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Simultaneous Quantification of Artemether and its Active Metabolite Dihydroartemisinin in Human Plasma by Liquid Chromatography-Tandem Mass Spectrometry

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ABSTRACT

A sensitive, selective and rapid liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated for the simultaneous determination of artemether (ART) and its active metabolite dihydroartemisinin (DHA) in human plasma using artemisinin (ARM) as the internal standard (IS). The analytes were extracted by liquid-liquid extraction and chromatographic separation was achieved on a reversed-phase Thermo HyPurity C18 (50 mm × 4.6 mm, 5 µm) column with a mobile phase consisting of 2 mM ammonium formate: methanol (15:85, *v/v*) in an isocratic mode at the flow rate of 0.600 mL/min. In order to quantify the analytes, triple quadrupole mass spectrometer equipped with electro spray ionization in the positive mode was used to obtain consistent precursor ammonium ion adducts $[M + NH_4]^+$ at *m/z* 316.5/163.5 for ART, 302.7/163.1 for DHA and 300.5/219.3 for ARM. The method was fully validated in the concentration range of 0.250 to 250 ng/mL using 50 µL human plasma. Intra- and inter-day accuracy and precision were within the acceptable limits of ±15 %. Stability experiments for ART and DHA were evaluated under different storage conditions. The assay was successfully applied to measure therapeutic plasma level of ART and DHA using COARTEM® (80 mg artemether and 480 mg lumefantrine) formulation in 12 healthy volunteers under fasting condition.

Keywords: Artemether; dihydroartemisinin; LC-MS/MS; human plasma; liquid-liquid extraction; bioequivalence study

INTRODUCTION

Artemisinin (ARM) based drugs are now considered as the first line antimalarials in the treatment and cure of falciparum malaria. In this regard, a potent combination of artemether and lumefantrine is extensively prescribed to treat patient worldwide. Artemether (ART) is a semi-synthetic methyl ether derivative of artemisinin (ARM)-a sesquiterpene lactone isolated from a Chinese herb, *Artimisia annua* L bearing a peroxide bridge which is believed to be responsible for the antimalarial activity. Both ART and ARM are used to cure severe acute infections caused by multi-drug resistant strains of *Plasmodium falciparum* [1, 2]. In comparison to ARM, ART has a superior bioavailability and antiplasma- dial activity and is most widely used clinically [1]. ART is hepatically metabolized to its active metabolite dihydroartemisinin (DHA) which exerts improved antimalarial activity compared to ARM and its derivatives [3, 4].

Several chromatographic methods, including LC with UV [5, 6], electrochemical detection [7, 8] and LC-MS/MS [9-17] have been reported for the simultaneous determination of ART and its metabolite DHA in human plasma. Two reports have described analysis for potent binary combination of ART and lumefantrine in human plasma using LC-MS/MS [18, 19]. In addition Duthaler et al. [20] have determined ART along with artesunate and their major metabolites in sheep plasma by LC-MS/MS. A comparative assessment of previously reported LC-MS/MS methods for ART and DHA in human plasma is presented in **Table 1**. Similarly, number of HPLC-UV [21, 22], LC using electrochemical detection [23] and LC-MS/MS [24-29] methods have also been reported for the determination of DHA with artesunate in human plasma. In addition to artesunate, DHA has also been determined with artemisinin [30] and other antimalarial drugs like mefloquine in human blood and plasma respectively by LC-MS/MS [31]. In order to investigate the pharmacokinetics of ART and DHA, there is a need to develop more selective and sensitive method to estimate their plasma concentrations.

Thus, the main objective of this work was to develop and validate a highly sensitive, reliable, rapid and robust method for the simultaneous estimation of ART and DHA in human plasma by LC-MS/MS. The method presents an efficient extraction procedure based on liquid-liquid extraction (LLE) which provides high and consistent recovery for both the analytes. Matrix effect was thoroughly assessed by estimating the IS-normalized matrix factors also through post-column analyte infusion. The proposed method was successfully applied to support a pharmacokinetic study of 80/480 mg artemether/lumefantrine formulation in 12 healthy Indian subjects under fasting.

Table 1 Comparison of salient features of LC-MS based methods developed for simultaneous determination of ART and DHA in human plasma

Sr. No.	Detection technique	Extraction procedure; Sample volume (µL)	Linear range (ng/mL); Retention time (min); Run time (min)	Application	Ref.
1.	LC-MS	LLE; 500	5.00-200; 10.4 and 3.6; 18.0	Pharmacokinetic drug–drug interaction study of co-artemether	9
2.	LC-MS	LLE; 1000 (human plasma), 2000 (human urine)	10.0-1000 for plasma & 5-500 for urine; 7.9 and 3.0; 10.0	ART and DHA quantification in human plasma and urine in a drug pharmacokinetic study	10
3.	LC-MS/MS	LLE; 100	5.0-500; 4.2 and 2.45; 6.0	Pharmacokinetic study of ART and DHA in healthy volunteers with a single oral dose of 200mg Artemedine tablets	11
4.	LC-MS/MS	SPE; 50	1.43-500; 2.8 and 1.3; 4.0	---	12
5.	LC-MS/MS	SPE; 500	2.00 -200; 4.9 and 2.5; 7.0	Pharmacokinetic studies of ARM-based antimalarial treatment in pediatric and co-infected patients with malaria	13
6.	LC-MS/MS	LLE; 100	2.00-500; 3.16 and 1.19; 8.00	Pharmacokinetic interaction study between the antimalarial combination ART/LUM & combination antiretroviral therapy including nevirapine in HIV-infected adults	15
7.	LC-MS/MS	SPE; 50	0.5-200; 5.6 and 2.9; 9.0	Determination of ART and DHA in human plasma with a new hydrogen peroxide stabilization method	16
8.	LC-MS/MS	Micro-elution SPE; 50	1.00-1000; 3.0 and 2.2; 4.0	Pharmacokinetic interaction between ETR at 200mg b.i.d. or DRV/RTV at 600/100 mg b.i.d. with ART/LUM at 80/480 mg for six doses in healthy subjects	17
9.	LC-MS/MS	LLE; 50	0.25-250; 1.14 and 0.82; 2.00	Bioequivalence study of ART and DHA using 80/480mg ART/LUM formulation in 12 healthy subjects	PM

ART: artemether; DHA: dihydroartemisinin; LUM: lumefantrine; ETR: etravirine; DRV: darunavir; RTV: ritonavir; LLE: liquid-liquid extraction; SPE: solid-phase extraction; PM: Present method

EXPERIMENTAL

Chemicals and materials

Reference standards of artemether (ART, 99.56 %), dihydroartemisinin (DHA, 98.96 %) and artemisinin (ARM, IS, 99.29 %) were obtained from Splendid Laboratory (Mumbai, India). HPLC grade methanol and ethyl acetate and analytical reagent (A.R.) grade *n*-hexane were procured from Merck Specialties Pvt. Ltd. (Mumbai, India). AR. grade ammonium formate was purchased from S.D. Fine Chemicals Ltd. (Mumbai, India). Water used in the present work was prepared using Milli-Q water purification system from Millipore (Bangalore, India). Drug free plasma containing K_3EDTA as an anticoagulant was obtained from Supratech Micropath (Ahmedabad, India) and stored at –20 °C until use.

Chromatography and mass spectrometric conditions

A Shimadzu HPLC system (Kyoto, Japan) equipped with LC-20AD pump was used for chromatographic analysis. The separation of ART, DHA and IS were performed on a Thermo Scientific HyPurity C18 (50 mm × 4.6 mm, 5 µm) column, maintained at 40 °C in a column oven. The mobile phase consisted of 2 mM ammonium formate: methanol (15:85, *v/v*) and was delivered at the flow rate of 0.600 mL/min. Mass spectrometric detection and quantitation was performed on MDS SCIEX API-4000 (Toronto, Canada), equipped with electro spray ionization and operating in positive ionization mode. The source dependent and compound dependent parameters optimized are shown in **Table 2**. Data acquisition was carried out using analyst classic software version 1.4.2.

Table 2 Mass parameters for analytes and IS

Mass Parameters	ART	DHA	ARM
Source parameters			
Source temperature (°C)	350		
Ion source voltage (V)	5000		
Curtain Gas (psi)	30		
Gas1, nebulizer gas (psi)	50		
Gas2, turbo gas (psi)	65		
Collision-activated dissociation (psi)	7		
Compound parameters			
Dwell time (msec)	200		
Entrance Potential (V)	10		
Declustering potential (V)	43	33	44
Collision Energy (eV)	15	23	17
Collision cell exit potential(V)	21	17	20
Q1 mass (amu)	163.5	302.7	300.5
Q3 mass (amu)	163.5	163.1	219.3

ART: artemether; DHA: dihydroartemisinin; ARM: artemisinin

Calibration standards and quality control samples

Standard stock solutions of 100 µg/mL of ART and DHA each was prepared by dissolving requisite amount in methanol, while the working solution (1000 ng/mL) was prepared in methanol:water (50:50, *v/v*). Calibration standards (CSs) and quality control (QC) samples were prepared by spiking blank plasma with working solutions. CSs were made at 0.250, 0.500, 1.50, 3.00, 6.00, 25.0, 65.0, 125.0, 190.0 and 250.0 ng/mL concentrations respectively, while quality control samples were prepared at five levels, viz., 220.0 ng/mL (HQC, high quality control), 100.0/15.00 ng/mL (MQC-1/2, medium quality control), 0.750 ng/mL (LQC, low quality control) and 0.250 ng/mL (LLOQ QC, lower limit of quantification quality control).

Stock solution (100 µg/mL) of the internal standard was prepared by dissolving 1 mg of ARM in 10.0 mL of methanol. Its working solution (100 ng/mL) was prepared by appropriate dilution of the stock solution in methanol: water (50:50, *v/v*). Standard stock and working solutions used for spiking were stored in refrigerator at 5 °C, while CSs and QC samples in plasma were kept at -70°C until use.

Sample extraction protocol

Prior to extraction, all frozen subject samples, CSs and QC samples were thawed in ice water bath and allowed to equilibrate at room temperature. A 50 µL of spiked/subject sample was transferred into pre-labelled tubes followed by addition of 50 µL of IS working solution (100 ng/mL) and vortex mixed for 10 s. Further, 200 µL of 10 mM ammonium acetate was added and vortex mixed for 20 s. Further, 2.5 mL ethyl acetate: *n*-hexane (80:20, *v/v*) binary solvent mixture was added and the sample was extracted on a rotary mixture for 15 min at 50 × g. Centrifugation was carried out for 5 min at 4000 × g. The supernatant were transferred to pre-conditioned vials and dried under a stream of nitrogen gas at room temperature. The sample was reconstituted with 100 µL of mobile phase and 5 µL was used for injection into the chromatographic system.

Validation procedures

The method was validated as per the USFDA guidelines to establish the accuracy and precision of the method [32]. The details of the parameters studied were similar to our previous report [33].

System suitability experiment was performed by injecting six consecutive injections, using extracted standard mixture of ART and DHA (100 ng/mL each) and ARM (100 ng/mL) at the start of each batch during method validation. System performance was studied by injecting one extracted LLOQ sample with IS at the beginning of each analytical batch and before re-injecting any sample during method validation. The carryover of analytes was experimentally determined by sequentially injecting the mobile phase solution → LLOQ sample → extracted blank plasma → upper limit of quantitation (ULOQ) sample → extracted blank plasma → LLOQ sample → extracted blank plasma.

The selectivity of the method toward endogenous plasma matrix components was assessed in 10 different batches which included 6 normal K_3EDTA, 2 haemolysed and 2 lipemic blank plasma sources. Interference of commonly used medications by human volunteers was checked for paracetamol, chlorpheniramine, caffeine, acetylsalicylic acid and ibuprofen. Their stock solutions (100 µg/mL) were prepared by dissolving requisite amount in methanol. Their working solutions (1000 ng/mL) were prepared and 5 µL was injected to check for any possible interference at the retention time of analytes.

The linearity of the method was ascertained by measuring the area ratio response (analyte/IS) for five calibration curves containing ten non-zero concentrations. Each calibration curve was analyzed individually by using least square weighted ($1/x^2$) linear regression. The lowest standard on the calibration curve having analyte response at least ten times more than that of drug free (blank) extracted plasma was accepted as the LLOQ.

For determining the intra-batch accuracy and precision, six replicates of QC samples along with calibration curve standards were analyzed on the same day. The inter-batch accuracy and precision were assessed by analyzing five precision and accuracy batches on three consecutive days. The precision (% CV) at each concentration level from the nominal concentration should not be greater than 15 % and the accuracy should be within ±15 %. Reinjection reproducibility was also checked by re-injecting one entire validation batch of 60 samples.

Ion suppression/enhancement effects on LC–MS/MS sensitivity were evaluated by post column analyte infusion experiment. A standard solution containing ART, DHA (at ULOQ level) and IS was infused post column via a 'T' connector into the mobile phase. Aliquots of 5 µL of extracted control (blank) plasma were then injected into the column and MRM chromatograms were acquired for analytes and IS.

The extraction recovery for the analytes and IS was calculated by comparing the mean area response of samples (n=6) spiked before extraction to that of extracts with post-spiked samples (spiked after extraction) at four QC levels. Matrix effect, expressed as matrix factors (MFs) was assessed by comparing the mean area response of post-spiked samples with samples prepared in mobile phase. IS-normalized MFs (analyte/IS) were calculated to access the variability of the assay due to matrix effects. Relative matrix effect was assessed from the precision (% CV) values of the slopes of the calibration curves prepared from eight plasma lots, which included haemolysed and lipemic plasma. To prove the absence of matrix interference, % CV should not be greater than 4 % [34].

All stability results were evaluated by measuring the area ratio response (analyte/IS) of stability samples against freshly prepared comparison standards at two QC levels. Stock solutions of analytes and IS were checked for short term and long term stability at 25 °C and 5 °C, respectively. The acceptance criterion was ±10.0 % deviation from the nominal value. The autosampler (wet extract), bench top (at 25 °C), dry extract, freeze–thaw (−20 °C and −70 °C) and long term (−20 °C and −70 °C) stabilities in plasma were also studied at both these levels. Whole blood stability was also determined to ascertain any enzymatic degradation by spiking blood samples with analytes at the LQC and HQC levels for 2.0 h in wet ice bath. The samples were considered stable if the deviation from the mean calculated concentration of freshly prepared quality control samples was within ±15.0%.

Method ruggedness was evaluated on two precision and accuracy batches. The first batch was analyzed by different analysts while the second batch was studied on two different columns (same make but different batch no.). Dilution reliability was determined by diluting the stock solution prepared as spiked standard at 500.0 ng/mL concentration for ART and DHA in the screened plasma. The precision and accuracy for dilution integrity standards at $1/5^{th}$ and $1/10^{th}$ dilution were determined by analyzing the samples against freshly prepared CSs.

Application of the method

The bioequivalence study was conducted with a single fixed dose of a test (80 mg artemether + 480 mg lumefantrine tablets from a Generic Company) and a reference (COARTEM®, 80/480 mg artemether/lumefantrine tablets from Novartis Pharmaceuticals Corporation, East Hanover, New Jersey, USA) formulation to 12 healthy adult Indian subjects under fasting. The design was an open label, balanced, randomized two-treatment, two-period, two-sequence, crossover study. The study was conducted as per International Conference on Harmonization, E6 Good Clinical Practice guidelines [35]. The subjects were orally administered a single dose of test and reference formulations with 240 mL of water after recommended wash out period of 7 days. Blood samples were collected at 0.00 (pre-dose), 0.33, 0.66, 1.00, 1.33, 1.66, 2.00, 2.33, 2.66, 3.00, 3.50, 4.00, 5.00, 6.00, 7.00, 8.00, 9.00, 10.0, 12.0, 16.0, 20.0 and 24.0 h after oral administration of test and reference formulation in labelled K_3EDTA-vacuettes. Plasma was separated by centrifugation and kept frozen at −70 °C until analysis. During study, subjects had a standard diet while water intake was unmonitored. The pharmacokinetic parameters of OB and DEOB were estimated by non-compartmental model using WinNonlin software version 5.2.1 (Pharsight Corporation, Sunnyvale, CA, USA).

RESULTS AND DISCUSSION

I. Method development

The current method was systematically developed based on the details available for evaluating antimalarial drugs in biological samples as reported previously [12, 16]. In the present method mass parameters were tuned using ESI in positive as well as negative polarity for best and reliable response for both the analytes. Many methods have stated that analysis of ART and DHA were done in positive electrospray ionization mode as both are basic in nature. The product ion mass spectra of ART and DHA showed a similar representative product ion peak at *m/z* 163.1 and *m/z* 219.3 for IS. The intensity of protonated precursor ions $[M + H]^+$ was relatively low. Higher intensity was observed for ammonium ion adduct $[M + NH_4]^+$ for both ART and DHA at *m/z* 316.5 and 302.7 respectively because of the presence of ammonium ions in the mobile phase. Similar ammonium effect was also observed for IS which gives rise to its ammonium adduct at *m/z* 300.5. The product ion mass spectra for ART, DHA and ARM as IS are shown in **Figure 1a-c**. A dwell time of 200 ms gave adequate data points for the quantitation of analytes and IS.

The separation of ART and DHA has been carried out on HyPurity C18 (50 × 4.6 mm, 5µm) analytical column under isocratic conditions to obtain adequate response and acceptable peak shape. Few existing methods have used acetonitrile and ammonium acetate/formate as mobile phase while other methods employed methanol instead of acetonitrile. To find the best eluting solvent system, trials were performed by using both the organic modifiers with buffers like ammonium acetate/formate in different composition.

Optimum chromatography in terms of baseline separation, the peaks shapes and response was much superior in methanol and ammonium formate solvent system. It was found that there was a substantial increase in the sensitivity in presence of ammonium formate as compared to ammonium acetate together with organic solvent. Additionally, their composition was varied by changing the ratio (organic: aqueous) from 50:50 to 90:10 (*v/v*) and flow rate

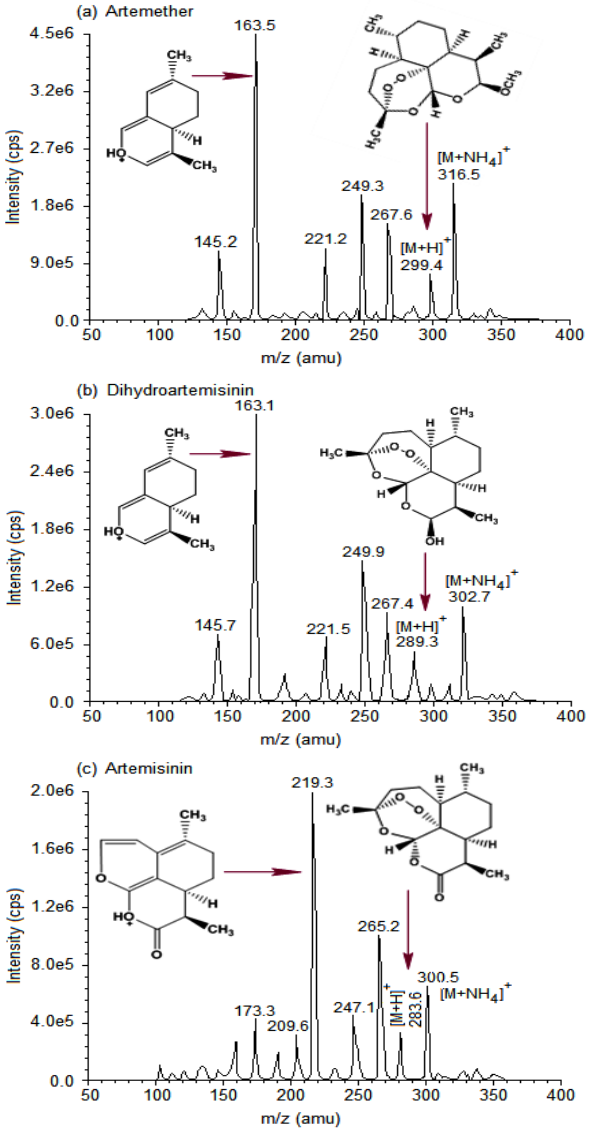


Figure 1 Product ion mass spectra of (a) artemether (*m/z* 316.5 → 163.5) (b) dihydroartemisinin (*m/z* 302.7 → 163.1) and (c) artemisinin, IS (*m/z* 300.5 → 219.3) in the positive ionization mode, scan range 50-400 amu.

from 0.400 to 0.700 mL/min to find the optimum mobile phase ratio. Best chromatographic conditions with respect to analyte response and peak shape were obtained by employing methanol-2.0 mM ammonium formate in water (85: 15, *v/v*) as the mobile phase at a flow rate of 0.600 mL/min. The overall chromatographic run time was 2.0 min with the retention time for ART, DHA and IS was 1.14, 0.87 and 1.02 min respectively. The MRM ion chromatograms of blank plasma spiked with IS, LLOQ sample and real subject sample at C_{max} concentration indicate absence of any interfering peaks at the retention of the analytes or IS and the ability of the method to quantify the analytes from endogenous components in the plasma matrix or other components in the sample (**Figures 2-4**).

In the previously reported method, LLE [6-11, 15] and SPE [12, 13, 16] have been employed to extract theses analytes from human plasma. Initially protein precipitation was carried out but due to higher protein binding of ART and DHA, which however resulted in very poor recovery [36, 37]. This led us to switch tor LLE by using common organic solvent either alone or in composition. Trials were conducted using ethyl acetate,

dichloromethane, methyl *tert*-butyl ether and *n*-hexane, which provided somewhat greater recovery (~60 % for ART and nearly 80 % for DHA) compared to protein precipitation (55 to 64 % for both the analytes) without any matrix interference. However by using 2.5 mL ethyl acetate: *n*-hexane (80:20, *v/v*) as an extracting solvent the mean recovery obtained were 90.1 %, 97.1 % and 95.9 % for ART, DHA and IS respectively. The extracts obtained were clear with no matrix interference and the recovery was consistent at all QC levels.

Assay results

System suitability, system performance and auto-sampler carryover

System suitability results showed a variation in the measurement of precision (% CV) of 0.53 to 1.06 % for the retention time and 0.42 to 1.97 % for the area response of the analytes. For both the analytes the signal to noise ratio for system performance was ≥ 20. The carry-over assessment was done to confirm that it does not affect the accuracy and the precision of the developed method. The change in the area response of blank sample after injection of highest calibration standard at the retention time of analytes and IS was negligible (≤ 0.92 %).

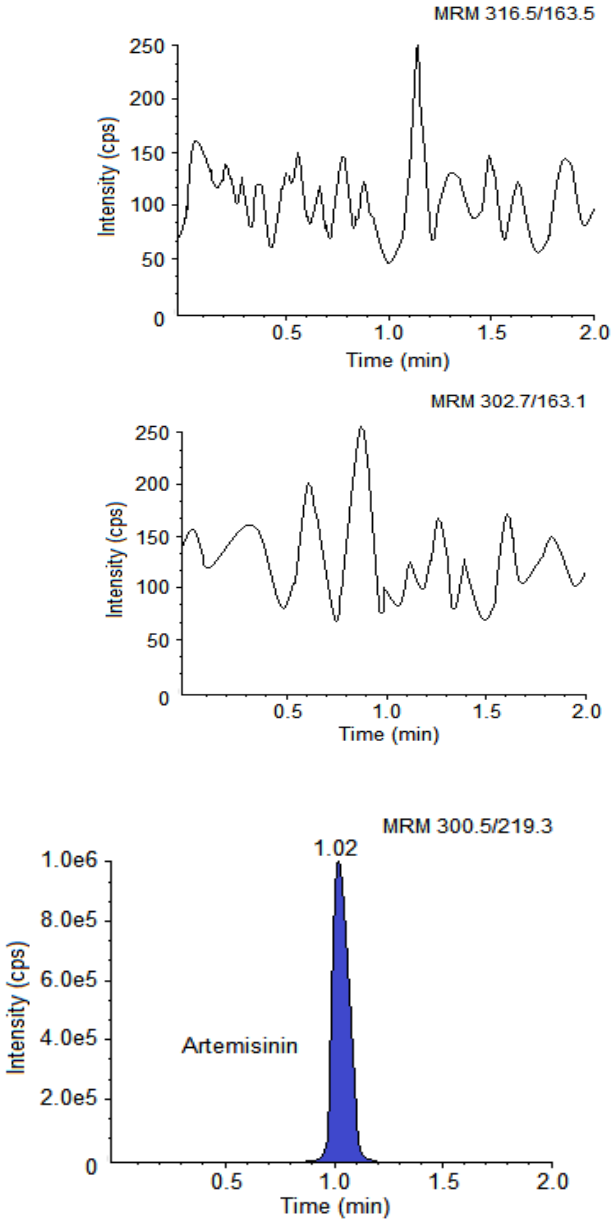


Figure 2 MRM ion-chromatograms of blank plasma for with artemisinin, IS.

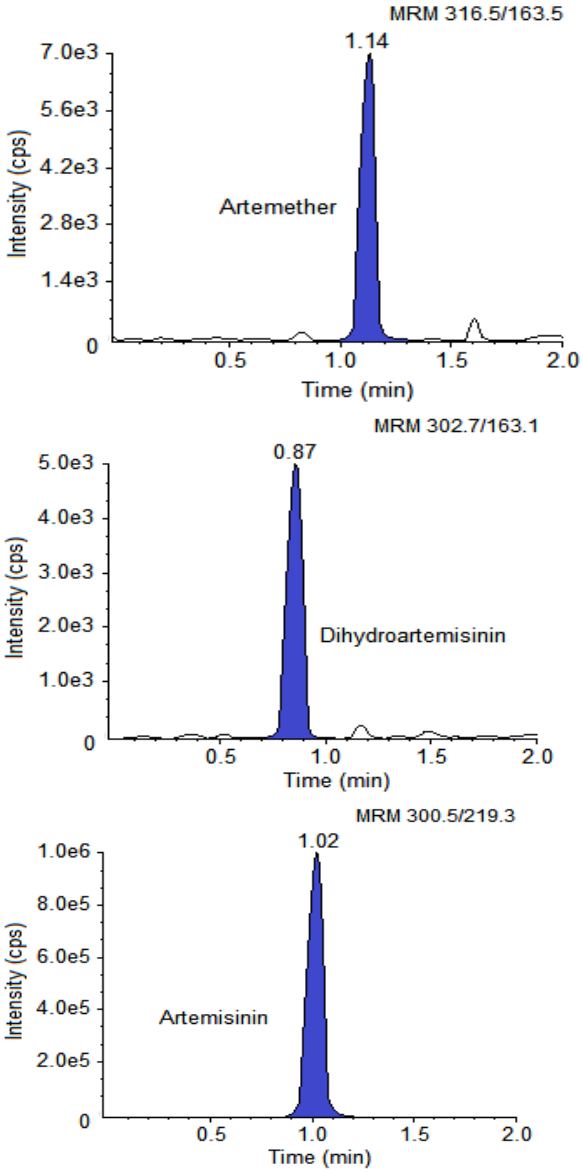
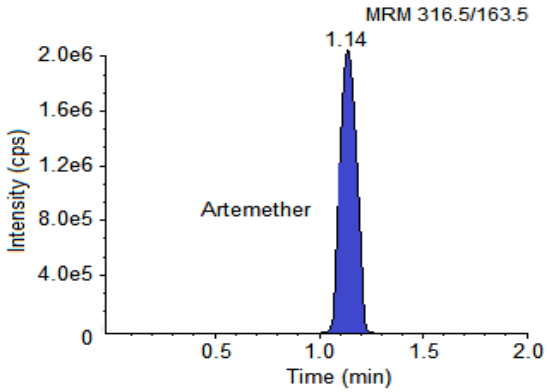


Figure 3 MRM ion-chromatograms of artemether (m/z 316.5 → 163.5) and dihydroartemisnin (m/z 302.7 → 163.1) at LLOQ and IS (m/z 300.5 → 219.3).



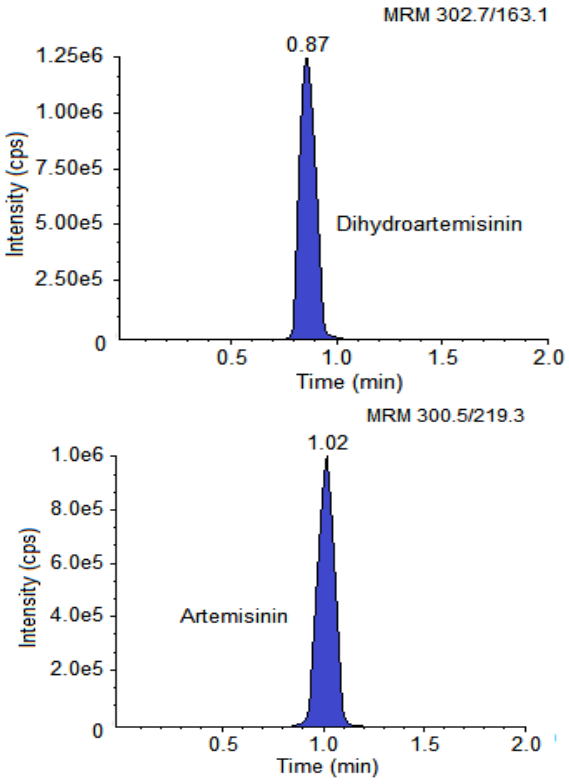


Figure 4 MRM ion-chromatograms of artemether and dihydroartemisnin in subject sample at Cmax after oral administration of 80/480 artemether/lumefantrine tablet formulation and artemisinin, IS.

Linearity, lower limit of quantification and accuracy & precision

The five calibration curves were linear over the concentration range of 0.25-250 ng/mL for both the analytes respectively, with a correlation coefficient (r2) • 0.9996 and 0.9997 for ART and DHA respectively. The mean linear equations obtained were y = (0. 0100 ± 0. 000023) x – (0. 000039 ± 0. 000060) and y = (0.00986 ± 0. 000095) x + (0. 000027± 0. 000014) for ART and DHA respectively. The accuracy and precision (% CV) observed for the calibration curve standards ranged from 97.2 to 102.3 % and 0.71 to 2.33 % for ART and 97.1 to 102.5 % and 0.52 to 3.35 % for DHA respectively. The lower limit of quantitation for both the analytes in plasma was obtained at a signal-to-noise ratio (S/N) of ≥ 20. The intra-batch and inter-batch precision and accuracy were established from validation runs performed at five QC levels and the results are presented in Table 3.

Extraction recovery and matrix effect

The extraction recovery of analytes from LLE ranged from 88.94-91.41 % for ART and 95.43-98.22 % for DHA. The mean recovery of ARM was 95.96 %. The presence of endogenous or exogenous components in biological fluids can lead to ion suppression or enhancement in the measurement of analyte signal, giving rise to matrix effect. The post-column infusion chromatograms in **Figure 5a-c** show negligible ion suppression or enhancement at the retention time of analytes and IS. The absolute matrix effect, expressed as matrix factor (MF) was evaluated at four QC levels. The MFs were calculated from the peak area response for the analytes and their IS separately and their ratios were then used to find the IS-normalized MF, which ranged from 0.965-1.050 across four QC levels for both the analytes (**Table 4**). Further, the relative matrix effect expressed as precision (% CV) in the measurement of the slopes of the calibration curves was < 2.5 % in eight different plasma sources.

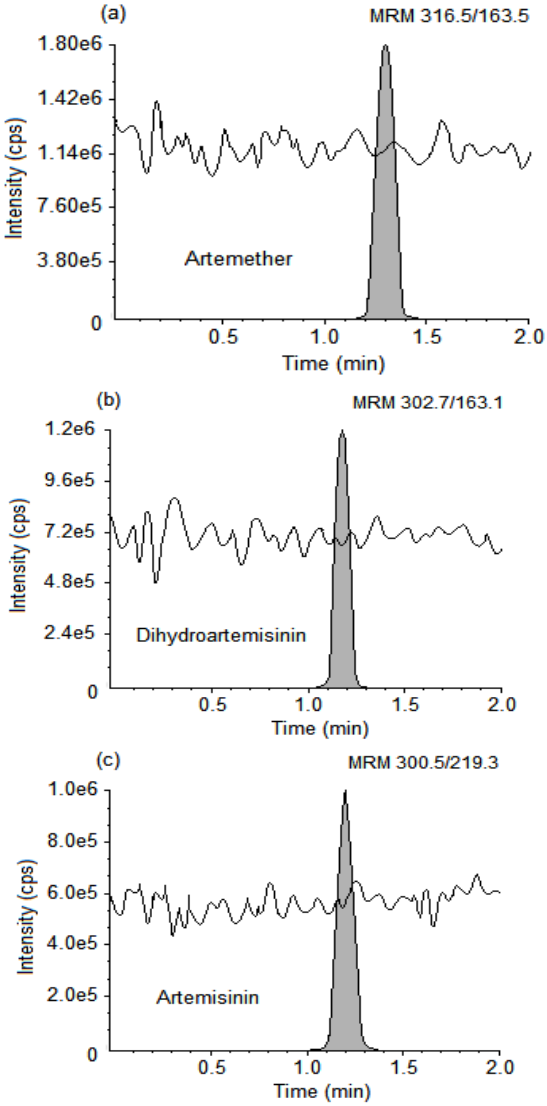


Figure 5 Post column analyte infusion MRM LC-MS/MS chromatograms for (a) artemether, (b) dihydroartemisinin and (c) artemisinin.

Stability results, method ruggedness and dilution reliability

Stock solutions kept for short-term and long-term stability as well as spiked plasma solutions showed no evidence of degradation under all studied conditions. No significant degradation of analytes was observed during sample storage and sample processing. The detailed results for stability studies are presented in **Table 5**. The precision (% CV) and accuracy values for two different columns for method ruggedness ranged from 1.9 to 4.5 % and 94.1 to 103.7 % respectively across five QC levels. For the experiment with different analysts, the results for precision and accuracy were within 1.2 to 3.5 % and 96.5 to 103.1 % respectively at these levels. For dilution reliability experiment the precision and accuracy values for $1/5^{th}$ and $1/10^{th}$ dilution ranged from 0.98-2.8 % and 94.7-102.4 % for both the analytes respectively.

Table 3 Intra and inter-batch precision and accuracy for artemether and dihydroartemisinin

Nominal conc. (ng/mL)	Intra-batch (n = 6; single batch)			Inter-batch (n = 30; 6 from each batch)		
	Mean conc. found (ng/mL)	% CV	% Accuracy	Mean conc. found (ng/mL)	% CV	% Accuracy
Artemether						
HQC (220.0)	222.7	1.42	101.2	220.9	0.98	100.4
MQC-1 (100.0)	100.7	4.58	100.7	99.3	2.16	99.3
MQC-2 (15.00)	15.04	3.08	100.2	15.15	1.70	101.0
LQC (0.750)	0.748	2.04	99.7	0.740	3.56	98.7
LLOQ QC (0.250)	0.246	4.79	98.3	0.254	4.72	101.6
Dihydroartemisinin						
HQC (220.0)	217.6	1.45	98.9	222.5	1.72	101.1
MQC-1 (100.0)	102.5	5.00	102.5	98.3	2.61	98.3
MQC-2 (15.00)	14.68	2.25	97.8	15.30	1.57	102.0
LQC (0.750)	0.761	1.41	101.5	0.752	1.26	100.3
LLOQ QC (0.250)	0.251	3.36	100.4	0.249	3.09	99.5

CV: Coefficient of variation; n: Number of replicates

Table 4 Extraction recovery and matrix effect for artemether and dihydroartemisinin

QC level	Area response (replicate, n = 6)			Extraction recovery, % (B/A)		Matrix factor		
	A	B	C	Analyte	IS	Analyte (A/C)	IS	IS-normalized
Artemether								
LQC	20399	19722	19395	88.94	95.03	1.05	1.009	1.041
MQC-2	423114	393395	435953	90.25	96.18	0.97	1.006	0.965
MQC-1	2933142	2554236	2957811	89.77	95.64	0.99	0.989	1.003
HQC	6039775	5691185	5853184	91.41	96.70	1.0	0.992	1.040
Dihydroartemisinin								
LQC	19722	18821	18952	95.43	95.03	0.98	1.009	0.971
MQC-2	393395	382679	389521	97.28	96.18	1.01	1.006	1.004
MQC-1	2554236	2488364	2556323	97.42	95.64	0.99	0.989	1.010
HQC	5691185	5590095	5462141	98.22	96.70	1.04	0.992	1.050

A: post- extraction spiking; B: pre-extraction spiking; C: neat samples in mobile phase; CV: coefficient of variation; n: Number of replicates; LQC: low quality control; MQC: medium quality control; HQC: high quality control

Table 5 Stability of artemether and dihydroartemisinin in plasma under different conditions

(n = 6)

Storage conditions	Artemether			Dihydroartemisinin	
	Nominal conc. (ng/mL)	Mean stability sample (ng/mL)± SD	% Change	Mean stability sample (ng/mL) ± SD	% Change
Bench top stability at 25 °C, 20 h	220.0	223.5 ± 2.8	1.59	224.9 ±7.1	2.21
	0.750	0.755 ± 0.018	0.69	0.745 ± 0.016	-0.72
Freeze & thaw stability at -20 °C	220.0	210.0 ± 6.6	-4.55	214.3 ± 11.6	-2.58
	0.750	0.742 ± 0.015	-1.12	0.735 ± 0.029	-2.03
Freeze & thaw stability at -70 °C	220.0	218.4 ± 10.5	-0.75	223.8 ± 9.6	1.73
	0.750	0.760 ± 0.030	1.33	0.737 ± 0.019	-1.79
Autosampler stability at 4°C, 16 h	220.0	224.1 ± 6.3	1.85	219.5 ± 10.7	-0.25
	0.750	0.758 ± 0.027	1.04	0.742 ± 0.018	-1.01
Dry extract stability at 2-8°C, 14 h	220.0	226.1 ± 9.3	2.76	215.4 ± 10.0	-2.10
	0.750	0.731 ± 0.010	-2.51	0.763 ± 0.023	1.79

Wet extract stability at 24 °C, 8 h	220.0	213.1 ± 6.8	-3.13	225.0 ± 7.1	2.25
	0.750	0.740 ± 0.034	-1.39	0.760 ± 0.024	1.36
Long term stability at -20 °C, 106 days	220.0	214.2 ± 6.3	-2.65	226.6 ± 9.9	3.01
	0.750	0.765 ± 0.021	1.89	0.742 ± 0.016	-1.04
Long term stability at -70 °C, 106 days	220.0	231.2 ± 9.5	5.11	211.7 ± 5.4	-3.75
	0.750	0.725 ± 0.017	-3.36	0.767 ± 0.014	2.24

SD: Standard deviation, *n*: Number of replicates

$$\%Change = \frac{Mean\ stability\ samples - Mean\ comparison\ samples}{Mean\ comparison\ samples} \times 100$$

Application of the method in healthy subjects and incurred sample reanalysis results

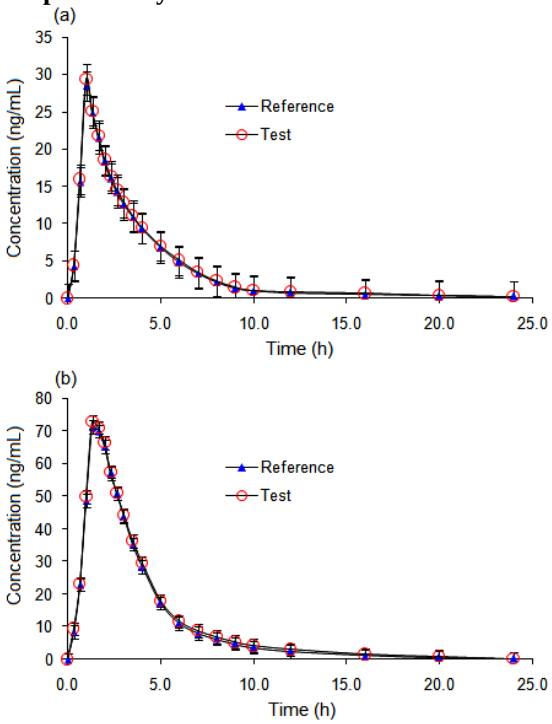


Figure 6 Mean plasma concentration-time profile of artemether and dihydroartemisinin after oral administration of test (80/480 mg artemether/lumefantrine tablets from a Generic Company) and a reference (COARTEM®, 80/480 mg artemether/lumefantrine from Novartis Pharmaceuticals Corporation, USA) formulation to 12 healthy volunteers. The validated method was successfully applied for the assay of ART and DHA in healthy Indian volunteers. The plasma concentration vs. time profile for ART and DHA under fasting is shown in Figure 6. Table 5 summarizes the mean pharmacokinetic parameters (Cmax: maximum plasma concentration, Tmax: time point of maximum plasma concentration, t1/2: half life of drug elimination during the terminal phase, AUC0-t: area under the plasma concentration-time curve from zero hour to 24 h; AUC0-inf: area under the plasma concentration -time curve from zero hour to infinity; Kel: elimination rate constant) for ART and DHA after oral administration of combination tablet of 85 mg ART/500 mg LUM test and reference formulation. Additionally, no statistically significant differences were found between the two formulations in any parameter. The ratios of mean log-transformed parameters (Cmax, AUC0-24h and AUC0-inf) and their 90 % CIs were all within the defined bioequivalence range of 80-125 % (Table 7). These observations confirm the bioequivalence of the test sample with the reference product in terms of rate and extent of absorption.

Table 6 Mean pharmacokinetic parameters following oral administration of 80/480 mg artemether/lumefantrine combination formulation in 12 healthy Indian subjects under fasting.

Parameter	**Artemether (Mean ±SD)**		**Dihydroartemisinin (Mean ±SD)**	
	Test	**Reference**	**Test**	**Reference**
C_{max} (ng/mL)	28.47 ± 4.18	29.32 ± 4.89	71.11 ± 12.08	72.70 ± 13.62
T_{max} (h)	1.06 ± 0.36	1.10 ± 0.47	1.48 ± 0.28	1.54 ± 0.32
$t_{1/2}$ (h)	2.55 ± 1.15	2.66 ± 1.31	3.59 ± 1.20	3.70 ± 1.11
AUC $_{0\text{-}24h}$(h.ng/mL)	91.31 ± 9.53	95.24 ± 9.72	255.88 ± 52.25	268.65 ± 61.32
AUC $_{0\text{-}inf}$ (h.ng/mL)	101.2 ± 7.18	107.5 ± 8.25	273.15 ± 56.74	288.31 ± 66.49
Kel (1/h)	0.272 ± 0.021	0.261 ± 0.017	0.151 ± 0.004	0.159 ± 0.004

SD: Standard deviation

Table 7 Comparison of treatment ratios and 90% CIs of natural log (Ln)-transformed parameters for test and reference formulations in 12 healthy subjects under fasting.

Parameter	Ratio (test/reference),%		90% CI (Lower – Upper)		Power		Intra subject variation, % CV	
	ART	DHA	ART	DHA	ART	DHA	ART	DHA
C_{max} (ng/mL)	97.1	97.8	93.2-101.9	93.3-102.5	0.9990	0.9994	8.51	7.77
AUC_{0-24h} (h.ng/mL)	95.9	95.2	91.3-99.5	91.5-99.9	0.9995	0.9997	6.37	5.39
AUC_{0-inf} (h.ng/mL)	94.1	94.7	89.7-100.2	90.7-98.2	0.9985	0.9992	4.65	2.51

ART: Artemther; DHA: Dihydroartemisinin; CI: confidence interval; CV: coefficient of variation

CONCLUSIONS

The proposed validated method for the estimation of ART and DHA in human plasma is highly selective, accurate and precise. The method offers significant advantages over those previously reported, in terms of lower sample requirements, sensitivity and analysis time. The efficiency of liquid-liquid extraction and a chromatographic run time of 2.0 min per sample make it an attractive procedure in high-throughput bioanalysis of these antimalarial derivatives. The linear dynamic range established was adequate to measure the plasma concentration of ART and DHA in a clinical study involving healthy subjects. In addition, matrix effect and stability of analytes in plasma was extensively studied.

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