Journal of Chromatography B, 876 (2008) 61–68

Contents lists available at ScienceDirect

Journal of Chromatography B

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

A liquid chromatographic-tandem mass spectrometric method for determination of artesunate and its metabolite dihydroartemisinin in human plasma

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ARTICLE INFO

Article history:
Received 7 August 2008
Accepted 9 October 2008
Available online 15 October 2008

Keywords:
Antimalarial
Artesunate
Dihydroartemisinin
High throughput
Liquid chromatography/tandem mass spectrometry (LC-MS/MS)
Stable isotope labeled (SIL) internal standard
Solid phase extraction (SPE)

ABSTRACT

A bioanalytical method for the analysis of artesunate and its metabolite dihydroartemisinin in human plasma using high throughput solid-phase extraction in the 96-wellplate format and liquid chromatography coupled to positive tandem mass spectroscopy has been developed and validated. The method was validated according to published FDA guidelines and showed excellent performance. The within-day and between-day precisions expressed as RSD, were lower than 7% at all tested concentrations including the lower limit of quantification. Using 50 μl plasma the calibration range was 1.19–728 ng/ml with a limit of detection at 0.5 ng/ml for artesunate and 1.96–2500 ng/ml with a limit of detection at 0.6 ng/ml for dihydroartemisinin. Using 250 μl of plasma sample the lower limit of quantification was decreased to 0.119 ng/ml for artesunate and 0.196 ng/ml dihydroartemisinin. Validation of over-curve samples in plasma ensured that accurate estimation would be possible with dilution if samples went outside the calibration range. The method was free from matrix effects as demonstrated both graphically and quantitatively.

1. Introduction

Plasmodium falciparum malaria, caused by a mosquito-borne protozoan parasite is the most important parasitic disease of man. Close to 500 million people are infected each year and death toll is highest in Africa where more than 1 child dies every 30 s [1,2]. Drug resistance is emerging to almost all antimalarial drugs except for the artemisinin derivatives, although recent reports indicate that even these essential antimalarials might be in jeopardy [3–5]. To delay the emergence of resistance the use of drug combinations (i.e. artemisinin based combination therapy) is now generally accepted and encouraged [6]. Artemisinin (ART) is a naturally occurring sesquiterpene lactone containing an endoperoxide group. It was first extracted from the Chinese herb *Artemisia annua.* Artesunate (ARS) is a hemisuccinate ester, which is rapidly and extensively hydrolysed *in vivo* to the active metabolite dihydroartemisinin (DHA), which has a relative antimalarial activity of approximately 1.4 compared to the parent compound [7]. ARS is the only derivative that is available as a parental formulation for intravenous administration. In the treatment of severe malaria its use was associated with a reduction in mortality by 35% compared to IV quinine [8]. The determination of ART and its derivatives in biological samples has traditionally been difficult for a number of reasons. The compounds lack chromophore groups which make ultraviolet (UV) detection unsuitable. Some analytical methods have acquired enhanced sensitivity by employing LC post-column on-line derivatization before the UV detection and reached limits of detection of approximately 20–30 ng/ml [9–11].

The endoperoxide configuration of the compounds can be used for electrochemical detection (ECD) in reductive mode. Several publications on the determination of artemisinin and its derivatives using ECD have been published [12–14]. The limit of detection for these methods is around 5–20 ng/ml using 0.5–1 ml plasma. The drawbacks of the ECD technique are that it requires rigorously controlled anaerobic conditions and deoxygenation of biologic samples as well as the mobile phase which can be very difficult to establish and maintain. The introduction of liquid chromatography mass spectrometry (LC-MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS) during the last 10 years has revolutionized drug analysis and is today considered the gold standard in

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doi:10.1016/j.jchromb.2008.10.018

the analysis of drugs in biological fluids. A sensitive LC-MS method for quantification of ARS/DHA in human plasma was able to reach limits of quantification of 1 ng/ml using 0.5 ml of plasma [15]. The drawbacks of the method were a long cycle time and that it was necessary to use two calibration curves over the anticipated therapeutic range. Recently a comparison between an ECD and a LC-MS/MS method indicated good correlation but superior sensitivity for the LC-MS/MS method reaching lower limits of quantification of 2 and 4 ng/ml for DHA and ARS, respectively, using 100 μl plasma [16].

The aim of this work was to develop a sensitive robust high throughput LC-MS/MS method using a low plasma volume (50 μl) to facilitate detailed pharmacokinetic studies and therapeutic drug monitoring. The method has been validated according to published FDA-guidelines [17,18].

2. Experimental section

2.1. Chemicals and materials

Artesunate (ARS) and dihydroartemisinin (DHA) were obtained from Guangzhou University of Traditional Chinese Medicine (Guangzhou, China). The stable isotopes labeled (SIL) internal standards (SIL-ARS and SIL-DHA) were obtained from Comipso (Bordeaux, France) and Novartis (Basel, Switzerland), respectively. The structures are shown in Fig. 1. Acetonitrile (HPLC-grade), methanol (pro analysis) and HPLC-water were obtained from JT Baker (Phillipsburg, USA). Ammonium acetate (LC-MS grade) was obtained from FLUKA (Sigma–Aldrich, St. Louis, USA). Ammonium acetate buffer solutions were prepared by dissolving appropriate amounts of ammonium acetate in HPLC-water and adjusting pH with acetic acid (Merck Darmstadt, Germany).

2.2. Instrumentation—liquid chromatography mass spectrometry

The LC system was an Agilent 1200 system consisting of a binary LC pump, a vacuum degasser, a temperature-controlled micro wellplate autosampler set at 4 °C and a thermostatted column compartment set at 40 °C (Agilent technologies, Santa Clara, USA). Data acquisition and quantification were performed using Analyst 1.4 (Applied Biosystems/MDS SCIEX, Foster City, USA). The compounds were analysed on a Hypersil Gold C18 (100 mm × 2.1 mm, 5 μm) column protected by a security guard column with a Hypersil Gold C18 (10 mm × 2.1 mm, 3 μm) guard cartridge (Thermo electron corporation, USA) using a mobile phase containing acetonitrile-ammonium acetate 10 mM pH 3.5 (50:50, v/v) at a flow rate of 500 μl/min with a wash out gradient. The complete LC gradient programme is listed in Table 1.

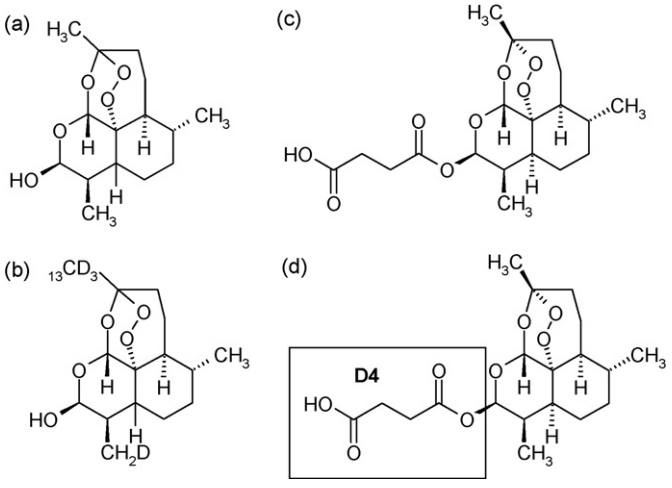

Fig. 1. Structures of DHA (a), SIL-DHA (b), ARS (c) and SIL-ARS (d).

Table 1
LC gradient programme.

Time (min)	% Solvent A	% Solvent B	Flow (μl/min)
0	100	0	500
0.3	100	0	500
0.8	0	100	500
2.2	0	100	500
2.4	0	100	1000
3.3	0	100	1000
3.5	100	0	750
5.2	100	0	750
5.4	100	0	500

Solvent A: Acetonitrile–aqueous ammonium acetate 10 mM (+1% acetic acid, v/v), 50–50 (v/v); Solvent B: Methanol–acetonitrile, 75–25 (v/v).

An API 5000 triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, USA), with a TurboV™ ionization source (TIS) interface operated in the positive ion mode, was used for the multiple reaction monitoring (MRM) LC-MS/MS analysis. The mass spectrometric conditions were optimized for the compounds by infusing a 100 ng/ml standard solution in mobile phase at 10 μl/min using a Harvard infusion pump (Harvard Apparatus, Holliston, USA) connected directly to the mass spectrometer. An additional tuning optimization was performed by continuously infusing the same standard solution at 10 μl/min via a "T" connector into the post-column mobile phase flow (500 μl/min). The TIS temperature was maintained at 475 °C and the TIS voltage was set at 4500 V. The curtain gas was set to 25.0 psi, and the nebulizer (GS1) and TIS (GS2) gases at 55.0 and 60.0 psi, respectively. The CAD gas in the collision cell was set to 5 psi. Quantification was performed using selected reaction monitoring (SRM) for the transitions *m/z* 402–267 and 406–163 for ARS and SIL-ARS, respectively, and 302–163 and 307–166 for DHA and SIL-DHA, respectively. The declustering potential (DP) was 50.0 V for ARS and SIL-ARS and 40.0 V for DHA and SIL-DHA.

2.3. Preparation of plasma standards

Stock solutions of all compounds (250–1000 μg/ml) were prepared in ethanol. Combined working solutions of DHA (ranging from 0.0978 to 125 μg/ml) and ARS (ranging from 0.0593 to 36.4 μg/ml) were prepared by serial dilution of the stock solutions in ethanol-water (50–50, v/v). A combined working solution of SIL-DHA/SIL-ARS (800/5000 ng/ml) was prepared in ethanol and stored in 200 μl aliquots at −86 °C until use. At the day of assay this working solution was diluted with a plasma-water solution (50–50, v/v containing sodium fluoride/potassium oxalate 2/3 mg/ml) down to 3.27/20.4 ng/ml. This solution was prepared and kept on ice during use. The stock solutions of SIL-DHA and SIL-ARS were stored at −86 °C until use. A 100 μl aliquot of each working solution (DHA/ARS) was added to blank fluoride/oxalate plasma (4900 μl) kept on ice to yield spiked calibration standards at 6 different concentrations ranging from 1.96 to 2500 ng/ml and 1.19–728 ng/ml for DHA and ARS, respectively. A calibration curve was constructed using 50 μl plasma of each standard. Linear regression with peak-area ratio (drug/internal standard response) against concentration with 1/concentration^2 (x^2) weighting was used for quantification. Quality control (QC) samples for determination of accuracy and precision in fluoride-oxalate plasma at three concentrations (5.87/2.90, 117/51.7 and 1880/546 ng/ml) were prepared in the same manner as the calibration standards and stored at −86 °C

until analysis. The amount of stock solution in all spiked samples was kept at 2% of the total sample volume to minimize any systematic errors between real samples and standards. The calibration standards and QC samples were stored in cryovials at −86 °C until analysis.

2.4. Analytical procedure

An eppendorf stream multistepper was used to add 150 μl ice-cold internal standard solution (3.27/20.4 ng/ml SIL-ARS/SIL-DHA in plasma-water (50–50, v/v) containing sodium fluoride/potassium oxalate 2/3 mg/ml) to 50 μl plasma in a 96-wellplate kept on ice. The samples were loaded onto a conditioned μ-elution HLB SPE 96-wellplate (Waters, USA). All steps in the solid plate extraction (SPE) procedure were conducted using a 12-channel pipette as follows: The SPE wells were activated and conditioned using 750 μl acetonitrile followed by 750 μl methanol and 200 μl water. The samples (200 μl) were loaded onto the SPE plate and drawn through using a low vacuum. The SPE wells were washed using 300 μl water drawn through using medium vacuum. Full vacuum was applied briefly before the SPE column tips were wiped dry with tissue paper and a 96-collection plate (0.5 ml) was inserted into the vacuum manifold. The wells were eluted using 100 μl methanol-acetonitrile (90:10, v/v) drawn through using a low vacuum followed by 100 μl water. The SPE eluates were briefly mixed on a mixmate™ (Eppendorf, Germany) at 600 rpm and stored at 4 °C for 15 h to enable α/β epimer equilibration before analysis. 5 μl was injected into the LC-MS/MS system.

2.5. Validation

Linearity and the calibration model were evaluated using calibration curves obtained during four days. Precision and accuracy throughout the calibration range were evaluated by analysis of 5 replicates quality control (QC) samples at three different concentrations daily for four days. Lower and upper limits of quantifications were evaluated by analysis of three replicates daily for four days. Carry-over effects for all the compounds were evaluated by injection of blank samples directly after injection of the highest point in the calibration curve. Over-curve dilution was evaluated by analysis of three replicates (10,000/2350 ng/ml DHA/ARS diluted 4.5 times with blank human plasma) daily for four days. Stability of stock

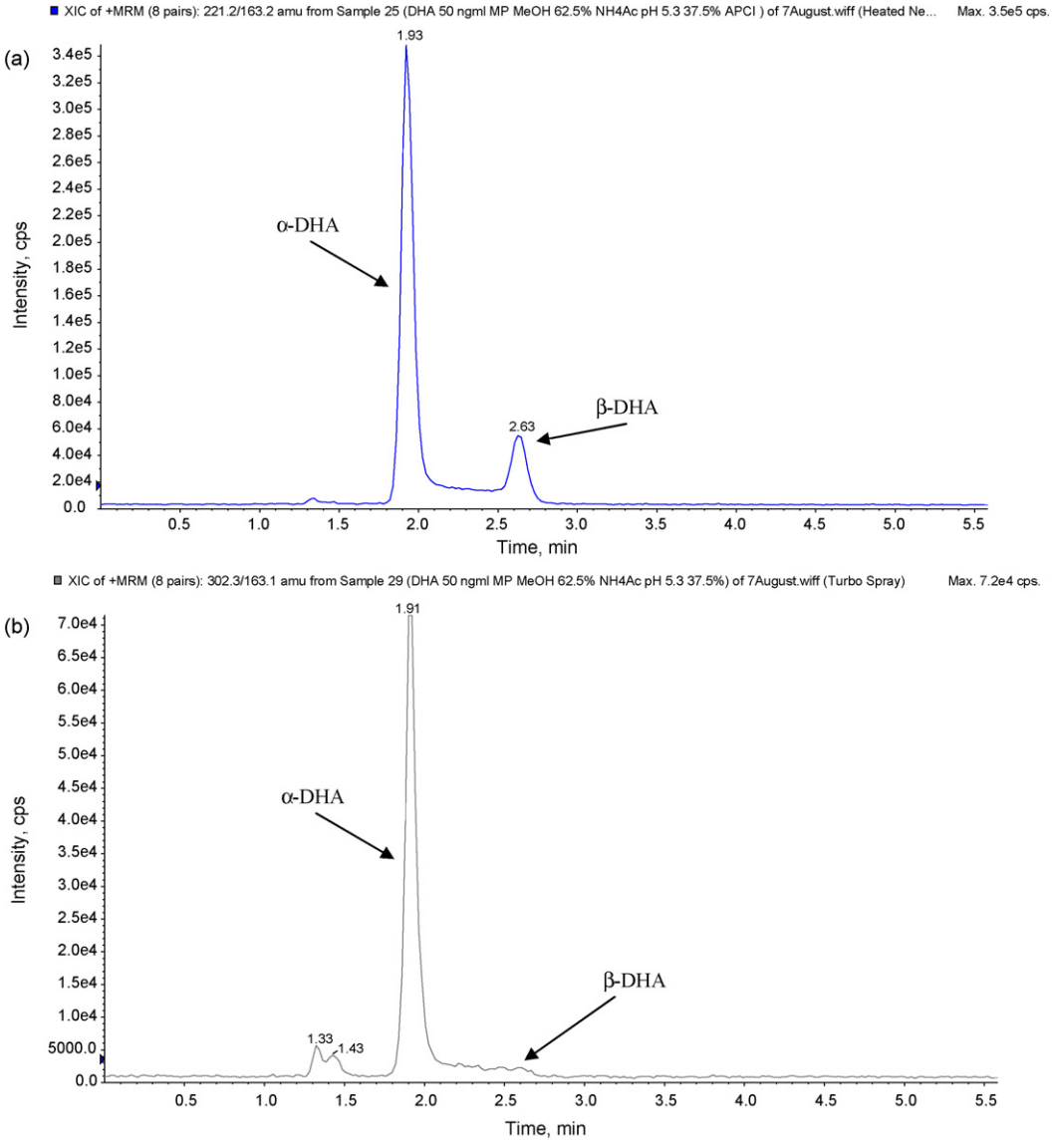

Fig. 2. Chromatogram of standard solution of DHA using APCI interface (a) and TIS interface (b). Mobile phase; methanol–ammonium acetate 10 mM pH 5.3 (62.5:37.5, v/v).

solutions in ethanol was evaluated at 4 °C and −86 °C. Stability of ARS/DHA in human plasma was evaluated during 3 freeze/thaw cycles, at ambient temperature for 2 h, on ice for 24 h, at 60 °C for 30 min and at −80 °C for 2 months. Bench-top stability of ARS/DHA in the autosampler was evaluated for 24 h. All stability studies in human plasma were conducted using three replicates at the lowest QC concentration (5.87/2.90 ng/ml DHA/ARS) and the highest QC concentration (1880/546 ng/ml DHA/ARS). The concentrations were determined with 1/amount^2 weighted linear regression using a calibration curve prepared each day. Intra-, inter- and total-assay precisions were calculated using analysis of variance (ANOVA). Selectivity was evaluated by analysis of blank plasma from six different donors. The potential interference between ARS/DHA and their internal standards was also evaluated. Recovery was determined by comparing the peak area for extracted QC samples with direct injected solution containing the same nominal concentration of the compounds as the QC samples after SPE. Matrix effects were thoroughly evaluated using blank plasma from six different donors. A quantitative estimation of the matrix effects was obtained by comparing the peak area for samples spiked in elution solution with extracted blank matrix spiked with the same nominal concentration of the compounds. A qualitative visualization of the matrix effects was obtained through post-column infusion experiments as described by others. Briefly, a continuous post-column infusion of 50/100 ng/ml ARS/DHA solution at 10 μl/min by a Harvard infusion pump through a T-connector was introduced to the mass spectrometer while samples to be tested were injected. The possibility of using more plasma to lower the lower limit of quantification (LLOQ to 0.112/0.196 ng/ml for ARS/DHA was evaluated through analysis of replicates over three days (totally 5 replicates). In addition, precision and accuracy for the modified method were investigated by analysis of triplicates at 0.290 and 54.6 ng/ml for ARS and 0.587 and 188 ng/ml for DHA over three days. The modified method used 250 μl (instead of 50 μl) plasma and the elution step consisted of 75 μl methanol-acetonitrile (90:10, v/v) + 50 μl water (instead of 100 μl methanol-acetonitrile (90:10, v/v) + 100 μl water).

3. Results and discussion

3.1. Method development

Both electrospray (TIS) and atmospheric pressure chemical ionization (APCI) have been used previously for quantification of artemisinin derivatives in biological fluids [15,16,19–22]. Both interfaces were evaluated on the basis of sensitivity and signal-to-noise ratios. The mass spectra of ARS and DHA using the APCI interface were characterized by an intense fragment ion at 221 *m/z* in line with previous reports [15]. The mass spectra of ARS and DHA using the TIS interface were characterized by several (NH_4, Na, NH_3 + Na) adducts with very low intensity for the protonated molecular ion $[M+H]^+$. The base peak for both drugs was produced by the $[M+NH_4]^+$ adduct. The absolute intensity was about 10 times greater with the APCI interface than with the TIS interface but the noise was increased proportionally leading to comparable sensitiv-

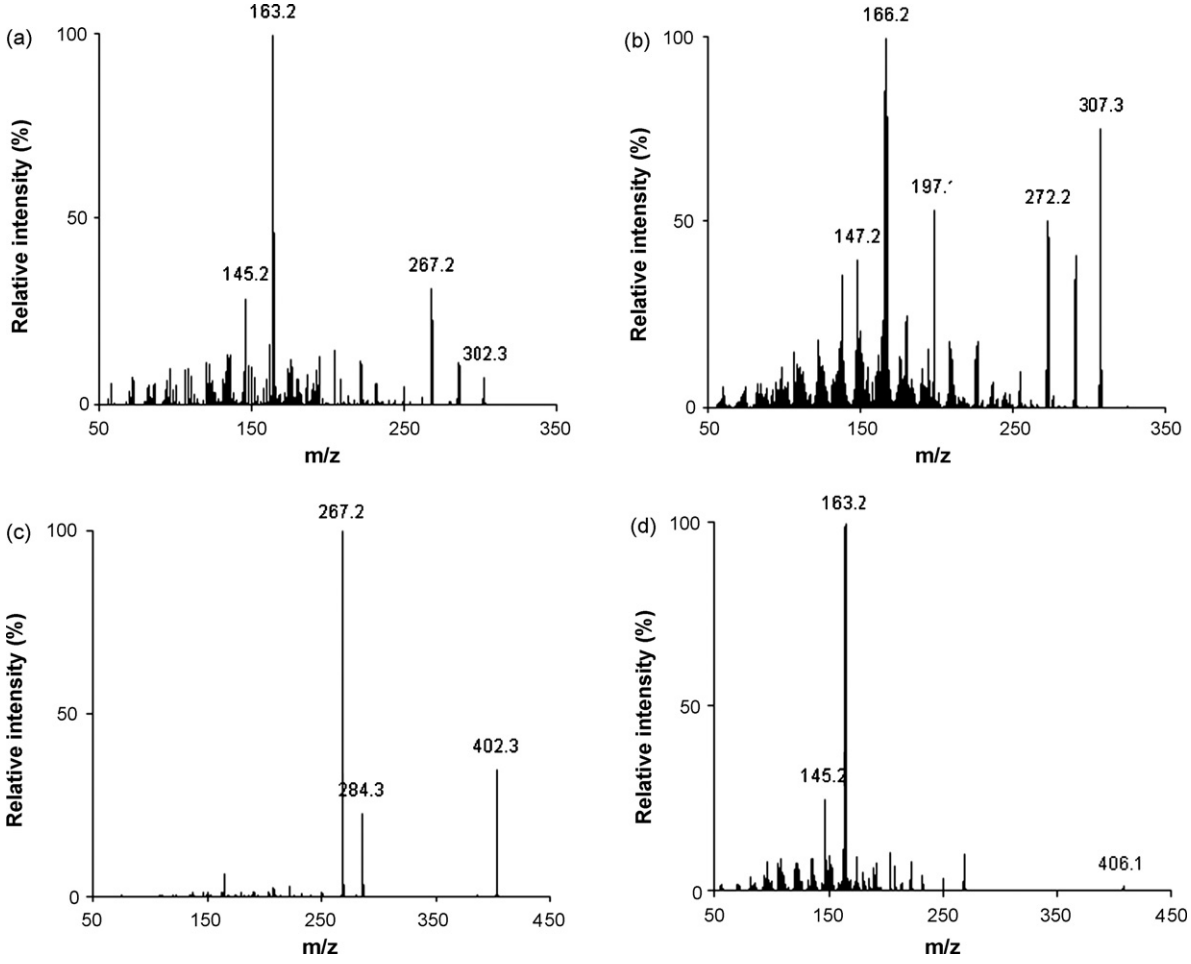

Fig. 3. Collision-induced dissociation mass spectra for DHA (a), SIL-DHA (b), ARS (c) and SIL-ARS (d). For experimental conditions see Section 2.2.

ity (i.e. same signal-to-noise ratio) for both interfaces. DHA exhibits tautomerism and the equilibrium between α- and β-DHA is influenced by temperature, solvent composition, and chromatographic conditions [23,24]. Traditionally only α-DHA is used for quantification of DHA concentrations. An interesting difference between the TIS interface and the APCI interface was their selectivity towards α- and β-DHA. The APCI interface (using SRM 221–163 *m*/*z*) produced two distinct peaks for α- and β-DHA but the TIS interface (using SRM 302–267 *m*/*z*) only displayed a peak for α-DHA (Fig. 2a and b). The exact reason for this phenomenon is unclear but it could possibly be explained by α-DHA being more prone to form an ammonium adduct than β-DHA for steric reasons. Finally the TIS interface with the ammonium adduct as pre-cursor ion was chosen for quantification as it would give equal sensitivity as the APCI interface but an increased linear range and straightforward integration of α-DHA. The collision-induced dissociation (CID) mass spectra (100–550 *m*/*z*) for DHA, SIL-DHA, ARS and SIL-ARS are shown in Fig. 3a-d. Both acetonitrile and methanol were evaluated as organic modifiers in the mobile phase. The peak shape was better when using acetonitrile compared to methanol while the TIS sensitivity was equivalent. We have previously reported problems with stability of the MS signal due to carbonization of the corona needle (APCI interface) when using mobile phases with high content of acetonitrile [25]. These problems are most likely interface related as no problems were detected when using the TIS interface in the present study. The retention of ARS but not DHA was affected by the pH of the mobile phase. ARS eluted before DHA when using high pH (pH ≥ 5) but after DHA when using lower pH (pH ≤ 3.5) in the mobile phase. A mobile phase containing acetonitrile-ammonium acetate 10 mM pH 3.5 (50:50, v/v) was finally chosen as this gave symmetrical peaks and high sensitivity. An efficient wash out gradient was included to minimize accumulation of non-polar phospholipids remaining after SPE [26]. The autosampler was kept at a temperature of 4 °C to minimize stability problems [27]. It was necessary to leave the samples in the autosampler for 15 h (data not shown) to enable full α/β DHA epimer equilibration which is in agreement with previous reports [14,28]. The column oven was kept at 40 °C to reduce back pressure and to increase peak efficiency and reduce separation times.

The goal was to develop a robust and selective sample preparation without compromising throughput for the final method. The Oasis μ-elution HLB SPE 96-wellplate was chosen as it facilitates low elution volumes and is particularly suitable when working with low sample volumes. Different elution solution compositions were evaluated to maximize recovery and facilitate direct injection from the eluate without the need for a tedious evaporation step. A 100 μl of the elution solution (methanol-acetonitrile, 90–10, v/v) was enough to completely elute the compounds from the SPE wells. A 100 μl of water was subsequently drawn through the SPE wells to dilute the organic solvent content in the samples which minimized band broadening of the peaks when injected into the LC-MS/MS system.

Table 3
Inter-, intra- and total-assay precision (ANOVA) for ARS and DHA in human plasma.

	Inter-assay (%)	Intra-assay (%)	Total-assay (%)
ARS			
QC 1, 2.90 ng/ml	4.55	3.62	3.78
QC 2, 51.7 ng/ml	4.38	2.00	2.53
QC 3, 546 ng/ml	1.90	2.49	2.40
DHA			
QC 1, 5.87 ng/ml	5.89	5.98	5.97
QC 2, 117 ng/ml	2.45	1.44	1.64
QC 3, 1880 ng/ml	4.40	2.49	2.88

$n = 5$, $h = 4$.

3.2. Validation

The concentrations of the internal standards were selected to be 3.27/20.4 ng/ml SIL-ARS/SIL-DHA) as these concentrations produced signals in the ARS and DHA traces lower than 20% of a response at LLOQ. The highest concentrations of ARS and DHA produced signals less than 2% of the working concentrations of SIL-ARS/SIL-DHA. Carry-over for ARS and DHA was less than 20% of a LLOQ sample and carry-over for SIL-ARS/SIL-DHA was not detectable with the chosen settings.

The back-calculated concentrations for the calibration standards and the results for the precision samples were used to choose the regression model. A calibration model using 1/amount^2 (x^2) weighted linear regression analysis was chosen as this generated an evenly distributed low error over the whole range (Table 2). Precision and accuracy for the QC samples during the validation are shown in Table 3. The lower limits of quantification (LLOQ) were determined to 1.19/1.96 ng/ml for ARS/DHA respectively with a precision and accuracy within 10%. The limits of detection (LOD) were 0.50/0.61 ng/ml for ARS/DHA respectively. The LOD was chosen as the lowest concentration that could be distinguished reliably from the background noise (i.e. ≥3 times the SD of a blank plasma sample). Precision and accuracy for LLOQ, upper limit of quantification and over-curve dilution samples are shown in Table 4.

All blank plasma sources produced signals that would contribute less than 20% of a standard at LLOQ. The recovery was high at all tested concentrations for ARS and DHA and the internal standards. The recovery (unadjusted for matrix effects) of ARS was

Table 2
Back-calculated concentrations of standard curves for ARS and DHA in human plasma.

	Nominal concentration					
	1.19 ng/ml	3.86 ng/ml	21.2 ng/ml	69 ng/ml	224 ng/ml	728 ng/ml
ARS						
Average ($n = 8$)	1.17	4.02	21.7	68.9	224	690
Sd	0.05	0.09	0.77	1.09	6.46	12.81
CV (%)	4.51	2.29	3.56	1.58	2.88	1.86
Accuracy	98.5	104.1	102.5	99.8	100.2	94.8
	Nominal concentration					
	1.96 ng/ml	7.82 ng/ml	39.1 ng/ml	156 ng/ml	625 ng/ml	2500 ng/ml
DHA						
Average ($n = 8$)	1.97	7.73	39.2	157	641	2430
Sd	0.09	0.17	1.14	4.37	20.45	47.79
CV (%)	4.79	2.26	2.91	2.79	3.19	1.96
Accuracy	100.3	98.8	100.4	100.5	102.6	97.4

Table 4
Inter-, intra- and total-assay precision (ANOVA) for lower limit of quantification, upper limit of quantification and diluted over-curve samples for ARS and DHA in human plasma.

	Inter-assay CV (%)	Intra-assay CV (%)	Total-assay CV (%)	Accuracy (%)
ARS				
LLOQ, 1.19 ng/ml	1.40	3.91	3.41	104
ULOQ, 728 ng/ml	2.29	2.00	2.08	95.5
Over-curve diluted to 519 ng/ml	4.63	1.97	2.95	90.5
DHA				
LLOQ, 1.96 ng/ml	4.23	7.09	6.44	103
ULOQ, 2500 ng/ml	3.43	1.85	2.39	96.7
Over-curve diluted to 2210 ng/ml	2.66	2.63	2.64	90.4

$n = 3$, $h = 4$.

Table 5
Matrix effects. ARS and DHA and internal standards spiked in extracted blank human plasma vs. spiked in elution solution.

	Blank A	Blank B	Blank C	Blank D	Blank E	Blank F	Average	SD	CV (%)
ARS									
ARS (QC1), 2.90 ng/ml	1.073	1.068	1.025	1.033	1.025	0.998	1.037	2.9	2.8
SIL-ARS (QC1), 3.3 ng/ml	1.053	1.026	1.023	1.047	1.063	1.026	1.040	1.7	1.6
Normalized response	1.019	1.041	1.002	0.986	0.965	0.972	0.998	2.9	2.9
ARS (QC 2), 51.7 ng/ml	1.031	0.993	0.964	1.037	0.991	1.019	1.006	2.8	2.8
SIL-ARS (QC2), 3.3 ng/ml	1.048	1.015	0.981	1.082	1.011	1.027	1.027	3.4	3.4
Normalized response	0.984	0.979	0.983	0.959	0.980	0.992	0.979	1.1	1.1
ARS (QC3), 546 ng/ml	1.009	0.976	0.984	0.983	0.967	0.962	0.980	1.6	1.7
SIL-ARS (QC3), 3.3 ng/ml	1.014	0.973	1.002	0.979	0.958	0.944	0.978	2.6	2.7
Normalized response	0.995	1.003	0.983	1.005	1.010	1.019	1.002	1.3	1.3
DHA									
DHA (QC1), 5.87 ng/ml	0.982	0.969	0.937	0.960	0.985	0.872	0.951	4.2	4.5
SIL-DHA (QC1), 20 ng/ml	1.036	0.998	0.998	1.026	1.036	0.948	1.007	3.4	3.3
Normalized response	0.948	0.972	0.939	0.935	0.952	0.920	0.944	1.8	1.9
DHA (QC2), 117 ng/ml	1.001	0.953	0.929	0.990	0.967	0.980	0.970	2.6	2.7
SIL-DHA (QC2), 20 ng/ml	1.027	0.961	0.950	1.017	0.978	0.988	0.987	3.0	3.1
Normalized response	0.975	0.992	0.977	0.974	0.989	0.992	0.983	0.9	0.9
DHA (QC3), 1880 ng/ml	1.011	0.997	0.987	1.005	0.997	0.987	0.997	0.9	1.0
SIL-DHA (QC3), 20 ng/ml	0.978	0.977	0.950	0.962	0.954	0.946	0.961	1.4	1.5
Normalized response	1.033	1.020	1.040	1.044	1.045	1.044	1.038	1.0	1.0

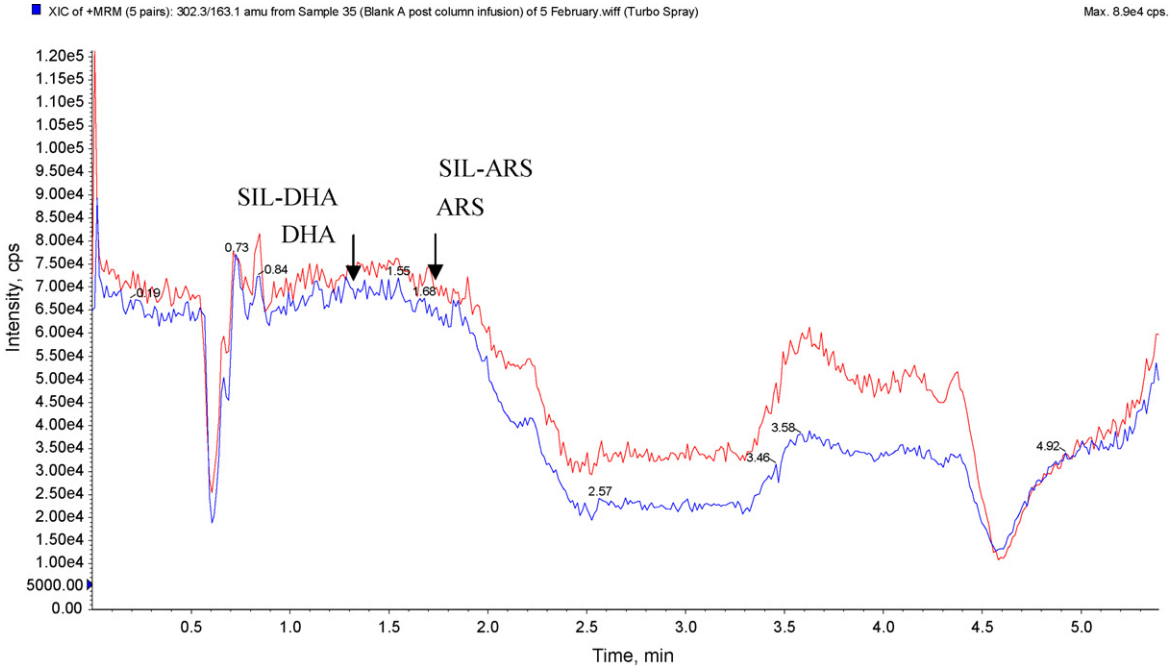

Fig. 4. Injection of extracted blank human plasma during post column infusion 10 μl/min of ARS/DHA, 50/100 ng/ml.

between 92.4 and 96.2% over the calibration range (2.90, 51.7 and 546 ng/ml) and the recovery of SIL-ARS was between 90.9 and 95.4% and independent of ARS concentration. The recovery (unadjusted for matrix effects) of DHA was between 100.1 and 102.6% over the calibration range (5.87, 117 and 1880 ng/ml) and the recovery of SIL-DHA was between 100.2 and 101.0% and independent of DHA concentration. No suppression/enhancement for ARS and DHA or the internal standards could be detected when references in neat injection solvent were compared with references in extracted blank biological matrix (Table 5). The normalized matrix effects (compound/internal standard) were close to 1 with a low variation in line with international recommendations [18]. Post-column infusion experiments confirmed the absence of regions with severe matrix effects (i.e. no sharp drops or increases in the response) for blank human plasma extracted with the developed method (Fig. 4).

Both ARS and DHA are thermolabile compounds and stability is significantly improved at lower temperatures [27]. When testing stability of these two compounds it is important to recognize that the primary route of ARS degradation is through formation of DHA. ARS can form DHA both through chemical hydrolysis and through plasma esterase mediated hydrolysis [11,29]. The stability of the drugs in different sources of plasma is very variable [30]. The anticoagulant fluoride-oxalate (i.e. fluoride) will inhibit plasma esterase mediated hydrolysis but not chemical hydrolysis. It is thus important to test the stability of these two compounds separately to avoid overestimation of remaining DHA concentrations (unless the ARS concentration is significantly lower than the DHA concentration). ARS had borderline stability for one hour in human fluoride-oxalate plasma at ambient temperature while DHA was considerably more unstable (Tables 6 and 7). It can also be seen in Table 6 that incorrect conclusions regarding the stability of DHA would have been drawn if they had been tested in the same samples at equivalent concentrations. In Table 6 it can be seen that ARS (original concentration 546 ng/ml) at 1 h have formed in average 34 ng/ml DHA and would thus have been overestimating the DHA concentration (i.e. if equivalent initial ARS/DHA concentrations with approximately 6%). The poor stability of the drugs, in particularly DHA, at ambient tem-

Table 6
Stability of ARS in human fluoride/oxalate plasma at ambient temperature.

	Fresh	1 h	2 h
ARS			
2.90 ng/ml	2.78	2.5	2.17
	2.85	2.46	2.29
	2.74	2.6	2.14
Average	2.79	2.52	2.20
Sd	0.06	0.07	0.08
CV (%)	2.00	2.86	3.61
Accuracy	96.21	86.90	75.86
Formation DHA			
	<LOD	<LLOQ	<LLOQ
	<LOD	<LLOQ	<LLOQ
	<LOD	<LLOQ	<LLOQ
546 ng/ml	515	474	409
	494	470	401
	499	471	396
Formation DHA			
	5.36	33.4	71.3
	5.46	32.5	73.1
	5.73	37.1	77.1
Average	503	472	402
Sd	10.97	2.08	6.56
CV (%)	2.18	0.44	1.63
Accuracy	92.06	86.39	73.63

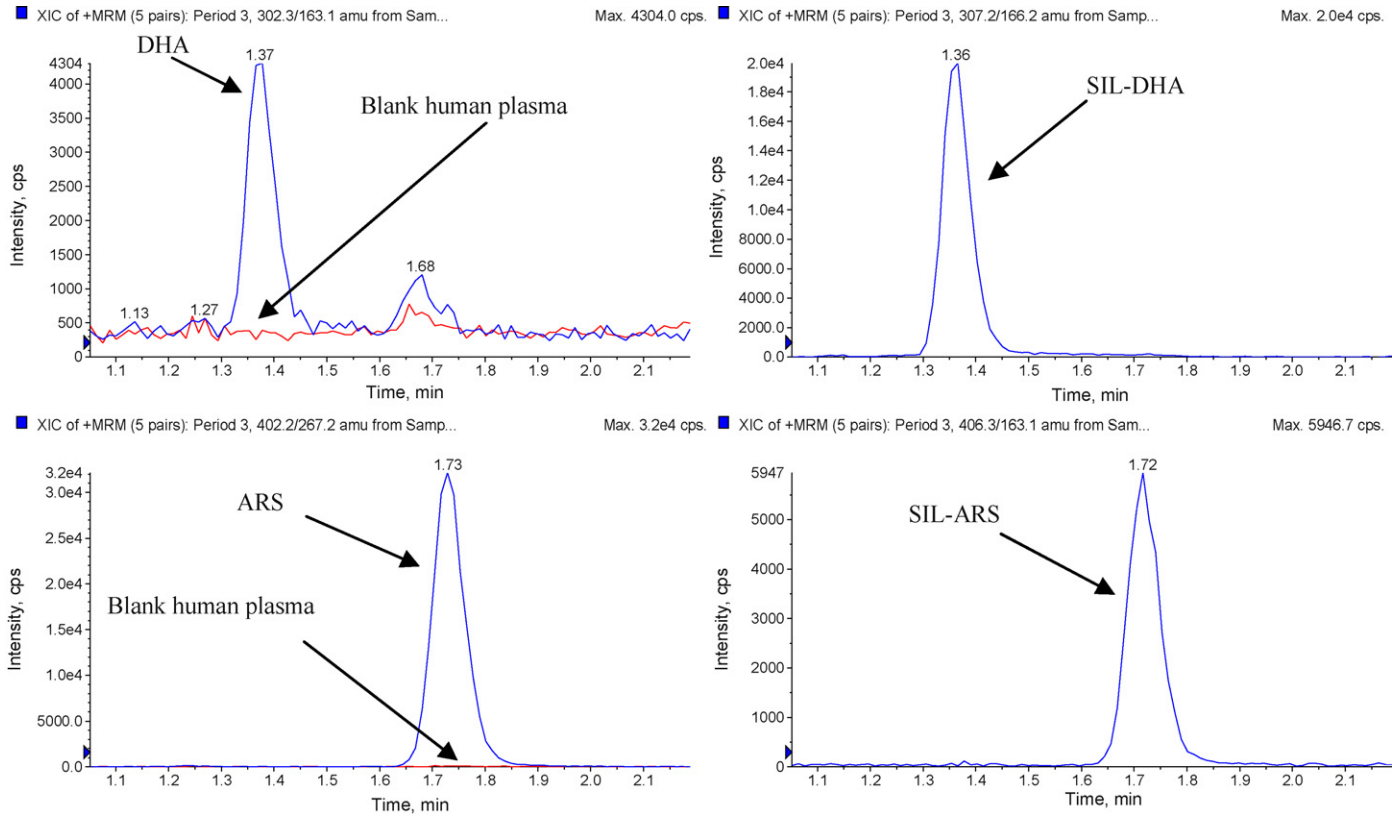

Fig. 5. Patient sample containing 13.4 ng/ml ARS and 3.60 ng/ml DHA. Overlay of blank plasma.

Table 7
Stability of DHA in human fluoride/oxalate plasma at ambient temperature.

DHA	Fresh	1 h	2 h
5.87 ng/ml	5.68	4.72	3.76
	5.32	4.55	3.51
	5.85	4.45	3.67
Average	5.62	4.57	3.65
Sd	0.27	0.14	0.13
CV (%)	4.82	2.98	3.47
Accuracy	95.68	77.91	62.12
1880 ng/ml	1790	1590	1180
	1820	1550	1210
	1800	1580	1210
Average	1803	1573	1200
Sd	15.28	20.82	17.32
CV (%)	0.85	1.32	1.44
Accuracy	95.92	83.69	63.83

perature in plasma became evident during method development and forced a decision to work entirely on ice throughout sample preparation [30]. Standards and QC samples were prepared on ice and clinical samples were thawed and aliquoted on ice. In contrast to the poor stability at ambient temperature both drugs showed good stability when stored on ice. The mean recovery ($n = 3$) after 24 hours on ice at 2.90 and 546 ng/ml for ARS were 96.3% (CV 1.8%) and 92.9% (CV 0.9%). The mean recovery ($n = 3$) after 24 h on ice at 5.87 and 1880 ng/ml for DHA were 91.6% (CV 3.9%) and 96.1% (CV 1.4%).

Both ARS and DHA were stable (i.e. within ±15% of initial concentration) during 3 freeze/thaw cycles and for at least 39 h (i.e. equilibration time 15 h + 24 h) in the autosampler. Neither ARS nor DHA were stable in human plasma kept at 60 °C with approximately 5% DHA and 45% ARS remaining after 30 min. The stock solutions in ethanol were stable for at least 6 days at 4 °C and for at least 100 days at −80 °C. Both ARS and DHA in human plasma were stable for at least 2 months at −80 °C. The assay was implemented for the analysis of clinical samples from a study in patients with uncomplicated malaria in Cambodia. Fig. 5 shows a chromatogram from a patient sample containing 13.4 ng/ml ARS and 3.60 ng/ml DHA with an overlay of a blank plasma sample. The patient sample was taken 15 min after the first dose of oral artesunate (4 mg/kg).

Using 250 μl plasma and small modifications to the method it was possible to obtain LLOQ's of 0.112/0.196 ng/ml for ARS/DHA. The mean concentrations ($n = 5$) found at LLOQ were 0.115 ng/ml (CV 12.7%) and 0.196 ng/ml (CV 10.1%) for ARS and DHA, respectively. The mean concentrations ($n = 9$) found at 0.290 and 54.6 ng/ml for ARS were 0.300 ng/ml (CV 2.6%) and 53.7 ng/ml (CV 3.9%). The mean concentrations ($n = 9$) found at 0.587 and 188 ng/ml for DHA were 0.574 ng/ml (CV 5.3%) and 188 ng/ml (CV 1.5%). The LC-MS/MS assay presented here uses a much lower sample volume (i.e. 50 μl) and shows much better sensitivity (i.e. LLOQ) than previously published methods for quantification of ARS and DHA in human plasma.

4. Conclusion

A sensitive high throughput LC-MS/MS method for the determination of ARS and DHA in fluoride/oxalate plasma has been developed and validated. The total analysis time for two batches (192 samples) is less than 24 h. The assay proved sensitive and reproducible and requires only 50 μl of plasma in the standard mode. The assay will be a very valuable tool for analyzing samples from clinical pharmacokinetic studies of ARS. It was shown that the stability of both ARS and DHA is poor at ambient and higher temperatures but that stability is guaranteed if all steps in sample preparation are conducted on ice.

Acknowledgement

This study was part of the SE Asian Influenza Clinical Trials Network supported by the US National Institute of Allergy and Infectious Diseases (NIH N01-AO-00042), and the Wellcome Trust-Mahidol University-Oxford Tropical Medicine Research Programme (077166/Z/05/Z) supported by the Wellcome Trust of Great Britain.

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