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A Rapid RP-HPLC Method for Determination of Dihydroartimisinine Derivatives Followed by LC-MS/MS Characterization to Confirm its Stability during Routine Analysis

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ABSTRACT:

Current research work describes rapid reverse phase high performance chromatographic method for estimation of dihydroartimisinine derivative namely arteether and artemether from bulk. This method was extended for liquid chromatography-mass spectrometry analysis to confirm the stability of component under analysis. The mentioned components were separated on HYPERSIL® ODS C18 column (200 mm× 4.6 mm). The components were eluted by application of Methanol: Acetonitrile (60:40 v/v) as mobile phase at 216 nm and flow rate of 1 ml/minute. Retention time for artemether and arteether was found to be 1.52 and 2.70 minutes respectively. For LC-MS/MS characterization mobile phase was spiked with 0.1% ammonium format and ESI was utilized as ionization source in combination with ion trap as mass analyzer. The components were found to be stable during and after their evaluation period. Method was found to be linear in concentration range of 10 to 50 µg/ml for both components. The method was further validated as per ICH guidelines to confirm its suitability for routine analysis**.**

KEY WORDS: Arteether, Artemether, Reverse phase - High performance Chromatography, Analytical Method validation, Hyphenated Tandem mass spectroscopy.

INTRODUCTION:

Artemether (ARM) is widely used artimisinine derivative use for treatment of uncomplicated malaria and arteether (ARE) is semisynthetic derivative of artimisinine and both the drug belongs WHO's essential medicine list [1-2]. ARE and ARM chemically belongs to the class of benzodioxepine (Figure 1)[3].

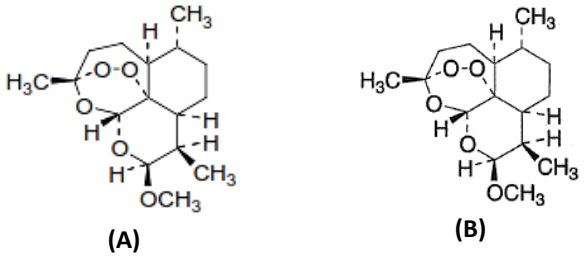


Figure 1: Structure of Artemether (A) and Arteether (B)

Review of literature had suggested that all dihydroartimisinine derivative are quite unstable and specifically prone to degradation in acidic media and media containing water by mechanism of hydrolysis [4]. So care must be taken that the estimation condition must avoid such degradation condition. Many RP HPLC methods are available for estimation of dihydroartimisinine

derivative but all method are unable to justify the stability of arteether and artemether during analysis as all methods has incorporated either water or acid as a component of mobile phase [5-9]. HTPLC methods are also available for determination of same but they are not sensitive enough and problem of stability still remains in question [10-11]. One LC-MS method is also available but they have incorporated glacial acetic acid as mobile phase component so again stability issue was unresolved [12-13]. Furthermore all reported methods were too much time consuming and requires complex reagents.

By keeping all problems in mind the main objective of work was to develop a RP- HPLC method which can separate and quantify ARM and ARE rapidly and also assures the stability. The stability of eluting components was monitored by LC-MS/MS and eluting components were found to be stable during and after analysis. Method was slightly modified by incorporating ammonium formate to mobile phase so that method can become compatible to MS. The developed method was further validated as per ICH Q2 R1 guidelines to adjudge its suitability for routine quality control use. The method was extremely quick in terms of elution and justification was made by evaluating column dead volume.

MATERIALS AND METHODS

Materials:

The gratis samples of dihydroartimisinine derivatives namely artemether (99.97% pure) and arteether (99.89 % pure) were provided by Osaka pharmaceuticals, Sakarda, Vasad. HPLC grade methanol and acetonitrile (MERCK) was provided by Smt. S.M. Shah Pharmacy College. Ammonium formate (Extra pure, HPLC grade) was procured from local market. MILI Q grade water was purchased from SICART, Vallabh Vidyanagar

Instrument and experimental condition:

Total method was developed on SHIMADZU LC 2010 system (Binary gradient with PDA detection). Elution was performed in isocratic mode on HYPERSIL® ODS C_{18} column (200 mm× 4.6 mm) at flow rate of 1 ml/minute. System was monitored by LC Solution software. LC-MS/MS was performed on Surveyor plus LC system equipped with ESI as ionization source and ion trap mass analyzer. Spraying voltage was kept at 4.5 kw and collision cell temperature was kept at 200ºC and system was monitored by X caliber software.

Preparation of working solutions:

About 100 mg of ARM and ARE was weighed and transferred to 100 ml volumetric flask covered with aluminum foil in order to protect drug from light. 50 ml of methanol was added to volumetric flask and it was sonicated for 2 minutes. Volume was made up to mark with methanol to give solution containing 1000 µg/ml of ARE and ARM. From above solution 1 ml aliquot was taken and was transferred to 10 ml volumetric flask and volume was made up to mark with methanol to give mixture containing 100 µg/ml of ARM and ARE respectively (Stock solution). From stock solution various aliquots ranging from 0.2 to 1.0 ml was taken and was transferred to 10 ml ambered colored volumetric flask and volume was made up to mark to give solutions containing 2-10 µg/ml of ARE and ARM.

Analytical Method Validation:

Linearity and Range:

For linearity and range studies various aliquots from stock solution100 µg/ml ranging from 0.2 to 1.0 ml was taken and was transferred to 10 ml ambered colored volumetric flask and volume was made up to mark to give solutions containing 2-10 µg/ml of ARE and ARM.. Resulting solutions were chromatographed as per optimized conditions and peak area and retention time were observed. Finally linear regression method was utilized to check linearity over the selected range (2-10 µg/ml of ARE and ARM).). Calibration curve was plotted for peak area (mV.sec) vs concentration (µg/ml).

Accuracy studies:

Accuracy of the method was assessed by spiking of blank (mobile phase) with standard at 50, 100 and 150 % of target concentration (4 µg/ml). Stock solution containing mixture of 100 µg/ml of ARE and ARM was prepared. Four 10 ml volumetric flask were prepared and marked as unspiked, 50 %, 100% and 150 %. Take 0.2 ml, 0.4 ml and 0.6 ml aliquots from stock solution and transfer it to 50%, 100 % and 150% flask respectively. Make volume to 10 ml with mobile phase. Unspiked flask only contains mobile phase. Inject 20 µl volume from each volumetric flask on to column and note the peak are for each drug at each level. Repeat the process for three time (n=3 determinations) and calculate RSD at each level for each drug. Calculate recovery of ARE and ARM by substituting values of peak area in linear regression equation.

Determination of LOD and LOQ:

For determination of LOD and LOQ statistical approach was used and data were compared with result directly from instrument.

$$Limit\ of\ Detection = 3.3 \times \frac{\sigma}{S}$$

$$Limit\ of\ Quantification = 10 \times \frac{\sigma}{S}$$

Where, σ = Standard deviation of Intercept and S= Mean of Slope

Instrumental results were obtained by visual inspection and from results of linearity and range. Comparison was made to check the reliability of results.

Robustness and ruggedness studies

For determining the robustness of the method various method parameters like flow rate, mobile phase composition were varies but still within the acceptable range. One cannot change detection wavelength for robustness studies as it is having significant effect on assay. Effect were also studied on retention time. Ruggedness of the method was assessed by varying method component like different column having same specification and different analyst. Change of such condition was observed for change in method results like assay value and retention time. 4 µg/ml solution was injected 1st on HYPERSIL ODS C_{18} (200 × 4.6 mm) column and same injection was made on to INERTSIL ODS C_{18} (200 × 4.6 mm) column and change of retention time and assay value was noted. Similarly solution were prepared by different analyst and injection were made to observe effect of different analyst on method reproducibility.

Assay studies:

For the assay of ARE and ARM solution containing mixture of 4 µg/ml of ARE and ARM was prepared and injected on to column. Procedure was repeated for 3 times and RSD was calculated. Peak area was substituted in to linear regression equation and concentration of ARE and ARM was found out.

Optimization of LC MS conditions:

Once the dihydroartimisinine derivatives were separated, the task was to make a compatible LC MS method. So trials where performed on Surveyor plus HPLC system (Component of Thermo scientific LC MS System with PDA Detector). For ionization of components EI source was utilized. Ion trap analyzer was used to monitor elute in range of 50-2000 amu (ITMS scan).To obtain a patent ion peak Ammonium formate was added to the solution From the obtained mass spectra stability of the ARE and ARM in given system after 24 hours was assessed.

RESULTS

Optimization of HPLC method:

Using optimized method the components were separated on Octa decyl Silane column (200 × 4.6 mm, 5 µm) column within 5 minutes of run time by application of Methanol: Acetonitrile (60:40, v/v) as mobile phase at flow rate of 1ml/minute and detection wavelength of 216 nm. The average elution time for ARE and ARM was found to be 1.52 ± 0.05 and 2.70 ± 0.07

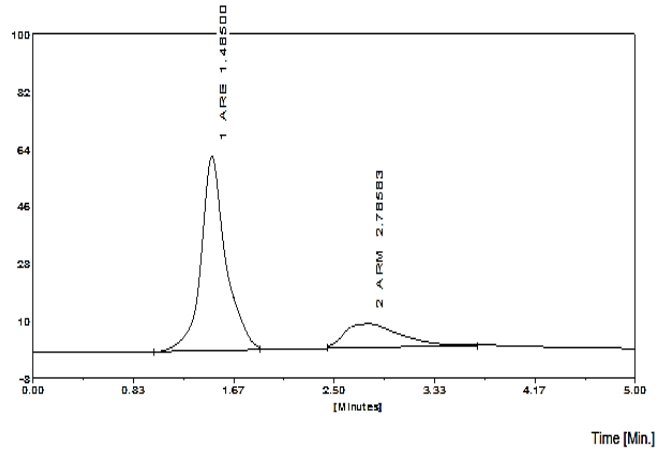
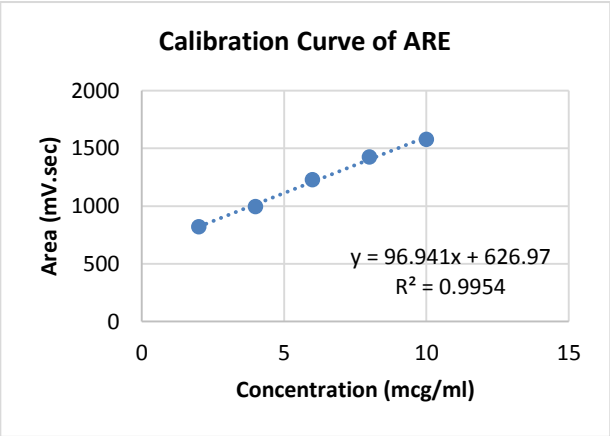


Figure 2: Chromatogram of ARE and ARM Using Optimized Conditions

Linearity and Range:

Method was found to be linear (Figure 2) over concentration range of 2-10 µg/ml of ARE and ARM with regression coefficient of 0.995 and 0.997 respectively (Table 1, Table 2).



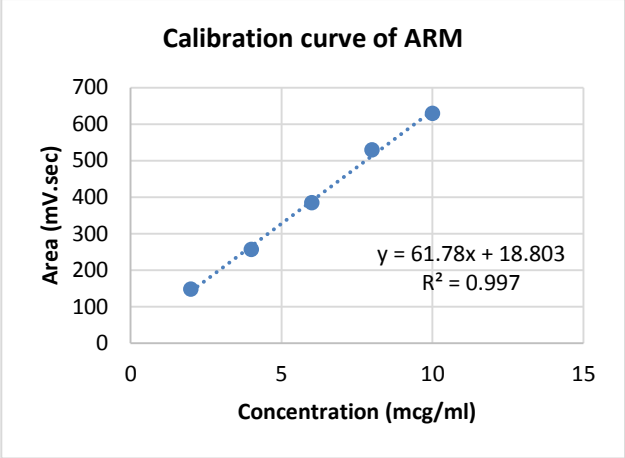


Figure 2: Linearity of ARE and ARM in the range of 2-10 µg/ml

Table 1: Linearity Studies of ARE

Concentration (µg/ml)	Area (mean ± S.D.)	R.S.D.
2 µg/ml	821.176 ± 7.23	0.88
4 µg/ml	993.93 ± 8.78	0.88
6 µg/ml	1228.13 ± 10.91	0.88
8 µg/ml	1424.57 ± 10.98	0.77
10 µg/ml	1575.26 ± 8.97	0.56
Regression Equation:	y = 96.941x + 626.97	
Linear Regression Coefficient :	r^2 = 0.9954	

Table 2: Linearity Studies of ARM

Concentration (µg/ml)	Area (mean ± S.D.)	R.S.D.
2 µg/ml	148.02± 1.36	0.92
4 µg/ml	256.18 ± 2.24	0.87
6 µg/ml	384.81 ± 3.14	0.81
8 µg/ml	528.96 ± 4.14	0.78
10 µg/ml	629.43 ± 4.63	0.73
Regression Equation:	y = 61.78x + 18.803	
Linear Regression Coefficient :	r^2 = 0.997	

Accuracy studies:

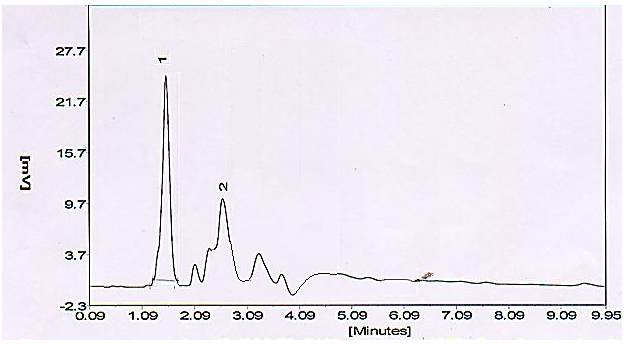
Method was found to be accurate with % recovery of 97.52 – 100.84 for ARE and 98.05-100.93 for ARM (Table 3)

Table 3: Accuracy data for ARE and ARM

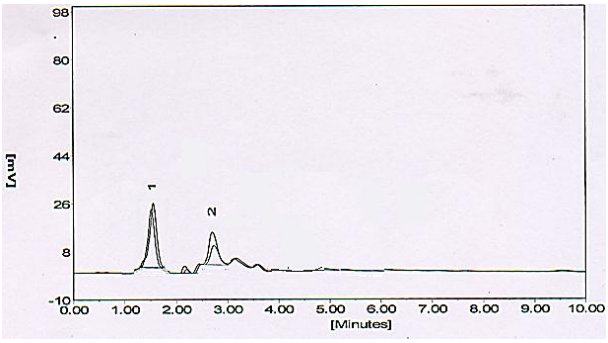
Drug	Level	Amount of Drug Present µg/ml	Amount of Drug Added µg/ml	Total mount of drug	Amount of drug recovered Mean ± S.D.	Mean Recovery ± S.D
ARE	**US**	0	-	-	-	-
	50%	0	2	2	1.97 ± 0.03	98.99 ± 2.39
	100%	0	4	4	3.90 ± 0.04	97.52 ± 0.66
	150%	0	6	6	6.05 ± 0.05	100.84 ± 0.86
ARM	**US**	0	-	-	-	-
	50%	0	2	2	2.01 ± 0.02	100.93 ± 1.01
	100%	0	4	4	3.02 ± 0.02	98.05 ± 0.68
	150%	0	6	6	5.91 ± 0.03	98.55 ± 0.64

Determination of LOD and LOQ:

Based upon mathematical equation LOD for ARE and ARM was found to be 0.22 and 0.04 µg/ml. LOQ for ARE and ARM based upon mathematical equation was found to be 0.66 and 0.13 respectively. Visual inspection was also performed to check LOD and LOQ (Figure 3)



(A)



(B)

Figure 3: (A) LOD for ARE and ARM at 0.4 ppm (B) LOQ for ARE and ARM at 0.8 ppm

Robustness and ruggedness studies

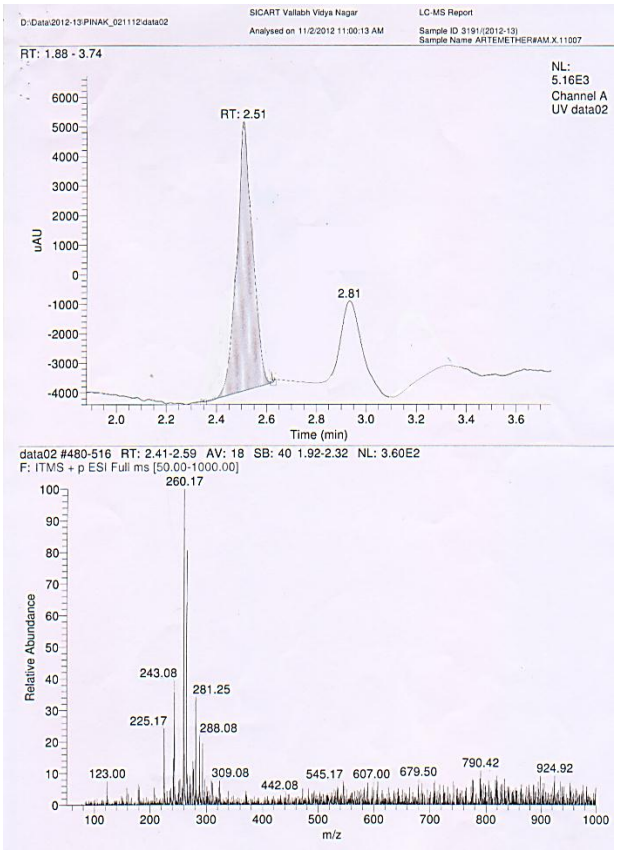
Method was found to be robust as there was no significant change by minor change in method parameters (Table 4). Method was found to be rugged as there was no significant difference observed when method was operated on different conditions (Table 5).

Table 4: Robustness studies

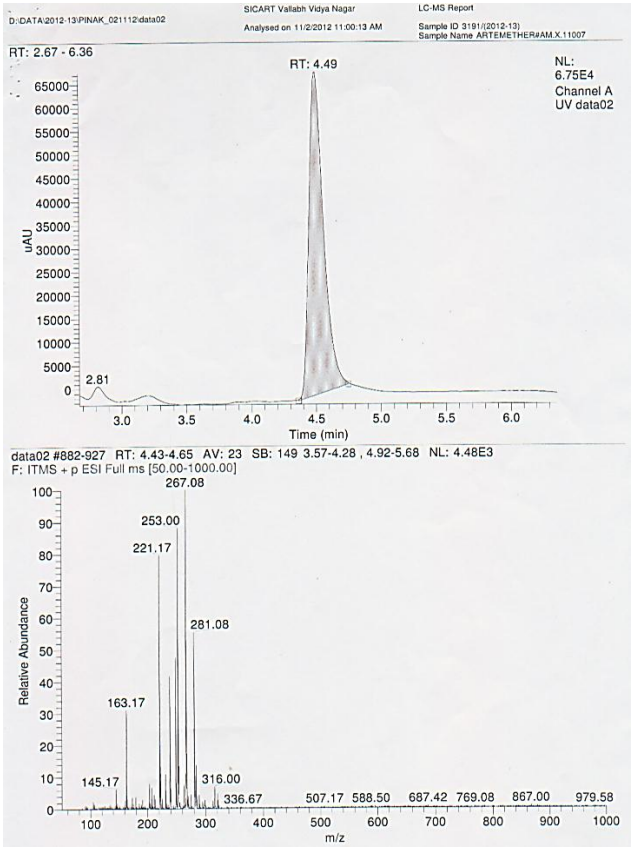
Parameter	Level of Change	Effect on assay value			
		ARE		ARM	
		Assay ± S.D.	CV	Assay ± S.D.	CV
Flow Rate	0.9 ml/min	94.47± 1.18	1.15	96.13 ± 1.24	1.29
	1.1 ml/min	97.05 ± 1.59	1.64	98.29 ± 1.15	1.18
Mobile Phase composition	58:42: 0.1	97.48 ± 1.73	1.78	98.56 ± 0.99	1.00
	62:38:0.1	96.59 ± 0.94	0.97	95.57 ± 0.51	0.54

Table 5: Ruggedness studies

Parameter Change		Result of T test	Value from table	Inference
Different Columns				
HYPERSIL	**INERTSIL**	0.64 (ARE)	2.78	No significant difference
		0.03 (ARM)	2.78	No significant difference
Different Analyst (same Column)				
ANALYST 1	**ANALYST 2**	0.69 (ARE)	2.78	No significant difference
		0.23 (ARM)	2.78	No significant difference



(A)



(B)

Figure 4: LC-MS/MS spectra after 24 hours (A) ARE (B) ARM

Assay studies:

When method was applied to synthetic mixture of ARE and ARM, % of ARE and ARM was found to be 98.29 ± 1.07 and 98.31 ± 0.80 respectively.

Matrix	Actual Concentration (µg/ml)		Amount obtained (µg/ml)		% Assay	
Synthetic mixture	ARE	ARM	ARE	ARM	ARE	ARM
	4	4	3.93 ± 0.03	3.93 ± 0.04	98.29 ± 1.07	98.31 ± 0.80

DISCUSSION:

In developing LC MS method it was initially found that the obtained M+1 peak in mass were too weak so ammonium formate was added to the system. Now ammonium adducts of ARE and ARM was confirmed by fragmentation pattern and after 24 hours the mentioned drugs was found to be stable. Based upon fragmentation pattern structures were identified and mass spectra was compared with reference spectra from literature. Method was originally developed on 200 mm RP C_{18} column but in LC MS studies 250 mm column was used. Repeatability on that column was ensured by taking chromatogram on same length column at laboratory.

Molecular weight of ARM is 298 mole but its ammonium adduct ($M+NH_4$) peak at 316 m/z. The precursor-product ion pairs at 316/267 was obtained for ARM. Ion observed at 281 is due to loss of water from [M+H] peak and most abundant ion at 267 appears due to opening of ring structure of ARM.

Molecular weight of ARE is 312 gm/mole and its ammonium adduct shows peak at 330 m/z. The precursor-product ion pairs at 316/267 was obtained for ARE. Furthermore literature says that ARE shows strong $[M - OR]^+$ peaks arising from the molecular ion adduct and the loss of the alkoxy group of the side chain. So here peak at 281 m/z corresponds to $[M-OCH_3]$ peak. Further loss of CH_2 (molecular mass=14) leads to production of precursor ion at 267 m/z. mostly base peak appears at 267 in case of artimisinine derivatives because there are several ways (fragmentation modes) by fragment of 267 m/z can be produced.

CONCLUSION:

For total study performed it was concluded that the method was extremely quick for the separation and estimation of ARE and ARM from bulk as both components were separated within 5 minutes. The method can assure the stability of components for period of 24 hours without any sign of degradation which was the major drawback of previously reported methods. Furthermore the method was validated as per ICH Q2R1 guidelines so that it can be applied for routine estimation of ARM and ARE from bulk.

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studies.

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