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Simultaneous determination of artemether and its major metabolite dihydroartemisinin in plasma by gas chromatography–mass spectrometry-selected ion monitoring

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Abstract

A sensitive, selective, and reproducible GC–MS–SIM method was developed for determination of artemether (ARM) and dihydroartemisinin (DHA) in plasma using artemisinin (ART) as internal standard. Solid phase extraction was performed using C_{18} Bond Elut cartridges. The analysis was carried out using a HP-5MS 5% phenylmethylsiloxane capillary column. The recoveries of ARM, DHA and ART were 94.9±1.6%, 92.2±4.1% and 81.3±1.2%, respectively. The limit of quantification in plasma was 5 ng/ml (C.V.≤17.4% for ARM and 15.2% for DHA). Calibration curves were linear with $R^2 \geq 0.988$. Within day coefficients of variation were 3–10.4% for ARM and 7.7–14.5% for DHA. Between day coefficients of variations were 6.5–15.4% and 7.6–14.1% for ARM and DHA. The method is currently being used for pharmacokinetic studies. Preliminary data on pharmacokinetics showed C_{max} of 245.2 and 35.6 ng/ml reached at 2 and 3 h and AUC_{0-8h} of 2463.6 and 111.8 ngh/ml for ARM and DHA, respectively.

Keywords: Artemether; Dihydroartemisinin

1. Introduction

Malaria continues to be a major health problem in many areas of the world. Parasite resistance to chloroquine, pyrimethamine and mefloquine is increasing rapidly throughout the world. Semisynthetic derivatives of qinghaosu (QHS), a natural product of the Chinese herb *Artemisia annua*, are highly effective against multiresistant strains of *Plasmodium falciparum*. QHS (artemisinin) derivatives are nitrogen-free sesquiterpenes that contain a peroxide linkage, which confers activity against the malaria parasite. These compounds are effective safe and well tolerated. They are rapidly metabolized to the active metabolite dihydroartemisinin. Artemether (Fig. 1) is one of these promising antimalarial compounds [1].

Only recently have literature reports appeared regarding metabolic, bioavailability and comparative pharmacokinetic studies of ART derivatives. This

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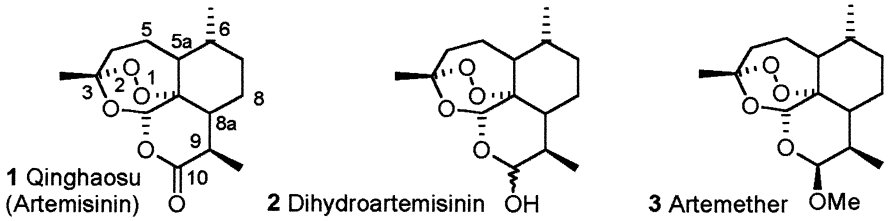

Fig. 1. Structure of artemisinin, artemether, and dihydroartemisinin.

can be attributed to the fact that these compounds do not have appropriate UV or fluorescent properties. This makes the development of suitable analytical methods for their measurement in nanogram concentrations in biological fluids difficult to achieve [2]. Nevertheless, several analytical techniques have been reported for the qualitative and quantitative determination of these compounds in biological matrices. High-performance liquid chromatography (HPLC) with post-column alkali [3–5] and pre-column acid [6–8] derivatization and UV detection were the most popular techniques. The presence of a peroxide bridge in the structure of these compounds offers the advantage of the use of HPLC with reductive electrochemical detection [9–12]. Combined HPLC with mass spectrometry (LC–MS) [13–15] and HPLC with chemiluminescent detection [16] is also reported. Gas chromatography (GC) alone [17] and combined GC–MS [18–20] has also been used for the quantitation of these compounds. Recently a method using capillary gas chromatography–chemical ionization mass spectrometry for the characterization of ARM and its metabolites in rat and dog blood has been reported [21]. Each method suffers its own limitations. Some HPLC–UV methods require lengthy derivatization procedure prior to sample preparation [8]. Some lack the required sensitivity to be used for measurement of blood samples obtained from clinical investigations [5]. While HPLC–EC is currently the most sensitive method for the determination of ART and its derivatives in biological matrices, it suffers from a need to maintain a constantly oxygen-free environment and a difficulty in quantifying oxygenated metabolites [12]. In this report we describe a novel GC–MS–SIM analytical method for the determination of ARM and DHA in plasma with application to pharmacokinetic studies.

2. Experimental

2.1. Materials

ARM, DHA and ART (internal standard) were obtained from SAPEC, Lugano, Switzerland. Ethyl acetate, methanol, 1-chlorobutane (high purity reagents, HPLC grade) and glacial acetic acid (analytical reagent) were obtained from Fisher (Loughborough, Leicestershire, UK). Solid phase extraction cartridges (SPE), Bond-Elut C_{18} octadecyl (100 mg/ml), were purchased from Varian (Harbor City, CA, USA). Collection tubes were silanized with dimethyldichlorosilane (Fluka, Buchs) in toluene (Fisher). ARM intramuscular (IM) injections (Paluther®) were obtained from, Rhone–Poulenc–Rorer (France).

2.2. Chromatography

A Hewlett-Packard 6890 GC (Palo Alto, CA, USA) was used with a capillary column containing 5% phenylmethylsiloxane (HP 5MS, 30 m×0.25 mm I.D., 0.25 μm film thickness). The system is equipped with a HP 7673 autosampler. Detection of the pyrolysed compounds was performed on a HP 6890 mass-selective detector. Purified helium (Purity 99.999%) was used as a carrier gas with a constant flow-rate of 0.9 ml/min, average velocity of 35 cm/s and pressure of 9.1 p.s.i.. Injection was performed with a splitless mode at 275°C in a HP deactivated glass liner (4 mm, Borosilicate). The temperature of the GC was set at 100°C initially for 2 min, ramped to 300°C at 16°C/min and kept at this temperature for further 6 min. The total run time was 20.5 min with a solvent delay time of 7.5 min.

Detection and quantification of ARM, DHA, and ART were achieved by monitoring the intensity of a

target ion and 2–3 qualifier ions using selected ion monitoring (SIM) as acquisition mode.

The chromatograms were recorded and analyzed with software provided with the instrument (Chemstation G1701 AA version A 02.00). Quantitation of ARM and DHA was achieved using peak area ratios of the selected ions of each compound to those of the internal standard and an appropriate calibration curve. The GC–MS was autotuned daily to ensure consistent performance.

2.3. Stability of ARM, DHA and ART in standard solutions

Standard solutions (1 mg/ml) of ART, ARM, and DHA were prepared in a mixture of 20% ethylacetate in 1-chlorobutane (E–C, 20:80, v/v). The standard solutions were prepared at different time points (about four months). Each solution was divided into two parts. One part was placed at room temperature and the second part was kept in the refrigerator until the date of analysis. A fresh standard solution (1 mg/ml) of each compound was prepared on the day of analysis. The standard solutions were then diluted to make appropriate working concentrations. For DHA two working concentrations (20 and 80 μg/ml) were prepared of the fresh and stored solutions. For ART and ARM duplicates of one working concentration were prepared (20 μg/ml). Calibration curves of the individual compounds were constructed in 20% E–C in the range of 5–200 μg/ml. The concentrations of the three compounds in fresh and stored solutions were determined. The stability (STABss%) of the compounds was calculated as percentage of the fresh solutions.

2.4. Extraction procedure

In order to minimize the possibility of glass binding, all glassware was silanized before use. The silanization procedure was carried out as follows: 1 ml of 5% (v/v) methyl dichlorosilane in toluene was added to all glass tubes. The tubes were then vortexed for 30 s and the silanization solution was removed. The tubes were rinsed with methanol (2 ml) with vortex mixing and the methanol was discarded. The tubes were then placed into an oven until dry.

Stock solutions (1 mg/ml) of the three compounds were prepared in a mixture of 20% E–C. Standard solutions (10 and 1 μg/ml) were prepared by dilution of stock solutions with the same solvent. Appropriate dilutions of standard solutions were used for preparation of calibration samples.

SPE procedure was carried out using a vacuum manifold (Vac Elut SPS 24™). Plasma (1 ml) was spiked with the appropriate amounts of ART as internal standard. The Bond Elut columns were conditioned with 1 ml of methanol followed by 1 ml of acetic acid (1 *M*). Plasma was added to the columns and washed twice with 1 ml of acetic acid and once with 1 ml of 20% methanol in acetic acid. The compounds were then eluted with two sequential applications of 1 ml of 20% E–C. A small amount of aqueous phase was aspirated and the organic phase was evaporated under a stream of N_2 at 40°C. The residue was reconstituted in 20 μl of 20% E–C and 4 μl of the mixture was injected into the column.

Calibration samples were prepared by spiking plasma (1 ml) with ART (100 ng for low range and 1000 ng for high range calibration curves) and the appropriate amount of ARM and DHA. The mixtures were vortexed and SPE was applied as above.

2.5. Recovery

The recoveries of the extraction procedure for ARM, DHA and ART was determined by spiking drug free plasma with a specified amount of each compound. SPE and chromatography procedures were as described previously. Recovery was determined by comparing the estimated concentrations of the compounds in plasma with directly injected solutions containing the same concentrations in 20% E–C.

2.6. Calibration, reproducibility and precision

Initially, calibration solutions of ARM, DHA and ART were prepared in 20% E–C in concentrations ranging from 0 to 4000 ng/ml. These solutions were injected directly into the GC–MS to assess detector linearity and the limit of detection of each compound. Peak areas of selected ions were plotted against their respective concentrations. Linear regression on the plots showed that all three compounds

were linear ($R^2 \geq 0.999$). The limit of detection was found to be 0.1 ng/ml for all compounds following direct injections.

Calibration curves in plasma were constructed by spiking drug-free plasma with standard solutions of ARM and DHA to produce concentrations of 2–1000 ng/ml. The internal standard was added to each concentration (10 μl of 10 ng/μl solution) and the mixture was vortexed. The calibration samples were extracted and chromatographed as described above. Peak area ratios of selected ions of ARM and DHA versus ART were plotted against their respective concentrations. The plots were subjected to linear regression analysis.

Reproducibility of the procedure was assessed by determination of inter and intra-day variability of the mixtures of ARM and DHA in plasma. Intra-assay (Within-day) precision and accuracy was determined by analyzing 6 replicates of 3 concentrations (20, 100, and 500 ng/ml) of each analyte in the same day. Interassay (day to day) variability was assessed by analyzing 4 replicates of 5 concentrations (20, 100, 500, 2000, 4000 ng/ml) of ARM and DHA every day for 3 days. A ten points calibration curve was constructed each day.

2.7. Selectivity

Injecting chloroquine, quinine, and arteether under same conditions assessed the selectivity of the method.

2.8. Clinical study

One healthy male Sudanese volunteer age 24 years and weight 55 Kg participated in the study. The volunteer was assessed for normal renal and liver functions. No other drugs were taken 7 days prior and during the clinical trial. The study was approved by the Ethics Committee of the National Health Laboratory, Khartoum, Sudan. Informed consent was signed by the volunteer. The volunteer was given an 80 mg IM dose of artemether (Paluther®, Rhone–Poulenc–Rorer, France) following an over night fast. A normal breakfast was served 3 h after injection. Venous blood samples (5 ml) were collected immediately before drug administration and at 0.5, 1, 2, 3, 4, 8 and 12 h after injection. Blood was collected in heparinized vaccutainers, centrifuged immediately and plasma was separated and stored at −20°C until transported to Liverpool for analysis. 10 μl of internal standard (10 μg/ml) was added to 1 ml of plasma. The mixture was analyzed as described above.

2.9. Data analysis

All linear regressions and predictions of unknown concentrations were determined by the on line data analysis Software provided with the GC–MS.

The stability of ARM, DHA and ART in standard solutions was calculated using the following equation:

$$\text{STABss\%} = \frac{\text{Mean conc. of stored solutions}}{\text{Mean conc. of the fresh solution}} \times 100$$

Pharmacokinetic parameters were calculated from plasma concentration-time profiles constructed for each compound using a non-compartmental approach. The elimination half-life was calculated by log-linear regression analysis of the terminal portion of the plasma concentration versus time curve. The area under the curve (AUC) was calculated by the trapezoidal rule. The maximum plasma concentration (C_{max}) and the time to reach this concentration (T_{max}) were noted directly. Other pharmacokinetic parameters (e.g. clearance and apparent volume of distribution) were calculated using standard model-independent equations.

3. Results and discussion

3.1. Chromatography

In a recent report Blum et al. [21] showed that capillary gas chromatography–chemical ionization mass spectrometry can be successfully used as a sensitive analytical method for mixture analysis of ARM and related compounds in pharmacological studies. In fact this approach was used in 1988 by Theoharides et al. [18] for the determination of DHA in blood. In the present study, we determined the concentration of ARM and DHA in human plasma by measuring their gas chromatographic decomposition products with GC–MS–SIM. Quantitation of

these decomposition products reflected the concentration of their parent compounds in plasma.

Once the sample is injected into the GC it is pyrolyzed in the injection port liner which is a part of the GC. Vapourized decomposition products are then transferred to the column where separation of compounds takes place. Upon exiting the column, the compounds are bombarded by a stream of electrons, which causes reproducible ionization of the molecules followed by reproducible fragmentation of the molecular ions (electron impact ionization). Mixtures of these ions can be detected and monitored in the mass spectrometer by scan or SIM mode. Data is then plotted either as chromatogram (abundance vs. time) or as mass spectra (abundance vs. m/z ratio).

In the present work, initial analysis was performed with the mass spectrometer in scan mode in order to identify the ART compounds and select the most abundant ions for monitoring with SIM mode. Full scans of the decomposition products of the three compounds were performed at m/z 50–500 (Fig. 2).

SIM was used as acquisition mode in order to increase the detector sensitivity of the measurement. When injected separately, ARM decomposed into two major and few minor products, DHA pyrolyzed into only one major product, and ART pyrolyzed into two products. These major decomposition products have different retention times and different mass spectra. The presence of only one product in each peak was confirmed by peak purity check for each compound. In the present analysis, three groups of ions were monitored in the same run for simultaneous determination of the three compounds. Group 1 (DHA) was monitored from $t=7.5$ to 10.2 min with m/z 152 as target ion and 123, 180 and 210 as qualifier ions (Dwell 100 ms). Group 2 (ARM) was monitored from 10.2 to 11.0 min with ion 138 as a target and 55, 96 and 165 as qualifiers (Dwell 35 ms). Group 3 (ART) started at 11 min till end of the run with m/z 137 as target ion and 151 and 166 as qualifier ions (Dwell 50 ms). Monitoring was performed at mass range of -0.3 to $+0.7$ amu.

Fig. 3 shows the extracted ion chromatogram of only the target ions of each compound, i.e. 152 for DHA, 138 for ARM, and 137 for ART. As can be seen in the figure, very good separation of the major decomposition products of the three compounds was achieved with reasonable retention times of 9.78, 10.3, and 11.5 min for DHA, ARM and ART, respectively. The peak at 12.1 min represents a minor decomposition product of ART. This elutes after the compounds of interest and does not affect the separation of the three compounds or the reproducibility of the analysis. Injection of the compounds was performed in splitless mode in order to increase the amount entering the column.

In the injector port, pyrolysis is complete. Contamination from previous injections does not affect the rate or extent of pyrolysis. This was ensured through baking out the column after each injection by ramping the oven temperature to 300°C and holding that temperature for 6 min. As can be seen from the chromatogram, the final compound of interest (ART, I.S.) eluted at 11.50 min and, because of column baking, the total run time is 20.5 min. As a further precaution, the injection port inlet liner was changed every week or whenever the autotune of the GC–MS was different from the expected value thus indicating that it was necessary to change the liner. Since DHA showed only one predominant product, we did not attempt to identify whether this decomposition product is related to the α or the β isomers.

3.2. Extraction and recovery

ART was chosen as internal standard because of its structural similarity to the investigated compounds. Moreover it elutes at a different retention time with different decomposition products and different qualifier and target ions

During development of the extraction procedure different types of solvents and mixtures of these solvents were tested (e.g. chlorobutane, ethylacetate, hexane) in attempt to choose the best solvent for liquid–liquid extraction. Protein precipitants i.e. perchloric acid and acetonitrile were also tested. These extraction procedures were found to be unsatisfactory because of poor recovery and the extracts were heavily contaminated with endogenous plasma constituents.

SPE procedure was found to be rapid and simple to perform with good recovery and less contamination. The mean recoveries ($\pm$SD, $n=4$) of ARM, DHA and ART were found to be $94.9\pm1.6\%$, $92.2\pm4.1\%$ and $81.3\pm1.2\%$, respectively.

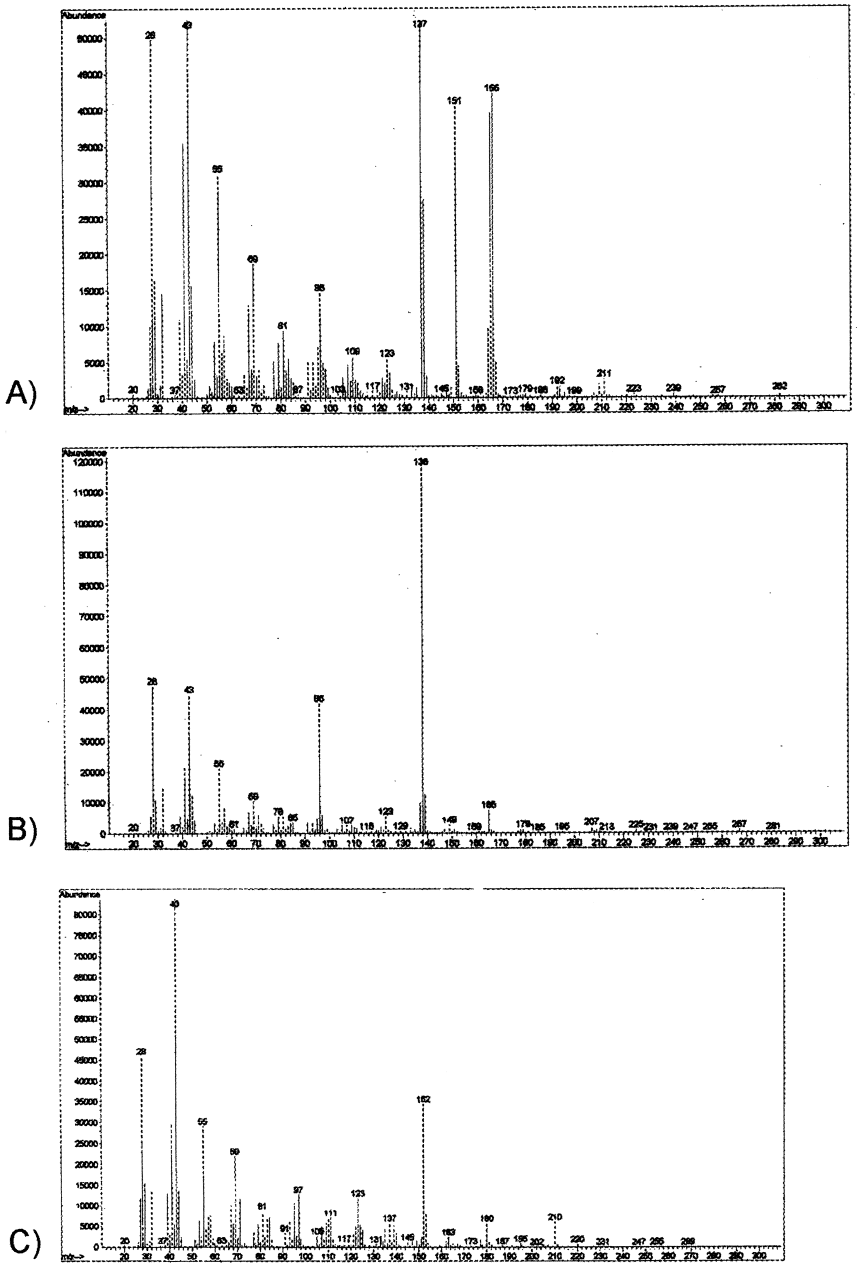

Fig. 2. Full scan mass spectra of the major decomposition products of (A) ART, (B) ARM, and (C) DHA.

The method showed high selectivity. Other antimalarial drugs such as chloroquine and quinine did not interfere with the analysis. Other artemisinin compounds e.g. arteether did not affect the elution of ART, ARM and DHA. Arteether eluted at 10.44 min when injected at the same conditions.

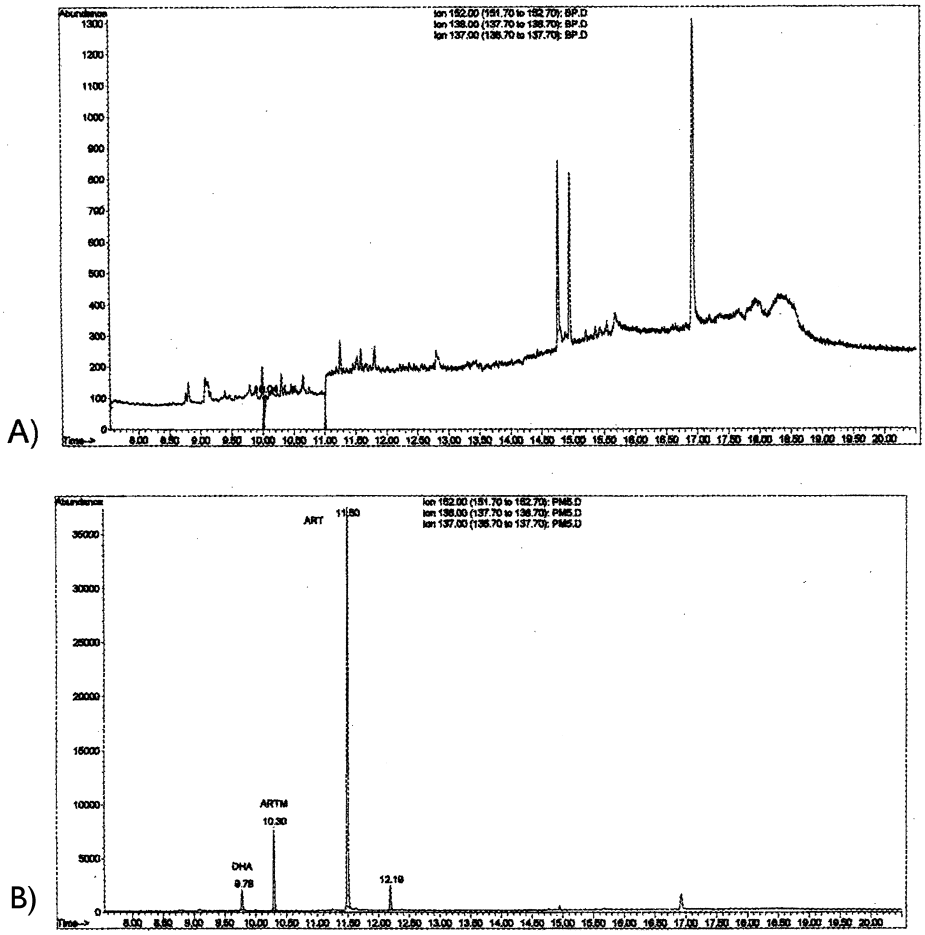

Fig. 3. Extracted ion chromatogram of A) drug free plasma and B) plasma spiked with 5 ng/ml of ARM and DHA and 50 ng/ml ART.

3.3. Stability

There is considerable body of evidence in the literature regarding the stability of ART derivatives when stored in plasma. Batty et al. [5] showed that artesunate and DHA can be stored at −20°C or lower for up to 6 months with no significant degradation. Melendez et al. [10] also carried out stability studies on DHA and arteether in plasma. These authors demonstrated that both compounds were stable when stored in plasma at −20°C or −80°C for a period of 8 months. However, we did not come across any report on the stability of ART derivatives in organic solvents, particularly the mixture of 20% ethylacetate in chlorobutane used to elute and reconstitute the artemisinin drugs in the current study. In the present study, the three compounds were found to be stable both at room temperature and in the refrigerator when prepared in a mixture of 20% E–C and stored for a period of four months (Table 1).

3.4. Limit of quantification

The minimum detectable concentration was 2 ng/ml in plasma for both ARM and DHA. The limit of quantification was determined by assessing the low-

Table 1
Stability of ART, ARM and DHA in standard solutions

Compound	STABss% mean±SD (range) Room temperature	STABss% mean±SD (range) Refrigerator
ART	106.4±5.2 (99.5−113.9%)	99.8±1.1 (98.32−101.2%)
ARM	111.9±7.7 (98.7−117.98%)	108.2±2.5 (105.3−111.6%)
DHA	109.1±3.3 (92.3−112.4%)	98.2±8.7 (88.9−111.8%)

est concentration that can be measured with a stated level of confidence. This was found to be 5 ng/ml for both compounds in plasma (C.V.≤17.4% for ARM and 15.2% for DHA, $n=6$). It is worth mentioning that with solutions prepared in E–C and injected directly into the GC the limit of detection is as low as 100 pg/ml. The limit of quantification of this method is lower than those previously published methods in which HPLC was used [5,7,8,10,22]. However, Navaratnam et al. [11] recently reported a limit of detection of 4 ng/ml for DHA when analyzed by HPLC–EC.

3.5. Calibration and assay precision

Calibration curves were constructed in the range of 0–100, 100–500 and 500–4000 ng/ml. Since detection of the compounds takes place as arbitrary area counts in relation to the highest peak present, the amount of the internal standard used was varied according the calibration curve range.

The correlation coefficient, R^2, of the standard curves was found to be >0.988 on all occasions for both compounds.

Table 2 shows the precision and accuracy of the calibration standards of ARM and DHA during the study.

The within day coefficient of variation ranged from 3–10.4% for ARM and 7.7–14.5% for DHA (Table 3). The day to day coefficient of variation was found to be 6.5–15.4% for ARM and 7.6–14.1% for DHA (Table 4).

3.6. Clinical study

Fig. 4 shows the plasma concentration-time profile of ARM and DHA following the administration of 80 mg artemether IM injection to a healthy volunteer. For ARM, a maximum concentration of 245.2 ng/ml was reached in 2 h and AUC_{0-8h} was found to be 2463.6 ngh/ml. The plasma clearance (Cl/F) and apparent volume of distribution were 0.03 l/h and 0.42 l, respectively. For DHA a C_{max} of 35.6 ng/ml was reached in 1 h and AUC_{0-8h} was 111.84 ngh/ml. The plasma clearance and Vd were 0.72 l/h and 0.85 l, respectively. Since the principal objective of this manuscript is to describe a novel method of analysis only one subject is included in

Table 2
Precision and accuracy of the calibration standards of ARM and DHA[a]

Spiked Conc. (ng/ml)	ARM					DHA				
	M Conc. (ng/ml)	SD	C.V. (%)	RE (%)	*n*	M Conc. (ng/ml)	SD (%)	C.V.	RE (%)	*n*
5	4.9	0.9	17.4	−2	6	5.2	0.8	15.2	4.3	6
10	8.9	1.2	13.1	−11.3	7	8.9	1.5	16.2	−10.7	6
40	38.0	6.7	17.6	−5.0	6	43.3	5.5	12.8	8.2	6
80	83.9	7.5	8.9	4.9	9	82.3	8.5	10.3	2.9	10
120	118.6	13.6	11.5	−3.5	5	118.2	17.3	14.7	−1.5	4
160	156.7	8.1	5.2	−2.1	8	159.9	15.2	9.5	−0.1	10
200	208.4	14.5	7.0	4.2	6	192.5	24.9	12.9	−3.8	4
300	302.2	28.4	9.4	0.7	4	311.3	3.0	1.0	3.8	2
320	340.1	37.3	11.0	6.3	4	299.2	18.2	6.1	−6.5	4
400	381.6	36.2	4.5	−4.6	6	411.2	18.3	4.5	4.0	9

[a] M Conc.=measured concentration; SD=standard deviation; C.V.=coefficient of variation; RE=residual error.

Table 3
Intra-assay variation for analysis of ARM and DHA in plasma ($n=6$)

Compound	Concentration added (ng/ml)	Concentration measured (mean ±SD) (ng/ml)	Coefficient of variation (%)
ARM	20	19.7±1.9	9.6
	100	102.6±3.1	3
	500	514.2±53.7	10.4
DHA	20	20.1±2.9	14.5
	100	119.4±9.2	7.7
	500	531.9±58.6	11

Table 4
Interassay variation for analysis of ARM and DHA in plasma ($n=12$)

Compound	Concentration added (ng/ml)	Concentration measured (mean±SD)	Coefficient of variation (%)
ARM	20 ng/ml	19.6±1.6	8.2
	100 ng/ml	107.2±7	6.5
	500 ng/ml	482.7±52.6	10.9
	2 μg/ml	2.3±0.4	15.4
	4 μg/ml	4.1±0.4	10.2
DHA	20 ng/ml	18.8±2.6	14.1
	100 ng/ml	117.8±10.3	8.8
	500 ng/ml	531.4±63.1	11.9
	2 μg/ml	2.1±0.2	7.6
	4 μg/ml	4.2±0.5	12.6

the pharmacokinetic study. The profile merely aims to demonstrate the ability of the method to measure plasma concentrations associated with therapeutic dosage. Since data are reported from only one subject, realistic pharmacokinetic comparisons cannot easily be made. Nevertheless, the pharmacokinetic parameters obtained from this subject lie within the range reported by other authors for ARM [23,24].

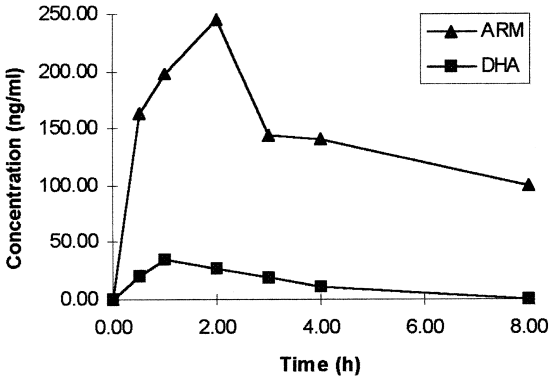

Fig. 4. Plasma concentration versus time profile of ARM and DHA following the administration of 80 mg ARM as a single IM injection to a healthy volunteer.

4. Conclusions

Artemisinin derivatives provide the best potential chemotherapy for resistant *falciparum* malaria. Therefore, the analysis of this class of compounds in biological fluids in low nanogram per milliliter range is very significant. The analytical method described in this paper represents a rapid, sensitive and selective procedure that is entirely suitable for the determination of ARM and DHA in plasma following therapeutic doses. It does not suffer from the difficulties associated with HPLC–EC and is five times as

sensitive as the HPLC–UV methods in the literature. The method may also be suitable for determination of the newer generation artemisinin derivatives where sensitivity might be an issue. The method is quantitative and can be successfully used in pharmacological, bioavailability and pharmacokinetic studies. Currently the method is being applied for the analysis of plasma sample following the administration of artemether to chloroquine resistant *falciparum* malaria patients and healthy controls.

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