DEVELOPMENT AND VALIDATION OF A LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY METHOD FOR THE SIMULTANEOUS QUANTIFICATION OF ARTESUNATE AND DIHYDROARTEMISININ IN HUMAN PLASMA

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Abstract. The present study describes the development and validation of a simple, sensitive, and specific liquid chromatography-mass spectrometry (LC-MS) analytical method used for the co-quantification of artesunate (ARS) and its active metabolite, dihydroartemisinin (DHA), in human plasma, using artemisinin (ARN) as an internal standard. The liquid-liquid extraction of samples was carried out using dichloromethane and *tert*.-methyl butyl ether (at a ratio of 8:2 v/v) and then evaporated to dryness by a stream of nitrogen gas at room temperature. Chromatographic separation and mass analysis were performed on the Agilent 1100 Series Liquid Chromatography/Mass Spectrometer Detector Trap system, using electrospray ionization as an interface. The stationary phase was an Elipse XDB-C18 column. The mobile phase contained acetonitrile and 0.003 M glacial acetic acid at a ratio of 62:38 (v/v) delivered at a flow rate of 0.5 ml per minute. Positive ion mode was selected to detect extracted ions at *m/z* 407 and 261 for ARS, at *m/z* 307 and 261 for DHA, and at *m/z* 305 for ARN. The retention times for α-DHA, ARS, β-DHA, and ARN were 6.6, 8.0, 9.2, and 10.8 minutes, respectively, and the total chromatography run time was 12 minutes. The limit of detection (LOD) was 2 ng/ml while the limit of quantification (LOQ) was 10 ng/ml for both ARS and DHA. In order to address any complications caused by the spontaneous non-catalytic breakdown of ARS to DHA, two calibration curves were prepared separately for both analytes. These graphs were found to be linear over the range of 10 to 3,200 ng/ml ($r^2 > 0.99$). The recoveries at concentrations of 100, 200, 400, and 800 ng/ml were 108, 106, 91, and 89%, respectively, for ARS and were 112, 95, 80, and 86%, respectively, for DHA. For ARN, the recoveries were 119, 119, and 90% for concentrations of 200, 400, and 800 ng/ml, respectively. ARS working solutions were not stable after two months of storage at 4ºC or after 21 days at room temperature. This newly developed LC-MS method was then applied for measuring of ARS and DHA concentrations in a healthy volunteer having received oral ARS at 200 mg once daily for 5 consecutive days. There was no decline in ARS concentration after repeated doses and the $C_{ss\text{-}max\text{-}average}$ for DHA was found to be 703 ± 94 ng/ml at $t_{ss\text{-}max}$ of 2 h.

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INTRODUCTION

Artemisinin (ARN) is a natural antimalarial product which can be isolated from the Chinese medicinal herb *Artemisia annua* (Klayman, 1985). It comprises a new generation of antimalarial compounds that feature a nitrogen-free sesquiterpene lactone. Artesunate (ARS),

a water-soluble semi-synthetic derivative of ARN, is very effective against *Plasmodium falciparum* strains that are already resistant to other antimalarials (Hassan *et al*, 1992; White, 1994). ARS was developed as a pro-drug for the treatment of both uncomplicated and severe *P. falciparum* malaria (Olliaro *et al*, 2001). It is available in both enteral and parenteral formulations. Once absorbed, ARS is rapidly metabolized to dihydroartemisinin (DHA) which then accounts for most of the antimalarial activity (Barradell and Fitton, 1995; Batty *et al*, 1998). Thus, the pharmacokinetic parameters of both the parent drug and its active metabolite are essential in monitoring the activity of ARS, and to permit the optimization of drug dosage regimens used in treating malaria.

To date, the pharmacokinetic data of ARN and its derivatives has generally been difficult to acquire due to the complexity involved in measuring their concentrations in biological fluids. The analytical methods for these drugs differ from those of the other antimalarials because the sesquiterpene compounds do not contain the chromophore responsible for ultraviolet or fluorescent absorbance (Edwards, 1994). High-performance liquid chromatography (HPLC) with post-column derivatization for measurement of ARS and DHA has been reported in a number of studies. The sensitivity of this technique is limited by its high LOD (20 - 50 ng/ml) (Batty *et al*, 1996; Newton *et al*, 2000; Taylor *et al*, 2000). The presence of a peroxide moiety in the chemical structure of ARN and its derivatives permits HPLC with a reductive electrochemical detector (HPLC-ECD) as a sensitive means of determining these drugs in biological fluids (Karbwang *et al*, 1997; Na-Bangchang *et al*, 1998; Olliaro *et al*, 2001; Teja-Isavadharm *et al*, 2001). However, it is a rather sophisticated technique that requires a rigorous deoxygenation system, assiduous equipment care, and is generally very time-consuming. Mass spectrometry has now become an indispensable analytical tool used across many disciplines. Indeed, gas chromatography-mass spectrometry has been used for the quantification of artemether (ARM) and DHA (Mohamed *et al,* 1999). More recently, Liquid chromatography-mass spectrometry (LC-MS) has been utilized for the measurement of ARN derivatives (Karunajeewa *et al*, 2004; Naik *et al*, 2005).

In the present study, we report a simple, sensitive, and specific LC-MS method for the concurrent quantification of ARS and DHA in human plasma using liquid-phase extraction, and incorporating ARN as an internal standard.

MATERIALS AND METHODS

Chemicals

ARN and DHA were obtained from the Pharmaceutical Factory N.1, Ha Noi, Vietnam. ARS was from an intravenous ARS formulation (60 mg per vial) marketing product from the Central Pharmaceutical Factory N.1, Ha Noi, Vietnam. HPLC-grade methanol, acetonitrile, dichloromethane, *ter*.-methyl butyl ether were purchased from Merck. Glacial acetic acid was obtained from Quangzhou Chemical Factory, China. Deionized filtered water was from the Biochemistry Department, Cho Ray Hospital. Blank plasma was provided from the blood bank of the Hematology Department, Cho Ray Hospital.

All standard solutions were prepared in methanol. The analytical balance (Adventurer™ Ohaus Corp, Pine Brook, NJ) with a scale sensitivity down to 0.0001 g was used to weigh DHA and ARN. Stock solutions of ARS, DHA, and ARN were at concentrations of 1 mg/ml. Working solutions of DHA and ARS were at concentrations of 1, 5, and 10 ng/μl. A working solution of ARN was at 10 ng/μl. Standard solutions were stored at -20ºC until use.

Instrumentation

The investigation of analyte mixtures, including chromatographic separation by liquid

chromatography (LC) and mass analysis by mass spectrometry (MS), were performed on Agilent 1100 Series Liquid Chromatography/ Mass Spectrometer Detector Trap (LC/MSD Trap) system, Agilent Technologies, USA. The entire system consisted of: autosampler, syringe pump for direct infusion work, analytical column, electrospray interface, ion trap mass analyzer, ion detector, vacuum system and complete electronics. The software included the operating system, the LC/MSD Trap software for data acquisition and data reduction, and the Agilent ChemStation software for sample automation and control of Agilent 1100 LC.

The stationary phase was an Elipse XDB-C18 column (4.6 mm x 150 mm, 5 μm), protected by an Eclipse XDB-C8 guard column (5.0 mm x 12.5 mm) (Agilent Technologies, USA). Mobile phase was the mixture of acetonitrile and 0.003 M glacial acetic acid (62:38 v/v). Flow rate was established at 0.5 ml per minute. Mass analysis for ARS, DHA, and ARN was performed by MSD Trap in the positive ion mode with electrospray ionization (ESI) as an interface. Drying gas was heated to 350ºC at a flow of 10.0 l/minute. The optimal pressure of the nebulizing gas was 30.0 psi.

Calibration curve

The nine-point calibration curves were prepared separately for ARS and DHA by spiking 1 ml of blank plasma with various quantities of ARS or DHA (10, 20, 50, 100, 200, 400, 800, 1,600, and 3,200 ng) and a fixed amount of internal standard (ARN; 1,000 ng). The quality control (QC) samples were prepared at concentrations of 100, 200, 400, 800, and 1,600 ng/ml for ARS and DHA in blank plasma. Sample extractions were conducted in clean 8-ml glass test tubes.

Sample preparation

Prior to analysis, samples were processed using a liquid-liquid extraction technique. The extraction solution mixture was dichloromethane and *tert*.-methyl butyl ether (DCM:MBE) at a ratio of 8:2 v/v. Each sample was extracted with 3.5 ml of DCM:MBE solution. After centrifugation at 4,000 rpm for 20 minutes, the yellow supernatant was aspirated and discarded. The remaining clear fluid was transferred to a fresh tube and evaporated under a stream of nitrogen gas at room temperature. Final residues were reconstituted with 60 μl of methanol for injection onto the HPLC column.

Validation

Limit of detection (LOD) and limit of quantification (LOQ). The LOD is defined as the smallest assignable peak detected by the chromatography system. It is described in terms of the signal-to-noise ratio (*S/N*) at 3, which compares detector response with baseline noise. The LOD can also be estimated as the response value calculated by adding three times the standard deviation of the background response to the average background response.

The LOQ defines the lowest amount or concentration of an analyte whose quantitative determination can be performed with suitable accuracy (80-120%) and precision (CV ≤ 20%). It is measured at *S/N* = 6. The LOQ also represents the addition of ten times the standard deviation of the background response to the average background response.

Precision and accuracy. The precision and accuracy of the method were evaluated by replicate analysis of various concentrations of ARS and DHA either on the same day or on different days. The intra-day repeatability and accuracy were determined by analyzing 5 replicates of 8 different concentrations (20, 50, 100, 200, 400, 800, 1,600, and 3,200 ng/ml) of ARS and DHA. The inter-day reproducibility and accuracy were assessed by analyzing 5 replicates of 8 different concentrations (20, 50, 100, 200, 400, 800, 1,600, and 3,200 ng/ml) of ARS and DHA per day for five days (*n* = 15). Precision was evaluated by the percent coefficient of variation (%CV) of measured concentrations. Accuracy was calculated

based on the difference between measured and true values.

Recovery. The efficiency of the extraction procedure was evaluated by comparing peak areas obtained from extracted samples with those from corresponding stock solutions that were measured directly without extraction. Replicate analyses were carried out on 100, 200, 400, and 800 ng/ml concentrations for ARS and DHA, and 200, 400, and 800 ng/ml for ARN.

Stability. The stabilities of the compounds throughout the period of this study were also determined. ARS working solutions were prepared at concentrations of 1, 5, and 10 ng/μl and stored at 4ºC for six months. Three concentrations (100, 400, and 1,600 ng/μl) of QC samples were measured after 1, 2, 3, and 6 months of storage and compared to standards at the appropriate concentrations measured on the first day of testing. The stability of the ARS working solution was reported as a concentration deviation percentage of QC sample from standard. Fresh ARS working solutions were prepared to establish new calibration curves. The peak areas of ARS and DHA over time were then simultaneously evaluated. The stability of ARS working solutions following room temperature storage was also analyzed following direct injection onto the HPLC column on Day 1 and then 1, 2, 3, and 6 months later.

Clinical application. The analytical method was applied to the investigation of the pharmacokinetics of ARS and DHA in a healthy male Vietnamese volunteer, age 50 years, with a weight of 56 kg. Informed consent was signed prior to inclusion into the study. Renal and hepatic functions were normal, and the subject was not taking any other medication. The volunteer received 200 mg oral doses of ARS once daily for 5 consecutive days. ARS (50 mg per tablet, batch 07001 FN) was purchased from the Mekophar Chemical Pharmaceutical Joint-Stock, Ho Chi Minh City, Vietnam. Three milliliters of whole blood was collected prior to drug administration and then at 0.5, 1, 2, 3, 4, and 6 hours (h) after dosage on Day 1 and Day 5, as well as at 1, 2, and 4 h after drug administration on Days 2, 3, and 4. Plasma was isolated immediately after blood collection and stored at -20ºC until analysis. The internal standard used was ARN, at a concentration of 1,000 ng/μl. The samples were analyzed using the method described above.

RESULTS

Mass spectra analysis

Fig 1 depicts the chemical structures of ARS, DHA, and ARN. Standard solutions for each compound were injected into the MSD Trap with an electrospray interface operated in the positive ion mode. The sodium-adducted ion mass species were recorded at *m/z* of 407 for ARS, 307 for DHA, and 305 for ARN. ARS and DHA had an additional fragment ion at an *m/z* of 261 (Fig 2).

Chromatography analysis

A stock solution containing ARS, DHA, and ARN at a concentration of 33.33 ng/μl was fractionated by HPLC after a manual injection. The data were plotted chromatographically (abundance versus time) and as mass spectra (abundance versus *m/z*). The retention times for α-DHA, ARS, β-DHA, and ARN were approximately 6.6, 8.0, 9.2, and 10.8 minutes, respectively. The total run time was 12 minutes (Fig 2). The α- and β-isomers of DHA were separated, however only the α-DHA isomer was used for DHA quantification because it consistently dominated the abundance of the α/β ratio by approximately five- to six-fold. The fragment ions of ARS and DHA exhibited a *m/z* 261 and the sum of both parent and daughter ion masses was used for drug quantification. The mass intensities of ARS and DHA (which have equivalent molecular weights) were approximately four-fold and two-fold higher than that of ARN, respectively.

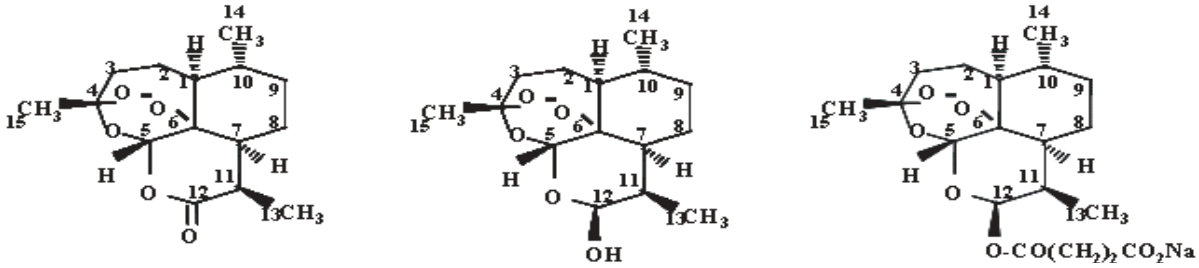

Fig 1–Chemical structures of (a) artemisinin (ARN), (b) dihydroartemisinin (DHA), and (c) artesunate (ARS).

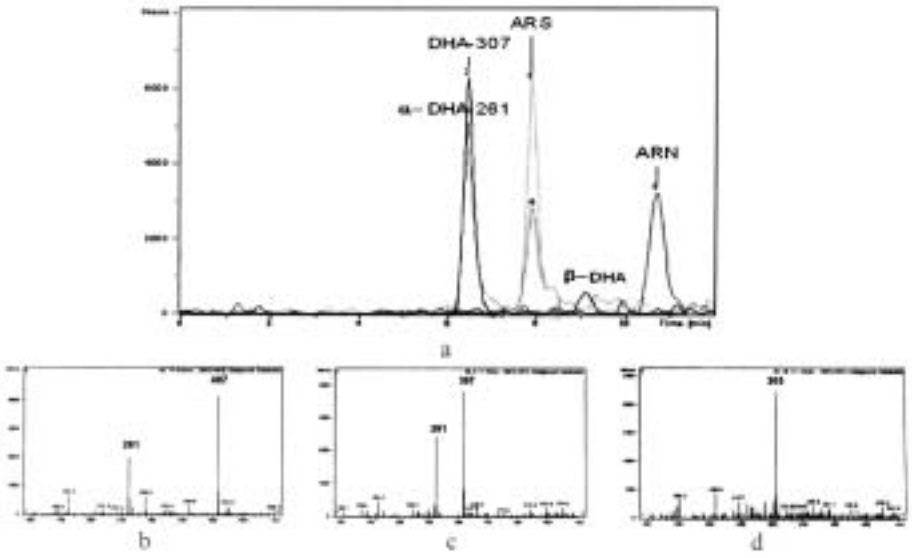

Fig 2–Chromatogram (a) and mass spectra (b, c, d) of ARS, DHA, and ARN.

LOD and LOQ

Table 1 shows the LOD and LOQ for ARS. The LOD for ARS was 2 ng/ml, with a peak area of 3,107 ± 140 and a signal-to-noise ratio of 3.39 ± 0.28 (mean ± SD). The LOQ for ARS was 10 ng/ml, with a peak area of 5,556 ± 286 and a signal-to-noise ratio of 6.07 ± 0.31 (mean ± SD). The LOQ for ARS was considered to be the lower limit of the calibration curve for ARS with a precision and accuracy of 21 and 84%, respectively. Table 2 presents the LOD and LOQ for DHA. The LOD for DHA was 2 ng/ml with a peak area of 2,144 ± 360 and a signal-to-noise ratio of 2.85 ± 0.47 (mean ± SD). The LOQ for DHA was 10 ng/ml with a peak area of 5,569 ± 547 and a signal-to-noise ratio of 7.42 ± 0.75 (mean ± SD). The LOQ for DHA was used as the lowest limit of the calibration curve for DHA with a precision and accuracy of 20 and 93%, respectively.

Calibration and linearity

Calibration curves were constructed separately for ARS and DHA, and prepared using nine different concentrations over the range of 10 to 3,200 ng/ml. The correlation coefficients were > 0.99. The slope of the ARS regression line was about two-fold higher than that of the DHA regression line.

Table 1
LOD and LOQ for ARS; data are presented as mean ± SD (*n* = 5).

	Peak area	Signal/noise ratio	Conclusion	Measured concentration (ng/ml)	Precision (%)	Accuracy (%)
Noise	915 ± 225	/	/	/	/	/
Nominal concentration (ng/ml)						
1	1,963 ± 126	2.15 ± 0.14	/	/	/	/
2	3,107 ± 140	3.39 ± 0.28	LOD	/	/	/
5	3,496 ± 518	3.82 ± 0.68	/	/	/	/
10	5,556 ± 286	6.07 ± 0.31	LOQ	8.4 ± 1.8	21.4	84.0

SD=standard deviation; *n*=number of replicates

Table 2
LOD and LOQ for DHA; data are presented as mean ± SD (*n* = 5).

	Peak area	Signal/noise ratio	Conclusion	Measured concentration (ng/ml)	Precision (%)	Accuracy (%)
Noise	751 ± 225	/	/	/	/	/
Nominal concentration (ng/ml)						
1	780 ± 69	1.04 ± 0.09	/	/	/	/
2	2,144 ± 360	2.85 ± 0.47	LOD	/	/	/
5	3,510 ± 730	4.67 ± 0.97	/	/	/	/
10	5,569 ± 547	7.42 ± 0.75	LOQ	9.3 ± 1.9	20.4	93.0

SD=standard deviation; *n*=number of replicates

Table 3
Intra-day and inter-day variation for ARS concentration (ng/ml); data are presented as mean ± SD.

Nominal concentration	Intra-day variation (n = 5)			Inter-day variation (n = 15)		
	Measured concentration	Precision (%)	Accuracy (%)	Measured concentration	Precision (%)	Accuracy (%)
20	17.6 ± 2.8	15.9	88.0	19.3 ± 4.0	20.7	96.5
50	46.1 ± 6.5	14.1	92.2	48.7 ± 8.9	18.3	97.4
100	93.9 ± 13.3	14.2	93.9	105.9 ± 15.4	14.5	105.9
200	218 ± 29	13.3	109.0	225 ± 20	8.9	112.5
400	403 ± 40	9.9	100.8	435 ± 47	10.8	108.8
800	801 ± 41	5.1	100.1	804 ± 47	5.8	100.5
1,600	1,495 ± 93	6.2	93.4	1,515 ± 84	5.5	94.7
3,200	3,183 ± 58	1.8	99.5	3,257 ± 119	3.7	101.8

SD=standard deviation; n=number of replicates

Table 4
Intra-day and inter-day variation for DHA concentration (ng/ml); data are presented as mean ± SD.

Nominal concentration	Intra-day variation (n = 5)			Inter-day variation (n = 15)		
	Measured concentration	Precision (%)	Accuracy (%)	Measured concentration	Precision (%)	Accuracy (%)
20	19.9 ± 4.0	20.1	99.5	23.8 ± 5.0	21.0	119
50	43.6 ± 7.2	16.5	87.2	48.4 ± 9.7	20.0	96.8
100	91.8 ± 9.6	10.5	91.8	110.2 ± 18.6	16.9	110.2
200	207 ± 27	13.0	103.5	207 ± 26	12.6	103.5
400	367 ± 36	9.8	91.8	373 ± 35	9.4	93.3
800	814 ± 55	6.8	101.8	791 ± 47	5.9	98.9
1,600	1,559 ± 63	4.0	97.4	1,591 ± 88	5.5	99.4
3,200	3,099 ± 85	2.7	96.8	3,178 ± 123	3.9	99.3

SD=standard deviation; n=number of replicates

Precision and accuracy

Tables 3 and 4 show the precision and accuracy of the assay at concentrations of 20, 50, 100, 200, 400, 800, 1,600, and 3,200 ng/ml for ARS and DHA. The intra-day precision was 1.8-15.9 and 2.7-20.1% for ARS and DHA, respectively. The inter-day precision was 3.7-20.7 and 3.9-21.0% for ARS and DHA, respectively. The accuracies of replicate analyses for each compound were within acceptable limits.

Recovery

The extraction recovery was evaluated by comparing the peak areas of extracted samples with those of pure reference analytes that did not go through the extraction procedure. Known concentrations of analytes were freshly spiked with the internal standard and replicate injections at each nominal concentration were performed. Table 5 presents the extraction recovery of ARS, DHA, and ARN. The recoveries of ARS at concentrations of 100, 200, 400, and 800 ng/ml were 108, 106, 91, and 89%, respectively. The recoveries of DHA at concentrations of 100, 200, 400, and 800 ng/ml were 112, 95, 80, and 86%, respectively. The recoveries of ARN at concentrations of 200, 400, and 800 ng/ml were 119, 119, and 90%, respectively.

Stability of ARS

Stability of ARS at 4ºC. Table 6 shows the changes in the measured concentrations of ARS in working solutions stored at 4ºC for six months. QC samples remained stable during the first month, and concentration deviation percentages from the standards measured on Day 1 were within 0-2%. Longer storage times resulted in decreased compound stability. The disparities increased to 6-18%, 25-37%, and 59-80% after 2, 3, and 6 months of storage, respectively. In addition, ARS concentrations in comparison to those of DHA throughout storage also varied, with peak area ratios of ARS to DHA being 11:1 on Days 1 and 30, and then decreasing to 5:1, 3:1, and 1:1 on Days 60, 90, and 180, respectively.

Stability of ARS at room temperature. Table 7 demonstrates the variations of the peak area ratios of ARS to DHA following direct injections of ARS working solutions that had been stored at room temperature for six months. Using ARS concentrations of 10 ng/μl, the peak area ratio of ARS to DHA was 7:1 on Day 1 and reduced gradually to 5:1, 4:1, 3:1, 2:1, 0.3:1, and 0.2:1 on Days 7, 15, 21, 30, 45, and 60, respectively. Complete conversion of ARS to DHA after six months of storage at room temperature reduced ARS abundances such that the amounts were barely detectable, yielding an ARS:DHA ratio of 0.07:1.

Clinical application

The above analytical method was used to measure plasma ARS and DHA concentrations in a healthy volunteer receiving a single daily oral administration of 200 mg ARS for five consecutive days. The blood was collected after drug administration at 0.5, 1, 2, 3, 4, and 6 h on Days 1 and 5; and at 1, 2, and 4 h on Days 2, 3, and 4. Plasma ARS concentrations at sampling times of 1 and 2 h were 34.4 ± 7.4 and 15.2 ± 4.5 ng/ml, with CVs of 21.5 and 29.6%, respectively. The concentrations at 4 h after drug dosage were lower than the LOD for this compound. Plasma DHA concentrations at sampling times of 1, 2, and 4 h were 354 ± 127, 703 ± 94.0 and 94.0 ± 5.0 ng/ml, with CVs of 35.9, 13.4, and 5.3%, respectively. Figs 3 and 4 show the concentration versus time profiles over the five days of treatment for ARS and DHA, respectively. There was little variation in DHA concentrations at 2 h between days and the corresponding $C_{ss\text{-}max\text{-}average}$ of DHA was 703 ± 94 ng/ml. The $t_{ss\text{-}max\text{-}average}$ of ARS was 0.5 h after drug administration and the $C_{ss\text{-}max\text{-}average}$ of ARS was 75.5 ± 7.8 ng/ml.

Table 8 presents the pharmacokinetic data for ARS and DHA during the treatment

Table 5
Extraction recovery; data are presented as mean ± SD (*n* = 5).

Concentration (ng/ml)	ARS		DHA		ARN	
	Recovery (%)	CV(%)	Recovery (%)	CV(%)	Recovery (%)	CV(%)
100	107.8 ± 29.0	26.9	112.2 ± 9.5	8.5	/	/
200	105.7 ± 20.4	19.3	94.7 ± 7.5	7.9	119.0 ± 4.8	4.0
400	90.9 ± 3.8	4.2	79.8 ± 3.3	4.1	119.0 ± 5.2	4.4
800	89.3 ± 13.2	14.8	85.5 ± 11.6	13.6	90.1 ± 16.6	18.4

SD=standard deviation; CV=coefficient of variation; *n*=number of replicates

Table 6
ARS stability at 4ºC; data are presented as mean ± SD (*n* = 5).

Nominal conc[a]	Day 1	Day 30		Day 60		Day 90		Day 180	
	Measured conc[a] of standard	Measured conc[a] of QCS	CDP(%)	Measured conc[a] of QCS	CDP(%)	Measured conc[a] of QCS	CDP(%)	Measured conc[a] of QCS	CDP(%)
100	105 ± 14	105 ± 14	0	99 ± 4	5.7	79 ± 9	24.8	33 ± 10	68.6
400	424 ± 32	417 ± 26	1.7	349 ± 37	17.7	296 ± 13	36.6	83 ± 9	80.4
1,600	1,567 ± 137	1,565 ± 121	0.1	1,296 ± 175	17.3	1,089 ± 76	30.5	643 ± 224	59.0
ARS/DHA peak area ratio	11.1 ± 7.4	11.3 ± 3.5		4.9 ± 1.3		2.7 ± 0.3		0.9 ± 0.9	

QCS=quality control sample; CDP=concentration deviation percentage from standard on Day 1; conc=concentration; [a]=ng/ml; SD=standard deviation; *n*=number of replicates

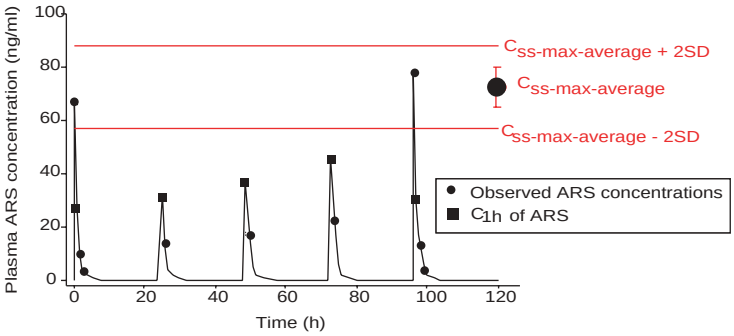

Fig 3–Plasma concentration-time profile for ARS over five days.

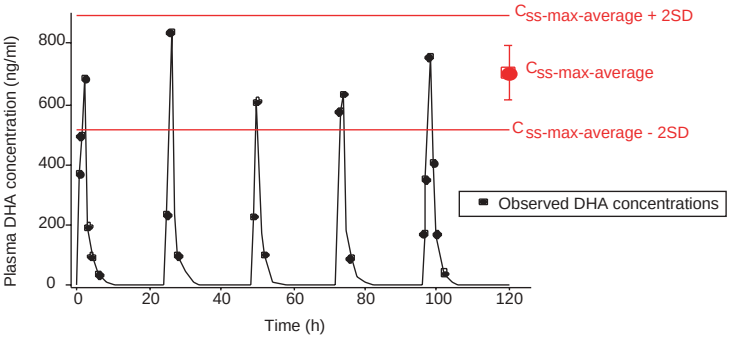

Fig 4–Plasma concentration-time profile for DHA over five days.

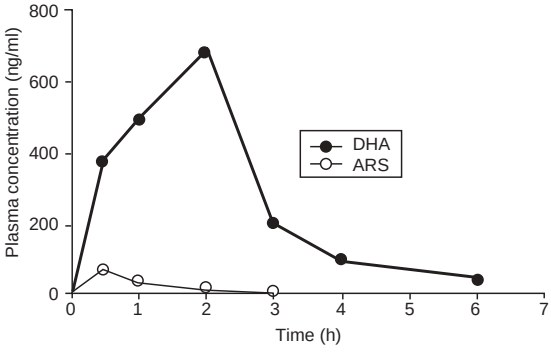

Fig 5–Plasma concentration-time profiles for ARS and DHA on Day 1.

period. There were no differences in pharmacokinetic parameters for either ARS or DHA between Days 1 and 5. The t_{max} for ARS was shorter than that recorded for DHA (0.5 h versus 2 h), while the C_{max} and AUC_{∞} of DHA were approximately 10 times and 20 times higher than those observed for ARS, respectively. DHA showed a longer elimination half-life than ARS (1 h versus 0.7 h). Conversely, the *Vd*/*F* and *Cl*/*F* values for ARS were approximately 13 times and 20 times higher than those for DHA, respectively. Fig 5 shows the plasma concentration-time profiles for ARS and DHA in a healthy volunteer following an oral dose of 200 mg ARS on day 1.

DISCUSSION

The use of solid-phase extraction (SPE) techniques has already been reported for ARM, ARS, ARN, and DHA (Mohamed *et al*, 1999; Karunajeewa *et al*, 2004; Naik *et al*, 2005). Although SPE is a versatile technique for the preparation of samples for analysis, it can be labor-intensive, particularly in high-throughput situations when large numbers of samples are to be processed. In the present work, a liquid-liquid extraction method was developed for pharmacokinetic studies. The advantages of this technique include facile manipulation of samples, low cost, and a consistent performance while using a large number of samples.

Several organic solvents were used to extract ARS and DHA simultaneously from samples. Hexane-dichloromethane, ethyl acetate-dichloromethane, and hexane-ethyl acetate used at different ratios were found to be unsuitable. However, dichloromethane–*ter.*-methyl butyl ether at a ratio of 8:2 v/v resulted in an improved separation, sensitivity, and recovery of ARS and DHA.

The mobile phase was selected for quick and efficient baseline separation of the com-

pounds. The solvents used were HPLC-grade to reduce the likelihood of contaminant peaks that might otherwise compromise the chromatogram. The eluent was filtered to ensure the removal of particulate matter which can damage the pump or block the column and tubing. Air bubbles dissolved in the solvents were removed by an on-line degassing system. A solvent mixture of 0.1% acetic acid:methanol:acetonitrile (38:46.5:15.5, v/v) has previously been implemented as a mobile phase for the analysis of ARS and DHA (Naik *et al*, 2005). An additional mobile phase has also been used to separate ARS and DHA, containing 55% 0.01 M LiOH (dissolved in water and adjusted to pH 4.8 with acetic acid) and 45% acetonitrile (Karunajeewa *et al*, 2004). In the present study, numerous solvents were tested to enhance chromatographic separation. Mixtures of methanol:acetonitrile or acetic acid:methanol:acetonitrile at different ratios were found to be unsuitable. The optimal mobile phase composition for separating ARS, DHA, and ARN was found to be 0.003 M glacial acetic acid:acetonitrile (38:62, v/v). The peaks of interest were separated, sharp, and symmetrical, with a total chromatography run time of 12 minutes.

ARS, DHA, and ARN were analyzed in the positive ion mode with adducted sodium ions to sample molecules. The analytes elicited a high sensitivity in acidic solution (pH = 5), with drying gas heated to 350ºC at a flow of 10.0 l/minutes and a nebulizing gas of 30.0 psi.

Highly purified ARN was used as an internal standard (IS) in the analytical procedure. Although the physical and chemical properties of ARN are similar to those of ARS and DHA, it was completely resolved from these other compounds. The retention times for α-DHA, ARS, β-DHA, and ARN were approximately 6.6, 8.0, 9.2, and 10.8 minutes, respectively. Together with ARS and DHA,

Table 7
ARS stability at room temperature; data are presented as mean ± SD (n = 5).

Day	ARS/DHA peak area ratio	Day	ARS/DHA peak area ratio
1	6.8 ± 1.7	30	1.8 ± 1.7
7	4.8 ± 0.3	45	0.3 ± 0.1
15	4.5 ± 0.8	60	0.2 ± 0.04
21	3.3 ± 0.6	180	0.07 ± 0.02

SD=standard deviation; *n*=number of replicates

Table 8
Pharmacokinetics of ARS and DHA in a healthy volunteer.

Pharmacokinetic parameters	ARS		DHA	
	Day 1	Day 5	Day 1	Day 5
t_{max} (h)	0.5	0.5	2	2
C_{max} (ng/ml)	67	78	685	755
$t_{1/2z}$ (h)	0.63	0.69	1.14	0.93
AUC_{∞} (ng.h/ml)	64	80	1,660	1,729
Vd/*F* (l/kg)	47.8	44.1	3.7	2.8
Cl/*F* (l/kg/h)	52.5	44.4	2.15	2.06

t_{max}=time for maximum concentration; C_{max}=maximum concentration; $t_{1/2z}$=elimination half-life; AUC_{∞}=infinitive area under concentration-time curve; *Vd*/*F*=volume of distribution; *Cl*/*F*=total clearance

ARN could be extensively recovered from plasma extraction (90-119%). Indeed, ARN has been used as an internal standard previously in studies involving ARN derivatives using HPLC-UVD and HPLC-ECD methodologies (Batty *et al*, 1996; Na-Bangchang *et al*, 1998; Taylor *et al*, 2000; Olliaro *et al*, 2001; Teja-Isavadharm *et al*, 2001).

The LCMS analysis of complex mixtures necessitates a combination of both good separation techniques and mass spectrometry to achieve the desired level of accuracy and reliability for the analysis of the results. The mass spectrometer is a universal detector possessing a high sensitivity and specificity. The LOD for both ARS and DHA in the present study was determined to be 2 ng/ml. It was much lower when compared to the LOD of 20-50 ng/ml obtained using an HPLC-ultraviolet detector (Batty *et al*, 1996; Taylor *et al*, 2000). The LOD for ARS and DHA using a previous LC-MS method was reported to be as low as 1 ng/ml (Naik *et al*, 2005). Another study, using the same Agilent 1100 Series Liquid Chromatography/Mass Spectrometry system, showed that the LOD for ARS and DHA were 20 and 3 ng/ml, respectively (Karunajeewa *et al*, 2004). The minimum detectable concentration for DHA was 3 ng/ml with a HPLC-ECD method (Karbwang *et al*, 1997).

The LOQ for both ARS and DHA in the present study was found to be 10 ng/ml. In another study using the same Agilent 1100 Series Liquid Chromatography/Mass Spectrometry system, the LOQ for ARS and DHA were 30 and 14 ng/ml, respectively (Karunajeewa *et al*, 2004). The LOQ for ARS and DHA have been reported as 3-5 ng/ml using the HPLC-ECD technique in a number of reports (Na-Bangchang *et al*, 1998; van Agmael *et al*, 1998; Newton *et al*, 2000; Nealon *et al*, 2002).

Calibration curves were generated over a concentration range from 10 to 3,200 ng/ml yielding a Pearson correlation coefficient of greater than 0.99 for both ARS and DHA. The quantitative relationship between concentration and response was characterized over the entire range of concentrations expected during this study, *eg*, maximum ARS and DHA concentrations of 1,700-2,200 ng/ml following 200-250 mg oral doses of ARS in malaria patients (Angus *et al*, 2002). In another study using an LC-MS method, the linearity was evaluated with two different calibration curves of ARS and DHA, *ie*, a lower calibration curve for concentrations from 1 to 600 ng/ml and a higher calibration curve for concentrations from 600 to 3,000 ng/ml, with the difference in slope parameters (Naik *et al*, 2005). Another report showed that the linearity of ARS and DHA could only be demonstrated from 50 to 800 ng/ml with their LC-MS method (Karunajeewa *et al*, 2004). The linearity of the calibration curves over a range from 100 to 1,600 ng/ml with HPLC-UVD (Taylor *et al*, 2000) and from 5 to 1,000 ng/ml with HPLC-ECD (Teja-Isavadharm *et al*, 2001) have been documented. In our study, the slope value of the ARS calibration curve was approximately twice that observed for DHA (0.0045 versus 0.0024). The slope of the ARS calibration curve was also reported to be higher than that for DHA (0.0038 versus 0.0028) in the study by Naik *et al* (2005), but not to the magnitude observed in the current study.

The performance of our LCMS equipment was tested using ARS and DHA at concentrations of 20, 50, 100, 200, 400, 800, 1,600, and 3,200 ng/ml. The intra-day and inter-day precision for ARS and DHA varied from 10 to 20% for concentrations of ≤ 200 ng/ml and 3 to 10% for concentrations ≥ 400 ng/ml. These values were within the acceptable ranges required for assay validation. The high recovery percentage of > 90% for all the analytes of interest indicates that the liquid-liquid extraction procedure of this method was suitable for these compounds.

In our study, spontaneous breakdown of ARS to DHA was observed. Fresh working

solutions of ARS on day 1 of preparation exhibited a small DHA peak with the peak area ratio of ARS to DHA of 7:1, following direct injection. The levels of ARS to DHA breakdown were evaluated periodically. ARS solutions were stored at concentrations of 100, 400, and 1,600 ng/ml at 4ºC and then analysed. In comparison with the drug on Day 1, the concentrations of ARS in QC samples were 98-100, 82-94, 63-75, and 20-41% following 1, 2, 3, and 6 months of storage, respectively. These findings suggest that new standard solutions of ARS must be prepared every two months. The rate of transformation of ARS to DHA increased considerably when the solutions were stored at room temperature. The peak area ratio of ARS to DHA reduced rapidly from a value of 7:1 on Day 1, to 2:1 on Day 30, followed by 0.2:1 and 0.07:1 at 2 and 6 months, respectively. In contrast, ARS stock solutions retained at -20ºC were stable for greater than 12 months, with the peak area ratio of ARS to DHA remaining at 7:1.

The enzymatic hydrolysis of ARS to DHA is catalyzed by an esterase enzyme in the blood and also by cytochrome P450 3A4 in the liver (Grace *et al*, 1999; White *et al*, 1999). In the present study, non-enzymatic conversion of ARS to DHA occurred in the solvent media (methanol). Furthermore, the rate of this transformation was much higher at room temperature. Thus, this simple hydrolysis reaction occurs as a function of temperature, and is the primary reason underlying the spontaneous transformation of ARS to DHA. This chemical reaction is also likely to occur in stomach fluid, which could explain the rapid appearance of high concentrations of circulating DHA following ARS administration.

Plasma concentrations of ARS and DHA in a healthy volunteer following the oral administration of 200 mg ARS once daily dose for five consecutive days were measured using our LC-MS method. There were no differences in the t_{max}, C_{max}, $t_{1/2z}$, AUC_{∞}, Vd/F, and Cl/F for ARS between Days 1 and 5. It appeared that the ARS pharmacokinetics were not time-dependent after repeated oral doses. This is not in keeping with previous reports in the literature regarding ARN in which it was shown to be time dependent (Ashton *et al*, 1998; Khanh *et al*, 1999). Thus, despite being in the same nitrogen-free sesquiterpene group, the pharmacokinetics of these substances may differ. The C_{max} and AUC_{∞} for DHA were around 10 times and 20 times higher than those for ARS, respectively. With a short half-life of around 1.0 h and a once daily dosing regimen, DHA reached maximal steady state concentrations at 2 h ($t_{ss\text{-}max}$) following the dosage, while the mean $C_{ss\text{-}max}$ value was 703 ± 94 ng/ml over the five days (CV = 13.4%). This LCMS method was used to measure the concentrations of ARS and DHA in a volunteer, with the lowest levels of detection being 3 and 33 ng/ml, respectively.

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