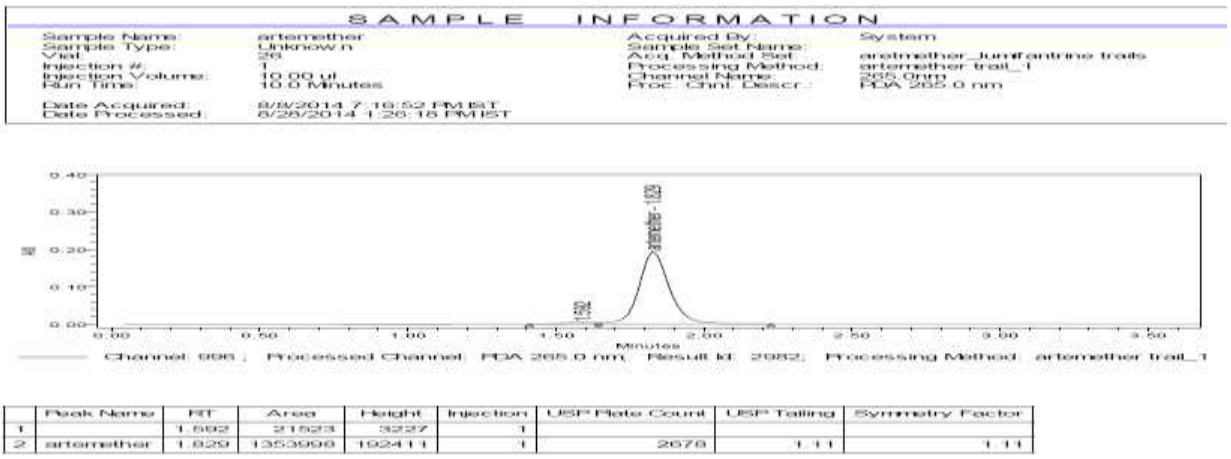
Column temperature : Ambient

Injection volume : 10 µl

Run time : 10 min

Mobile phase : TEA buffer : Methanol (30: 70)

Rt : 1.8



	Peak Name	RT	Area	Height	Injection	USP Plate Count	USP Tailing	Symmetry Factor
1		1.592	21523	3237	1			
2	artemether	1.829	1353998	192411	1	2678	1.11	1.11

Fig no:1 chromatogram trail-1

Observation: Individual component was eluted but it has low plate count and poor tailing

TRAIL-2

CHROMATOGRAPHIC CONDITIONS

Flow rate : **1** ml/min

Column : Symmetry C_{18} (4.6 x 150 mm, 5 µm)

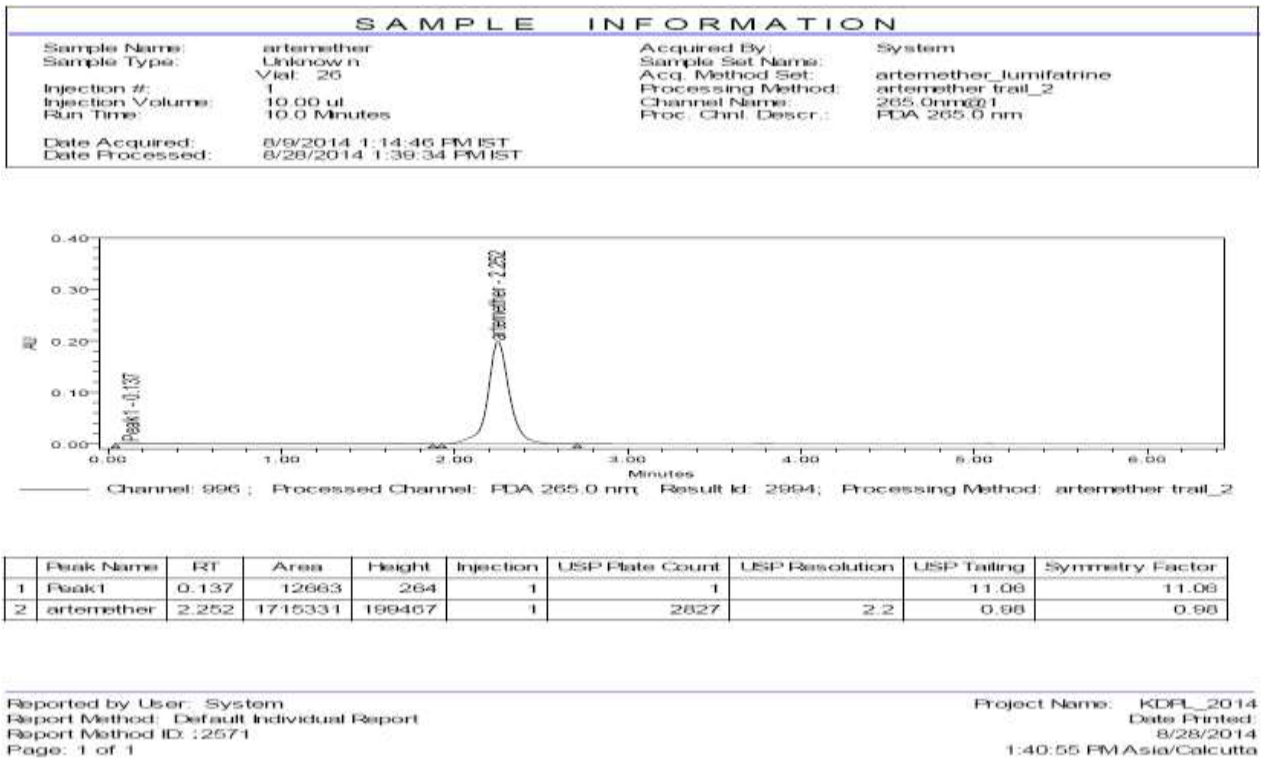
Detector wave length : 265 nm

Column temperature : Ambient

Injection volume : 10µl

Run time : 10 min

Mobile phase : TEA buffer: Methanol (30: 70)



	Peak Name	RT	Area	Height	Injection	USP Plate Count	USP Resolution	USP Tailing	Symmetry Factor
1	Peak1	0.137	12883	264	1	1		11.08	11.08
2	artemether	2.252	1715331	199467	1	2827	2.2	0.98	0.98

Fig no: 2 chromatogram trail- 2

Observation: individual component was separate

TRAIL-3

CHROMATOGRAPHIC CONDITIONS

Flow rate : **0.8** ml/min

Column : Symmetry C_{18} (4.6 x 150 mm, 5 µm,)

Detector wave length : 295nm

Column temperature : Ambient

Injection volume : 10 µl

Run time : 10 min

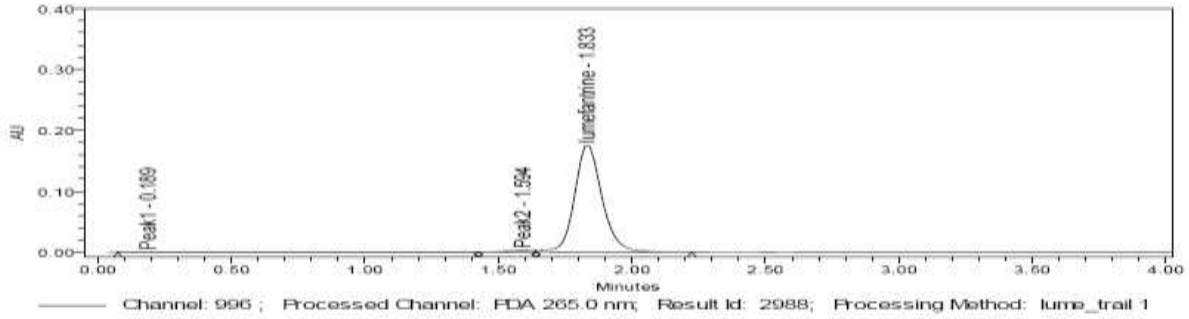
Mobile phase : TEA buffer : methanol (20: 80)

Empower 2 software

Default Individual Report

SAMPLE INFORMATION

Sample Name:	lumefantrine	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	
Vial:	25	Acq. Method Set:	aretmether_lumifantrine trails
Injection #:	1	Processing Method:	lume_trail 1
Injection Volume:	10.00 ul	Channel Name:	265.0nm
Run Time:	10.0 Minutes	Proc. Chnl. Descr.:	PDA 265.0 nm
Date Acquired:	8/8/2014 7:21:50 PM IST		
Date Processed:	8/28/2014 1:31:46 PM IST		



	Peak Name	RT	Area	Height	Injection	USP Plate Count	USP Tailing	Symmetry Factor
1	Peak1	0.189	13406	234	1	0		
2	Peak2	1.594	20242	2944	1			
3	lumefantrine	1.833	1251935	176922	1	2607	1.15	1.15

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Report Method ID: ;2571
Page: 1 of 1

Project Name: KDPL_2014
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Fig 3: Chromatogram of Trial-3

Observation; individual component was eluted but it has low plate count and tailing

TRIAL-4

CHROMATOGRAPHIC CONDITIONS

Flow rate : **1** ml/min

Column : symmetry C_{18} (150 × 4.6 × 5 µm)

Detector wave length : 295 nm

Column temperature : Ambient

Injection volume : 10µl

Run time : 10 min

Mobile phase : TEA buffer : methanol (40: 60)

PH : 6

Rt : 3.568

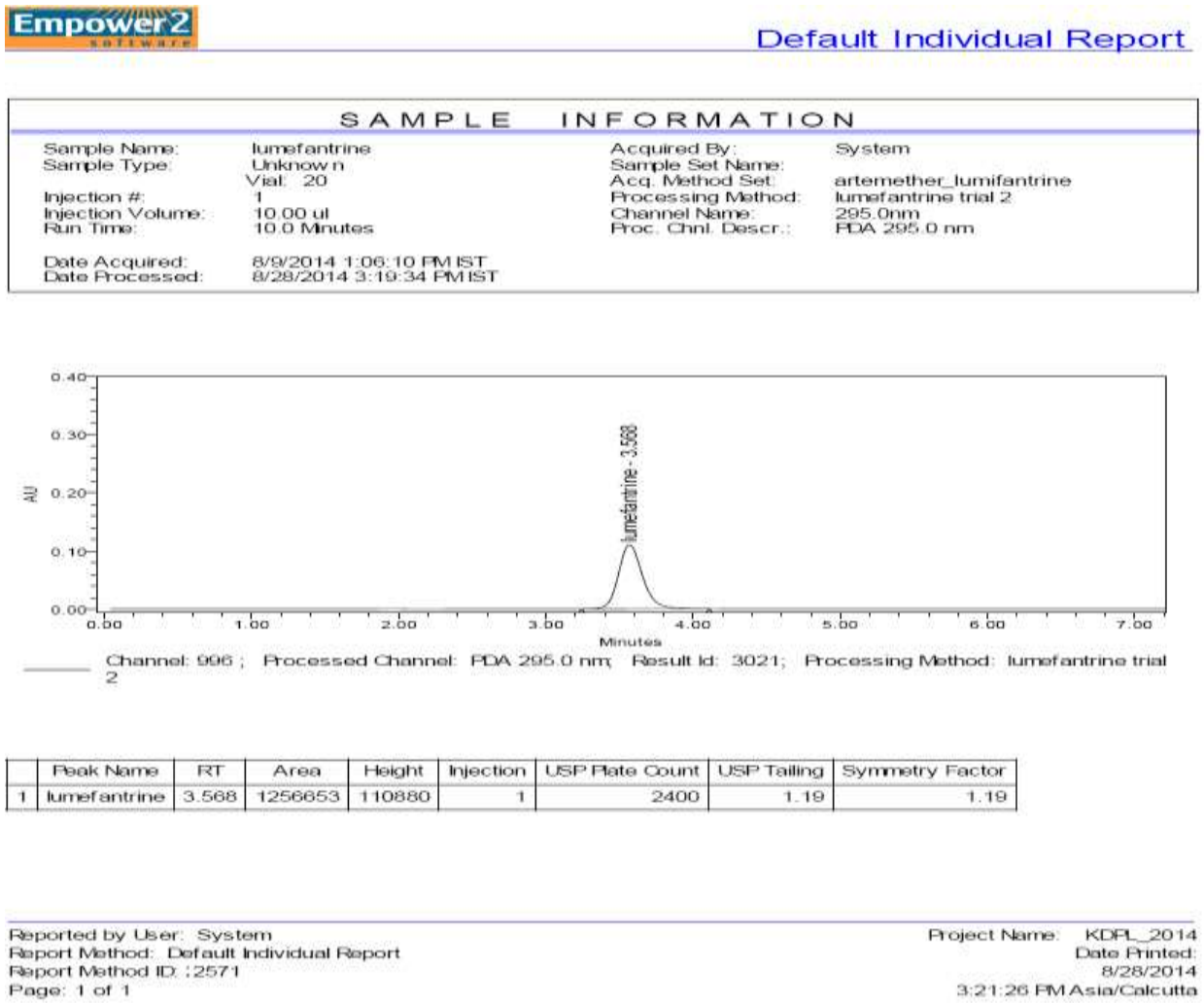


Fig 4: Chromatogram of Trial-4

Observation: individual component was eluted

TRIAL-5

CHROMATOGRAPHIC CONDITIONS

Flow rate **:** **1** ml/min

Column **:** Kromasil C_{18} (4.6 x 150 mm, 5 µm,)

Detector wave length **:** 290 nm

Column temperature **:** Ambient

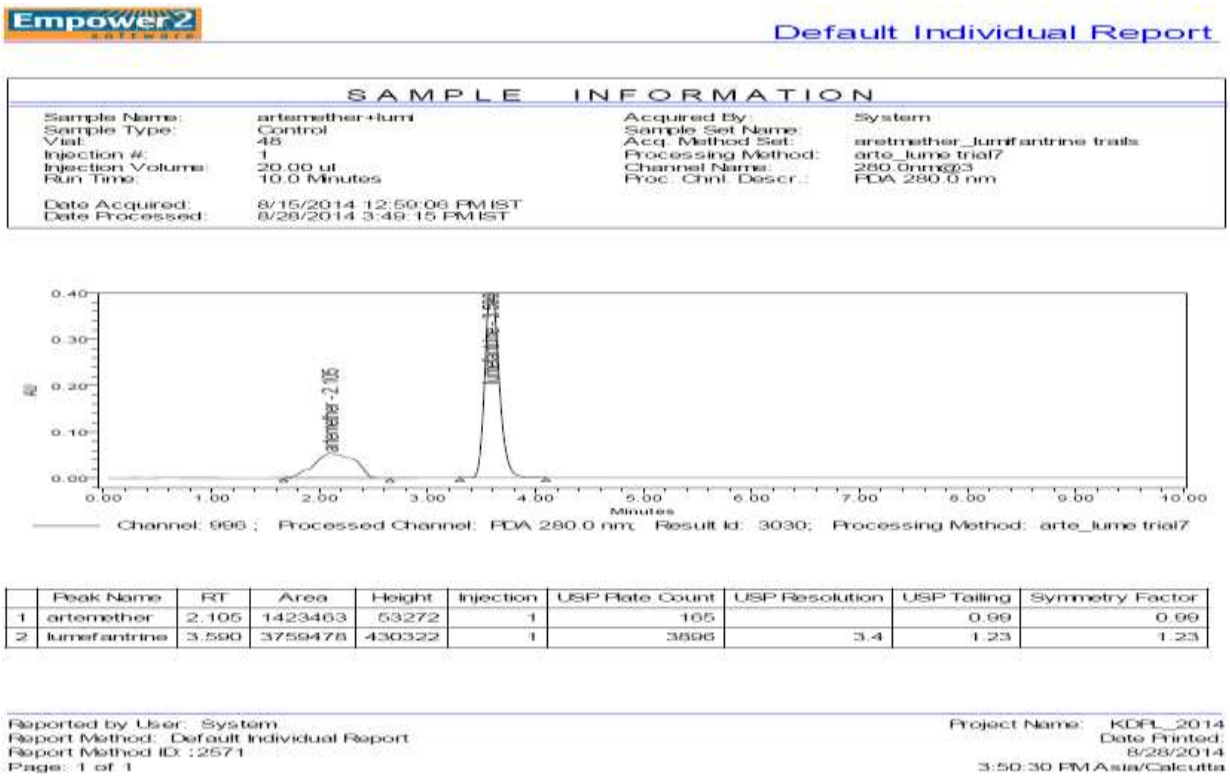
Injection volume **:** 20 µl

Run time **:** 10 min

Mobile phase **:** Ammonium acetate buffer : Methanol (20: 80)

pH **:** 6

Rt : 2.1 , 3.5.



	Peak Name	RT	Area	Height	Injection	USP Plate Count	USP Resolution	USP Tailing	Symmetry Factor
1	artemether	2.105	1423463	53272	1	165		0.99	0.99
2	lumefantrine	3.590	3759478	430322	1	3896	3.4	1.23	1.23

Fig 5: Chromatogram of Trial-5

Observation: Artemether compound wsa not eluted properly

TRIAL-6

CHROMATOGRAPHIC CONDITIONS

Flow rate **:** **1** ml/min

Column **:** Kromasil C_{18} (4.6 x 150 mm, 5 µm,)

Detector wave length **:** 290 nm

Column temperature **:** Ambient

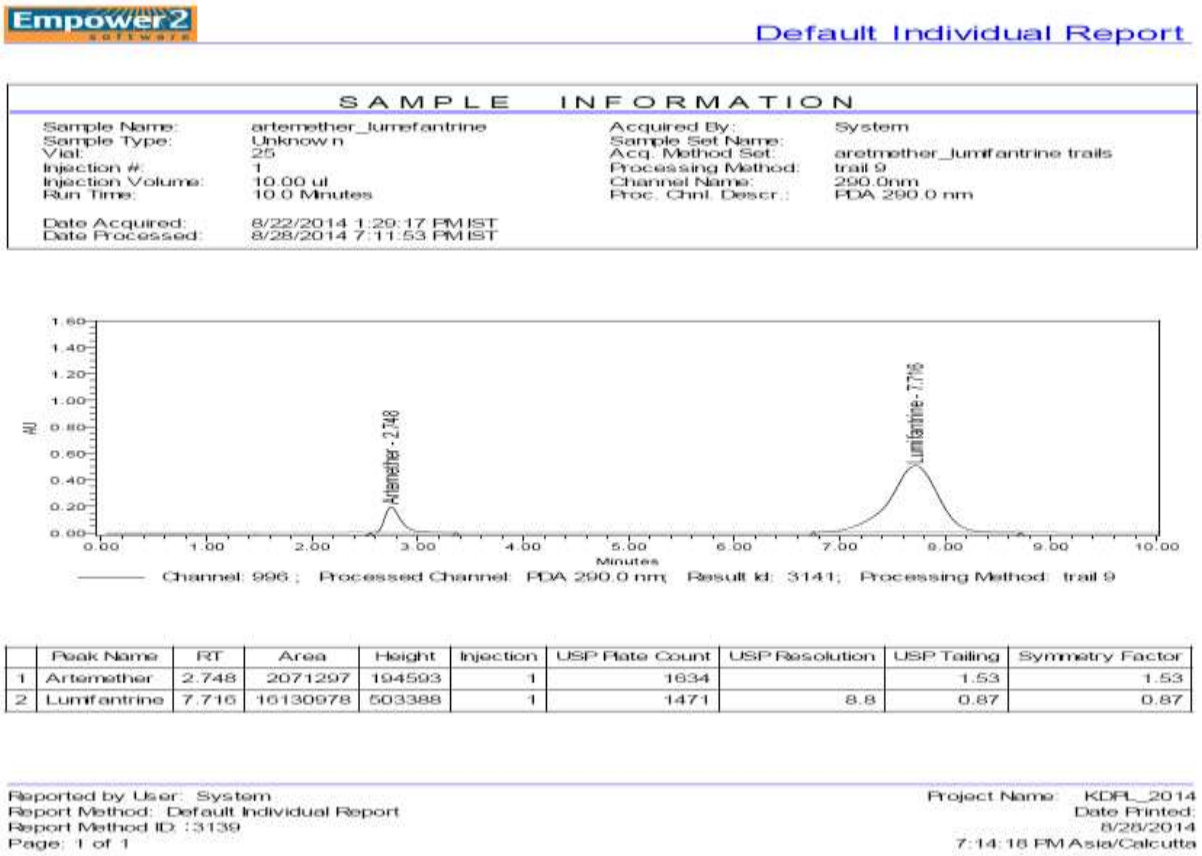
Injection volume **:** 10 µl

Run time **:** 10 min

Mobile phase **:** Phosphate buffer : Methanol (30: 70)

pH **:** 5

Rt : 1.45 ,2.8



	Peak Name	RT	Area	Height	Injection	USP Plate Count	USP Resolution	USP Tailing	Symmetry Factor
1	Artemether	2.748	2071297	194593	1	1634		1.53	1.53
2	Lumifantrine	7.716	16130978	503388	1	1471	8.8	0.87	0.87

Fig 6: Chromatogram of Trial-6

Observation :components was separated but plate count was less

OPTIMIZED METHOD

Reagents and Standard:

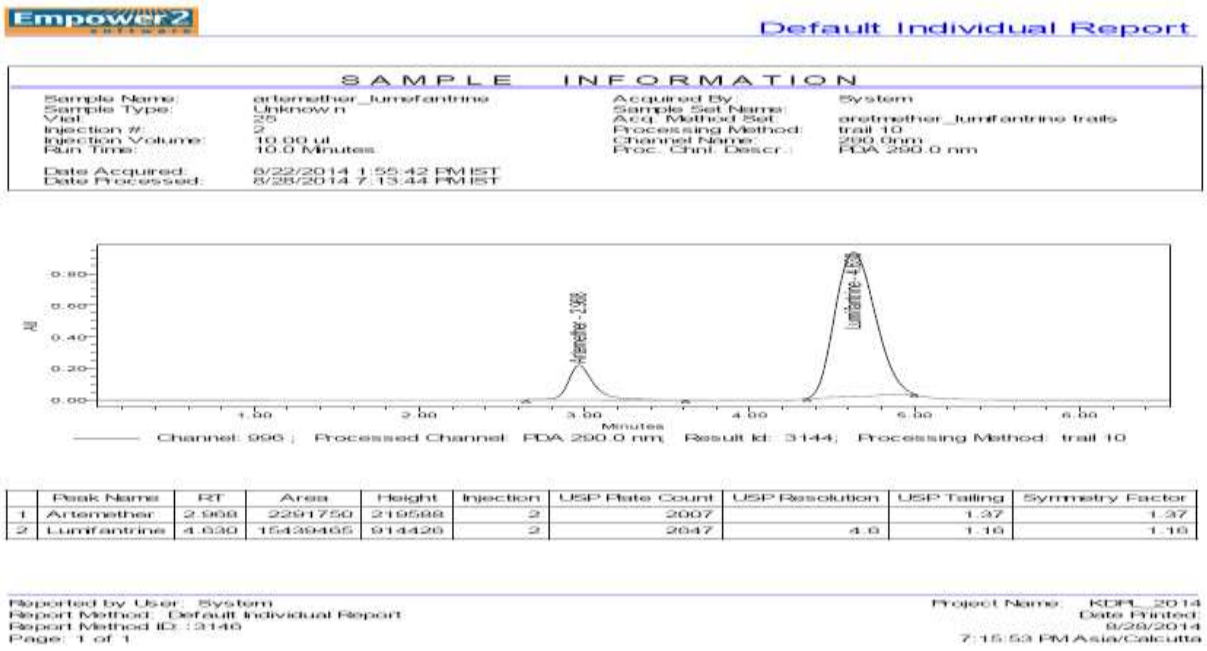
1. Artemether and Lumefantrine Tablets
2. Water HPLC Grade.
3. Artemether and Lumefantrine Working Standards.
4. Methanol HPLC Grade.
5. Ortho phosphoric acid.

Chromatographic Parameters:

Equipment	: High performance liquid chromatography equipped with Auto Sampler and PDA detector.
Column	: Symmetry C_{18} (4.6 x 150 mm, 5 µm)
Flow rate	: 0.8 ml per min.
Wavelength	: 290 nm.
Injection volume	: 10 µl

Column oven : Ambient

Run time : 10 min



	Peak Name	RT	Area	Height	Injection	USP Plate Count	USP Resolution	USP Tailing	Symmetry Factor
1	Artemether	2.958	2291750	219588	2	2007		1.37	1.37
2	Lumifantrine	4.030	15439465	914426	2	2647	4.0	1.16	1.16

Fig 7: Chromatogram of Trial-7

Observation : components was well separated with good resolution and plate count

Preparation of TEA buffer:

Weigh accurately 1ml of tri ethylamine dissolved in 10 ml HPLC water .and make up the volume up to250 ml with the same . Adjust the ph 6 with ortho phosphoric acid filtrate by 0.45µ filter by vacuum filtration . then sonicate

Preparation of mobile phase:

Mix a mixture of above buffer 20 ml (20 %) and 80 ml of Methanol HPLC (80%) and degas in0 ultrasonic water bath for 5 minutes. Filter through 0.45 µ filter under vacuum filtration. ***Diluent Preparation:***

The Mobile phase is used as Diluent.

Preparation of the Artemether and Lumefantrine Standard & Sample Solution:

Standard Solution Preparation:

Accurately weigh and transfer 10 mg of Artemether & 60 mg of Lumefantrine working standard into a 100 ml clean dry volumetric flask add diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Pipette 1 ml of Artemether & Lumefantrine of the above stock solution into a 10 ml volumetric 0.flask and make up to the mark with diluent.

Sample Solution Preparation:

Accurately weigh and transfer equivalent to 10 mg of Artemether & 60 mg of Lumefantrine sample into a 100 ml clean dry volumetric flask add about 70 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Pipette 1 ml of Artemether & Lumefantrine the above stock solution into a 10 ml volumetric flask and make up to the mark with diluent.

Procedure:

Inject 10 µl of the sample into the chromatographic system and measure the areas for Artemether & Lumefantrine peaks and calculate the % Assay by using the formulae.

System Suitability:

Tailing factor for the peaks due to Artemether & Lumefantrine in Standard solution should not be more than 1.5.

Theoretical plates for the Artemether & Lumefantrine peaks in Standard solution should not be less than 2000.

Observation:

Resolution between two analytes is good. No peak asymmetry was observed. All the results were found to be within the acceptance criteria. Hence the method was considered to be optimized.

METHOD VALIDATION SUMMARY

1) PRECISION

Preparation of stock solution:

Accurately weigh and transfer 10 mg of Artemether & 60 mg of Lumefantrine working standard into a 100 ml clean dry volumetric flask add about 70 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Pipette 1 ml of Artemether and Lumefantrine the above stock solution into a 10 ml volumetric flask and make up to the mark with diluent.

Procedure:

The standard solution was injected for six times and measured the area for all six injections in HPLC. The % RSD for the area of six replicate injections was found to be within the specified limits.

Acceptance Criteria: The % RSD for the area of six standard injections results should not be more than 2 %.

2.**SYSTEM PRECISION**

Preparation of Stock Solution: Accurately weigh and transfer equivalent to 10 mg of Artemether & 10 mg of Lumefantrine standard into a 100 ml clean dry volumetric flask add about 70 ml Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Pipette 1 ml of Artemether & Lumefantrine the above stock solution into a10 ml volumetric flask and make up to the mark with diluent.

Procedure:

The standard solution was injected for six times and measured the area for all six injections in HPLC. The % RSD for the area of six replicate injections was found to be within the specified limits.

Acceptance Criteria: The % RSD for the area of six standard injections results should not be more than 2 %.

2) METHOD PRECISION

To evaluate the method precision of the method, Precision was performed on different day.

Preparation of stock solution:

Accurately weigh and transfer 10 mg of Artemether & 60 mg Sample into a 100 ml clean dry volumetric flask add about 70 ml diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Pipette 1 ml of Artemether & Lumefantrine the above stock solution into a 10 ml volumetric flask and make up to the mark with diluent.

Procedure:

The standard solution was injected for six times and measured the area for all six injections in HPLC. The % RSD for the area of six replicate injections was found to be within the specified limits.

Acceptance Criteria: The % RSD for the area of six standard injections results should not be more than 2 %.

3) SPECIFICITY

The effect of wide range of excipients and other additives usually present in the combination of Artemether and Lumefantrine in the determinations under optimum conditions were investigated. Placebo solution, sample and standard solutions were analysed individually as per the method to examine interference. Chromatograms of placebo, standard, and sample

Acceptance criteria:

No elution of the interfering peaks should take place, which shows that the peak of analyte was pure and excipients in the combination should not interfere with the analyte.

4) ACCURACY

Assay was performed in triplicate for various concentrations of Artemether and Lumefantrine equivalent to 50, 100, and 150 % of the standard amount was injected into the HPLC system as per the test procedure.

Preparation of Standard stock solution:

10 mg of Artemether and 60 mg of Lumefantrine accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicated to dissolve it completely and volume was made up to the mark with the same solventto give the concentration of 1000 µg/ml and 6000 µg/ml (Stock solution)

Preparation Sample solutions:

Preparation of 50% solution (5 µg/ml of Artemether and 30µg/ml ofLumefantrine):

From the above stock solutions take 0.5 ml into 10 ml dry volumetric flask, make up to the mark with diluent**.**

Preparation of 100% solution (10 µg/ml of Artemether and 60µg/ml of Lumefantrine):

From the above stock solutions take 1 ml into 10 ml dry volumetric flask, make up to the mark with diluent

Preparation of 150% solution (15 µg/ml of Artemether and 90 µg/ml of Lumefantrine):

From the above stock solutions take 1.5 ml into 10 ml dry volumetric flask, make up to the mark with diluent.

These solutions were filtered through 0.45µ membrane and then each concentration; three replicate injections were made under the optimized conditions

Procedure :

The standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions were injected. The amount found and amount added for Artemether and Lumefantrine individual recovery and mean recovery values were calculated and the results were summarized

Acceptance criteria

The mean % recovery of the Artemether and lumefantrine each spike level should be not less than 98.0 % and not more than 102.0 %.

Table 1: Accuracy results for Artemether

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	714347	5.0	4.98	99.6%	99.76%
50%	714366	5.0	4.98	99.6%	

50%	714328	5.0	5.01	100.1%	
100%	1428698	10.0	9.98	99.80%	
100%	1428732	10.0	9.98	99.80%	99.89%
100%	1428649	10.0	9.99	99.90%	
150%	2143044	15.0	14.99	99.93%	
150%	2143098	15.0	14.97	99.93%	100.15%
150%	2142976	15.0	15.01	100.06%	

Acceptance Criteria: The % Recovery for each level should be between 98.0 to 102.0%.

Table 2: Accuracy results for Lumefantrine

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	1102061	30	29.9	99.94%	100.17%
50%	1102186	30	29.9	99.94%	
50%	1102128	30	30.1	100.64%	
100%	2204123	60	59.9	99.22%	
100%	2204372	60	59.9	99.22%	100.02%
100%	2204257	60	60.3	101.6%	
150%	3306184	90	88.7	99.10%	
150%	3306556	90	89.7	100.2%	
150%	3306385	90	88.9	99.10%	99.46.%

Acceptance Criteria: The % Recovery for each level should be between 98.0 to 102.0%.

5) LINEARITY

Preparation of stock solution:

Accurately weigh and transfer 10 mg of Artemether & 60 mg of Lumefantrine working standard into a 100 ml clean dry volumetric flask add about 70 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Preparation of Level – I (5 ppm & 30 ppm of Artemether & Lumefantrine):

0.5 ml of stock solution has taken in 10 ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – II (7.5 ppm & 45 ppm of Artemether & Lumefantrine):

0.75 ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – III (10 ppm & 60 ppm of Artemether & Lumefantrine):

1.0 ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – IV (12.5 ppm & 75 ppm of Artemether & Lumefantrine)

1.25 ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – V (17.5 ppm & 90 ppm of Artemether & Lumefantrine)

1.75 ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with diluent.

Procedure:

Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

Table 3: Linearity Results for Artemether

S.No	Linearity Level	Concentration	Area
1	I	5 ppm	731950
2	II	7.5 ppm	1097923
3	III	10 ppm	1463895
4	IV	12.5 ppm	1829903
5	V	17.5 ppm	2200875
Correlation Coefficient			0.999

Acceptance Criteria: Correlation coefficient should be not less than 0.999.

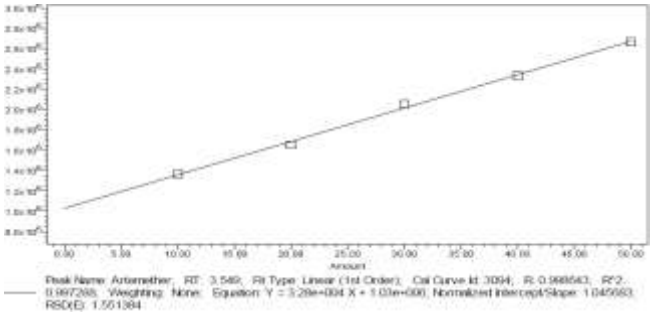


Fig 11: Linearity of Artemether

Table 4: Linearity Results for Lumefantrine

S.No	Linearity Level	Concentration	Area
1	I	30 ppm	1242612
2	II	45 ppm	1863950
3	III	60 ppm	2485000
4	IV	75 ppm	3106400
5	V	90 ppm	3728612
Correlation Coefficient			0.999

Acceptance Criteria: Correlation coefficient should be not less than 0.999.

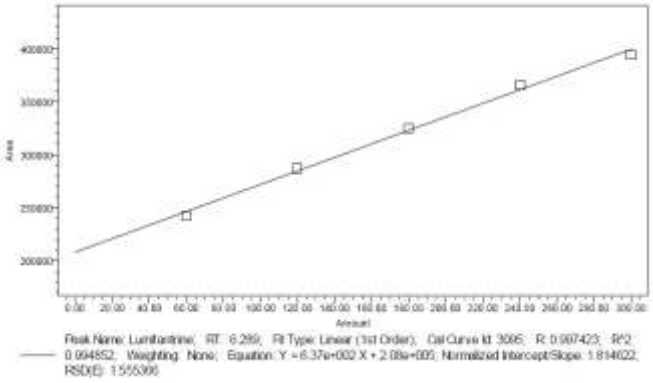


Fig 12: Linearity of Lumefantrine

6) RUGGEDNESS

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day.

Preparation of sample solution:

Accurately weigh and transfer 10 mg of Artemether & 60 mg of lumefantrine Sample into a 100 ml clean dry volumetric flask add about 70 ml diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Pipette 1 ml of Artemether & Lumefantrine the above stock solution into a 10 ml volumetric flask and make up to the mark with diluent.

Procedure: The standard solution was injected for six times and measured the area for all six injections in HPLC. The % RSD for the area of six replicate injections was found to be within the specified limits.

Acceptance Criteria: The % RSD for the area of six standard injections results should not be more than 2 %.

7) ROBUSTNESS

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

a). The flow rate was varied at 0.8 ml/min to 1.2 ml/min. Standard solution 10 ppm of Artemether & 60 ppm Lumefantrine prepared and analysed using the varied flow rates along with method flow rate.

Results:

On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10 %.

The method is robust only in less flow condition.

Results for actual flow (1.0 ml/min) have been considered from Assay standard.

b). The Organic composition in the Mobile phase was varied from 30 % to 50 %. Standard solutions 10 µg/ml of Artemether & 12.5 µg/ml of Lumefantrine were prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method

8) LIMIT OF DETECTION

FOR ARTEMETHER & LUMEFANTRINE: Limit of detection is the lowest concentration of

the analyte that can be detected by injecting decreasing amount, not necessarily quantity by the method, under the stated experimental conditions. It is determined by based on the standard deviation of response and the slope. The detection limit may be expressed as

$$LOD=3.3\ \sigma/S$$

Where:

σ = standard deviaton of standard.

S = Slope of caliberation curve.

9) LIMIT OF QUANTIFICATION :

FOR ARTEMETHER & LUMEFANTRINE:

Limit of quantitation is the lowest concentration of the analyte in a sample that can be estimated quantitatively by injecting decreasing amount of drug with acceptable precision and accuracy under the stated experimental conditions of the method . Based on the standard deviation of the response and the slope. Limit of Quantification (LOQ) may be expressed as

$$LOQ=10\sigma/S$$

Where:

σ = standard deviaton of standard , S = Slope of caliberation curve.

DEGRADATION STUDIES

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing to be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on Artemether and Lumefantrine using the proposed method.

Standard solution preparation

Accurately weigh and transfer about 10 mg Artemether and 60 mg Lumefantrine of standard into a 100 ml dry volumetric flask add about 70 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Hydrolytic degradation under acidic condition:

1.0 ml of Artemether, 6.0 ml of Lumefantrine above stock solution and 3 ml of 0.1N HCl was added in 10 ml of volumetric flask. The volumetric flask was kept at normal condition for 90 minutes and then neutralized with 0.1 N NaOH and make up to 10ml with diluent. Filter the solution with 0.45 microns syringe filters and place in vials.

Hydrolytic degradation under alkaline condition:

1.0 ml of Artemether, 6.0 ml of Lumefantrine above stock solution and 3 ml of 0.1N NaOH was

added in 10 ml of volumetric flask. The volumetric flask was kept at normal condition for 90 minutes and then neutralized with 0.1 N HCL and make up to 10 ml with diluent. Filter the solution with 0.45 microns syringe filters and place in vials.

Thermal induced degradation:

1.0 ml of Artemether, 6.0 ml of Lumefantrine above stock solution and 3 ml of diluent was added in 10 ml of volumetric flask. The volumetric flask was kept at reflex condition for 60 minutes and make up to 10 ml with diluent. Filter the solution with 0.45 microns syringe filters and place in vials.

Oxidative degradation:

1.0 ml of Artemether, 6.0 ml of Lumefantrine above stock solution and 1 ml of 3 % w/v of hydrogen peroxide added in 10 ml of volumetric flask and the volume was made up to the mark with diluent . The volumetric flask was then kept at room temperature for 15 min. Filter the solution with 0.45 microns syringe filters and place in vials.

Photolytic degradation:

1.0 ml of Artemether and 6.0 ml of Lumefantrine above stock solution taken in 10 ml of volumetric flask and the volume was made up to the mark with diluents. The volumetric flask was then exposed to light for 12 hr. Filter the solution with 0.45 microns syringe filters and place in vials.

Table 5: Results of Degradation for Artemether

	Area	***% Assay***	***% Degradated***
Acid	8142946	94.95	5.05
Base	7287545	94.31	5.69
Thermal	5644250	93.83	6.17
peroxide	76612671	87.98	12.02
Photolyitc	4967331	88.23	11.77

Table 6: Results of Degradation for Lumefantrine

	Area	***% Assay***	***% Degradated***
Acid	58430128	91.31	5.96
Base	106022049	88.90	11.1
Thermal	48496666	91.49	8.51
peroxide	48172177	88.18	11.82
Photolyitc	43811634	80.20	19.8

RESULTS AND DISCUSSION

The result from development activity is that a suitable, easy, less time-consuming validated method has been developed for Artemether and Lumefantrine. So the results of chromatographic conditions are:

FIXED CHROMATOGRAPHIC CONDITIONS

Chromatographic conditions

OPTIMIZED CHROMATOGRAPHIC CONDITIONS	
Mobile phase	Solvent-A: TEA buffer Ph-4.5 Solvent-B: Methanol
Column	Symmetry (4.6 x 150mm, 5µm)
Flow rate	0.8 ml/min
Detection Wavelength	290 nm
Injection volume	10 µl
Column oven temperature	Ambient

The retention times of Artemether and Lumefantrine in the standard solution having the concentration of 10 µg/ml of Artemether and 60 µg/ml of Lumefantrine were found to be around 2.9 min and 4.6 min respectively.

Artemether and Lumefantrine shows the percentage purity values are 99. 43 % w/v and 100.59 % w/v respectively.

1) SPECIFICITY

The specificity of the method was confirmed by injecting the placebo and placebo spiked standard and observed that there was no shift in wavelength interference due to placebo. This confirms the specificity of the proposed method**.**

2) PRECISION

Precision

The Precision was determined by replicate injections of mixed standard solution. The R.S.D of Area is present within the Acceptance criteria of 2 %.

System precision

The precision of the system was determined by replicate injections of mixed standard solution. The % R.S.D of Area is present within the Acceptance criteria of 2 %. The results are reported

Method Precision

The precision of the method was determined by replicate injections of sample solution. The % R.S.D of Area is present within the acceptance criteria of 2 %.

Thus the proposed method was found to be high degree of precision and reproducibility.

3) ACCURACY

The validation of the proposed method was further verified by recovery studies. Acceptance criteria are 98 - 102 % w/v..

- This serves as a good index of the accuracy and reproducibility of the proposed method.

4) LINEARITY AND RANGE

Linearity was carried out by injecting each level into the chromatographic system and measures the peak area. A graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) was plotted and correlation coefficient was calculated

5) RUGGEDNESS

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day by using different column on same dimensions. The standard solution was injected for six times and measured the area for all six injections in HPLC. The % RSD for the area of six replicate injections was found to be within the specified limits.

6).**ROBUSTNESS**

Robustness was determined by carrying out the assay during which flow rate and organic phase were altered slightly..

% RSD values for robustness indicated that the method is robust and does not show variations in the results on slight variations in flow rate and detection wavelength.

6) LIMIT OF DETECTION

The limit of detection of Artemether and Lumefantrine were calculated and found to be 0.46 and 0.099 respectively. The results are reported in

7) **LIMIT OF QUANTIFICATION**

The limit of quantification of Artemether and Lumefantrine were calculated and found to be 1.4 and 0.3 respectively. The results are reported in

8) DEGRADATION STUDIES

The sample solutions were subjected to acidic, basic, peroxide, temperature and light. In acidic the degradation for Artemether and Lumefantrine were found to be -5.05 % and -5.96 % respectively. In basic the degradation for Artemether and Lumefantrine were found to be -5.69 % and -11.1 % respectively. Degradation by peroxide for Artemether and Lumefantrine were found to be -12.02 % and -11.82 %. The solid sample was subjected to light for 7 days and then the degradation of Artemether and Lumefantrine were found to be -11.77% and -19.8 %. Thermal degradation for Artemether and Lumefantrine were found to be -6.17 % and -8.51 %. The results are reported

CONCLUSION

1. A wavelength of 290 nm was selected and the mobile phase consists of triehtylamine buffer (P^H 6 adjusted with ortho phosphoric acid) and methanol in 20:80 % v/v ratios at a flow rate of 0.8 ml/min were found to be optimum conditions for analysis. The peaks were well resolved

with symmetry C_{18} column. System suitability studies were also carried out which includes theoretical plates, resolution and tailing factors etc.

2. The accuracy studies were shown as % recovery for Artemether and Lumefantrine at 50 %, 100 % and 150 %. The limits of % recovered should be in the range of 98-102 %. The results obtained for Artemether and Lumefantrine were found to be within the limits. Hence the method was found to be accurate. The accuracy studies showing % recovery of Artemether were found to be 99.9 %, 99 % and 99.9. % respectively and the % recovery of Lumefantrine were found to be 99.9%, 99.9 % and 98.9 % respectively.
3. In the System precision study, the % RSD was found to be less than 2 %. For Artemether 1.7 and Lumefantrine 1.2 which indicates that the system has good reproducibility.
4. For ID precision studies six replicate injections of Artemether and Lumefantrine were performed. The % RSD was determined for peak areas of Artemether and Lumefantrine. The acceptance limit should not be more than 2 % and the results were found to be within the acceptance limits.
5. Using the optimized chromatographic conditions, chromatograms of mixed standard solutions which contained Artemether and Lumefantrine were recorded. Retention times of Artemether and Lumefantrine were found to be 2.9 min and 4.6 min respectively.
6. Calibration curves were obtained by using peak area vs. concentration. Artemether and Lumefantrine show linearity in the range of 0.1 – 0.5 µg/ml and 0.6 – 3.0 µ/ml.

Calibration curve was plotted and correlation co-efficient for both the drugs Artemether and Lumefantrine were found to be 0.999 and 0.999 respectively

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