MONITORING OF ADHERENCE TO THE 14-DAY COURSE OF PRIMAQUINE IN PATIENTS WITH *PLASMODIUM VIVAX* MALARIA: APPLICATION OF LIQUID CHROMATOGRAPHY MASS SPECTROMETRY (LC/MS/MS) FOR DETERMINATION OF PRIMAQUINE IN DRIED BLOOD SPOT SAMPLES

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การติดตามการรักษาด้วยยาไพรมาควินในช่วงเวลา 14 วันในผู้ป่วยติดเชื้อพลาสโมเดียมไวแวกซ์ : การประยุกต์ใช้ลิควิดโครมาโทกราฟี-แมสส์สเปคโทรเมตรีสำหรับการวิเคราะห์หายาไพรมาควินในตัวอย่างเลือดแห้ง

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ไพรมาควินเป็นยาต้านมาลาเรียเพียงชนิดเดียวที่รักษาการกลับเป็นซ้ำของโรคมาลาเรียในผู้ป่วยมาลาเรียชนิดไวแวกซ์ได้ ซึ่งต้องใช้ระยะเวลารักษานาน 14 วัน การยึดหมั่นต่อการรักษาของผู้ป่วยจึงส่งผลต่อประสิทธิภาพในการรักษา ดังนั้นการประเมินการยึดมั่นต่อการรักษาด้วยไพรมาควินจึงมีความสำคัญ การศึกษานี้มีวัตถุประสงค์เพื่อพัฒนาและทดสอบความใช้ได้ของวิธีการวิเคราะห์ความเข้มข้นของไพรมาควินจากหยดเลือดแห้งบนกระดาษกรอง (DBS) โดยใช้เครื่องโครมาโทกราฟีชนิดของเหลวร่วมกับการวิเคราะห์มวลสาร (LC-MS/MS) เพื่อเป็นเครื่องมือในการประเมินการยึดมั่นต่อการรักษาด้วยไพรมาควิน การเตรียมตัวอย่างโดยการสกัดตัวทำละลายอินทรีย์ และแยกผ่านคอลัมน์ C_{18} (50 x 4.6 มิลลิเมตร) ด้วยวัฏภาคเคลื่อนที่ประกอบด้วย 0.1% กรดฟอร์มิคและเมทานอล (80:20 โดยปริมาตร) ที่อัตราการไหล 0.5 มิลลิลิตร/นาที และเกิดเป็นไอออนโดยเทคนิคอิเล็กโทรสเปรย์ และวิเคราะห์การแตกตัวเป็นไอออนของโมเลกุล จำนวน 2 คู่ (multiple reaction monitoring) ซึ่งตรวจวัดไอออน 243.3 จากการแตกตัวของไพรมาควินไอออน 260.1 และไอออน 233.1 จากการแตกตัวของไพริเมทามีนไอออน 249.1 ความเข้มข้นต่ำสุดของการทดสอบเชิงปริมาณคือ 1 นาโนกรัมต่อมิลลิลิตร มีความถูกต้องและความแม่นยำสูง (ภายในวันเดียวและระหว่างวัน) มีประสิทธิภาพการสกัดมากกว่าร้อยละ 80 และความเข้มข้นของไพรมาควินจากตัวอย่างเลือดครบส่วนและหยดเลือดแห้งบนกระดาษกรองมีความสอดคล้องกัน (r = 0.991) จึงเป็นเครื่องมือที่เหมาะสมในการประเมินความยึดมั่นของผู้ป่วยมาลาเรียต่อการรักษาด้วยไพรมาควิน การประเมินการยึดมั่นของผู้ป่วยต่อการรักษาด้วยไพรมาควินจากระดับความเข้มข้นของไพรมาควินในหยดเลือดแห้งบนกระดาษกรอง แบบสอบถาม และการสัมภาษณ์ พบว่าการรักษาโรคมาลาเรียชนิดไวแวกซ์ด้วยคลอโรควิน 3 วัน ร่วมกับไพรมาควิน 14 วัน มีประสิทธิภาพในการรักษาร้อยละ 100 หลังติดตามการรักษานาน 42 วัน และการวิเคราะห์ความเข้มข้นไพรมาควินจากหยดเลือดแห้งบนกระดาษกรองของผู้ป่วยในวันที่ 3, 7 และ 14 ของการรักษา พบว่าอัตราการยึดมั่นของผู้ป่วยต่อการรักษาด้วยไพรมาควินร้อยละ 95-98

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ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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ANURAK CHEOYMANG: MONITORING OF ADHERENCE TO THE 14-DAY COURSE OF PRIMAQUINEIN PATIENTS WITH *PLASMODIUM VIVAX* MALARIA: APPLICATION OF LIQUID CHROMATOGRAPHY MASS SPECTROMETRY (LC/MS/MS) FOR DETERMINATION OF PRIMAQUINE IN DRIED BLOOD SPOT SAMPLES. ADVISOR: ASST. PROF. KANCHANA RUNGSIHIRUNRAT, Ph.D., CO-ADVISOR: PROF. KESARA NA-BANGCHANG, Ph.D., 81 pp.

Primaquine (PQ) is only antimalarial drug for treatment relapse malaria in vivax malaria patient. The long treatment course of primaquine (14 days) related to limits of patient's compliance which affect to treatment efficacy. Therefore, There are monitoring of adherence to the 14-day course of primaquine in vivax malaria patients. This study aims to the develop and validate a liquid chromatography-mass spectrometry method for determination of primaquine in dried blood spot (DBS) samples. Sample preparation was performed by two-step liquid-liquid extraction with organic solvents. The separation was carried out on a reversed-phase column (50 x 4.6 mm.I.D.) with the mobile phase consisting of 0.1% formic acid and methanol (80:20, v:v) running at a flow rate of 0.5 ml/min. The mass spectrometry was operated with positive electrospray ionization and multiple reactions monitoring (MRM) mode. The ion transitions of PQ and IS were 260.1 m/z to 243.3 m/z and 249.1 m/z to 233.1 m/z, respectively. The limits of quantification of PQ were 1 ng ml−1. Good precision and accuracy (both within-day and day-to-day assays) with good linearity (r^2>0.997) and high recovery were over 80 %. Excellent correlation (r = 0.991) was observed between the analysis of PQ in paired whole blood and DBS samples. Patients' adherence to primaquine therapy was assessed based on primaquine concentrations in finger-prick dried blood spot (DBS) samples and interview questionnaires. Results suggest that the 14-day primaquine when given as an anti-relapse, together with a 3- day blood schizontocidal chloroquine, remains an effective treatment for *P. vivax* infection in this area with a cure rate of virtually 100% during the 42-day follow-up. Based on the analysis of primaquine concentrations in DBS collected from patients on days 3, 7, and 14 of treatment, adherence rates of as high as 95-98% was observed.

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Student's Signature .....
Advisor's Signature .....
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LIST OF ABBREVIATIONS

APCI	-	Atmospheric pressure chemical ionisation
API	-	Atmospheric pressure ionization
AUC0-t	-	The Area under the concentration-time curve to the time of the last quantifiable concentration
AUC0-∞	-	The Area under the concentration-time curve extrapolated to infinity
CI	-	Chemical ionization
$C_{L/F}$	-	Apparent total body clearance
C_{max}	-	Maximum concentration
$C_{min\text{-}ave}$	-	The Average concentration at steady state.
$C_{trough\text{-}obs}$	-	The maximum observed concentration
$C_{ss\text{-}ave}$	-	The average steady-state drug concentration
CV	-	Coefficient of variation
CQ	-	Chloroquine
DBS	-	Dried blood spots
DMV	-	Deviation of mean value
DNA	-	Deoxyribonucleic acid
e.g.	-	for exsample
et al	-	and other
ESI	-	Electrospray ionisation
FDA	-	US food and drug administration
g	-	Gram
$H3O^+$	-	Hydronium ion (Water protonated)
$(H2O)^nH+$	-	Water protonated
HPEC	-	High performance capillary electrophoresis
HPLC	-	High performance liquid chromatography
h	-	Hour(s)
ICH	-	International conference of harmonization
IPP	-	Intermediates isopentenyl pyrophosphate

kPa	-	Kilopascal
Kg	-	Kilogram
LC	-	Liquid chromatography
LC-MS	-	Liquid chromatography-mass spectrometry
LC-MS/MS	-	Liquid chromatography - tandem mass spectrometry
LOD	-	Limit of detection
LOQ	-	Limit of quantification
LLOQ	-	Lower limit of quantification
M	-	Molar
MΩ/cm	-	Mega ohms/centimeter
mg	-	Milligram
min	-	Minute(s)
ml	-	Milliliter
mm	-	Millimeter
MRM	-	Multi Reaction Monitoring
MS	-	Mass spectrometry
m/z	-	Mass to charge ratio
ng	-	Nanogram
nl	-	Nanolittre
nm	-	Nanometer
O.D.	-	Optical Density
PCR	-	Polymerase Chain Reaction
pH	-	Potential of Hydrogen ion
PQ	-	Primaquine
PYR	-	Pyrimethamine
QC	-	Quality control
r	-	The correlation coefficient
r^2	-	The coefficient of determination
Rs	-	Resolution
RSD	-	Relative standard deviation
S	-	Slope

SNR	-	Signal-to-noise ratio
SD	-	Standard deviation
SPE	-	Solid phase extraction
SRM	-	Selected reaction monitoring
$t_{1/2}$	-	Elimination half life
$T_{1/2z}$	-	The terminal phase elimination half-life
t_{max}	-	Time to reach maximum concentration
UV/UV-VIS	-	Ultraviolet and visible
µA	-	Micro angstrom
µg	-	Microgram
µl	-	Microliter
V	-	Volt
$V_{Z/F}$	-	Apparent volume of distribution
v/v	-	Volume ratio
WHO	-	World health organization
°C	-	Degree Celsius

CHAPTER I
INTRODUCTION

Background

Malaria remains one of the major global public health problems. The most recent Malaria Report in 2013 found an estimated 3.3 billion people at risk, 198 million confirmed cases, and 584,000 deaths, of which 90% occurred in Africa and an estimated 437 000 died are African children below fifth years olds (World Health Organization, 2014). Five Plasmodium species infected in humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi*. Efforts have been made by various authorities and research groups for effective malaria control particularly by the Global Malaria Eradication Program, the World Health Organization (WHO) (World Health Organization, 2012). Malaria has been completely eliminated in low levels of transmission and good health care infrastructure areas. Case management is important for malaria control to reduce morbidity and mortality by using rapid diagnosis and effective management of acute clinical periods with antimalarial drugs (World Health Organization, 2012). Chemotherapy with effective antimalarial drugs plays an important role either in the phase of the control program to reduce transmission and in the later stage to maintain interruption of transmission, prevent re-introduction of malaria, and eliminate the last residual foci of infection.

In Thailand, the proportion of *P. vivax* infection has been increasing in the past decade. In 2012, 32,231 microscopically-confirmed cases of malaria were reported, of which 35.8% and 54.3% were due to *Plasmodium falciparum* and *Plasmodium vivax* (World Health Organization, 2013). Although rarely fatal, the persistence of its dormant stage hypnozoite in the liver, can provoke relapse, weeks, months, or even years after the initial infection (Baird and Rieckmann, 2003). This makes patients' adherence to medication a potential problem for the elimination of the latent hypnozoite reservoir after symptoms subside. Primaquine is currently the only antimalarial drug available for eradicating the hypnozoite stage of *P. vivax* to prevent the disease from recurring (John, *et al.*, 2012). Occasional failure of the standard

primaquine therapy to prevent relapse has been observed, but drug resistance has not been confirmed (Duarte, *et al.*, 2001; Jelinek, *et al.*, 1995; Kitchener, *et al.*, 2000; Schwartz, *et al.*, 2000; Smoak, *et al.*, 1997; Spudick, *et al.*, 2005; Yi, *et al.*, 1998). In Thailand, the drug is prescribed to all adult patients with *P. vivax* infection at a dose of 15 mg base (0.25 mg base/kg body weight) given daily for 14 days starting on the first or second day of the blood schizontocide chloroquine (25 mg base/kg body over 3 days) (Vijaykadga, *et al.*, 2004). Relatively high relapse rates of > 18% within 1-6 months were reported in several studies in Thailand following this standard regimen (S. Pukrittayakamee, *et al.*, 2004). The shorter course of 7 days with higher doses of 30 or 60 mg reduced the relapse rates to 12 and 4%, respectively (S. Pukrittayakamee, *et al.*, 2010). However, the application of these short course regimens may be limited by concerns over the potential for severe intravascular hemolysis. The problem with long-course therapy of 14 days on the other hand, is compliance after clinical symptoms have been abated. Patients' adherence to treatment therefore, remains one of the cornerstones of successful treatment. The consequence of non-adherence to antimalarial medication does not only adversely affect the health of the individual patient, but also the dramatic spreading of *P. vivax* resistant strains. This consequence would complicate the control of the infection and in addition, increase the financial cost for public health services. To ensure clinical effectiveness of primaquine in the unsupervised 14-day primaquie course, monitoring patients' adherence to medication is necessary.

A number of tools have been applied for monitoring patients' adherence to treatment (Osterberg and Blaschke, 2005; Pullar, 1991). Clinical assessment and drug level monitoring have been considered as gold standard methods because the questionnaire data can provide marked erroneous results due to bias. The aim of the current study was to assess patients' adherence and clinical effectiveness of the unsupervised standard 14-day primaquine regimen when given together with a 3-day choroquine in patients with *P. vivax* malaria in a malaria endemic area along the Thai-Myanmar border. Primaquine concentrations on days 3, 7 and 14 determined from finger-prick dried blood spot (DBS) samples were used as markers of non-adherence, alongside patients' self-reporting on drug administration as well as pill counting

methods. The analytical method for determination of primaquine in DBS samples was developed and validated before applying to clinical samples.

Research gap

The problem with long-course therapy of the 14 days course of primaquine as an antirelapse for *P. vivax* is patients' adherence to medication. This would significantly affect successful control and effectiveness of the drug when used in combination with the standard 3-days course of chlrooquine. Practical approach/tool for monitoring of patients' adherence to primaquine course is necessary for application in the malaria field settings.

Research Question

1. Is the LC-MS/MS method developed, a sensitive, accurate, and reliable analytical method for determination of primaquine in DBS samples using 80 µl blood?

2. Is whole blood concentrations of primaquine measured using dried blood spot (DBS) samples is a reliable tool for monitoring patients' adherence to a 14 days course of primaquine?

Research Hypothesis

1. The LC-MS/MS method developed is a sensitive, specific, accurate, and reliable analytical method for determination of primaquine in DBS samples using 80 µL blood.

2. Whole blood concentration of primaquine measured using DBS samples is a reliable tool for monitoring patients' adherence to a 14 days course of primaquine.

Objective

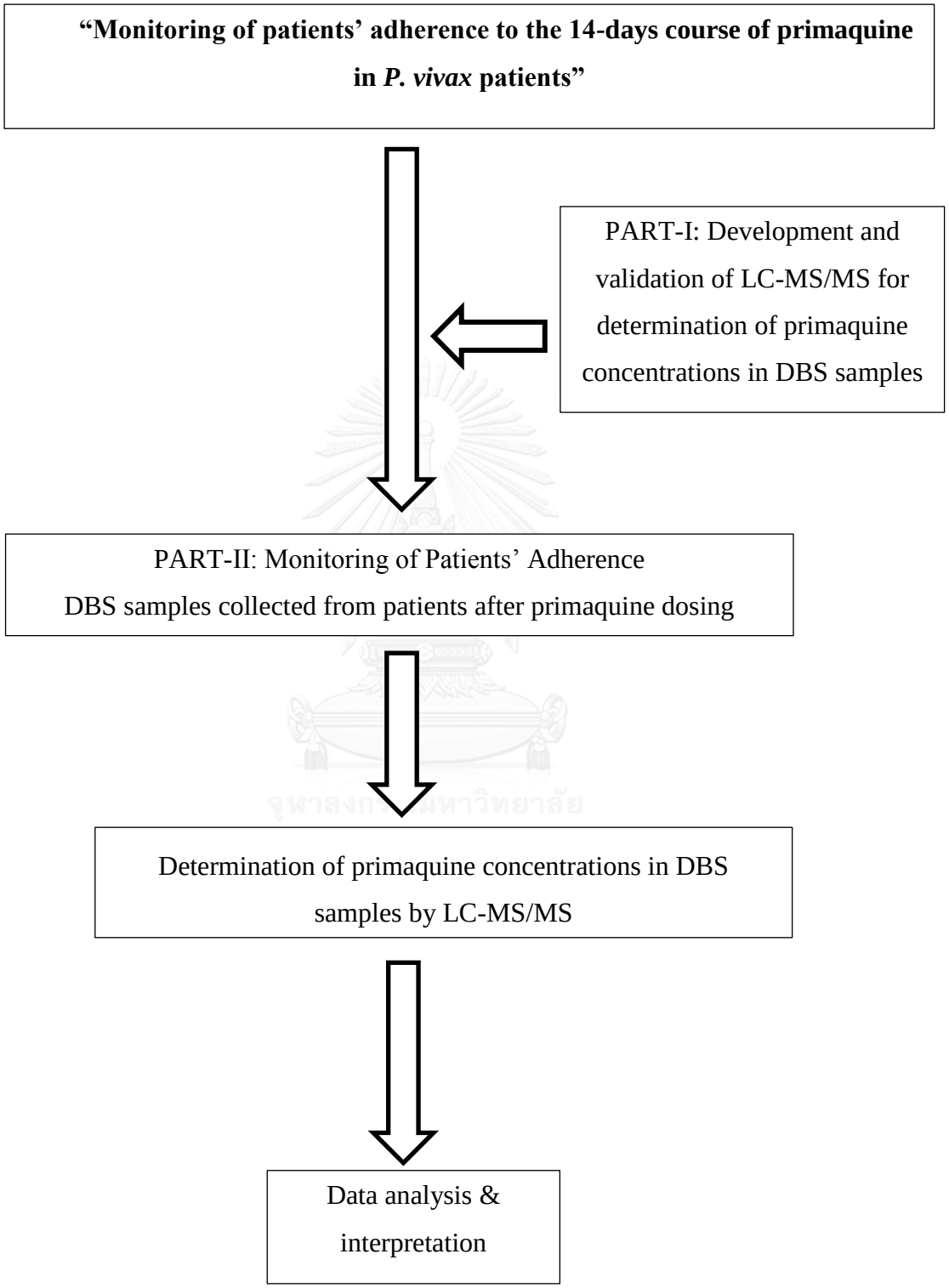
The objective of the study is to assess patients' adherence and clinical effectiveness of the unsupervised standard 14-day primaquine regimen when given together with a 3-day choroquine in patients with *P. vivax* malaria in a malaria endemic area along the Thai-Myanmar border. Primaquine concentrations on days 3, 7 and 14 determined from finger-prick dried blood spot (DBS) samples are used as markers of non-adherence, alongside patients' self-reporting on drug administration as well as pill counting.

Specific objectives include:

1. To establish a sensitive, specific, accurate, and reliable analytical method for determination of primaquine in blood samples collected onto filter paper (DBS) using liquid chromatography mass-spectrometry (LC/MS/MS)

2. To apply the established assay method for monitoring of patients' adherence to the 14 days course of primaquine in *P. vivax* malaria patients.

Conceptual Framework



CHAPTER II
REVIEW OF RELATED LITERATURES

1. Malaria

Malaria is a tropical disease caused by the protozoa parasites infection, *Plasmodium* and a importance global public health problem. It is prevalence in tropical and subtropical regions, including parts of the Americas (22 countries), Africa, and Asia., There are new malaria approximately 250 million cases and causing the deaths approximately 860,000 cases in each year (World Health Organization, 2012). The mortality rate of malaria diseases is between 20-50%. More than 40% of the world's population is at risk of malaria. In Thailand, malaria is found mostly in the western of Thailand especially in province on the Thai-Myanmar border. Annually, there is malaria infection approximately 100,000 cases and around 800 cases dead due to this infection. Malaria has lifelong effects on cognitive development, education and productivity levels. Indeed, the most virulent of the disease, caused by the parasite *Plasmodium falciparum* and *Plasmodium vivax* are also the commonest of the disease in this area due to transmission that is associated with forest where is breeding sites of Anopheles that is malaria parasites vector (Somboon, *et al.*, 1998) Moreover, there are movement of population of migrant or refugees, socioeconomic factors (Butraporn, *et al.*, 1995; Fungladda, *et al.*, 1998) and high degree of drug resistance malaria (Wernsdorfer, 1994).

1.1 Malaria Parasite

Malaria is an infection of the blood caused by parasitic protozoa in the genus *Plasmodium*. There are five types of *Plasmodium* species infected human as malaria, *Plasmodium vivax, Plasmodium malariae*, *Plasmodium falciparum Plasmodium ovale* and *Plasmodium knowlesi*. The most serious forms of the disease are caused by *Plasmodium falciparum* and *Plasmodium vivax*, but other related species (*Plasmodium ovale* and *Plasmodium malariae*) can also infect humans. This

group of human pathogenic *Plasmodium* species is usually referred to as malaria parasites.

1.2 Malaria life cycle

The ecology of malaria involves 2 types of host, humans and female mosquito (Anopheles). The malaria life cycle started during a blood meal of malaria-infected mosquito, the sporozoites are injected with the mosquito's saliva into human host. Sporozoites grow and multiply into schizonts in the liver cells, which release merozoites to blood stream. In *P. vivax* and *P. ovale*, a dormant stage (hypnozoites) can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later. After the merozoites release from the liver cells, the merozoites infect red blood cells to undergo asexual multiplication. In the red blood cell, the ring stage trophozoites mature into schizonts and break red blood cell to release merozoites that continue the cycle by infecting the other red blood cell. Some parasites differentiate into sexual erythrocytic stages (gametocytes). Blood stage parasites are responsible for the clinic manifestations of the disease. In Figure 1 (World Health Organization, 2008) There are two forms as female (macrogametocytes) in the sexual blood stages (gametocytes) that are picked by a female anopheles mosquito during a blood meal. At the same time the microgametes penetrate the macrogametes to generate zygotes in the mosquito's stomach. The zygotes become elongated and motile (ookinetes) to invade the midgut wall of the mosquito where they develop into oocysts. These oocysts grow and release sporozoites to the mosquito's salivary glands. The sporozoites is inoculated into a new human host.

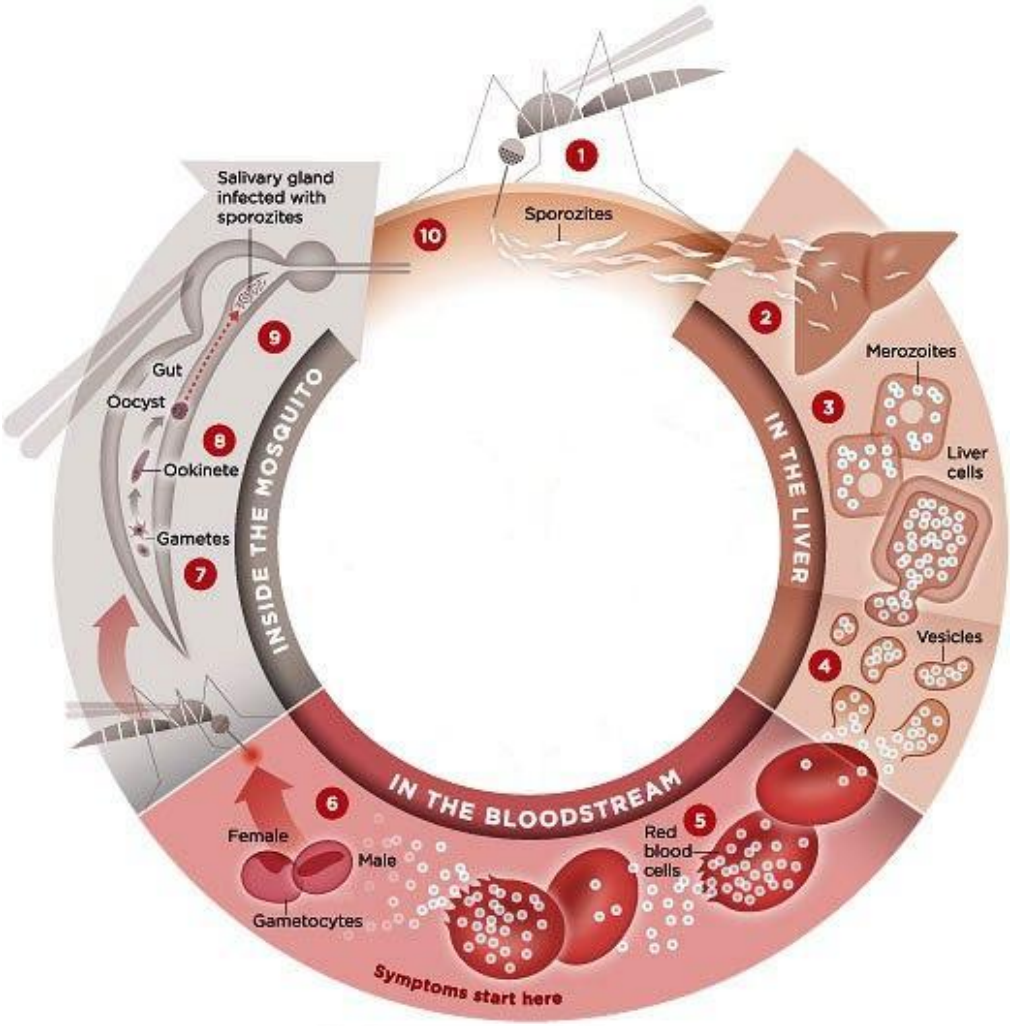


Figure 1 Malaria lifecycle (Malaria Vaccine Initiative, 2013)

1.3 Malaria symptoms

Malaria symptoms usually develop during 10 to 35 days depending on *plasmodium* species after mosquito bite and inject its saliva mix with the parasites to the human blood stream and invade the red blood cells. In red blood cells the parasites grow and multiply. After they have matured, the infected red blood cells break to release the parasites to invade other red blood cells as a result to heamolysis and hemoglobinuria. Its main cause of the malaria symptoms includes fever, shivering, arthralgia (joint pain), vomiting, anemia, and convulsions. There may be the feeling of tingling in the skin, particularly with malaria caused by *P. falciparum*. The classical symptom of malaria is cyclical occurrence of sudden coldness followed by rigor and then fever and sweating lasting 4 to 6 h, occurring every two days in *P. vivax* and *P. ovale* infections, while every 3 days for *P. malariae*. *P. falciparum* can have recurrent fever every 36-48 h or a less pronounced and almost continuous fever. The parasites mature and the red blood break due to jaundice (yellowing of the skin or whites of the eyes) and this is often accompanied by anemia.

1.4 Malaria diagnosis

Malaria diagnosis is determination of malaria infection by identifying *Plasmodium* parasites or antigens/antibodies in patient blood. Malaria diagnosis is essential for effective malaria treatment Although this may seem simple, the diagnostic efficacy is subject to many factors (Sutherland and Hallett, 2009).

1.4.1 Symptomatic diagnosis

The symptom are used to diagnosis malaria in areas where cannot afford simple laboratory diagnostic tests. They often use a history of patient fever as the indication to treat malaria. There is one study have been reported clinical predictors (rectal temperature, nail bed pallor and splenomegaly) were used as treatment indications, rather than using only a history of patient fevers, a correct diagnosis increased from 21% to 41% of cases, and unnecessary treatment for malaria was significantly decreased (Redd, *et al.*, 1996).

1.4.2 Microscopic examination

The microscopic examination remain gold standard testing for diagnosis malaria because it is simple and reliable to identifying specific characteristics in each species of five major malaria parasite. There are 2 types of blood films, Thick films and thin film. Thick film is a larger volume of blood film and allow to measure levels of infection because there are more sensitive than the thin film about 11 times. However the appearance of the parasite is much more distorted therefore it is more difficult to differentiate plasmodium species. The advantage and dis advantage of both thick and thin smears taken into consideration, it is necessary to use both smears to make an accurate diagnosis. (Mirdha, *et al.*, 1997).

1.4.3 Antigen tests

The antigen detection tests is one choice to diagnosis malaria infection especially in areas that microscope is not available, or non-specialized laboratory staffs for using microscopic examination to diagnosis malaria (Pattanasin, *et al.*, 2003). Immunochromatographic tests or antigen detection tests have been developed validated and apply on field. These tests can use both finger-prick blood and venous blood, using shot time for testing around 15–20 minutes, and the results can read visually as the presence or absence of colored stripes on the dipstick therefore suitable for use in the field. The sensitivity of antigen tests is in the range of 100 parasites/µl of blood (about 0.002% to 0.1% parasitemia), It is more sensitive than thick film microscopy 5 times. However the antigen tests are qualitative but not quantitative. They can determine if parasites are present in the blood but can not determine how many.

1.4.4 Molecular methods

Molecular methods are technique to detect biomarker (specific genetic code) in the genome (DNA or RNA) that associated with disease. Molecular diagnostic techniques are high sensitivity and specificity, recent developments in molecular biological technologies, *e.g.* PCR (the polymerase chain reaction), loop-mediated isothermal amplification (LAMP) (Mens, *et al.*, 2006).

PCR offer more sensitivity and specificity than microscopy examination. Moreover, levels of parasitemia are not necessarily correlative with the progression of disease, particularly when the parasite is able to adhere to blood vessel walls. Therefore the diagnosis tools need to be developed to more sensitive for detecting low levels of parasitemia in the field (Redd, *et al.*, 1996). However, it is more expensive and requires a specialized laboratory staff (Bloland, 2001)

2. Antimalarial drugs currently used for treatment of *Plasmodium vivax*

2.1 Chloroquine

Chloroquine (Figure 2a) remains the mainly anti-malarial drug for treatment *of P. ovale*, *P. vivax* and *P. malariae* infections, although chloroquine resistant *P. vivax* have been reported from several areas in the world (Loyola and Rodriguez, 1992; Myat Phone, *et al.*, 1993; Soto, *et al.*, 2001). For eradicating parasite in blood stage, chloroquine is given at 10 mg/kg of base followed by 5 mg/kg of base at 6, 24 and 48 h (total of four doses). The intravenous quinine or quinidine can be used, when oral treatment causes nausea. This regimen generally produces acute symptoms and clearing of parasitemia although a substantial number of relapses subsequently develop (Bunnag, *et al.*, 1994; Looareesuwan, *et al.*, 1997; Wilairatana, *et al.*, 1999). For the prevention of relapse malaria, the primaquine is given at 15 mg daily for 14 days to eradicate hypnozoites of *P.vivax* and *P.ovale* in liver stage. All antimalarial treatment for *P. vivax* malaria in normal glucose-6-phosphate-dehydrogenase (G6PD) patient concluse in Table 1 (2010). For A 14-day treatment course is required and adherence to such this long treatment is likelsy to be unsuccess. This coupled with avoid primaquine in patients with G6PD deficiency account for high rate of relapse. Chloroquine resistant isolates can be treated with mefloquine or quinine sulfate plus doxycycline (Inion, *et al.*, 2003).

2.2 Primaquine and other 8-aminoquinolines

Primaquine is anti-malarial drug**,** a member of the aminoquinoline group (Figure 2b) and remain use for treatment malaria and *Pneumocystis pneumonia.* There are four clinical used for treatment malaria including (i) Severe treatment of infection with *P. vivax* or *P. ovale* malaria; (ii) presumptive anti-relapse therapy (PART; terminal prophylaxis) in persons with epidemic area of *P. vivax* or *P. ovale* parasites; (iii) primary prophylaxis against all malaria species; (iv) and gametocytocidal or interrupt transmission agent for *P. falciparum* (Table 2). Primaquine is only anti-malarial drug that is currently used for eradicating the hypnozoites, relapsing liver stage *of P. vivax* and *P.ovale* (Baird, *et al.*, 2003). The mode of action of primaquine and optimal usage are limited. It severely interrupt the metabolic processes of plasmodial mitochondria. The anti-malarial activity is probably attributable to interference with the function of ubiquinone as an electron carrier in the respiratory chain. Another potential mechanism of action against plasmodia is the production of highly reactive metabolites that generate toxic intracellular oxidative potentials (Hill, *et al.*, 2006).

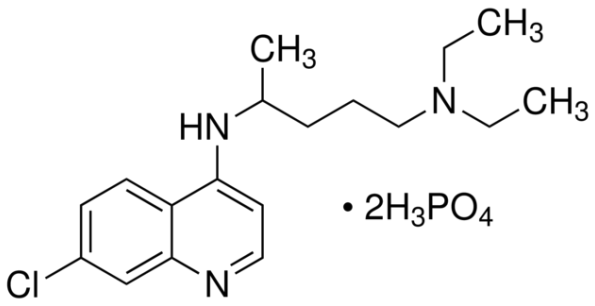
Table 1 Antimalarial treatment for *P. vivax* malaria

Drugs	Adult dose	Pediatric dose
Areas with chloroquine-sensitive *P. vivax, P. ovale and P. malariae*		
Chloroquine phosphate	1 g (600 mg base), then 500 mg (300 mg base) 6 h later, then 500 mg (300 mg base) at 24 and 48 h	10 mg base/kg (max. 600 mg base), then 5 mg base/kg 6 h later, then 5 mg base/kg at 24 and 48 h
plus Primaquine	15 mg base daily × 14d	0.3 mg/kg/d × 14d
Areas with chloroquine-resistant *P. vivax*		
Chloroquine	25 mg base/kg in 3 doses over 48 h	25 mg base/kg in 3 doses over 48 h
plus Primaquine	30 mg base daily × 14d	0.6 mg/kg/d × 14d
Quinine sulfate	650 mg q8h × 3-7d	30 mg/kg/d in 3 doses × 3-7d
plus Doxycycline	100 mg bid × 7d	4 mg/kg/d in 2 doses × 7d
Mefloquine	750 mg followed 12 h later by 500 mg	15 mg/kg followed 12 h later by 10 mg/kg

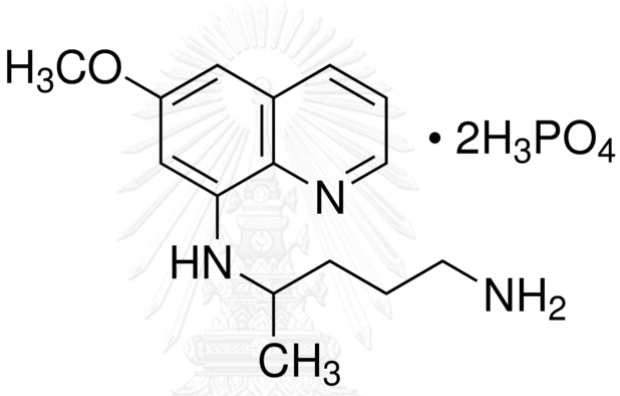
Table 2 Recommended dose regimens for primaquine (Hill, *et al.*, 2006; World Health Organization, 2010)

Indication	**Dose regimen**
Radical cure of *P. vivax* or *P . ovale*	15 mg daily (0.25 mg/kg/d) for 14 days* (World Health Organization, 2010)
Presumptive anti-relapse therapy (PART)	30 mg base (0.5 mg/kg) follow 14 days, started on return from a malarious region and overlapping with a blood schizonticide (Hill, *et al.*, 2006)
Primary prophylaxis for all malaria species	30 mg (0.5 mg/kg) daily begun 1-2 days before traveling, continued once daily during staying in endemic area, and continued for 7 days after returning (World Health Organization, 2010)
Transmission blocking agents for *P. falciparum*	45 mg once per week for 8 weeks

**Although effective treatment for preventing relapse with P. vivax from many areas of the world, some strains of P. vivax (principally found in Southeast Asia and South Pacific) may not be eradicated at this regiment.*



(a)



(b)

Figure 2 Chemical structures of (a) chloroquine and (b) primaquine

2.1.1 Radical cure

Primaquine is only anti-malarial drug sed to treat the *P. vivax* or *P. ovale* malaria that they are hypnozoites remain in the liver after it has been released into the bloodstream, which must be eradicated by given a 14 day course of primaquine (Baird and Rieckmann, 2003). This process is called a radical cure. If patients do not receive primaquine for preventing *P. vivax* or *P. ovale* relapse infection, there is a very high risk of relapse within weeks or months or sometimes years after initial infection. The doses for radical cure are : 30 mg once daily for 14 days (Baird and Hoffman, 2004) for *P. vivax* and 15 mg follow 14 days for *P. ovale.*

2.2.2 Primary prophylaxis

Primaquine is not routinely anti-malarial drug for preventing malaria in travelers, but it is only used as such when no other alternatives are appropriate. Primaquine is the recommended prophylactic drug by the CDC in some instances. (Steinhardt, *et al.*, 2011)

2.2.3 Interruption of transmission

Primaquine has high potent and rapid to gametocytocidal activity of *P. falciparum*. Primaquine treatment can eradicate the asexual blood stage and the sexual blood stage of *P. falciparum* in the same time, so it appropriate to reduce transmission in rare transmission areas. However the dose-response relationship of action is not clear and possible toxicity, it should be careful before given primaquine to patients.

2.2.4 Pneumocystis pneumonia

Primaquine is also used for the *Pneumocystis pneumonia* treatment, a fungal infection commonly found in people with AIDS and, more rarely, in those taking immunosuppressive drugs. it is usually combined with clindamycin for increasing effectively of pneumocystis pneumonia treatment

.

2.2.5 Pharmacokinetics

Primaquine is rapidly absorbed in the gastrointestinal tract and concentrated in the liver, brain, heart, lungs, and skeletal muscle. It crosses the placenta. The mean volume of distribution (V_d) is 3 L/kg. Primaquine is highest concentration (C_{max}) in plasma ~70 mg/mL and maximum time (T_{max}) with in 1- 3 h after administer medication. It is rapidly excreted in urine with a plasma half-life ($t_{1/2}$) of 4-9 h. Its metabolism is complex and poorly understood (Brueckner, *et al.*, 2001). The many its metabolites has not been reported about the activity against Plasmodium parasite.

2.2.6 Adverse reactions

The common side effects of primaquine during treatment is nausea, vomiting and stomach cramps and the other adverse effects that occasionally occur are headache, visual disturbances and intense itching. In addition, the severe adverse effect of primaquine is cause of hemolysis in patients with glucose-6-phosphate dehydrogenase deficiency (G6PD) (Hill, *et al.*, 2006).

Normally, primaquine induces methemoglobinemia and hemolytic anemia in all patients, but G6PD patients can occur severe levels of methemoglobinemia (Fernando, *et al.*, 2011).

3. Medication adherence

The medical development and technology, the drugs discovery and development including new medical innovation lead to more effective treatment However, medical adherence (compliance) remains importance factor for patient outcome.

In term of “medication adherence” WHO is definition that the extent to which a person’ s behavior—taking medication, following a diet, or making healthy lifestyle changes—corresponds with agreed-upon recommendations from a health-care provider (World Health Organization, 2003). Often, the terms adherence and compliance are used interchangeably. However, their meaning is relatively different.

adherence implies the patient's agreement with the recommendations, whereas compliance implies patient passivity.

3.1 Incident of non-adherence

Poor-adherence is a important worldwide problem. It is key factor related with the effectiveness medicinal therapies especially, critical for long time treatment for chronic conditions. WHO report published, adherence to long time treatment rates average about 50%. It is lower in developed countries, (World Health Organization, 2003).

3.2 Measurement of adherence

Adherence of medication has been monitored since the time of Hippocrates, they record the effects of various potions with symbols that the patient had taken them or not. Even today, patients' self-reports continue to be a simply and effectively measure adherence method (Haynes, *et al.*, 1980). There are many methods available for measuring adherence which are divided into direct and indirect methods, each method has advantages and disadvantages, however no method is considered the gold standard (Alcoba, *et al.*, 2003; Wagner, *et al.*, 2001).

3.2.1 Direct measurement adherence methods

Direct measurment adherence method, is approach to measure the concentration of the drug or its metabolites directly from biological samples (blood or urine or saliva) of patients. In addition, A measuring a biologic marker that added into the drug formulation, is used one approach for measuring adherence. The concentration of these medication can reflect to adherence level of patient directly and effective dose strengths. For examples, the serum concentration of antiepileptic drugs such as phenytoin or valproic acid. Although, direct method is a good and commonly used to assess adherence. These approaches are expensive, require professional staff, and risk to non-cooperate by the patient.

3.2.2 Indirect measurement adherence method

Indirect methods for measuring adherence, are many approach such as assessing clinical response, measuring physiological markers, performing pill counts, using electronic medication monitors, collecting patient questionnaires, asking the patient to keep a medication diary, and asking the help of a caregiver, school nurse, or teacher for assessing children's adherence including patients' self-reports. All methods are very easy to use. However, Questioning the patient for a questionnaire and interviews, patient diaries, and asking patients self-report can be easy to bias and result in the health care provider's overestimating the patient's adherence.

The clinical response as a measure is confounded by many factors other than adherence to a medication that can account for clinical outcome. Other than patient questioning, pill counts (counting the number of pills, capsule and bottles or vials for the patient's medication) is the most common method for measuring adherence. Due to the simplicity and empirical nature, this method are interest to investigators. However, patients can switch medicines between bottles or vials and discard pills or capsule before visits for following the regimen. Therefore, pill counting method should not appropriate to be a good measure of adherence (Rudd, *et al.*, 1988). In addition, this method can not provides information of taking medications, such as dose timing and drug holidays (taking dose interval), which is important to determine clinical outcomes.

4. Chromatography methods for determination of drug and its metabolite.

4.1 Liquid chromatography (LC)

Liquid chromatography (LC) is analytical chemical technique, it is used commonly for separation, qualification, and quantification of chemicals or drugs (McDowall, 1989). The principal of separation is based on the interaction of interested analyte with the stationary phase (packing material in the column) and the mobile phase (the liquid flow through the column). Example, the reversed phase LC, a non-polar packing material is used stationary phase and the mobile phase consists of an aqueous organic solvent mixture that contains the sample solution including

interested analyte, flows through the column. The compounds in the sample solution are chemically bonded to the non-polar packing material in the column. The bond strength depends on degree of polar in each compound. So the most polar compounds will be eluted first when the components of mobile phase decrease polarities. The polarity can be useful to predict the compounds elution order in reversed phase LC

4.2 The liquid chromatography system

High performance liquid chromatography (HPLC) system consists: A solvent reservoir with the mobile phase. A high-pressure pump is important part of LC system; it is used to generate a specified flowing mobile phase deliver in all part of the system. An injector takes the sample into the flowing mobile phase that carries the sample into the HPLC column. The column contains the packing material needed to separate the compounds mixture in the sample. A detector is used to determine the eluted compound from the HPLC column in form of the electrical signal. A integrator is used to records the electrical signal and generate the chromatogram to identify and quantitate the concentration of the sample. All components are shown in Figure 3. Since properties of each compound are very different, a suitable detectors is importance. For example, a UV- detector for compound that is sensitivity of ultraviolet light absorption property. Like with a fluorescence detector for compound that is emitted-fluorescence light property and a more universal type of detector such as a mass spectrometry detector for the compound does not have either of these characteristics.

4.3 Detector systems

4.1.1 UV/UV-VIS Detectors

UV/UV-VIS detector is used commonly to analysis compounds and drugs by measuring an absorption spectrum of the ultraviolet or visible region. Detection is based on the differentiation of UV light absorption in each compound at a specific wavelength (Figure 4). The Detection is performed by UV light is irradiated on a clear color-less glass cell (the flow cell) that contains compound or drug and determine the intensity of UV light after pass the flow cell in term of electrical

signals. The intensity are difference depend on the property of each compound. Thus, The different of absorption spectrum of each compound is used to measure the amount of sample. The UV absorbance also differs depend on what wavelength is used, it relates sensitivity and specificity, a suitable wavelength of the type of compound is importance.

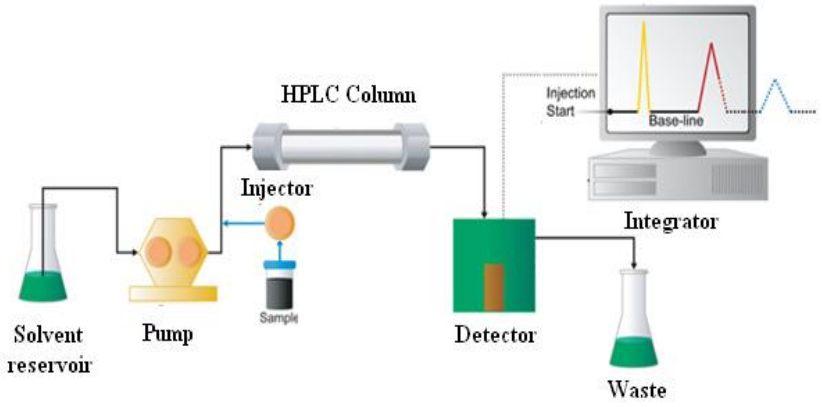


Figure 3 High performance liquid chromatography (HPLC) system **(Waters, 2014)**

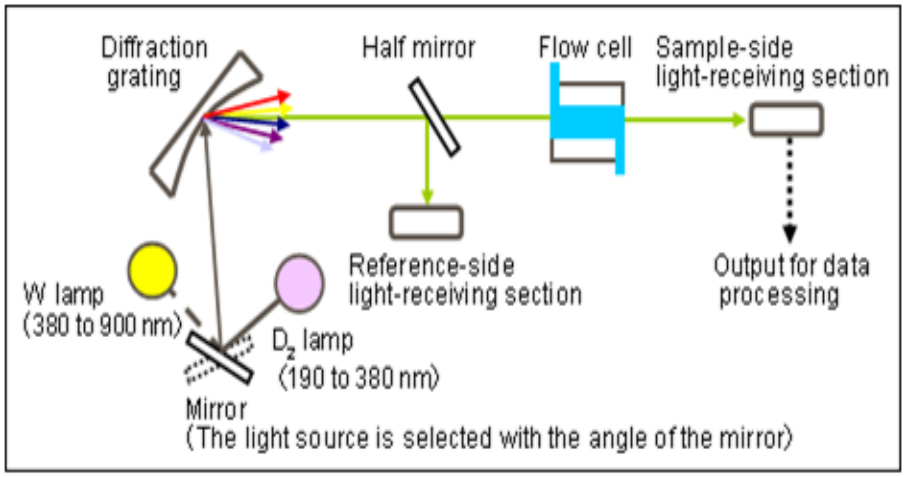


Figure 4 Diagrammatic illustration of a UV-VIS detector optical system (Hitachi High-Technologies Corporation, 2003)

4.3.2 Fluorescence Detector:

The principle of fluorescence detector is a phenomenon that the UV light is absorbed at one wavelength by some compounds, and emits light at another wavelength. The emitted light is fluorescence. A compound absorbs light until a high-energy level after that emits light to return to its original energy level. Moreover each compound has specific wavelengths to absorb (excitation wavelengths) and emits (emission wavelengths). Examples, fluorescent light at the bottom of fireflies.

4.3.3 Mass spectrometry Detector:

Mass spectrometry is a powerful technique for qualitative and quantitative includes identifying unknown compounds, determining the isotopic composition of elements in a molecule and determining the structure of a compound by observing its fragmentation. The quantifying the amount of a compound in a sample or studying the fundamentals of gas phase ion chemistry separating and identifying molecules. Mass spectrometry principle based on determines the mass of a molecule by measuring the mass-to-charge ratio (m/z) of its ion. Ions are generated by inducing either the loss or gain of a charge from a neutral species. Ions are electrostatically directed into a mass analyzer where they are separated according to m/z and finally detected. The result of molecular ionization, ion separation, and ion detection is a spectrum that can provide molecular mass and even structural information. A mass spectrometer 3 consist modules (Figure 5):

(1) An ion source, which can convert gas phase sample molecules into ions (In the case of electrospray ionization, move ions that exist in solution into the gas phase)

(2) Mass analyzer, which sorts the ions by their masses by applying electromagnetic fields

(3) A detector, which measures the value of an indicator quantity and thus provides data for calculating the abundances of each ion present

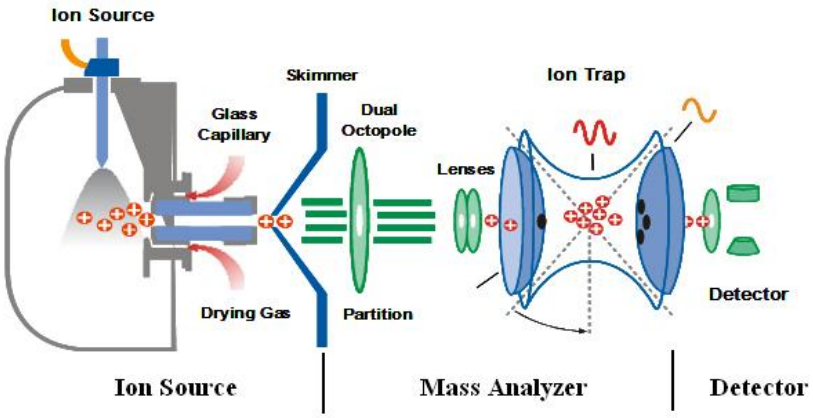


Figure 5 Diagrammatic illustration of a mass spectrometry system (Bruker Daltonics, 2013)

5. Determination of primaquine in biological fluids

Many analytical methods have been reported for measuring PQ and its metabolite carboxyprimaquine (CPQ) in biological fluids: plasma, urine, red blood cells. (Baker, *et al.*, 1985; Dua, *et al.*, 1996; Ward, *et al.*, 1985). There are two studies have been conducted using high-performance liquid chromatography (HPLC) with electrochemical detection of both analytes simultaneously, following protein precipitation with acetonitrile (Dean, *et al.*, 1994; Kim, *et al.*, 2004). Recovery and LOQ of primaquine and carboxyprimaquine were 75% and 90%, and 5 µg/L and 20 µg/L, respectively with a run time more than 30 min (Dean, *et al.*, 1994).

There pharmacokinetic studies of primaquine and its principal metabolite, carboxyprimaquine, have used a HPLC method with liquid–liquid extraction and UV detection (Elmes, *et al.*, 2006; Mihaly, *et al.*, 1985; Ward, *et al.*, 1984). In the most recent studies, the analytes were extracted separately, requiring more than 750 µL of plasma, and the limits of quantitation (LOQs) for primaquine and carboxyprimaquine were 5 µg/L and 25 µg/L, respectively (Binh, *et al.*, 2009; Cuong, *et al.*, 2006).

LC-MS/MS assays offer high specificity and high sensitivity more than immunoassays or conventional HPLC. Moreover, it requires less amount of sample for analysis, but have the disadvantages of complexity of operation, require special staff and high cost of instrumentation. Liquid chromatography mass spectrometry method has been reported that Liquid chromatography tandem mass spectrometry for measuring primaquine with solid phase extraction from human plasma (Dua, *et al.*, 1996) and Liquid chromatography with time-of-flight detection (LC–MS/TOF) for the measuring primaquine in spiked simian, murine and human plasma (Avula, *et al.*, 2011; Nitin, *et al.*, 2003)

Although blood sample is necessary for Clinical pharmacology studies of PQ and its main metabolite, it obtained from invasive technique and requires expert staff. Currently, there is alternative biological fluid sampling as the use of capillary sampling blood from a finger prick spot on filter paper. Filter paper sampling has been proposed to collect blood in hard conditions, especially in remote areas or poor countries (Kolawole and Mustapha, 2000). It is called “dried blood samples” (DBS),

this sampling is less invasive than a venipuncture and non-require s expert staff. Furthermore, it is a cost-effective choice for both sampling and storage during transport to the laboratory. Several methods have been published on the use of DBS for various anti-malarial drug assays, including quinine (Kolawole and Mustapha, 2000), pyrimethamine (Minzi, *et al.*, 2005), and chloroquine (Cheomung and Na-Bangchang, 2011).

CHAPTER III
METHODOLOGY

1. Research Design

The research design is a cross-sectional study in a total of 85 patients with *Plasmodium vivax* infection. The LC-MS/MS method for determination of primaquine in dried blood spot (DBS) samples was established and applied for monitoring of patients' adherence to the 14-day course of primaquine in patients with *P. vivax* infections.

2. Study Population

The study will be conducted at Mae Tao clinic for migrant workers, Mae Sot district, Tak province, Thailand on March 2008 - February 2009. The study area is situated approximately 100 km west of Bangkok near the Thai-Myanmar border. Malaria is a serious imported medical problem in this area with peak incidence during May-August and November-January of each year. Most patients are adult males and approximately 45-50% of malaria cases are caused by *P. vivax*. All age groups are affected and nearly all the *P. vivax* cases are symptomatic (Muhamad, *et al.*, 2011). The study was approved by the Ethics Committee of the Department of Disease Control, Ministry of Public Health of Thailand. Written informed consents were obtained from all the patients before study participation.

3. Sample size

The estimated 1% prevalence of malaria in Thailand (World Health Organization, 2013) so the estimate prevalence of *P. vivax* malaria is 0.5%. The appropriate sample size was calculated according to the follow formula

$$n = \frac{z_{\alpha/2}^{\ 2} pq}{d^2}$$

Where; p = The prevalence = 0.05

q = 1- p = 0.05

d = Allowable error = 0.05

$Z_{\alpha/2}$ = Probability of type I error = 1.96

$$n = \frac{(1.96)^2(0.05)(0.95)}{(0.05)^2}$$

$$n = 72.99$$

Give the 0.5 % prevalence of vivax malaria in Thai population, The least of target sample size is 72.99 that is sufficient at the precision and confidence level of 95% and significant level (α) of 5%. Therefore, the target sample size for the study is at least 75 patients.

4. Sampling Technique

Simple random sampling technique are used to select samples from the retrospectively storage blood samples (Muhamad, *et al.*, 2011).

Inclusion Criteria

1. Patients with microscopically confirmed mono-infection with *P. vivax* malaria.
2. No symptom of severe disease.
3. No anti-malarial drug during the preceding 4 weeks.

Exclusion Criteria

1. Pregnancy.
2. History of G6PD deficiency and dark urine or significant hemoglobinuria.

5. Measurement Tools

This study use Liquid Chromatography-Mass Spectrometry (LC-MS/MS) for measuring primaquine concentration from vivax malaria infected dried blood spot samples.

Liquid Chromatography-Mass Spectrometry (LC-MS/MS) is a advance technique for separation, qualitative, and quantitative any interest compound or chemical from biological fluid such as whole blood, plasma and dried blood spot (DBS) that high sensitivity and high specificity. Developed LC/MS/MS method will be validated in term of sensitivity, linearity, recovery, precision and accuracy for monitoring primaquine.

Part-I: Establishment of Analytical Methods for Determination of Primaquine in Whole Blood and Dried Blood Spot Samples

In this study, method development was to develop simple, rapid, specific and sensitive LC-MS/MS method for determination of primaquine from dried blood spot. Extraction procedures are simple, short and use small amounts of reagent and solvent.

1. Chemicals

Primaquine (PQ) and the internal standard pyrimethamine (PYR) were obtained from Sigma-Aldrich Inc. (St. Louis. MO, USA). All solvents are HPLC grade, were purchased from Fisher Scientific Equipment (Bishop Meadow Road, Loughborough, UK). Hydrochloric acid and diethylamine were obtained from Sigma Chemical (St. Louis, M.O., USA). Ultrapure analytical grade Type I water (r >18 MΩ/cm) was prepared by using a Milli-Q PlusTM water system (Millipore Corporation, Bedford, MA, USA) and be filtrated with membrane 0.45 micron.

2. Standard stock solutions:

PQ and PYR were prepared stock solutions by dissolving appropriate amounts of PQ and PYR with distilled water in volumetric flasks. Stock solutions were prepared at 1,000 ng/μl. The working solutions were prepared at 2 ng/μl for

PQ and 30 ng/μl for PYR by diluting the stock solutions. Stock solutions and Standard solutions were stored at -80°C until use.

3. Chromatography:

For PQ assay in whole blood, the analytical method was developed and validated on a LC-MS/MS system consisting of the solvent delivery (AgilentTM 1260 Infinity Quaternary Pump VL G1311C), equipped with solvent degasser (AgilentTM 1260 Infinity G1322A), an auto-sampler (AgilentTM 1260 Infinity ALS G1329B). The separation was carried out on a reversed-phase column (Thermo Hypersil Gold C18, 250 X 4.6 mm i.d., 5 μm, Fisher Scientific, CA, USA). The mobile phase consisted of acetonitrile and 0.25 % diethylamine (3:7, v/v) (pH 3.0) running at the flow rate of 1.0 ml/min. The chromatographic analysis was operated at 30 ºC. The detection system used was a UV DAD detector (Agilent™ 1260 DADVL G1315D) set at the wavelength of 263 nm

For PQ assay in DBS samples, the method was developed on a LC-MS/MS system consisting of the solvent delivery (Agilent^{TM} 1260 Infinity Quaternary Pump VL G1311C), equipped with solvent degasser (Agilent^{TM} 1260 Infinity G1322A), an auto-sampler (Agilent^{TM} 1260 Infinity ALS G1329B). The separation was performed through a reversed-phase column. The mobile phase was optimized the ratio of methanol and 0.1% formic acid to associate with flow rate and injection volume. The detection system was Mass Specmeter (AB SCIEX QTRAP$^{®}$5500) couple with Ion Source (Turbo V™ source and Electrospray Ionization; ESI). Ion source parameter and ion analyzer were optimized to suitable for measuring of PQ from dried blood spot (DBS).

The separation, LC C_{18} revers phase column was selected to associate with a LC-MS/MS system and mobile phase. The mobile phase were optimized to suitable for separating of primaquine and IS from impurity. Variable mass ion were chosen to suitable for primaquine and pyrimethamine (IS) base on high sensitivity and specificity.

4. Sample Preparation

Sample preparation was procedures to remove interfere and to concentrate sample. The extraction procedures were optimized to simple and given highest recovery without interferes at eluted times.

Whole Blood

The procedure was validated on specimens using 500 µl of spiked human whole blood. Human whole blood was obtained from the Blood Bank of Thammasat Chalermprakiet Hospital and was kept frozen in aliquots at −20 ºC until use. To 150 µl of whole blood, 3,000 ng/ml of IS was added. After mixing, 1 ml of 2 M sodium hydroxide and 5 ml of hexane and tert-butyl methyl ether (9:1, v:v) were added and gently mixed for 30 min on a rotating mixer. The organic phase (supernatant) was separated by centrifugation at 1,300 x g for 30 min (4 ºC). The supernatant was transferred into a clean silanized glass tube and evaporated to dryness under nitrogen stream at 37 ºC. The residue was reconstituted with 100 µl of the mobile phase and 80 µl injected into the column.

Dry Blood Spot, DBS

The procedure was developed and validated on specimens spiked dried blood spot (DBS). Outdated human whole blood were obtained from the Blood Bank of Thammasat Chalermprakiet Hospital, and were stored frozen in aliquots at -20ºC. Dried blood spot (DBS) was prepared by spotting 80 µl of PQ-free blood sample onto filter paper (Whatman No 3, GE Healthcare, CA, USA). Each dried blood spot (DBS) was prepared by cutting in small piece and put in a 15 ml screw-cap glass tube. The buffer were selected and optimized for eluting blood from filter paper and PYR (internal standard) was added. Adjusted pH until PQ is in neutral form and move from aqueous phase to organic phase by adding appropriate basidic solution and Organic solvent. The extraction procedures were selected and optimized to suitable for measuring of PQ from Dried Blood Spot (DBS).

5. Calibration curves

Whole Blood

Calibration curves were prepared by replicate analysis of seven whole blood samples (150 µl each) at vary concentrations of (25, 50, 100, 250, 500, 1,000 and 1,500 ng/ml) of PQ and a fixed concentration of the internal standard (3,000 ng). Calibration curves samples were conducted by using the ratio of peak height of PQ and IS as y-axis against x-axis that primaquine concentration (described below).

Dry Blood Spot, DBS

Standard curve was prepared by replicate analysis of ten DBS (80 µl of capillary blood each) at vary concentrations of (1, 2, 5, 10, 25, 50, 100, 250, 500, and 1,000 ng/ml) of PQ and a fixed the IS concentration. Calibration curve covered a expected concentration. Calibration curves samples were observed the peak height of PQ and IS for conducting calibration curves as described below.

6. Data analysis

A calibration curve was generated regression equation by conducting a linear regression relationship between concentration and the peak height ratio (peak height of PQ/peak height of IS), obtained from calibration sample. Concentrations of PQ in DBS samples were calculated by the regression equation, where X was the concentration of the PQ in ng/ml, and Y was the peak ratio of the PQ. Conducting chromatogram, integrating interested peaks, conducting the calibration curve fitting and calculating sample concentrations were performed by using Analyst® 1.6 software.

7. Method validation

Method validation is procedures to assess the developed LC-MS/MS method that it is suitable for establishment method by following to the procedures described in ICH guidelines Q2 (R1) (ICH harmonized tripartite guideline, 2005). The validation characteristics were evaluated: linearity, accuracy, precision, limited of qualification and quality control.

Linearity

Linearity was estimated by preparing DBS (80 µl of capillary blood each) at concentrations range 1 - 1,000 ng/ml of PQ and a fixed the IS concentration. All sample were injected into the LC-MS/MS system to assess linearity by determining peak heights of each sample and plotting a linear regression relationship between concentration and the peak height ratio (peak height of PQ/peak height of IS). The coefficients of determination (r^2) should to be more than 0. 99.

Precision

The precision based on repeatability (within-day) were evaluated by preparing six sets of triplicate DBS samples spiked with six various concentrations of PQ. DBS samples were determined at the concentrations of 2.5, 75 and 750 ng/ml in one day. The reproducibility (day-to-day variation) was evaluated by using the same concentration of DBS samples. Each concentration set was performed on each day for six days. All of concentration level were calculated the Coefficient of variation (CV) by using the ratios of standard deviation (SD) from mean of PQ concentration to of PQ concentration in each concentration level. The precision will be reported as the percentage of Coefficient of Variation (%CV)

$$\%\text{CV} = \frac{\text{standard deviation from mean of PQ concentration}}{\text{mean of PQ concentration}} \times 100$$

The precision, the coefficient of variation (%CV) at each concentration level should not exceed 15%

Accuracy

Accuracy was evaluated by preparing six sets of triplicate DBS samples spiked with six various concentration of PQ. DBS samples was performed at the concentrations of 2.5, 75 and 750 ng/ml., respectively in one day for the within-batch. The between-batch was evaluated by using the same concentration of DBS samples but each set of concentrations were performed on each day for six days. All of concentration level were calculated the accuracy by comparing the difference between spiked value and actually found (theoretical value). Accuracy was reported as the percentage deviation of mean value from theoretical value (%DMV)

$$\%\text{DMV} = \frac{(\text{Measured PQconcentration} - \text{True PQ concentration})}{\text{True PQconcentration}} \times 100$$

The accuracy, the deviation of mean value from theoretical value (%DMV) should be within ±15% of the actual value except.

Recovery

The recovery is used to estimate the sample preparation procedures by preparing triplicate analysis of PQ. DBS samples was performed at the concentrations (low, med, high) of 2.5, 75 and 750 ng/ml and distilled water will be prepared in same concentration. All of the peak heights will be calculated % recovery by using the ratios of Peak height from the extracted spiked PQ and peak height from the un-extracted spiked PQ. The recovery was reported as the percentage of recovery

$$\%\text{Recovery} = \frac{\text{Peak height from the extracted spiked PQ}}{\text{Peak height from the unextracted spiked PQ}} \times 100$$

The matrix effect

The matrix effect was determined by comparing the peak heights of the post-extracted spiked PQ with the standards of PQ prepared in the mobile phase, respectively. The assays were performed at the concentrations (low, med, high)

of 2.5, 75 and 750 ng/ml in five replicate analyses. The matrix effect was reported as the percentage of matrix effect

$$\%\text{matrix effect} = \frac{\text{Peak height of the post} - \text{extracted spiked PQ}}{\text{Peak height of PQ in the mobile phase}} \times 100$$

Selectivity

The selectivity was verified by comparing chromatograms between spiked-PQ DBS and spiked-PQ DBS with interfere such as commonly used drugs, *i.e.*,antimalarials (mefloquine, artemisinin, chloroquine), anthelminthics (albendazole,), paracetamol and dimenhydrinate. All compounds should be separated from the analyzed peak (PQ) by $Rs \geq 2$.

Limit of quantification

The limit of quantification (LOQ) of PQ in a DBS sample (80μl) was estimated by observing the lowest concentration to generate a signal to noise ratio(S/N) ≥ 10 at verify precision and accuracy pass accepted criteria.

Stability

The stability of PQ in DBS samples were estimated by storing one set of triplicate spiked DBS samples 2.5, 75 and 750 ng/ml in a plastic zipper bags with a desiccant pack at room temperature and other set were kept in -20ºC until analysis for one month. Concentrations were measured periodically (1, 15 days, 1, and 3 month) and were calculated the stability by comparing the difference between spiked value at several period and actually found (theoretical value).

Quality Control

Quality control (QC) samples for PQ were made up in whole blood and DBS samples using a stock solution separate from that used to prepare the calibration curves at the concentrations of 35, 250 and 1,250 ng/ml and 2.5, 75 and 750 ng/ml for whole blood and DBS samples, respectively (triplicate analysis each).

Whole blood samples were aliquotted into cryovials and stored frozen at −80 ºC for use with each analytical run. DBS samples were stored in plastic zipper bags in a desiccator at room temperature (25 ^{o}C). The results of the QC samples provided the basis of accepting or rejecting the run. At least four of every six QC samples had to be within $\pm$15 % of their respective nominal values. Two of the six QC samples could be outside $\pm$15 % of their respective nominal values, but not at the same concentration.

Application of the Analytical Methods for Clinical Samples

The 500 µl whole blood were shared from retrospectively blood sample at Day 0 and during follow up (Day 3, 7, and 14) for determination of primaquine concentrations. For each sample, two aliquots (80 µl each) of blood was spotted onto filter paper (Whatman number 3). DBS samples were stored in a plastic zipper bags with a desiccant pack until analysis. Blood smear film were performed for malaria diagnosis.

The developed and validated LC-MS/MS method was applied to measure PQ from dried blood spot samples and whole blood at D 0, 3, 7, and 14. The concentration of PQ were calculated the correlation between DBS and whole blood.

The methods were applied for the investigation of the concentration- time profiles of PQ in whole blood and DBS samples in a total of ten Burmese patients with *P. vivax* malaria (aged 20–28 years, weighing 50–62 kg) following the administration of a 3-day chloroquine given at a total dose of 2,000 mg and PQ given at 15 mg/kg body weight (base) for 14 days starting from the second day of chloroquine treatment and then once daily until day 14. The study was conducted at Mae Sot Hospital, Tak Province, Thailand, during 2008–2009. Approval of the study protocol was obtained from the Ethics Committee of the Ministry of Public Health of Thailand. Written informed consents for study participation were obtained from all patients. Venous blood samples (2 ml) were collected into heparinized plastic tubes at the following time points: day 0 (0, 1, 6, 12 h), day 1 (0, 1, 6, 12 h), day 2 (0, 1 h), day 3, 6, 7, 10, 14, 21, 28, 35 and 42. Finger-prick capillary blood (for DBS) samples were also collected from each patient at the same schedule. Whole blood samples were stored immediately after collection at −80 ºC until analysis. DBS samples were stored in plastic zipper bags in a desiccator until analysis.

Correlation between PQ concentrations in whole blood and DBS samples was determined from paired samples of whole blood and DBS obtained from all patients.

Part-II: Monitoring of Patients' Adherence to the 14-days Course of Primaquine in Patients with *Plasmodium vivax*

1. Study Site and Patients

The study will be conducted at Mae Tao clinic for migrant workers, Mae Sot district, Tak province, Thailand on March 2008-February 2009. The study area is situated approximately 100 km west of Bangkok near the Thai-Myanmar border. Malaria is a serious imported medical problem in this area with peak incidence during May-August and November-January of each year. Most patients are adult males and approximately 45-50% of malaria cases are caused by *P. vivax*. All age groups are affected and nearly all the *P. vivax* cases are symptomatic (Muhamad, *et al.*, 2011). The study was approved by the Ethics Committee of the Department of Disease Control, Ministry of Public Health of Thailand.

Patients with mono-infection with *P. vivax* malaria of all age groups and genders were included in the study. Inclusion criteria included a parasitemia of 1,000-100,000 parasites/μL blood, with no signs of severe disease, no antimalarial treatment during the preceding four weeks. All participants had residential areas in the Mae Sot district. Written informed consents were obtained from all the patients before study participation.

2. Treatment

Upon diagnosis, all patients were treated with the national standard for *P. vivax* malaria, *i.e.*, a 3-day chloroquine (Government Pharmaceutical Organization of Thailand, 250 mg chloroquine phosphate *per* tablet) given at a total dose of 25 mg base/kg body weight over 3 days (10 and 5 mg/kg at 0 and 6-12 hours on day 0, and 5 mg/kg each on day 1 and day 2) and primaquine (Government Pharmaceutical Organization of Thailand, 15 mg base *per* tablet) given at daily doses of 15 mg base/kg body weight daily for 14 days starting from the second day (day 1) of chloroquine treatment. Chloroquine and primaquine dose administration during the first 3 days (days 0, 1, and 2) were administered with a glass of 250 ml drinking water under the supervision of a medical staff. Patients were closely observed for at least 30 minutes after drug ingestion. Primaquine doses from day 3 to day 14 (12 tablets)

were given to each patient for home treatment and patients were instructed to take medication in the morning before meal. Written instructions were also provided on the plastic zipper bags containing the drugs.

3. Treatment follow-up

All patients were requested to return to the clinic for follow-up in the morning of the fourth day of treatment (day 3), and on days 7, 14, 21, 28, 35, and 42, or at any time whenever signs and symptoms suggestive of malaria occurred. At each visit, a parasite count (from finger-prick blood samples) was performed and a structured questionnaire for general symptom (during the 42 day follow-up) and drug administration (during day 3 to day 14) was recorded. Blood films were stained with Giemsa and examined under a light microscope. Asexual stage *P. vivax* was counted against 1,000 erythrocytes in thin blood films or against 200 white blood cells in thick film. Examination was reported as negative only after at least 200 fields of the thick film had been examined without encountering a parasite.

The classification of treatment outcomes was based on an assessment of the parasitological and clinical outcome of antimalarial treatment according to the World Health Organization guideline (World Health Organization, 2008).

4. Sample collection and determination of primaquine concentrations

Finger-prick blood samples (80 µl) were collected from each patient before primaquine dosing on day 0 (primaquine dose under supervision), and about 2-4 hours after dosing on days 3, 7, and 14 (self-medication of primaquine doses) of the initial treatment for the determination of primaquine concentrations. Samples were spotted onto filter paper (Whatman no. 3, GE Healthcare, CA, USA) and stored in plastic zipper bags in a desiccator until analysis. Concentrations of primaquine in DBS samples were determined using LC-MS/MS according to the method described in Part-I.

Quality control (QC) samples for PQ were made up in whole blood and DBS samples using a stock solution separate from that used to prepare the calibration curves at the concentrations of 35, 250 and 1,250 ng/ml and 2.5, 75 and 750 ng/ml

for whole blood and DBS samples, respectively (triplicate analysis each). Whole blood samples were aliquotted into cryovials and stored frozen at −80 ºC for use with each analytical run. DBS samples were stored in plastic zipper bags in a desiccator at room temperature (25 ^{o}C). The results of the QC samples provided the basis of accepting or rejecting the run. At least four of every six QC samples had to be within ±15 % of their respective nominal values. Two of the six QC samples could be outside ±15 % of their respective nominal values, but not at the same concentration.

5. Assessment of Patients' Adherence to Medication

Patients' adherence to treatment medication was defined as patients' reliability in using an exact prescribed medication, together with the levels of the drug taken within the acceptable limits. Adherence to the 14-day primaquine regimen was assessed based on (i) primaquine concentrations in DBS collected from each patient on days 3, 7, and 14 of treatment; (ii) patients' self-reporting on drug administration, and (iii) pill counting (the number of the remaining tablets on days 3, 7, and 14 of treatment). Box and Whisker plot was applied to identify outlier and extreme primaquine concentrations on days 3, 7, and 14. Outlier and extreme cases of non-adherence were defined as patients who had primaquine concentrations lower than 1.5 and 3 box length apart from the lower limit line of the 1st quartile or 25th percentile (Q1-1.5IQR and Q1-3IQR, respectively).

6. Data analysis (statistical analysis)

The reliability of the analysis of primaquine concentration in whole blood and DBS sample were evaluated using spearman correlation. Box and Whisker plot was applied to identify outlier and extreme primaquine concentrations on days 3, 7, and 14. Outlier and extreme cases of non-adherence were defined as patients who had primaquine concentrations lower than 1.5 and 3 box length apart from the lower limit line of the 1st quartile or 25th percentile (Q1-1.5IQR and Q1-3IQR, respectively). (Na-Bangchang *et al.,* 1997; Congpuong *et al.,* 2010). All test set statistical significance set at $\alpha = 0.05$ (SPSS version 16; SPSS, Chicago, Illinois, USA).

7. Ethical consideration

The study was reviewed and approved by the Ethical Committees for research in human subject, Department of Diseases Control, Ministry of Public Health. Reference No. 3/52-293. Informed written consent was obtained from patient. All results will be kept confidential and be unlinked to any identifying information. Whole blood sample, dried blood spot and blood smear were shared with other researchers for the purpose of investigating the malaria resistance in Thailand.

8. Limitation

None

9. Expected Benefit & Application

The expected benefit of the research is the established analytical methods for determination of PQ in whole blood and dried blood spot samples that are sensitive, specific and reliable. The analytical methods can be applied effectively as a monitoring tool for assessing adherence of patients with the 14-day course of primaquine.

12. Administration & Time Schedule

Research/Project Activities	Time Frame (Month)											
	1	2	3	4	5	6	7	8	9	10	11	12
Literature review	←	—	—	→								
Development and optimization of LC-MS/MS condition for measuring of primaquine in DBS		←	—	—	→							
Validation of LC-MS/MS method for measuring of primaquine in DBS					←	—	—	→				
Application of LC/MS/MS method to measure primaquine concentration from retrospectively DBS sample							←	—	—	→		
Data analysis									←	—	—	→

13. Budget

List	Cost (Baht)
- Standard drug HPLC solvent and chemical	35,000.00
- HPLC column and equipment	30,000.00
- Documentation and data analysis	10,000.00
- Manuscripts and publication	10,000.00
- Miscellaneous	5,000.00
Total	100,000.00

CHAPTER IV
RESULTS

Part-I: Establishment of Analytical Methods for Determination of Primaquine in Whole Blood and Dried Blood Spot Samples

1. LC-MS/MS optimization

The standard PQ and PYR (IS) were injected to a mass spectrometer system. Their compounds were induced to ions with an electrospray ionization (ESI: Turbo V™ Ion Source) in the positive ionization mode. The mono-isotopic masses of PQ and PYR are 259.168 and 248.082, respectively. During Q1 setting up, the masses of their molecular ions were protonated to be 260.1 m/z (parent ion) for PQ and 249.1 m/z (parent ion) for PYR. After that their protonated molecular ions were induced to dissociation to fragment ions using collision energy, the most abundant and stable fragment ions (Product ions) were at m/z 243.3 for PQ and at m/z 233.1 for IS (Figure 6)

Therefore, the MRM transitions of PQ (m/z $260.1 \rightarrow 243.3$) and PYR (m/z $249.1 \rightarrow 233.1$) were selected for the quantitative analysis. All parameters were optimized to the most sensitive ion transitions

(A) (B)

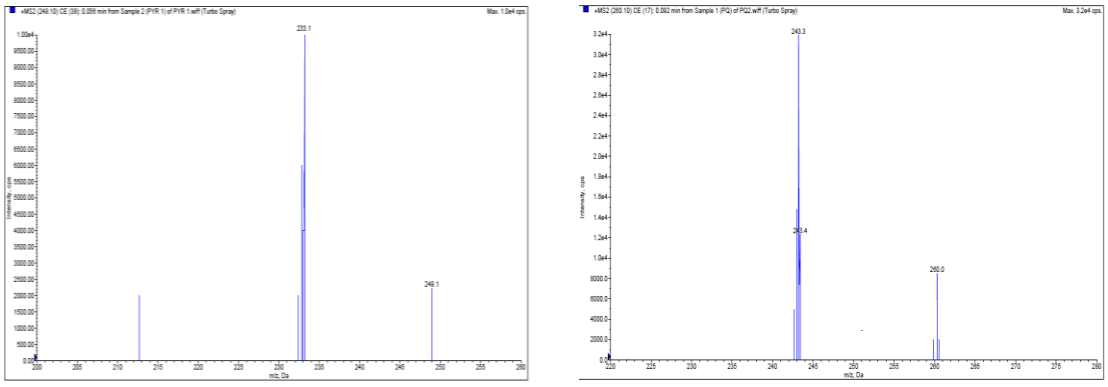


Figure 6 Production spectra on MS/MS of (A) Primaquine at m/z 260.1 to 243.3 and (B) Pyrimethamine at m/z 249.1 to 233.1

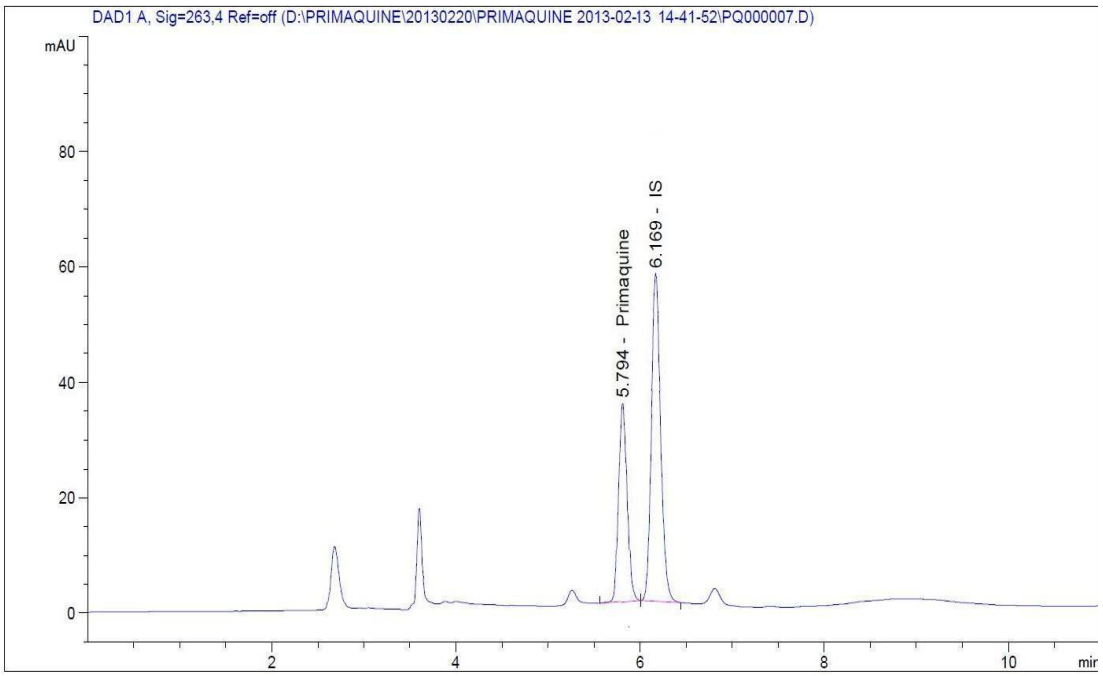
2. Chromatographic Separation

A number of liquid chromatographic systems were investigated to optimize the separation of PQ and the internal standard PYR by HPLC and LC MS/MS. The chromatograms showed a good baseline separation. The chromatogram of the standard solution of PQ and PYR is shown in Figure 7A (HPLC) and 7B (LC-MS/MS). For HPLC analysis, the retention maps were generated for PQ and PYR as a function of stationary and mobile phases. Optimal separation was achieved using the mobile phase consisting of acetonitrile and 0.25 % diethylamine (3:7, v:v) running at the flowrate of 1.0 ml/min and a reversed phase column (5 µm, 250 x 4.6 mm; Thermo Hypersil Gold, Fisher Scientific, CA, USA). The retention times of PQ and PYR were approximately 5.79 and 6.16 min, respectively.

For LC-MS/MS analysis, the LC separation condition was separated on a reversed-phase column (Thermo Hypersil Gold C18, 50 × 2.1 mm i.d., 5 µm, Fisher Scientific, CA, USA) at 30 °C. The optimal mobile phase was achieved from various ratios of solvent (methanol, acetonitrile) and volatile buffer (formic acid, ammonium format, etc.) by observing the efficacy of their MS ionization, the variability of retention time, and the shape of the peak. The best chromatogram was obtained the mobile phase which consist of methanol: 0.1% formic acid; 80:20, v/v running at a flow rate of 500 µl /min with injection volume was 3 µl.

The optimal LC-MS/MS condition resulted in the best shape and resolution of chromatographic peak with highest intensity ionization and a stable retention time. The retention times of PYR and PQ were approximately 2.66 and 2.16 min, respectively.

(A)



(B)

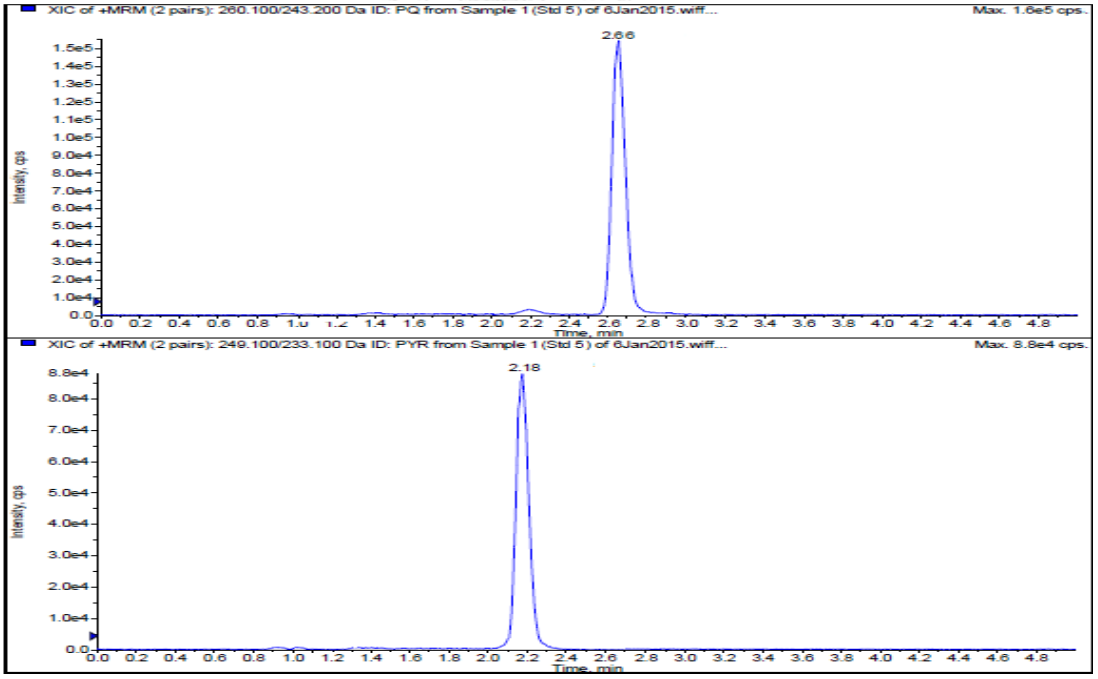


Figure 7 The chromatograms of of PQ and PYR (IS) (A) HPLC (B) LC-MS/MS

3. Sample preparation

The sample preparation in this study, sample preparation procedures was investigated to optimize the extraction of PQ and PYR from whole blood and DBS samples.

For whole blood was simple, involving only a single step liquid-liquid extraction by organic solvents (5 ml of hexane: tertbutyl methylether = 9:1, v:v). After centrifuged at 10,000×g (4°C) for 10 min, the mixture was separated into two phase. The upper organic layer was transferred to a polypropylene tube and evaporated under a stream of nitrogen at 40°C. The residue will be reconstituted with 200 µl of mobile phase

For DBS involved, the optimal sample preparation procedure are two-step extraction. Dried blood spot (DBS) was cut in small piece and placed in a 2.0 ml polypropylene micro centrifuge tube. Primaquine was eluted from filter paper by using 200 µl of methanol and followed by 20% sodium hydroxide, due to PQ and IS are strong base so particle diffuse into organic phase under alkaline condition. Added 1.5 ml of the mixture of hexane and tert-butyl methyl ether (1:1, v/v), After followed whole extraction that descript above. This condition found to be the most optimal condition from the sample as the resulted in a clean chromatogram.

4. Method Validation

4.1 Calibration curve

Whole blood analysis of PQ was calibrated using the concentration range of 25–1,500 ng/ml, whereas DBS sample analysis was calibrated using the concentration range of 1-1,000 ng/ml. All calibration ranges yielded linear relationships with coefficients of determination (r^2) of 0.999 or better.

4.2 Precision

Precision of PQ assays was evaluated as coefficients of variation (%CV) of six replication during six days at low (2.5 ng/mL), medium (75 ng/mL) and high (750 ng/mL) for DBS and 35, 250 and 1,250 ng/ml for whole blood. All coefficients of variation (%CV) (intra-assay and inter-assay) variation of PQ assay was less than 4.74% that are summarized in Table 3

4.3 Accuracy

The accuracy was observed from six replication of tree level of PQ concentrations (2.5, 75 and 750 ng/ml for DBS and 35, 250 and 1,250 ng/ml for whole blood.) both the intra- or interday assays, as indicated by the minimal deviation of mean values found with measured samples from that of the theoretical values (actual amount added), with % DMV. All accuracy (intra-assay and inter-assay) of primaquine assay are lower than 4.08 % at all investigated concentrations (Table 3)

Table 3 Summary of precision and accuracy (intra- and inter assay) of PQ assay in whole blood and DBS samples

Concentration added of primaquine (ng ml^{-1})	Precision (% CV)		Accuracy (%DMV)	
	Within day	Between day	Within day	Between day
Whole Blood				
35	2.25	2.86	2.10	2.28
250	0.72	2.53	-0.33	0.81
1,250	0.89	0.38	-0.69	-0.73
Dried blood spot				
2.5	3.89	4.74	2.33	4.08
75	0.67	1.29	1.18	2.19
750	1.19	1.22	0.83	1.30

4.4 Recovery

The mean recoveries of PQ in whole blood and DBS samples at the investigated concentrations were 78.2-83.8 and 71.8-76.6 %, respectively. The mean recoveries of PYR at the concentration of 3,000 ng/ml (whole blood) and 300 ng/ml (DBS) were greater than 70 %. Since the volume of blood adsorbed on the filter paper was lower than 100 μl and because finger-prick specimens were subject to dilution by interstitial fluid, the recovery of extraction (liquid-liquid) of PQ from DBS samples was considered satisfactory. The results reflected essentially high recovery for all compounds from the spiked whole blood and DBS and indicate a lack of interference from the sample preparation procedure.

4.5 Selectivity

Selectivity of the chromatographic separation was demonstrated by the absence of interference from endogenous peaks and commonly used drugs in whole blood and DBS samples in both spiked samples and samples collected from one patient after 1 h after the first dose of PQ on the first day (Figure 8).

4.6 Matrix Effect

The mean (%CV) absolute matrix effects calculated for PQ at the concentrations of 2.5, 75 and 750 ng/ml were 97.2 (2.16), 96.3 (2.01) and 95.0 (4.40) %, respectively. For the IS PYR, mean (%CV) absolute matrix effect at the concentration of 300 ng was 95.44 (3.78) %. These results indicate comparable responses in the reconstitution solution and DBS extract, and no absolute matrix effect was observed. No ion suppression was observed.

4.7 Limit of Quantification

The limit of quantification (LOQ) of the PQ assay procedure was determined from the lowest concentration of PQ was considered base on a signal to noise ratio, The lowest concentration of primaquine that gave a peak height ten times to the baseline noise Therefor, The limit of quantification (LOQ) of the PQ assay procedure in whole blood and DBS samples was accepted as 25 ng/ml using 500 µl whole blood and 1 ng/ml using 80 µl DBS samples.

4.8 Stability

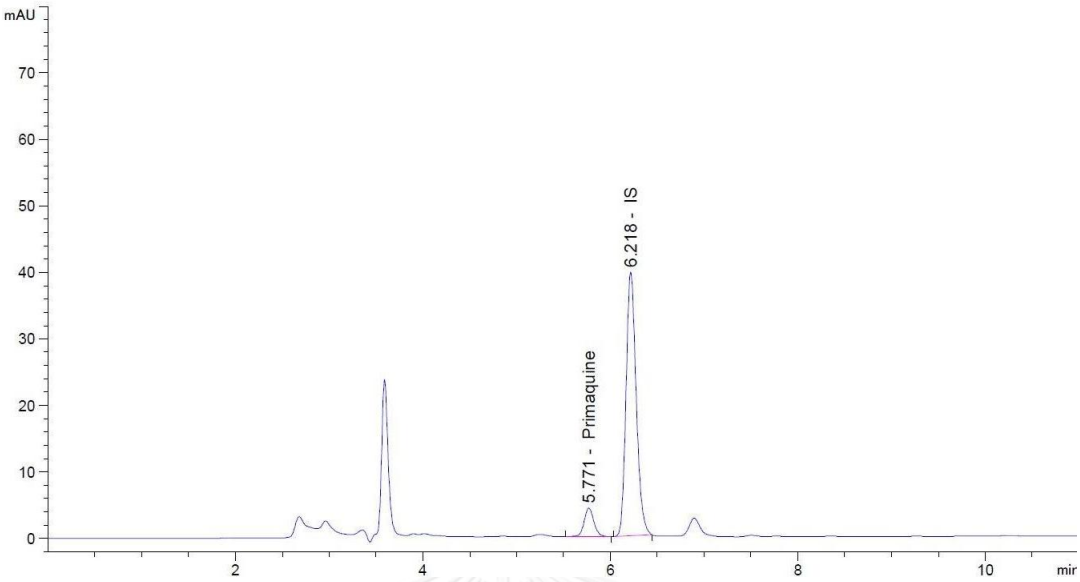
Whole blood samples containing PQ at the concentrations of 35, 250 and 1,250 ng/ml were found to be stable when stored in a −80 ^{o}C freezer for a minimum of 3 months without significant decomposition. DBS samples containing PQ at the concentrations of 2.5, 75 and 750 ng/ml were also found to be stable when stored in plastic zipper bags in a desiccator at room temperature (25 ^{o}C) for a minimum of 3 months without significant decomposition. DBS samples produced enhanced drug stability because of dehydration of the sample on the filter paper and consequent minimization of enzymatic and chemical hydrolyses of the drug under

investigation. Nevertheless, the specimens should be dried at ambient temperature and humidity for at least 3–4 h before storage.

4.9 Application of Assay and Analysis of Clinical Samples

The methods for analysis of PQ in whole blood and DBS samples developed in this study were applied for the analysis of samples collected for a clinical pharmacology study with good accuracy and precision. Figure 8. shows chromatograms of whole blood (analyzed by HPLC) and DBS (analyzed by LC-MS/MS) samples collected 1 h after administration of a single oral dose of 30 mg PQ in one patients with *P. vivax* malaria. Excellent correlation (r = 0.991, Spearman's rank correlation test) between the whole blood and DBS assays was also found. The concentration-time profiles of PQ (Figure 9.) in both types of blood samples following a daily dose of 15 mg/kg body weight PQ for 14 days in Thai patients with *P. vivax* infection were similar to those reported previously (Fletcher, *et al.*, 1981; Kim, *et al.*, 2004).

(A)



(B)

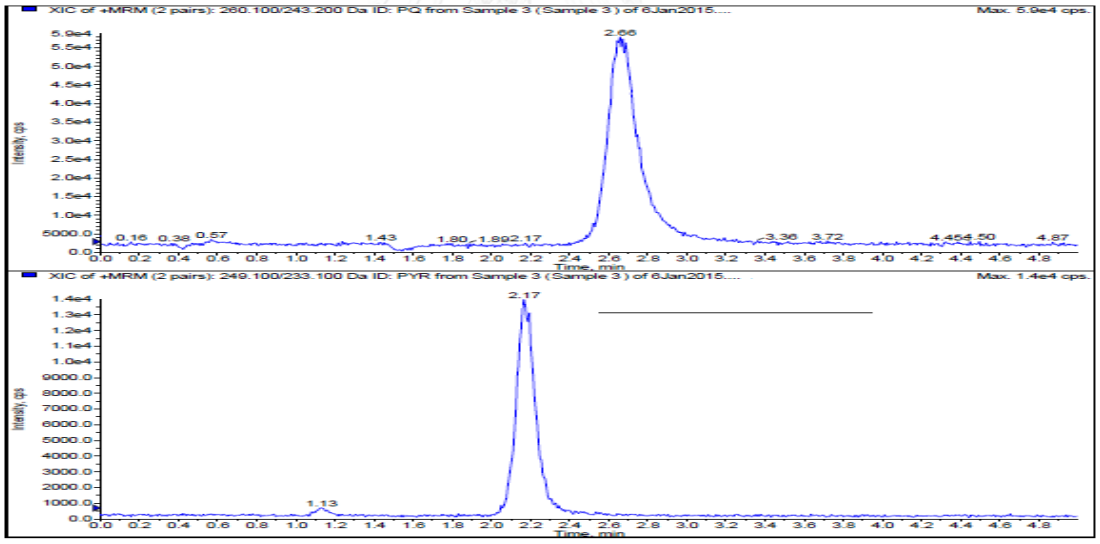


Figure 8 The chromatograms of (A) whole blood (analyzed by HPLC) and (B) DBS (analyzed by LC-MS/MS) samples collected 1 h after administration of a single oral dose of 30 mg PQ in one patients

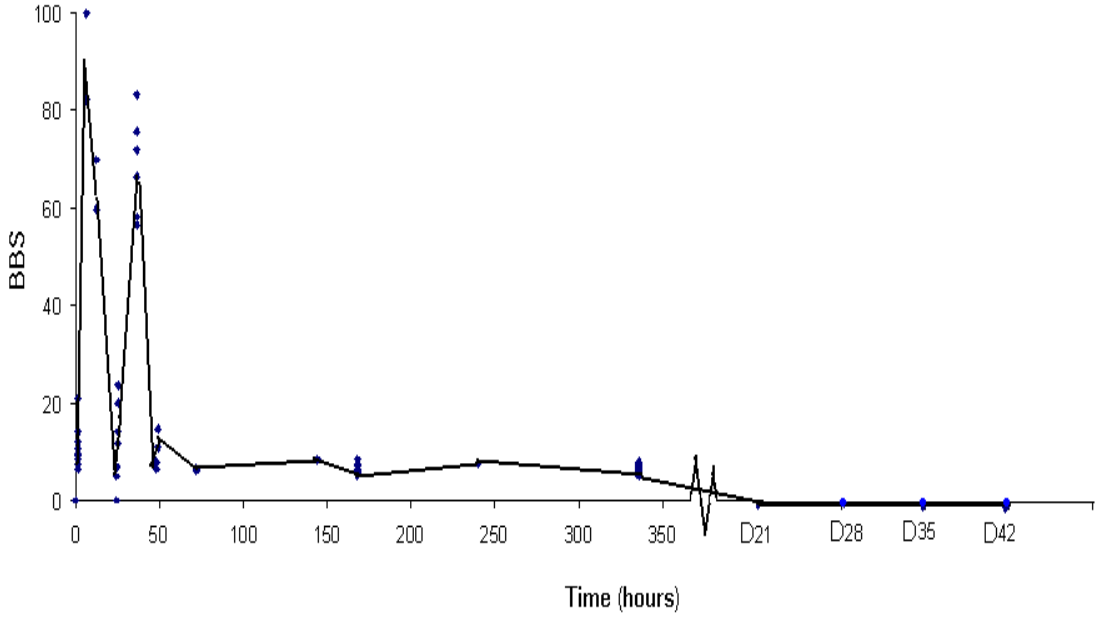


Figure 9 The concentration-time profiles of PQ in Dried blood Spot samples following a daily dose of 15 mg/kg body weight PQ for 14 days in Thai patients with *P. vivax* infection

Part-II: Monitoring of Patients' Adherence to the 14-days Course of Primaquine in Patients with *Plasmodium vivax*

A total of 85 patients (5 Thais and 80 Burmeses, 56 males and 29 females, aged 20-28 years) with mono-infection with *P. vivax* malaria (admission parasitemia 1,200-50,000 parasites/μL blood) participated in the current study. All participants completed the 42-day follow-up period. All had a rapid initial response to treatment with parasitemia cleared from their peripheral blood within the first three days of treatment. Based on patients' self-reporting on drug intake and the counting of primaquine tablets remaining in the plastic bag, full compliance to therapy of 100% was obtained.

A total of 85, 78, 85, and 85 DBS samples were collected from patients on days 0 (baseline), 3, 7, and 14 of treatment, respectively. All had undetectable (lower than the lower limit of quantification) baseline primaquine concentrations. Figure 10 represents the Box and Whisker plot of DBS primaquine concentrations on days 3 (1 day after the second dose), 7 (1 day after the sixth dose), and 14 (1 day after the thirteenth dose). Median (interquartile range: IQR, and minimum-maximum values) of primaquine concentrations in DBS samples collected on days 3, 7, and 14 were 5.07 (1.87, and 2.76-7.62), 4.76 (2.06, and 1.98-8.77), and 4.92 (1.97, and 1.89-8.52) ng/mL, respectively. The 1.5 box length apart from the lower limits of the 1st quartile or 25th percentile (Q1–1.5IQR) on days 3, 7 and 14 were 1.07, 0.84 and 0.96 ng/mL, respectively. The 1.5 box length apart from the upper limits of the 3rd quartile or 75th percentile (Q3+1.5IQR) on days 3, 7 and 14 were 8.54, 9.10 and 8.83 ng/mL, respectively. The 3 box length apart from the upper limits of the 3rd quartile or 75th percentile (Q3+3IQR) on days 3, 7 and 14 were 11.35, 12.19 and 11.78 ng/mL, respectively. Two, one, and four patients had outlier primaquine concentrations lower than the 1st quartile (Q1-1.5IQR) on days 3, 7, and 14, respectively. Based on the primaquine concentrations, patients' adherence rates to the 14-day primaquine regimen on days 3, 7, and 14 were 97.43% (76/78), 98.82% (84/85), and 95.29% (81/85), respectively. Two patients had outlier primaquine concentrations above the 3rd quartile on day 7 and one patient had extreme concentration above the 3rd quartile on day 14.

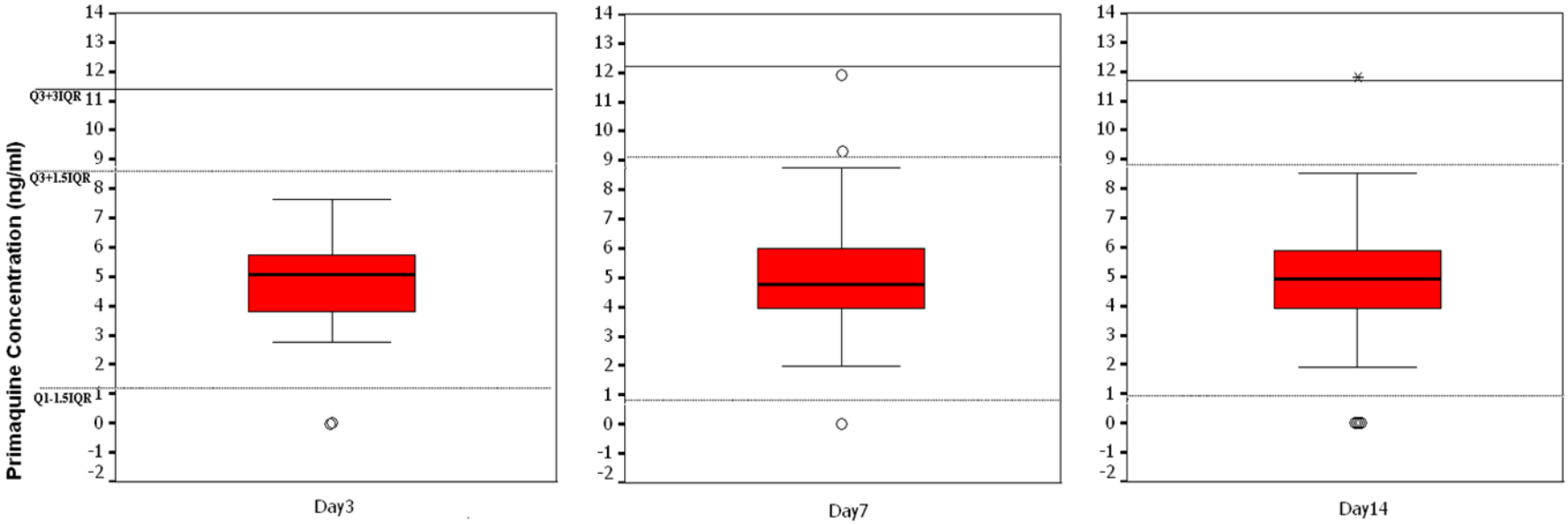


Figure 10 Box and whisker plot of primaquine concentrations on days 3, 7, and 14 of the initial treatment. The box indicates the lower (1st quartile or 25th percentile), upper (3rd quartile or 75th percentile), and the median line. The vertical lines represent the minimum and maximum plasma primaquine concentrations in patients on each day. The lower horizontal dot line indicates cut-off line for outlier concentrations (1.5 box length apart from the 1st quartile: Q1-1.5IQR). The upper horizontal dot line indicates cut-off line for outlier concentrations (1.5 box length apart from the 3rd quartile: Q3+1.5IQR). The upper horizontal filled line indicates cut-off line for extreme concentrations (3 box length apart from the 3rd quartile: Q3+3IQR). Each individual circle represents the case with outlier concentration below or above the Q1-1.5IQR or Q3+1.5IQR lines and the asterisk indicates a case with extreme concentration above the Q3+3IQR.

CHAPTER V
DISCUSSION

Part-I: Establishment of Analytical Methods for Determination of Primaquine in Whole Blood and Dried Blood Spot Sample

The analytical methods for the determination of PQ in whole blood and DBS samples established in this study meet the US FDA criteria for application to routine clinical drug level monitoring or pharmacological study (FDA, 2001). The advantage of the methods over previously reported ones are basically their rapidity, simplicity, and high sensitivity and specificity with simple UV (HPLC for whole blood) and MS/MS (LC-MS/MS for DBS) detection. In addition, the sample preparation procedures are simple, faster and less expensive. No absolute matrix effect was observed. The LOQ of PQ in whole blood and DBS samples was accepted as 25 ng/ml using 500 µl whole blood and 1 ng/ml using 80 µl DBS samples. With UV and MS/MS detection, sensitivity was also comparable to that with electrochemical detection (Nora, *et al.*, 1984). Sampling finger-prick capillary blood on to filter paper is considered easier to perform, requires less training (finger puncture with a lancet), is less invasive than conventional venous collection, and also involves little discomfort or risk to the participant. In addition, storage and transportation of samples do not require a cold chain or concern about degradation as observed for other plasma, serum or whole blood samples. There is also very limited biohazard risk for laboratory workers.

Good accuracy was observed from both the intra- or interday assays, as indicated by the minimal deviation of mean values found with measured samples from that of the theoretical values (actual amount added), with % DMV of lower than 4.08 % at all investigated concentrations.

The mean recoveries of PQ in whole blood and DBS samples at the investigated concentrations were 78.2-83.8 and 71.8-76.6 %, respectively. The mean recoveries of PYR at the concentration of 3,000 ng/ml (whole blood) and 300 ng/ml (DBS) were greater than 70 %. Since the volume of blood adsorbed on the filter paper was lower than 100 µl and because finger-prick specimens were subject to dilution by

interstitial fluid, the recovery of extraction (liquid-liquid) of PQ from DBS samples was considered satisfactory. The results reflected essentially high recovery for all compounds from the spiked whole blood and DBS and indicate a lack of interference from the sample preparation procedure.

Selectivity of the chromatographic separation was demonstrated by the absence of interference from endogenous peaks and commonly used drugs in whole blood and DBS samples in both spiked samples and samples collected from one patient after 1 h after the first dose of PQ on the first day.

Whole blood or DBS samples containing PQ were found to be stable at the concentration ranges observed when stored in a −80 ºC freezer or in plastic zipper bags in a desiccator at room temperature (25 ºC) for a minimum of 3 months without significant decomposition. DBS samples produced enhanced drug stability because of dehydration of the sample on the filter paper and consequent minimization of enzymatic and chemical hydrolyses of the drug under investigation. Nevertheless, the specimens should be dried at ambient temperature and humidity for at least 3–4 h before storage.

Part-II: Monitoring of Patients' Adherence to the 14-days Course of Primaquine in Patients with *Plasmodium vivax*

A number of factors associated with relapse, or the reappearance of *P. vivax* has been documented. These include inadequate primaquine dosage and/or drug concentrations in blood, high admission parasitemia, short duration of symptoms prior to diagnosis, presence of gametocytes on admission, age, gender, and patients' adherence to therapy (Prasad, *et al.*, 1991; Srivastava, *et al.*, 1996) (Duarte, *et al.*, 2001; Luxemburger, *et al.*, 1999; S. Pukrittayakamee, *et al.*, 2010). Inadequate adherence to therapy is considered one of the most significant factors that greatly affect the prevention of relapse particularly with non-supervision medication. Several methods have been applied for monitoring adherence to therapy particularly in the treatment of chronic diseases. The procedures involve either direct approach (directly observed therapy or DOT, measurement of concentrations of a drug or its metabolite in blood or urine, and detection or measurement in blood of a biologic marker added

to the drug formulation) or indirect approach (collection of patient questionnaires, assessment of clinical response, performing pill counts, ascertainment of rates of refilling prescriptions, application of electronic medication monitoring devices, measurement of physiologic markers, and assessment of patients' medication diaries) (Osterberg and Blaschke, 2005; Pullar, 1991). DOT is an effective strategy to ensure patient adherence to long-term chemotherapy. However, the procedure is unsustainable owing to the need for large numbers of health workers.

Questionnaire interview method suffers from its subjective evaluation. Pill counting is suitable for monitoring patients' adherence to treatment in remote areas and has been considered as a reference method in several studies. Clinical assessment and drug level monitoring have been considered as benchmark methods. Measurement of antimalarial drug concentrations in blood has been applied satisfactorily for monitoring patients' compliance to a 2-day artemether-mefloquine and a 3-day artesunate-mefloquine combination when adapted to field application in this malaria endemic area of Tak province along the Thai-Myanmar border (Congpuong, *et al.*, 2010; Na-Bangchang, *et al.*, 1997). Measurement of drugs other than the antimalarial itself, e.g., low dose phenobarbital, has also been applied for monitoring compliance to short course treatment with antimalarial regimens (Fungladda, *et al.*, 1998; Karbwang, *et al.*, 1998).

High patients' adherence rate to the 14-day primaquine regimen of 95-98% was found based on the cut-off primaquine concentrations on days 3, 7, and 14 of the initial treatment. This adherence rate was similar to what was observed with patient's self- reporting of drug intake and pill counting methods that were applied for the evaluation of patients' adherence to medication (100%). Two, one, and four cases with outlier primaquine levels below the 1st quartile on days 3, 7, and 14, respectively, could be due either to the non-adherence to primaquine doses or inadequate systemic drug exposure (due to impaired drug absorption or vomiting). The observed concentration-time profile of primaquine was in agreement with other previous reports in similar populations indicating relatively short systemic exposure of primaquine in human blood (Bangchang, *et al.*, 1994; Sasithon Pukrittayakamee, *et al.*, 2014). Capillary sampling blood from a finger prick spot on filter paper (DBS) provides a valid tool for monitoring patients' adherence to therapy (Kissinger, 2011;

Robijns, *et al.*, 2014) as it is simple, less invasive, and requires less experienced staff (finger puncture with a lancet) compared with the conventional venous blood collection. In addition, it is easy for transportation and storage. Terminal phase elimination half-life of primaquine is relatively short (5.1-10.3 hours) (S. Pukrittayakamee, *et al.*, 2004). Plasma concentration of primaquine at 25-30 hours after a single oral dose has been reported to be lower than 10 of the maximum concentration (Moore et al., 2014). Blood samples for primaquine concentrations on days 3, 7 and 14 in the current study were collected at about 2-4 hours after dosing when patients were present at the clinic for follow-up. The minimum concentrations observed in the study were approximately 1-3 ng/mL. Monitoring patients' adherence to therapy based on primaquine concentrations therefore requires a sensitive assay method with lower limit of quantification of at least 1 ng/mL, which in most cases is not possible (Dua, *et al.*, 1996; Dwivedi, *et al.*, 2003; Jin, *et al.*, 2014; Kim, *et al.*, 2004; Page-Sharp, *et al.*, 2012; Paliwal and Gupta, 1998). The use of primaquine together with carboxy-primaquine (major plasma metabolite of primaquine) as biological markers would increase the sensitivity of adherence monitoring as the half-life of this metabolite is relatively longer (33 hours) (Sasithon Pukrittayakamee, *et al.*, 2014). Unfortunately, measurement of carboxy-primaquine concentrations was not possible in the current study due to limitation of the analytical method used.

Studies to measure compliance with the treatment of *P. vivax* malaria with chloroquine for 3 days and primaquine for 7 or 14 days, have been conducted in several malaria endemic regions such as the Brazilian Amazon, Venezuela, Ecquador, and Sri-Lanka (Duarte and Gyorkos, 2003; Goller, *et al.*, 2007; Grietens, *et al.*, 2010; Leslie, *et al.*, 2004; Pereira, *et al.*, 2011; Reilley, *et al.*, 2002; Solari-Soto L, *et al.*, 2002; Yepez, *et al.*, 2000; Yeung and White, 2005). In general, results suggest higher adherence levels for shorter and simpler therapy regimens. Adherence rates following the 14-day primaquine have previously been reported in two studies in *P. vivax* patients in areas along the Thai-Myanmar border (Khantikul, *et al.*, 2009; Takeuchi, *et al.*, 2010). In the study conducted in the Mae-Hongsorn province (northwestern border) in unsupervised patients with mixed ethnic groups (Thai, Karen, and Shan), adherence rate based on patients' self-reporting was shown to be markedly low (76.2%) (Khantikul, *et al.*, 2009). In another study carried out in the Rachaburi

province (southwestern border) also in unsupervised patients with mixed ethnic groups (Thai, Karen, Mon, and Burmese), the adherence rates based on patients' self-reporting during weeks 1 and 2 were 94% and 88%, respectively. The non-adherence rate (12%) during week 2 was double of that during week 1 (6%). The median number of missed doses on day 14 was about four times of day 7 (Takeuchi, *et al.*, 2010).

Factors associated with patients' low or non-adherence to the treatment of malaria include poor education and knowledge on disease and medication, inadequate understanding of the treatment instruction, disintegration or loss of medication tablets, poor patients' care conditions at the healthcare center, inadequate prescription and drug dispensing by health professionals, incorrect perception of the potential of benefits (treatment efficacy) and risks (occurrence of side effects) of treatment, poor memory (forgetting to take the medication), and complexity of the treatment regimen (long course and non-standardized regimen). Other factors include social and economic situations, and cultural factors. The patient's lack of knowledge about the delayed hypnozoitocidal effect of primaquine and the lack of adequate prescription instructions may result in the early suspension of the drugs and also constitute a negative influence on adherence. The relatively fast clinical improvement and disappearance of the symptoms after the completion of the 3-day chloroquine has also been reported as an important reason for the early suspension of the treatment with primaquine. The high adherence rate of almost 100% observed in the current study was in agreement with the clinical effectiveness (100%) of this standard chloroquine and primaquine therapy. As antimalarial drug resistance develops and spreads rapidly, it is noted however that the figures may not reflect the current situation since the study was conducted 7-8 years ago (2008-2009). The impact of patients' adherence on treatment outcome following a 3-day chloroquine and 14-day primaquine was shown in patients with *P. vivax* malaria in the Thai-Myanmar border (Takeuchi, *et al.*, 2010). The *P. vivax* rate was found to be significantly lower in the DOT group than the self-administered therapy group (3.4 *vs*. 13.5 *per* 10,000 persons *per* day). Results from the study conducted during 2005-2006 in the Rachanuri province in the Thai-Myanmar border also suggest that patients' compliance with the 14-day primaquine (DOT) improves the outcome of *P. vivax* malaria treatment

(Maneeboonyang, *et al.*, 2011). The relatively high adherence rate of almost 100% in patients with self-medication in the current study compared with other similar studies conducted in the Thai-Myanmar border could be due mainly to the fact that blood samples for monitoring primaquine concentrations were collected on the appointment follow-up (days 3, 7, and 14). Patients' apprehension about appointment follow-up may have significantly influenced their behavior (*i.e.*, better compliance). Another limitation is that all study participants were in the age range of adulthood (20-28 years) which does not represent the actual population at risk. They had a tendency to comply with treatment medication compared with young or elderly patients (Takeuchi, *et al.*, 2010). Furthermore, good acceptability of patients at the clinic where the diagnosis was made with no cost and a relatively good healthcare condition may have also contributed to high adherence rate to therapy.

CHAPTER VI
CONCLUSION

The LC-MS/MS analytical methods for the determination of PQ in DBS samples that was developed in this study meet the US FDA criteria (FDA, 2001; ICH harmonized tripartite guideline, 2005; Nowatzke and Woolf, 2007). The advantage of the methods over previously reported ones are simplicity, rapidity, high sensitivity and specificity. The developed LC-MS/MS analytical method is suitable for application to monitoring of drug adherence in malaria patient.

In conclusion, the results of the current study suggest that a 14-day primaquine when given as an anti-relapse, together with the 3-day blood schizontocidal chloroquine was an effective treatment for *P. vivax* infection in the area along the Thai-Myanmar border during the period of 2008-2009, with a 42-day cure rate of 100%. The adherence rate for medication was high (95-100%). Nevertheless, careful monitoring of patients' adherence to medication is still required to prevent treatment failure from relapsing, as well as the development and spread of drug resistance. The potential problem related to patients' adherence to the 14-day primaquine therapy in unsupervised patients may be overcome with clear and simple instructions for drug administration combined with standardization of dose packaging (Leslie, *et al.*, 2004). Determination of plasma concentrations of primaquine in finger-prick DBS samples should be applied as a tool for monitoring patients' adherence to therapy alongside patients' self-reporting on drug intake as well as pill counting methods.

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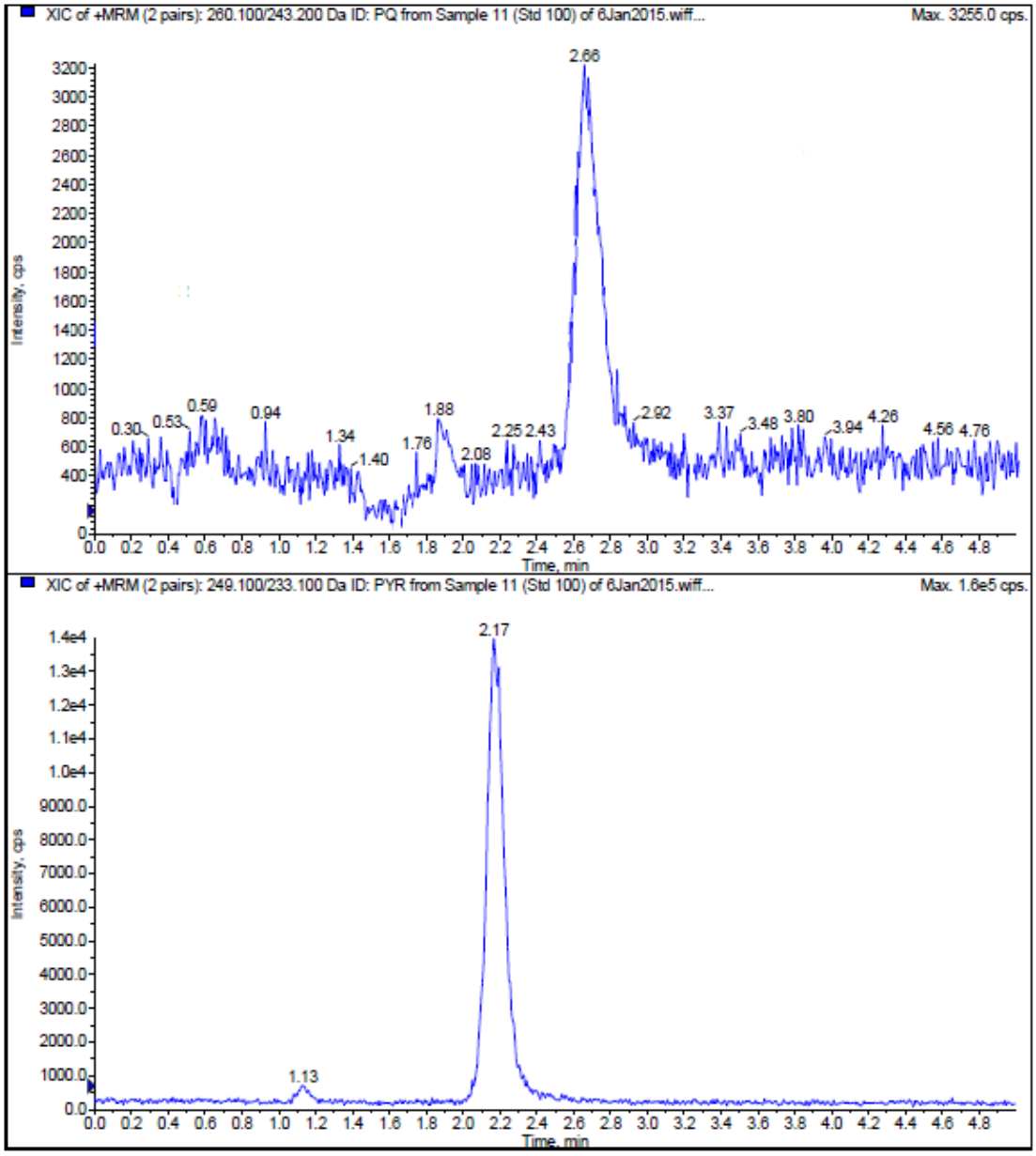
APPENDIX

Appendix A Parameters of AB SCIEX Q TRAP®5500 system for primaquine and IS

Parameter	Primaquine	Pyrimethamine (IS)
Q1 mass (Da)	260.1 $[M+H]^+$	249.1 $[M+H]^+$
Q3 mass (Da)	243.3	233.1
Collision Energy	17.0	38.0
DP (V)	50.0	286.0
EP (V)	8.0	10.0
CXP (v)	16.0	16.0

Appendix B The Limit Of Quantification chromatogram of PQ at 1 ng/ml



Appendix C Short-term stability data of PQ in whole blood

Time period	Concentration added of Primaquine (ng/ml)	Measured concentration of Primaquine		
		Mean	SD	%DMV
1 week	2.5	2.59	0.04	+3.63
	75	76.03	0.73	+1.38
	750	749.83	6.38	-0.02
2 week	2.5	2.60	0.06	+4.10
	75	76.48	0.38	+1.98
	750	753.74	2.70	+0.50
1 Month	2.5	2.51	0.07	+0.26
	75	75.80	0.55	+1.06
	750	754.29	3.87	+0.57
1 Month	2.5	2.50	0.06	-0.03
	75	75.51	0.30	+0.68
	750	747.36	4.79	-0.35

Appendix D The primaquine concentrations compared between from DBS sample and whole blood from retrospectively sample at Day-7 and D-14

No	Sample Name	Primaquine concentration (ng/ml)	
		DBS sample	Whole blood
1	D-7 MSV014	4.38	3.78
2	D-7 MSV015	5.56	5.76
3	D-7 MSV017	3.44	3.44
4	D-7 MSV018	9.20	10.00
5	D-7 MSV019	11.91	12.09
6	D-7 MSV020	8.01	8.51
7	D-7 MSV021	3.94	4.14
8	D-7 MSV023	5.38	4.98
9	D-7 MSV024	4.73	4.81
10	D-7 MSV025	4.27	4.47
11	D-7 MSV026	3.93	4.03
12	D-7 MSV027	6.45	6.68
13	D-7 MSV028	3.01	3.21
14	D-7 MSV029	6.83	6.73
15	D-7 MSV031	3.92	4.12
16	D-7 MSV032	4.96	4.66
17	D-7 MSV033	3.49	3.99
18	D-7 MSV034	7.32	7.72
19	D-7 MSV035	4.49	4.49
20	D-7 MSV036	4.65	4.15
21	D-14 MSV014	8.52	8.12
22	D-14 MSV015	17.81	17.91
23	D-14 MSV017	5.32	5.12
24	D-14 MSV018	8.91	7.51
25	D-14 MSV019	6.89	6.89

No	Sample Name	Primaquine concentration (ng/ml)	
		DBS sample	Whole blood
26	D-14 MSV020	4.78	4.78
27	D-14 MSV021	7.12	7.12
28	D-14 MSV023	4.13	4.13
29	D-14 MSV024	13.41	12.41
30	D-14 MSV025	4.90	4.90
31	D-14 MSV026	4.75	4.75
32	D-14 MSV027	6.22	6.22
33	D-14 MSV028	5.96	5.86
34	D-14 MSV029	6.36	6.06
35	D-14 MSV031	3.35	3.25
36	D-14 MSV032	13.27	14.27
37	D-14 MSV033	5.88	5.98
38	D-14 MSV034	3.95	4.35
39	D-14 MSV035	6.02	6.12
40	D-14 MSV036	5.54	5.74

Appendix E The primaquine concentrations of 85 patients on Day-3, Day-7 and Day-14

No	Sample Name	Primaquine concentration (ng/ml)			
		H0	Day-3	Day-7	Day-14
1	MSV012	0	5.22	7.16	-
2	MSV013	0	-	3.80	5.54
3	MSV014	0	6.01	4.38	8.52
4	MSV015	0	3.32	5.56	7.81
5	MSV017	0	3.94	3.44	5.32
6	MSV018	0	7.31	9.20	6.91
7	MSV019	0	5.22	11.91	6.89
8	MSV020	0	6.67	8.01	4.78
9	MSV021	0	4.73	3.94	7.12
10	MSV022	0	6.47	7.43	-
11	MSV023	0	3.96	5.38	4.13
12	MSV024	0	3.69	4.73	3.41
13	MSV025	0	5.35	4.27	4.90
14	MSV026	0	2.89	3.93	4.75
15	MSV027	0	7.62	6.45	6.22
16	MSV028	0	5.71	3.01	5.96
17	MSV029	0	4.22	6.83	6.36
18	MSV030	0	-	4.46	-
19	MSV031	0	2.76	3.92	3.35
20	MSV032	0	5.73	4.96	3.27
21	MSV033	0	4.80	3.49	5.88
22	MSV034	0	5.15	7.32	3.95
23	MSV035	0	3.53	4.49	6.02
24	MSV036	0	4.98	4.65	5.54
25	MSV037	0	5.36	6.00	4.90

No	Sample Name	Primaquine concentration (ng/ml)			
		H0	Day-3	Day-7	Day-14
26	MSV038	0	4.30	3.97	3.84
27	MSV039	0	6.96	4.71	7.14
28	MSV040	0	5.78	3.98	4.74
29	MSV041	0	7.16	-	5.85
30	MSV042	0	3.80	2.86	3.19
31	MSV043	0	4.38	6.94	7.26
32	MSV044	0	5.56	5.38	4.57
33	MSV045	0	3.44	4.50	5.74
34	MSV046	0	9.20	5.76	6.07
35	MSV047	0	11.91	6.46	5.51
36	MSV048	0	9.32	3.69	6.98
37	MSV049	0	5.08	6.00	5.43
38	MSV050	0	3.33	5.37	4.03
39	MSV052	0	5.92	3.85	5.67
40	MSV053	0	3.27	5.56	3.90
41	MSV054	0	-	4.32	5.16
42	MSV055	0	2.96	5.70	4.89
43	MSV056	0	4.53	5.92	6.37
44	MSV057	0	3.01	4.87	5.56
45	MSV058	0	2.94	1.98	3.56
46	MSV059	0	5.12	5.15	3.97
47	MSV060	0	-	4.54	5.26
48	MSV061	0	8.51	4.03	5.98
49	MSV062	0	10.88	5.59	5.14
50	MSV063	0	6.90	3.16	4.73
51	MSV064	0	5.16	8.22	6.36
52	MSV065	0	3.33	4.13	5.87
53	MSV066	0	-	4.63	3.21

No	Sample Name	Primaquine concentration (ng/ml)			
		H0	Day-3	Day-7	Day-14
54	MSV067	0	3.09	2.17	4.94
55	MSV068	0	4.33	5.30	6.85
56	MSV069	0	3.49	6.29	3.02
57	MSV070	0	7.23	5.90	4.35
58	MSV071	0	4.49	2.73	3.91
59	MSV072	0	4.65	4.41	5.73
60	MSV074	0	6.00	6.32	5.81
61	MSV075	0	-	8.77	4.34
62	MSV076	0	-	5.94	2.94
63	MSV077	0	5.66	5.35	-
64	MSV078	0	10.48	6.42	4.13
65	MSV079	0	8.05	5.77	2.44
66	MSV080	0	4.41	4.45	5.33
67	MSV081	0	7.87	7.16	11.79
68	MSV082	0	6.25	3.46	2.34
69	MSV083	0	6.11	5.71	5.46
70	MSV084	0	3.55	5.38	4.15
71	MSV085	0	3.02	4.20	1.89
72	MSV086	0	-	6.57	4.92
73	MSV087	0	5.70	3.93	3.22
74	MSV088	0	5.92	6.86	7.04
75	MSV089	0	4.87	4.46	3.46
76	MSV090	0	1.98	3.48	2.84
77	MSV091	0	5.15	7.66	4.31
78	MSV092	0	3.28	3.05	5.47
79	MSV093	0	5.98	6.36	4.53
80	MSV094	0	5.14	4.76	5.35
81	MSV095	0	4.73	3.06	3.97
82	MSV096	0	6.36	4.15	5.96

No	Sample Name	Primaquine concentration (ng/ml)			
		H0	Day-3	Day-7	Day-14
83	MSV097	0	3.50	3.33	6.31
84	MSV098	0	4.69	5.92	3.06
85	MSV099	0	-	3.27	4.58

VITA

Anurak Cheoymang was born and raised in Nongkhaem district; the suburb of Bangkok, Thailand. He has a Bachelor of Science degree. (Medical Technology) from Thammasat University in 2007. After completed a bachelor's degree, he has been working at Faculty of allied health Sciences, Thammasat University since 2007. He is particularly proud of his work as scientists where he worked on Biomedical research and apply to help people. Currently, he is studying a master's degree in public health.