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An analytical method with a single extraction procedure and two separate high performance liquid chromatographic systems for the determination of artesunate, dihydroartemisinin and mefloquine in human plasma for application in clinical pharmacological studies of the drug combination

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Abstract

The combination of two sensitive, selective and reproducible reversed phase liquid chromatographic (RP-HPLC) methods was developed for the determination of artesunate (AS), its active metabolite dihydroartemisinin (DHA) and mefloquine (MQ) in human plasma. Solid phase extraction (SPE) of the plasma samples was carried out on Supelclean LC-18 extraction cartridges. Chromatographic separation of AS, DHA and the internal standard, artemisinin (QHS) was obtained on a Hypersil C4 column with mobile phase consisting of acetonitrile–0.05 M acetic acid adjusted to pH 5.2 with 1.0 M NaOH (42:58, v/v) at the flow rate of 1.50 ml/min. The analytes were detected using an electrochemical detector operating in the reductive mode. Chromatography of MQ and the internal standard, chlorpromazine hydrochloride (CPM) was carried out on an Inertsil C8-3 column using methanol–acetonitrile–0.05 M potassium dihydrogen phosphate adjusted to pH 3.9 with 0.5% orthophosphoric acid (50:8:42, v/v/v) at a flow rate of 1.00 ml/min with ultraviolet detection at 284 nm. The mean recoveries of AS and DHA over a concentration range of 30–750 ng/0.5 ml plasma and MQ over a concentration of 75–1500 ng/0.5 ml plasma were above 80% and the accuracy ranged from 91.1 to 103.5%. The within-day coefficients of variation were 1.0–1.4% for AS, 0.4–3.4% for DHA and 0.7–1.5% for MQ. The day-to-day coefficients of variation were 1.3–7.6%, 1.8–7.8% and 2.0–3.4%, respectively. Both the lower limit of quantifications for AS and DHA were at 10 ng/0.5 ml and the lower limit of quantification for MQ was at 25 ng/0.5 ml. The limit of detections were 4 ng/0.5 ml for AS and DHA and 15 ng/0.5 ml for MQ. The method was found to be suitable for use in clinical pharmacological studies.
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Keywords: Artesunate; Dihydroartemisinin; Mefloquine; SPE; RP-HPLC-EC; RP-HPLC-UV; Pharmacological studies

1. Introduction

The World Health Organization (WHO) recommends now that uncomplicated falciparum malaria be treated with effective Artemisinin-containing Combination Therapies (ACTs) [1].

There is vast experience with the combination of artesunate (AS) and mefloquine (MQ), which has been in use for several years in South-East Asia, particularly on the Thai-Myanmar borders [2]. Various regimens have been tried with the two separately formulated drugs (AS usually as 50 mg tablets, MQ as 250 mg base tablets). The regimen most commonly used has AS at 4 mg/kg/d over three days, while MQ is given at 15 mg/kg with the second AS dose and 10 mg/kg with the third dose. This regimen is very effective in areas of multidrug resistance and well tolerated (except dose and age-related vomiting) but is difficult to comply with. In order to improve patient's adherence, AS and MQ have been co-packaged into a single blister, and more recently co-formulated in a fixed-dose oral form (AS:MQ ratio 1:2). The latter allows daily doses of AS and MQ of 4 and 8 mg/kg for a total of 12 and 24 mg/kg over 3 days. This regimen was shown to be as effective as and better tolerated than the reference treatment above [3].

With the use of this combination and the number of products expanding, there is an increased need for a sensitive, reliable

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and robust analytical method to assess drug bioavailability and disposition. However, so far there is no published analytical method for AS and MQ when used in combination. Current methods are for quantifying AS and MQ individually, but most of them are not sensitive enough thus limiting the application to pharmacokinetic studies [4–13]. The HPLC method with electrochemical detection (EC) described by Na-Bangchang et al. [14] can detect AS and DHA at concentrations as low as 5 ng/ml and 3 ng/ml respectively, but requires a large sample volume (1 ml of plasma) and involves liquid–liquid extraction, which is not suitable for the AS–MQ drug combination. Equally sensitive is also the HPLC-EC method by Navaratnam et al. [15], but interferences resulting from the endogenous substances makes it unsuitable for simultaneous extraction. A LC–MS method reported recently requires the construction of two calibration curves due to the absence of linearity throughout the whole analytical concentration range [16].

In view of these practical limitations, the present study was undertaken to develop and validate an analytical method for the determination of AS and its active metabolite dihydroartemisinin (DHA), as well as MQ in human plasma. The method involves a simple sample preparation procedure whereby AS and DHA are separated from MQ and analysed in two HPLC systems—one coupled to an EC detector for the analysis of AS and DHA and the other coupled to an ultraviolet (UV) detector for the analysis of MQ. Two separate assays are used because adequate sensitivity for the detection of AS/DHA is only possible with an EC detector operating in the reductive mode. This method was found to be selective, sensitive, accurate and reproducible.

2. Experimental

2.1. Chemicals and reagents

Artesunate (AS) (batch no. 129.5), dihydroartemisinin (DHA) (batch no. 1.03) and artemisinin (QHS) (batch no. 99.4) were obtained from Knoll AG (Liestal, Switzerland). Mefloquine hydrochloride (MQ) (batch no. 82031A) was contributed by Farmanguinhos, Brazil, who obtained it from F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Chlorpromazine hydrochloride (CPM) was bought from Sigma (St. Louis, MO, USA). Acetonitrile (ACN) was purchased from Fisher Scientific (Loughborough, UK), methanol (MeOH) and acetic acid was purchased from BDH Laboratory (Poole, England), ethanol (EtOH) was purchased from Scharlau (Barcelona, Spain), while sodium hydroxide, orthophosphoric acid and potassium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany). All chemicals used were of analytical or HPLC grade. Human plasma was obtained from Hospital Pulau Pinang, Malaysia.

2.2. Preparation of standards and quality control sample

The primary stock solutions of AS (200 μg/ml), DHA (200 μg/ml) and MQ (500 μg/ml), as well as the internal standards QHS (25 μg/ml) and CPM (200 μg/ml) were prepared in EtOH and kept at −20 °C until the time of use. Our studies showed that all analytes were stable for a period of 1 month (data not shown). The stock solutions were diluted with EtOH to intermediate stock solutions before spiking with 500 μl of drug free plasma to obtain calibration curve of 10, 15, 20, 50, 100, 200, 400, 600 and 800 ng/0.5 ml plasma for AS and DHA, and 25, 50, 75, 100, 200, 400, 800, 1200 and 1600 ng/0.5 ml plasma for MQ. Fixed amounts of the internal standards QHS and CPM were added to all the samples before making up with deionised water to produce a total volume of 1 ml. The quality control (QC) samples at concentrations of 30, 450 and 750 ng/0.5 ml plasma for AS and DHA and 75, 750, 1500 ng/0.5 ml for MQ were prepared independently from different sets of stock solutions and intermediate stock solutions. The QC samples were also spiked with the same amount of internal standards and plasma and made up with deionised water to a total volume of 1 ml.

2.3. Solid phase extraction (SPE)

To prevent drug adsorption, all glassware was silanised with 5% (v/v) dichloromethylsilane (DCMS) in toluene before use. The SPE extraction was carried out on Supelclean^{TM} LC-18 SPE tubes, 1 ml, 100 mg sorbent (Bellefonte, PA, USA).

Each SPE tube was conditioned with 1 ml of ACN followed by 1 ml of MeOH and 1 ml of deionised water prior to sample loading. After the samples had been loaded onto the cartridges, 1 ml of 0.05 M acetate buffer (pH 5.2) was used for washing followed by 1 ml of 10% ACN in deionised water to prevent emulsification. AS, DHA and QHS were eluted with two consecutive aliquots of 0.5 ml ACN–MeOH (90:10, v/v) into a test tube. At this stage, MQ and CPM were still retained in the SPE tubes. Before these two compounds were eluted, the sorbent was conditioned to plasma pH by adding 1 ml of 0.05 M phosphate buffer (pH 7.4) followed by 1 ml of 10% ACN in deionised water. MQ and CPM were then eluted with two consecutive aliquots of 0.5 ml 1% NH_3 in MeOH.

Extracts of both AS with DHA and MQ were dried under a gentle flow of nitrogen gas at room temperature (25 °C). A volume of 100 μl ethanol–water (50:50 v/v) was used to reconstitute the dried samples before storing them at 4 °C prior to analysis. For the extracts which contained DHA, analyses were only performed after 18 h to ensure that the tautomerisation of α and β isomers of DHA reaches equilibrium [15]. Before this sample was injected into the HPLC-EC system, rigorous sample deoxygenation with purified helium was performed.

2.4. Instrumentation and chromatography

Analysis of AS and DHA was carried out on a BAS 200 HPLC system (Bioanalytical Systems, West Lafayette, IN, USA). This system consisted of three mobile phase reservoirs, a dual piston pump and a detector system made up of a thin-layer dual glassy carbon electrode and a Ag/AgCl reference electrode operating in the reductive mode at −1000 mV. Manual sample injections were performed on a Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA) with a sample loop of 20 μl capacity. Chromatographic separation was achieved on Hypersil

C4, 250 × 4.6 mm I.D., 5 μm particle size (Thermo Hypersil-Keystone, Bellefonte, PA, USA). The mobile phase which consisted of ACN and 0.05 M acetic acid adjusted to an apparent pH 5.2 with 0.1 M NaOH (42:58, v/v) was filtered through a 0.2 μm nylon filter before rigorous deoxygenation with purified helium (purity, 99.99%) for 2 h. The flow rate was then set to 1.50 ml/min.

MQ was analysed on a HPLC system which consisted of a Waters in-line degasser, a Waters 515 dual piston pump, a Waters 680 automated gradient controller and a Waters 2487 dual wavelength absorbance detector (Waters Corp., Milford, MA, USA). Injection was done on a Rheodyne 7725i manual sample injector (Rheodyne, Cotati, CA, USA) with a 50 μl sample loop. The analytical column was Inertsil C8-3, 150 × 4.6 mm I.D., 5 μm particle size (GL Sciences Inc., Tokyo, Japan). The mobile phase which consisted of MeOH, ACN and 0.05 M KH_2PO_4 adjusted to an apparent pH 3.9 with 0.5% orthophosphoric acid (50:8:42, v/v/v) was filtered through a 0.2 μm nylon filter. The flow rate was 1.00 ml/min and the detection was at 284 nm.

2.5. Method validation

2.5.1. Linearity and range

Linearity of the analytes was evaluated using freshly prepared samples covering the range as described in Section 2.2. The calibration curve of AS and MQ was constructed by plotting the peak height ratios of the respective analyte to the internal standard (y) against the analyte concentration (x). In the case of DHA, the calibration curve was constructed by plotting the peak height ratios of α-DHA/QHS against α-DHA concentrations. The quantification of DHA using the predominant peak of α isomer of DHA was done after storing for 18 h at 4 °C in the reconstituted solvent for equilibration of the two isomers [15]. The calibration curve follows the equation of $y = bx + a$, where b and a refer to the slope and y-intercept, respectively. Five such calibration curves were plotted and the values of b, a, as well as the correlation coefficient, r were determined using the SPSS software (Version 10.0 for Windows, Chicago, IL, USA) with the weighting scheme of $1/y^2$. The analyte concentration, x was determined from the calibration curve using the formula: $x = (y - a)/b$.

The lowest concentration of the analytes with more than five times signal to noise ratio and an accuracy of 80–120%, as well as the coefficient of variation of <20% was regarded as the lowest limit of quantification (LLOQ) [17]. This concentration was used as the first calibration point for the calibration curve. The lowest detectable analyte concentration with approximately three times signal to noise ratio was regarded as the limit of detection (LOD).

2.5.2. Selectivity

Six different sources of drug-free plasma were processed using the sample preparation and analytical procedures mentioned above. Selectivity of the method was evaluated by examining the extent to which endogenous substances, combination drug and degradation products could possibly interfere with the retention time of the analyte.

2.5.3. Accuracy and precision

The accuracy of the method was determined by comparing the assayed concentrations with the actual concentrations of the analytes at low, medium and high concentration levels. Five replicates of each of the concentrations were analysed. Within-day precision was evaluated by extracting and analyzing five replicates of a single homogenous sample within the same run in the same day. This was also done at low, medium and high concentrations. The day-to-day precision was evaluated by analyzing a sample of each of the low, medium and high concentrations on 5 different days.

2.5.4. Extraction recovery

The recovery of the samples was determined by directly comparing the peak heights of the analytes in the extracted samples with the peak height of the corresponding pure standards of the same concentrations. Five replicates of each low, medium and high concentration were used. For the internal standard QHS and CPM, recovery was determined at the working concentrations only.

2.6. Stability studies

2.6.1. Storage stability

The stability of AS, DHA and MQ in human plasma was investigated over a period of 12 months. Spiked samples at low, medium and high QC concentrations were prepared with drug free plasma and stored at −80 °C. Aliquots at each level were thawed and analysed in triplicate at 0, 3 and 12 months. A standard calibration curve was freshly prepared on the day of the analysis and concentration levels were determined.

2.6.2. Freeze and thaw stability

Analysis in triplicate was performed on low, medium and high concentrations of QC samples of AS, DHA and MQ. QC samples at each level were divided into two aliquots and stored at −80 °C for 48 h. The first aliquot served as the freeze–thaw samples and the second aliquot was used as the control. The first aliquot was thawed unassisted at room temperature. When completely thawed, the samples were refrozen for about 24 h at −80 °C. This freeze–thaw cycle was repeated twice and analysed on the third cycle along with the second aliquot of QC samples to determine whether there is any variation due to thawing of the samples.

2.6.3. Bench stability

Samples were prepared at low, medium and high QC concentrations of AS, DHA and MQ in drug free plasma and kept on working bench at ambient temperature (ca. 25 °C) for 1 and 3 h to determine whether degradation occurred during sample preparation.

2.7. Pharmacokinetic application

2.7.1. Study protocol

Four healthy normal volunteers (1 male/3 female) aged 23, 29, 35 and 40 and weighing from 49 to 67 kg participated in this

study carried out at the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. The study protocol was approved by the research and ethics committee on bioavailability studies of Mahidol University. All the volunteers were assessed prior to the study and were found to be fit according to their medical history, physical examinations (i.e. no liver, cardiovascular and peripheral neuropathy abnormalities), haematological examinations, electrocardiogram test and pregnancy test (for female). The investigation was carried out with informed written consent from each subject. The volunteers were not on other anti-malarial medication 2 months prior to and during the clinical trial. No alcohol was taken by the volunteers 48 h before and after the study. The subjects were given two AS-MQ co-formulated tablets (Farmanguinhos, Brazil, batch no. 070008), each consisting of 100 mg artesunate base and 200 mg mefloquine base with 200 ml of water following an overnight fast. Venous blood (5 ml) was taken at pre-dose, and thereafter at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 48, 72 h after dosing. Blood samples of day 5, 7, 14, 21, 28, 42, 56, 70 and 90 after oral administration were collected by direct venipuncture. All the blood samples were collected in heparinised Vacutainer™ tubes. After centrifuging the blood sample at 3500 r.p.m. for 15 min, the plasma layer was removed and stored at −80 °C in 5 ml cryovials. The plasma sample was then transferred to Centre for Drug Research in dry ice and stored at −80 °C until the time of analysis.

2.7.2. Pharmacokinetic analysis

In order to demonstrate the applicability of the assay method in bioavailability studies, a non-compartmental analysis was used to derive the basic pharmacokinetic parameters. The elimination half-life ($T_{1/2}$) was calculated by using the equation $\ln 2/\lambda_z$, where λ_z was determined by linear regression from the terminal log-linear (disposition) phase of the plasma concentration versus time curve. The area under the plasma concentration–time curve from 0 to t ($AUC_{0\rightarrow t}$) was calculated by utilizing the linear trapezoidal rule: $\{(C_1 + C_2)/2(t_2 - t_1)\} + \ldots + \{(C_{n-1} + C_n)/2(t_n - t_{n-1})\}$. The $AUC_{0\rightarrow\infty}$ (up to infinite) was calculated by the formula $AUC_{0\rightarrow t} + Ct/\lambda_z$ where Ct is the concentration at the last quantifiable time. Maximum concentration (C_{max}) and time to reach maximum concentration (T_{max}) were the observed values from the plasma concentration versus time curve.

3. Results

3.1. Method validation

3.1.1. Linearity and range

The calibration curve of AS and DHA was linear from 10 to 800 ng/0.5 ml plasma. The detector response was also linear for MQ from 25 to 1600 ng/0.5 ml. The mean regression equation of five calibration curves and their correlation coefficient (r) were as follows: (i) AS: $y = (0.0009 \pm 0.0004)x + (0.0019 \pm 0.0023)$, $r = 0.9996 \pm 0.0003$; (ii) α-DHA: $y = (0.0014 \pm 0.0006)x + (0.0021 \pm 0.0014)$, $r = 0.9994 \pm 0.0007$; (iii) MQ: $y = (0.0010 \pm 0.0000)x + (0.0032 \pm 0.0021)$, $r = 0.9995 \pm 0.0004$.

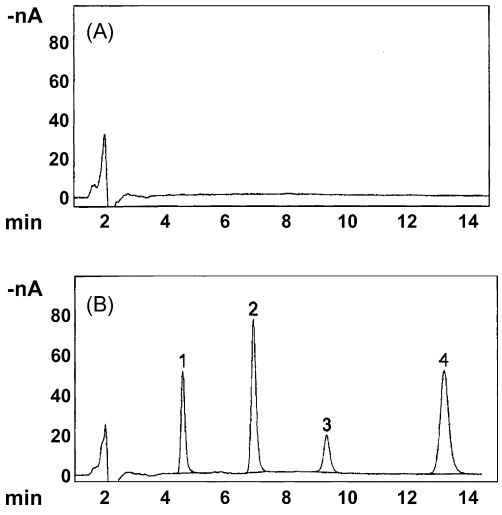

Fig. 1. HPLC chromatogram of (A) extracted blank (drug-free) human plasma; (B) extracted human plasma containing AS, DHA and QHS. The retention times for the analytes are: peak 1, AS = 4.57; peak 2, α-DHA = 6.92; peak 3, β-DHA = 9.34; peak 4, QHS = 13.24.

The LLOQ of AS was 10 ng/0.5 ml plasma with an accuracy of 109.9% and the coefficient of variation (CV) of 7.2%. The LLOQ of DHA was 10 ng/0.5 ml plasma with an accuracy of 99.1% and CV of 5.9%. The LLOQ of MQ was 25 ng/0.5 ml with an accuracy of 98.9% and CV of 8.0%. The LOD for AS and DHA were 4 ng/0.5 ml while the LOD for MQ was 15 ng/0.5 ml.

3.1.2. Selectivity

Both the HPLC methods used for determining AS with DHA and MQ were found to be selective. No endogenous substances or interfering peaks were observed at the retention time of the analytes. Fig. 1 shows the representative chromatograms of extracted blank human plasma and the plasma extract which contained AS, DHA and QHS obtained from the HPLC-EC system. Fig. 2 shows the representative chromatograms of

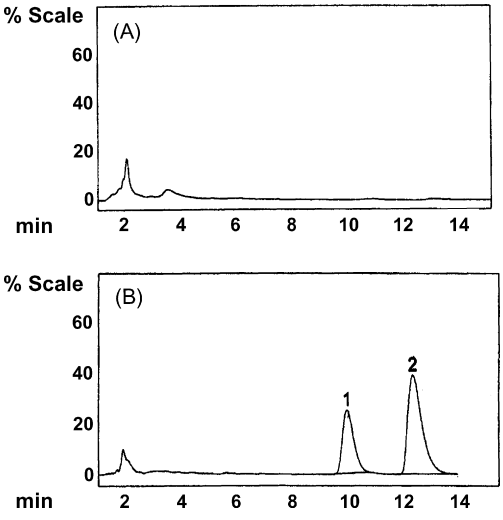

Fig. 2. HPLC chromatogram of (A) extracted blank (drug-free) human plasma; (B) extracted human plasma containing CPM and MQ. The retention times for the analytes are: peak 1, CPM = 10.33; peak 2, MQ = 12.88.

Table 1
Accuracy of the determination of AS, DHA and MQ in human plasma ($n = 5$)

Analyte	C_{nom} (ng/0.5 ml)	C_{act} (ng/0.5 ml)	C_{est} (ng/0.5 ml) (mean ± SD)	Accuracy (%)
AS	30	30.2	31.0 ± 2.7	102.7
	450	452.7	465.8 ± 10.9	102.9
	750	754.5	725.6 ± 41.5	96.2
DHA	30	29.6	29.7 ± 0.52	100.5
	450	443.5	446.5 ± 10.3	100.7
	750	739.2	739.9 ± 58.2	100.1
MQ	75	74.6	68.0 ± 2.1	91.1
	750	746.4	772.5 ± 18.6	103.5
	1500	1492.7	1477.6 ± 32.6	99.0

C_{nom}, nominal concentration; C_{act}, actual concentration; C_{est}, estimated concentration; SD, standard deviation.

Table 2
Precision of the determination of AS, DHA and MQ in human plasma ($n = 5$)

Analyte	C_{nom} (ng/0.5 ml)	Within-day		Day-to-day	
		C_{est} (ng/0.5 ml) (mean ± SD)	CV (%)	C_{est} (ng/0.5 ml) (mean ± SD)	CV (%)
AS	30	32.9 ± 0.3	1.0	30.3 ± 2.3	7.6
	450	469.8 ± 6.6	1.4	460.0 ± 5.8	1.3
	750	842.1 ± 9.6	1.1	767.4 ± 38.0	5.0
DHA	30	30.5 ± 1.0	3.4	31.1 ± 2.4	7.8
	450	467.2 ± 1.8	0.4	442.9 ± 7.9	1.8
	750	783.9 ± 6.0	0.8	756.3 ± 16.3	2.2
MQ	75	72.0 ± 0.9	1.2	74.5 ± 1.9	2.5
	750	766.2 ± 5.1	0.7	757.5 ± 25.7	3.4
	1500	1497.5 ± 22.2	1.5	1518.1 ± 30.9	2.0

C_{nom}, nominal concentration; C_{est}, estimated concentration; SD, standard deviation.

the extracted blank human plasma and the plasma extract which contained MQ and CPM obtained from the HPLC-UV system.

3.1.3. Accuracy and precision

The accuracy of the method ranged between 91.1 and 103.5%. The results are given in Table 1. The within-day coefficient of variations for AS, DHA and MQ were less than 3.5% for all the three concentrations, while the day-to-day coefficient of variations for AS, DHA and MQ were less than 8% as indicated in Table 2.

3.1.4. Extraction recovery

The mean extraction recoveries of the analytes and the internal standards were good with AS: 86.3–92.0%; DHA: 89.4–91.9%; QHS: 90.2%; MQ: 79.2–87.3% and CPM: 83.2%. The data are shown in Table 3.

3.2. Stability

3.2.1. Storage stability

The storage stability of AS, DHA and MQ at −80 °C were investigated. The percentage of the remaining drugs was calculated with reference to the initial amount determined pre-storage. The results in Table 4 indicated that the drugs were stable at −80 °C for up to 3 months and showed only slight degradation after a period of 12 months.

3.2.2. Freeze and thaw stability

The freeze and thaw stability of AS, DHA and MQ was determined by comparing the mean concentration of the samples that underwent freeze–thaw cycles with that of the QC samples. The results of the freeze and thaw stability shown in Table 4 indicate that there is no variation due to thawing of the samples.

3.2.3. Bench stability

All three analytes were found to be stable at room temperature after 1 h. However, a slight reduction in the levels of AS and

Table 3
Extraction recovery of AS, DHA and MQ in human plasma ($n = 5$)

Analyte	C_{nom} (ng/0.5 ml)	Recovery (%) (mean ± SD)	CV (%)
AS	30	92.0 ± 5.6	6.1
	450	88.3 ± 9.1	10.3
	750	86.3 ± 5.5	6.3
DHA	30	91.3 ± 9.9	10.9
	450	89.4 ± 8.9	10.0
	750	91.9 ± 5.8	6.3
QHS	250	90.2 ± 6.7	7.4
MQ	75	81.8 ± 5.9	7.2
	750	79.2 ± 3.1	3.9
	1500	87.3 ± 2.4	2.7
CPM	2000	83.2 ± 1.6	1.9

C_{nom}, nominal concentration; SD, standard deviation.

Table 4
Stability of AS, DHA and MQ in spiked plasma samples ($n = 3$)

Stability		Remained (%) (mean ± SD)		
		Low 30[a] 75[b]	Medium 450[a] 750[b]	High 750[a] 1500[b]
Storage stability (−80 °C)				
AS	3 months	97.46 ± 3.17	102.17 ± 4.90	92.23 ± 7.58
	12 months	92.38 ± 6.15	86.44 ± 3.10	80.13 ± 0.35
DHA	3 months	91.27 ± 6.16	97.43 ± 1.99	95.22 ± 0.69
	12 months	77.74 ± 5.40	81.57 ± 0.88	81.45 ± 4.71
MQ	3 months	100.75 ± 3.75	94.51 ± 2.03	91.84 ± 1.18
	12 months	96.44 ± 11.95	89.74 ± 1.61	83.93 ± 2.98
Freeze and thaw stability (−80 °C and 25 °C)				
AS		104.07 ± 4.93	107.31 ± 2.34	96.72 ± 0.87
DHA		94.51 ± 1.04	103.05 ± 0.62	94.70 ± 1.92
MQ		95.82 ± 1.87	91.66 ± 4.50	94.78 ± 1.56
Bench stability (25 °C)				
AS	1 h	95.41 ± 6.21	99.52 ± 1.50	98.58 ± 0.49
	3 h	97.06 ± 7.81	94.02 ± 2.04	93.35 ± 2.60
DHA	1 h	97.25 ± 3.49	101.82 ± 4.84	95.81 ± 2.01
	3 h	92.91 ± 4.22	95.26 ± 1.95	89.86 ± 2.81
MQ	1 h	98.03 ± 1.05	100.50 ± 3.16	99.16 ± 0.98
	3 h	99.74 ± 2.70	99.54 ± 2.81	104.50 ± 1.34

[a] AS and DHA (ng/0.5 ml).
[b] MQ (ng/0.5 ml).

DHA was observed after 3 h. MQ was found to be stable even up to 3 h. Data for the percentage of the drugs remained for low, medium and high concentrations are given in Table 4.

3.3. Pharmacokinetic application

The validated method for spiked plasma was used to study the pharmacokinetics of AS and MQ in healthy volunteers following administration of a single dose of oral combination formulation consisting of 200 mg AS and 400 mg MQ. The plasma concentration–time profiles of AS with DHA and MQ are shown in Figs. 3 and 4, respectively. Plasma concentrations were measured up to 12 h for AS and DHA and up to 2160 h for MQ. The pharmacokinetic parameters are given in Table 5.

4. Discussion

The method was found to be sensitive, selective, accurate and reproducible for the determination of AS, DHA and MQ in human plasma. All the calibration curves had r values of >0.999, thus indicating their linearity over the range. The chromatograms were found to be free from interferences and all the analyte peaks were resolved to baseline. The extraction procedure yielded high recovery of analytes (mostly >80%), thus enabling the compounds to be quantified at very low concentration levels. Accuracy and precision of the method was found to be within the acceptable range laid down by the USFDA guidelines for the validation of a bioanalytical method [17], where the determined concentrations should be within 85–115% of the actual values and the CVs should be <15%.

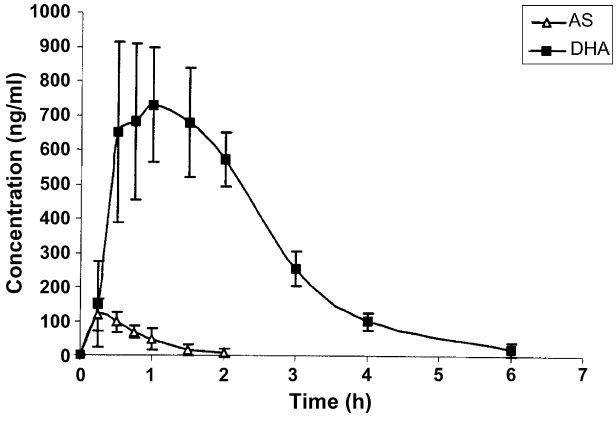

Fig. 3. Mean plasma concentration–time curves of AS and DHA after oral administration to four healthy human volunteers.

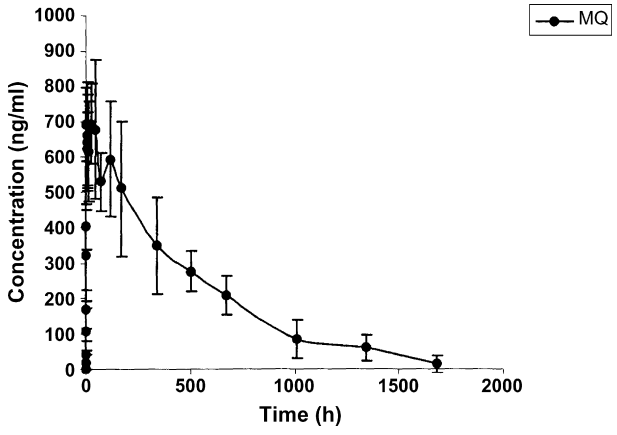

Fig. 4. Mean plasma concentration–time curve of MQ after oral administration to four healthy human volunteers.

Table 5
Pharmacokinetic parameters of AS, DHA and MQ following oral administration of AS–MQ combination tablet at 200 mg and 400 mg, respectively ($n = 4$)

Analyte	AS	DHA	MQ
$T_{1/2}$ (h)	0.47 ± 0.14	0.75 ± 0.25	345.88 ± 57.69
$AUC_{0\rightarrow t}$ (ng h/ml)	91.29 ± 26.24	1432.56 ± 260.26	270848.93 ± 128900.52
$AUC_{0\rightarrow\infty}$ (ng h/ml)	116.40 ± 25.83	1498.90 ± 285.88	314987.41 ± 112188.82
C_{max} (ng/ml)	135.08 ± 37.97	618.50 ± 221.61	612.40 ± 148.75
T_{max} (h)	0.38 ± 0.14	0.43 ± 0.70	15.80 ± 21.17

To the best of our knowledge, this is the first analytical method described for the assay of AS–MQ combination. The sample extraction procedure using SPE cartridges was simple and less laborious when compared to the previously described liquid–liquid extraction methods for AS, DHA and MQ [4,6–13]. Furthermore, this extraction method was able to separate both AS and DHA from MQ for separate assays without compromising on its sensitivity and selectivity (the sensitivity is comparable to the HPLC-EC method described by Na-Bangchang et al. [14] and Navaratnam et al. [15]). Although the HPLC-EC system requires meticulous maintenance of an oxygen-free environment, no better alternatives are available at present for the quantification of AS and DHA for pharmacokinetic studies. The LC–MS method developed by Naik et al. [16] involves comparatively high capital expenditure and quantifies both AS and DHA using the fragment ion at *m/z* 221. Moreover, the requirement to construct two calibration curves makes it less practical for routine analysis. The previous analytical methods for the quantification of MQ in clinical samples were generally less sensitive (LLOQ at 50 ng/ml or higher) compared to the present method [8–13].

Stability studies were carried out to demonstrate the applicability of the validated method for pharmacokinetic studies by estimating the maximum length of storage at the intended temperature of the samples in order to preserve the integrity of the samples containing the drug combination. The storage stability results showed that the samples could be kept at −80 °C for up to 3 months but slight degradation occurred after a period of 12 months. Freeze–thaw analysis showed that the samples could be thawed and refrozen for at least up to three cycles. Results of the bench stability study indicated that AS and DHA were not stable at room temperature for up to 3 h and need to be processed within 1 h from the time it is thawed.

Finally, our study demonstrated that the validated method is suitable for pharmacokinetic studies.

5. Conclusion

The validated method satisfies all the criteria that are necessary for a bioanalytical method. The SPE extraction method which provides a simple and relatively fast sample clean-up procedure when compared to conventional liquid–liquid extractions, is suitable for routine use. More significantly, this method is able to separate AS and DHA from MQ in order to be analysed separately, while maintaining the required sensitivity and selectivity. Thus, the assay method can reliably quantitate AS, DHA and MQ in human plasma for clinical pharmacological study of AS–MQ drug combination.

Conflict of interest

The authors declare no conflict of interest.

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