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HPLC study of related substances in artemether and Lumefantrine tablets

Manisha U.Phadke[1]*, Vivek K.Jadhav[2], Rajendra K.Jadhav[1], Yatish K.Bansal[1], Dattatray Patil[1], Shraddha Pawar[1], Pranita Padwal[1]

[1]Ipca Laboratories Ltd, Corporate Analytical development Lab, Charkop, Kandivali (W), Mumbai-400067, (INDIA)

[2]Vibha Pharma, Andheri (West), Mumbai-400056, (INDIA)

E-mail : manishap@ipca.co.in

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ABSTRACT

A simple, sensitive reverse phase liquid chromatographic method involving flow gradient and wave length gradient has been developed for detection and quantitative determination of related compounds namely dihydroartemisinin, alpha artemether, artemisinin, impurity A, PCB and DBA in Artemether and Lumefantrine tablets, used as an antimalarial fixed dose combination. Efficient chromatographic separation was achieved on 250 × 4.6mm, 5µm particle size, Water Symmetry C18 column, with mobile phase combination containing buffer with decane sulphonic acid sodium salt, sodium dihydrogen phosphate monohydrate and triethylamine in 1000ml water. Adjust the pH to 2.3 with Orthophosphoric Acid. and acetonitrile delivered in gradient mode and quantification carried out at wavelength 210nm and 380 nm at the flow rate of 1.0 mL min^{-1} upto 25 mins and then increased to 2.0 mL min^{-} upto 50 mins. The chromatographic conditions were optimized to avoid interferences from the excipients as well to achieve acceptable resolution between dihydroartemisinin, imput\rity A and and artemether and also well between PCB and DBA and Lumefantrine. The developed method, validated according to the ICH Q2R guidelines, met the pharmaceutical analysis requirements and can be successfully applied for intended purpose to establish the product quality.

KEYWORDS

Artemether;
Lumefantrine;
ACT method validation;
Column liquid chromatography;
Pharmaceutical preparation;
Degradants.

INTRODUCTION

Malaria, the most important parasitic disease of humans, remains a major health and economic burden in most tropical countries. Malaria is a major cause of death equal with HIV/AIDS and tuberculosis. The mortality and morbidity associated with malaria have a crippling effect on the economies of endemic countries[1].

It afflicts more than 500 million people, causing from 1.7 million to 2.5 million deaths each year[2]. It occurs in over 90 countries worldwide.

According to NAMP, total malaria cases in 2000 was 2.02 million, out of which 1.05 million was the total P. falciparum cases. Thus there has been increase in P. falciparum percentage from 26% in 1965 to 50% in 2000[3].

Plasmodium falciparum is responsible for most morbidity and mortality. It causes serious complications

like jaundice, renal failure, cerebral malaria. The main obstacle to malaria control is the emergence of drug resistant strains of Plasmodium falciparum. Emergence of resistance in P. falciparum to antimalarial drugs increases malaria morbidity, mortality and treatment cost. Chloroquine resistance is a major contributor to the increasing malaria-related morbidity and mortality. Malaria control efforts have been greatly affected by the emergence and spread of chloroquine resistance.

Increasing drug resistance limits the choice of efficacious chemotherapy against Plasmodium falciparum malaria. Combination therapy can both improve treatment and provide important public health benefits if it curbs the spread of parasites harbouring resistance genes. Thus, drug combinations must be identified which minimise gametocyte emergence in treated cases, and so prevent selective transmission of parasites resistant to any of the partner drugs.

Artemisinin and its derivatives are renowned for their potent antimalarial activity. The clinical efficacy of this group of drugs is characterised by an almost immediate onset and rapid reduction of parasitemia, with complete clearance in most cases within 48 hours. Efficacy is high even in areas with multidrug resistant parasite strains[4]. At present, it is the only group of antimalarial drugs to which resistance to P.falciparum has not yet developed in the field. Toxicological studies in animals have shown that the toxicity of artemisinin, artemether and artesunate is much less than that of chloroquine. Prospective clinical studies of over 10,000 patients and the use of artemisinin drugs in several million patients, has not shown any serious drug related adverse effects

As per WHO, to improve efficacy and delay the onset of resistance, artemisinin drugs should always be used in combination with another effective antimalarial.

Artemisinin (qinghaosu), artesunate, artemether and dihydroartemisinin have all been used in combination with other antimalarial drugs for the treatment of malaria. Of all of these drugs, artesunate has the most documented clinical information.

There is a growing interest in using antimalarial combinations containing an artemisinin derivative as first-line treatment. The aim is to provide efficacious and safe antimalarial drug treatment while probably delaying the onset and spread of resistance to both drugs in the combination.

ACTs(artemetemisinin combination therapy) combine the rapid schizontocidal activity of an artemisinin derivative (artesunate, artemether or dihydroartemisinin) with a longer-half-life partner drug[5].

It is the most rational way to use the few antimalarials available, maximising the benefits to the patients while minimising the risk of losing efficacy, secondary to the development of resistance. When used in combination with other effective antimalarials, the artemisinin derivatives (most artesunate and artemether) have constantly achieved very high parasitological cure rates even against multidrug resistant strains. In these emergencies when mortality is high, artemisinin derivatives save lives because of their speed of action. Given orally, they are superior to intravenous quinine in patients with uncomplicated hyperparasitaemia.

Because of the short half-life of artemisinin derivatives, their use as monotherapy requires daily doses over a period of 7 days. Combination of one of these drugs with a longer half-life partner antimalarial drug allows a reduction in the duration of antimalarial treatment while at the same time enhancing efficacy and reducing the likelihood of resistance development. The major immediate effect of the artemisinin component is to reduce the parasite biomass. The residual biomass is exposed to maximum concentrations of the partner drug, well above its minimum inhibitory concentration, resulting in a lesser likelihood of resistant mutations breaking through[6].

In addition, the impact of artemisinin derivative on gametocyte carriage means that even if a parasite has survived the double action of the drugs, the probability that it will be transmitted is low.

The World Health Organization has endorsed ACT as first-line treatment where the potentially life-threatening parasite Plasmodium falciparum is the predominant infecting species.

The particular features of ACT relate to the unique mode of action of the artemisinin component, which includes the following:

- Rapid and substantial reduction of the parasite biomass,
- Rapid parasite clearance,
- Rapid resolution of clinical symptoms,

Therefore, it is especially important to ensure the quality of anti-malarial drugs . A combination tablet for-

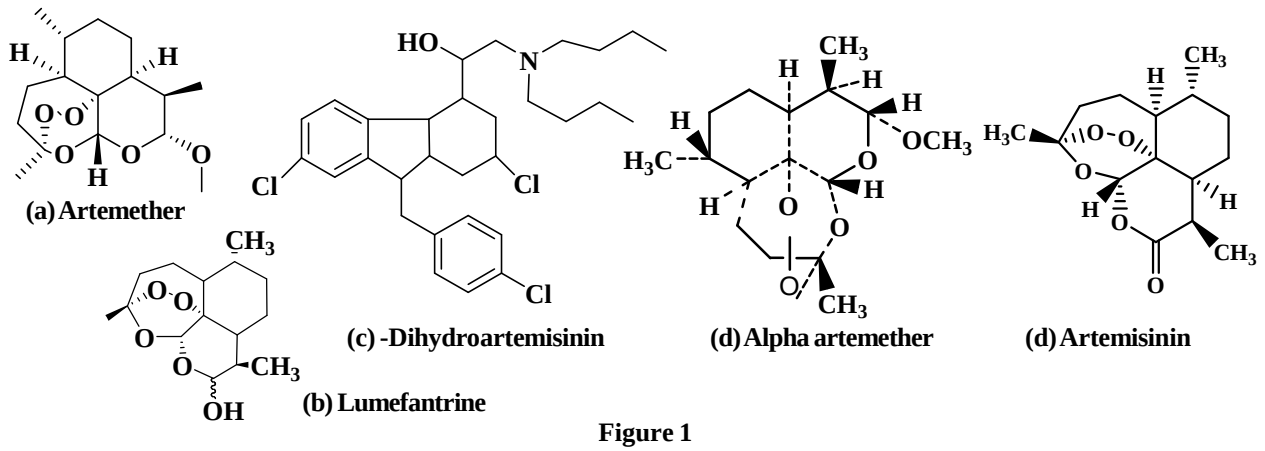

Figure 1

mulation is beneficial in terms of its convenience and patient compliance. Artemether (AM), 3R,5aS,6R, 8aS,9R,10S,12R,12aR)-Decahydro-10-methoxy-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin., Its molecular formula is $C_{16}H_{26}O_5$ and its molecular weight is 298.4. is used in the treatment of malaria(Figure 1a) and Lumefantrine chemically 1R,S)-2-Dibutylamino-1-{2,7-dichloro-9-[(Z)(4-chloro benzylidene)-9H-fluoren-4-yl}-ethanol.and its molecular weight is 528.3. (Figure 1b), is also antimalarial active substance.

The safety of a drug is dependent not only on the toxicological properties of the active substance itself, but also on its pharmaceutical impurities, which consist of reaction by-products, generated during synthesis of drug substances and degradation products formed during the formulation manufacturing process and/or storage of drug substances or formulated products. Determinations of drug impurity and drug degradation products are very important from both pharmacological and toxicological perspectives. Establishment of monitoring methods for impurities and degradation products during pharmaceutical development is necessary because of their potential toxicity[7,8]. High performance liquid chromatography (HPLC) is an extensively used technique in the pharmaceutical industry due to the availability of fully automated systems, excellent quantitative precision, accuracy, broad linear dynamic range and availability of a wide variety of column stationary phases. The aim of this study was to develop LC method for simultaneous determination of known impurities along with unknown impurities of AM and LU in the combination pharmaceutical drug product.

Dihydroartemisinin (DHA), 3R,5aS,6R,8aS,9R, 12S,12aR)-octahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4.3-j]-1,2-benzodioxepin-10(3H)-one. [Figure 1c], alpha artemether chemically described as 3R,5aS,6R,8aS,9R,10R,12aR)-10-methoxy-3,6,9-trimethyldecahydro-3,12-epoxy-12H-pyrano[4,3]1,2-benzodioxepine, [Figure 1d], artemisinin,[Figure 1e][9] and Lumefantrine are known impurities of AM and LU respectively and hence considered for development. Thin layer chromatographic methods have been reported for the determination of DHA,Artemeisin,alpha artemether, impurity A in artemether active substance and artemether and lumefantrine tablets in Intenational Pharmacopoea. Literature search revealed that several analytical methods are available of determination of AM separately in formulations, in biological fluids and in presence of other anti-malarial combinations agents[10-14]. Recently published method for the determination of AM along with its impurities by TLC and LU by liquid chromatography limits its application by separately carrying out the determination[15], A TLC method is also reported for identification and determination of lumefantrine and its impurities in active drug substance[16]. If the reported individual methods are applied for the related substances analysis of tablets containing AM and LU, it would require double time of analysis, method would not be rapid, less expensive or economical, whereas simultaneous determination of related substances would save analysis time and also economy. So far, to our present knowledge, there is no method for concomitant determination of impurities of AM and LU in the combination product using single chromatographic conditions. In the work, discussed in this paper, we therefore fo-

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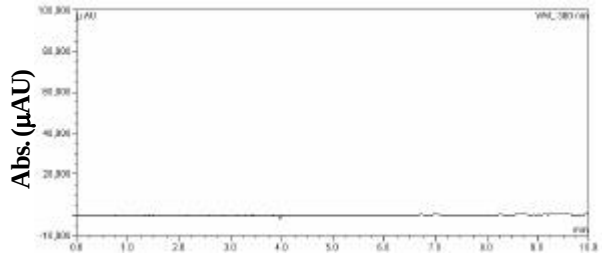
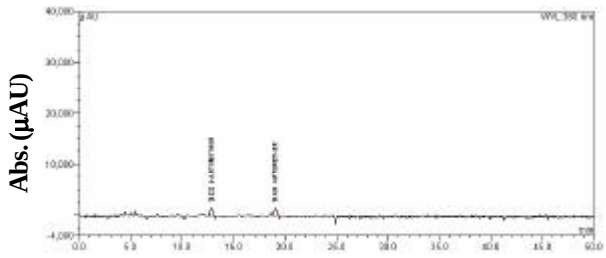
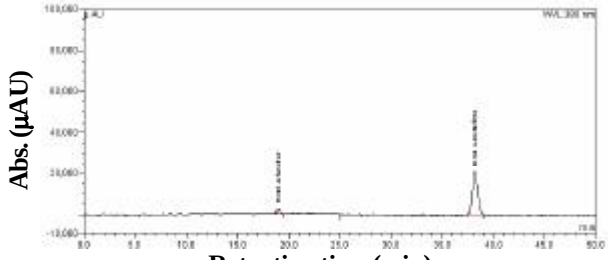


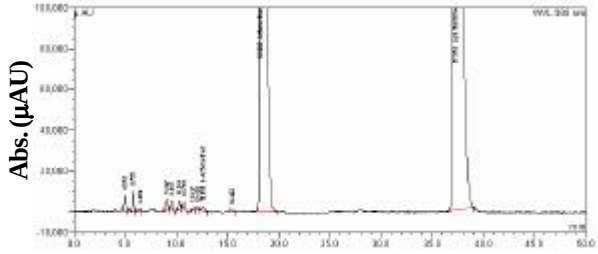
(a)-Typical HPLC chromatogram of resolution solution



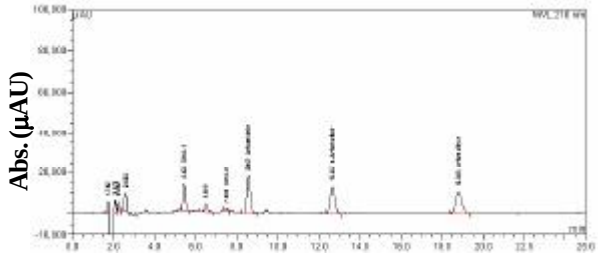
(a)-Typical HPLC chromatogram of resolution solution



(c) -Typical HPLC chromatogram of diluted standard solution



(d)-Typical HPLC chromatogram of sample solution



(e)- Typical HPLC chromatogram of impurity mixture

Figure 2

cused on finding optimum HPLC conditions with flow and wave length gradient elution for separation and quantitation of their potential impurities in AM and LU in fixed dosage form and validation as per ICH guidance documents. The investigated validation elements showed the method has acceptable specificity, accuracy, linearity, precision, robustness and high sensitivity with quantitation limits ranging from 0.744µg mL^{-1}, 0.636µg mL^{-1} and 0.468µg mL^{-1} dihydroartemisini, artemether and lumefantrine respectively. The method is carried out with commercially available and conventional HPLC equipment with easy sample preparation .It is simple, accurate and reproducible for the quantitation of the impurities from the formulation. Figure 2 (a-d) represent specimen chromatograms of diluent,resolution,diluted standard and sample preparations.

MATERIALS AND METHODS

Chemical and reagents

All working standards of AM, LU and impurities like DHA, alpha artemether, artemisinin, impurity A of AM, PCB and DBA of LU were procured from Ipca laboratories Ltd, Mumbai, India .Combination product of AM and LU (label claim : AM 80mg and LU 480mg) of Ipca Laboratories Ltd, were used for the development and validation. Acetonitrile of HPLC grades, decane sulphonic acid sodium salt, sodium dihydrogen phosphate monohydrate and triethylamine were procured from Merck (India). Milli-Q water was used. GF/C filter paper was obtained from Whatmann. All dilutions were prepared in standard volumetric flasks.

Instrumentation and chromatographic conditions

Chromatography was performed with Waters Alliance system, Waters 2695 separation module and Waters 2996 photo diode array detector. The output signal was monitored and processed using chromeleon software. A column Waters symmetry C18 column, (250 × 4.6mm dimensions) having particle size 5µm was used for the separation as a stationary phase. The buffer was prepared by dissolving 5.6g of decane sulphonic acid sodium salt, 2.8g sodium dihydrogen phosphate monohydrate and 5ml of triethylamine in 1000mL water, pH adjusted to 2.3 with ortho-phophoric acid AR grade

(88%) and diluting to 1000mL with water, filtered through GF/C filter and degassed in ultrasonic bath prior to use as mobile phase A. Acetonitrile was used as mobile phase B. The flow rate was 1.0mL min^{-1}. The injection volume amounted to 50µl The analysis was

Time	Buffer	Acetonitrile	Flow rate
0.0 min.	40	60	1.0 ml / min.
25.0 min.	40	60	1.0 ml / min.
26.0 min.	40	60	2.0 ml / min.
45.0 min.	40	60	2.0 ml / min.
46.0 min.	40	60	1.0 ml / min.
50.0 min.	40	60	1.0 ml / min.

carried out under gradient condition as follows,

Time in minutes	Wavelength program
0 – 4 min	380nm
4 – 25 min	210nm
25 – 50 min	380nm

Gradient program

Wavelength programming

Detection was monitored at a wavelength of 210nm and 380 nm. A mixture of buffer (Buffer Preparation for Diluent: Dissolve 2.8 g of Sodium Dihydrogen Phosphate Monohydrate, in 1000ml water. Adjust the pH to 2.3 with Orthophosphoric Acid.) and acetonitrile in the ratio of (25:75 v/v) was used as diluent in the preparation of analytical solutions.

System suitability solution

Resolution solution of 24µg mL^{-1} of artemether and 12µg mL^{-1} of α-artemether was used as system suitability solution.

Diluted standard solution

Standard stock solution of artemether (2400 µg mL^{-1}), lumefantrine (1440 µg mL^{-1}, DHA (960µg mL^{-1}) are prepared in diluent. Further stocks are mixed and diluted with diluent in such way that it has concentration of each of artemether, lumefantrine and DHA as 24µg mL^{-1}(equivalent to 0.5% with respect to artemether· 72µg mL^{-1} (equivalent to 0.25% of Lumefantrine w.r.t Lumefantrine in test sample) and 96µg mL^{-1}(equivalent to 2.0% of Dihydroartemisinin w.r.t Artemether in test sample.

Sample preparation

Twenty tablets were weighed and crushed to homogenous powder using a mortar and pestle. An accurately weighed portion of the powder, equivalent to 480mg of artemether into a 100ml volumetric flask, added 50ml diluent, and shaken for about 15 minutes. Then dispersed with the aid of ultrasound for 10 minutes with intermittent swirling The flask was further shaken with the means of mechanical shaker for 15 minutes and allowed to reach the ambient room temperature The volume was made up to 100mL with diluent and mixed. Filtered the solution GF/C.

RESULTS AND DISCUSSION

The main target of the chromatographic method is to detect and quantify the known impurities DHA, Artemeisin, alpha artemether and impurity A of artemether and PCB and DBA of lumefantrine in combination tablets by utilizing same chromatographic setup in single run. Optimization of conditions for simple, accurate and reproducible analysis involves analyzing system suitability solution on varying stationary phase, strength of aqueous phase, pH, and proportion of acetonitrile-aqueous phase, flow rate and column temperature. Our preliminary experiments indicated that using different concentration of acetonitrile and even different pHs of the buffers did not produce suitable separation of DHA I and II and artemether. Hence, ion pair reagent (oppositely charged ion) in the mobile phase was used which reacts with them to form neutral ion pair enabling to retain on non-polar stationary phase. When different ion pairs were used like pentane salt, hexane salt, heptane salt and octane salt of sulphonic acid shows no proper resolution of artemisinin and artemether and peak shape of lumefantrine gets distorted hence Sodium salt of decane sulfonic acid was used as ion pair agent. Further both artemether and lumefantrine being strongly basic in nature, it becomes important to select suitable pH for simultaneously retaining and separating artemether and lumefantrine from its impurities. Due to the ionization capacity of these charged analytes, pH played an important role. It is determined by the pH of the mobile phase that in which form they exist and whether they can react with negative ion of sodium decane sulfonic acid to form a neutral ion pair to retain on non polar stationary phase. The

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TABLE 1: Validation summary report

Performance parameter	Evaluation parameters	Acceptance Limit	Results
System suitability	%RSD for 6 replicate injections of diluted standard solution.	NMT 10.0%	Artemether : 7.76% Lumefantrine : 1.91%
	Resolution between α-Artemether and Artemether.	NLT 5.0	11.46
	Theoretical plates for Artemether peak in resolution solultion	NLT 5000	16430
Determination of relative retention Time (RRT) for known impurities	To be determined from a synthetic mixture preparation containing active at 100% level and all known impurities at 1.0 % level	Report RRT considering principle peak of active as RRT 1.0.	**Impurities** / **RRT** (a) α-Artemether 0.686 (b) Artemisinin 0.464 (c) DHA 1 0.290 (d) DHA 2 0.407 (e) Imp A 0.266 (f) PCB 0.311 (g) DBA 0.583 (h) Lumefantrine 2.026 (i) Artemether 1.000
Determination of relative response factor (RRF)/Correction factor (C.F.)	Mixture of 100 ppm concentration each of DHA, α-Artemether and Artemisinin along with active Artemether and PCB and DBA along with active Lumefantrine is injected and responses of each impurity against the respective active is calculated.	Report the values.	**Impurities** / **RRF** / **C.F.** (a) α-Artemether 0.91 1.1 (b) Artemisinin 0.96 1.0 (c) DHA 0.57 1.8 (d) PCB 28.51 0.04 (e) DBA 24.56 0.04
	The retention time of the of α-Artemether , Artemisinin, DHA, PCB, DBA, and active Artemether and Lumefantrine.	Report the Retention time	α-Artemether : 12.771mins Artemisinin : 8.642 mins DHA1 : 5.396 mins DHA2 : 7.571 mins Imp A : 4.967 mins PCB : 5.783 mins DBA : 10.850 mins Artemether : 18.617 mins Lumefantrine : 37.721 mins
Specificity study	Evaluation of interference with active peak and related substance peaks by excipients.	A placebo solution should not exhibit any peak at the Retention time of the active peak and impurities	NIL
	Evaluation of interference with active peak by excipients and degradation product	In all degraded samples of finished product of assay preparation the active peak analyzed by PDA should be proved spectrally pure; no co-elution should be observed.	Pure
Accuracy Study (In terms of % recovery) of Known impurity	% Recovery at 50%,100% and 150% level of known impurity at the specified limit in the dosage form with three preparation at each level and one injection each ii) 95% Confidence level	Mean % over all recovery values (n=9) for each impurity 85.0% - 115.0% 95% Confidence level	DHA impurity : 98.3% DHA impurity : 2.94
System Precision	% RSD of six replicate injections of a diluted standard	RSD NMT 10.0 %	Diluted Artemether : 7.76% Diluted Lumefantrine : 1.91%
Method precision (repeatability)	i	Evaluation of six sample preparation as per the 100% recovery study for % known impurity	(i) Report mean impurity values
	ii	Calculate % RSD for six 100% recovery samples	(ii) RSD (n=6) NMT 15.0% for eacl known, unknown and total impuritie
	iii	95% Confidence level	95% Confidence level

choice of pH 2.3 for the mobile phase was made for excellent separation and reasonable retention time, also for longer column life.

Because of the high dependence on the mobile

TABLE 2 : Validation summary report

Performance parameter		Evaluation parameters	Acceptance Limit	Results		
					Analyst 1	Analyst 2
Method Precision (Intermediate Precision/ Ruggedness)	i	%Difference between mean of the two of known, unknown and total impurities values obtained by two different analyst on a different day using different instrument from the individual mean value	(i) NMT± 15.0%	DHA impurity :	-0.02	0.02
				α-Artemether :	0.04	-0.04
	ii	Calculate % RSD (n=12) for known, unknown and total impurities	(ii) RSD NMT 15.0% for each known, unknown and total impurities	DHA impurity : 2.11 α- Artemether : 0.50		
Limit of detection (LOD) (a)Active (b) Known impurity	i	Active shall be serially diluted at lower concentration range, extended fairly close to the expected LOD. The detection responses were calculated from the calibration curve using the formula i.e. DL = [3.3∗ SyX/Slope]	(i)Report value	DHA impurity 248µg Lumefantrine : 156µg Artemether : 212µg		
Limit of Quantitation (LOQ) (a)Active (b) Known impurity	i	Active shall be serially diluted at lower concentration range, extended fairly close to the expected LOQ. The detection responses were calculated from the calibration curve using the formula i.e. DL = [10∗ SyX/Slope].	(i) Report value	DHA impurity : 0.744µg Lumefantrine : 0.636µg Artemether: 0.468µg		
	ii	%RSD of six replicate injection of LOQ level	(ii) RSD (n=6) NMT 15.0%	DHA impurity : 4.95% Lumefantrine : 9.89% Artemether : 12.54%		
	iii	100% Recovery at LOQ level (if LOQ by Linearity method is 0.01 ppm which is equivalent to 0.05% then that concentration impurity solution shall be spiked in the blank solution and recovery shall be determined. One preparation 3 injections and calculated as per recovery study)	% Recovery – 80% - 120%	DHA impurity : 84.72%		

phase composition, the attempts to improve the selectivity and peak shapes by altering buffer and acetonitrile composition in isocratic mode were successful. Further to reduce the total run time or to reduce the retention time flow gradient mode of separation was chosen by altering the flow to double after elution of artemether which reduces total run time to 50 mins. Investigation of column selectivity of the method[17] showed improvement in the peak profile of artemether, DHA, artemisinin and its impurities more significantly on C_{18} column than on C_8 column while studying the different concentration of ion pair reagent, keeping pH constant (2.3). Finally, Waters Symmetry C_{18} column was utilized as separation unit. Waters Symmetry C18 was selected since this being packed with particles of silica gel, surface of which has been modified with chemically bonded octadecylsilyl groups was well fitted to the two studied drugs which are cationic species in the acidic mobile phase. Further, column with 5µ particle size gave better resolution between

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TABLE 3 : Validation summary report

Performance parameter	Evaluation parameters	Acceptance limit	Results
Filter paper Interference	Absolute difference from the solution filtered through centrifuged solution. If the centrifuge solution is unclear, then comparison against specified filter to be done	Absolute difference should be within ± 0.05	**Absolute difference**

Type of filter paper	DHA	α-Artemether	Artemisinin	PCB	DBA	Unk. Max. Imp.	Total Impurities excluding DHA
Whatman-1	0.000	0.000	0.000	0.000	0.000	BLQ	0.000
Centrifuged	0.000	-0.044	0.000	0.005	0.001	BLQ	-0.032
PVDF-	-0.686	-0.055	0.000	0.003	0.003	-0.053	-0.102
GF/C	0.000	0.000	0.000	0.004	0.001	BLQ	0.021

All the above mentioned filters are suitable except for PVDF.

			Diluted Standard Solution :	
			Time in hrs	**Diluted artemether**	**Diluted lumefantrine**
	(a) Mean area response of reference solution after 24 hrs with at least 2 intermediate time points	(a)% difference within ±10% of a freshly prepared standard (Injected at 0 hour.)	0	--	--
			11	-0.1	1.3
			18	-1.3	0.1
			23	0.3	-1.2
			29	-1.1	1.3
			34	-0.6	0.9
			40	3.3	1.3
			45	8.9	0.7
			50	8.7	0.4
			55	14.2	0.8
			Artemether standard solution is stable for 50 hrs and Lumefantrine standard solution is stable for 55hrs.		

Solution Stability (a) Diluted solution (b) Sample Solution

(b) % the known, unknown and total impurities, values after 24 hrs with minimum of two intermediate time points

(1) Absolute difference from initial for known and unknown shall not be more than 0.05 and should be within the specification. (2) Absolute difference from initial for total impurities is not more than 0.2. and should be within the specification. And no change in impurity profile shall be observed.

(b) Sample solution

Time in hrs	DHA	α-artemether	Artemisinin	PCB	DBA	Unk. Max. Impurity	Total Impurities excluding DHA
0	0.000	0.000	0.000	0.000	0.000	BLQ	0.000
9	0.000	-0.055	0.000	0.012	0.000	-0.093	-0.097
17	0.000	0.000	0.000	0.001	-0.001	BLQ	0.028
21	0.000	0.000	0.000	0.010	-0.002	-0.074	-0.036
25	0.000	-0.049	0.000	0.010	-0.002	BLQ	-0.030
32	0.000	0.000	0.000	0.010	-0.002	BLQ	0.026
36	0.000	0.000	0.000	-0.001	-0.003	BLQ	-0.019

Sample solution is stable for 36 hrs

dihydromisinin a degradant of artemether, and artemether compared to 10µ particle size column where there was merging of the both the peaks observed Challenge for selection of wavelength was due to the six fold concentration difference in the dosage form that is 80 mg Artemether and 480 mg of Lumefantrine. UV absorption spectra of artemether, lumefantrine and its impurities recorded in HPLC system using photodiode array detection it is observed that UV absorption maxima of artemether showed optimum UV absorption at 210 nm and lumefantrine shows UV absorption in a wide range ie.210-400nm. The response of Lumefantrine decreases with increase in wavelength. Hence it is concluded to use dual wavelength for simultaneous detec-

TABLE 4 : Validation summary report

Performance parameter	Evaluation parameters	Acceptance limit	Results		
Linearity of diluted solution of DHA , Artemether, and Lumefantrine From 50% to 150 % of the standard concentration (3 injections for each preparation)			**Diluted artemether**	**Diluted lumefantrine**	**DHA impurity**
	Correlation coefficient	Record the value	0.9988	0.9996	0.9978
	Residual sum of the squares	$r^2 \geq 0.99$	0.9976	0.9992	0.9957
	Slope	Record the value	22.86	182.06	21.4128
	Intercept	Record the value	49.5793	-214.6391	-59.0322
Range From LOQ to 200% of the specified limit of known impurity and of the active(s) (3 injections for each concentration)	Graph shall be plotted for each solution against their known concentrations.	Graph is visually examined.	Diluted artemether	Diluted lumefantrine	DHA impurity
			9.372ppm - 48ppm	12.729ppm-144ppm	14.890ppm-144ppm
Robustness. (Any one critical system suitability parameter can be tested)	Run system after deliberate change **HPLC conditions** 1. pH of Mobile phase (± 0.2units) 2. Composition of Mobile phase (±5% of absolute o 10% at actual)	System passes the system suitability criteria	**Mobile phase composition**		
			Parameters	**34:66**	**40:60**	**44:56**
			%RSD of Artemether	8.62	7.76	5.23
			%RSD of Lumefantrine	2.15	1.91	0.68
			Resolution between α-artemether and artemether	11.41	11.46	11.44
			Theoretical plates of artemether in resolution solution	19232	16430	19885
			Mobile phase pH			
			Parameters	**2.1**	**2.3**	**2.5**
			%RSD of Artemether	9.10	7.76	9.39
			%RSD of Lumefantrine	1.47	1.91	1.53
			Resolution between α-artemether and artemether	13.44	11.46	13.51
			Theoretical plates of artemether in resolution solution	20263	16430	20770

tion of artemether and lumefantrine. Wavelength 210 nm for artemether content determination and 380 nm for lumefantrine content determination in the combined dosage form. The wave length programming is adjusted in such way that all impurities of AM are separated at 210nm still to avoid the response of placebo the wave length was kept at 380 nm for initial 4 minutes then it is switched over to 210nm till 25 minutes and artemether and its impurities are well separated at flow rate of 1mL min^{-1} then after from 26 mins to 45 mins wavelength is switched to again 380nm and flow rate is increased to 2.0 mL min^{-1}. During this lumefantrine and its impurities are well separated. System is set back to 1mL min^{-1} flow 46 to 50 mins. Sampler temperature is maintained at 8-10 ^{o}C . A typical chromatogram showing the separation of the impurities in the sample was given in figure 3.

Method validation

The method was validated for Specificity, linearity, accuracy, precision, range, robustness, system suitability and reproducibility according to the International Conference on Harmonization (ICH) guidelines[17,18]. The summary of validation results are tabulated in TABLES (1-4).

CONCLUSION

The proposed flow gradient RP-HPLC method for the simultaneous detection and quantitation of DHA, artemisinin, and unspecified impurities in artemether and

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lumefantrine tablets is highly sensitive, accurate and precise. This procedure can be easily adopted for the routine quality control analysis of tablet dosage form without any interference from the excipients or each other. Method was validated for its performance parameters such as Specificity (placebo interference), Linearity and range, Recovery, LOD, LOQ, Precision and Ruggedness. The investigated validation elements showed the method has acceptable specificity, accuracy, linearity, precision, robustness and high sensitivity with the quantitation limits ranging from 0.744µg mL^{-1}, 0.636µg mL^{-1} and 0.468µg mL^{-1} dihydroartemisinin, artemether and lumefantrine respectively. The method is carried out with commercially available and conventional HPLC equipment with easy sample preparation. It is simple, accurate and reproducible for the quantization of the impurities from the formulation.

It was concluded that the developed method offers several advantages such as single chromatographic condition for the determination of impurities of two drugs, simple mobile phase and sample preparation steps, improved sensitivity makes it specific and reliable for its intended use. Additionally the method is applicable to all the strengths and all types of formulations such as dispersible tablets, artemether and lumefantrine dry syrup and strengths like 20+120, 40+240 mg etc.

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