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Comparison of HPLC with electrochemical detection and LC–MS/MS for the separation and validation of artesunate and dihydroartemisinin in animal and human plasma

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ABSTRACT

High-performance liquid chromatography with reductive electrochemical detection (HPLC-ECD) method has been used for assaying artemisinins since 1985. Although the methods have been remarkably improved, tandem mass spectrometry (LC–MS/MS) systems with significant advantages have gradually replaced HPLC-ECD to analyze artesunate and dihydroartemisinin in plasma. In the present study, the two methods were evaluated for linearity, quantitation limits, selectivity, precision, and accuracy. The HPLC-ECD performed well in terms of various validation parameters, and showed a good agreement with the LC–MS/MS when calibrated in plasma. However, the major benefit of LC–MS/MS is that it requires only one-tenth the plasma volume needed by HPLC-ECD assay.

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1. Introduction

Artemisinin (QHS) is a naturally occurring, sesquiterpene lactone endoperoxide isolated from *Artemesia annua* L. and chemically characterized by Chinese scientists during the 1970s and early 1980s [1,2]. QHS and several synthetic analogs constitute a new class of antimalarial drug that has been shown to be effective against the erythrocytic stages of the plasmodial parasite, even against strains that have developed resistance to currently available therapies such as chloroquine [3]. QHS and its developed derivatives, dihydroartemisinin (DHA), artemether, arteether, and artesunate (AS), are in use for the treatment of uncomplicated, complicated or severe malarias, including multidrug resistant falciparum malaria.

Sensitive methods for determining QHS and its derivatives in biological fluids are needed in conducting therapeutic drug monitoring, pharmacokinetic and metabolic studies. The development of sensitive and selective analytical methods for QHS and its analogues and metabolites has been a challenging problem. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) as the analytical approaches have proven difficult because this class of compounds is thermally labile [4] and does not contain an ultraviolet (UV) visible or fluorescent chromophore. Acid or base hydrolysis of QHS producing an UV-chromophore prior to HPLC analysis has been employed [5,6], but this approach lacks specificity [7,8]. Another less satisfying and low sensitivity approach for determining QHS and its derivatives in biological fluids uses supercritical fluid chromatography with electron-capture detection [9,10].

A technique that best met the sensitivity and specificity requirements is HPLC with reductive electrochemical detection (HPLC-ECD) system [11,12], which was firstly reported from our laboratory in 1985 [13]. The limitations of the approach include the requirement for rigorous temperature control and automated deoxygenation, as well as expensive and dedicated equipment. In order to keep high sensitivity, the system needs its electrochemical detector cleaned very often (e.g. after each batch of samples or approximately 50 injections). In addition, the mobile phase and flow path must be maintained oxygen-free constantly in order for the system to operate in the reductive mode [14]. An HPLC-tandem mass spectrometry (LC–MS/MS) system meeting our analytical requirements is now being used for pharmacokinetic and metabolic studies [15–17]. Currently, several research groups are able to use these systems routinely to assay AS and DHA even though the systems are expensive and complex in operation and maintenance [18–20].

The main purpose of this study was to evaluate and validate three systems of LC-MS/MS, BAS HPLC-ECD, and Agilent-ESA HPLC-ECD, which uses porous graphite electrodes. This type of probe can

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be used to measure reductive agents for extended periods before any electrode maintenance is required [21]. A second objective was to use pharmacokinetic samples to compare LC–MS/MS systems, which are widely used to assay AS and DHA in animal and human plasma.

2. Experimental

2.1. Chemicals

Artesunic acid (4-(10′ dihydro-artemisinin-oxymethyl) succinate) was manufactured by Knoll AG (Switzerland) and rebottled by BASF Pharmaceuticals. The drug was contained in sterilized bottles with 110 mg of AS per bottle. The buffer for the AS was manufactured as the phosphate salt with 0.3 M PBS at a pH of 8.1 by Stanford Research Institute (SRI, Menlo Park, CA). Injections were administered within 1 h of reconstitution of AS. Dihydroartemisinin (DHA) was obtained from the Walter Reed Army Institute of Research (WRAIR) and was used as an external standard for HPLC assay. Artemisinin was used for internal calibration in the quality control process for the HPLC assay. Saline (0.9%) was purchased from Abbott Labs (Chicago, IL). Pentobarbital, heparin, D-glucose, and methanol were purchased from Sigma Chemical Co.

2.2. Animals

Twelve adult male beagle dogs 0.9–1.1 years old and weighing 7.3–14.4 kg were obtained from Harland Sprague-Dawley (Alder Ridge Farms, Lakewood, PA, USA). Prior to initiation of the study, animals were acclimated to the laboratory environment for at least 2 weeks. During the 2-week pre-study period dogs received a complete physical examination by an attending veterinarian which included a complete blood chemistry, hematology, fecal, and urinalysis evaluation. Animals were housed in an aluminum run measuring 4 × 10 feet and fed a measured amount of commercial laboratory canine ration (Canine diet as provided by Vet Med, PMI Feeds, Inc., St. Louis, USA), and provided water ad libitum by lixit valve.

Eight-week old Sprague-Dawley (SD) male rats were purchased from Charles River Laboratories (Raleigh, NC, USA). All animals were individually housed and maintained in a stable environment at 21 °C with 50–60% humidity and 12 h day/night cycles. Standard rodent feed was provided during the day cycle from 8:00 am to 4.00 pm and deprived at all other times. Tap water was provided ad lib. All rats were randomly assigned to be euthanized for taking plasma samples.

2.3. BAS HPLC-ECD analysis

HPLC with reductive electrochemical detection was performed utilizing a model BAS 200B liquid chromatography system (Bioanalytical Systems, West Lafayette, IN). This system has three mobile phase reservoirs, solenoid proportioning valves, a dual piston pump, a pulse dampener, a column and detector oven, dual thin-layer electrodes with Ag/AgCl reference electrode, and a Rheodyne injector for manual injection that was modified for reductive work [14]. The system is also equipped for mobile phase heating (35 °C) and sparging. Stainless steel connectors and tubing were used throughout the system. For simultaneous determination of AS and its hydrolytic metabolite DHA (artemisinin as an internal standard), the Waters μBondapack, CN column (4.6 mm × 30 cm), and a mobile phase consisting of 30% acetonitrile: 70% 0.1 M acetic acid/NaOH buffer (pH 4) was used. Compounds were detected via Reductive Electrochemical Detection, as described previously [14]. Data was acquired and analyzed using a Waters model 820-chromatography data system, Empower program (Waters Associates, Milford, MA).

2.4. Agilent-ESA HPLC-ECD analysis

The analysis was performed using an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA) with an ESA Coulochem III multi-electrode detector (ESA, Chelmsford, MA). The Agilent HPLC was equipped with a quaternary pump and degasser, a thermostated autosampler and column compartment (35 °C), a multiple wavelength detector, and ChemStation software. The ESA Coulochem III electrochemical detector was provided with a variety of cells, which were consisted of a high-sensitivity analytical cell (Model 5011A) and guard cell (Model 5020) with an in-line filter containing a filter element. For simultaneous determination of AS and DHA (artemisinin as an internal standard), the Agilent Eclipse XDB-C18 column 5 μm (4.6 mm × 150 mm) was used with an isocratic mobile phase consisting of 40% acetonitrile: 60% ESA Acid Metabolite A (70-4835) in the reductive mode (−400 mV) for the quantification of AS and DHA.

2.5. LC–MS/MS assay and sample preparation

An LC–MS/MS method for the quantitation of AS and DHA in dog and human was validated from 2.0 to 500 ng/ml for AS and DHA. Dog and human plasma samples (20–100 μl) fortified with AS and DHA were analyzed to validate the method. The proteins in the dog plasma were precipitated with 100 μl of acetonitrile containing indomethacin as the internal standard. For extraction method, the human plasma samples (50–100 μl) fortified with AS and DHA were analyzed to validate the method. The analytes were extracted from human plasma with ethyl acetate. These extracts were dried and reconstituted in 50:50 (v/v) acetonitrile:water containing indomethacin as the internal standard. The supernatant was combined with 50 μl of water and analyzed on a Micromass Quattro II Mass Spectrometer in the positive ion electrospray ionization (+ESI) mode. DHA, AS, and indomethacin were monitored in the multiple reaction monitoring (MRM) modes. This method employed a Varian Pursuit C18 column (150 mm × 2.0 mm, 5-μm particle size) and a gradient elution with the following mobile phases: A: acetonitrile and B: 10 mM ammonium acetate in water for the chromatographic separation and the gradient was performed in 65% B for 4 min; to 95% B in 0.01 min, hold at 95% B for 2.99 min; to 65% B in 0.01 min, hold at 65% B for 2.99 min with flow rate of 0.2 ml/min.

2.6. Validation

The analytical method using HPLC-ECD for the determination of the AS and DHA in human plasma were validated in within-day precision and accuracy, day-to-day precision and accuracy, sensitivity evaluation recovery measurement, linear range, and stability test.

2.6.1. Calibration

Calibration curves were prepared in the drug concentration range of 0.5–1000 (n = 5, at each level) for AS and DHA from plasma. Serial dilutions (v/v) of plasma were made from same pool plasma that was obtained from blank control dog or humans. The initial concentrations of the AS and DHA (1000 ng/ml in HPLC-ECD method and 400 ng/ml in LC–MS/MS assay) were serially diluted down to a theoretical 0.5 ng/ml of drugs with internal standard (IS). The lower calibration curve extended from 0.5 to 7.8 ng/ml of concentration of AS and DHA with standard values at 0.5, 1.0, 2.0, 3.9, and 7.8 ng/ml, and the higher calibration curve extended

from 50 to 500 with standard values at 50, 100, 200, and 500 ng/ml. Least-squares linear regression using the individual samples and a weighting factor x^{-2} (reversed square of concentration) were employed to define the calibration curves using the ratios of the peak area of the analyte and IS in each calibration sample. Concentration in lower limit of detection (LLD) and lower limit of quantitation (LLQ) was calculated from the calibration curve of AS and DHA. The LLD of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The LLQ is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy.

2.6.2. Precision and accuracy

Precision is a standard analytical parameter that measures the reproducibility of data points from a single sample and analyzed in duplicate at least 10 times. To validate the HPLC-ECD and LC–MS/MS for AS and DHA concentrations, intra-day accuracy and precision (reproducibility) were evaluated by analysis at various percentage levels on the same day. The 1000 ng/ml AS and DHA stock solution was used to obtain validation (quality control; QC) samples in HPLC-ECD assay at 1000 (QC-high), 200 (QC-med), 25 (QC-low) and 5 ng/ml (QC-LLQ), and in LC–MS/MS analysis at 400 (QC-high), 100 (QC-med), 4 (QC-low) and 2 ng/ml (QC-LLQ). The concentrations of AS and DHA were calculated from the calibration curves, and the lower limits of detection and quantitation were given. To assess the inter-day accuracy and precision (reproducibility), the intra-day assay was repeated on 3 different days.

2.6.3. Recovery

The recovery (extraction efficiency) of AS and DHA was measured by comparing the analytical results of drugs extracted from rat, dog and human plasma samples at three concentrations equal to the Low QC (25 ng/ml for AS and DHA), Mid QC (100 ng/ml for AS and DHA), and High QC (400–500 ng/ml for AS and DHA) concentrations with the results from post-spiked (un-extracted) matrix standards. The results from the un-extracted samples represented 100%. The IS was tested identically at the concentration used in the assay in all plasma samples.

2.7. Sample evaluation with HPLC-ECD and LC–MS/MS

Blood samples were collected from six beagle dogs treated intravenously at AS 20 mg/kg from a safety pharmacology and tolerant study. Simultaneous determinations of AS and its metabolite DHA was be performed in both Agilent-ESA HPLC-ECD and LC–MS/MS systems. To facilitate the multiple blood draws, a sampling intravenous catheter was placed in the anterior cephalic vein of the dogs on the first (day 0) and last (day 2) treatment days. The blood samples (2 ml each) was collected from the intravenous catheter of each dog prior to dosing (0 h), and at 5, 15, 30, 45 min, 1, 1.5, 2, 4, 6, 24, 48, 48.08, 48.25, 48.5, 48.75, 49, 49.5, 50, 52, and 54 h post-dosing (measured from the end of the injection) after the first and last treatments. Commercial vacutainer sample tubes containing 1% heparin as an anticoagulant were used for blood drawing. The samples were placed immediately on wet ice for up to 30 min after which they were spun down using a refrigerated centrifuge and the plasmas removed. The plasma samples were then frozen at a −80 °C until the assay was performed.

3. Results and discussion

The HPLC-ECD and LC–MS/MS methods for the simultaneous estimation of AS and DHA in animal and human plasma have been optimized and validated in the present studies. Also, the methods of liquid–liquid extraction and protein precipitation have been evaluated for extracting AS, DHA and internal standards from plasma samples.

3.1. Optimization of HPLC-ECD

The sensitivity of detection from calibration curves of AS and DHA by using a new Agilent-ESA HPLC-ECD are shown in Fig. 1. The 4.28 ng/ml LLQ for AS and 2.31 ng/ml LLQ for DHA were two-fold higher than the values obtained using (9.51 and 4.62 ng/ml, respectively) the BAS system (Table 1). The main reason for the loss of sensitivity by the BAS HPLC-ECD relates to the stainless steel mobile phase heater inside the reservoir, which could increase oxides and raise the background noise. When the heater was removed from the system, the sensitivity was easily increased. However, it was still very hard to maintain due to the unstable temperature of the mobile phase.

HPLC-ECD system consisting of an Agilent HPLC coupled to an ESA electrochemical detector, which uses porous graphite electrodes, was also evaluated in this report. This type of probe can be used to measure reductive agents for extended periods before any electrode maintenance is required [21]. To minimize the background noise arising from the reduction of oxides from mobile phase, the handling of samples, tubing and column was performed as per instructions provided by the ESA. Additional steps were necessary to minimize the nano-amperes response required for reductive electrochemical reaction. This included the following:

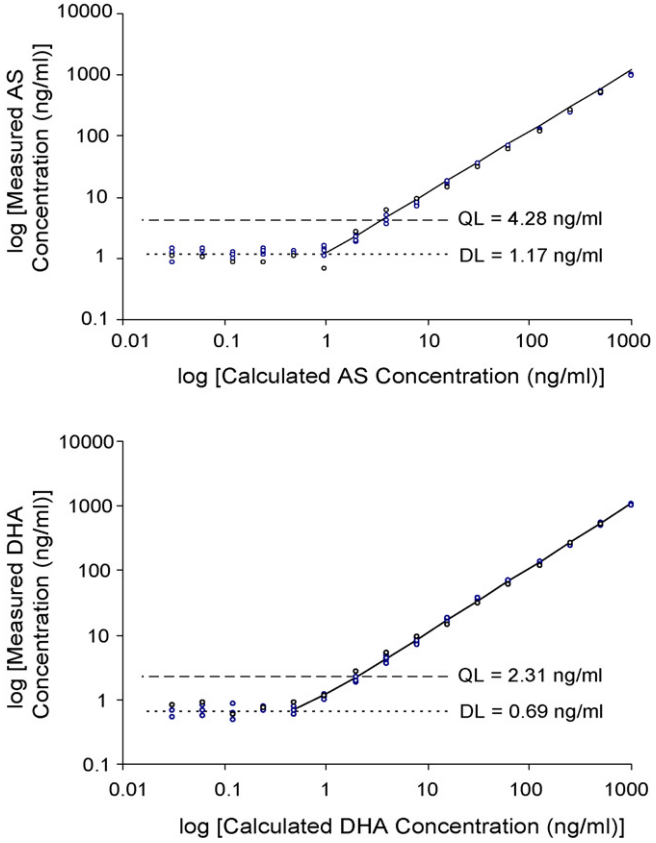

Fig. 1. Sensitivity of detection from calibration curves of artesunate (AS, top) and dihydroartemisinin (DHA, bottom) measured by Agilent-ESA HPLC with reductive electrochemical detector (ECD) from five separate assay. The mean lower limit of detection (LLD) and lower limit of quantitation (LLQ) was determined from the background using the FDA recommended calculation method (25) with the decimal logarithm of the mean of duplicate samples for each dilution tested ($n = 5$).

(1) In-line filters of 0.22 μm were used to prevent clogging the electrode and impede the flow of the mobile phase;
(2) Kept environment temperature stable when running samples;
(3) Protected from contamination by microorganism in mobile phase and samples by cleaning glassware with methanol prior to mobile phase preparation;
(4) Mobile phase was prepared freshly every day;
(5) Protected metals from oxidizing by using solvents of the highest purity commercially available;
(6) Used air filters to prevent particulate contamination from helium tanks.

The electronic reaction was also stabilized following ESA's recommendations. The objective was to achieve a more stable baseline in the chromatography which would significantly increase the sensitivity. It is important to recognize that the background noise increases as the applied potential is increased. This increase may reduce the signal-to-noise ratio and hence the sensitivity of the assay. Measures taken for electronic stabilization included the following:

(1) Using of a regulated power supply;
(2) Maintaining constant pH condition;
(3) Ascertaining the oxidation potential for each of the components of the mobile phase (cyclic voltammetry or hydrodynamic voltammetry);
(4) Removing dissolved oxygen by sparging or degassing techniques;
(5) Locating a guard cell between the pumps and the injector as to lower the concentration of possible impurities in the mobile phase or mobile phase modifiers;
(6) Monitoring metallic components of the system for corrosion which may contribute to higher noise and loss of signal.

Several parameters of artemisinin assay were optimized in this project in order to minimize the background noise. It is a common knowledge that the electrochemistry involves the use of an applied potential to effect a chemical reaction; the potential is characteristic of the compound of interest and its environment. In our study, the current that is measured in an electrochemical reaction is proportional to the concentration of artemisinins being oxidized. In an electrochemical detector, the eluent passes through a flow cell that provides the appropriate potentials and monitors the current. Typically, this potential is used for analysis, as the signal will be maximized. It should be noted; however, that limit of detection for an assay, which has the ability to detect the lowest possible concentration, is a function of both the signal and the noise. With coulometric electrodes the signal may already be maximized. In this case the characteristics that produce noise should be minimized so that the maximum sensitivity can be obtained.

3.2. Optimization of LC–MS/MS

The LC–MS/MS is a highly sensitive system, which has slowly replaced ECD-based assays for the quantitative determination of AS and DHA in animal and human plasma in our laboratory during the past 3 years. In this work, protein precipitation of the dog plasma samples using pure acetonitrile was done prior to LC–MS/MS analysis. Although the recovery efficiencies of AS and DHA were high (81.6–118.7%), the sensitivities were low; with 10.4 and 4.9 ng/ml LLQ for AS and DHA, respectively. In the human clinical trials a method involving liquid–liquid extraction was performed for extracting AS and DHA. The extraction efficiencies of AS and DHA were rather low; 56.5–81.3% for AS and 76.0–121.6% for DHA. However, the sensitivities were much higher than those obtained by the protein-precipitation method. The LLQ values with the liquid extraction method from human plasma samples were 3.4–4.3 ng/ml for AS and 1.7–2.6 ng/ml for DHA (Table 1), which were 2–3 folds higher than for the dog study.

The quantitative method for AS and DHA in human samples was designed to reach the lowest limit of quantification possible. Currently, the best LLD and LLQ of LC–MS/MS methods are ranged from 1 to 5 ng/ml for both of AS and DHA [22,23]. In the present report, the better LLQs were achieved by liquid–liquid extraction

Table 1
Mean value of validation parameters for artesunate (AS) and dihydroartemisinin (DHA) in animal and human plasma-based calibration by BAS HPLC-ECD, Agilent-ESA HPLC-ECD, and LC–MS/MS analyses

Validation parameters	BAS HPLC-ECD		Agilent-ESA HPLC-ECD		LC–MS/MS		LC–MS/MS	
	Rat/dog plasma (ng/ml)		Dog plasma (ng/ml)		Dog plasma (ng/ml)		Human plasma (ng/ml)	
	AS 2–1000	DHA 2–1000	AS 2–1000	DHA 2–1000	AS 10–500	DHA 5–500	AS 4–400	DHA 2–200
Plasma sample volume (μl)	500–1000		500–1000		20–50	20–50	50–100	50–100
Sample preparation	Extracted in1:9 EE/BC		Extracted in 1:9 EE/BC		Precipitated in acetonitrile		Extracted in ethyl acetate	
Absolute recovery (%)	83.2–87.5		82.8–86.8		101.7–118.7	81.6–85.6	56.5–81.3	76.0–121.6
Linear range (5–1000 or 2–400 ng/ml)								
Y-Intercept	0.04–0.19	0.04–0.26	0.08–0.82	0.12–0.86	0.01–0.98	0.08–0.84	0.14–0.98	0.07–0.89
Slope	1.2–26.9	0.8–3.41	1.51–2.47	0.52–4.05	0.17–6.20	0.64–3.04	0.22–3.24	0.02–1.62
Regression coefficient (r^2)	>0.992	>0.985	>0.998	>0.989	>0.987	>0.982	>0.998	> 0.996
Assay performance (n = 8)								
Intra-day precision (%)	5.5 to 7.0	10.2 to 7.4	3.9 to 7.4	4.7 to 7.8	5.7 to12.7	6.4 to 15.1	3.1 to 12.9	3.2 to 10.6
Intra-accuracy (%CV)	−3.3 to 6.2	−8.2 to 2.1	11.4 to 8.1	−3.1 to −2.6	0.0 to 6.0	−13.3 to −6.0	−5.3 to 19.3	−8.1 to 4.0
Inter-day precision (%)	8.3 to 6.2	7.5 to 10.1	5.6 to 6.4	6.6 to 7.5	10.6 to 13.2	9.6 to 11.8	9.5 to 14.1	6.4 to 12.1
Inter-accuracy (%CV)	10.7 to 5.2	3.5 to 4.7	1.4 to −9.2	2.5 to −6.8	−6.3 to 4.0	−12.4 to −1.2	−2.5 to 9.7	−4.0 to 0.9
Calibration (n = 6–10)								
LLD (ng/ml)	3.04	1.36	1.17	0.69	NA		NA	
LLQ (ng/ml)	9.51	4.62	4.28	2.31	10.4–10.6	4.9–5.1	3.4–4.3	1.7–2.6
Carryover test (% of LLQ)	6.5	9.1	5.6	7.2	15.4	16.8	18.6	19.0
Re-injection reproducibility (%)	2.2	4.3	1.6	3.9	2.9	5.7	1.7	5.9
Low QC (4–31 ng/ml)								
High QC (50–250 ng/ml)								

LLD = lower limit of detection; LLQ = lower limit of quantitation; QC = quality control; 1:9 EE/BC = 1:9 ethyl acetate/*n*-butyl chloride.

Table 2
Mean pharmacokinetics (PK) parameters comparison of artesunate (AS) and its active metabolite, dihydroartemisinin (DHA) in beagle dogs following a daily intravenous dosed of AS at 20 mg/kg/day for 3 days (n = 6 dogs)

	Artesunate (AS)			Dihydroartemisinin (DHA)		
	HPLC-ECD[a]	LC–MS/MS[a]	t-Test[a] P=	HPLC-ECD[a]	LC–MS/MS[a]	t-Test[a] P=
Day 1						
C_{max} (ng/ml)	32148 ± 14385	40605 ± 4228	0.1010	5008 ± 676	4528 ± 1173	0.2480
T_{max} (h)	0.08	0.08		0.14 ± 0.09	0.17 ± 0.08	0.1816
$AUC_{inf.}$ (ng h/ml)	6161 ± 2825	6736 ± 1816	0.2177	3548 ± 663	3262 ± 701	0.2871
$t_{1/2}$ distribution (h)	0.04 ± 0.01	0.03 ± 0.01	0.1682	0.12 ± 0.02	0.05 ± 0.03	0.0462
$t_{1/2}$ elimination (h)	0.30 ± 0.08	0.30 ± 0.09	0.4164	1.08 ± 0.43	0.75 ± 0.12	0.0156
Vss (ml/kg)	459.9 ± 149.1	398.4 ± 115.2	0.0414			
CL (ml/min/kg)	52.8 ± 13.3	46.2 ± 9.9	0.2332			
MRT (h)	0.17 ± 0.07	0.12 ± 0.02	0.0733	0.72 ± 0.16	0.69 ± 0.14	0.0579
Day 3						
C_{max} (ng/ml)	46042 ± 20071	53101 ± 12987	0.3048	6371 ± 2228	6172 ± 2025	0.4415
T_{max} (h)	0.08	0.08		0.13 ± 0.07	0.17 ± 0.08	0.1816
$AUC_{inf.}$ (ng h/ml)	8494 ± 2525	9231 ± 3713	0.1623	4487 ± 1569	3854 ± 971	0.0907
$t_{1/2}$ distribution (h)	0.12 ± 0.05	0.07 ± 0.04	0.0751	0.09 ± 0.03	0.07 ± 0.05	0.2162
$t_{1/2}$ elimination (h)	0.33 ± 0.06	0.34 ± 0.08	0.4628	0.77 ± 0.27	0.67 ± 0.10	0.1987
Vss (ml/kg)	378.6 ± 126.8	374.3 ± 276.3	0.4888			
CL (ml/min/kg)	62.6 ± 44.3	53.6 ± 14.7	0.1176			
MRT (h)	0.15 ± 0.03	0.17 ± 0.06	0.3145	0.75 ± 0.34	0.69 ± 0.13	0.2183
Total $AUC_{1\text{-}3D}$ (ng h/ml)	21775 ± 6154	26197 ± 3062	0.0760	12772 ± 3881	11055 ± 2585	0.0953
AUC ratio of D3/D1	1.70 ± 0.93	1.56 ± 0.73	0.4211	1.26 ± 0.31	1.12 ± 0.16	0.1012
C_{max} ratio of D3/D1	1.98 ± 1.56	1.64 ± 1.02	0.2780	1.29 ± 0.24	1.23 ± 0.36	0.3418

MRT = mean residence time; D = days.
[a] PK parameters.

with ethyl acetate, which in combination with mass spectrometry and selected reaction monitoring gave sufficient selectivity and sensitivity. A less selective sample preparation method, such as protein precipitation, would not provide sufficient sensitivity [24]. The complexity of plasma proteomes is a challenge to achieving high-sensitivity quantification of AS and DHA in plasma. In the absolute quantification of AS and DHA, the protein is precipitated to have no major interference or high noise level from plasma, which eliminates the noise from the high-abundance proteins. However, to detect low-concentration samples, the noise from low-level proteins now becomes significant. Therefore, the strategy to improve the signal-to-noise ratio (S/N) should be through the use of a nonspecific cleanup which covers low-level proteins as well.

In this study, the liquid–liquid extraction method removed the matrix components from the plasma. Therefore, the complexity of the plasma protein was reduced, and this minimized the burden on the LC separation and MS/MS selection to provide an improvement in the S/N ratio.

3.3. Main validations of two methods

After optimization of the HPLC-ECD assay, we were able to improve its sensitivity and specificity while minimizing the number of times the electrode needed to be cleaned. This provided the relatively constant oxygen-free condition required in the reductive mode and facilitated the simultaneous determination of AS and DHA in plasma. Similar improvements were observed with the LC–MS/MS method due to a reduced S/N ratio after liquid–liquid extraction. The improved HPLC-ECD and LC–MS/MS conditions were validated and compared using a batch of 34 runs. The batch was composed of duplicate runs for each QC sample at high, medium and low concentration, 10 runs for calibration curve, and eight runs for assay performances including blank samples (Table 1). The intra- and inter-day precision for plasma for all levels were within the acceptable range required for validation of the assay.

There was good reproducibility (1.6–5.9%) and a low quantitation limit of 4 ng/ml for AS and 2 ng/ml for DHA was achieved. The inter- and intra-day coefficient of variation for accuracy and precision was within ±13%. The inter-day coefficients of variation (precision) for AS and DHA samples measured by both methods were ranged from 3.1 to 10.2%. The CV% accuracy for AS and DHA analyzed by HPLC-ECD and LC–MS/MS were in a range of −13.3–11.4%. The data obtained for AS and DHA obtained by both methods were well within the acceptable limits to meet guidelines for bioanalytical methods validation [25].

Acceptable LLQ (defined as the lowest concentration on the standard curve that can be quantitated with accuracy within ±15% of nominal and precision not exceeding 15% CV), were achieved in the present studies. Calibration curves were constructed by plotting the peak areas (AS or DHA) of plasma standards versus nominal concentration. Calibration curves for AS or DHA were linear using weighted (1/concentration) linear regression in the concentration range of 0.2–1000 ng/ml on five sample sets with a mean correlation coefficient (r^2) greater than or equal to 0.981 for all curves. Different concentrations of AS and DHA were acquired to establish the limits of sensitivity for the two techniques. When using the HPLC-ECD approach, the standard curves were found to be linear and the LLDs for AS and DHA were 1.17–3.04ng/ml and 0.69–1.36 ng/ml, respectively (Table 1, and Fig. 1). The LLQs in HPLC-ECD were calculated as 4.28–9.51 ng/ml for AS and 2.31–4.62 ng/ml for DHA, which were measured as the concentration of AS or DHA to be detected about 3-fold over the LLD in accordance with FDA Guidance (25). When using the LC–MS/MS approach, the LLQs were 10.4–10.6 ng/ml for AS and 4.9–5.1 ng/ml for DHA as determined from a dog study involving the protein-precipitation technique. In comparison, LLQs of 3.4–4.3 ng/ml for AS and 1.7–2.6 ng/ml for DHA were observed in conjunction with a human clinical trials in which liquid–liquid extraction was used.

The LC–MS/MS assay showed improved sensitivity, and the results from the validation of in vitro study samples were in agreement with the data obtained by HPLC-ECD method (Table 1). Although the LC–MS/MS and HPLC-ECD systems reflect similar val-

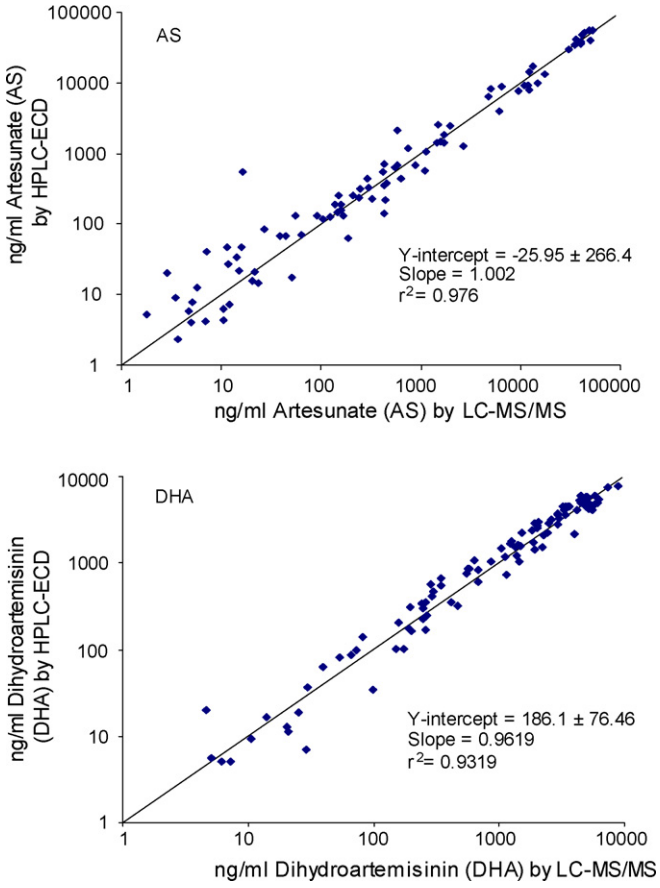

Fig. 2. Correlations (r^2 = 0.932–0.976) are shown between concentrations of artesunate (AS, top) and dihydroartemisinin (DHA, bottom) in dog plasma by using HPLC-ECD and LC–MS/MS methods. Samples (markers) were taken from beagle dogs treated with a daily intravenous dose of AS at 20 mg/kg for 3 days in a tolerant study (n = 6).

idation parameters analysis using the LC–MS/MS assay needs only 50–100 μl volume of plasma while the HPLC-ECD assay requires 500–1000 μl, which is 10-fold higher. The results from this study indicate that the LC–MS/MS method is a more feasible approach for the analysis of AS and DHA to support *in vivo* studies during drug discovery and development.

3.4. Applicability of the analytical methods

To validate the two methods, a steady-state pharmacokinetic parameters and tolerant studies of AS dissolved in 0.3 M PBS were evaluated in 12 male beagle dogs following a daily intravenous administrations of the drug at 20 mg/kg for 3 consecutive days. A series blood sampling during 72 h was performed, and drug concentrations were analyzed by Agilent-ESA HPLC-ECD and LC–MS/MS with positive electrospray ionization in the MRM mode. The summary of the main pharmacokinetic parameters of the AS and DHA by two methods is shown in Table 2. The two data sets are very similar between the two methods without significant difference. Regression analysis was performed to determine the correspondence between methods. The resulting r^2 values of 1.000 indicated a high degree of linear correlation between these two methodologies. Concentration results for AS and DHA obtained from both methods are exhibited in Fig. 2. The measurements of 324 per drug and per each method were done on samples taken from 3 individual days, and showed a direct correlation (r^2 = 0.932–0.976) between HPLC-ECD and LC–MS/MS determinations for both AS and DHA (Fig. 2).

3.5. Conclusion

This paper describes the method optimizations and major validation of AS and DHA by using HPLC-ECD and LC/MS/MS. Based on the validation, it was possible to successfully apply the assays and assess the AS and DHA in animal and human studies. Both methods used in the present study are reliable, easy and fast to perform. They are also characterized with adequate accuracy, precision, selectivity and sensitivity. The HPLC-ECD performed well in terms of various validation parameters, and showed a good agreement with the LC–MS/MS when calibrated in plasma. However, the major benefit of LC–MS/MS is that it requires only one-tenth of plasma volume needed by HPLC-ECD assay. The challenge of future work will be to improve the assay sensitivity to meet the assay requirements for AS and DHA associated with low-dose clinical studies.

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