Journal of Chromatography B, 877 (2009) 558–562

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Validation of high performance liquid chromatography–electrochemical detection methods with simultaneous extraction procedure for the determination of artesunate, dihydroartemisinin, amodiaquine and desethylamodiaquine in human plasma for application in clinical pharmacological studies of artesunate–amodiaquine drug combination

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ARTICLE INFO

Article history:
Received 17 July 2008
Accepted 16 December 2008
Available online 25 December 2008

Keywords:
Artesunate
Dihydroartemisinin
Amodiaquine
Desethylamodiaquine
Drug combination
Solid phase extraction
HPLC–EC
Pharmacokinetics
Bioavailability

ABSTRACT

With the expanded use of the combination of artesunate (AS) and amodiaquine (AQ) for the treatment of falciparum malaria and the abundance of products on the market, comes the need for rapid and reliable bioanalytical methods for the determination of the parent compounds and their metabolites. While the existing methods were developed for the determination of either AS or AQ in biological fluids, the current validated method allows simultaneous extraction and determination of AS and AQ in human plasma. Extraction is carried out on Supelclean LC-18 extraction cartridges where AS, its metabolite dihydroartemisinin (DHA) and the internal standard artemisinin (QHS) are separated from AQ, its metabolite desethylamodiaquine (DeAQ) and the internal standard, an isobutyl analogue of desethylamodiaquine (IB-DeAQ). AS, DHA and QHS are then analysed using Hypersil C4 column with acetonitrile–acetic acid (0.05 M adjusted to pH 5.2 with 1.00 M NaOH) (42:58, v/v) as mobile phase at flow rate 1.50 ml/min. The analytes are detected with an electrochemical detector operating in the reductive mode. Chromatography of AQ, DeAQ and IB-DeAQ is carried out on an Inertsil C4 column with acetonitrile–KH_2PO_4 (pH 4.0, 0.05 M) (11:89, v/v) as mobile phase at flow rate 1.00 ml/min. The analytes are detected by an electrochemical detector operating in the oxidative mode. The recoveries of AS, DHA, AQ and DeAQ vary between 79.1% and 104.0% over the concentration range of 50–1400 ng/ml plasma. The accuracies of the determination of all the analytes are 96.8–103.9%, while the variation for within-day and day-to-day analysis are <15%. The lower limit of quantification for all the analytes is 20 ng/ml and limit of detection is 8 ng/ml. The method is sensitive, selective, accurate, reproducible and suited particularly for pharmacokinetic study of AS–AQ drug combination and can also be used to compare the bioavailability of different formulations, including a fixed-dose AS–AQ co-formulation.

1. Introduction

The World Health Organization (WHO) currently recommends the use of artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated falciparum malaria [1] and malaria endemic countries are following this recommendation [2]. One of these ACTs, artesunate–amodiaquine (AS–AQ), has been adopted so far by 18 countries as first-line treatment and is until today the second most used ACT after the fixed combination of artemether and lumefantrine (Coartem®) [2]. The standard regimen has AS (4 mg/(kg d) and AQ (10 mg/(kg d)) for 3 days and is given as either loose formulations of the two individually formulated and packaged products, co-blistered products, or, more recently, a co-formulated (fixed combination) product. With the expanded use and the abundance of brands, comes the need for reliable assays to assess and compare the bioavailability and disposition of AS–AQ formulations. The existing HPLC–UV methods used for the quantification of AS [3–6] and AQ [7–10] are generally limited by sensitivity. The LC–MS method for the determination of AS not only involves high capital expenditure but also requires the construction of two calibration curves [11]. HPLC with electrochemical detection (EC) remains the standard as it is affordable and sensitive enough for pharmacokinetic studies. However, the existing HPLC–EC methods for the quantification of AS [12,13] and AQ [14,15] are unsuited for extract-

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doi:10.1016/j.jchromb.2008.12.037

ing AS, AQ and their metabolites simultaneously. Hence, this article reports a validated analytical method which allows simultaneous extraction of AS, AQ and their metabolites from human plasma, followed by the determination of these analytes using HPLC–EC systems. Due to the chemical difference of AS and AQ, both drugs have to be analysed separately in order to achieve adequate sensitivity, i.e., AS and DHA in reductive mode; AQ and DeAQ in the oxidative mode.

2. Experimental

2.1. Chemicals and reagents

AS, DHA and the internal standard (I.S.), artemisinin (QHS) were obtained from Knoll AG (Liestal, Switzerland). AQ was obtained from The United States Pharmacopeial Convention Inc. (Rockville, MD, USA). DeAQ and the I.S., IB-DeAQ were given by University of Liverpool (Liverpool, UK) and Pfizer Inc. (Groton, CT, USA). 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 samples

Stock solutions of AS (200 μg/ml), DHA (200 μg/ml) and QHS (50 μg/ml) were prepared in ethanol (EtOH), while the stock solutions of AQ (200 μg/ml), DeAQ (200 μg/ml) as well as IB-DeAQ (50 μg/ml) were prepared in methanol (MeOH). The stock solutions of the standards were diluted in ethanol–water (1:1, v/v) to produce intermediate standard solutions of 1, 5 and 20 μg/ml. The I.S. stock solutions were mixed equally by volume to produce a solution consisted of 25 μg/ml QHS and IB-DeAQ. Following that 10–40 μl of the standard solutions plus 10 μl of the I.S. were spiked into 500 μl of plasma and then made up to 1 ml with KH_2PO_4 (pH 4.0; 0.05 M) to obtain calibration standards of 20, 40, 100, 200, 400, 800, 1200 and 1600 ng/ml. The quality control (QC) samples of concentrations 50, 600 and 1400 ng/ml for AS, DHA, AQ and DeAQ were prepared from the QC stock solutions in the same way.

2.3. Solid phase extraction (SPE)

The SPE extraction was carried out on Supelclean™ LC-18 SPE tubes with 1 ml capacity and 100 mg sorbent (Bellefonte, PA, USA). To condition the extraction cartridges, 1 ml each of acetonitrile (ACN), MeOH and KH_2PO_4 buffer (pH 4.0; 0.05 M) were added in sequence. Plasma samples were then loaded. The tubes were washed with 1 ml of KH_2PO_4 (pH 4.0; 0.05 M) 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 (95:5, v/v) and subsequently AQ, DeAQ and IB-DeAQ were eluted with three consecutive aliquots of 0.5 ml 5% NH_3 in MeOH. The extracts were dried under a gentle flow of nitrogen gas at room temperature (*ca.* 25 °C) using Reacti-Vap™ 18780 drying system (Pierce, Rockford, IL, USA). The dried extracts containing AS, DHA and QHS were reconstituted with 100 μl of ethanol–water (1:1, v/v) and kept at 4 °C for at least 18 h before analysis to ensure that the tautomerization of α and β isomers of DHA reaches equilibrium [4,12,13,16]. Dried extracts of AQ, DeAQ and IB-DeAQ were reconstituted with 100 μl of 0.01 M HCl.

2.4. Instrumentation and chromatography

The analysis of AS and DHA was done by using the same instrumentation and chromatographic conditions as those previously described for AS–MQ drug combination [17]. The chromatographic run time was 14.5 min.

AQ and DeAQ were also analysed on BAS 200 HPLC–EC system (Bioanalytical Systems, West Lafayette, IN, USA) but operating in the oxidative mode at +800 mV. The EC detector was equipped with a thin-layer dual glassy carbon electrode with reference to the Ag/AgCl electrode. Manual sample injections were performed on a Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA) with a 20 μl sample loop. The analytical column was Inertsil C4, 4.6 mm × 100 mm, 5 μm particle size (GL Sciences Inc., Tokyo, Japan) and the mobile phase consisted of ACN–KH_2PO_4 (pH 4.0; 0.05 M) (11:89, v/v). The flow rate was 1.00 ml/min and the chromatographic run time was 17 min.

2.5. Method validation

The analytical method for the determination of AS, DHA, AQ and DeAQ in human plasma was validated according to the requirements of USFDA [18].

For evaluation of method selectivity, six different sources of drug-free human plasma were extracted and analysed to check for possible interference with the retention time of the analytes.

The linearity of the detector response for AS, DHA, AQ and DeAQ was evaluated from 20 to 1600 ng/ml. For DHA quantification, the calibration curve was constructed based on the predominant α-DHA peak after tautomerization of the isomers reaches equilibrium. Regression parameters were calculated for five calibration curves using SPSS software, Version 10.0 for Windows (SPSS Inc., Chicago, IL, USA) with the weighting scheme of $1/x^2$.

The lower limit of quantification (LLOQ) was the lowest concentration of the analyte with >5 times signal-to-noise ratio with an accuracy between 80% and 120% and a coefficient of variation (CVs) of <20%. This was used as the first point for the calibration curve. The limit of detection (LOD) was the lowest detectable analyte concentration with approximately three times signal to noise ratio.

The accuracy of the method was determined by comparing the calculated analyte concentrations of five replicates of independently spiked plasma samples with the theoretical concentrations. The precision of the method was evaluated by comparing the coefficient of variations of five replicates of a single homogenous sample within the same day and from day-to-day for five consecutive days.

Sample recoveries were determined by comparing the peak height of the analytes in the extracted samples with the peak height of the corresponding unextracted analytical standards.

2.6. Stability

Long term stability of AS, DHA, AQ and DeAQ in human plasma was investigated over a period of 6 months at −80 °C. Freeze and thaw stability was evaluated for 3 cycles. The stability of the analytes on bench at room temperature (25 °C) was evaluated for only 1 h since our previous study demonstrated that AS and DHA were unstable after being left for 3 h on the bench [17].

2.7. Application to pharmacokinetic study

The method was tested on plasma samples obtained from three healthy normal volunteers with approval from the research and ethics committee of Universiti Sains Malaysia. Two AS–AQ tablets (each consisted of 100 mg artesunate and 270 mg amodiaquine base) were given to the volunteers with 200 ml of water following an overnight fast. Venous blood (5 ml) was taken at pre-dose, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 48 and 72 h after dosing. On day 5, 7, 14, 21, 28, 35, 42 and 60, blood samples were collected by direct venipuncture. All the samples were collected in heparinised Vacutainer™ tubes which contained lithium heparin. Plasma layers were separated from the blood after centrifuging at 3500 r.p.m. for

15 min. The samples were stored at −80 °C until the time of analysis. The pharmacokinetic parameters were derived with EquivTest/PK (Statistical Solutions, Saugus, MA, USA) using a non-compartmental analysis. The elimination half-life ($T_{1/2}$) was calculated by regression analysis of the log-linear portion of the plasma concentration versus time curve with the use of at least three time points. The area under plasma concentration versus time curve was calculated by utilizing the linear trapezoidal rule. The maximum concentration (C_{max}) and the 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. Selectivity

The analytical method was found to be selective for all the analytes and no endogenous substances were found to interfere with the compound separation. Representative chromatograms of the blank human plasma extract and the plasma extract containing AS, DHA and QHS obtained through the HPLC–EC (reductive mode) method is shown in Fig. 1. Similarly, representative chromatograms of the blank human plasma extract and the plasma extract containing AQ, DeAQ and IB-DeAQ obtained through the HPLC–EC (oxidative mode) method is shown in Fig. 2.

3.1.2. Linearity and range

The calibration curves of AS, DHA, AQ and DeAQ were found to be linear between 20 and 1600 ng/ml with correlation coefficient >0.997. The typical equation for the calibration curves were as follows: AS, $y = 0.0010x + 0.0051$ ($r = 0.9977$); α-DHA, $y = 0.0018x + 0.0071$ ($r = 0.9992$); AQ, $y = 0.0061x - 0.0086$ ($r = 0.9991$); DeAQ, $y = 0.0057x - 0.0062$ ($r = 0.9991$), where y is the peak height ratio of the analyte to the internal standard and x is the concentration of the analyte. The LLOQs of AS, DHA, AQ and DeAQ were 20 ng/ml with accuracies in the range of 99.1–109.9% and the coefficient of variations were <8.0%. The LOD for AS, DHA, AQ and DeAQ were 8 ng/ml.

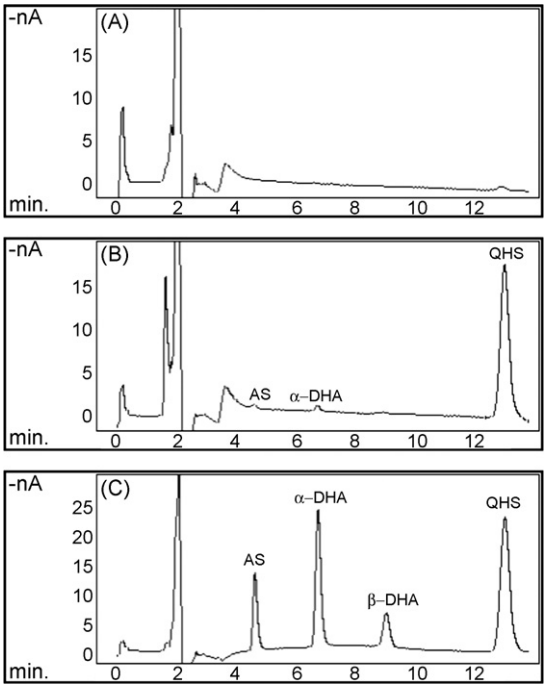

Fig. 1. HPLC chromatogram of (A) extracted blank human plasma; (B) extracted human plasma containing AS and DHA at 20 ng/ml (LLOQ) with QHS; (C) extracted human plasma containing AS and DHA at 1400 ng/ml with QHS. The retention times for the analytes are: AS (4.5 min); α-DHA (6.6 min); β-DHA (8.9 min); QHS (12.8 min).

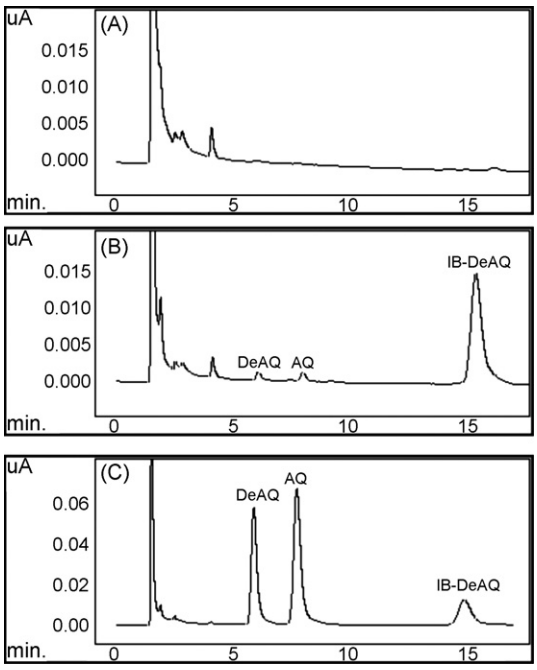

Fig. 2. HPLC chromatogram of (A) extracted blank human plasma; (B) extracted human plasma containing AQ and DeAQ at 20 ng/ml (LLOQ) with IB-DeAQ; (C) extracted human plasma containing AQ and DeAQ at 1400 ng/ml with IB-DeAQ. The retention times for the analytes are: DeAQ (5.8 min); AQ (7.6 min); IB-DeAQ (14.7 min).

3.1.3. Accuracy, precision and extraction recovery

The method was found to have good accuracies and reproducibility for all the analytes at all three level of concentrations. The mean accuracies for the analysis of AS, DHA, AQ and DeAQ are between 96.8% and 103.9% while the within-day and day-to-day precision have coefficient of variation of <15%. The extraction method was able to produce high recoveries (>79%) for all the analytes as well as the internal standards. Further details of the accuracy, precision and extraction recovery are shown in Table 1.

3.2. Stability studies

Stability studies carried out to determine whether any sample deterioration occurred during storage and handling showed that the samples can be kept at −80 °C for up to 6 months without degradation. The analytes were also found to be stable after 3 freeze–thaw cycles as well as 1 h on the bench at room temperature. The stability data are shown in Table 2.

3.3. Application to pharmacokinetic study

The validated analytical method has been successfully applied to pharmacokinetic studies. Pharmacokinetic profiles were obtained by plotting the drug concentration in plasma versus time after drug administration. Plasma concentrations were measured up to 12 h for AS and DHA while AQ and DeAQ were measured up to 1440 h. The pharmacokinetic profiles for AS and DHA are shown in Fig. 3A while the profiles for AQ and DeAQ are shown in Fig. 3B. The pharmacokinetic parameters are given in Table 3.

Table 1
Accuracy, precision and the extraction recovery for the determination of AS, DHA, AQ and DeAQ in human plasma ($n = 5$).

Analyte	C_{theo} (ng/ml)	C_{cal} (ng/ml)	Accuracy (%)	Precision (CV, %)		Recovery (%)
				Within-day	Day-to-day	
AS	50.0	48.4	96.8	7.6	12.2	79.1 ± 10.0
	600.4	611.6	101.9	3.4	4.9	96.4 ± 9.5
	1401.2	1409.6	100.6	4.8	4.3	91.0 ± 10.0
DHA	49.4	46.8	94.7	14.2	8.4	88.9 ± 8.5
	592.4	591.6	99.9	4.1	5.6	103.4 ± 10.6
	1382.4	1374.6	99.4	5.7	6.5	97.9 ± 10.7
AQ	50.2	50.0	99.6	4.3	4.5	104.0 ± 11.3
	601.2	619.4	103.0	0.6	2.4	90.2 ± 7.7
	1402.4	1457.2	103.9	4.8	2.8	84.8 ± 3.4
DeAQ	50.0	49.8	99.5	3.6	3.6	96.6 ± 8.2
	600.6	608.8	101.4	1.5	3.2	84.7 ± 7.4
	1401.4	1454.4	103.8	2.9	2.2	80.2 ± 3.9
QHS	250.0					97.4 ± 8.0
IB-DeAQ	250.0					80.1 ± 2.1

C_{theo} = theoretical concentration; C_{cal} = calculated concentration; CV = coefficient of variation.

Table 2
Stability of AS, DHA, AQ and DeAQ in spiked plasma samples ($n = 3$).

Stability	Remained (mean ± S.D.) (%)		
	Low 50 ng/ml	Medium 600 ng/ml	High 1400 ng/ml
Storage stability (−80 °C)			
AS			
3 months	98.24 ± 4.51	99.75 ± 6.21	104.08 ± 3.76
6 months	97.05 ± 1.32	102.64 ± 3.89	98.25 ± 0.49
DHA			
3 months	100.33 ± 7.18	97.48 ± 2.97	95.06 ± 2.40
6 months	94.46 ± 5.43	96.73 ± 7.61	97.64 ± 1.52
AQ			
3 months	99.57 ± 0.84	101.03 ± 4.06	94.66 ± 5.37
6 months	102.11 ± 2.18	97.21 ± 1.73	96.50 ± 1.69
DeAQ			
3 months	108.54 ± 2.49	103.22 ± 5.15	98.18 ± 0.42
6 months	101.93 ± 1.04	96.86 ± 0.25	93.83 ± 6.28
Freeze and thaw stability (−80 °C and 25 °C)			
AS	106.95 ± 8.37	96.17 ± 4.10	96.53 ± 0.55
DHA	93.47 ± 3.48	98.59 ± 1.03	97.84 ± 1.78
AQ	94.18 ± 0.91	96.47 ± 4.75	101.71 ± 7.17
DeAQ	95.75 ± 2.68	101.98 ± 2.70	102.11 ± 1.83
Bench stability (25 °C, 1 h)			
AS	95.41 ± 6.21	99.52 ± 1.50	98.58 ± 0.49
DHA	97.25 ± 3.49	101.82 ± 4.84	95.81 ± 2.01
AQ	98.03 ± 1.05	100.50 ± 3.16	99.16 ± 0.98
DeAQ	107.44 ± 5.09	99.96 ± 1.51	101.89 ± 9.32

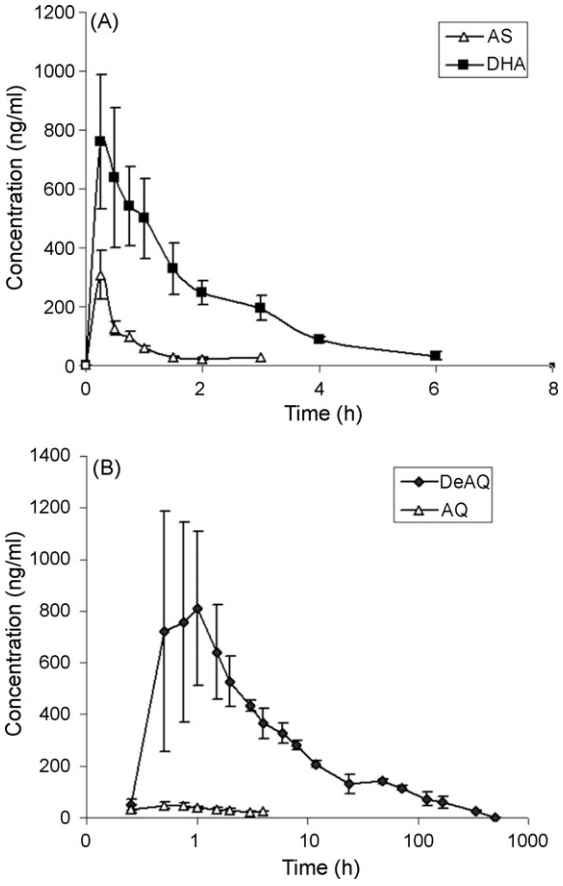

Fig. 3. Mean plasma concentration–time curves of (A) AS and DHA; (B) AQ and DeAQ (in log scale), after oral administration to three healthy normal volunteers.

Table 3
Pharmacokinetic parameters of AS, DHA, AQ and DeAQ following oral administration of AS–AQ combination tablet with a dose of 200 mg AS and 540 mg AQ ($n = 3$).

Analyte	AS	DHA	AQ	DeAQ
$T_{1/2}$ (h)	0.41 ± 0.18	1.22 ± 0.27	2.07 ± 1.29	114.14 ± 77.67
$AUC_{0\to t}$ (ng h/ml)	121.56 ± 36.26	1003.83 ± 418.35	78.94 ± 14.16	30,290.79 ± 16,440.59
$AUC_{0\to\infty}$ (ng h/ml)	133.62 ± 41.04	1094.27 ± 366.94	144.09 ± 44.18	34,900.06 ± 15,142.79
C_{max} (ng/ml)	242.60 ± 24.16	573.33 ± 231.57	47.57 ± 14.77	802.47 ± 501.23
T_{max} (h)	0.25 ± 0.00	0.25 ± 0.00	0.58 ± 0.14	0.83 ± 0.29

4. Discussion and conclusion

This analytical method satisfies the criteria for a reliable bioanalytical method as it is specific, reproducible, accurate, and linear within the calibration range of 20–1600 ng/ml. The extraction procedure is rapid and simple and allows the separation of AS and DHA from AQ and DeAQ while retaining the analytical sensitivity which is comparable to the existing methods. For AS and DHA determination, the LLOQ of the method reported by Na-Bangchang et al. was 10 ng/ml using 1 ml plasma volume [12], while the LLOQ of the method reported by Navaratnam et al. was 12.5 ng/ml using 0.5 ml plasma volume [13]. For AQ and DeAQ determination, the LLOQ of the method developed by Pussard et al. was 14 ng/ml, with 1 ml sample volume [14]. The method developed by Mount et al., although claimed to have LOD of 1 ng/ml, used 39.4 ng/ml as the first point of the calibration curve which implied that the LLOQ was high [15]. The current method has LLOQ of 20 ng/ml and requires only 0.5 ml plasma volume for AS, DHA, AQ and DeAQ determinations. The current method has improved efficiency, in that it permits the analysis of all the analytes of the drug combination with just one sample preparation procedure instead of two sample preparations which is not just beneficial in terms of time, cost and labour, it also cuts down on the total plasma volume by half. Small sample volumes are particularly important when conducting pharmacokinetic studies in children.

Pharmacokinetic study has been carried out on three healthy volunteers in order to demonstrate the applicability of the validated method. The present data obtained for AS and DHA is in agreement with the previously published data, where the reported range of $T_{1/2}$, C_{max}, T_{max} and $AUC_{0\rightarrow t}$ for AS were 0.3–0.5 h, 30–577 ng/ml, 0.25–0.66 h and 119–1050 ng h/ml, respectively, and that for DHA were 0.4–1.6 h, 290–1141 ng/ml, 0.5–2 h and 490–1340 ng h/ml, respectively, following a single oral dose of 200–300 mg AS [12,13,19,20]. Similarly, the present data for AQ and DeAQ is close to the previously published data, where the reported range of $T_{1/2}$, C_{max}, T_{max} and $AUC_{0\rightarrow t}$ for AQ were 3.9–14.5 h, 15–32 ng/ml, 0.5–2.0 h and 78–108 ng h/ml, respectively, and that for DeAQ were 137–149 h, 181–1092 ng/ml, 2.0–3.4 h and 2304–31,793 ng h/ml, respectively, following a single oral dose of 600 mg AQ [10,21,22]. It is clear from the published data that there is wide inter-individual variation in the pharmacokinetic data. This has been attributed to the rapid biotransformation of AS and AQ to its active metabolites which is greatly affected by the rate of metabolisms of individuals [23,24].

In conclusion, the current HPLC–EC method with simultaneous extraction procedure for the determination of AS, DHA, AQ and DeAQ has been fully validated and found suitable for use in pharmacokinetic studies. The method can also be used to assess and compare the bioavailabilities of different formulations of either separately formulated or co-formulated AS–AQ combinations and thus help identify suitable products for malaria control.

Conflict of interest

None declared.

Acknowledgements

This project was sponsored by the Drugs for Neglected Diseases initiative (DNDi)/INCO-DEV ("International Role of Community Research for Development"). The authors would like to express gratitude to Mr. Arunachalam for his technical assistance. P.O. is a staff member of the WHO; the authors alone are responsible for the views expressed in this publication and they do not necessarily represent the decisions, policy or views of the WHO.

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