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ANALYTICAL METHOD DEVELOPMENT BY RP-HPLC FOR QUANTITATIVE ESTIMATION OF ANTIMALARIAL DRUG AND ITS VALIDATION IN BULK AND PHARMACEUTICAL DOSAGE FORM

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Abstract

A simple, rapid, precise and accurate reversed phase high performance liquid chromatographic Method has been developed for the quantitative determination of Artemether. This Method uses a Zorbax C8 (250x4.6mm, 5µ particle Size) analytical column, a mobile phase of Phosphate buffer (pH – 3.0) and methanol in ratio of (30:70 v/v). The instrumental settings are a flow rate of 0.8 ml/min and VWD detector wavelength at 210 nm. The retention time for Artemether is 2.997 ± 0.2 min. The Method is validated and shown to be linear. The linearity range for Artemether 10-50 µg/mL. The Percentage recovery for Artemether was found to be 99.99 %. The correlation coefficients of Artemether 0.999. The relative standard deviation for six replicates is always less than 2%. The Statistical analysis proves that the Method is suitable for analysis of Artemether as a bulk drug and in pharmaceutical formulation with out any interference from the excipient.

Key Words:

Artemether, RP-HPLC, Antimalarial, Method Development, Phosphate buffer and Validation.

Introduction:

Artemether is chemically described as: (1R,4S,5R,8S,9R,10S,12R,13R)-10-ARThoxy-1,5,9-triARThyl-11,14,15,16-tetraoxatetracyclo[10.3.1.0^{4,13}.0^{8,13}]hexadecane with molecular formula $C_{16}H_{26}O_5$ and molecular weight is 298.26 gm/mol (**Fig. 1**). Artemether is Artemisinin derivatives mainly used in the management of Malaria infection caused by one-celled parasite *Plasmodium*. It is also used for antiprotozoal activity in low doses[21-23]. The literature survey revealed several analytical methods developed for estimation of Artemether by UV spectrophotometric, HPTLC and RP-HPLC methods[24-43].

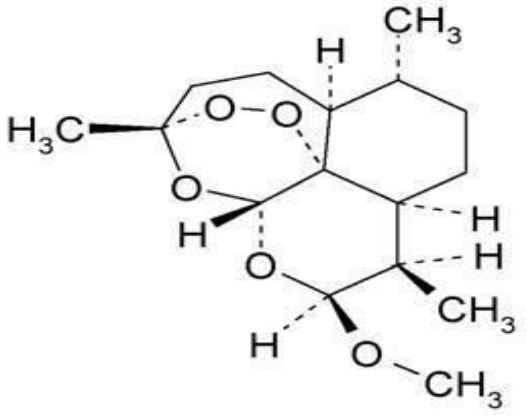

Fig. 1: Structure of Artemether.

The injection form is available in market (Larither). The present drug has promising effect to control Malaria. From the literature survey it was clear that no Method have been developed and validated to access stability of ART in bulk and injection dosage form. To establish stability indicating nature of the RP-HPLC Method, forced degradation of drug substances were performed under stress conditions (oxidation, acid and base hydrolysis, thermal, and photolytic). The proposed Method was optimized and validated as per ICH guidelines[13-19]

The present research work describes a rapid, accurate, sensitive and reproducible stability indicating RP-HPLC Method for quantitative estimation of Artemether from bulk and injection formulation.

Experimental Work

Chemicals and Reagents

The pure drug sample of Artemether was obtained as gift sample from, Micro Labs Ltd., Mumbai. Double Distilled Water (HPLC grade), Methanol (HPLC grade), orthophosphoric acid and Potassium dihydrogen ortho phosphate were purchased from Research–Lab Fine Chem Industries, Mumbai. The injection containing Artemether (Larither) having 80mg of ART was purchased from local market (Manufactured by IPCA Laboratories Ltd., Mumbai).

Instrumentation and Equipments

The HPLC analysis was accomplished on Agilent 1220 high pressure liquid chromatography System with an Infinity Isocratic LC Manual Injector, Zorbax Eclipse plus C8 column (250mm×4.6mm) analytical column reversed -phase material of 5µ size with Variable wavelength detector. All the parameters of HPLC were controlled by Ezchrom Elite software. Other instruments used were UV –Vis Double Beam Spectrophotometer Schimadzu 1800 with UV probe Software, Contech electronic balance, pH meter (Elico india) and sonicator (Bio Technics India).

Analytical Method Development

a) Selection of Detection Wavelength

The Detection of wavelength was done in UV Schimadzu 1800 instrument. An ideal wavelength is the one that gives good response for the drugs that are to be detected. In the present study standard drug solutions of 10 µg/mL Artemether was, therefore, prepared in solvent mixtures of Phosphate buffer (pH 3.0): methanol (30:70). This drug solution was scanned in the UV region of 200-400 nm. The wavelength selected for the HPLC analysis was 210 nm to which the drug showed significant absorbance and very good resolution. The overlain UV spectrum of ART is shown in **Fig. 2**.

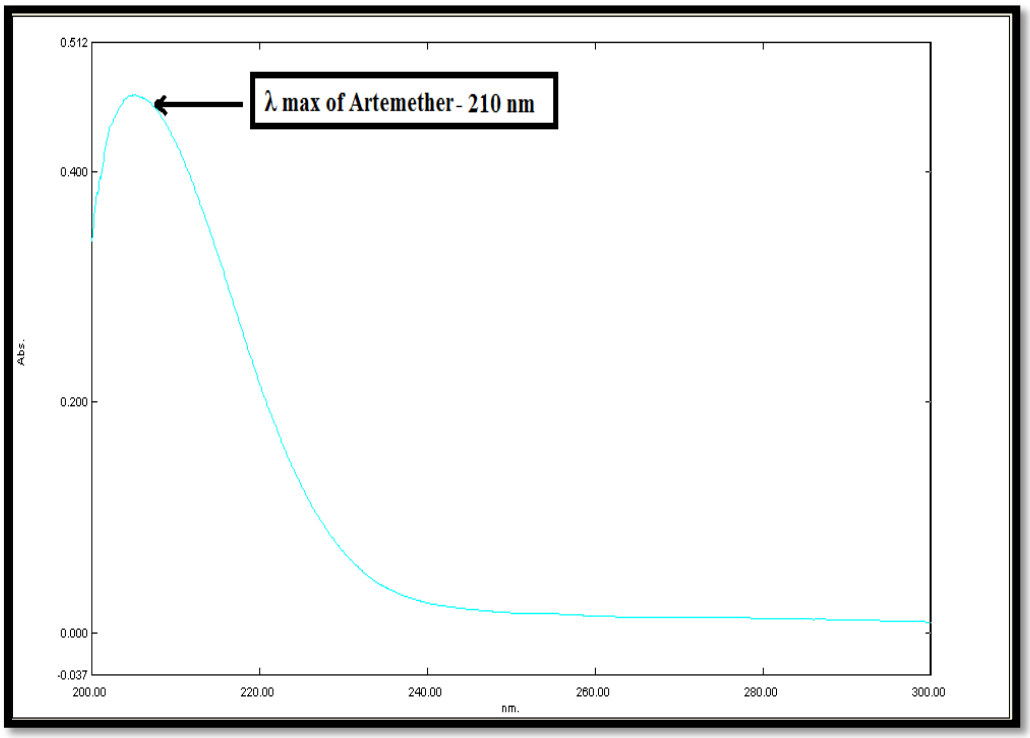

Fig. 2: Analytical wavelength of Artemether (210 nm).

b) Selection of Mobile Phase

After assessing the solubility of drugs in different solvents as well on the basis of literature survey, the standard solution of Artemether was injected into the HPLC system by using different solvent systems. Different mobile phases were tried in order to find the best conditions for the separation of both the drugs. It was found that of potassium dihydrogen orthophosphate buffer (pH adjusted to 3.0 with orthophosphoric acid) and methanol in the ratio of 30:70%v/v showed satisfactory results as compared to other mobile phases. Prior to use, the mobile phase was degassed by an ultrasonic bath and filtered through 0.45µm filter paper. Final optimized chromatographic parameters are shown in **Table 1**.

Table-1: Final Optimized Chromatographic Condition.

Column	Zorbax Eclipse Plus C8 (4.6 × 250 mm)
Mobile Phase	Phosphate buffer: Methanol (30:70) pH adjusted to 3.0 with ortho phosphoric acid (OPA).
Flow Rate	0.8 mL/min.
Detection Wavelength	210 nm
Injection Volume	20 µL
Run Time	5 min
Column Temperature	Ambient Temperature

c) Preparation of mobile phase

300 mL of Buffer (pH 3.0), 700 mL methanol (HPLC grade) were mixed and filtered through 0.45µm filter paper, sonicated for 10 minutes to degas and used as mobile phase.

d) Preparation of Standard Solution

Standard stock solution of ART was prepared by dissolving 10 mg of ART in 100 mL volumetric flask with 70 mL mobile phase. It was sonicated to dissolve completely and made volume up to the mark with the same diluents to get concentration of 100 µg/mL (Stock solution). From this, 1 mL of the solution was pipette out into another 10 mL volumetric flask and diluted up to the mark with diluents to get a working standard solution of 10 µg/mL concentration.

e) Preparation of Sample Solution:

Transfer about 0.12 mL of Artemether injection to a 100 mL volumetric flask, add 50 mL of mobile phase, mix, and make up the volume with mobile phase and sonicate for 15 min. (100µg/mL). Transfer 1mL of this solution to 10 mL of volumetric flask and make up with mobile phase (10µg/mL).

Procedure

20 µL of the standard and sample solutions were injected into the chromatographic system and areas for the Artemether peak was measured. % Assay was calculated by using the formula. Result of assay of ART is shown in Table 2.

Table-2: Results of Assay.

Sample	Amount Taken	Area	% Purity	% Assay
Standard Drug (Artemether)	10 mg in 100mL	6097714	99.8	**101.20**
Sample (Artemether) injection	0.12 mL in 100 mL	6177452	99.8	

$$\%\textbf{Assay} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{100}{LC}$$

Where,

AT : Average area from the Sample solution chromatogram.

AS : Average area from the Standard solution chromatogram.

WS : Weight of ART standard in mg.

DT : Dilution of sample in mL.

DS : Dilution of standard in mL.

WT : Volume of sample taken in mL.

P : Potency of ART reference standard (%).

LC : Label claim in (mg).

Analytical Method Validation

To develop a precise, accurate and reproducible HPLC method for the estimation of ART various mobile phases, stationary phases and sample preparation methods were employed and the proposed chromatographic condition was found to be appropriate for the quantitative determination. After optimization of the analytical conditions, the evaluation of the fundamental parameters, such as system suitability test, linearity, precision, accuracy, recovery selectivity, and stability were performed for the method validation.

System Suitability Test

To verify the analytical system is working properly and can give accurate and precise results, the system suitability parameters are to be set. The standard solution which is prepared as per the procedure is injected six injections of diluted drug in the linear region of the calibration curve and measuring the relative standard deviation in percentage (% RSD) to check the instrument is giving consistent results.

Accuracy

The accuracy of an analytical method validation is the closeness of test results obtained by that method to the true value (Standard value). Spike known quantity of Artemether standard at 50%, 100%, and 150% of assay specification limit. Analyze these samples in triplicate for each level. From the results, the % recovery was calculated.

Precision

The system precision of the method was verified by six replicate injections of standard solution containing ART. The precision for repeatability (intra-day and Inter-days precision) was carried out the analyte six times in a day in order to record any intra-day variations in the results. For Inter-days variations studies, analysis was carried out on three different days with same concentrations of ART.

Linearity and Range

The linearity was determined for ART. Linearity of the method was studied by injecting six concentrations of drug prepared in methanol and calibration curves were constructed by plotting peak area against the respective concentrations. Concentration range will be selected for ART 10-60 µg/mL.

Limit of detection and Limit of quantitation

Sensitivity of the proposed method was estimated in terms of Limit of Detection (LOD) and Limit of Quantitation (LOQ). The LOD and LOQ were calculated according to the formula given by the ICH guidelines as described below,

LOD is calculated from the formula: -

$$\mathbf{LOD} = \frac{\mathbf{3.3}\boldsymbol{\sigma}}{\mathbf{S}}$$

LOQ is calculated from the formula: -

$$\mathbf{LOQ} = \frac{\mathbf{10}\boldsymbol{\sigma}}{\mathbf{S}}$$

Where,
σ = Standard deviation of the response of calibration curve.
S = Slope of the calibration curve.

Robustness

Robustness was evaluated by making deliberate variations in method parameters such as variation of wavelength, flow rate, mobile phase ratio and change in pH of mobile phase. The robustness of the method was studied for ART.

Stability of Solution

Evaluate the stability in analytical solution by injecting the standard preparation and sample preparation at regular interval. The stability of solution is carried out at 0, 3, 6, 12, 24, 48 hrs.

Forced Degradation Study

Sample Stock solutions containing 100µg/mL Artemether was prepared in diluents. This solution was used for forced degradation to provide an indication of the stability indicating property of proposed method. In all degradation studies the average peak area of ART sample after analysis were recorded in order to study the degradation products of both the drugs.

A. **Acidic and Basic degradation:** To 2 mL of sample stock solution of drugs, 2 mL of each of 5 N HCl and 5 N NaOH were added. Acidic and Basic mixture of drug was placed in the water bath for 30 min. at 80 ^{o}C. The solutions were neutralized as needed (5 N NaOH or 5 N HCl).

B. **Hydrogen peroxide degradation:** To 2 mL of sample solution of drugs, 2 mL of each 3% w/v of hydrogen peroxide (H_2O_2) was added. The solutions were placed in the water bath for 3 hr at 80 ^{o}C.

C. **Neutral hydrolysis:** To 2 mL of sample solution of drugs, 2 mL of distilled water was added. The solutions were placed in the water bath for 4 hr at 80 ^{o}C to study the degradation under neutral conditions.

D. **Thermal degradation:** To carry out thermal degradation of sample solution, take 2 mL of sample solutions of drug and placed in the hot air oven for 4 hr at 80 ^{o}C.

E. **Photolytic degradation:** As per ICH guidelines for photo-stability testing of new drug substances and products, 2 mL samples should be exposed to direct sunlight for 4 hrs.

From the above solutions, each sample was taken in 10 mL volumetric flask, cooled to room temperature and dilute up to with diluents. Filter and sonicate for 10 min. and analyze this solution in HPLC.

Results and Discussion

Selection of Chromatographic Conditions and Optimization of Mobile Phase

Mobile phase was optimized to separate ART using reverse phase Zorbax Eclipse Plus C8 column (250mm×4.6mm), with mobile phase consist of phosphate buffer (KH_2PO_4): methanol (30:70v/v) at pH 3.0 adjusted with ortho-phosphoric acid, the flow rate was 0.8 mL/min and the detection was carried at 210 nm. A typical chromatogram of blank solution is as shown in **Figure 3**. Blank sample solution was screened and interference of endogenous substances was not observed at retention time of ART which represented the selectivity of the method. Under optimum chromatographic conditions, the retention time for ART standard stock solution was found to be 2.997 min. A typical chromatogram drug in standard and formulation is shown in **Fig. 4 and 5**. The method is simple, accurate,

and reproducible and can be used for quantitative analysis of Artemether. All of the analytical validation parameters for this proposed method were determined according to ICH guidelines[53].

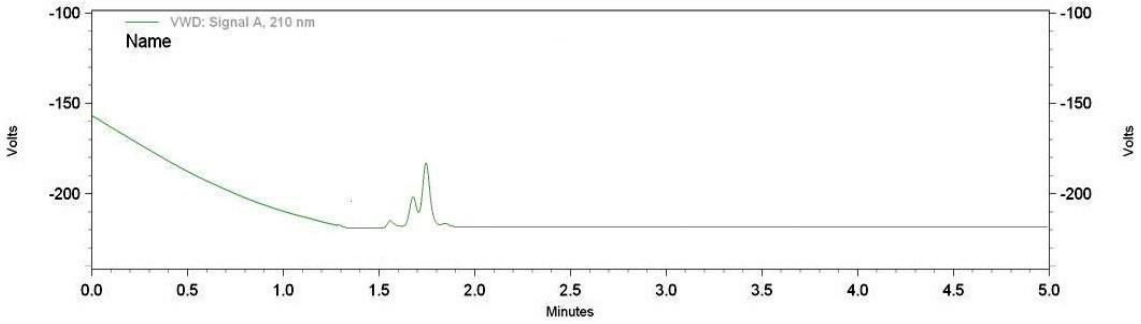

Fig. 3: Chromatogram for Blank.

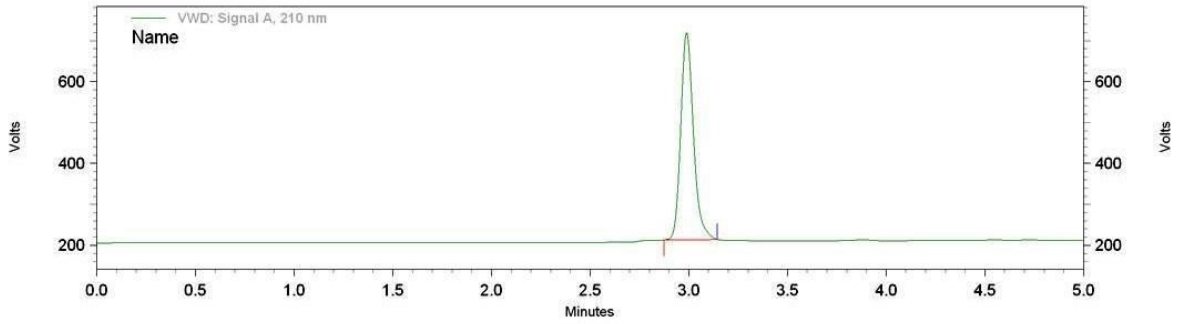

Fig. 4: Chromatogram of Standard ART.

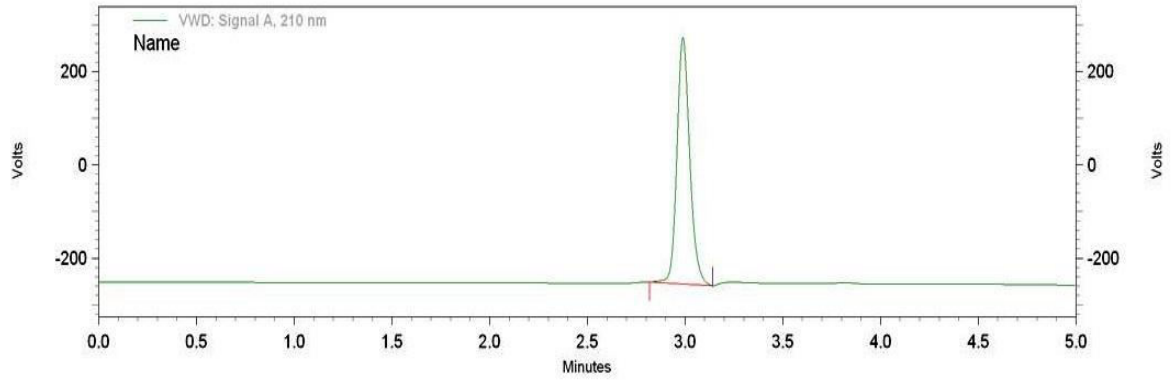

Fig. 5: Chromatogram of ART formulation (Injection).

System Suitability Test

The results of system suitability test are shown in **Table 3**. The % RSD calculated for the method was found to be less than 2%, which revealed the suitability of the developed method and the optimized chromatographic conditions.

Table-3: Results of System Suitability Test.

Sr. No.	Sample	Area	Retention Time
1	STD 1	5791714	2.957
2	STD 2	5777522	2.96
3	STD 3	5855268	2.96
4	STD 4	5745734	2.96
5	STD 5	5951084	2.96
Average		**5824264.4**	**2.9594**
SD		**81326.25**	**0.001341641**
% RSD		**1.396335201**	**0.045334892**
Tailing Factor		**1.328**	
Theoretical plates		**10297**	

Accuracy studies

The accuracy of the method in term of recovery was studied at three different concentration levels i.e. 50%, 100 % and 150 % showed acceptable % recoveries. The results are shown in **Table 4**.

Table-4: Results of % Recovery of ART.

Spike Level in %	Area	Amt. of Sample Added (µg/mL)	Amount of STD Added (µg/mL)	Total Amount Added (µg/mL)	Total Amount Found (µg/mL)	% Recovery	Mean	SD	%RSD
50%	4020472	10	5	15	15.04	**100.26**	**100.3**	**0.13**	**0.13**
	3928073	10	5	15	15.05	**100.33**			
	4053343	10	5	15	15.08	**100.53**			
100%	5298776	10	10	20	19.59	**97.95**	**98.85**	**0.80**	**0.81**
	5430902	10	10	20	19.9	**99.5**			
	5423268	10	10	20	19.82	**99.1**			
	6932465	10	15	25	25.2	**100.8**	**100.4**	**0.31**	**0.31**

150%	7028406	10	15	25	25.05	**100.2**			
	6948366	10	15	25	25.08	**100.32**			

Precision

The precision of the method was measured by the percentage relative standard deviation (% RSD) over the concentration range of high, middle and low QC samples respectively of drug during course of validation. The precision study was evaluated on the basis of % RSD value was found to be within limit. The % RSD values were found to be 1.80 ART stating that the developed method is precise. Results of precision study are shown in **Table 5**. Intra-day precision of the method was found to be 0.704 % RSD (**Table 6**). Inter-days precision of the method was found to be 0.930 % RSD (**Table 7**).

Table-5: Results of System Precision.

Sr. NO.	**Sample**	**Area**	**RT**
1	STD 1	4256321	2.961
2	STD 2	4311892	2.962
3	STD 3	4200369	2.966
4	STD 4	4298561	2.963
5	STD 5	4420152	2.963
6	STD 6	4365984	2.963
Average		**4308879**	**2.963**
SD		**257762.67**	**0.0053072**
% RSD		**1.80**	**0.61**
Tailing Factor		**1.22**	
Theoretical plates		**9491**	

Table-6: Intra-day Precision of ART

Time Interval	Conc. (µg/mL)	Area	Mean (n=3)	SD	% RSD
11.00 am	10 µg/mL	41316517	41972859.33	**568698.278**	**1.354919076**
		42282898			
		42319163			
	50 µg/mL	25236323	25107499.67	**118905.1295**	**0.473584113**
		25084220			
		25001956			
02.00 pm	10 µg/mL	40349131	40295031.33	**95784.11582**	**0.237707014**
		40351525			
		40184438			
	50 µg/mL	23218251	23121985.33	**194348.7766**	**0.840536718**
		22898293			
		23249412			
05.00 pm	10 µg/mL	38349131	38295031.33	**95784.11581**	**0.250121524**
		38351525			
		38184438			
	50 µg/mL	18218251	18121985.33	**194348.7766**	**1.072447489**
		17898293			
		18249412			

Table-7: Inter-days Precision of ART

Time Interval	Conc. (µg/mL)	Area	Mean (n=3)	SD	% RSD
11.00 am	10 µg/mL	41316517	41972859.33	**568698.278**	**1.354919076**
		42282898			

		42319163			
	50 µg/mL	25236323	25107499.67	**118905.1295**	**0.473584113**
		25084220			
		25001956			
02.00 pm	**10 µg/mL**	40349131	40295031.33	**95784.11582**	**0.237707014**
		40351525			
		40184438			
	50 µg/mL	23218251	23121985.33	**194348.7766**	**0.840536718**
		22898293			
		23249412			
05.00 pm	**10 µg/mL**	38349131	38295031.33	**95784.11581**	**0.250121524**
		38351525			
		38184438			
	50 µg/mL	18218251	18121985.33	**194348.7766**	**1.072447489**
		17898293			
		18249412			

Linearity and Range

The linearity was determined for ART. Linearity of the method was studied by injecting six concentrations of drug prepared in mobile phase and calibration curves were constructed by plotting peak area against the concentrations. The ART followed linearity in the concentration range of 10-60 µg/mL (**Table 8 and Fig. 6**).

Table-8: Results of Linearity Study of ART

Concentration (µg/mL)	**Area**
10	1629408
20	3699226
30	5773333
40	7777041

50	9900464
Intercept	**-43008**
Slope	**20319**
Correlation Coefficient (r^2)	**0.999**

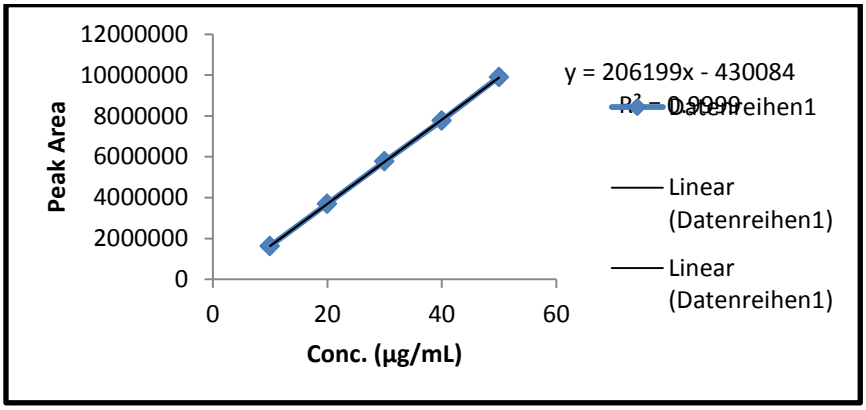

Fig. 6: Results of Linearity Study of ART.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD for ART was found to be 0.44 µg/mL. The LOQ for ART was found to be 1.44 µg/mL. The low values of LOD and LOQ indicates high sensitivity of the method (**Table 9**).

Table-9: LOD and LOQ of ART

Drug	LOD	LOQ
ART	0.4777 µg/mL	1.4478 µg/mL

Robustness study

Robustness of the method was studied by making deliberate changes in the chromatographic conditions (change in flow rate, wavelength, mobile phase ratio and pH) and the effects on the results were examined. The low value changes of theoretical plates, tailing factor indicating robustness of the method (**Table 10, 11, 12, and 13**).

Table-10: Results of Robustness - Change in flow rate.

Sample	As Such (0.8 mL/min)	0.6 mL/min	1.0 mL/min
STD 1	5791714	4629696	2784339
STD 2	5777522	4447745	2797167
STD 3	5855268	4537108	2772137
STD 4	5745734	4526541	2737624

STD 5	5951084	4578955	2738639
Average	**5824264.4**	**4544009**	**2765981.2**
SD	**81326.25**	**67422.17**	**26922.03**
% RSD	**1.396335**	**1.483759**	**0.973326**
Tailing Factor	**1.328**	**1.318**	**1.23**
Theoretical Plates	**10297**	**11586**	**8501**

Table-11: Results of Robustness - Change in Wavelength.

Sample	As Such (210 nm)	208 nm	212 nm
STD 1	5791714	2107378	1261390
STD 2	5777522	2183105	1286935
STD 3	5855268	2125802	1294763
STD 4	5745734	2161841	1274754
STD 5	5951084	2148611	1288314
Average	**5824264.4**	**2145347.4**	**1281231.2**
SD	**81326.25**	**29715.418**	**13242.321**
% RSD	**1.396335**	**1.3851098**	**1.0335622**
Tailing Factor	**1.328**	**1.36**	**1.32**
Theoretical Plates	**10297**	**10318**	**10407**

Table-12: Results of Robustness - Change in Mobile Phase Ratio.

Sample	As Such (30:70)	32:68	28:72
STD 1	5791714	14474678	3829379
STD 2	5777522	14764781	3858057
STD 3	5855268	14462216	3782636
STD 4	5745734	15005261	3777319
STD 5	5951084	14740984	3816731
Average	**5824264.4**	**14689584**	**3812824.4**
SD	**81326.25**	**226842.07**	**33568.129**
% RSD	**1.396335**	**1.5442375**	**0.8804006**
Tailing Factor	**1.328**	**1.248**	**1.266**
Theoretical Plates	**10297**	**9825**	**10157**

Table-13: Results of Robustness - Change in pH.

Sample	As Such Buffer- 3.0	Buffer- 2.8	Buffer- 3.2
STD 1	5791714	4474678	4829379
STD 2	5777522	4564781	4858057
STD 3	5855268	4462216	4782636
STD 4	5745734	4505261	4777319
STD 5	5951084	4540984	4816731
Average	**5824264.4**	**4509584**	**4812824.4**
SD	**81326.25**	**43340.2625**	**33568.12972**
% RSD	**1.396335201**	**0.96107008**	**0.697472563**
Tailing Factor	**1.328**	**1.236**	**1.25**
Theoretical Plates	**10297**	**10825.4**	**12157.6**

Stability of Solution

Short-term stability study indicated that sample solutions remained stable for 24 h even at room temperature (25^{o}C). The results of table top stability of solution are shown in **Table 14**.

Table-14: Stability of ART in Sample Solution (n=6).

Stability in hours	% Assay
0	101.8
3	102.1
6	102.38
12	102.49
24	102.64
Average	**102.28**
SD	**0.3340**
% RSD	**0.3266**

Forced Degradation Study

In acidic conditions it was found that around 3.09 % of the drugs content was degraded (**Fig. 7-8**) whereas in alkaline condition it was found to be around 2.18 % of the drugs content was degraded and impurity peak was found at 3.85 min (**Fig. 9-10**). Major degradation was observed in oxidative condition in which drug products were degraded up to 49.99 %. (**Fig. 11-12**). While slightly degradation was observed under the neutral condition (**Fig. 13-14**). In thermal

condition the drugs were placed in Hot Air Oven and the degradation was found to be 4.34 % **(Fig. 15-16).** In photolytic condition where the drugs were directly exposed to sunlight the degradation was found to be 17.95 % **(Fig. 17-18).** The results of forced degradation study are shown in **Table 15**.

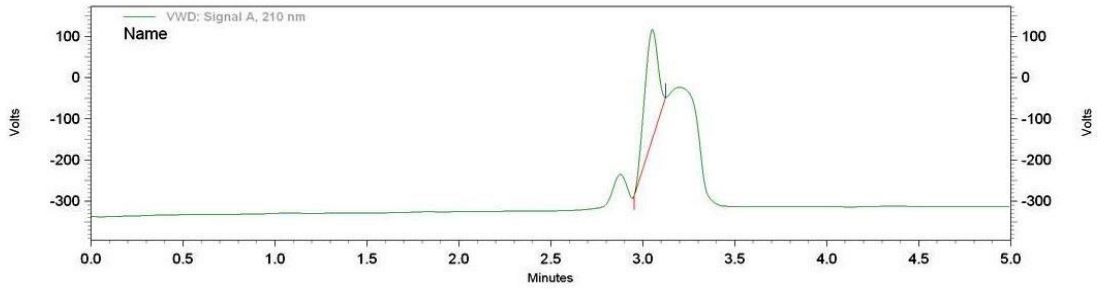

Fig. 7: Chromatogram for standard drug in 5 N HCl.

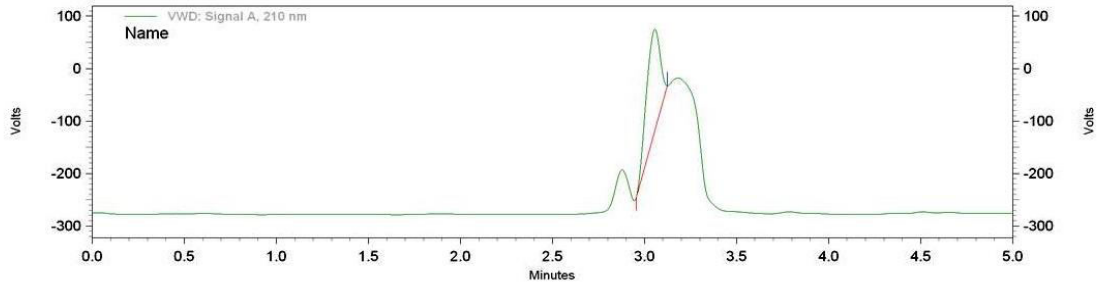

Fig. 8: Chromatogram for formulation in 5 N HCl.

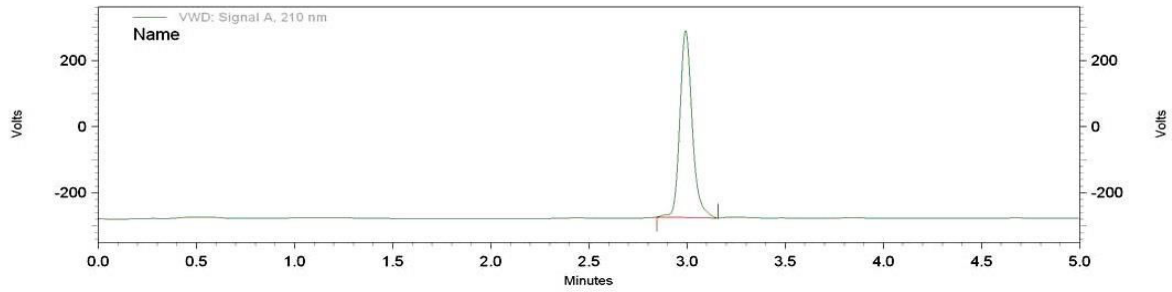

Fig. 9: Chromatogram for standard drug in 5 N NaOH.

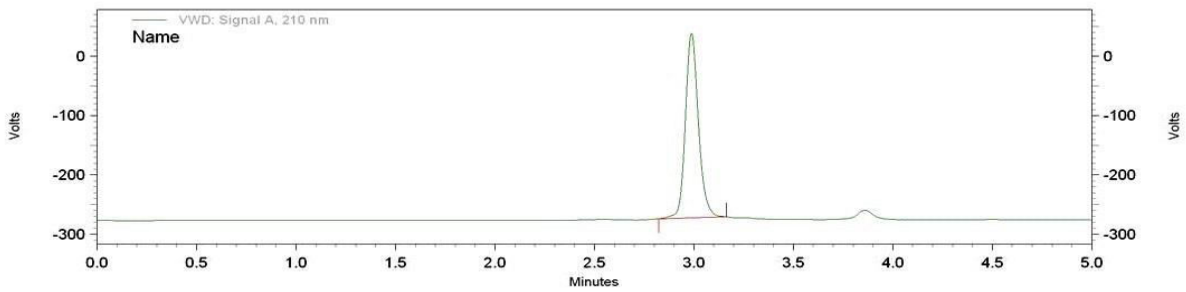

Fig. 10: Chromatogram for formulation in 5 N NaOH.

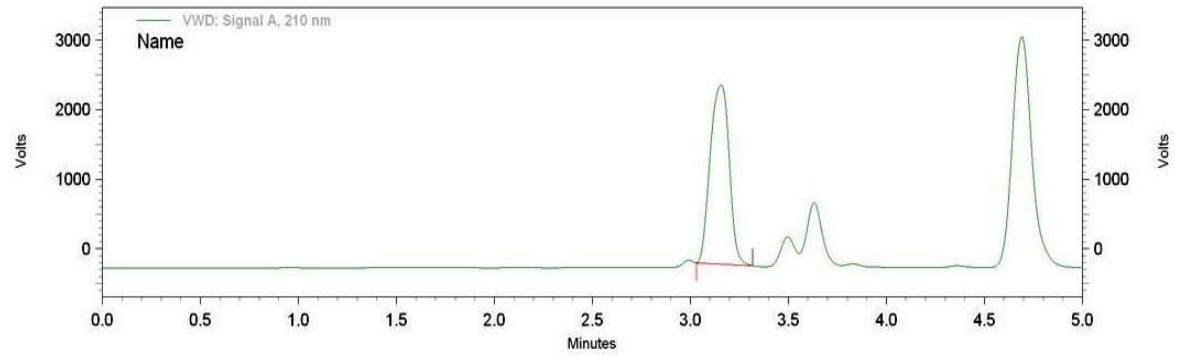

Fig. 11: Chromatogram for standard in 3% H_2O_2.

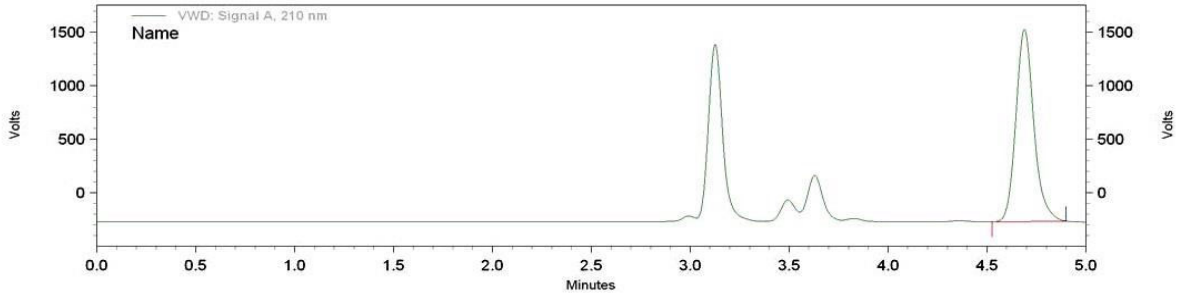

Fig. 12: Chromatogram for formulation in 3% H_2O_2.

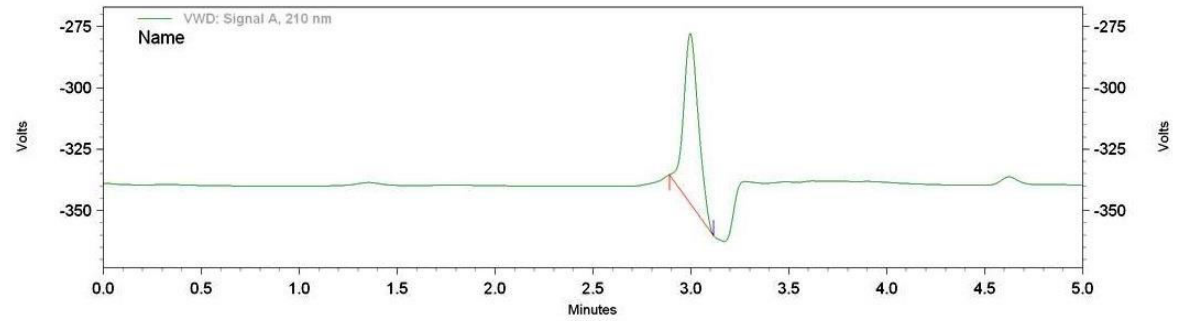

Fig. 13: Chromatogram for standard in Neutral degradation.

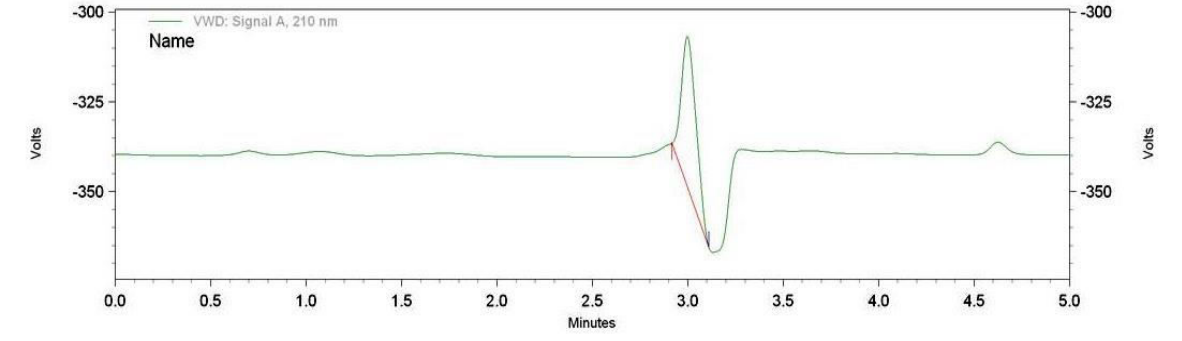

Fig. 14: Chromatogram for formulation in Neutral degradation.

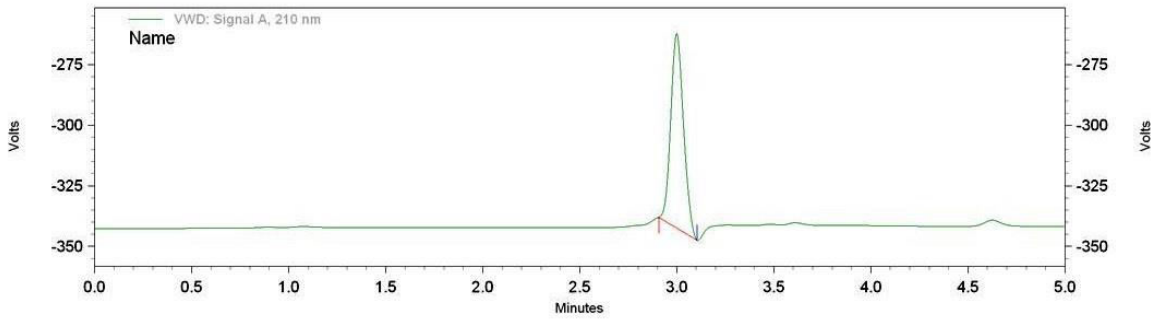

Fig. 15: Chromatogram for standard in hot air oven.

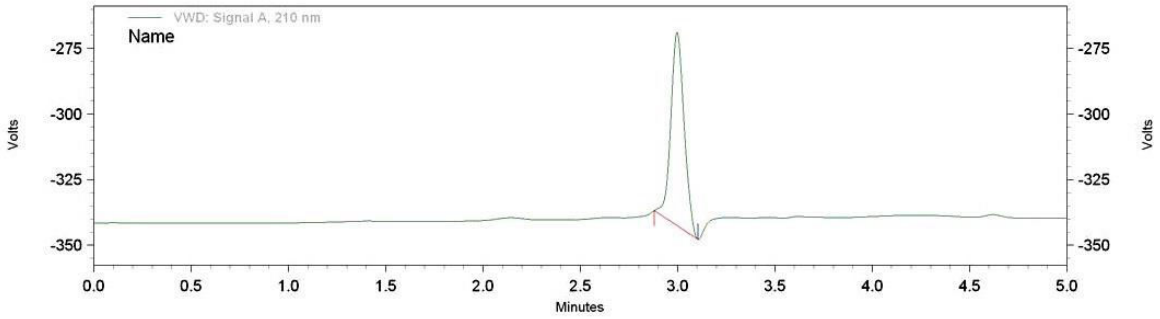

Fig. 16: Chromatogram for formulation in hot air oven.

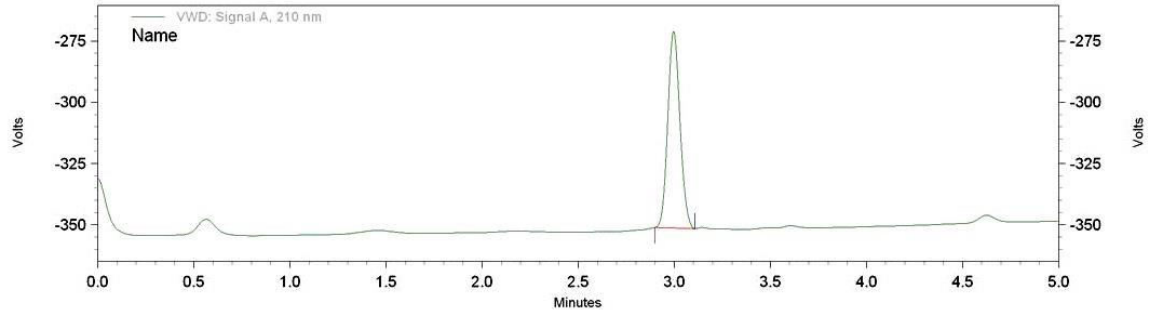

Fig. 17: Chromatogram for standard in direct sunlight.

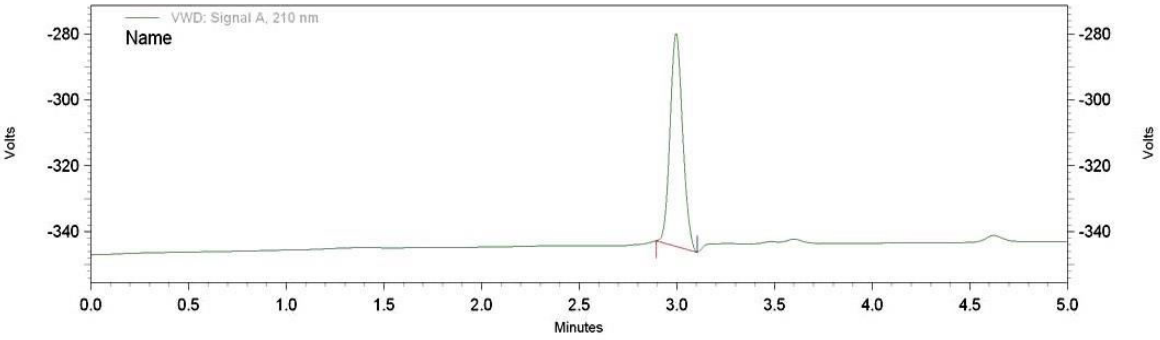

Fig. 18: Chromatogram for formulation in direct sunlight.

Table 15: Results of Forced Degradation Study of Sample Solution.

Stress Condition	Drug	% Assay of drug after degradation	% Degradation
Acidic Degradation	Artemether	96.90268207	3.097317932
Alkali Degradation	Artemether	97.81784273	2.182157274
Peroxide Degradation	Artemether	50.00142967	49.99857033
Photolytic Degradation	Artemether	82.04381042	17.95618958
Thermal Degradation	Artemether	95.65129441	4.348705585
Neutral Degradation	Artemether	62.08950058	37.91049942

Conclusion

The research work represents a simple and validated HPLC method for quantitative estimation of Artemether in the presence of degradation products. The developed method is specific, accurate, precise and robust. The method was linear response in stated range and is accurate and precise. All the degradation products formed during forced decomposition studies were well separated from the analyte peaks demonstrating that the developed method is specific and stability indicating**.** The method could be applied with success even to the analysis of marketed products of ART injection formulation, as no interference was observed due to excipients along with the degradants present therein.

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