ABC Research Alert
Vol 6, Number 3
Published Online: 23 November 2018
http://abcreal.weebly.com/

A selective LC-MS/MS method for simultaneous quantification of Artemether, Lumefantrine and their principle metabolites in human plasma

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Abstrac

We have developed and validated a sensitive, selective and reproducible reversed-phase high-performance liquid chromatography method coupled with electrospray ionization mass spectrometry (HPLC–ESI-MS/MS) for the simultaneous quantitation of artemether (ART), dihydroartemisinin (DHA), lumefantrine (LUM) and desbutyl-lumefantrine (DBL) in human plasma. Mefloquine was used as an internal standard (IS). The analytes were extracted by protein precipitation procedure and separated on a reversed-phase Zorbax SB-Ciano column with a mobile phase composed of acetonitrile and 20mM aqueous ammonium formate containing 0.5% (v/v) formic acid. Multiple reaction monitoring was performed in the positive ion mode using the transitions *m/z* 316.3→*m/z* 163.1 (ART), *m/z* 302.3→*m/z* 163.1 (DHA), *m/z* 530.3→*m/z* 512.2. (LUM), *m/z* 472.2→*m/z* 454.1 (DBL) and *m/z* 379.1→*m/z* 361.1(MQ) to quantify the drugs. Calibration curves in spiked plasma were linear ($r^2 \geq 0.9992$) over the range of 5–1500 ng/mL for ART/ DHA and 5–5,000 ng/mL for LUM/DBL. The lower limit of quantitation (LLOQ) was 10 ng/mL ART/ DHA and 5 ng/mL for LUM/ DBL. The mean R.S.D. values for the intra-run precision were 2.2% , 3.8%, 1.9% and 4.7% and for the inter-run precision were 3.2%, 3.6% , 4.4% and 3.5% for ART, DHA, LUM and DBL, respectively. The mean percentage recovery values were 93.2%, 98.5%, 97.1% and 99.4% for ART, DHA, LUM and DBL, respectively. No matrix effect was detected for all the analytes and the IS. The validated method was successfully applied to determine the plasma concentrations of ART, DHA, LUM and DBL in pregnant and non-pregnant women volunteers in a multiple-dose pharmacokinetics study over the course of 336 hours.

Keywords

Artemether; Lumefantrine; Metabolites; LC-MS/MS; Pharmacokinetics; Human plasma.

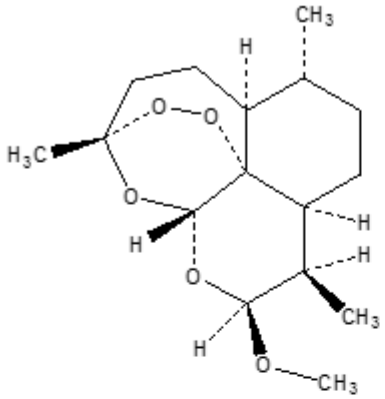
INTRODUCTION

Plasmodium falciparum has always developed widespread resistance to widely deployed antimalarial drugs requiring close monitoring of their efficacy (Martensson A. *et al*., 2005). The World Health Organization currently recommends artemisinin-based combination therapy (ACT), a combination of artemisinin derivative and another structurally-unrelated and slowly eliminated antimalarial for treatment of uncomplicated malaria (Global report 2010).

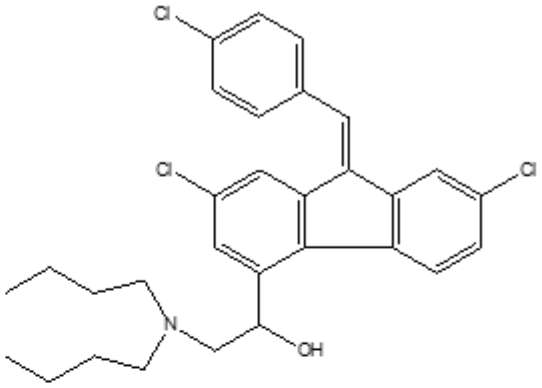
Artemether (ART) 20 mg and lumefantrine (LUM) 120 mg (Figure 1) is the most common ACT used in malaria endemic Africa (Omari A. *et al*., 2004). Whereas dihydroartemisinin (DHA) and desbutyl-lumefantrine (DBL) have been reported as active metabolites of ART and LUM respectively, with abilities to influence AL's treatment outcomes (Mugoyela, and Omary, 2011), no method has been validated to quantitate these drugs simultaneously. A quantitative assessment of ART, DHA, LUM and DBL in plasma in the course of AL treatment is essential in order to evaluate the bioavailability and pharmacokinetics of these co-formulated antimalarial compounds and how their potent metabolites influence treatment outcome. Previous studies have suggested that the area under the plasma Lumefantrine concentration–time curve was the principal determinant of curing malaria (Ezzet F. et al., 2000), and artemether formulations with a high bioavailability are considerably important to their rapid clinical efficacy (Karbwang J. *et al*., 1997).

Many methods have been reported for quantitation of either artemether (Navaratnam V. *et al*., 1995; Sandrenan N. *et al*., 1997; Souppart C. *et al*., 2002; Shi B. *et al*., 2006) or lumefantrine (Mansor M. *et al*., 1996; Zeng M. *et al*., 1996; Lindegårdh N. *et al*., 2005; Annerberg A. *et al*., 2005; Ashley A. *et al*., Wahajuddin, *et al*., 2009) in human plasma. Mc Gready *et al*., 2006 evaluated the pharmacokinetics of both drugs in pregnant women; however, the ART and LUM analysis were carried out separately. César *et al*., 2008 developed an LC-UV method for the simultaneous quantitation of artemether and lumefantrine in fixed-dose combination tablets. Nevertheless, ultraviolet detection is not adequate for ART quantitation in a biological matrix due to its low sensitivity and selectivity. Hodel *et al*., 2009 developed an HPLC–ESI–MS/MS method for the quantitation of 14 antimalarial drugs in human plasma, including ART and LUM. However, the method developed in our study involves a shorter sample processing time; absence of the drying and sample reconstitution with a shorter chromatographic run time. In addition, this is the first report providing simultaneous quantitation of ART, LUM and their metabolites with a pharmacokinetics application. This follows on César et al's simultaneous quantitation of ART and LUM.

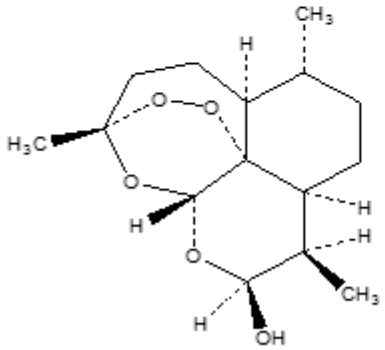
In this study, we report an HPLC-ESI-MS/MS method for simultaneous quantitation of ART and LUM with their principle metabolites in human plasma. The method was successfully applied in a pharmacokinetic study of AL in pregnant and non-pregnant women who received a fixed- dose combination tablets during the course of their treatment for uncomplicated malaria.



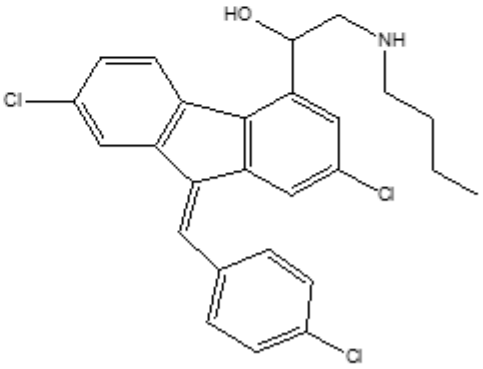
Artemether 298 g/mol



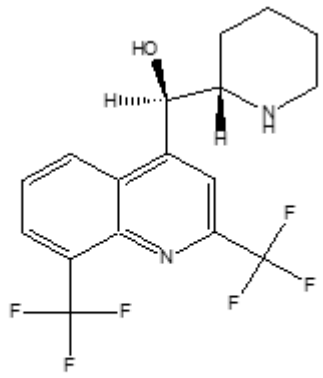
Lumefantrine 529 g/mol



Dihydroartemisinin 284 g/mol



Desbutyl-lumefantrine 472 g/mol



Mefloquine 378 g/mol (IS)

Figure 1. Chemical structures and molar masses of ART, LUM, DHA, BDL and Mefloquine (MQ), IS

METHODOLOGY

Chemicals and reagents

ART, LUM and MQ (IS) reference standards were purchased from United States Pharmacopeia (Rockville, USA), Dihydroartemisinin and Desbutyl-lumefantrine reference standards were

kindly donated by the National Quality Control Laboratory (Nairobi, Kenya). Coartem® tablets were purchased from Novartis (Basel, Switzerland), deionized water was prepared using a Smart2 Pure™ water purification system (Thermo-scientific, Niederelbert, Germany). Blank human plasma with Li-heparin for the preparation of calibrators and quality controls was obtained from Kenya Medical Research Institute, Centre for Clinical Research (Nairobi, Kenya). Acetonitrile (HPLC), methanol (HPLC) and formic acid (analytical grade) were purchased from Sigma Aldrich (St Louis, MO, USA). Glacial acetic acid HiperSolv® grade was from BDH (Polle, UK) and ammonium formate, analytical grade (Fisher Scientific, UK).

Instrumentation and analytical conditions

The separation and quantitation of ART, LUM and metabolites were carried out on an Agilent technology HPLC-ESI-MS/MS system (Santa Clara, CA, USA), composed of a 1260µ binary pump, 1260 auto sampler and 1260 thermosetting column compartment (TCC) and an Agilent technology 6410 triple quadrupole mass spectrometer, equipped with an electrospray ion source. LC separation was performed on a Zorbax Eclipse XDB-CN (75mm x 4.6 mm I.D; 3.5µm particle size) from Agilent (Santa Clara, CA, USA) with a similar Zorbax RRHD security guard column (5mm x 4.6mm, 3.5µm) maintained at 30 ºC. The mobile phase consisted of (A) 0.5% formic acid in 20mM aqueous ammonium formate and (B) 0.5% formic acid in acetonitrile.

A linear gradient elution was used to deliver the mobile phase, 60% acetonitrile (solvent B) at time 0 min, and 75% from 2 min, to 5 min, and back to 60% from 6 min to 8 min, (re-equilibration step). The flow rate was set at 0.5mL / min, the autosampler was conditioned at 18 ºC, an injection volume of 20 µL was used to optimize the drug signals and for analysis. Mass spectrometric detection was operated using electrospray ionization in the positive mode. Nitrogen was used as the nebulizing, desolvation and collision gas (1.4×10^{-3} to 1.6×10^{-3} mbar). The ion source parameters were: capillary 3.5 kV, exit potential 5V, RF lens 0.5V, source temperature was 100 ºC and desolvation temperature 250 ºC. Multiple reaction monitoring (MRM) was employed for the data acquisition. The precursor ions of artemether and DHA were the ammonium adduct $[M+NH_4]^+$ and the protonated molecular ion $[M+H]^+$ was the precursor ion of AL and DBL. The MRM was employed for data acquisition, analytical parameters optimized for the compounds were declustering potentials (DP) and collision energies (CE) (Table 1), the scan dwell time was set at 500 ms for each channel. Data acquisition and analysis were accomplished with Mass Hunter software (version A.02.00; Agilent Technologies).

Compound	Precursor Ion	MRM (Q1/Q3) (amu)	Dwell time (ms)	DP (V)	CE (eV)
ART	$[M+NH_4]^+$	316.3→163.1	500	92	10
DHA	$[M+NH_4]^+$	302.3→ 163.1	500	90	10
LUM	$[M+H]^+$	530.3→ 512.2	500	150	30
DBL	$[M+H]^+$	472.2→ 454.1	500	110	18
MQ	$[M+H]^+$	379.1→ 361.1	500	60	30

Table 1. Compound optimization parameters for artemether (ART), dihydroartemisinin (DHA), lumefantrine (LUM), desbutyl-lumefantrine (DBL) and mefloquine (MQ) and MRM transitions.

Preparation of standard solutions

Stock solutions of ART, DHA and MQ (1mg/ mL) were prepared by dissolving the accurately weighed reference substances in methanol. LUM and DBL stock solutions (1000µg/ mL) were prepared in a similar way using methanol and glacial acetic acid (100:2) as a solvent. The working solution of MQ (IS) was prepared by diluting the stock solution with methanol to a final concentration of 10µg/ mL. All of the stock solutions were prepared and stored in amber glass vials at nominal 20 ºC till use.

Preparation of calibration and QC samples

The working solutions containing both ART /DHA and LUM/ DBL were prepared using serial dilutions of the stock solutions with methanol and water (50:50, v/v). Nine calibration samples were prepared by spiking the appropriate amounts of these working solutions into blank plasma obtained from healthy volunteers.
The concentration of the calibration samples in plasma were 5, 25, 50, 100, 300, 500, 700, 1000 and 1500 ng/mL for ART/ DHA and 5, 25, 100, 300, 900, 1500, 2500, 4000 and 5000 ng/mL for LUM/ DBL. Quality control (QC) samples in plasma were prepared in a similar way, at high, middle and low concentrations: 25, 350 and 560 ng/mL for ART/ DHA and 25, 1600 and 3200 ng/mL for LUM/ DBL.

Sample preparation

A 50µL aliquot of the IS solution (10µg/mL of MQ in methanol) was added to 150µL of the plasma sample in a clean 1.5 mL polypropylene tube. A 300µL aliquot of glacial acetic acid 0.5% (v/v) in methanol was added, the sample was vortex-mixed for 40 seconds followed by centrifugation (3500 x g; 10 min.; 4 ºC). The supernatant was transferred into an autosampler vial and submitted for analysis with a volume of 20µL being injected into the chromatographic system.

METHOD VALIDATION

The validation process was carried out according to Guidance for Industry–Bioanalytical Method Validation, recommended by the US Food and Drug Administration (Van A. *et al*., 2013). The validation parameters evaluated were selectivity, linearity and sensitivity, extraction recovery, accuracy and precision, stability, matrix effect and carry over.

Selectivity

Selectivity of the method was assessed and assured by analysis of six independent blank plasma from different sources, each blank sample was tested for interference using the proposed extraction procedure and chromatographic/mass spectrometric conditions and compared to those containing ART, DHA, LUM and DBL at lower limits of quantitation (LLOQ) or MQ at 1000 ng/mL. In addition, plasma samples spiked with commonly used antimalarials and analgesics (sulfadoxine, artesunate and paracetamol each at 1000 ng/mL) were also evaluated to ensure there were no interferences in the method.

Linearity and LLOQ

Linearity of the calibration curves were assessed assaying standard plasma samples at nine concentrations in the range of 5-1500 ng/mL for ART/DHA and 5-5000 ng/mL for LUM/DBL on three consecutive days. The curves were constructed by plotting the peak-area ratio of each antimalarial drug to that of the IS verses the nominal concentration of the drug. The curves were evaluated and fitted by weighted (1/x) linear regression. The LLOQ was defined as the lowest concentration on the calibration curve, at which an acceptable accuracy (relative error, RE) within ± 20% and a precision (relative standard deviation, RSD) below 15% can be obtained by means of analyses of at least five replicates from a homogenous sample.

Accuracy and precision

The intra-day accuracy and precision of the method were evaluated by analyzing six replicates of QC samples at three concentration levels (25, 350, 560 ng/mL for ART/DHA and 25, 1600, 3200 ng/mL for LUM/DBL) on the same day. Inter-day accuracy and precision was determined by analyzing freshly prepared QC samples (six replicates) at three concentration levels on three consecutive days. Each validation run consisted of one set of calibration standards and six replicates of QC samples at three concentration levels.

Extraction recovery and matrix effect

The extraction recovery of the method was determined by analyzing six replicates of plasma samples at three QC concentration levels of 25, 350, 560 ng/mL (ART/DHA) and 25, 1600, 3200 ng/mL (LUM/DBL) corresponding to low, medium and high QCs. The recovery was calculated by comparing the peak areas obtained from extracted spiked samples (A) with those of samples spiked post-extraction (B) at corresponding concentrations. Matrix effect was evaluated to verify whether ion suppression or enhancement due to the co-elution matrix components existed in the analysis. The peak areas of the analytes and the IS from the post-extraction (protein precipitation) matrix spike samples were compared to those of the standard solutions in the mobile phase at the same concentrations. This experiment was carried out with blank plasma samples from six different sources at low and high QC concentrations of ART, DHA, LUM and DBL. The extraction recovery and matrix effect of IS at a single concentration of 1000 ng/mL were also evaluated using the same procedure.

Stability

The stability of the analytes in human plasma was assessed by analyzing six replicates of low and high QC samples under different temperature and time conditions. The freeze–thaw stability was performed by subjecting QC plasma samples to three freeze (−20 ˚C) – thaw cycles at ambient temperature (24 ◦C). QC samples were stored frozen at −20 ˚C for 60 days and analyzed after allowing to thaw unassisted at ambient temperature to determine the long-term stability. Short-term stability was assessed by keeping QC samples at ambient temperatures for 8 h before processing and analyses. The post-preparation stability was studied by analyzing the processed QC samples kept in the autosampler at 4 ˚C for 15 h. Sub-stock solution stability was evaluated for the analytes, by comparing the response

generated from the same solution at preparation and after being stored at −20 ◦C for a period of 28 days. The IS was freshly prepared on every day of analysis. The analytes were considered stable when the concentrations found were within ± 15% of the initial concentration.

$$ST\% = \frac{c_t}{c_0} \times 100\%$$

Where c_0 is the initial concentration, determined without introducing any extra pauses in the analysis process; c_t is the concentration obtained after the storage period with time *t*.

Pharmacokinetics study

The validated method was used to determine the plasma concentration of ART, LUM and their principle metabolites in a pharmacokinetic study with fixed-dose combination tablets in three healthy volunteers. The volunteers received single oral dose of 80mg ART and 480mg LUM, corresponding to four tablets of the fixed-dose combination with milk. Blood samples (2 mL) were collected into heparinized tubes at 0, 0.5, 1, 1.5, 2, 3, 6, 8, 10, and 12h. Dosing was administered just after the pre dose sampling. Plasma samples were obtained by centrifugation at 2000 rpm for 10 min and frozen at −80 ◦C until the analyses.

RESULTS AND DISCUSSION

Optimization of mass spectrometry

The LC–MS/MS operation parameters were carefully optimized for the simultaneous determination of ART, LUM and their principle metabolites. The mass spectrometer was tuned in the positive ionization mode with ESI for optimum response of all the analytes as previous attempts to use atmospheric pressure chemical ionization (APCI) resulted to low intensities of ART precursor ions and poor detection of LUM (Sandrenan N. *et al*., 1997; Souppart C. *et al*., 2002). Parameters such as desolvation temperature, ESI source temperature, capillary and cone voltage, flow rate of desolvation gas and cone gas were optimized to obtain the highest intensity of the molecular ions of the analytes and IS. Whereas Xing *et al*., 2006 and Sabarinath *et al*., 2003 had performed successful quantitation of artemisinin derivatives by LC–MS/MS using ESI by monitoring the ammonium adduct $[M+NH_4]^+$ as the precursor ion, the 0.5% formic acid in 20mM ammonium formate used in this method produced desirable precursor ions intensities $[M+NH_4]^+$ of ART m/z 316 and DBL m/z 302 while at the same time facilitating the protonation $[M+H]^+$ of LUM precursor ion m/z 530 and DBL m/z 472.

ART and DHA presented the same intense product ion at m/z 163 at a collision energy of 10 eV. For LUM, the major product ion was m/z 512, at a collision energy of 30 eV. The product ion mass spectra of ART, LUM and their principle metabolites are presented in Figure 2.

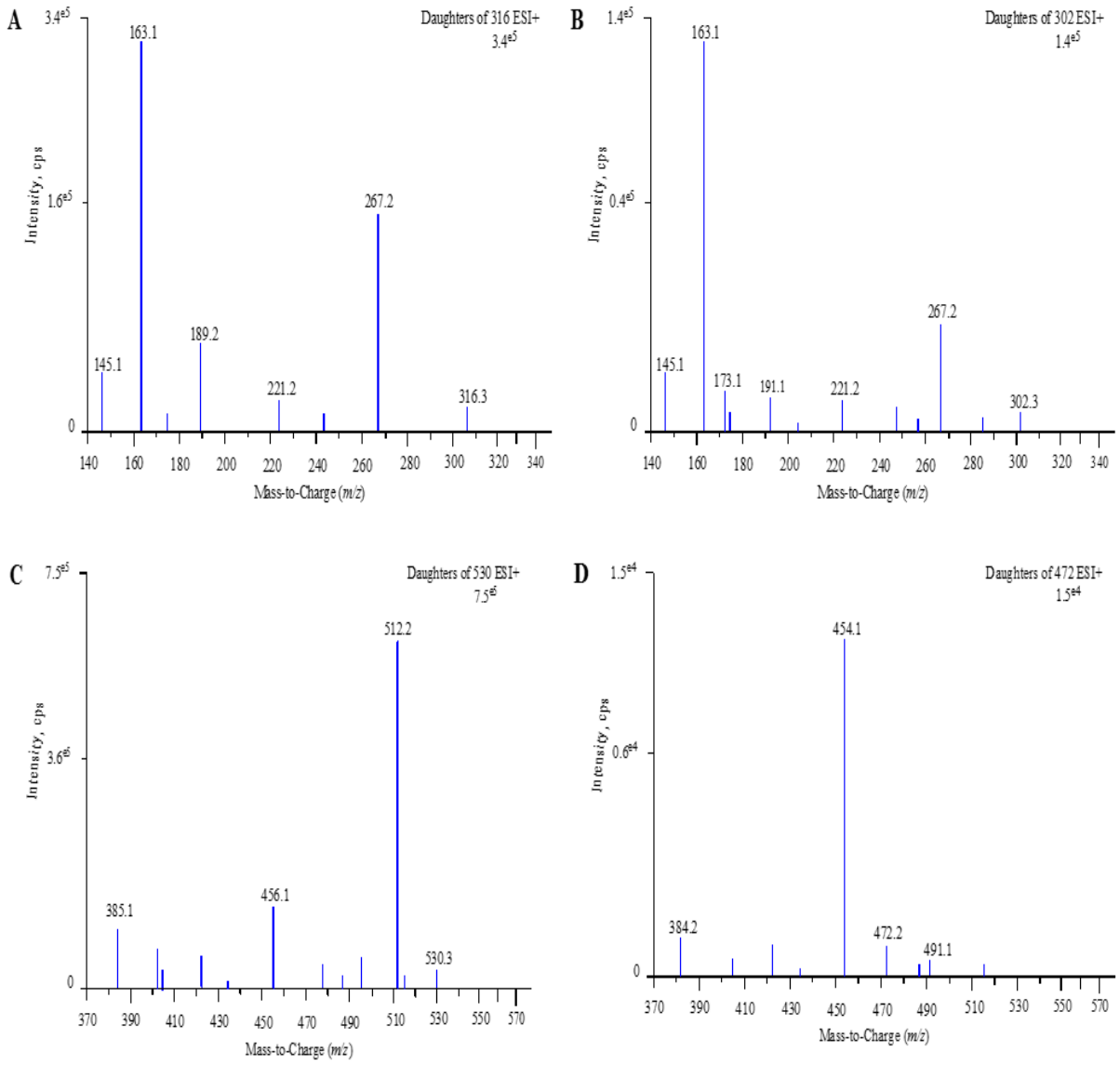


Figure 2. Product ion mass spectra for artemether (A), dihydroartemisinin (B), lumefantrine (C) and desbutyl-lumefantrine (D). The most intense ions were monitored and used for quantitation.

Optimization of chromatography

During the method development, several chromatographic conditions were optimized to ensure good peak symmetry, sharp peaks and well resolved peaks. The use of 20mM ammonium formate buffer in the mobile phase was necessitated for detection of the ammonium adducts $[M+NH_4]^+$ of ART and DHA. The mobile phase acidification with formic acid was to ensure adequate LUM and DBL peak shape and to promote the ionization of the analytes in the positive mode by protonation $[M+H]^+$. Various ratios (20:80, 70:30, 60:40 v/v of 20mM ammonium formate buffer: methanol) were evaluated, high recovery percentages due to an ionization enhancement of the analytes was encountered for LUM and DBL due to matrix effect. Thus, a linear gradient elution program was employed, using 60% acetonitrile (solvent B) at 0 min and 75% B at 2 minutes, so that no matrix effect was verified in this optimized

condition. The re-equilibration phase lasted 2 minutes, with 60% acetonitrile with a total run time of 5 minutes. The retention times were about 1.3 (DHA), 1.7 (ART and MQ), 3.0 (DBL) and 3.9 min for LUM. The chromatograms obtained with this developed method are shown in Figures 3.

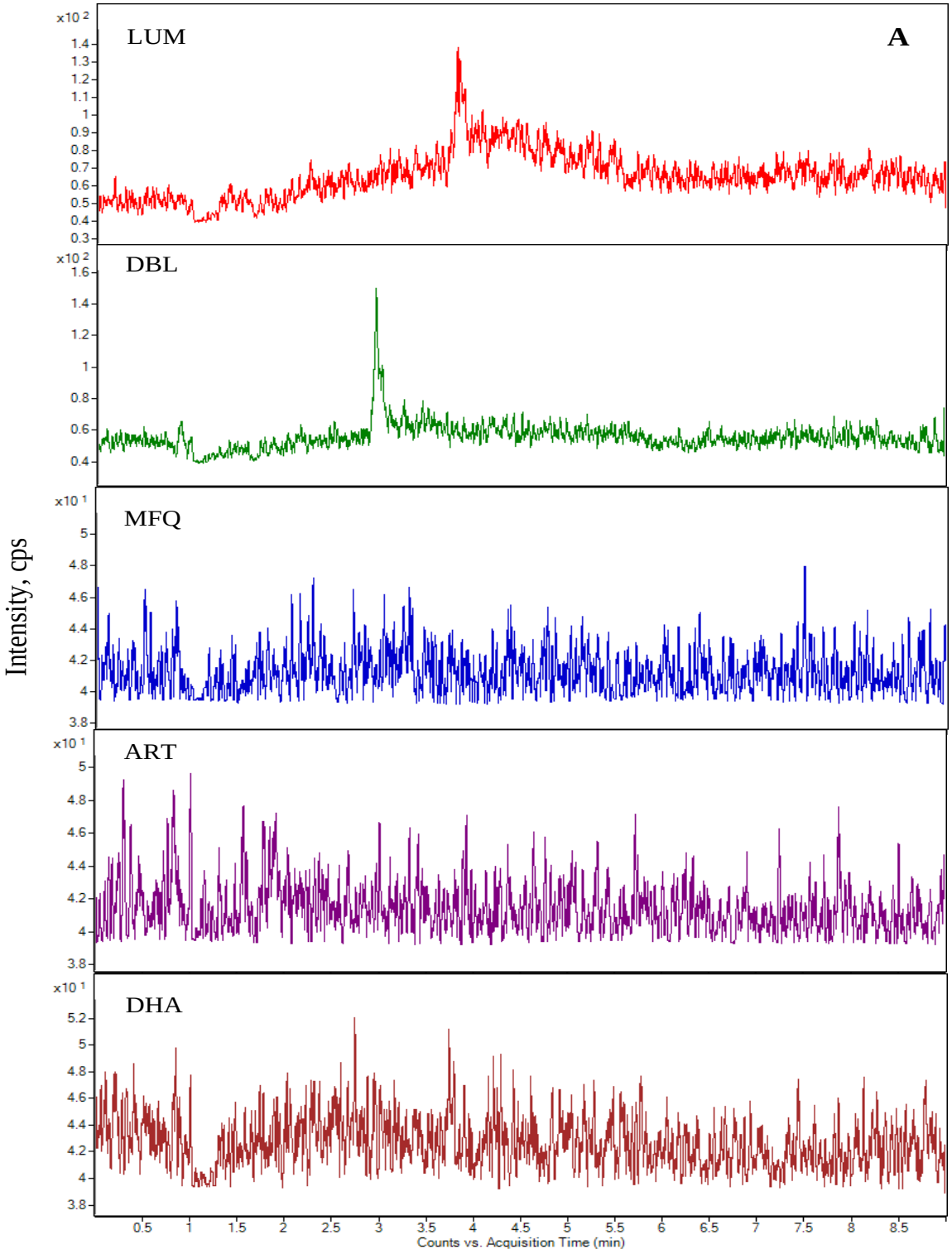


Figure 3A. Representative chromatograms of processed blank plasma at for DHA, ART, MFQ, DBL and LUM

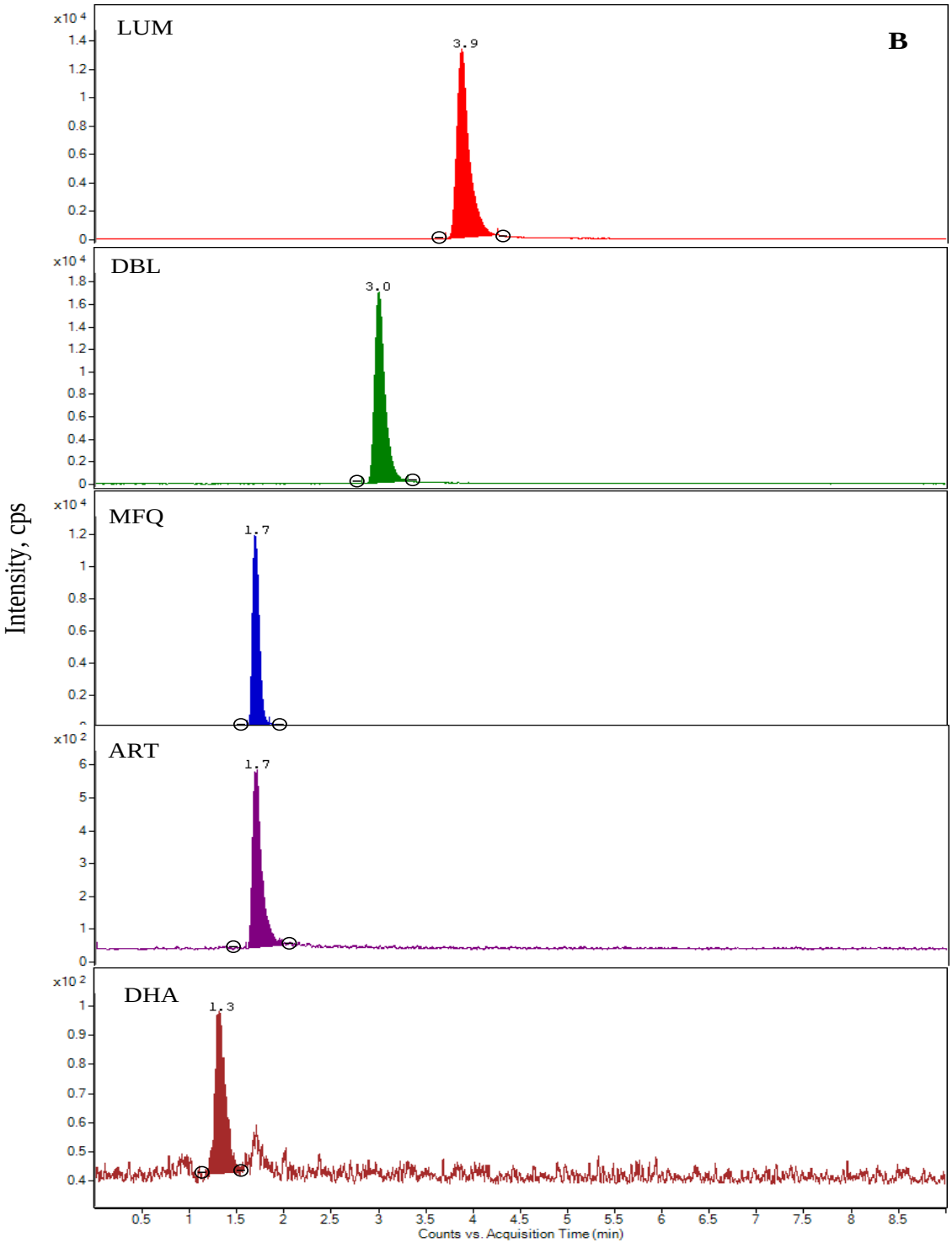


Figure 3B. Representative chromatograms of processed spiked plasma at LOQ of 25ng/mL for DHA (RT 1.3 min), ART (RT 1.7 min), DBL (RT 3.0 min) and LUM (RT 3.9 min).

Selection of extraction method

Several extraction methods were tried, since all the analytes and the IS significantly differs in drug-plasma binding and physicochemical properties, it was difficult to optimize extraction

procedure for all the analytes and IS from plasma. Several approaches were tried based on the previously reported methods such as protein precipitation (PPT) (Hodel *et al*., 2009), solid phase extraction (SPE) and liquid-liquid extraction (LLE) (Khuda *et al*., 2016). Finally PPT procedure with methanol was chosen. This was found to be economical and considerably less laborious compared to the LLE used in previous publications. The validated protein-precipitation procedure is simple and resulted in high recovery percentage for all the drugs, the procedure does not require sophisticated apparatus and thus applicable even within resource-limited laboratories.

Method validation

All the lots of blank plasma used for selectivity studies met the acceptance criteria. Figure 3 show the typical chromatograms of extracted blank plasma (A) a spiked plasma sample with the analytes at LLOQ (B). These results support the high selectivity and specificity of this method as there were no interfering peaks from endogenous compounds observed at the retention times of the analytes and the IS, nor any interfering peaks from the commonly used antimalarials and analgesics (sulfadoxine, artesunate and paracetamol) at the ions selected for analyte quantitation.

The calibration curves for analytes spiked in plasma were found to be linear over the concentration ranges of 5-1500 ng/mL (ART/DHA) and 5-5000 ng/mL (LUM/DBL) with a regression coefficient greater than 0.9992. A weighted (1/x) linear regression model was used by determining the best fit of the peak-area ratios (peak area of analyte/ peak area of IS) vs analyte concentration and conformed to y= mx + c (where y- peak area ratio; x- concentration; c- slope of the curve). The choice of this regression model was based on all available data from the validation phase, in light of this the method proved to be reliable in terms of accuracy and reproducibility over the entire calibration range. Typical regression parameters for the calibration curves are summarized (Table S1). The LLOQs were determined by the needs of the method and defined as the lowest standards on the calibration curve with identifiable, discrete, and reproducible with a precision ≤ 20% and accuracy within 85%-115%. The limits of detection (LODs) were determined as the lowest concentration of the analyte at which the signal to noise (S/N) ratio exceeded 3:1 (Van A. *et al*., 2013), these were 3 ng/mL (ART/DHA) and 2 ng/mL for (LUM/DBL).

Intra-assay (n=6)	Analyte	Nominal concentration (ng/ mL)	Mean estimated concentration (ng/ mL) ±SD	Precision (CV %)	Accuracy (%)
	ART	25	23.7 ± 1.6	2.2	94.8
		350	322.6 ± 6.7	5.7	92.2
		560	531.3 ± 4.0	2.7	94.9
	DHA	25	24.2 ± 4.4	6.1	96.7
		350	526.7 ± 5.9	1.8	93.3
		560	527.4 ± 4.3	3.3	94.2
	LUM	25	23.5 ± 1.8	2.5	93.8
		1600	1566.0 ± 1.5	1.6	97.9
		3200	2948.2 ± 4.3	1.5	92.1
	DBL	25	26.2 ± 5.4	6.8	104.9
		1600	1743.4 ± 1.5	3.8	109.0
		3200	3025.6 ± 4.3	3.4	94.5
Inter- assay (n=18)	ART	25	24.5 ± 3.3	3.4	97.8
		350	328.3 ± 3.0	3.2	93.8
		560	531.4 ± 2.8	2.9	99.3
	DHA	25	24.2 ± 4.3	4.4	96.8
		350	322.4 ± 3.4	3.7	91.1
		560	521.9 ± 2.7	2.9	93.2
	LUM	25	25.1 ± 6.1	6.1	100.7
		1600	1668.8 ± 4.9	4.7	104.3
		3200	3027.2 ± 2.4	2.5	94.6
	DBL	25	26.7 ± 5.5	5.1	106.9
		1600	1723.2 ± 2.8	2.6	107.7
		3200	3024.0 ± 2.6	2.8	94.5

Table 2. Intra-assay and inter-assay accuracy and precision of artemether (ART), dihydroartemisinin (DHA), lumefantrine (LUM) and desbutyl-lumefantrine (DBL) in plasma at LOQ, MOQ and HOQ.

Stability parameters	Spiked conc. (ng/mL)	ART		DHA		LUM		DBL	
		25	560	25	560	25	3200	25	3200
Bench-top stability in matrix (ambient temperature, 8 h)	Mean stability of samples	23.4	518.0	26.3	536.5	24.6	3203.2	24.6	3027.2
	CV %	3.0	2.1	2.2	4.6	5.6	8.4	6.9	3.6
	ST %	93.6	92.5	105.3	95.8	98.5	100.1	98.3	94.6
Freeze-thaw stability (3 freeze-thaw cycles at – 20 °C)	Mean stability of samples	24.0	553.8	23.0	527.0	23.1	3270.4	24.7	3052.8
	CV %	4.1	8.5	3.0	4.7	3.4	6.1	3.9	6.9
	ST %	96.0	98.9	91.9	94.1	92.3	102.2	98.8	95.4
Post-preparative stability (15 h at 18 °C)	Mean stability of samples	25.1	521.4	24.1	524.2	25.4	3078.4	24.2	3058.7
	CV %	3.4	2.4	3.0	2.2	3.6	1.8	3.8	3.0
	ST %	100.5	93.1	96.5	93.6	101.7	96.2	96.9	95.6
Long-term stability (60 days at -20°C)	Mean stability of samples	21.4	518.0	24.0	554.4	24.2	3145.6	24.9	3152.0
	CV %	2.2	2.7	6.1	3.3	2.5	3.8	3.8	3.0
	ST %	85.5	92.5	96.0	99.0	96.9	98.3	99.6	98.5
Sub-stock solution stability (28 days at – 20 °C)	**Nominal Conc. (µg/mL)**		**10**		**10**		**50**		**50**
	Mean stability of samples		9.0		9.4		47.9		46.8
	CV %		3.2		2.8		3.6		2.9
	ST %		90.2		94.0		95.8		93.5

Table 3. Stability (ST %) of artemether (ART), dihydroartemisinin (DHA), lumefantrine (LUM) and desbutyl-lumefantrine (DBL) in plasma with the coefficient of variation (CV %) (n=6).

To evaluate the inter-assay precision and accuracy, six replicates of quality control (LQC, MQC and HQC) plasma samples were analyzed together with one independent calibration standard curve, this was done in three consecutive days; while intra-assay precision and accuracy were evaluated by analysis of quality control plasma samples in replicate of six in the same day. Inter-assay and intra-assay precision were expressed as coefficient of variation (CV %). The accuracy was expressed as the percent ratio between the experimental concentrations and the nominal concentration for each sample. Assay accuracy of the method was above 92.1% and precision values did not exceed 6.8% for both intra and inter-assay experiments (Table 2). The accuracy and precision data evidently show that the method meet the acceptance criteria of accuracy and precision hence reliable in quantifying the drugs.

The protein precipitation method of extraction with acidified methanol yielded high recoveries with better reproducibility of the analytes than in the previously reported publications (Navaratnam V. et al., 1995; Sandrenan N. et al., 1997; Souppart C. et al., 2002; Shi B. et al., 2006). The addition of acetic to methanol for precipitation improved the recoveries of LUM and DBL considerably (Table S2). Matrix effect experiments carried out by direct pre-column infusion of processed blank plasma lots revealed no significant ion enhancement or suppression to the transition ions chosen for analysis in this method. Figure S1 is an overlaid chromatogram of post-column infusion of processed blank plasma and plasma sample at LLOQ of the analytes. In this present method therefore, the matrix effects on all the analytes were not significant.

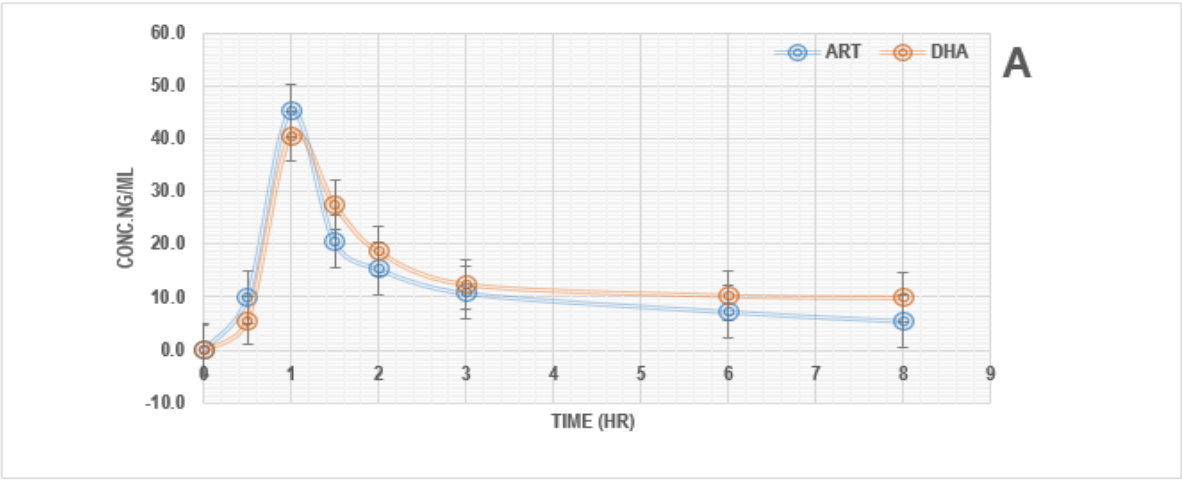
The results of all the stability studies experiments were well within the acceptable limits of accuracy (± 15%) and precision (CV ≤ 15%). The long-term stability duration (60 days) under which the study was conducted was necessitated by the need to minimize the period for storage of the real study samples. Whereas César et al., 2008 and Hodel et al., 2009 have studied the stabilities of these drugs over a longer period, this method focused on their stability under storage at -20 oC which is the common setup with resource-limited facilities. Nonetheless, all the analytes indicated acceptable stability at the storage temperature, 85.5 – 99.6% of the original concentration was found after the storage period of 60 days. Artemether however recorded the lowest value due to its labile endoperoxide bridge (Table 3). Bench-top and post-processing stability results indicate that all the drugs were stable through the investigated period of experiments.

Therefore the sample processing procedure reported here can be used in analyses of large number of samples without the risk of sample degradation due to room temperature exposure, similarly re-analysis of the processed samples can be initiated in case of equipment failure during an analytical run without compromising sample integrity. All the analytes met the stability criteria after the three freeze and thaw cycles, the sub-stock solutions were also found stable (90.2 – 95.8%), and fresh stock solutions were prepared after the 28 days.

Application of the method to a clinical pharmacokinetics study

The validated method was successfully applied to a pharmacokinetic study of ART, LUM and their principle metabolites in healthy volunteers following a single oral dose with Coartem® (80 mg ART and 480 mg LUM) over a course of 12h. The sensitivity and specificity of the method showed to be adequate for accurately characterizing the pharmacokinetics of ART, LUM and their metabolites. The mean plasma concentrations of ART /DHA and LUM/LUM after an oral administration of a single dose of 80mg of ART and 480mg of LUM were highest at around 1h and 6h respectively (Figure 4). The chromatograms of patient samples at the two sampling times are shown in (Figure S2). These results are consistent with those previously reported (Ezzet F. *et al*., 2000 ; Shi B. *et al*., 2006), which demonstrate a rapid absorption and elimination of ART, while LUM presented a long elimination half-life and could be detected in the patients' plasma for up to 48h post dose.

This method requires very small plasma volumes and will allow the assay of drug and metabolite concentrations following administration ART/LUM in people who can only provide small volumes of blood such as infants and children.



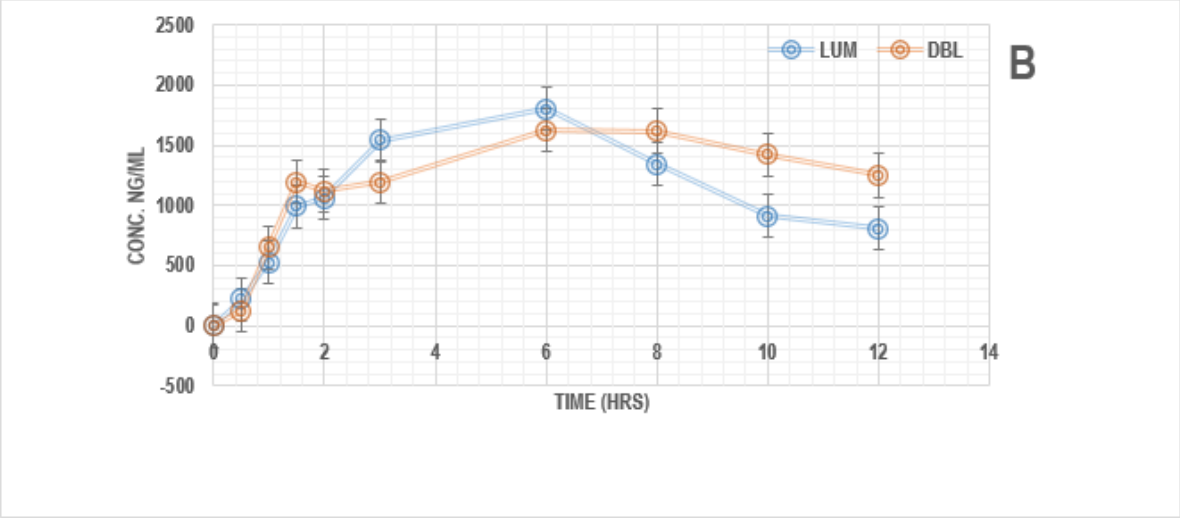


Figure 4. Mean plasma concentrations of ART, DHA (A) and LUM, DBL (B) after an oral administration of a single dose of 80mg artemether and 480mg lumefantrine to healthy volunteers, the bars represents a ±2 standard error value on the mean concentrations.

CONCLUSION

The validated HPLC–ESI–MS/MS method allowed the simultaneous quantitation of ART, LUM and their principle metabolites from only 150µL human plasma, provided simple and rapid analyses, as well as sensitive and reliable results. Therefore, this method proved to be suitable for routine high-throughput analyses and may be successfully applied to pharmacokinetic and bioequivalence of multiple doses evaluated in the present in human subjects.

Acknowledgments

This work is published with the permission of the Director of the Kenya Medical Research Institute (KEMRI). We wish to thank the Consortium for National Health Research (CNHR) who provided the study grant that enabled the conduct and publication of this study.

Conflict of Interest

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript, apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

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