Chalmers University of Technology

# Protein binding determination of dihydroartemisinin (DHA) in human plasma by HPLC using post-column on-line alkali derivatization and UV detection

**Master of Science thesis in biotechnology**

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## Abstract

Artemisinin and its derivatives are considered as a very important new class of antimalarials and becoming more and more commonly used throughout the world. Dihydroartemisinin (DHA) is the main bioactive metabolite of artemisinin in clinical use and has greater intrinsic antimalarial activity. Although pharmacokinetic research *in vivo* and *in vitro* has been done and conventional pharmacokinetic parameters for DHA are well documented, data relating to parameter of protein binding of DHA is still inconsistent. In this project, equilibrium dialysis and ultrafiltration methods were carried out to determine the protein binding percentage of DHA in healthy human plasma. At the same time, HPLC conditions for DHA quantification were optimized during experiments. Protein binding fraction of DHA was reported here as 80%- 84% considering the volume shifts in equilibrium dialysis and 88%-91% in various DHA concentrations in ultrafiltration.

## Key words

Dihydroartemisinin; protein binding; equilibrium dialysis; ultrafiltration; HPLC-UV

## Abbreviation:

| | |
|---|---|
| ACN | acetonitrile |
| ACT | artemisinin-based combination |
| ARTS | artesunate |
| DHA | dihydroartemisinin |
| DHA-PIP | dihydroartemisinin-piperaquine |
| Fu | free drug fraction |
| Fb | degree of drug binding |
| HPLC | high-performed liquid chromatography |
| HPLC-ECD | high-performed liquid chromatography with reductive electrochemical Detection |
| HPLC-MS | high-performed liquid chromatography with mass spectrometry |
| HPLC-UV | high-performed liquid chromatography with ultraviolet detection |
| LLOQ | lower limit of quantification |
| RAM | restricted-access material |
| $T_{1/2}$ | half-life |

# 1. Introduction

## 1.1. Artemisinin and its derivatives

Malarial is a life threatening parasitic diseases transmitted by mosquitoes. It is a common and serious tropical disease which induces a major and public health problems throughout most of the developing world (1).

Artemisinin (qinghaosu), a sesquiterpene lactone endoperoxide, has been used in Chinese medicine for the treatment of malaria for more than 2000 years (2, 3). In ancient China, artemisinin was used traditionally for treating fevers. In 1967, Chinese scientists screened a series of traditional remedies for drug activities, and found that the extracts of qinghao had potent antimalarial activity (2). In 1972, the active ingredient was purified from the Chinese medicinal herb qinghao, Artemis ia annua Linn (asteraceae), later renamed artemisinin (4). Artemisinin derivatives—dihydroartemisinin (DHA), artemether and arteether (Fig. 1.) —were used widely in China by 1980s. By the early 1990s, artemisinin derivatives were being widely used in Thailand, Burma and Vietnam. Now, several artemisinin derivatives are being developed by western pharmaceutical companies and regarded as the most promising choice against both drug-sensitive and drug resistant strains of plasmodium falciparum and are of use in the treatment of life-threatening cerebral malaria (2). Artemisinin and its derivates have little adverse effects in patients treated. In a prospective study of over 3,500 patients in Thailand, there was no evidence for serious adverse effects (3). Artemisinin and its derivates also appear to be safe in pregnant women, but not recommended for women in early pregnancy (2, 4).

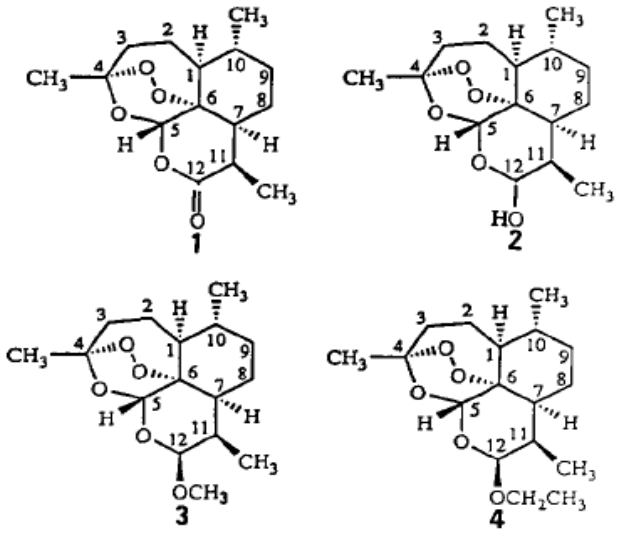


Fig. 1. Structure of artemisinin (1), dihydroartemisinin (2), artemether (3) and arteether (4).

The malarial community has argued that the use of artemisinin-based combination is the only practical solution to controlling malarial and limiting the evolution and spread of resistance (5). Since artemisinin and its derivates are short life-time drugs on the malarial treatment, the monotherapy of this antimalarial medicine has high rate of recrudescent infection observed in several clinical studies (16). Nowadays, the artemisinin-based combination therapy (ACT) is regarded and recommended as better choice on malarial

treatments. In ACT, artemisinin or its derivates combining with another antimalarial drug with relatively long half-life is considered as a standard choice. Artemether-lumefantrine (lumefantrine, a new Chinese drug previously known as benflumetol) is the only coformulated ACT currently manufactured to European Union Good Manufacturing Process standards and widely registered (13). Artemether-lumefantrine treatment is safe and effective against multidrug resistant infectious. The combination of dihydroartemisinin-piperaquine (DHA-PIP) is relatively inexpensive and found to be highly efficacious and well tolerated. Notably, DHA-PIP was highly effective against multidrug-resistant falciparum malaria in Asia (17).

## 1.2. Theoretical background

### *1.2.1. Dihydroartemisinin and its clinical treatments*

Dihydroartemisinin (DHA) is the derivative of artemisinin with the C-10 lactone group replaced by hemiacetal (6). It has greater antimalarial activity than artemisinin in clinical use and it is the active metabolite of a number of artemisinin derivatives. (7,8). A study demonstrated that only DHA was rapidly effective against all stages of parasite life cycle and completely inhibited the parasite growth within the shortest exposure time when compared to all other artemisinin drugs (9). The endoperoxide bridge is necessary for its antimalarial activity. This structure was able to form toxic free radicals in malarial parasites by reacting with a ferrous iron. In 2001, data from a study also indicated that DHA was able to be used as an anticancer drug (10). This finding gave the ancient medicine herb a promising and exciting future in clinical treatment.

There are two anomers of DHA: α-DHA and β-DHA. They reach equilibrium after preparation at least 18 hours after reconstitution in solutions at 4℃. The ratios of α-DHA and β-DHA vary in different solutions. Navaratnam et al (11), noted that the equilibrium ratio of α: β DHA anomers (4.5:1) took at least 18 h in 50% (v: v) methanol and water solution. In an earlier study, the ratio of α: β DHA anomers was reported approximately 5:1 in 50% (v: v) methanol and water solution (7).

The pharmacokinetics of DHA has been characterized by methods based on high-performed liquid chromatography (HPLC) in a few studies with varying routes of administration and disease states. It is consistent that the time reaching to the maximum dihydroartemisinin drug concentration ($C_{max}$) is approximately 1-2hours of dosing and elimination half-life ($t_{½}$) is estimated to be in the range of 0.8-1.5 hours (60-90min) in healthy human, much longer than that of 19-25min in mice (5, 12). In pregnant women, $C_{max}$ was reported 9 times lower than non-pregnant adults (13). There are no marked sex differences in DHA pharmacokinetics in oral DHA (14). Pharmacokinetic parameters of patients are obviously different comparing that of healthy human. Table below summarized results obtained from clinical researches.

**Table 1**
Pharmacokinetic parameters for dihydroartemisinin (DHA) following intravenous (120 mg; 312.5 mmol) and oral (100 mg*; 260.4 mmol) administration of ARTS in patients (15)

| | n | t½ (min) | MRT (min) | AUC (µmol.hr/L) | Cmax (mM) | Tmax (min) |
|---|---|---|---|---|---|---|
| **Intravenous ARTS** | 12 | 36.7 (30.3–43.1) | 54.2 (45.3–63.1) | 6.49 (5.18–7.80) | 7.71 (6.90–9.78) | 8 (7–12) |
| **Oral ARTS** | 11 | 40.1 (31.4–48.9) | 101 (81–120) | 4.08 (3.33–4.84) | 2.99 (1.98–3.48) | 75 (52–98) |

*Actual dose 5 90 mg (234 mmol)

### *1.2.2. Drug protein binding in human plasma*

Plasma protein binding is a very important factor in establishing the pharmacokinetic and pharmacodynamic properties of a drug. It indicates how much of the total amount of a drug in plasma or tissue is bound to plasma or tissue proteins. Although there are many components in plasma that are capable of binding drugs, albumin and α-acid glycoprotein are the two major plasma proteins which are capable of binding various drugs with sufficient affinity (18). The binding of a drug to proteins can be viewed as reversible and rapid equilibrium process (19). Normally, this process can be described in the following scheme:

$$[D] + [P] \rightleftharpoons [DP] \qquad \textit{Equ.1}$$

Where [D] is the free drug concentration and [P]is the concentration of plasma proteins free of drug; [DP] is the concentration of the drug-protein complex. At equilibrium, the ratio of unbound drug and total plasma drug concentration (fu) is

$$fu = [D]/([D] + [DP]) \qquad \textit{Equ.2}$$

The free drug hypothesis states the unbound drug equilibrates beteween tissues and binds to drug targets. Free (unbound) drug concentration ([D] in *Equ.1*) is generally used to predict parameters like drug clearances, volumes of distribution and half-lives of drugs (19, 20), so the kowledge of unbinding percentages (fu) is of clinical importance, expecially for high protein bound drugs.

Based on calculation of free drug fraction (fu) above, protein binding percentage (fb) in plasma is:

$$fb = 1 - Fu \qquad \textit{Equ.3}$$

Potential limatations and factors influencing a drug-to-plasma protien binding determination can be divided into individual differences and inevitable changes during experiments. The individual differences include pregnancy, hepatic disease, age and racial or ethnic differences (21-23). The inevitable changes during experiments contains drug loss by drug binding to the memberanes and walls of the devices, volume shifts as well as pH and

temperature changes during experiments (24-26). Several studies also indicated that different buffers chosen for equilibrum dialysis appeared to cause different protein binding for the same drug (27).

The other question which needs to be answered is whether it is concentration dependent binding to plasma. In the albumin binding studies, the free fraction of DHA was not concentrated-depended binding at different albumin concentration (data not shown) (7). But in 2009, an article revealed that there was a concentration dependent decrease in protein binding of DHA in human plasma (Fig. 2). The maximum binding percentage occurred in the concentration of 0.15 – 462 ng/ml (9).

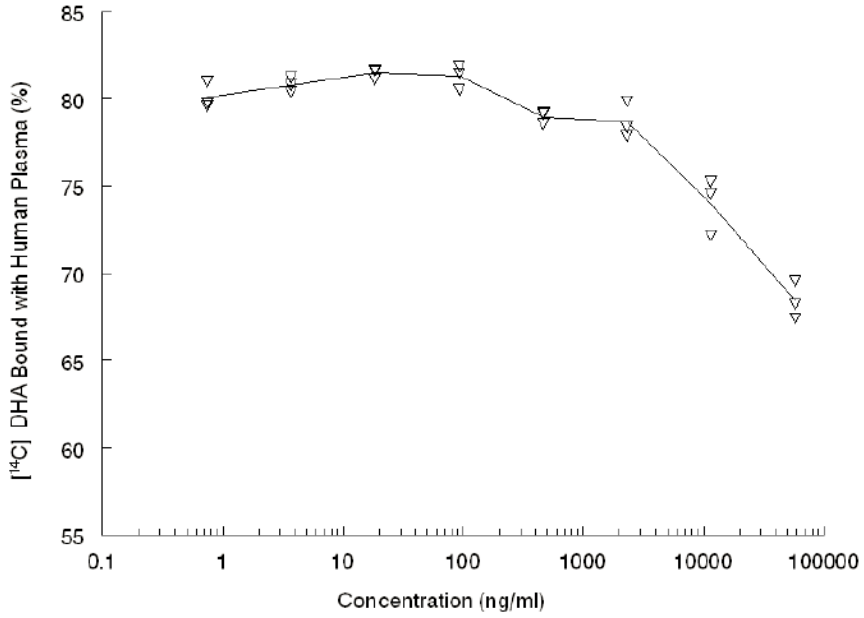



**Fig. 2.** Protein binding of DHA in human plasma at variable concentrations of DHA (0.15, 0.74, 3.7, 18.5, 92.5, 462, 2312, 11560 and 57800 ng/ml) at 37℃ for 5 hours determined by equilibrium dialysis (9)

### *1.2.3. Methods for protein binding determination*

There are several *in vitro* methods for determing the degree of drug protein binding in plasma, including equilibrum dialysis, ultrafiltration, gelfiltration and albumin column (19). Among these methods, equilibrum dialysis and ultrafiltration are the two most predominant techniques.

Equilibrum dialysis, accepted as the 'gold standard', is based on the establishment of an equilibrium state between plasma containing certain drug and buffer after a period of incubation at a fixed temperature (usually 37 ℃). Sodium or potassium phosphate buffers at pH7.4 are the ones most commonly used on the buffer side. At equilibrium, the drug

concentration on the buffer side is a reflection of free drug on the plasma side of the membrane and on the plasma side is represent of the total drug concentration of plasma. Based on *Equ.2,* free drug fraction (fu) can be calculated as:

$$fu=\frac{[\mathrm{Drug}]\mathrm{buffer}}{[\mathrm{Drug}]\mathrm{plasma}} \qquad \textit{Equ.4}$$

During the equilibrium process, water molecules from the buffer side moving into plasma side can induce a volume shift, and free drug fraction (fu) can be overestimated using the equation above. Considering the volume shift during incubation, the Fu can be determined by the following equation:

$$fu=\frac{[\mathrm{Db}]}{([\mathrm{Dp}]-[\mathrm{Db}])*(\mathrm{Vpa}/\mathrm{Vba})+[\mathrm{Db}]} \qquad \textit{Equ.5}$$

Where, $[\mathrm{Db}]$ is the concentration of drug on buffer side after equilibrium dialysis; $[\mathrm{Dp}]$ is the drug concentration on plasma side. $V_{pa}$ and $V_{ba}$are the volumes on plasma and buffer side respectively after equilibrium dialysis.

Ultrafiltration, regarded as the 'fast method' (15-45 min being a typical range), is based on the physical separation of free drug molecules in plasma water from drug bound to plasma proteins by filtering plasma samples through a semipermeable membrane under a positive pressure generated by centrifugation (19). After ultrafiltration, drug concentration in the ultrafiltrate is the concentration of the free drug in plasma and the fraction of unbound drug can be measured using the equation below:

$$fu=\frac{[\mathrm{Drug}]\mathrm{ultrafiltrate}}{[\mathrm{Drug}]\mathrm{original\ plasma\ (total\ drug\ in\ plasma)}} \qquad \textit{Equ.6}$$

This technique is very simple to use, because commercial devices are now available. Since adsorption to the UF device and the filter as well as protein leakage across the filter may appear during ultrafiltration (28), the sample recovery is also regarded as an important parameter on free drug fraction determination. If sample recovery is below 90%, the free drug fraction is calculated based on the following item (19):

$$fu=\frac{[\mathrm{Drug}]\mathrm{ultrafiltrate}}{[\mathrm{Drug}]\mathrm{original\ plasma\ (total\ drug\ in\ plasma)}*\mathrm{Recovery}} \qquad \textit{Equ.7}$$

Advantages and disadvantages of equilibrium dialysis and ultrafiltration for measuring drug protein binding to plasma can be summarized in the Table below:

**Table 2**
Advantages and disadvantages of equilibrium dialysis and ultrafiltration method (19, 28)

| | Equilibrium dialysis | Ultrafiltration |
|---|---|---|
| **Advantages** | ● Considered as a standard method<br>● Temperature controlled<br>● Non-specific adsorptions can be compensated | ● Needs small amount of sample (<1ml)<br>● Fast (less than 30min)<br>● No buffer needed<br>● Small changes in drug concentration |
| **Disadvantages** | ● Long time to reach equilibrium<br>● Need of buffer<br>● Degradation of unstable compounds<br>● Volume shift<br>● pH changes<br>● Donnan ion effect*<br>● Not suitable to concentration dependent drug protein binding | ● Nonspecific binding<br>● Volume of ultrafiltration maybe not sufficient for drug assay<br>● Usually not temperature controlled<br>● Donnan ion effect<br>● pH changes |

*The Donnan-ion effect, due to the fact that charged proteins cannot pass through the membrane, which results in flow of small ions across the membrane to achieve electroneutrality. This problem is able to be reduced by using an isotone phosphate buffer to which electrolytes (i.e. NaCl) are added (28).

### *1.2.4. HPLC methods for DHA detection and quantification*

There are several methods for DHA detection and quantification based on HPLC, such as HPLC with reductive electrochemical detection, HPLC with mass spectrometry as well as HPLC with ultraviolet detection.

The technique of HPLC with reductive electrochemical detection (HPLC-ECD) system was firstly reported in 1985 (30). This method best meets the sensitivity and specificity requirements in detection and quantification. The limitations of this approach include the requirement of rigorous deoxygenation of system and temperature control (19, 30, and 31). In order to keep high sensitivity, the electrochemical detector needs to be cleaned very often (e.g. after approximately 50 injections).

Liquid chromatography coupled with mass spectrometry (LC-MS) is a powerful separation and detection technique in a large number of analytical fields (5, 30, and 31). This method has higher sensitivity compared to HPLC with ultraviolet (UV) detection (HPLC-UV) but requires special and expensive laboratory facilities.

High-performance liquid chromatography (HPLC) followed by ultraviolet (UV) detection (Fig. 3.) is an economical and efficient method for DHA detection and quantification. Since DHA lacks ultraviolet absorbent or fluorescent chromophores which are necessary for conventional UV detection method, several methods combined with HPLC-UV method were

developed, such as post-column alkali and pre-column acid decomposition (11, 19, 20, 31 and 32). In this project, HPLC-UV with post-column decomposition was chosen.

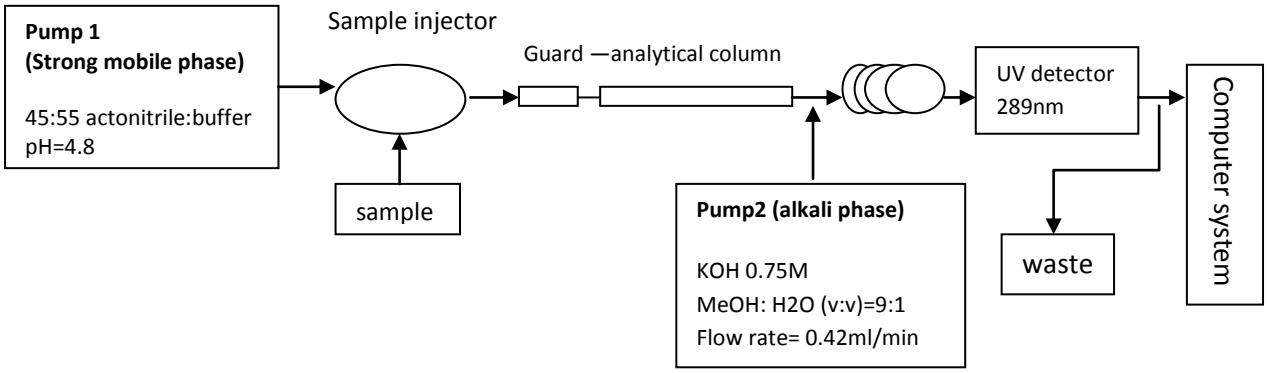



**Fig. 3.** HPLC- UV system Mobile phases were degassed more than 30 min before use in ultrasonic bath. The sample inject volume is 100µl each time by sample injector. The UV-detector was set at 289nm with 0.01 AUFS and AUX range of 2

## 2.1. Reagents and materials

Dihydroartemisinin was obtained from Vietnam. Human plasma was from Sahlgrenska Hospital. Acetonitrile, methanol and acetic acid were HPLC grade and purchased from Kemetyl (Stockholm, Sweden). All other reagents were analytical grade unless otherwise noted. Water for the HPLC system and the whole process of the experiment was Milli-Q water.

Equilibrium dialysis cells and membranes with a molecular weight cut-off of 6,000Da were purchased from Scienceware (USA). Syringes with 1.0 volume and 0.8x40 mm needles were obtained from Terumo (Leuven, Belgilim). pH after ED and UF was estimated by pH meters.

## 2.2. Instruments

Two pumps used in HPLC system were LC-10 AD from Schimadzu (Kyoto, Japan). Sample injector with a 200µl loop was Endurance Spark (Emmer, Netherlands). The separation column, protected by a guard column was a reversed-phase C18 from Agilent (USA). The post-column reaction took place in a 5-m knitted Teflon-tube coil, di=0.46mm (Coricon, Knivsta, Sweden) immersed in a 70℃ water bath (Grant instruments, Cambridge, UK). An APD-10A UV detector from Shimadzu was used for signal detection. Signals were transferred to and evaluated by software of Chromatographic System Window (CSW32). The instrument used for centrifugation was purchased from Thermo (Germany).

## 2.3. Chromatographic conditions

Chromatographic conditions are shown below (table 3). Both mobile phases were degassed more than 30 min before use in ultrasonic bath.

**Table 3**
Chromatographic conditions

| Mobile phase | Function | Composition | Flow rate |
|---|---|---|---|
| **Strong mobile phase** | separation | ACN: acetic buffer (v:v)=45:55 pH=4.8 | 0.7ml/min |
| **Alkali phase** | Post-column derivatization | KOH 0.75M, MeOH:H2O(v:v)=9:1 | 0.42ml/min |

.

## 2.4. Standards preparation

Three dihydroartemisinin (DHA) working stock solutions were prepared by dissolving DHA in 50% methanol:water (v:v). Two of them were used for calibration curve preparation and another was for quality control determination. All stock solutions were prepared monthly and stored at -25℃. No obvious adsorption of DHA to containers was seen from 50% methanol: water (v: v). Therefore, containers for stock solutions were not silianized.

Phosphate buffer was prepared from reagents of analytical grade: 1.78g KH2PO4, 7.61g NA2HPO4, 9g NaCl in 1L of Milli-Q water and pH was adjusted to pH 7.4 with 3M/l NaOH (32). Plasma from hospital was stored at -25℃ and re-thawed in room temperature before use.

### *2.4.1. Calibration curve in buffer (free drug)*

Six calibration standards prepared in buffer were 20, 50, 150, 250, 750, 1500, 2000ng/ml. Each standard was made by adding 1.00ml appropriate DHA stock solution to 9.00ml buffer. Standard curve in buffer was determined from the average of three samples at each concentration. The step of calibration curve determination in buffer was performed on three separate occasions. Stock solutions and six calibration standard solutions were prepared monthly and stored at -25℃.

### *2.4.2. Calibration curve in plasma (total drug)*

Six calibration standards prepared in plasma were 250, 500, 750, 1500, 2000 and 3000ng/ml. Each standard was made by adding 1.00ml appropriate DHA stock solution in 9.00ml plasma. Standard curves in plasma were determined from the average of three samples in each concentration. The step of calibration curve determination in plasma was performed on three separate occasions. Peak areas for α-DHA and β – DHA were calculated for each sample. Six calibration standard solutions were prepared weekly and stored at -25℃. Before analysis, each 150μl plasma sample was added to 2 times volume acetonitrile in 500μl tube

and centrifuged 5mins at 11,000g at room temperature. After centrifugation, 100μl supernatant fluid was analyzed by HPLC system.

## 2.5. Equilibrium dialysis

Equilibrium dialysis was performed using two-chambered Plexiglas dialysis cells with 1-ml capacity. The cells were separated by a membrane with a molecular weight cut-off of 6,000. Before use, the membranes were immersed in Milli-Q water for 15min and thereafter washed in phosphate buffer for 30min at room temperature. Phosphate buffer and plasma with 2000ng/ml DHA were placed at opposite sides of the membrane in each cell. Equilibrium dialysis temperature was fixed at 37℃ in a water bath. Dialysis for 1, 1.5, 2, 3, 4, 5, and 6h was evaluated. At the end of dialysis, the volume on each side of chamber was measured (the procedure is summarized in Fig. 4.). All samples were analyzed within 2 hours or stored immediately at -25℃ before analyzed. Each calculation of free fraction was based on the results from at least duplicated dialysis samples at each time. Unbound fraction rose with increasing times of dialysis to a maximum at 4 h and remained unchanged with longer dialysis times. Therefore, 4h was finally used in all analyses.

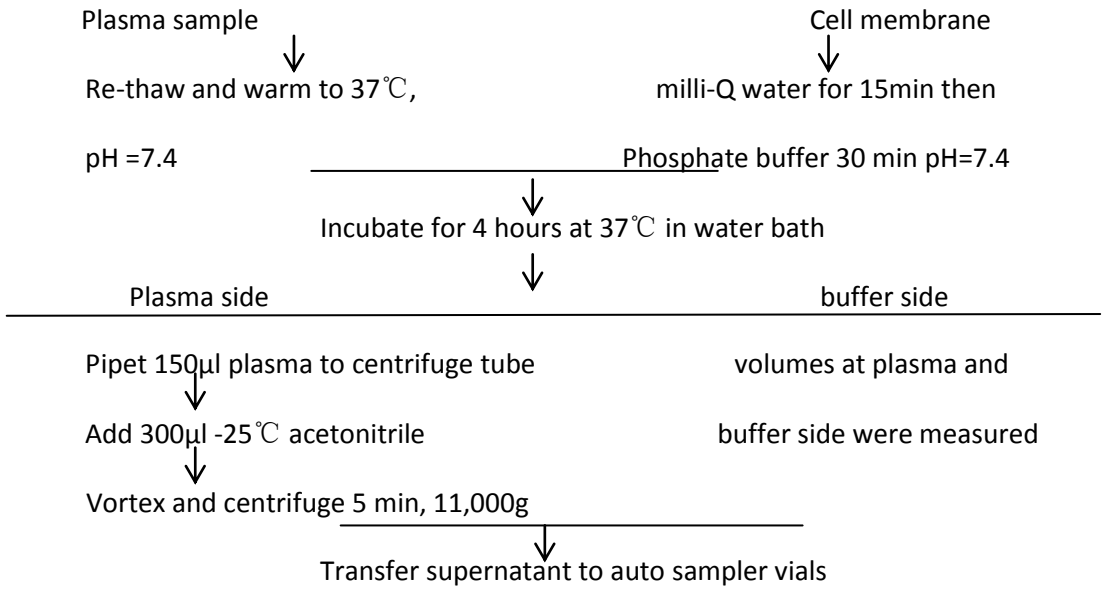



**Fig. 4**. Processes on equilibrium dialysis

## 2.6. Ultrafiltration

500μl of plasma, to which DHA had been added, was added to ultrafiltration tube (3,000NMWL, Amicon Ultra, USA). The device was centrifuged at 14,000g for 15 minutes. After centrifugation, samples from filtrate were analyzed by HPLC-UV system. The data obtained from ultrafiltrate represented the free drug concentration of DHA in plasma. Recoveries were calculated according to the guide book (Amicon Ultra, USA).

## 2.7. Determination of ratio of α-DHA and β – DHA in different solutions

The ratio of α-DHA and β–DHA was determined in 2000ng/ml in solution of undiluted plasma, phosphate buffer (pH=7.4) and 50%methanol: water (v: v).

## 2.8. Method optimization

### *2.8.1. Column selection*

Agilent C18 column (250×4.6mm, Agilent technology, USA), Agilent C18 column( 150×4.6mm, Agilent technology, USA) and Chromolith C18 column( 100×4.6mm, Merck, Germany) were evaliated together with mobile phase optimization step to obtain the best condition for DHA quantification in human plasma.

### *2.8.2. Mobile phase optimization*

Four different compositions of mobile phase at two pH values (Table 4) were tested in order to get best separation of α and β anomers of DHA.

**Table 4**
Mobile phase optimization

| | Mobile phase composition | pH |
|---|---|---|
| **A** | Acetonitrile:water=60:40 | 4.8 |
| **B** | Acetonitrile:water=55:45 | 4.8 |
| **C** | Acetonitrile:water=50:50 | 4.8 |
| **D** | Acetonitrile:water=45:55 | 4.8 |

∗ Mobile phases are marked as A, B, C and D with different mobile phase compositions.

## 2.9. Validation

### *2.9.1. Accuracy and precision*

The lower limit of quantification (LLOQ) of DHA in plasma or buffer was assessed based on average of six samples of each concentration and with a within-day coefficient of variation less than 20%.

Accuracy and precision for quantification of DHA in plasma at concentrations of 250, 750 and 1500ng/ml were determined within day and between days. In buffer, concentrations of 50, 150 and 1500ng/ml were analyzed for accuracy and precision for quantification of DHA within and between days.

### *2.9.2. Freeze and thaw analysis*

Standard samples of 250, 750 and 1500ng/ml of plasma were thawed and kept for 8 hours at room temperature then frozen again. This process was performed three times and at the last time, samples were guaranteed by HPLC-UV.

# 3. Results

## 3.1. Method optimization

### *3.1.1. Mobile phase optimization*

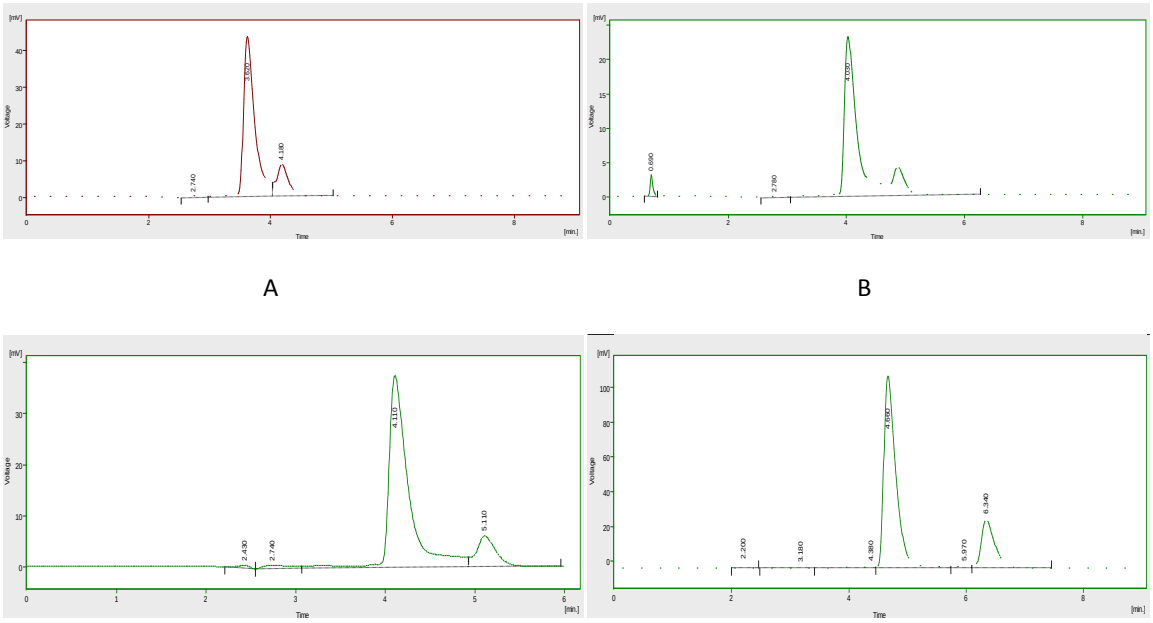



**Fig. 5.** Optimization of mobile phase composition—seperation of α-DHA and β–DHA using different composition of mobile phase: (A) pH=4.8, ACN: water=60:40; (B) pH=4.8, ACN:water=55:45; (C) pH=4.8, ACN:water= 50:50; (D) pH=4.8, ACN:water= 45:55.

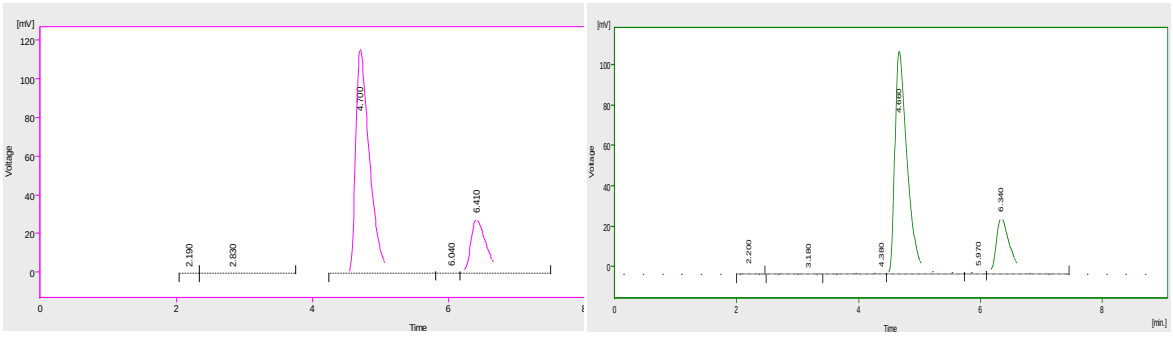
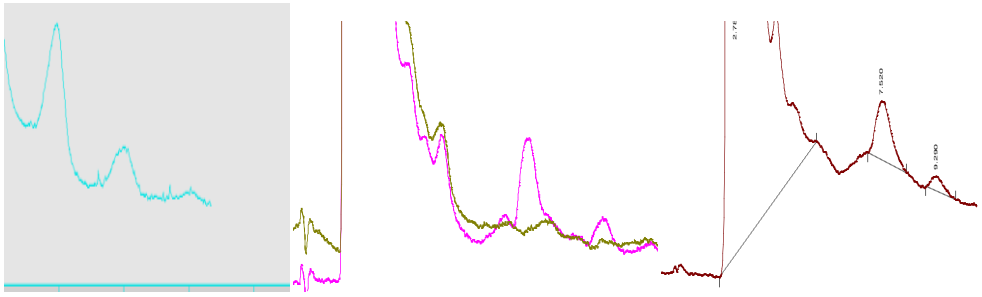



**Fig. 6.** Optimization of mobile phase pH: (A) pH=5.0, ACN: water= 45:55; (B) pH=4.8, ACN: water= 45:55.

α-DHA and β-DHA were able to be best separated when ACN:water= 45:55. pH = 4.8 and 5.0 did not have any appreciable effects on separation of α-DHA and β–DHA and their retention times when ACN:water= 45:55.

### *3.1.2. Column selection on DHA quantification in plasma*



A.100x4.6mm B. 150x4.6mm C.250x4.6mm

**Fig. 7.** Results of different columns: (A) Chromolith C18 column (100×4.6mm, Merck, Germany); (B) Agilent C18 column (150×4.6mm, Agilent technology, USA); (C) Agilent C18 column (250×4.6mm, Agilent technology, USA). Strong mobile phase was ACN: water= 45:55, pH = 4.8, flow rate=0.7ml/min; alkali phase was KOH 0.75M, MeOH:H2O(v:v)=9:1, flow rate=0.42ml/min.

The 100x4.6mm column was not able to separate α-DHA with the front plasma peak. The 250x4.6mm column was able to separate well, but it required longer separation time. The 150x4.6mm column was able to get satisfying separation of α-DHA and β – DHA in plasma with relative short retention time comparing with 250x4.6 column. There was a small disturbing peak at the same retention time of α-DHA with injecting blank plasma, but not large enough to affect DHA quantization.

## 3.2. Validation

### *3.2.1. Accuracy and precision*

The lowest concentration of quality control was determined as 200 ng/ml (CV=9.1%) for plasma and 10 ng/ml (CV= 8.2%) for buffer.

Accuracy and precision for quantification of DHA in plasma at different concentrations within a day and between days were calculated and summarized in table8.

**Table 5**
Within-day and between-days accuracy and precision of DHA in human plasma

| plasma | Concentrations (ng/ml) | Analyzed average concentrations (ng/ml) | CV (%) | Accuracy (%) |
|---|---|---|---|---|
| a | 250 | 241 | 11 | -0.36 |
| | 750 | 799 | 5.6 | +6.5 |
| | 1500 | 1550 | 7.8 | +3.3 |
| b | 250 | 284 | 10.7 | +9.6 |
| | 750 | 692 | 9 | -7.7 |
| | 1500 | 1569 | 5.3 | +4.6 |

*a is accuracy and precision of DHA in human plasma within a day; b is accuracy and precision of DHA in human plasma between days

### *3.2.2. Freeze and thaw analysis*

Plasma samples (n=3) at concentrations of 250, 750 and 1500 ng/ml were performed freeze-thaw process; results are shown in table 6.

**Table 6**
Accuracy and precision of DHA in human plasma

| Plasma concentration (ng/ml) | Analyzed average concentrations (ng/ml) | CV (%) | Accuracy (%) |
|---|---|---|---|
| 250 | 248.6 | 7.6 | -0.56 |
| 750 | 682.7 | 6.4 | +8.9 |
| 1500 | 1565.5 | 8.4 | +4.4 |

*Average concentration was obtained from 6 individual occasions

## 3.3. Calibration curve

### *3.3.1. Calibration curve for α-DHA and β – DHA in plasma*

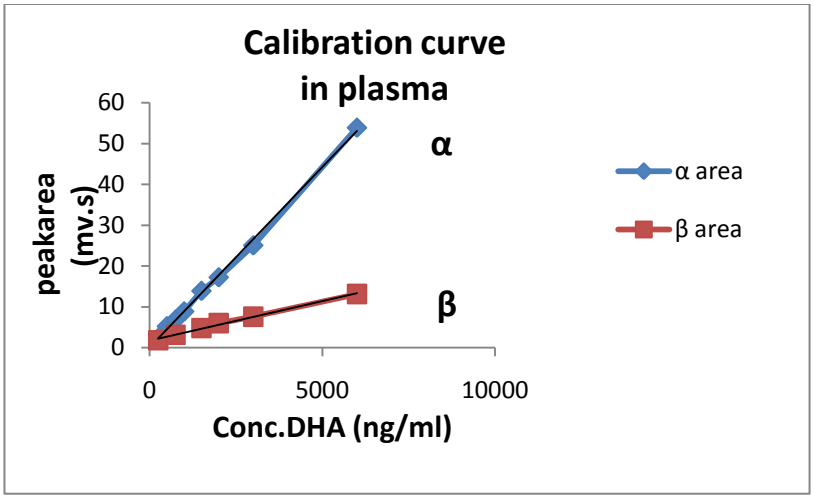



**Fig. 8**. Calibration curve for DHA in plasma: concentrations of 6 dots were 250, 500, 750, 1500, 2000 and 3000ng/ml. Standard curves of DHA in plasma: y = 0.0088x + 0.085 ($R^2$ = 0.9979) for α-DHA; y = 0.0019x + 1.7315 ($R^2$ = 0.9953) for β – DHA.

### *3.3.2. Calibration curve for DHA in buffer*

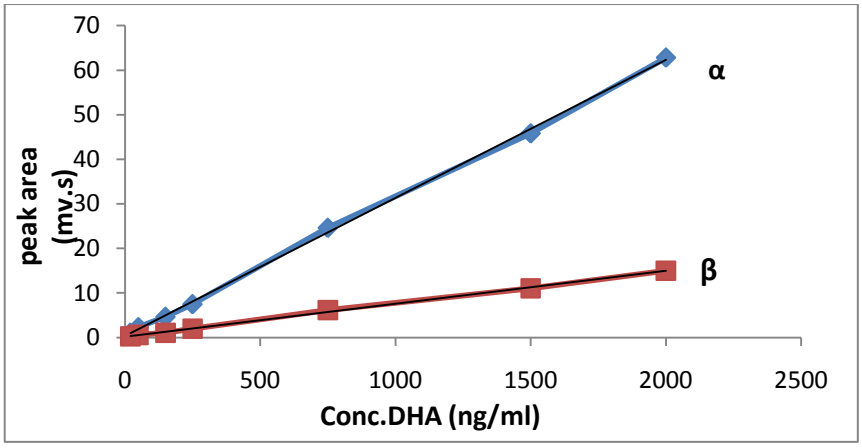



**Fig. 9.** Calibration curve for DHA in buffer: concentrations of 6 dots were 20, 50, 150, 250, 750, 1500, 2000ng/ml. Standard curves of DHA in buffer: y = 0.031x + 0.3675($R^2$ = 0.9992) for α-DHA, y = 0.0074x + 0.1611 ($R^2$ = 0.9986) for β – DHA.

### 3.4. Protein binding of α-DHA by equilibrium dialysis and ultrafiltration

#### *3.4.1. Protein binding of α-DHA in Equilibrium dialysis*

During two separate six-hour equilibrium dialysis, samples from both plasma and buffer sides at 1, 1.5, 2, 3, 4, 5 and 6 hours were analyzed by HPLC and concentrations were calculated based on peak area of the sample (Fig. 10). After 6$^{th}$ hour, final volumes were on the buffer side 0.9 ml and approximately 1.1 ml on the plasma side.

The free drug fractions in the 6-hour samples were calculated to be 0.223 and 0.198 without consideration of volume shifts during incubation respectively for the two experiments.

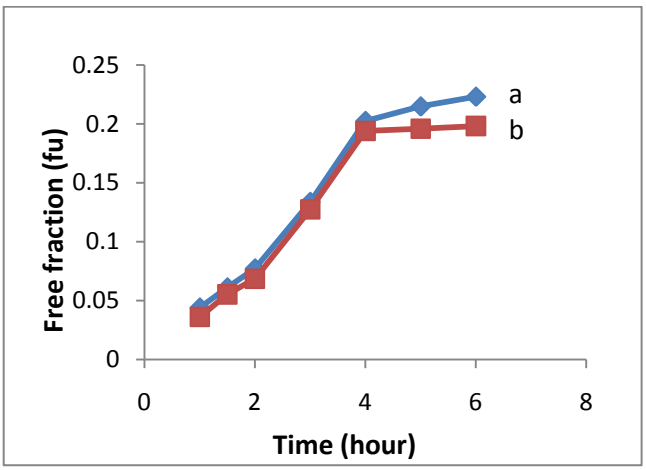



**Fig. 10.** Six-hour Equilibrium dialysis: Unbound fraction rose with increasing times of dialysis to a maximum at 4 h and remained unchanged with longer dialysis times. Free fractions of a and b were 0.223 and 0.198 without consideration of volume shift during incubation respectively.

It was observed that the free drug percentage (fu) remains same at approximately 4$^{th}$ hour in equilibrium dialysis; the final free drug percentage (fu) and protein binding percentage (fb) before and after correction for volume shifts during equilibrium dialysis were compared in table 7.

**Table 7**
Fu and Fb before and after correction

| | 1$^{st}$ experiment | | 2$^{nd}$ experiment | |
|---|---|---|---|---|
| | **uncorrected** | **corrected** | **uncorrected** | **corrected** |
| **Fu** | 0.223 | 0.190 | 0.198 | 0.168 |
| **Fb** | 0.777 | 0.810 | 0.802 | 0.832 |

Equilibrium dialysis was repeated in duplicate with DHA plasma concentrations varying at 750, 1500 and 2000ng/ml. The incubation time was 4 hours since the previous experiment had indicated equilibrium to have been reached by this time. Free drug percentages (fu) and protein binding percentages (fb) were calculated thereafter.

**Table 8**
fu and fb at different concentration before and after correction

| | 750ng/ml | | 1500ng/ml | | 2000ng/ml | |
|---|---|---|---|---|---|---|
| | **uncorrected** | **corrected** | **uncorrected** | **corrected** | **uncorrected** | **corrected** |
| **Average Fu** | 0.214 | 0.183 | 0.195719196 | 0.166 | 0.212122689 | 0.181 |
| **Average Fb** | 0.786 | 0.817 | 0.804 | 0.834 | 0.788 | 0.819 |

* Free fractions at different DHA concentrations of 750, 1500 and 2000ng/ml in plasma

### *3.4.2. Protein binding of α-DHA in ultrafiltration*

Ultrafiltration was repeated in duplicates with DHA plasma concentration varying at 750, 1000, 1500 and 2000ng/ml. The recovery of ultrafiltration was approximately 85%. pH of plasma before ultrafiltration was 7.5. After centrifugation, the pH in filtrate was about 8 and in concentrate, the pH was about 9.

**Table 9**
fu and fb in ultrafiltration

| Conc.DHA (ng/ml) | 750ng/ml | 1000ng/ml | 1500ng/ml | 2000ng/ml |
|---|---|---|---|---|
| **fu** | 0,110 | 0.115 | 0.097 | 0.112 |
| **fb** | 0.890 | 0.885 | 0.903 | 0.888 |

***** fu and fb were obtained from 6 samples at 750, 1000 1500 and 2000ng/ml concentrations

### 3.5. Protein binding of β –DHA in human plasma

Due to lower assay sensitivity and much lower concentrations of β–DHA in plasma, Results for β–DHA might be inaccurate. Fu of β –DHA was only determined at DHA concentration of 2000ng/ml in human plasma. The final Fu of β –DHA was approximately 0.24 in human plasma without consideration of volume shift.

### 3.6. Determination of ratio of α-DHA and β –DHA in different solutions

**Table 10**
The ratios of α-DHA and β – DHA various in different solutions

| | plasma | buffer | 50% methanol |
|---|---|---|---|
| Ratio of α-DHA and β – DHA | 3:1 | 4:1 | 4.2:1 |

* Ratio of α-DHA and β – DHA were determined by peak area in different solutions, the drug concentration was 2000ng/ml in plasma, buffer and 50% methanol

## 4. Discussion

### 4.1. Method optimization on DHA quantification in HPLC based methods

On method optimization, several columns were investigated at the start of this project to identify the suitable column and mobile phase to optimize the chromatography. In DHA analysis, 100mm and 150mm columns are commonly used, some articles even use 200mmor 250mm column in order to get satisfying separation. In this study, it indicated that 150mm was the best choice. Its retention time was shorter (approximately 10 min) comparing 250mm column with better separation (comparing with 100mm column). Although there was a small disturbing peak at the same retention time of α-DHA with injecting blank plasma, but not large enough to affect DHA quantization.

Different mobile phase compositions were tested to increase the sensitivity and obtain better separation and sharper peaks. In DHA analysis by HPLC-UV, samples were able to be analyzed within 20min and α-DHA was able to be separated well with the front peak from plasma in mobile phase of ACN: water= 45:55(v: v)at pH of 4.8 or 5.0 in the strong mobile phase.

New methods based on HPLC have been performed recently, in order to get higher sensitivities on DHA determination. Toufigh Gordi et al (34) reported that artemisinin was able to be analyzed directly in plasma and saliva using a HPLC-UV system with a restricted-access material (RAM) pre-column. The advantage of this method was that precipitation of plasma proteins could be avoided before sample analysis, directly enhancing the sensitivity. This method was also tested in this project, but it was abandoned for its low recoveries. This problem might be caused for two reasons. Firstly, the restricted-access material (RAM) pre-column used was old and maybe not able to retain DHA effectively. Secondly, since DHA was more soluble than artemisinin in water (in weak mobile phase, 90% was water), DHA did not retain in restricted-access pre-column.

HPLC-UV with post-column alkali decomposition is an economic and convenient method on protein binding of DHA in human plasma. The disadvantage of this method is its relative low sensitivity compared to HPLC-MS. It is even harder to obtain accurate data for protein binding of β-DHA in human plasma at low drug concentration. Although fb of DHA is still inconsistent, there is no doubt that, DHA is a high protein binding drug (fb>80%). Considering all of reasons above, higher sensitivity analysis systems, such as HPLC-MS or HPLC-MS/MS, are better techniques for pharmacokinetic studies. Especially exact data in low concentration or studies on β-DHA in human plasma is needed (In HPLC-MS/MS, the detection limits is about 2ng/ml in human plasma) (30).

### 4.2. Protein binding of dihydroartemisinin in healthy human plasma

The fraction of protein binding was 80%- 84%, using equilibrium dialysis after adjusting for volume shifts and that of 88%-91% in ultrafiltration. At $6^{th}$ hour, the volume in plasma side was 1.1ml and 0.9ml in buffer side. Plasma was diluted to approximately 90% of its original concentration. This percentage was almost consistent with the data of 87% shown in the earlier study (35). pH measured in both sides of the chamber in equilibrium dialysis was almost the same (7.4-7.5) before and after equilibrium dialysis. After ultrafiltration, pH in concentrate increased to 9.

Recent publications have also proved that pH upon ultrafiltration experiment cannot be assumed to remain constant (26). This phenomenon can be explained by the equation below:

$$H_2O+CO_2 \longleftrightarrow H_2CO_3 \longleftrightarrow HCO_3 +H \longleftrightarrow CO_3+2H$$

During centrifugation, water in concentrate gradually crosses to filtrate from filter device. This caused the loss of carbon dioxide ($CO_2$) from biological samples with time and induced the pH increases in the concentrate. The pH increase in concentrate might explain the higher binding percentage in ultrafiltration compared to equilibrium dialysis, since at higher pH the fraction of unbound DHA is always less than at lower pH (26). Data indicated that, for

some drugs, 30% or more decease in free fraction was observed if pH increased in plasma (36).

In ultrafiltration, the disadvantage of volume insufficient for drug assay was obvious, especially for determining DHA concentration in concentrate on recovery calculation. The recoveries in ultrafiltration were almost 10% lower than that described in instruction (95% in guide book, Amicon, USA). Recovery lower than 90% should also be considered in protein binding fraction determination using the ultrafiltration method.

Data in this study indicated that the bound fraction was concentration independent when DHA concentration was in the range of 750-2000ng/ml in plasma. This was consistent with the conclusion in the article reported in 2009 (9). However, this article also illustrated that protein binding of DHA was concentration dependent when DHA content in plasma was higher than 2312ng/ml or lower than 462ng/ml (9). Conclusion from the other study proved that, in the albumin binding studies, the free fraction of DHA was not concentrated-depended (data not shown) (7). If the conclusion that DHA is concentration dependent can be proven in future studies, the volume shift must be considered in equilibrium dialysis as the drug concentration is diluted in human plasma side by shift volume effect.

Methods for determining both α-DHA and β–DHA protein binding fraction in human plasma was discussed in an earlier study (8), it suggested using ratio of α-DHA and β–DHA in various solutions to determine not only fb for α-DHA but also for β–DHA. This suggestion should be doubted, since it is not certain that α-DHA and β–DHA obey the same rule on protein binding. Moreover, the ratio for α-DHA and β–DHA is still inconsistent in different studies (7, 12). This may directly induce different Fu after calculation for β–DHA. Considering this, best the way to quantify β–DHA in plasma is still enhancing the sensitivity of the HPLC analysis system.

In a word, when carrying out equilibrium dialysis or ultrafiltration, it is critically important to keep track of pH changes as this will affect the protein binding results. This may be used to explain the inconsistent of Fu in different articles. The final volume of plasma and buffer on each side of the membrane in equilibrium dialysis should also be measured. The Final Fu should be marked by with or without volume shift correction during calculation. If DHA is concentration dependent protein binding drug, plasma dilution may induce Fu change during incubation. New methods with higher sensitivity are expected in pharmacokinetic study of DHA in lower concentration as well as Fu determination of β–DHA in human plasma

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## Reference


1. Himanshu Naik, Dary J Murry, and L. E. Kirsch. Development and validation of a high-performance liquid chromatography-mass spectroscopy assay for determination of artesunate and dihydroartemisinin in human plasma. *Journal of chromatography* 816 (2005) 233-242
2. Steven R. Meshnick. Artemisinin: mechanisms of action, resistance and toxicity. *International Journal for Parasitology* 32 (2002) 1655-1660
3. Igor R.S. Magalhaes, Valquiria A.P. Jabor, Anizio M. Faria. Determination of β-artemether and its main metabolite dihydroartemisinin in plasma employing liquid-phase micro extraction prior to liquid chromatographic-tandem mass spectrometric analysis. *Talanta* 81(2010) 941-947
4. Monica Longo, Sara Zanoncelli, Paola Della Torre. In vivo and in vitro investigations of the effects of the antimalarial drug dihydroartemisinin (DHA) on rats embryos. *Reproductive Toxicology* 22 (2006) 797-810
5. Kevin T. Batty, Peter L. Gibbons, and Timothy M. E. Davis. Short Report: Pharmacokinetics of dihydroartemisinin in a murine malaria model. *The American Society of Tropical Medicine and Hygiene* 78(2008) 641–642
6. Phantip Vattanaviboon, Noppadol Stiritanarakul and Jidapa Ketpirune. Membrane heme as a host factor in reducing effectiveness of dihydroartemisinin. *Biochemical Pharmacology* 64 (2002) 91-98
7. Kevin T. Batty, Kenneth F. llett, Timothy M. E. Davis. Protein binding and α: β anomer ratio of dihydroartemisinin in vivo. *British Journal of Clinical Pharmacology* 57 (2004) 529-533
8. Batty, K.T. Pharmacokinetic studies of artesunate and dihydroartemisinin PhD thesis. (1999)
9. Lisa H Xie, Qigui Li, Jing Zhang. Pharmacokinetics, tissue distribution and mass balance of radiolabeled dihydroartemisinin in male rats. *Malaria Journal* 8 (2009) 112
10. Narendra P. Singh, Henry Lai. Selective toxicology of dihydroartemisinin and holotransferrin towards human breast cancer cells. *Life Science* 70 (2001) 49-56
11. Navaratnam V, Mordi MN, Mansor SM. Simultaneous determination of artesunic acid and dihydroartemisinin in blood plasma by HPLC for application in clinical pharmacology studies. *Journal of Chromatography* B 692 (1997) 157-162
12. K. Na-bangchang, K. Congpuong, L. N. Hung, and P. Molunto. Simple high-performance liquid chromatographic method with electrochemical detection for the simultaneous delectation of artesunate and dihydroartemisinin in biological fluids. *Journal of Chromatography* 708 (1998) 201-207
13. Stephen A Ward, Esperanca P seven, and Lan M Hastings. Antimalarial drugs and pregnancy: safety, pharmacokinetics, and pharmacovigilance. *Lancet infect discovery* 7 (2007) 136-144
14. K Na-Bangchang, S Krudsood, and U Silachamroon. The pharmacokinetics of oral dihydroartemisinin and artesunate in healthy thai volunteers. *Southeast Asian j trop med public health* 35 (2004) 575-582
15. Kevin T. Batty, Le Thi Anh Thu, Kenneth F. llett, Nguyen Phuc Tien. A pharmacokinetic and pharmacodynamic study of artesunate for vivax malaria. *The American society of tropical medicine and hygiene* 59 (1998) 823-827
16. Gabrielle Holmgren. Plasmodium falciparum resistance to amodiaquine in monotherapy and in combination therapy with artesunate, PhD thesis. *Karolinska Institute* (2010)
17. H.Y. Myint, E.A. Ashley, N.P.J. Day, and Nostena. Efficacy and safety of dihydroartemisinin-piperaquine. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 101 (2007) 858—866
18. George L Trainor. The importance of plasma protein binding in drug discovery. *Drug Discovery* 2 (2007) 51-64
19. Younggil Kwon. Handbook of essential pharmacokinetics, pharmacodynamics, and drug metabolism for industrial scientists. (2001)

20. C.G. Thomas, S.A. Ward, G. Edwars. Selective determination, in plasma, of artemether and its major metabolite, dihydroartemisinin, by high-performance liquid chromatography with ultraviolet detection. *Journal of chromatography* 583 (1992) 131-136
21. Claude Meistelman, Dan Benhamou, Jerome Barre. Effect of age on plasma protein binding of sufentanil. *Anesthesiology* 72 (1990) 470-473
22. J.A. Johnson, T.N. Livington. Differences between blacks and whites in plasma binding of drugs. *Europe Journal Clinical Pharmacol*ogy 51 (1997) 485-488
23. Beresford Stock, Margaret Dean, Gerhard Levy. Serum protein binding of drugs during and after pregnancy in rats. *The Journal of Pharmacology and experimental therapeutics* 212 (1979) 264-268
24. Claudia Herforth, Anura L. Jayewardene, Terrence F. Blaschke. Determination of nelfinavir free drug concentrations in plasma by equilibrium dialysis and liquid chromatography/ tandem mass spectrometry: important factors for method optimization. *European Journal of pharmaceutical sciences* 15 (2002) 185-195
25. M. Andrew Levitt, John B Sullivan, S. Michael Owens Amitriptyline. Plasma protein binding: effect of plasma PH and relevance to clinical overdose. *American Journal of emergency Medicine* 4 (1986).
26. Christopher J. Kochansky, Daniel R. McMasters, Ping Lu. Impact of pH on plasma protein binding in equilibrium dialysis. *Molecular Pharmaceutics* 5 (2008) 438-448
27. Chrisian Bruun Kristensen, Lars F. Gram. Equilibrium dialysis for determination of protein binding of imipramine- evaluation of a method. *Acta pharmacol* 50 (1982) 130-136
28. Mats A. L. Eriksson, johan Gabrielesson, and Lars B. Nilsson. Studies of drug binding to plasma proteins using a variant of equilibrium dialysis. *Journal of pharmaceutical and biomedical analysis* 38 (2005) 381-389
29. Karbwang, K. Na-bangchang, P. Molumnto. Determination of artemether and its major metabolite, dihydroartemisinin, in plasma using high-performance liquid chromatography with electrochemical detection. *Journal of Chromatography* B 690 (1997) 259-265
30. Yuanchao Guo, Qing Li, and Victor Melendez. Comparison of HPLC with electrochemical detection and LC-MS for the separation and validation of artesunate and dihydroartemisinin in animal and human plasma. *Journal of chromatography* 867 (2008) 213-218
31. M. Rajanikanth, K. P. Madhusudanan, R. C. Gupta. An HPLC-MS method for simultaneous estimation of α, β- arteether and its metabolite dihydroartemisinin in rat plasma for application to pharmacokinetic study. *Biomedical chromatography* 17 (2003) 440-446
32. Kevin T. batty, Timothy M.E. Davis, Le Thi Anh Thu. Selective high-performance liquid chromatographic determination of artsunate and α, β dihydroartemisinin in patients with falciparum malaria. *Journal of chromatography* 677 (1996) 345-350
33. Danielle Colussi, Carole Parisot, Francois Legay. Binding of artemether and lumefantrine to plasma and erythrocytes. *European Journal of Pharmaceutical Science* 9 (1999) 9-16
34. Tough Gordi, Elisabet Nilsen, Zuoxiang Yu. Direct analysis of artemisinin in plasma and saliva using coupled-column HPLC with a restricted –access materials pre-column. *Journal of Chromatography*B 742 (2000) 155-162
35. Michael J. Banker, Tracey H. Clark, John A. Williams. Development and validation of a 96-well equilibrium dialysis apparatus for measuring plasma protein binding. *Journal of pharmaceutical science* 92 (2003) 967-974
36. O.Brors, S.Jacobsen. PH lability in serum during equilibrium dialysis. *Br.J.clin.Pharmac*. 20 (1985) 85-88