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RESEARCH ARTICLE

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Validation of a Liquid Chromatography/Tandem Mass Spectrometry Assay for the Quantification of Plasma Dihydroartemisinin

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Background: Insufficient plasma level of dihydroartemisinin (DHA) can select resistance and will further hinder malaria elimination program. We investigated clinical applicability of a validated liquid chromatography/tandem mass spectrometry (LC-MS/MS) assay to quantify plasma concentration of DHA in healthy subjects from a single oral administration of fixed dose combination of Dihydroartemisinin-Piperaquine.

Materials and Methods: Micro-elution solid-phase extraction in a 96-well plate format was used to prepare the samples. DHA separation happened in Acquity UPLC™ BEH C18 column (50 × 2.1 mm, 1.7 µm). Mobile phase was a mixture of acetonitrile-ammonium acetate 10 mM pH 3.5 (50:50, v/v) at 0.3 mL/minute flow rate. Waters Acquity UPLC™ H-Class system coupled with triple quadruple mass spectrometry in positive electrospray ionization mode was used for detection. The internal standard was a stable isotope labelled DHA.

Results: Calibration curve was linear with a correlation coefficient >0.995 over a concentration range of 1–1,000 ng/mL. Bias and variation for accuracy and precision were in the range of 15% (20% at the lower limit of quantification). Using 5 µL sample, lower limit of quantification was 1 ng. Matrix effect was less than 15%. The method was successfully applied to investigate the pharmacokinetics of DHA from five healthy subjects, although carry over and the role of anticoagulants were not tested.

Conclusion: The LC-MS/MS assay for the quantification of plasma DHA was validated for selectivity, linearity, lower limit of quantitation, accuracy, precision, matrix effect and stability. Although clinical applicability was demonstrated, this method was to be improved to address the not-tested validation parameters.

Keywords*: dihydroartemisinin, liquid chromatography/tandem mass spectrometry assay (LC-MS/MS), malaria, Indonesia*

Introduction

Artemisinin-based Combination Therapy (ACT) is a WHO-recommended treatment for uncomplicated malaria. Dihydroartemisinin (DHA) together with piperaquine have been used as the first line ACT in Indonesia since 2008.[1,2] Dihydroartemisinin is a reduced lactol derivative of artemisinin with a common pharmacopore of 1,2,4-trioxane.

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It is the main metabolite of artesunate and artemether, as well as an antimalarial compound on its own, which exhibits tautomerism of α and β epimerization in solution and is chemically unstable in ambient temperature.[3] DHA acts like amorphous powder with melting point of 164°C, and with pKa 12.6, DHA is neutral at physiological pH and has moderate lipophilicity with four values of water solubility (8.4; 50; 62; 168 mg/L) and two values of octanol-water partition coefficient (Log P) of 2.35 and 2.9.[4]

As artemisinin derivative, analytical methods to quantify plasma DHA have been studied since the earliest roll out of ACTs and some pitfalls have been identified.[5-13] High performance liquid chromatography (HPLC) with ultraviolet (UV) detection was proven resilient for most drugs, however it was difficult to be applied to DHA since the compound lack of chromophore moieties. Several methods to yield UV-absorbing products were able to increase selectivity and sensitivity, but with a set of drawbacks which requires rigorously controlled anaerobic conditions and deoxygenation of the samples and mobile phases. The use of tandem mass spectrometry greatly improves selectivity and sensitivity.

The emergence of resistant *Plasmodium falciparum* to artemisinin in five neighboring countries, *i.e.*, Cambodia, Thailand, Myanmar, Vietnam, and Lao PDR has generated concerns to the National Malaria Control Program (NMCP) as it would hamper malaria elimination program.[14,15] Pharmacokinetic (PK) study or in essence, a drug-level study, is particularly of importance in order to distinguish treatment failures due to true resistance or inadequate drug concentrations. However, this kind of study is not commonly done in Indonesia due to limited laboratory capability. In line with the artemisinin resistance monitoring program, this study was conducted with the goal of building laboratory capacity at the National Institute of Health Research and Development (NIHRD), Ministry of Health of Indonesia. We investigated clinical applicability of a validated liquid chromatography/tandem mass spectrometry (LC-MS/MS) assay to quantify plasma concentration of DHA in healthy subjects from a single oral administration of fixed dose combination of Dihydroartemisinin-Piperaquine.

Materials and methods

This study was ethically approved by the Ethics Committee of Faculty of Medicine, University of Indonesia (No. 360/UN2.F1/ETIK/2016). The selected reference assay was validated for the determination of DHA and artemether in human plasma and published in previous article.[16]

Chemicals and Reagents

DHA and stable isotope-labeled (SIL) internal standard (SIL-DHA) was acquired from the WorldWide Antimalarial Resistance Network (WWARN) for their reference material program. The structures as shown on the certificate of analysis are in Figure 1. Acetonitrile (HPLC-grade), methanol (pro analysis) and HPLC-water were obtained from Merck Chemicals and Life Sciences (Darmstadt, Germany). Ammonium acetate (LC-MS grade) was obtained from Sigma-Aldrich (Darmstadt, Germany). Ammonium acetate in HPLC-water was mixed to obtained ammonium acetate buffer solutions and the pH was adjusted with acetic acid from Merck Chemicals and Life Sciences (Darmstadt, Germany). Blank human plasma (common plasma with no DHA) was obtained from the Indonesian Red Cross (Jakarta, Indonesia) and citrate phosphate dextrose with adenine (CPDA-1) solution was used as anticoagulant.

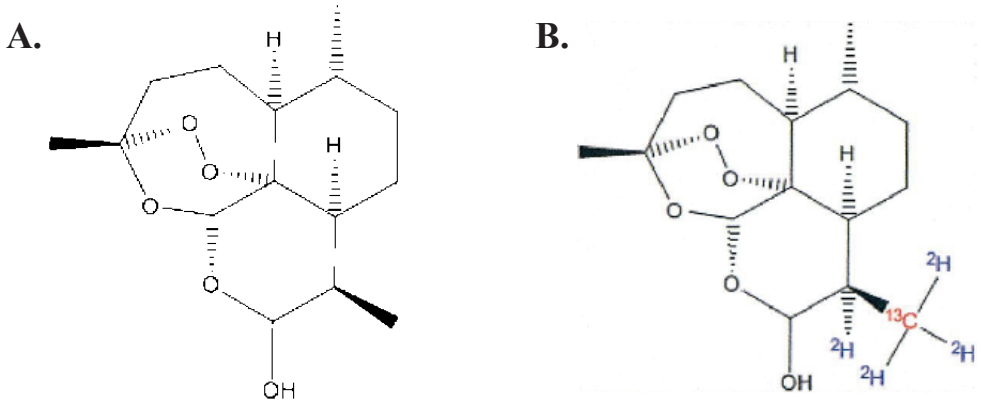

Figure 1. Structures of DHA (A) and SIL-DHA (B).

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Instrumentations

Waters Acquity UPLC™ H-Class system coupled with triple quadruple (TQD) mass spectrometry (Waters Corp., Milford, USA) was used for the validation experiments. The LC system consisted of a quaternary (binary pump with two channels in each pump) LC pump with vacuum degasser, an autosampler with flow-through needle, a column with active pre-heater properties. MassLynx™ Mass Spectrometry Software was used for data acquisition and quantification. The stationary phase was Waters Acquity UPLC™ BEH C18 (50 × 2.1 mm, 1.7 µm, Waters Corp., Milford, USA) column. The mobile phase was consistent with the reference assay, which is a mixture of acetonitrile and ammonium acetate 10 mM pH 3.5 (50:50, v/v). It was set at a flow rate of 0.3 mL/minute in isocratic condition.

The MS conditions were optimized by directly injecting 1000 ng/mL standard solution of DHA mixed with 100 ng/mL SIL-DHA solution to the MS. Argon gas was set to 7 psi and nitrogen gas at 90 – 100 psi. Electrospray ionization (ESI) was set in positive ionization mode. The MS console was set on autotune. Multiple reaction monitoring (MRM) was used for the quantification of DHA and SIL-DHA at transitions of m/z 302 to 163 and m/z 307 to 272, respectively with dwell time 0.2 second. Electrospray ionization (ESI) was set in positive ionization mode. The cone voltage was 12 V for DHA and 14 V for SIL-DHA. The collision energy was 20 eV for DHA and 12 eV for SIL-DHA.

Stock and Working Solutions, Calibration Standards and Quality Control Samples

Stock solutions for DHA and SIL-DHA were prepared at concentration of 1,000 µg/mL in ethanol. Working solution of SIL-DHA was prepared by dilution of stock solution to achieve concentration of 100 µg/mL in ethanol-water (50-50, v/v). Working solutions of DHA ranging from 100 ng/mL to 100 µg/mL were also prepared by serial dilution of stock solution in ethanol-water (50-50, v/v). These solutions were kept on ice during use. On the day of assay, a fix concentration of SIL-DHA at 100 ng/mL was obtained by diluting working solution of SIL-DHA with plasma-water (50-50, v/v containing sodium fluoride/potassium oxalate 2/3 mg/mL).

A ten-point calibration curve was prepared for DHA by spiking blank plasma to yield calibration concentration of 1, 3, 5, 10, 25, 50, 100, 250, 500, and 1,000 ng/mL, in addition to the blank and zero plasma. Quality control (QC) samples for DHA (low, medium, high) were prepared similarly for concentrations of 5, 402 and 800 ng/mL. The QC low (QCL) concentration was 5 times the LLOQ, QC high (QCH) concentration was 80% of the highest concentration of the standard solution. The QC medium (QCM) concentration was obtained by adding QC low and high concentrations divided by two. These solutions were kept at the minimum temperature of 4°C until day of the assay.

Sample Preparation

Plasma samples were prepared according to the reference assay using solid-phase extraction (SPE) method in µ-elution Oasis® HLB 96-wellplate (Waters, USA) with slight modification to the plasma volume. One hundred and fifty microliters of internal standard solution (100 ng/mL SIL-DHA in plasma-water (50:50, v/v) containing sodium fluoride/potassium oxalate 2/3 mg/mL) was added to 100 µL plasma in polypropylene tube on ice. Acetonitrile (750 µL), followed by methanol (750 µL) and water (200 µL) were loaded to activate and conditioned the SPE wells. The samples (250 µL each) were pipetted onto the activated wells and drawn through with low pressure vacuum. Then, water (300 µL) was loaded to wash the wells, drawn through with medium pressure vacuum.

The pressure was increased briefly to full vacuum to make sure all liquids were drawn through. The SPE column tips were dried with tissue paper before inserting a 96-collection plate (0.5 mL) into the vacuum manifold. Methanol-acetonitrile (100 µL, 90:10 v/v) followed by water (100 µL) were used to elute the wells with low pressure vacuum. The eluates were briefly mixed before refrigeration at 4°C for 15 hours. As stated on the reference assay, this step was needed to enable α/β-DHA epimer equilibration. Five µL of eluates was injected into the LC-MS/MS system.

Assay Validations

Partial validation experiments were performed according to the European Medicines Agency (EMA) guideline.[17] The method was tested against several validation parameters, which include linearity, lower limit of quantitation (LLOQ), selectivity, accuracy, precision, matrix effects, and stability. Linearity was evaluated by preparing calibration standards of the previously mentioned concentrations in four replicates. Peak area ratios of the analyte to the IS versus the nominal concentrations in quadruplet were plotted to obtained calibration standards over the range of 1-1,000 ng/mL concentrations. The calibration curve with $1/x^2$ weighed

linear regression was chosen for quantification, since it was expected that the residual errors will not be randomly distributed. It is generally acceptable when the correlation coefficient (r) of the calibration curves was greater than 0.99 and the bias of the calculated concentrations was within ±15% of the nominal concentrations, except at the LLOQ concentration of which the deviation can be within ±20%.

LLOQ was established by analyzing six blank plasma samples, first by spiking it with the LLOQ concentration of the reference assay and analyzing the response. The analyte response should be reliably precise and accurate (acceptable if each criterion is less than 20%). When the analyte response was within acceptable range, the concentration was lowered by half and the response was analyzed once again.

Selectivity was evaluated by analyzing six blank plasma samples in comparison to spiked plasma at LLOQ concentration. The response of endogenous or other components in the plasma should be less than 20% of the LLOQ for the analyte and 5% for the IS.

Intra-assay accuracy and precision experiments were determined by repeating the analysis of each QC standard five times. This was performed at least in three different batches (inter-assay) and the deviation within ±15% of the true value was acceptable, except at the lower LLOQ concentration of which the deviation within ±20% was acceptable.

Matrix effect of analyte and IS was evaluated in six lots of blank plasma from different donors, extracted and spiked at QCL, QCM and QCH concentrations, in addition to pure standard solutions in equivalent concentrations. Peak area in post-extraction samples spiked with analyte or IS were compared with peak areas of standard solutions in equivalent concentrations to obtain matrix factor for each analyte and IS (matrix factor (MF) = peak response in presence of matrix ions / peak response in absence of matrix ions). IS-normalized MF were calculated by dividing the MF of analyte to the MF of IS. Variation of IS-normalized MF from six lots of plasma should not exceed 15%.

Stability was evaluated with blank matrix spiked with QCL and QCH concentrations in triplicates right after the preparation, at 6 hours and 12 hours after refrigeration in 4ºC. The mean concentration should be within ±15% of the nominal concentration.

Plasma DHA Sample Collection from Healthy Adults

Five healthy male subjects between 18 and 40 years of age with body mass index (BMI) range between 18.5 and 24.9 kg/m^2, normal vital signs, normal standard physical examination, normal values of routine hematology, blood glucose, liver and renal function tests, and normal electrocardiogram (ECG) readings were consented and recruited in this study. They did not have any history of having allergic/hypersensitivity reaction to either DHA or PIP, active psychiatric condition, or alcohol or drug dependence, and did not take any concomitant medications or under long-term treatment.

The study drug was a fixed-dose oral formulation (tablet) containing 40 mg DHA and 320 mg PIP phosphate obtained from the Indonesian NMCP. Drug dosage was individually adjusted by body weight (DHA 2-4 mg/kg body weight/day and PIP 16-32 mg/kg body weight/day) and rounded up to the nearest quarter. The drug dose was administered once with water under the supervision of study physician at least 3 hours after their last meal. The participants continued to fast up to 3 hours after the dose and monitored for adverse events. No specific diet restriction was applied to the participants.

Venous blood was collected just before drug administration (time 0) and at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 3, 4, 6, and 8 hours after drug administration in ethylenediaminetetraacetic acid (EDTA) tubes. Within 30 minutes after collection, the blood samples were centrifuged for 15 minutes at 3000 rpm. The resulting plasma were aliquoted, labelled and stored at -70ºC or lower prior to analysis.

The PK parameters were modelled using non-compartment analysis (NCA) using Stata® software version 14 (StataCorp, Texas, USA). Peak drug concentration (C_{max}) and the actual time when the peak concentration was measured (t_{max}) was observed directly from the plotted mean plasma drug concentrations versus time. Other parameters were calculated from the logarithmic plot of mean plasma concentrations versus time, such as the elimination rate constant and half-life ($t_{½}$), volume of distribution (V_d) and clearance (CL). The areas under the curve (AUC) from time 0 to t were calculated using log-linear trapezoidal rule.

Results

Sample Preparation

Several experiments (n=3) of sample preparation using 50 µL plasma as stated in the reference assay failed to produce sufficient peak when injected to the LC-MS/MS system.

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Doubling the volume of plasma to 100 µL was able to correct the peak and subsequently chosen to be used in this study.

Mass Spectra Analysis and Chromatographic Separation

The optimized chromatographic condition was achieved by autotune. Full scan mass spectra were acquired in positive ion mode. The cone voltage was 12 V for DHA and 14 V for SIL-DHA. The collision energy was 20 V for DHA and 12 V for SIL-DHA. In the direct infusion experiment, the mass spectra for DHA and SIL-DHA revealed peaks at mass to charge ratio (m/z) 302 and 307, respectively. The most stable abundant fragment ion observed product MS/MS spectrum was at m/z 163 for DHA and 272 for SIL-DHA. Figure 2 showed the collision-induced dissociation (CID) mass spectra for DHA (mass transition 302 →163 m/z) and SIL-DHA (mass transition 307 → 272 m/z).

The total ion chromatogram for standard solution of DHA is shown in Figure 3 using mobile phase of acetonitrile and ammonium acetate 10 mM pH 3.5 (50:50, v/v) at flow rate 0.3 mL/min. Since the separation of α- and β-DHA was not clearly defined, both peaks were used for the quantification of DHA. Run time was less than 10 minutes.

Assay Validations: Linearity, Lower Limit of Quantification and Selectivity

Linearity of the assay was observed on the calibration curve at the concentration range of 1-1,000 ng/mL with correlation coefficient (r2) >0.99. The mean accuracy and precision at each concentration of the standard in the calibration curve are shown in Table 1.

The LLOQ for DHA was 1 ng/mL with coefficient of variation (%CV) and bias (standard deviation /SD) of back calculated of each concentration were 4.83% and 0.05, respectively. All blank plasma sources produced signals that contributed lower than 20% of a standard at LLOQ and less than 5% for IS.

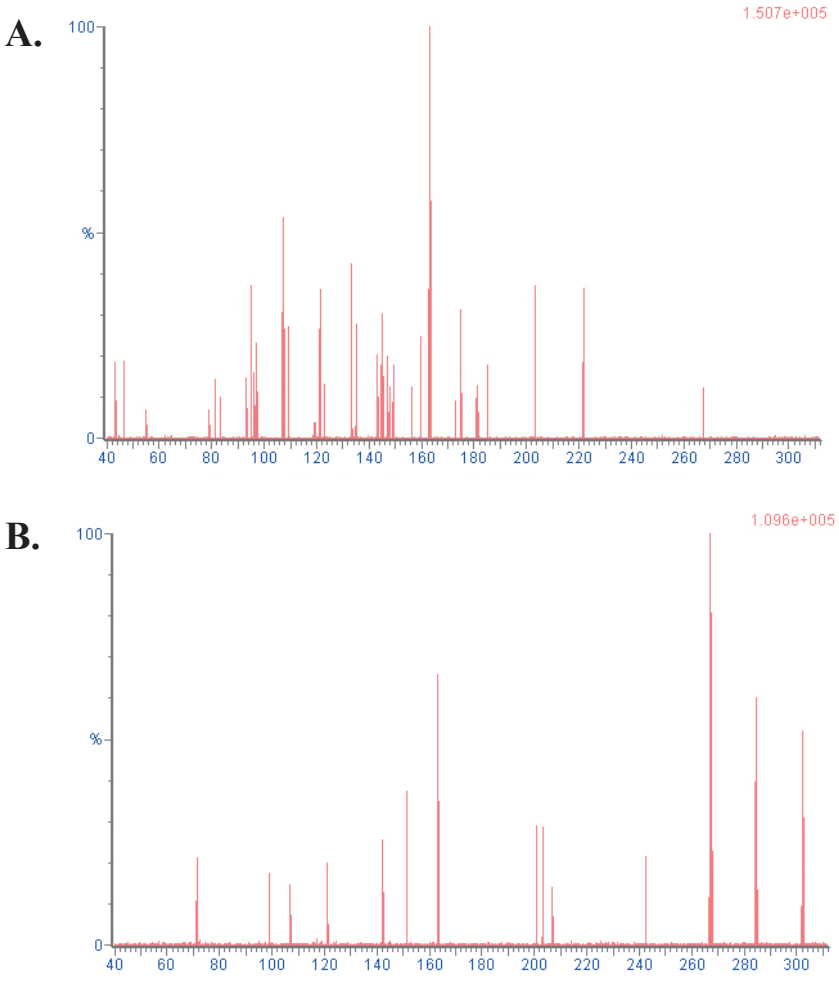

Figure 2. Product ion spectra (positive ESI, m/z 40-350) of DHA (302/163) (A) and SIL-DHA (307/272) (B).

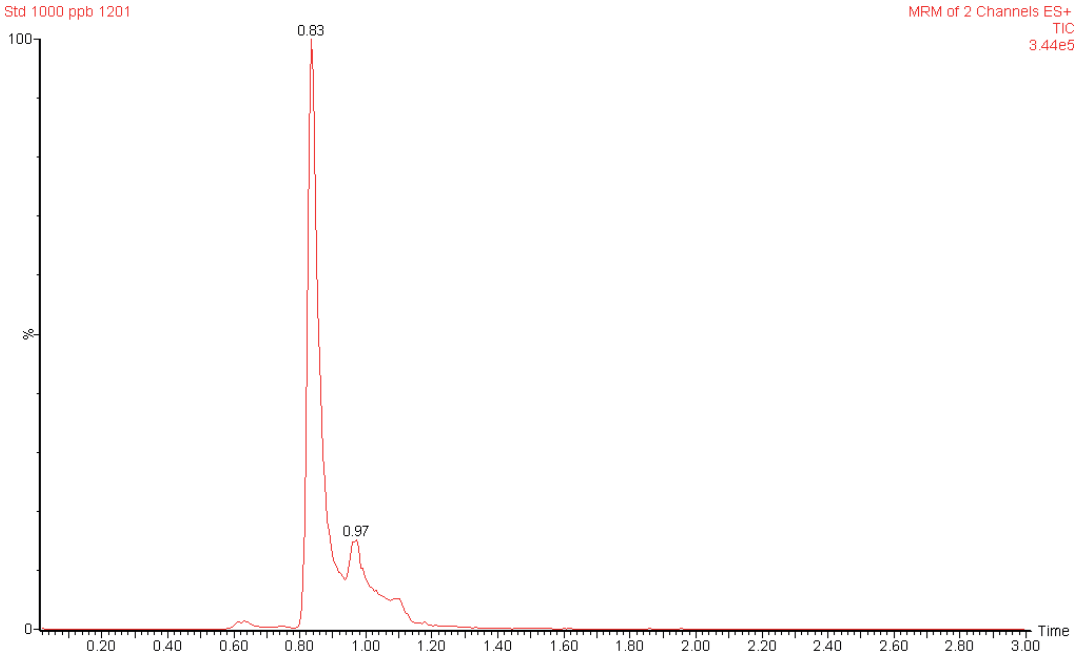

Figure 3. The total ion chromatogram of dihydroartemisinin from standard solution on the Waters Acquity UPLC™ BEH C18 column (50 × 2.1 mm, 1.7 µm) at flow rate 0.3 mL/min with mobile phase containing a mixture of acetonitrile and ammonium acetate 10 mM pH 3.5 (50:50, v/v).

Accuracy and Precision

The results of intra- and inter-day precision and accuracy of the assay method are summarized in Table 2. Intra- and inter-day accuracies were 99.99-102.85% and 96.45-107.47%, respectively. Intra- and inter-day precisions (relative standard deviation /RSD) were 2.79-4.16% and 1.46-3.04%, respectively. Results show that the accuracy and precision values were within the acceptable criteria.

Matrix Effects

The normalized matrix effect of DHA to IS were close to 1 with acceptable variations (below 15%), summarized in Table 3. These data showed that under the current conditions, the event of ion suppression or enhancement (matrix effect) from human plasma can be ignored.

Stability

Poor stability of DHA at ambient temperature has been demonstrated. According to the reference method, working on ice throughout the sample preparation is required. The mean recovery (n=5) after 24 hours refrigeration at 4ºC at low concentration was 4.83 ng/mL (3.44% CV) and 781.34 ng/mL at high concentration (2.19% CV). The stability test results of DHA in human plasma samples are summarized in Table 4. Results indicate adequate stability of DHA enduring sample preparation process and storage conditions.

Application of the Assay to Plasma Samples of DHA from Healthy Subjects

To describe clinical application of the assay, the pharmacokinetics of DHA following single oral

Table 1. Back-calculated concentrations of standard curve for DHA in human plasma.

DHA	Nominal Concentration (ng/mL)									
	1	3	5	10	25	50	100	250	500	1000
Average (n=4)	0.97	2.93	4.75	9.26	23.68	47.67	99.83	249.38	510.43	995.12
SD	0.03	0.21	0.12	0.14	1.16	0.8	1.1	8.92	3.25	24.49
CV (%)	3.27	7.19	2.49	1.46	4.88	1.67	1.11	3.58	0.64	2.46
Accuracy (%)	96.67	97.61	94.92	92.64	94.72	95.34	99.83	99.75	102.09	99.51

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Table 2. Intra- and inter-day accuracy and precision of DHA in human plasma.

DHA	Inter-day		Intra-day	
	Precision (%RSD)	Accuracy (%)	Precision (%RSD)	Accuracy (%)
LLOQ, 1 ng/mL	4.16	102.85	2.4	104.11
QCL, 5 ng/mL	3.67	101.43	3.04	96.45
QCM, 402 ng/mL	3.16	99.99	1.46	107.47
QCH, 800 ng/mL	2.79	100.53	2.66	99.45

administration of fixed-dose combination of 40 mg DHA and 320 mg PIP in 5 healthy male adults was characterized. The median age was 23 years (range 18–39) and median body mass index was 23.25 kg/m^2 (range 18.75–24.63). The average dose of DHA was given at 2.3 mg/kg body weight.

The mean plasma concentration-time profiles of DHA in 5 healthy subjects is shown in Figure 4. A C_{max} of 300.4213 ng/mL was reached at 1.5 h (t_{max}) after drug administration. The elimination rate constant was 0.85/h and the $t_{½}$ was 0.81 hour. The $AUC_{0\text{-last}}$ of the mean plasma concentration-time curve of DHA was 611.79 ng/mL and $AUC_{0\text{-}\infty}$ was 613.680 ng/mL. The CL was 3.75 L/h/kg and the apparent volume of distribution (V_d) was 4.36 L/kg.

Discussion

This study aimed to established laboratory readiness for pharmacokinetic or drug level study, therefore we used an already-validated assay as the reference and did not develop novel methods for the quantification of DHA for practical reasons. The reference assay was replicated to the extent possible, but some modifications were inevitable due to the differences in the LC-MS/MS detection system and anticoagulants used in the reference assay, plasma and clinical samples. Partial validation was required according to the EMA guideline, but the specific parameters were not rigidly defined. As such, they can range from within-run precision accuracy evaluation to near full validation.

In this study, the following parameters were evaluated, *i.e.*, linearity, LLOQ, selectivity, accuracy, precision, stability and matrix effects. Carry over and the effect of different anticoagulant to plasma and clinical samples were later deemed necessary, but not done in this study due to shortage of materials and reagents. On the other hand, stability was an optional parameter that was not required but done in this study. Our first lesson learned from this study was to better-plan the assay validation experiments. The parameters for partial validations should be carefully selected based on risk-based approach, considering the impact of the modifications to the method and whether the changes considered significant.[18]

This study used plasma samples from the Indonesian Red Cross and citrate phosphate dextrose (CPDA-1) solution was used as anticoagulant, whereas the reference assay was validated in fluoro-oxalate plasma and blood samples from healthy adults were collected in ethylenediamine tetra-

Table 3. Matrix effects DHA and SIL-DHA 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
DHA, 5 ng/mL	3.061	2.463	2.296	2.458	2.512	2.086	2.479	0.3	13.1
SIL-DHA, 100 ng/mL	3.169	2.436	2.377	2.487	2.506	2.131	2.518	0.3	13.8
Normalized response	0.966	1.011	0.966	0.988	1.002	0.979	0.986	0	1.9
DHA, 402 ng/mL	3.85	3.19	2.96	3.07	3.32	3.81	3.367	0.4	11.2
SIL-DHA, 100 ng/mL	2.601	2.246	1.973	2.06	2.228	2.683	2.299	0.3	12.5
Normalized response	1.48	1.42	1.5	1.49	1.49	1.42	1.467	0	2.5
DHA, 800 ng/mL	2.307	2.049	1.699	1.866	1.744	1.694	1.893	0.2	12.9
SIL-DHA, 100 ng/mL	2.271	2.033	1.683	1.856	1.681	1.759	1.88	0.2	12.3
Normalized response	1.016	1.008	1.009	1.006	1.037	0.963	1.007	0	2.4

Table 4. Stability of DHA in human plasma samples in temperature of 4ºC.

	DHA	Fresh	6 h	24 h
5 ng/mL	Average	5.26	4.96	4.83
	SD	0.22	0.25	0.17
	CV (%)	4.2	4.96	3.44
	Accuracy	105.26	99.27	96.54
800 ng/mL	Average	807.51	808.89	781.34
	SD	18.6	19.26	17.08
	CV (%)	2.3	2.38	2.19
	Accuracy	100.94	101.11	97.67

acetic acid (EDTA) tubes. The EMA Guideline stated that partial validation is required when there were changes in anticoagulants. To our knowledge, this area has not been widely investigated since only one study investigated DHA concentration in fluoro-oxalate plasma to heparin plasma.[19] A separate validation experiments to address this issue would have been a valuable insight, thus our second lesson learned from this study.

This study's DHA chromatogram showed peak asymmetry in the form of marginal peak tailing. Peak asymmetry is a common sign of LC separation problem and several possible causes were identified. The first was the difference in the stationary phase. The reference assay used Hypersil Gold C18 (100 × 2.1 mm, 5 µm), while this study used Waters Acquity UPLC™ BEH C18 column (50 × 2.1 mm, 1.7 µm) column. Both columns have different intrinsic characteristics, such as relative hydrophobicity, relative polarity, efficiency, *etc*., that may have influenced chromatographic separation.[20]

The mobile phase of acetonitrile and ammonium acetate 10 mM pH 3.5 (50:50, v/v) and injection volume of 5 µL was directly transferred from the reference assay without further adjustments to the composition of the mobile phase (ratio of aqueous to organic solvent) to correct the peak. This was identified as the second cause for peak asymmetry in this study. In addition, the reference assay used gradient condition that can further minimized tailing, while this study used isocratic condition, this was the third cause for peak asymmetry in this study.

Low sensitivity of the MS detection to DHA in this study was observed with the doubling of plasma volume to 100 µL. The reference assay used API 5000 triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, USA), while this study used Waters

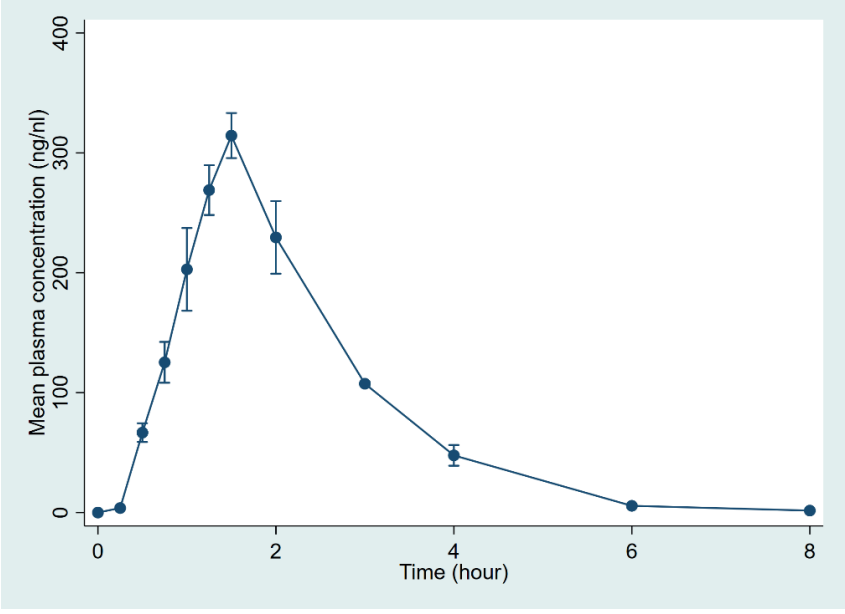

Figure 4. Mean plasma concentration-time profiles of dihydroartemisinin in five healthy male adults after single oral administration of fixed dose combination of Dihydroartemisinin-Piperaquine.

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TQ Mass Spectrometers (Waters Corp., Milford, USA). During optimization, the MS condition in this study was achieved by autotune program, which means the MS tuning parameter was set according to factory setting, except for the cone voltage and collision energy. No adjustments were made to the temperature and gas settings that could have improved ionization. The Sciex API 5000 used by the reference assay might also be more sensitive than Waters TQ Mass Spectrometers, even though the latter was more user friendly for both their software and hardware.

The assay performance for DHA was incompletely validated against several parameters such as linearity, lower limit of quantitation (LLOQ), selectivity, accuracy, precision, stability and matrix effects; while leaving out other required parameters such as carry over and anticoagulants. Calibration curve was shown to be linear with a correlation coefficient greater than 0.995 over a concentration range of 1–1,000 ng/mL. Bias and variation for accuracy and precision were in the range of 15% (20% at the lower limit of quantification). Matrix effect from human plasma was negligible (less than 15%).

Clinical applicability was demonstrated when analyzing plasma drug concentrations after oral administration of fixed drug combination of DHA-PIP in 5 healthy subjects. Several studies addressing the PK of DHA using NCA following oral fixed dose formulation of DHA-PIP in healthy young adults in Asian countries showed fairly similar results with this study.[21-23] The Cmax was between 300 and 387 ng/ml and tmax was achieved within 1–1.5 hours after drug administration. The half-life was also consistent with other studies, between 0.98 and 2.03 hour. However, there appeared to be variability in the apparent volume of distribution of DHA *i.e*., 3.37; 4.38; 5.53 and 6.31 L/kg. The same variability was noted in the clearance of DHA *i.e.*, 1.32; 2.21; 3.75 and 4.54 L/h/kg that could have been caused by the differences in the subjects's body weight, but overall DHA was cleared after 8 hours.

Conclusion

Validation experiments to the LC-MS/MS method for the quantification of plasma DHA was successful for selectivity, linearity, lower limit of quantitation, accuracy, precision, matrix effect and stability parameters. Other validation parameters such as carry over and the role of anticoagulants were not tested in this study. Although clinical applicability was demonstrated, this method needed to be improved with additional measures to address the not-tested validation parameters. Additional evaluation to artesunate might be good to explore, since this could be done with the same-already-in-placed LC condition as DHA.

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