การวิเคราะห์ปริมาณไดไฮโดรอาร์ติมิซินินในพลาสมาคนโดยเทคนิคไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโทกราฟี-แทนเดมแมสสเปกโทรเมทรี

นางสาว นิภา ประดิษฐ์เทียมผล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2546

ISBN 974-17-4527-3

DETERMINATION OF DIHYDROARTEMISININ IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Biotechnology

Faculty of Science

Chulalongkorn University

Academic Year 2003

ISBN 974-17-4527-3

Thesis Title	Determination of dihydroartemisinin in human plasma by high - performance liquid chromatography –tandem mass spectrometry
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นิภา ประดิษฐ์เทียมผล: การวิเคราะห์ปริมาณไดไฮโดรอาร์ติมิซินินในพลาสมาคนโดยเทคนิคไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโทกราฟี-แทนเดมแมสสเปกโทรเมทรี (DETERMINATION OF DIHYDROARTEMISININ IN HUMAN PLASMA BY HIGH - PERFORMANCE LIQUID CHROMATOGRAPHY - TANDEM MASS SPECTROMETRY) อ.ที่ปรึกษา: ผศ. ดร. พลกฤษณ์ แสงวณิช, อ.ที่ปรึกษาร่วม: รศ. นพ. สุพรชัย กองพัฒนากูล, 68 หน้า. ISBN 974-17-4527-3.

ได้ทำการพัฒนาวิธีวิเคราะห์ปริมาณสารไดไฮโดรอาร์ติมิซินินในพลาสมาคน โดยทำการสกัดด้วยสารผสมของ 1-คลอโรบิวเทนกับไอโซออกเทนในอัตราส่วน 55:45 จากนั้นวิเคราะห์ด้วยเทคนิคไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโทกราฟี-แทนเดมแมสสเปกโทรเมทรี (LC-MS/MS) โดยมีอิเล็กโทรสเปรย์เป็นเทคนิคไอออไนเซชัน ในระบบมัลติเพิล รีแอคชัน มอนิเทอร์ริง (multiple reaction monitoring) ซึ่งเป็นระบบที่มีความจำเพาะเจาะจงกับสารที่ตรวจวิเคราะห์ เป็นอย่างสูง การวิเคราะห์ปริมาณไดไฮโดรอาร์ติมิซินินนั้นทำการตรวจวัดไอออน 261 และ163 ที่เกิดจากการแตกตัวของไดไฮโดรอาร์ติมิซินิน และการวิเคราะห์ปริมาณอาร์ติมิซินินซึ่งเป็น อินเทอร์นอลสแตนดาร์ดนั้นทำการตรวจวัดไอออน 151 ที่เกิดจากการแตกตัวของอาร์ติมิซินิน โครมาโทแกรมถูกแยกบนคอลัมน์ลูนาขนาด 150 x 2.00 มิลลิเมตร และใช้เฟสเคลื่อนที่เป็นสารผสมของอะซิโตไนไตร์กับน้ำในอัตราส่วน 50:50 ด้วยอัตราการไหล 0.2 มิลลิลิตรต่อนาที จากการทดลองพบว่า กราฟมาตรฐานเป็นเส้นตรงในช่วงความเข้มข้น 5-200 นาโนกรัมต่อมิลลิลิตร โดยเปอร์เซ็นต์ความถูกต้องและความเที่ยงตรงของวิธีวิเคราะห์ใน 1 วันและระหว่างวัน ซึ่งแสดงในรูปค่าสัมประสิทธิ์ความแปรปรวนมีค่าอยู่ในช่วงที่ยอมรับได้ เทคนิควิเคราะห์นี้สามารถนำไปใช้วิเคราะห์ปริมาณไดไฮโดรอาร์ติมิซินินในพลาสมาคนเพื่อใช้ในการศึกษาเภสัชจลนพลศาสตร์ต่อไป

หลักสูตร…เทคโนโลยีชีวภาพ.....	ลายมือชื่อนิสิต……..
สาขาวิชา…เทคโนโลยีชีวภาพ……	ลายมือชื่ออาจารย์ที่ปรึกษา……
ปีการศึกษา…2546…….....	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม……..

4572606823 : MAJOR BIOTECHNOLOGY

KEYWORD: Dihydroartemisinin / LC-MS/MS / MRM

NIPA PRADITTIEMPHON: DETERMINATION OF DIHYDROARTEMISININ IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY. THESIS ADVISOR: ASST. PROF. POLKIT SANGVANICH, Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. SUPORNCHAI KONGPATANAKUL, Ph.D., 68 pp. ISBN 974-17-4527-3.

A sensitive and highly specific assay method was developed for the determination of dihydroartemisinin in human plasma by liquid chromatography - tandem mass spectrometry (LC-MS/MS) using electrospray ionization (ESI) as an interface. Artemisinin was used as internal standard. A simple liquid-liquid extraction with 1-chlorobutane-isooctane (55:45,v/v) was used to isolate dihydroartemisinin from plasma. The mobile phase was composed of acetonitrile-water (50:50,v/v) and chromatographic separations were achieved on a luna 3 μm C_{18} column (150 x 2.00 mm). The quantitation was operated in positive ion multiple reaction monitoring (MRM) mode. Dihydroartemisinin produced a precursor ion ($[M+Na]^+$) at m/z of 307 and corresponding product ion at m/z of 261 and 163. And internal standard (artemisinin) produced a precursor ion ($[M+Na]^+$) at m/z of 305 and corresponding product ion at m/z of 151. The calibration curve was linear over a concentration range of 5-200 ng/ml, with intra- and inter day accuracy and precision were within the acceptable range. The method was applied to the quantification of dihydroartemisinin in human plasma for pharmacokinetic analyses.

Program …Biotechnology……	Student's signature…….……...
Field of study….Biotechnology……	Advisor's signature……
Academic year…….2003……..	Co-advisor's signature……...

ACKNOWLEDGEMENT

I would like to express my deepest gratitude to my advisor, Assistant Professor Dr. Polkit Sangvanich, and co-advisor, Associate Professor Dr. Supornchai Kongpatanakul for useful guidance, kind helps, and suggestions in my thesis.

I am grateful to Professor Dr. Sophon Roengsumran, Associate Professor Dr. Amorn Petsom and Assistant Professor Dr. Nattaya Ngamrojnavanich for their advice as the thesis committee.

I am thank to Department of Pharmacology, Faculty of Medicine, Siriraj hospital, Mahidol University for offering laboratory facilities in the research.

Special thanks are given to Missis Piyapat Pongnarin and staffs of the Department of Pharmacology, Faculty of Medicine, Siriraj hospital, Mahidol University for their helps during my study. I would like to express my deepest appreciation to Miss Supaporn Sawettaporn for her friendship.

I would like to thank HM. KING RAMA IX 72th ANNIVERSARY, Thailand Tropical Diseases Research Programme and Graduate School, Chulalongkorn University for financial support.

Finally, I wish to express extremely grateful for the constant encouragement received from my parents for their supporting, infinite love and understanding.

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LIST OF ABBREVIATIONS

α	=	Alpha
β	=	Beta
API	=	Atmospheric pressure ionization
APCI	=	Atmospheric pressure chemical ionization
^{o}C	=	Temperature in degree of Celsious
CE	=	Collision energy
Cs	=	Calibration standards
C.V.	=	Coefficient of variation
CAD	=	Collision activated dissociation
CID	=	Collision induced dissociation
eV	=	Electron voltage
ESI	=	Electrospray ionization
GPO	=	Government Pharmaceutical Organization
h	=	Hour
I.S.	=	Internal standard
kV	=	Kilovoltage
LC	=	Liquid chromatography
LOD	=	Limit of detection
LOQ	=	Limit of quantitation
MS	=	Mass spectrometry
Min	=	Minute
m/z	=	Mass to charge ratio
MRM	=	Multiple reaction monitoring
n	=	Number of determination
ng/ml	=	Nanogram per mililitre

LIST OF ABBREVIATIONS (continued)

QCs	=	Quality control samples
Q1	=	First quadrupole
Q3	=	Third quadrupole
RSD	=	Relative standard deviation
S.D.	=	Standard deviation
TIC	=	Total ion counting
µl	=	Microlitre
µm	=	Micrometer
v/v	=	Volume by volume

CHAPTER I

INTRODUCTION

Malaria continues to be a major health problem in many areas of the world. Malaria was caused by parasitic protozoa in the genus *Plasmodium*. Human malaria parasites are *P. falciparum, P. malariae, P. vivax and P. ovale.* Among these four species, *P. falciparum* is the overwhelming cause of serious disease and death. The occurrence and spread of resistance to the classical antimalarial drugs in *P. falciparum* has stimulated the search for alternative medicaments. Artemisinin and its derivatives represent a new class of antimalarials that is effective against drug-resistant *P. falciparum* strains and therefore, they are of utmost importance in the current antimalarial campaign. Dihydroartemisinin is the active metabolite and its derivatives of artemisinin. The use of dihydroartemisinin instead of the other artemisinin derivatives has advantages. The drug is easy to produce with less synthetic steps thus a lower cost and to exert the highest antimalarial activity in this class.

Optimization of oral as well as parenteral dosage regiments for this drug will therefore, only be correctly obtained when more information on the pharmacokinetics of DHA becomes available. This requires a sensitive and specific drug assay method in biological fluids. Several methods have been reported for measurement of artemisinin and derivatives in biological fluids including high-performance liquid chromatography with ultraviolet detection (LC-UV), high-performance liquid chromatography with electrochemical detection (LC-EC) in reductive mode, a gas chromatography with mass spectrometric detection (GC-MS) and liquid chromatography with mass spectrometric detection (LC-MS).

The tandem mass spectrometry combines two mass spectrometer (MS/MS) has proved to be selective, even specific, for target analytes than single mass

spectrometers. And detection of dihydroartemisinin using liquid chromatography tandem mass spectrometry (LC-MS/MS) has yet to reported.

Therefore, the purpose of this study to develop and validate method for determination of dihydroartemisinin in human plasma by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS) using the multiple reaction monitoring (MRM) mode with positive ion detection. Details on these techniques will be described in later chapters.

Objective

1. Determination dihydroartemisinin in human plasma using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in multiple reaction monitoring (MRM) mode.
2. Validate in terms of recovery, linearity, accuracy and precision.

CHAPTER II

LITERATURE REVIEW

1. Malaria

About 40% of the world's population is at risk of malaria infection. Each year, more than 250 million people experience a malarial illness, and over 1.5 million individuals (mostly African children) die. In patients with severe and complicated disease, the mortality rate is between 20 and 50% [1]. In Thailand, malaria is found mostly in the west region and approximately 100,000 people are with this disease and around 800 people die from the disease annually [2].

1.1. Malarial Parasites

Malaria was caused by parasitic protozoa in the genus *Plasmodium*, that there are some 100 species of these protozoa but only four are responsible for the disease in humans; *P. falciparum*, *P. malariae*, *P. vivax* and *P. ovale*. Of the four human malaria parasites, *P. falciparum* is the overwhelming cause of serious disease and death. Human are affected by inoculation with infected blood of by infected female *Anopheles* mosquito.

1.2. Malaria Symptoms

Malaria symptoms usually develop 10 to 35 days after a person was infected by mosquito's bite. Frequently, the first symptoms are mild fever, headache, muscleaches, and chills, together with a general feeling of illness. Sometimes symptoms begin with shaking chills followed by fever. These symptoms last 2 or 3 days and are very similar to those of the flu. Subsequent symptoms and patterns of disease vary among the four types of malaria. For vivax and ovale malaria, an attack may begin abruptly with a shaking chill, followed by sweating and a fever. Within a week, the typical pattern of intermittent attacks is established. A period of headache or of feeling ill may be

followed by a shaking chill. After the fever subsides, the person feels well until the next chill. New attacks tend to occur every 48 hours in vivax malaria. For malariae malaria, an attack often begins abruptly. The attack is similar to that of vivax malaria but recurs every 72 hours. For falciparum malaria, an attack may begin as chills. The person's temperature rises gradually, then falls suddenly. The attack may last for 20 to 36 hours. The person may feel more ill than with vivax malaria and have a severe headache. Between attacks, during intervals that vary from 36 to 72 hours, the person usually feels miserable and has a mild fever.

In falciparum malaria, the parasite causes red blood cells to stick to the walls of arteries, which can result in severe anaemia, kidney failure, water in the lungs, shock or jaundice. It can also cause cerebral malaria with symptoms of a fever with at least 40^{o}C, severe headache, drowsiness, delirium, and confusion. Cerebral malaria can be fatal as a result of red blood cells sticking to the arteries of the brain and so cutting off blood supply. It most commonly occurs in infants, pregnant women, and travelers to high-risk areas.

Malaria can be diagnosed by the clinical symptoms and microscopic examination of the blood. It can normally be cured by antimalarial drugs. The symptoms quickly disappear once the parasites are killed. If the person is untreated, the symptoms of vivax, ovale, or malariae malaria subside spontaneously in 10 to 30 days but may recur at variable intervals. Untreated falciparum malaria is fatal in up to 20 percent of patients.

2. Antimalarial drugs

Quinine (Figure 1a), the alkaloid from the bark of cinchona tree, which was the first-line treatment for malaria. During the World War II (1939-1945) quinine was no longer available. Therefore, in order to search for active compounds, some extensive antimalarial reseach programs were established in many countries.

From the investigation by the cooperative program of antimalarial research in the United States, chloroquine (Figure 1b) was found to be a very effective drug and fewer side effects [3]. In the early 1960s the chloroquine-resistant strains of *P. falciparum* were reported in South America and South East Asia [4].

The fixed combination of sulfadoxine (Figure 1c) and pyrimethamine (Figure 1d) has replaced chloroquine as the therapy for uncomplicated falciparum malaria in several African countries [5]. However, the therapeutic lifespan of sulfadoxine/pyrimethamine will be limited by the rapid emergence of parasites resistant to this combination.

This prompted to develop new effective antimalarial drugs. Mefloquine (Figure 1e) displayd high activity against the chloroquine and the pyrimethamine resistant strains [6]. Due to its highly effectiveness, mefloquine was wide spreadingly used since the late 1970s. However, the resistance to this drug since the early 1980s and its severe side effects [7] reduced its use.

(a) (b)

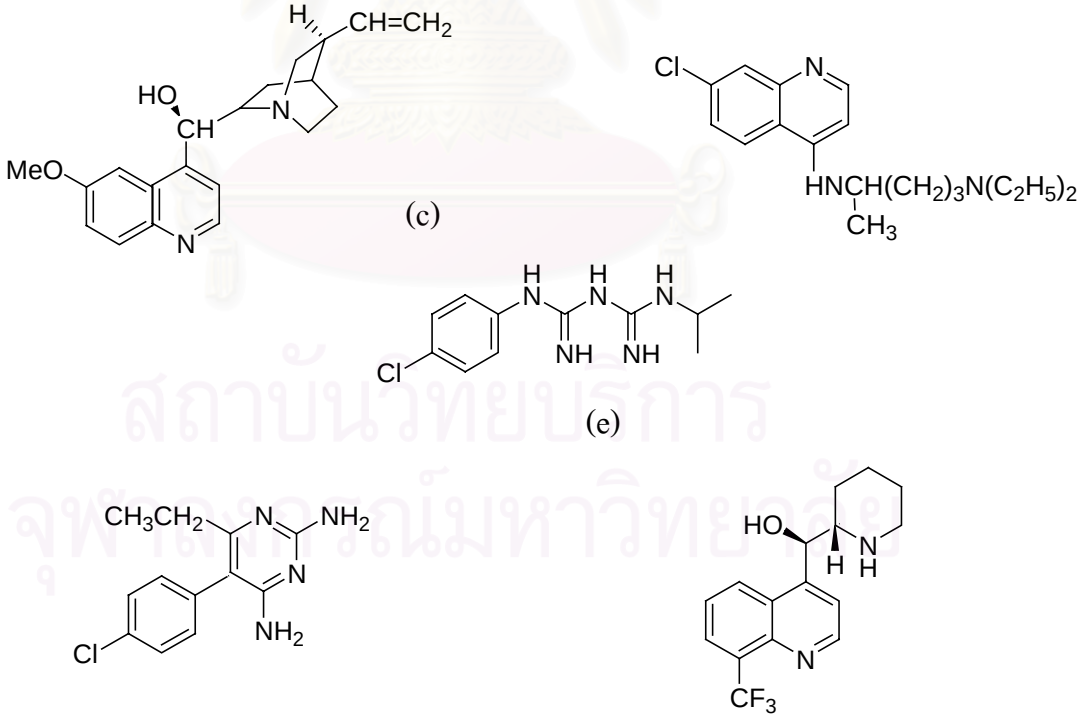


Figure 1. Structures of antimalarial drugs (a) quinine, (b) chloroquine, (c) sulfadoxine, (d) pyrimethamine, (e) mefloquine

Parasites have developed resistance to antimalarial drugs, e.g., chloroquine, quinine, sulfa/pyrimethanine combination, and mefloquine have been reported in many parts of the world [8-10]. Therefore, there is a dire need for new drugs in the prophylaxis and treatment of malaria.

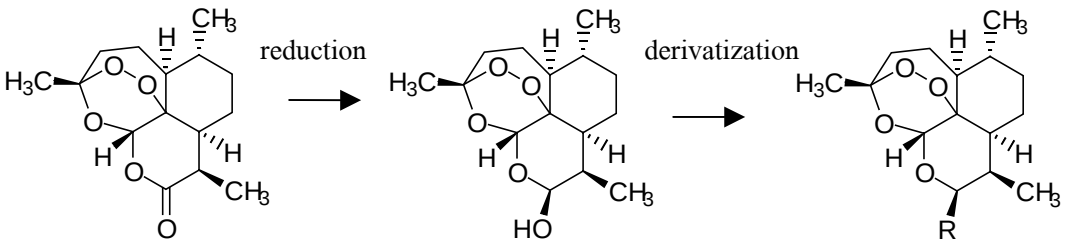
Artemisinin and its derivatives represent a very important new class of antimalarials that is effective against drug-resistant *P. falciparum* strains and therefore they are of utmost importance in the current antimalarial campaign [11].

3. Artemisinin and its derivatives

The herb *Artemisia annua L.* (sweet or annual wormwood), a native plant in China, has been used in traditional Chinese medicine as a remedy for chills and fevers for more than 2000 years. Artemisinin or qinghaosu is the active principal of *Artemisia annua, L.* was isolated by Chinese researchers in 1972, and its structure was elucidated in 1979 [12]. Chemically, artemisinin is a sesquiterpene trioxane lactone containing a peroxide bridge (Figure 2a), which is essential for its activity [13]. The drawbacks of artemisinin are also contributed from its insolubility in both water and oil [14], its poor efficacy by oral administration [15], and short plasma half-life [12].

In order to solve the problem of its poor solubility, many derivatives were synthesized [14]. The lactone can easily be reduced (with sodium borohydride), resulting in the formulation of dihydroartemisinin (Figure 2b), which produced artemether (Figure 2c) and arteether (Figure 2d) as oil soluble analogues, artesunic acid (Figure 2e) and artelinic acid (Figure 2f) as water-soluble analogues [16].

These derivatives were found to be more active than artemisinin and hence, they are now increasingly being used for malaria treatment against drug resistant strains of *P. falciparum* [17-19]. All the artemisinin derivatives are metabolized rapidly to the active metabolite dihydroartemisinin [20].



(a) (b) (c) R = OCH_3

(d) R = OCH_2CH_3

(e) R = $OCOCH_2CH_2COOH$

(f) R = $OCH_2C_6H_5CO_2H$

Figure 2. Structures of artemisinin and its derivatives (a) artemisinin, (b) dihydroartemisinin, (c) artemether, (d) arteether, (e) artesunic acid, (f) artelinic acid

Dihydroartemisinin

Dihydroartemisinin is the active metabolite and its derivatives of artemisinin. Dihydroartemisinin is a potent antimalarial drug that has even more antimalarial activity *in vitro* than artemisinin [21] and can reduce parasitaemia by 90% within 24 hours of administration [12]. The use of dihydroartemisinin instead of the substitute compounds (e.g. artesunate or artemether) has advantages. The drug is easy to produce with less synthetic steps thus a lower cost and to exert the highest antimalarial activity in this class.

3.1. Mechanism of action

The endoperoxide group in artemisinin and its derivatives was necessary for antimalarial activity [22-23]. The evidence is that deoxyartemisinin analogs, which lack the endoperoxide moiety, are devoid of antimalarial activity [13,24]. The endoperoxide moiety is believed to produce free radicals which are important for mediating the effects. The mechanism of action was proposed to involve two sequential steps, i.e., activation and alkylation steps [25].

(a) Activation step

The malaria parasite is rich in heme-iron, derived from the proteolysis of host cell hemoglobin [26]. Artemisinin interacted with intraparasitic heme and that intraparasitic heme or iron might function to activate artemisinin inside the parasite into toxic free radicals [27]. Posner *et al*. [28]. proposed that the iron attacks the compound at the O_2 position (A) and produces a free radical at the O_1 position. It is then rearranged to form a C_4 free radical. This radical (B) was suggested to be an important substance for antimalarial activity [29]. The radical (B) is then changed to the vinyl ether (C) by a beta-scission reaction, which also generates the Fe (IV)=O as another product. Subsequently, the intermolecular reaction between compound (C) and Fe (IV)=O leads to an epoxide compound (D). Alternatively, the epoxide (D) could be derived derectly from the radical (B) by a direct expulsion of iron. Finally, a C4-hydroxylated product (E) is formed (see Figure 3). The compound (D) is able to alkylate the specific proteins of the malarial parasites and possibly causes damage to the parasites [30].

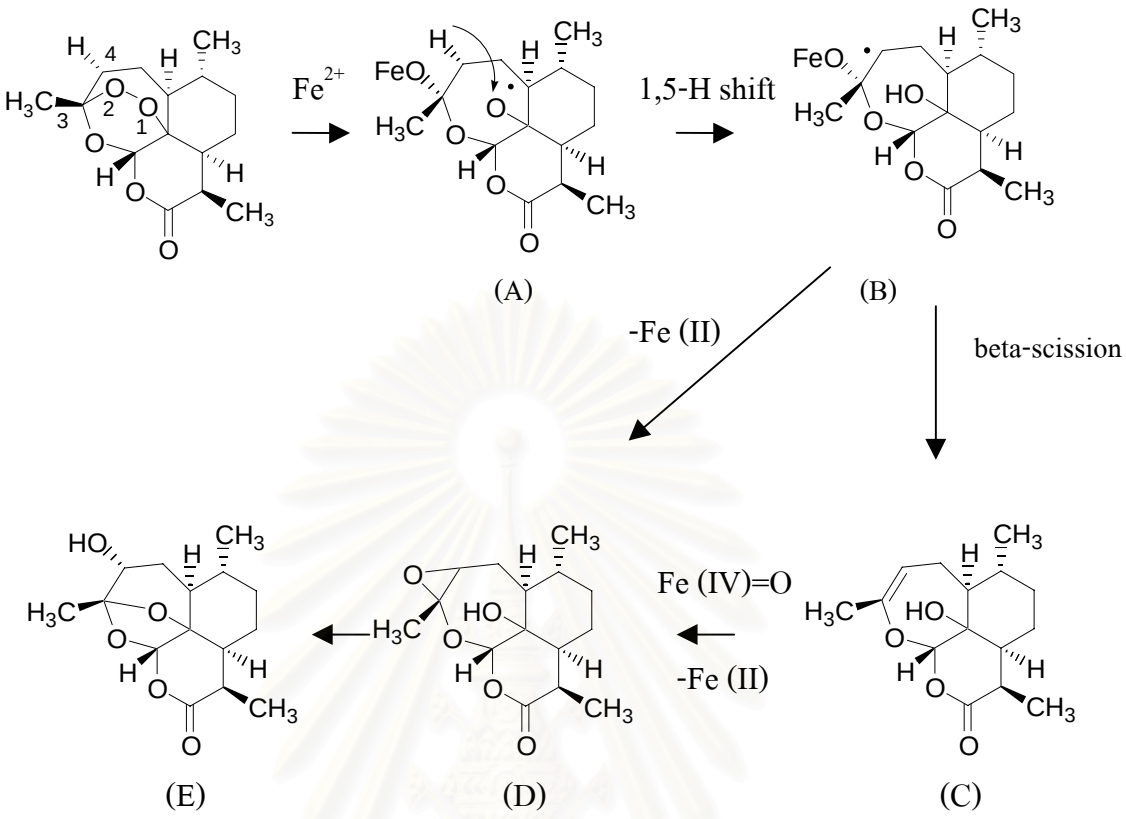


Figure 3. The mechanism of action of artemisinin compound as proposed by Posner *et al* [28].

(b) Alkylation step

Radicals and reactive intermediates formed from the activation step would rapidly react with nearby molecules due to their high reactivity. Artemisinin forms covalent adducts with protein, but not with DNA [31-32]. Protein alkylation is heme-dependent and covalent bonds form with protein, preferentially to heme.

4. Pharmacokinetics studies

The recent demand for monitoring trace levels of drugs and metabolites in animals of or humans to support drug discovery has increased dramatically. Pharmacokinetics is the study of the uptake, distribution, and excretion of drugs with respect to time. It is an extremely important subject area from a clinical view, as the intensity and duration of action of a drug are related to what concentration of drug is present at the active site. Optimization of oral as well as parenteral dosage regimens for this drug will therefore, only be correctly obtained when more information on the pharmacokinetics becomes available. This requires a sensitive and specific drug assay method in biological fluids.

5. Analytical method for determination of dihydroartemisinin

Several methods have been reported for measurement of artemisinin and derivatives in biological fluids including high-performance liquid chromatography with ultraviolet detection (LC-UV) [33-34], high performance liquid chromatography with electrochemical detection (LC-EC) in reductive mode [35-37], a gas chromatography with mass spectrometric detection (GC-MS) [38] and liquid chromatography with mass spectrometric detection (LC-MS) [39-41].

5.1. Liquid chromatography with ultraviolet detection (LC-UV)

Thomas C.G. *et al.* (1992) [33] reported a method for the determination of artemether and its major metabolite, dihydroartemisinin using LC-UV detection.This involved extraction of plasma with dichloromethane and acid decomposition with hydrochloric acid to produce a UV absorbing product, an α, β-unsaturated decalone [8-methyl-5- (2-propanalyl) decalin-4-ene-3-one, see Figure 4). Examination of their chemical structure suggests that artemether and dihydroartemisinin would be expected to undergo this reaction when incubated with acid under the same conditions. Therefore to determine selectivity artemether and dihydroartemisinin in plasma requires physical separation prior to acid derivatization.

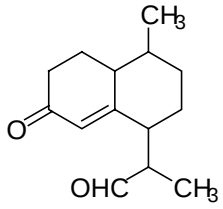


Figure 4. The acid decomposition product of both artemether and dihydroartemisinin.

Muhia, D.K. *et al.* (1994) [34] described a method for separation of artemether from its metabolite dihydroartemisinin. The basis of the separation is differential extraction of the drugs from plasma as a function of plasma pH. Hexane extracted artemether from basified plasma and both artemether and dihydroartemisinin from normal plasma. Derivatized extracts were chromatographed on a 5 μm ODS column with water-acetonitrile (40:60) as mobile phase and detected at 254 nm.

5.2. Liquid chromatography with electrochemical detection (LC-EC)

Melendez *et al*. (1991) [35] reported a method for determination of artemether and dihydroartemisinin in plasma using liquid-liquid extraction and HPLC with reductive amperometric detection. This detection was performed with a glassy carbon electrode versus Ag/AgCl. This detection system also required rigorous sample and mobile phase deoxygenation. In addition, manual and electropolishing at -2.0 v for 2 hour were necessary to maintain the sensitivity and stability of glassy carbon electrode. A new automated sample deoxygenation and injection system was used to greatly increase sample throughput.

Karbwang, J. *et al.* (1997) [36] described HPLC with reductive electrochemical detection for quantitative determination of artemether and its plasma metabolite, dihydroartemisinin in plasma. Several extraction solvents were tested in this work. It was found that the mixture of dichloromethane-*tert*.-methybutyl ether (1:1, v/v) and of *n*-butyl chloride-ethyl acetate (9:1, v/v) resulted in a clean extract. The minimum detectable concentrations for artemether and α-dihydroartemisinin in spiked plasma samples were 5 and 3 ng/ml, respectively.

Sandrenan, N. *et al.* (1997) [37] reported the determination of artemether and its major metabolite, dihydroartemisinin in plasma by LC-EC in the reductive mode. The limit of quantitation (LOQ) of artemether and dihydroartemisinin were 10.9 ng/ml and 11.2 ng/ml, respectively.

The drawback of the technique is difficult to use routine analysis due to the very rigorous conditions that requires deoxygenation of the samples and mobile phase to prevent dissolved oxygen from entering the flowcell. And a difficulty in quantifying oxygenated metabolites. Therefore, the development of a new, easier to use and sensitive method to be necessary.

5.3. Gas chromatography- mass spectrometry (GC-MS)

Mohamed, S.S. *et al*. (1999) [38] reported a gas chromatography mass spectrometry (GC-MS) method to determine artemether and dihydroartemisinin in plasma using artemisinin as internal standard. Solid phase extraction (SPE) was performed using C_{18} Bond Elut cartridges. The SPE procedure was found to be rapid and simple to perform with good recovery and minimal contamination. The LOQ was 5 ng/ml.

5.4. Liquid chromatography- mass spectrometry (LC-MS)

Liquid chromatography coupled to mass spectrometry (LC-MS) is known to be a powerful separation and detection technique in a large number of analytical fields, and particularly for the detection of drug in biological fluids [42-43]. Some attractive features of LC-MS are its good sensitivity, reliability, and specificity for a wide variety of compounds, with minimal sample handling. Because of these advantages, LC-MS has become widely adopted for studies of drug metabolism and pharmacokinetics.

In the literature, Stefansson, M. *et al*. (1996) [39] reported electrospray mass spectrometric (ESI) method for studies on some uncharged antimalarial drugs, extensive multimer formation was observed. The concentration-dependent aggregation

behavior, including species up to tetramers, resulted in highly non-linear calibration curves. For some analytes, these effects were operating down into the nanomolar level.

Sahai, P. *et al.* (1998) [40] reported LC-MS using ESI method for the determination of artemisinin, without pre- or postcolumn derivatization. The method has been shown to be particularly suitable for analysis of artemisinin in biosynthetic stable isotope labeling experiments and quantitative analysis in the nanogram range in crude biological samples such as *Artemisia annua* plant extracts. But none of them were validated in plasma.

More recently Souppart, C. *et al*. (2002) [41] described LC-MS using atmospheric pressure chemical ionization (APCI) method for the determination of artemether and its active dihydroartemisinin metabolite in human plasma. The method consists of sample preparation by liquid-liquid extraction, followed by chromatographic separation on a C_{18} column and detected in the selected ion monitoring mode. The LOQ was 5 ng/ml.

6. Combining LC with API-MS

6.1. Atmospheric pressure ionization (API) technique

The development of the atmospheric pressure ionization (API) source is an important breakthrough that has enabled coupling LC with MS conveniently, has been extremely successful because

1. API approaches can handle volumes of liquid typically used in LC.
2. API is suitable for the analysis of nonvolatile, polar compounds typically analyzed by LC.
3. API-MS systems are sensitive, offering comparable or better detection limits than achieved by GC/MS.
4. API systems are very rugged and relatively easy to use.

For these reasons, LC-API-MS instruments are found in most commercial and government laboratories that perform research involving compounds that require LC separations [44].

Atmospheric pressure ionization (API) techniques consist of (1) Atmospheric-pressure chemical ionization (APCI) and (2) Electrospray ionization (ESI).

API when operated in the electrospray ionization (ESI) format is unique in that it has a great potential for the analysis of a variety of both small and large molecules at femtomole sensitivities. The utility of HPLC-ESI-MS has been demonstrated for a wide range of applications in bioanalytical, pharmaceutical, and environmental fields.

6.1.1 Electrospray ionization (ESI)

(a) Electrospray process

The essence of the electrospray process can be described with simplicity. A solution of the analyte is passed through a capillary which is held at high potential. The effect of the high electric field as the solution emerges is to generate a mist of highly charged droplets which pass down a potential and pressure gradient towards the analyser portion of the mass spectrometer. During that transition, the droplets reduce in size by evaporation of the solvent or by droplet subdivision resulting from the high charge density. Ultimately, fully desolvated ions result from complete evaporation of the solvent or by field desorption from the charged droplets. Nebulization of the solution emerging from the capillary may be facilitated by a sheath flow of nebulizer gas. Sampling of the fully or partially desolvated ions is made using a capillary or a skimmer device. The essential features of the experimental arrangement are shown in Figure 5 [45].

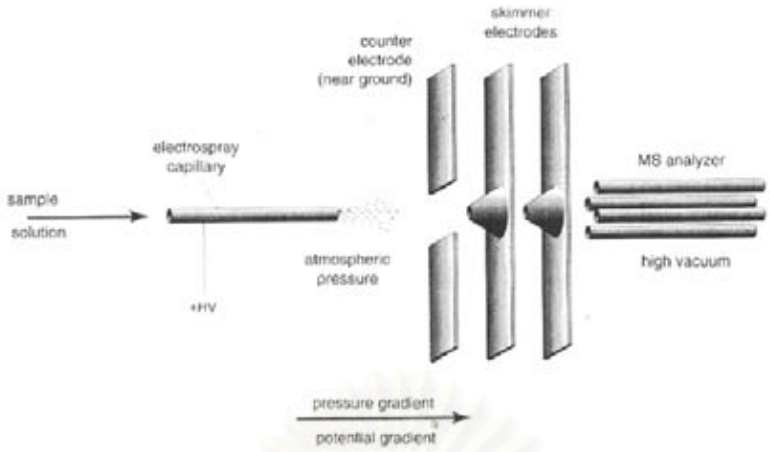


Figure 5. Essential features of the electrospray interface.

(b) Production of gas-phase ions by electrospray

There are three major steps in the production by electrospray of gas-phase ions from electrolyte ions in solution: (1) production of charged droplets at the ES capillary tip; (2) shrinkage of the charged droplets by solvent evaporation and repeated droplet disintegrations, leading utimately to very small highly charged droplets capable of producting gas-phase ions, and (3) the actual mechanism by which gas-phase ions are produced from the very small and highly charged droplets (Figure 6) [44].

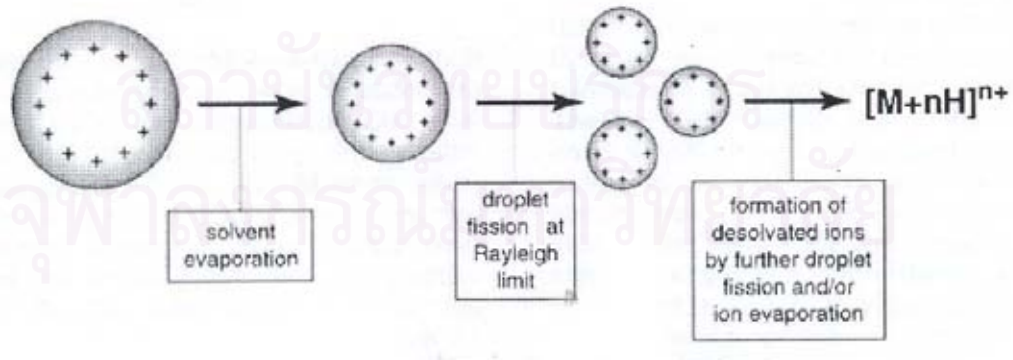


Figure 6. Droplet production in the electrospray interface.

6.2 Quadrupole mass analyzer

A quadrupole consists of a focusing lens stack and four cylindrical metal rods which act as the electrodes of the mass filter (Figure 7). Ions are accelerated and focused into the space between the rods by the lens stack. Quadrupoles operate using both radio frequency (r.f.) and d.c. voltages applied to the four rods of the analyzer. Using the appropriate amplitude and sign of the applied voltages, the trajectories of ions of a range of mass-to-charge ratios (m/z) can be made to be stable in both x- and y-directions as they travel through the rod assembly. The quadrupole operates as a mass analyzer when conditions are selected such that only ions of a single m/z have stable trajectories, all others being unstable in the x- and/or y-directions and hence being lost from the two-dimensional trapping field. By scanning the applied d.c. (U) and r.f. (V) voltages applied to the rods at a fixed U/V ratio, a full mass spectrum can be obtained as the trajectories of ions corresponding to a single m/z in turn become stable under the operating conditions and pass along the axis of the rods to the detector. The mass analysis equation:

$$m/z = \frac{2V}{q_x \omega^2 r_0^{\,2}}$$

shows how m/z is related to the applied r.f. voltage (V), the Mathieu stability parameter ($q_x = q_y$), the angular frequency of the applied r.f. (ω) and the inscribed radius of the rod assembly (r_0). A scan of V (with U also scanned such that V/U is constant) therefore yields a mass-selective stability mass spectrum [46].

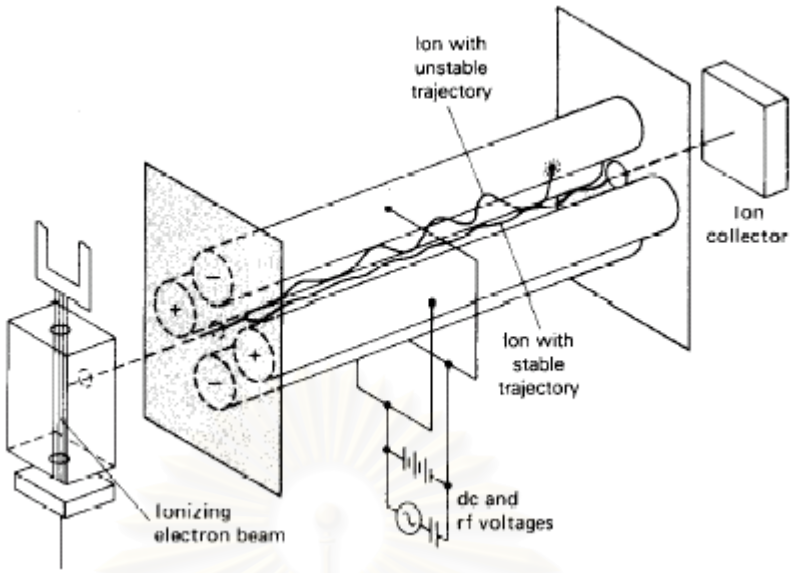


Figure 7. Schematic diagram of a quadrupole mass analyzer.

7. Tandem mass-spectrometry (MS/MS)

The tandem mass spectrometry is the instrument which consisted of two mass analysers (MS/MS) used in quantitative analysis has proved to be selective, even specific, for target analysis. The triple quadrupole is the most widely used tandem mass spectrometer. It is a linear assembly of three quadrupoles as shown in Figure 8. Only the first and the third quadrupoles are mass analysers, necessary for mass selection. The second quadrupole; the central one, is used as a collision cell with ion focusing properties.

Collisional activation

To achieve collisional activation in MS/MS instruments with spatially separate analysers, a collision cell is placed between the two mass analysers to promote fragmentation. This cell, often simply a small chamber with entrance and egress apertures, contains an inert target gas such as argon or xenon at a pressure sufficient for collisions with ions to occur. The acronyms CAD, for collision-activated dissociation, and CID, for collision-induced dissociation, are both in use to describe this process. The process is a two-step. The first step is collision between the ion and

the target molecules. Ion translational energy is converted to internal energy so that the ion is elevated from the ground state to an excited state. The second step is the unimolecular decomposition of the activated ion.

(a) High-energy collisions

This refers to collisions where the precursor ion is accelerated to kinetic energies of approximately one kilovolt or higher, resulting in the excitation of electronic states in the precursor ion. High-energy collisions produce a broad internal energy distribution. Virtually all structurally possible fragmentations have some probability of occurring.The target mass does not have a large influence on the MS/MS spectrum for high-energy CID because the center-of-mass energy is a small fraction of a large kinetic energy. This means that changes in the collision conditions (collision gas, pressure, and temperature) do not produce large changes in the product-ion mass spectrum. Therefore, high-energy CID is very reproducible. Helium is often used as the target gas for high-energy CID because it is inexpensive, it has a high ionization potential, and it does not cause large scattering of the precursor ions.

(b) Low-energy collisions

This refers to collisions where the precursor ions have kinetic energies in the range of a few eV to a few hundred eV. Low-energy collisions are thought to excite vibrational states, and they produce narrower internal energy distributions. The product ions that are observed depend strongly on the internal energy distribution. Increasing the collision energy shifts the center of the internal energy distribution to a higher value, and changes the product ions that are observed. A product-ion mass spectrum resulting from 10 eV collisions can be dramatically different from one resulting from 25 eV collisions. Xenon and argon are often used as target gases for low-energy CID to increase the center-of-mass energy and increase the probability of observing high-energy fragments.

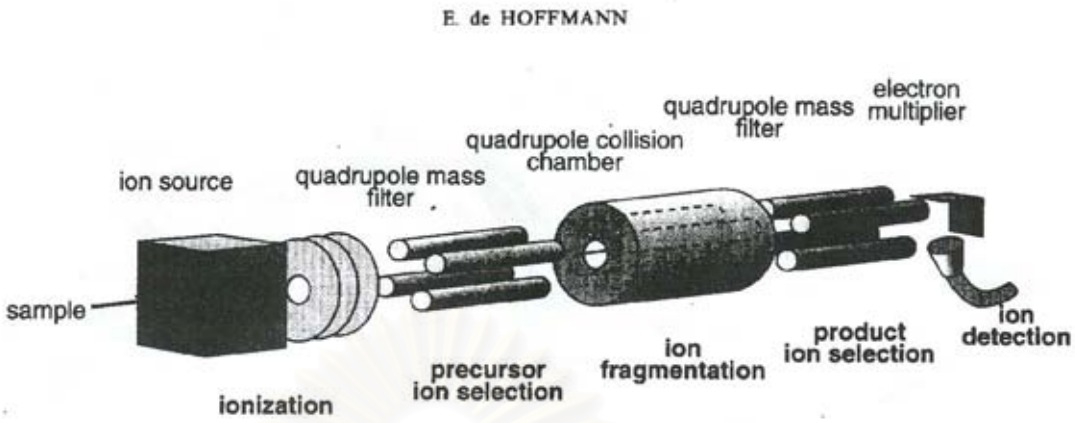


Figure 8. Schematic diagram of a triple quadrupole mass spectrometer.

7.1. Mode of tandem mass spectrometry

(a) Product ion scan (Qualitative analysis)

In this scan mode, ions of a given mass to charge ratio (m/z) value are selected with the first mass spectrometer. The selected ions are passed into the collision cell. The ions are activated by collision, and therefore are induced to fragment. The product ions are then analysed with the second mass spectrometer, which is set to scan over an appropriate mass range.

(b) Precursor ion scan (Qualitative analysis)

In this scan mode, the second mass spectrometer is set to pass only ions with a particular, selected m/z value. The first mass spectrometer is scanned over a chosen mass range, with a collision gas present in the instrument. Ions which pass through the first mass spectrometer will be detected.

(c) Neutral loss scan (Qualitative analysis)

Both analysers are now scanned together, but with a constant m/z difference between the two spectrometers. This scan allows the selective recognition of all ions which, by fragmentation, lead to the loss of a given neutral fragment.

(d) Multiple reaction monitoring (MRM) (Quantitative)

In the MRM method, a limited set or sets of precursor ion/product ion pairs are monitored. Detection is targeted only on compounds with the chosen precursor ions, and each precursor ion should also fragment to form a product ion of the chosen m/z. MRM is the specificity is highly method.

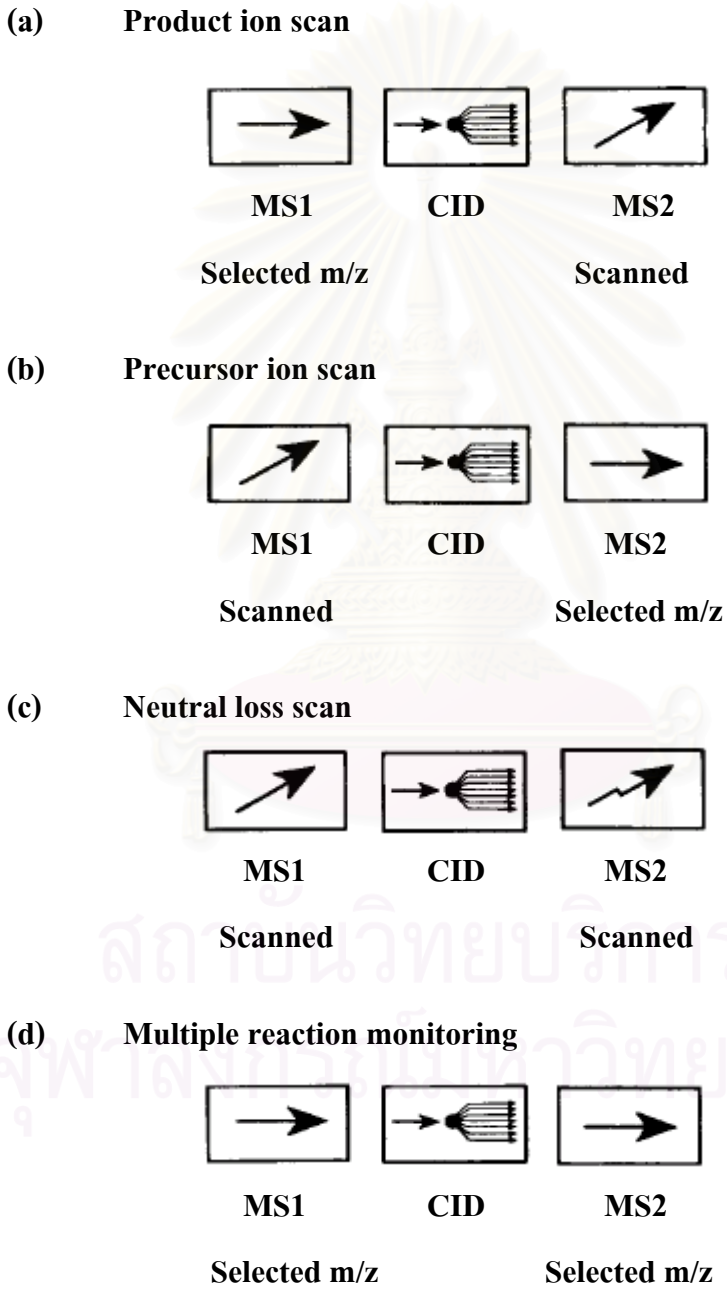


Figure 9. Mode of tandem mass spectrometry (MS/MS).

CHAPTER III

EXPERIMENTATION

1. Chemicals and Instruments

1.1 Dihydroartemisinin (Government Pharmaceutical Organization (GPO), Thailand)

1.2 Artemisinin (GPO, Thailand)

1.3 Drug-free human plasma was obtained from "Blood Blank Siriraj"

1.4 Acetonitrile, HPLC grade (Labscan, Thailand)

1.5 Methanol, HPLC grade (Labscan, Thailand)

1.6 Methanol, AnalaR grade (Labscan, Thailand)

1.7 Ethyl alcohol , AnalaR grade (Merck, Germany)

1.8 1-Chlorobutane, AnalaR grade (Labscan, Thailand)

1.9 2,2,4-Trimethylpentane, AnalaR grade (Labscan, Thailand)

1.10 Dimethyldichlorosilane, AnalaR grade (Labscan, Thailand)

1.11 Toluene, AnalaR grade (Labscan, Thailand)

1.12 Sodium chloride, AnalaR grade (Labscan, Thailand)

1.13 Water was deionized , filtered and purified on a Milli-Q Reagent grade

1.14 Micropipet (Eppendorf, Research 3111, Germany)

1.15 Vortex (Vortex-genie 2, Sciencetific Industries)

1.16 Centrifuge (Jouan BR 3.11, France)

1.17 High performance liquid chromatography (HPLC) (Water, Co., Ltd.)

1.18 Mass spectrometer (Model Quatto Micro)

2. Method

2.1. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) Optimization

2.1.1. Liquid chromatography condition

The HPLC system consisted of a Waters Model 2795 pump, an autosampler with temperature control. The system was programmed at 30 ^{o}C for the column, 5 ^{o}C for the sample oven (autosampler). Chromatographic separation was performed on luna 3 μm C_{18} column (150 x 2.00 mm) fitted with a guard column of the same packing material (4.0 x 2.0 mm). The injection volume was 20 μl, flow-rate was 0.2 ml/min.

Mobile phase

A search for the optimum mobile phase composition to provide the best chromatographic separation of dihydroartemisinin from internal standard and endogenous substance in plasma.

2.1.2. Tandem mass spectrometer (MS/MS) condition

Tandem mass spectrometry (MS/MS) was performed on the Quattro micro triple quadrupole mass spectrometer equipped with electrospray ion source. Data acquistion and analysis were performed using Mass Lynx version 3.5 software.

The following instrument settings were used: nitrogen nebulization, desolvation gas flow 500 l/h; cone gas flow 20 l/h; extractor at 2 V; RF lens at 0 V; multiplier at 650 V. The source temperature was set at 120 ^{o}C, desolvation temperature 300 ^{o}C and argon was used as collision gas at pressure of $3x10^{-3}$ millibars. In detecting dihydroartemisinin, dwell time for each transition was 0.3 s. In detecting artremisinin; dwell time was 0.6 s.

To find out the optimal parameters, by direct flow line injection of dihydroartemisinin and artemisinin (I.S.) in ethanol-water (50:50,v/v) solution, each at 500 ng/ml concentrations. Based on the findings of positive first quadrupole (Q1) scan and product ion scan, the following ion transitions are used for final MRM detection.

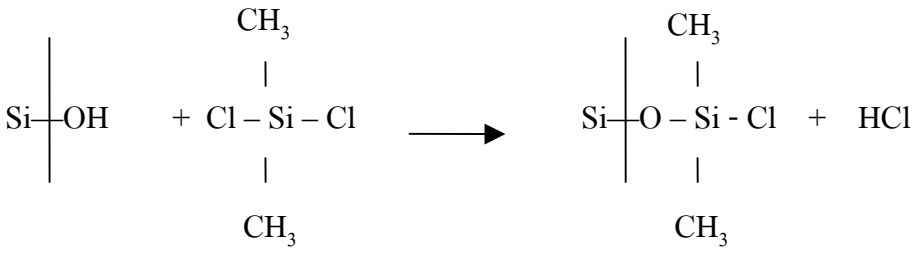
Cone voltage and capillary voltage were optimized for dihydroartemisinin and artemisinin (I.S.) by performing full scan acquistions.

Collision energies for fragmentation of precursor to product ion were optimized by flow injection analysis, varying the collision energies for each of the analytes to obtain the most intense precursor to product ion transitions.

2.2. Silanization procedure

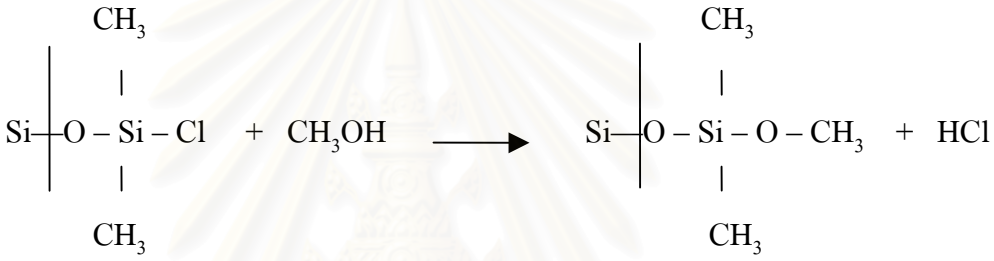
Diatomaceous earth is a chemical material that is essentially SiO_2. However, there are many Si-OH groups (Silanol groups) on the surface of this material. Samples with -OH groups (hydroxyl groups, such as dihydroartemisinin, ethanol) form hydrogen bonds with these silanol groups. To prevent this, the Si-OH group is chemically treated so that it cannot form hydrogen bonds. This treatment is called silanization. All glassware was silanized before use. The silanization procedure was carried out as follows: 5% (v/v) dimethyldichlorosilane (DMCS) in toluene was added to all glass tubes. The glassware were then vortexed mixing and glass the silanization solution was removed. The glassware were rinsed with methanol and the methanol was discarded and placed into an oven until dry. The reaction is shown in the Figure 10.

Step I



Glass surface **DMCS**

Step II



Glass surface **Methanol**

Figure 10. Silanization of glass surfaces.

2.3. Internal standard method

In the internal standard method, some standard material is added to the sample. The material added to the sample is called the internal standard (I.S.). The calibration curve shows the relationship between the concentration ratio and the peak area or height ratio of the target component to internal standard.

The internal standard should be selected such that it fulfills the following conditions:

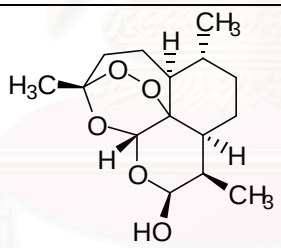
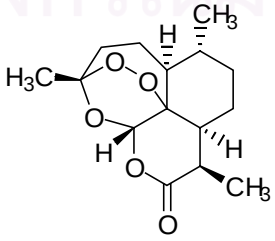
1. The peak of the internal standard should be separate and distinct from components in the sample.
2. The peak of the internal standard should be close to the peak of the target component.

3. The internal standard should have chemical properties similar to those of the target component.

Compared to the absolute calibration curve method, the internal standard method takes more time and care. But, since the calibration curve is generated based on relative sensitivity and volume is determined from peak area ratios, there is little effect from error introduced by differences in injection technique. For this reason, the internal standard method is often used for highly accurate quantitative analysis.

In this experiment, artemisinin was chosen as internal standard because artemisinin have chemical properties similar to dihydroartemisinin, can separate from components in the sample, and not present in original plasma.

Table 1. Properties of dihydroartemisinin and artemisinin (I.S.)

Analytes	Structure	Molecular weight	Molecular formular	Solubility
Dihydroartemisinin		284.35	$C_{15}H_{24}O_5$	Soluble in chloroform; acetone and ethanol; insoluble in water
Artemisinin (I.S.)		282.17	$C_{15}H_{22}O_5$	Soluble in acetone; methanol; ethanol; insoluble in water

2.4. Preparation of calibration standards and quality control samples

Individual standard stock solutions of dihydroartemisinin and artemisinin (I.S.) were prepared by accurately weighing required amounts into separate volumetric flasks and dissolving in appropriate volumes of ethanol to obtain the required concentrations. Working solutions of dihydroartemisinin in the range 1,250-50,000 ng/ml and I.S. at a concentration of 125,000 ng/ml were prepared by dilute of the stock solutions with ethanol-water (50:50,v/v). The working solutions were used to prepare the calibration standards (Cs) and quality control samples (QCs). All the solutions were prepared in polypropylene flasks and stored in darkness about 0-4 ^{o}C. Cs and QCs were prepared for calibration, accuracy and precision, quality control and stability assessment by adding appropriate volume of working solution to 0.5 ml of drug-free human plasma.

2.5. Sample preparation

A simple liquid-liquid extraction to isolate dihydroartemisinin from 0.5 ml plasma.

10 µl of artemisinin (I.S.) 500 ng/ml was added to calibration standard (Cs), quality control samples (QCs) or actual samples

↓

Add 0.125 ml of NaCl saturated solution and
2.5 ml of 1-chlorobutane-isooctane (55:45,v/v)

↓

Vortex mixing for 5 min and following centrifugation for 15 min at 3000 g at 15 ^{o}C

↓

1 ml of organic layer was transferred into another tube

↓

The solvent was evaporated to dryness under a nitrogen stream

↓

The residue was dissolved in 1 ml of ethanol-water (50:50,v/v) by vortex mixing and injected 20 µl onto the LC-MS-MS method which obtained from section 2.1.

3. Analytical method validation

3.1. Specificity

The specificity was defined as non-interferene in the regions of interest with the endogenous substances, drug metabolites of dihydroartemisinin in the determination of the concentration. Six human plasma from six healthy donors receiving no medication were extracted and analysed for the assessment of potential interferences with endogenous substances.

3.2. Recovery

The recovery in sample preparation method is an important parameter that affects quantitative such as sensitivity, limit of detection (LOD) and even the range of quantitation.

The analytical recoveries of the extraction procedure for dihydroartemisinin and artemisinin (I.S.) were estimated by comparing the peak areas obtained from an extracted sample with those from non-extracted standards at the same concentration. The concentrations used were 5, 50, 200 ng/ml for dihydroartemisinin and 500 ng/ml for artemisinin (I.S.).

3.3. Limit of detection (LOD)

The limit of detection (LOD) is defined as the lowest concentration that can be discriminated from the baseline level, with signal intensity at least three times greater than the baseline noise (S/N > 3). The LOD was carried out by injection of low concentration of dihydroartemisinin solutions under the optimal LC-MS/MS parameters. The signal value of 3 times from injected 1 ng/ml of dihydroartemisinin for six replicates was converted to the concentration to give the detection limit.

3.4. Linearity and calibration range

Linearity was assessed by six standard solution in the range 5 to 200 ng/ml were extracted described in section 2.4 and analyzed using the condition LC-ESI-MS/MS described in section 2.1. Calibration curve ($y = mx + b$), represented by the plots of the peak areas ratio (y) of DHA to I.S versus the concentration (x) of the calibration standards. The calibration was constructed using weighting (1/x) linear regression model. Linear calibration curves were obtained with a coefficient of correlation (r) usually higher than 0.995. Samples with concentrations above this upper limit of quantitation (ULOQ) should be diluted prior to re-analysis.

3.5. Accuracy and Precision

All measurements are accompanied by a certain amount of error, and an estimate of its magnitude is necessary to validate results. The error cannot be eliminated completely, although its magnitude and nature can be characterized. It can also be reduced with improved techniques.

Intra-day accuracy and precision was determined by injection of calibration samples (Cs) and quality control samples (QCs) at levels of 5, 10, 50 and 200 ng/ml (n = 3 at each level). To assess the inter-day accuracy and precision, the intra-day assays were repeated on three different days and overall performance was calculated.

Accuracy was determined by calculating the mean recovery for the concentrations found in % of the nominal concentration in standard samples.

Precision is measure of reproducibility and is affected by random error. Since all measurements contain random error, the result from a single measurement cannot be accepted as the true value. An estimate of this error is necessary to predict within what range the true value may lie, and this is done by repeating a measurement several times. Two important parameters, the *average value* and the *variability of the measurement*, are obtained from this process. The most widely used measure of average value is the arithmetic mean, :

$$\bar{x} = \frac{\sum x_i}{n}$$

where $\sum xi$ is the sum of the replicate measurements and n is the total number of measurements. Since random errors are normally ditributed, the common measure of variability (or precision) is the standard deviation, σ .

This is calculated as

$$\sigma = \frac{\sqrt{\sum (xi - \bar{x})^2}}{n}$$

When the data set is limited, the mean is often approximated as the true value, and the standard deviation may be underestimated. In that case, the unbiased estimate of σ, which is designated s, is computed as follows:

$$s = \sqrt{\frac{\sum (xi - \overline{x})^2}{n-1}}$$

As the number of data points becomes larger, the value of s approaches that of σ. When n becomes as large as 20, the equation for σ may be used. Another term commonly used to measure variability is the coefficient of variation (C.V.) or relative standard deviation (RSD), which may also be expressed as a percentage:

$$\% \text{ C.V.} = \frac{s}{x} \text{x } 100 \qquad \text{or} \qquad \% \text{ RSD} = \frac{s}{x} \text{x} 100$$

The following validation criteria for accuracy and precision were used to assess the method suitability: mean recoveries must be within 85-115% except at the limit of quantitation (LOQ) where it should be within 80- 120%; % C.V. should not exceed 15% except at the LOQ where it should not exceed 20%.

3.6. Stability

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. Stability procedures was evaluate the stability of the analytes during sample collection and handling after going through freeze and thaw cycles and the analytical process. Conditions used in stability experiments was reflect situations likely to be encountered during actual sample handling and analysis. The procedure was also include an evaluation of analyte stability in stock solution.

3.6.1. Stock solution stability

3.6.1.1 Dihydroartemisinin in working solutions at concentration of 200, 1,250, 5,000 ng/ml were stored at 0-4 ^{o}C for 4 weeks, the stability were tested by comparing the response with that of freshly prepared solutions.

3.6.1.2 Stability of working solutions of dihydroartemisinin were evaluated at room temperature for 6 hours, the working solution at concentration of 50 and 200 ng/ml were stored at room temperature in darkness for 6 hours and then analyzed.

3.6.2. Long-term stability

Stability of spiked sample at the concentration of 10 and 200 ng/ml after long term (3 months) storage at -80 ^{o}C were determined by repeat analysis of three replicate. Stability was expressed as recovery (%) of dihydroartemisinin by comparing the obtained concentrations of stored samples with those of freshly prepared samples.

3.6.3. Freeze and thaw stability

The effect of three freeze-thaw cycles, were determined by repeat analysis of six replicate sample at concentration of 20 ng/ml and 200 ng/ml were stored at -80 ^{o}C for 24 hours and thawed at room temperature. When completely thawed, the samples were refrozen for 21 hours under the same conditions. Then analyzed on the third cycle.

3.6.4. Post – preparative stability

The plasma sample extracts at 10 and 100 ng/ml (n=3) were stored at 5 ^{o}C in autosampler, the stability were assessed by determining concentrations on original calibration standards. The repeatability was assessed.

CHAPTER IV

RESULT AND DISCUSSION

1. Liquid chromatography - tandem mass spectrometry (LC-MS/MS) optimization

1.1. Tandem mass spectrometry (MS/MS) optimization

1.1.1. Mass spectra analysis

Dihydroartemisinin and artemisinin (I.S.) in 50% ethanol solution at concentration of 500 ng/ml was directly infused to the ESI-mass spectrometer at a flow rate of 10 μl/min using a syringe pump. First quadrupole (Q1) scan was performed to scan the mass range of m/z of 100-350 for positive modes. Protonated specie $(MH)^+$ at m/z of 285 for dihydroartemisinin and m/z of 283 for artemisinin were not detected. A sodium adduct ion specie $(M+Na)^+$ were predominant at m/z of 307 for dihydroartemisinin and at m/z of 305 for artemisinin on the ESI mass spectra are shown in Figure 11 and Figure 12, respectively. Consequently, the precursor ion $(M+Na)^+$ at m/z of 307 for dihydroartemisinin and 305 for artemisinin were selected at first quadrupole (Q1).

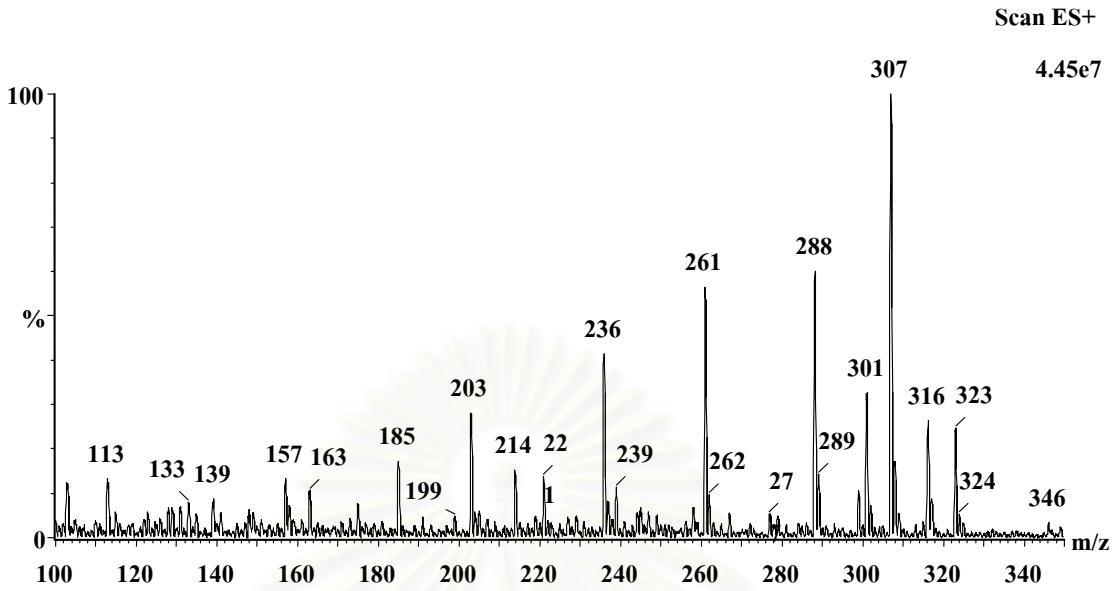


Figure 11. Conventional mass spectra of dihydroartemisinin.

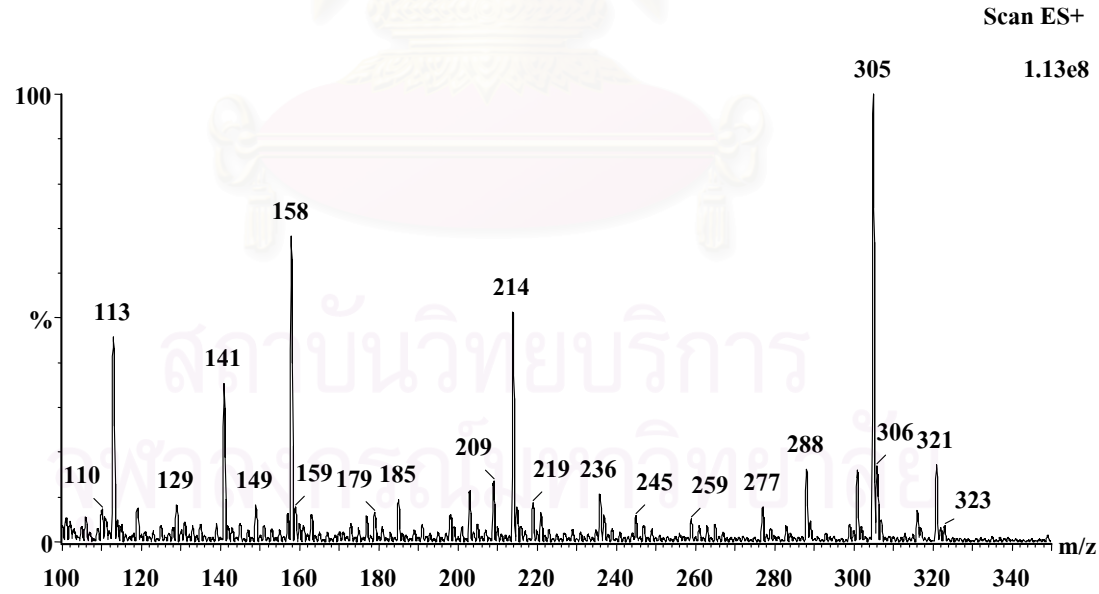


Figure 12. Conventional mass spectra of artemisinin.

1.1.2. Optimization of parameters

1.1.2.1. *Capillary voltage*

The capillary voltages were optimized to obtain the highest signal for selected ion $(M+Na)^+$ of dihydroartemisinin at m/z of 307 and artemisinin at m/z of 305 are shown in Figure 13. The results show a sodium adducts specie ion $(M+Na)^+$ at m/z of 307 of dihydroartemisinin and m/z of 305 of artemisinin were found to be most intense at capillary voltage around 3.2 kV.

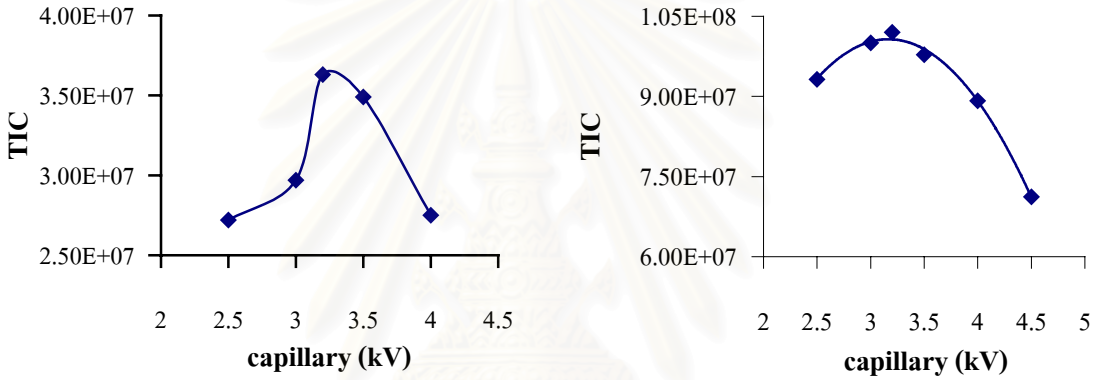


Figure 13. Optimization of capillary voltage for $(M+Na)^+$ of (a) dihydroartemisinin; (b) artemisinin.

1.1.2.2. *Cone voltages*

The cone voltages were optimized to obtain the highest signal for selected ion $(M+Na)^+$ of dihydroartemisinin at m/z of 307 and artemisinin at m/z of 305 are shown in Figure 14. The results show a sodium adducts specie ion $(M+Na)^+$ at m/z of 307 of dihydroartemisinin and m/z of 305 of artemisinin were found to be most intense at cone voltage of 25 V, and 30 V, respectively.

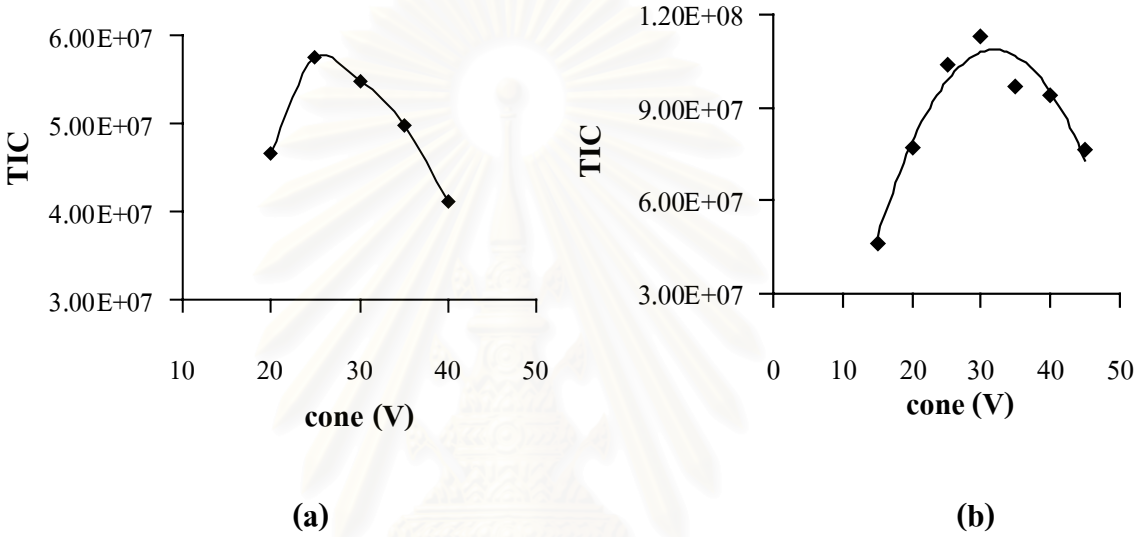


Figure 14. Optimization of cone voltage for $(M+Na)^+$ of (a) dihydroartemisinin; (b) artemisinin.

1.1.2.3. *Collision energy*

Product ions were generated through fragmentation of the precursor ion using argon as collision gas.

For dihydroartemisinin, when the precursor ion at m/z of 307 $(M+Na)^+$ was selected at first quadrupole (Q1). The selected ions (m/z of 307) are passed into the collision cell. The ions at m/z of 307 are activated by collision, induced to fragment. The product ions are then analysed with the third quadrupole (Q3), the major product ion at m/z of 261 and secondary product ion at m/z of 163 were generated (Figure 15). Collisional energies for fragmentation of precursor to product ions were optimized at 10 eV for most intense ion 261 and 18 eV for most intense ion 163 are shown in Figure

16. The proposed structures of product ion at m/z of 261 and m/z of 163 from precursor ion at m/z of 307 are showed in Figure 17.

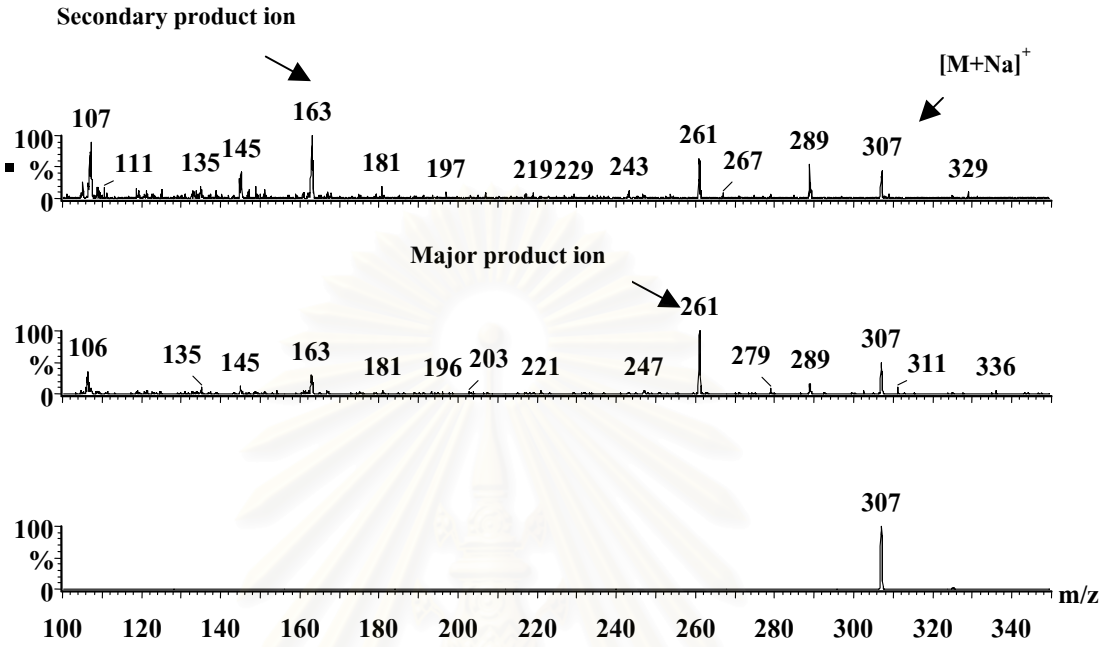


Figure 15. Product ion spectra on MS-MS of the $(M+Na)^+$ at m/z of 307 of dihydroartemisinin.

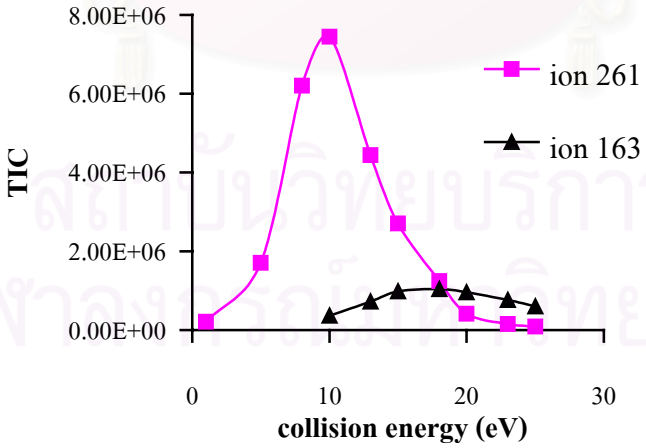
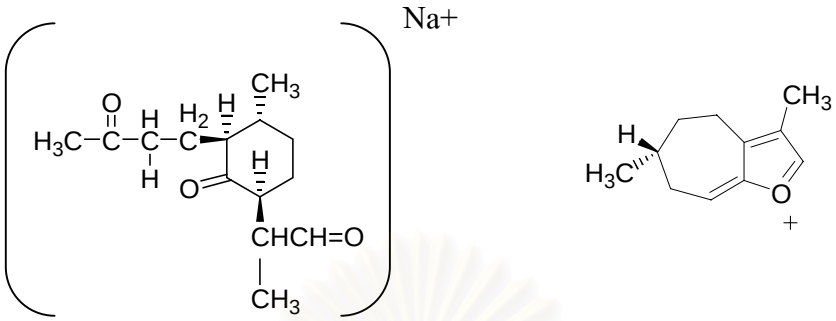


Figure 16. Optimization collision energies for product ion at m/z of 261 and 163 of $(M+Na)^+$ at m/z of 307.



(a) (b)

Figure 17. Proposed structure of product ion at : (a) m/z of 261 and (b) m/z of 163 from precursor ion at m/z of 307.

For artemisinin, when the precursor ion at m/z of 305 $(M+Na)^+$ was selected at first quadrupole (Q1) and collided in collision cell, the most intense product ion at m/z of 151 was generated (Figure 16). Collision energies were optimized at 18 eV to obtain the most intense ion 151 is shown in Figure 19.

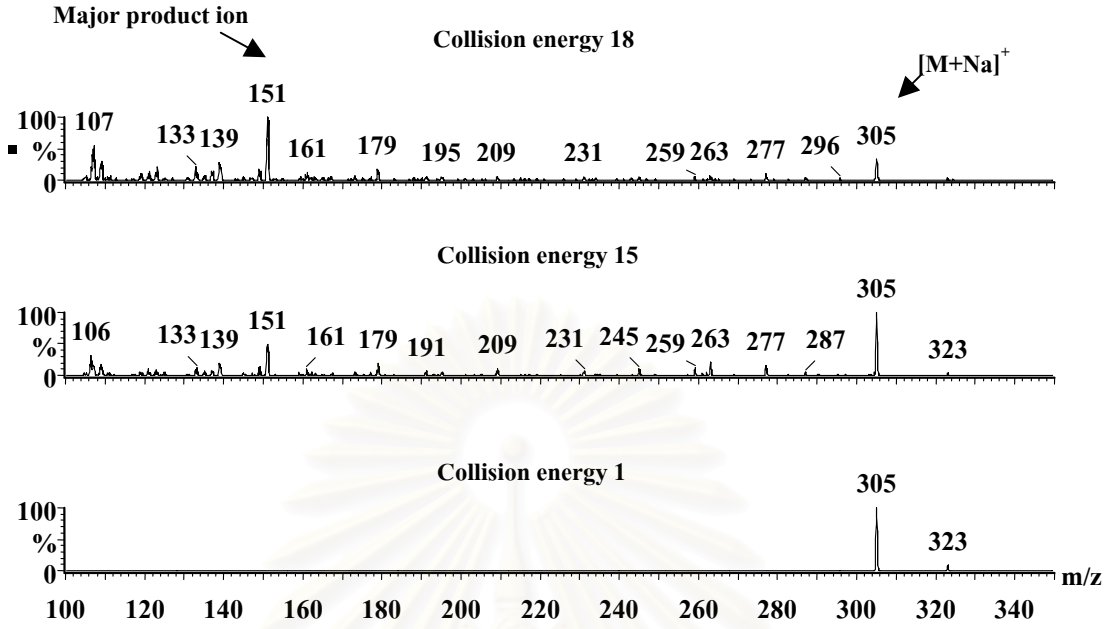


Figure 18. Product ion spectra on MS-MS of the $(M+Na)^+$ at m/z of 305 of artemisinin (I.S).

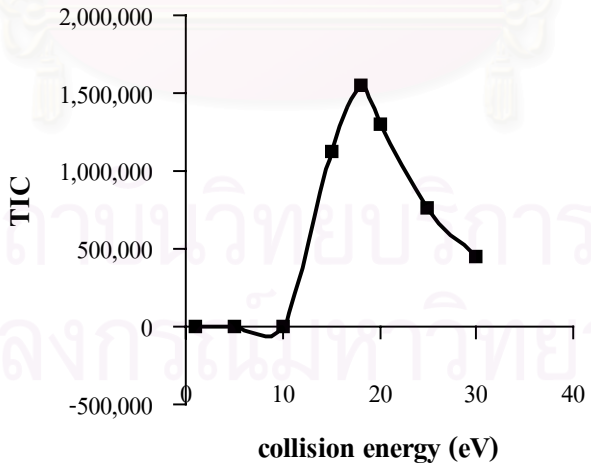


Figure 19. Optimization collision energies for product ion at m/z of 151 of $(M+Na)^+$ m/z of 305.

The two selection for dihydroartemisinin fragmentation; m/z of 307 to 261 and 307 to 163, one selection for artemisinin (I.S.) fragmentation; m/z of 305 to 151 were monitored in single function were considered for quantitation for both analytes.

The optimization of LC-ESI-MS/MS in multiple reaction monitoring (MRM) mode, are summarized in Table 2.

Table 2. MRM conditions for dihydroartemisinin and artemisinin.

Analyte	Funtion	Channel	Precursor ion (m/z)	Product ion (m/z)	Cone (V)	Capillary (kV)	Collision energy (eV)
DHA	MRM of 2	1	307	261	25	3.2	10
	mass pairs	2	307	163	25	3.2	18
Artemisinin	MRM of 1	1	305	151	30	3.2	18
(I.S.)	mass pairs						

1.2. Chromatographic condition

The optimal composition of acetonitrile and water in ratio of 50:50 (v/v) was used, the peak of dihydroartemisinin cloud be separated from internal standard and endogenous substance in plasma. The obtained chromatogram is shown in Figure 20. Retention times were about 6.67, 9.70, 12.70 for α, β-dihydroartemisinin and I.S., respectively. The duration of the analytical time was 15 min. Only the α-tautomer (the predominant peak) of DHA was taken into account for quantitation, the constant ratio α versus β tautomer being around 28 in working solution and around 68 in plasma extract sample.

LC-tandem mass spectrometric in multiple reaction monitoring (MRM) mode were used in the optimal condition as describe above for dihydroartemisinin analysis.

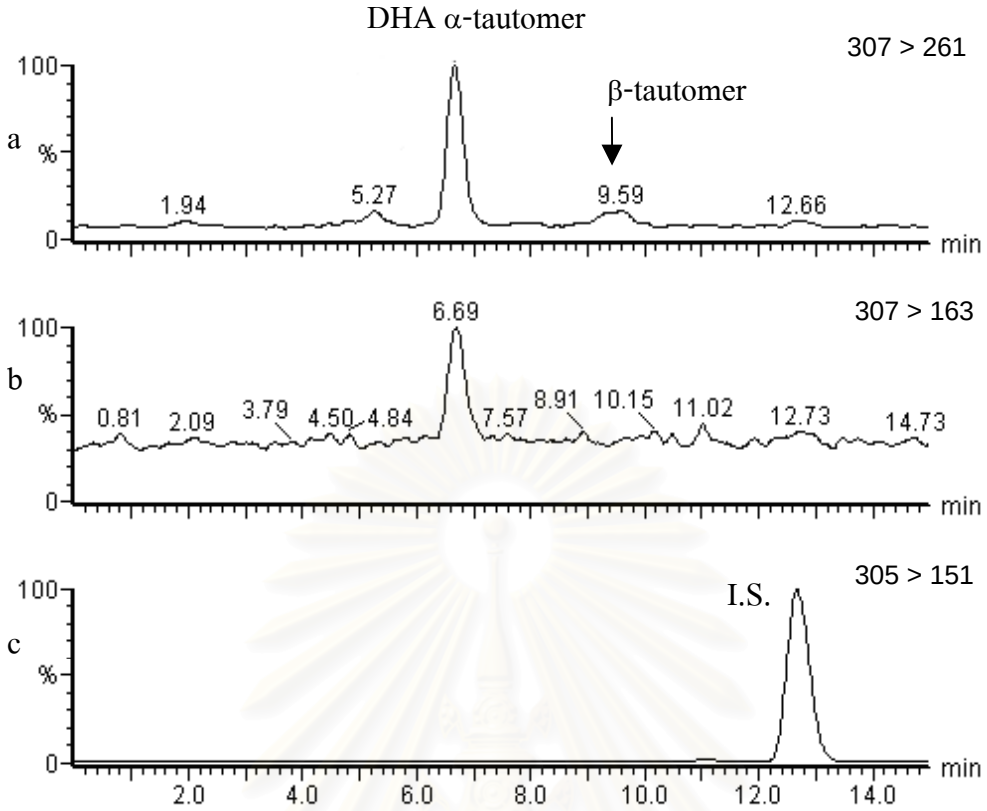


Figure 20. LC-MRM chromatograms of (a, b) dihydroartemisinin (5 ng/ml); and (c) artemisinin (I.S) (500 ng/ml) in human plasma.

2. Analytical method validation

2.1. Specificity

LC-MS/MS analysis of the six different plasma samples from volunteers with no administration showed no endogenous peak at the retention times of DHA and I.S. Chromatograms of extracts of drug free six human plasma and plasma sample spiked with DHA and I.S. are shown in Figure 21.

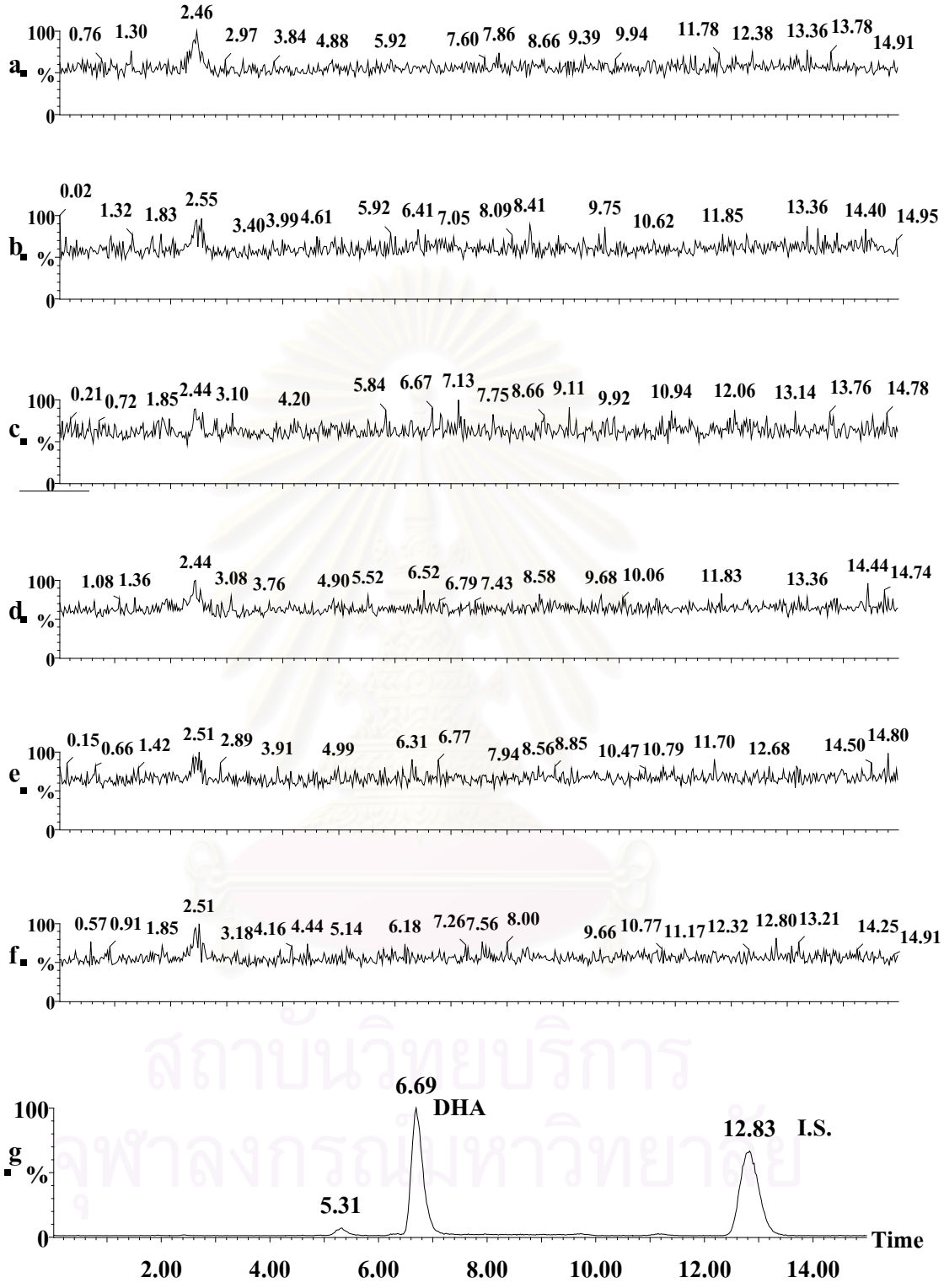


Figure 21. LC-MRM chromatograms of (a-f) six different of blank plasma; (g) plasma standard spiked with 50 ng/ml of dihydroartemisinin and 500 ng/ml of artemisinin (I.S.).

2.2. Recovery

Average recoveries for dihydroartemisinin in plasma over the concentration range of 5-200 ng/ml are shown in Table 3. The results show mean ± S.D. recoveries after liquid-liquid extraction were range from 69.9 - 89.2 % for dihydroartemisinin and 89.9 % for artemisinin (I.S.) (n=9) at concentration of 500 ng/ml.

Table 3. Mean recoveries of dihydroartemisinin from spiked human plasma

Analyte	Concentration (ng/ml)	Absolute recovery (mean ± SD) (%)
Dihydroartemisinin (n=3)	5	69.9 ± 13.2
	50	81.4 ± 2.7
	200	89.2 ± 3.2
Artemisinin (I.S) (n=9)	500	89.9 ± 7.5

2.3. Limit of detection (LOD)

The limit of detection (LOD) was determined under the definition of three times of signals to noise ratio that is 3 S/N. The LOD of this method was 0.16 ± 0.02 ng/ml. The obtained LOD is sufficiently sensitive for detection.

2.4. Linearity and calibration range

Calibration standard samples at levels of 5, 10, 20, 50, 100 and 200 ng/ml were used in the linearity determination. When plotting the peak area ratio (y) of dihydroartemisinin to I.S. versus the dihydroartemisinin concentration (x), the calibration curve was found to be linear over the range of concentration observed. Calibration curves gave relevant regression lines of y = 0.0189422x + 0.0109263. Correlation coefficient was 0.9984 as shown in Figure 22. A new calibration curve is prepared every working day.

For each calibration standard level, the concentration was back-calculated from the linear regression curve equation. Inter-day variability on three different days is presented in Table 4. The precision was ≤ 12.6% and mean accuracies was within 7.0 % of the nominal values for dihydroartemisinin.

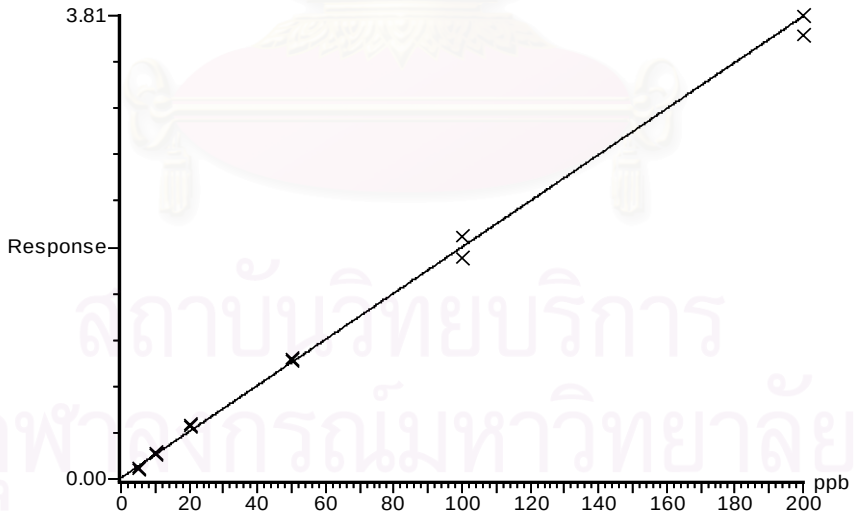


Figure 22. Calibration curve from the method validation.

Table 4. Back-calculated concentrations from calibration curves.

Nominal conc.(ng/ml)	5	10	20	50	100	200
Dihydroartemisinin						
Intra-day accuracy(%)(day1)	99.3	100.3	99.9	107.4	105.1	95.7
Intra-day precision(%)(day1)	-	10.9	2.8	4.2	1.3	3.3
N	1	2	2	2	2	2
Intra-day accuracy (%)(day2)	107.0	110.5	101.2	98.9	108.2	95.0
Intra-day precision (%)(day2)	-	12.2	0.1	-	6.1	1.5
N	1	2	2	1	2	2
Intra-day accuracy (%)(day3)	83.9	108.6	112.5	102.8	100.0	98.1
Intra-day precision (%)(day3)	5.1	4.6	2.0	1.1	6.5	3.1
N	2	2	2	2	2	2
Inter-day accuracy (%)	93.5	106.5	104.5	103.8	104.4	96.2
Inter-day precision (%)	12.6	8.8	6.1	4.1	5.3	2.6
N	4	6	6	5	6	6

2.5. Accuracy and Precision

Intra-, Inter-day accuracy and precision at the four concentrations (5, 10, 50 and 200 ng/ml) are presented in Table 5. The result show that the analytical method is highly accurate; the intra-, inter-day accuracy is within the acceptance limits of ± 20% at low concentration and ±15% for all the concentration levels studied. The intra-, inter-day %C.V. was found to be less than 14.2%.

Table 5. Accuracy and precision of dihydroartemisinin in human plasma

QC conc. (ng/ml)	Intra -day 1 (n=3)		Intra -day 2 (n=3)		Intra-day 3 (n=3)		Inter-day (n=9)	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
5	101.5	8.1	95.7	7.5	120.0	13.1	105.7	13.2
10	110.2	14.2	113.0	3.6	99.7	4.2	107.6	9.1
50	111.2	9.0	104.4	2.6	92.7	1.6	102.7	9.1
200	101.2	2.7	101.3	0.9	107.3	1.2	103.3	3.3

2.6. Stability

2.6.1. Stock solution stability

2.6.1.1 Working solution of dihydroartemisinin were found to be stable for at least 4 weeks at about 0-4 ^{o}C, the mean recovery (n=3) from comparing the response with that of freshly prepared solutions were 94.8%, 91.6%, and 89.8% for dihydroartemisinin at concentration of 200, 1,250 and 5,000 ng/ml, respectively.

2.6.1.2 Dihydroartemisinin in working solutions were found to be stable for at least 6 hours at room temperature in darkness; the mean recovery (n=3) from comparing the response with that of freshly prepared solutions were 112.8% and 120.3% for dihydroartemisin at concentration of 50 and 200 ng/ml, respectively.

2.6.2. Long-term stability

Dihydroartemisinin was stable for at least 3 months in spiked human plasma samples when frozen at -80 ^{o}C. The mean recovery (n=3) were 101.7% and 99.9% at concentration of 10 and 200 ng/ml, respectively.

2.6.3. Freeze and thaw stability

Dihydroartemisinin mean recovery values (n=6) after three freeze-thaw cycles were 106.7% and 89.5% of the nominal value for concentration of 20 and 200 ng/ml, respectively. The results shown mean recovery was within ± 10% indicating dihydroartemisinin was stable after three freeze-thaw cycles.

2.6.4. Post-Preparative stability

The plasma sample extracts were found to be stable during at least 26 hours in the autosampler at about 5 ^{o}C, the concentrations following the storage period (n=3) were 96.9% and 86.6% of the nominal concentration of 10 and 100 ng/ml, respectively.

CHAPTER V

SUMMARY

A novel liquid chromatography-tandem mass spectrometry (LC-MS/MS) method with high sensitivity and specificity was developed for analysis of dihydroartemisinin in human plasma. The method consists of sample preparation by liquid-liquid extraction with 1-chlorobutane-isooctane (55:45,v/v) followed by chromatographic separation on a C_{18} column and detection with positive electrospray ionization (ESI) using the multiple reaction monitoring (MRM) mode.

A liquid-liquid extraction with 1-chlorobutane-isooctane that gave high recovery for dihydroartemisinin and artemisinin (I.S.). The mean recovery was ranged from 69.9-89.2% and 89.9% for dihydroartemisinin and artemisinin (I.S), respectively.

The optimal mobile phase of acetronitrile-water (50:50,v/v), could be separated dihydroartemisinin from internal standard (I.S) and no interference from endogenous substance. Electrospray ionization (ESI) technique has proven effective in generating ions with sufficient intensity to be monitoring quantitatively. In multiple reaction monitoring (MRM) method, dihydroartemisinin was quantified of the summed product ion at m/z of 261 and m/z of 163 from precursor ion at m/z of 307 $[M+Na]^+$. And artemisinin (I.S.) was quantified of product ion at m/z of 151 from precursor ion at m/z of 305 $[M+Na]^+$. This technique offers low noise baseline as well.

Stability of dihydroartemisinin in spiked human plasma samples were found to be stable after three freeze-thaw cycles and after 3 months when storage at -80 ^{o}C. The plasma sample extracts were found to be stable during at least 26 hours in the autosampler at about 5 ^{o}C. The working solutions were found to be stable for at least 4 weeks at about 0-4 ^{o}C.

The method is specific, accurate and precise. Calibration curve was linear over the range 5-200 ng/ml using 0.5 ml of human plasma per assay. The detection limit was 0.16 ng/ml, which sensitive enough for the determination of dihydroartemisinin in human plasma for pharmacokinetic analyses.

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APPENDIX

Table 6. Relationship between peak area ratios of dihydroartemisinin to internal standard and concentration of dihydroartemisinin in human plasma.

Concentration (ng/ml)	Peak area ratios (n=2)	
	Mean	% C.V.
5	0.0905	3.9
10	0.2165	4.3
20	0.4370	1.9
50	0.9850	1.2
100	1.9050	6.5
200	3.7245	3.1

Table 7. Peak areas of dihydroartemisinin in ethanol-water (50:50, v/v) solutions at concentration of 200, 1,250 and 5,000 ng/ml were stored at 0-4 ^{o}C for 4 weeks and freshly prepared solutions.

Concentration (ng/ml)	Peak areas of dihydroartemisinin (n=3)					
	200		1,250		5,000	
	fresh	4 weeks	fresh	4 weeks	fresh	4 weeks
	21876	23522	129628	118634	327761	300487
	25764	24607	127088	114374	341903	292369
	28618	24235	125435	117411	331183	306027
Mean	25419	24121	127384	116806	333616	299628
Mean recoveries		94.8%		91.6%		89.8%

$$\text{Mean recoveries} = \frac{\text{Peak areas of DHA were stored at 0-4 } ^{o}\text{C for 4 weeks}}{\text{Peak areas of DHA freshly prepared solutions.}} \times 100$$

Table 8. Peak areas of dihydroartemisinin in ethanol-water (50:50, v/v) solutions at concentration of 50 and 200 ng/ml were stored at room temperature in darkness for 6 hours and freshly prepared solutions.

Concentration (ng/ml)	Peak areas of dihydroartemisinin (n=3)			
	50		200	
	fresh	6 hours	fresh	6 hours
	12669	13416	43633	52553
	12407	14601	44830	52900
	13060	15017	43283	53083
Mean	12712	14344	43915	52845
Mean recoveries		112.8%		120.3%

$$\text{Mean recoveries} = \frac{\text{Peak areas of DHA were stored at room temperature for 6 h. x100}}{\text{Peak areas of DHA freshly prepared solutions.}}$$

Table 9. Stability of dihydroartemisinin in spiked human plasma samples at concentration of 10 and 200 ng/ml after 3 months storage at - 80 ^{o}C

Concentration (ng/ml)	Observed concentration (ng/ml)	Recovery (%)	Accuracy: mean recovery (%)	Precision: C.V. (%)
10	11.0 9.7 9.7	110.5 97.1 97.4	101.7	7.5
200	197.1 198.5 204.1	98.5 99.2 102.1	99.9	1.9

Table 10. Stability of dihydroartemisinin in spiked human plasma samples at concentration of 20 and 200 ng/ml after three freeze-thaw cycles.

Concentration (ng/ml)	Observed concentration (ng/ml)	Recovery (%)	Accuracy: mean recovery (%)	Precision: C.V. (%)
20	24.8	123.8	106.7	11.4
	19.9	99.4		
	22.8	114.0		
	18.1	90.5		
	22.4	112.1		
	20.0	100.1		
200	176.6	88.3	89.5	4.0
	187.2	93.6		
	184.9	92.5		
	170.0	85.0		
	182.9	91.4		
	171.8	85.9		

Table 11. Stability of plasma sample extracts at concentration of 10 and 100 ng/ml after stored at 5 ^{o}C in autosampler for 26 hours.

Concentration (ng/ml)	Observed concentration (ng/ml)	Recovery (%)	Accuracy: mean recovery (%)	Precision: C.V. (%)
10	9.3 10.2 9.6	93.4 101.9 95.6	96.9	4.6
100	91.9 82.4 85.4	91.9 82.4 85.4	86.6	5.6

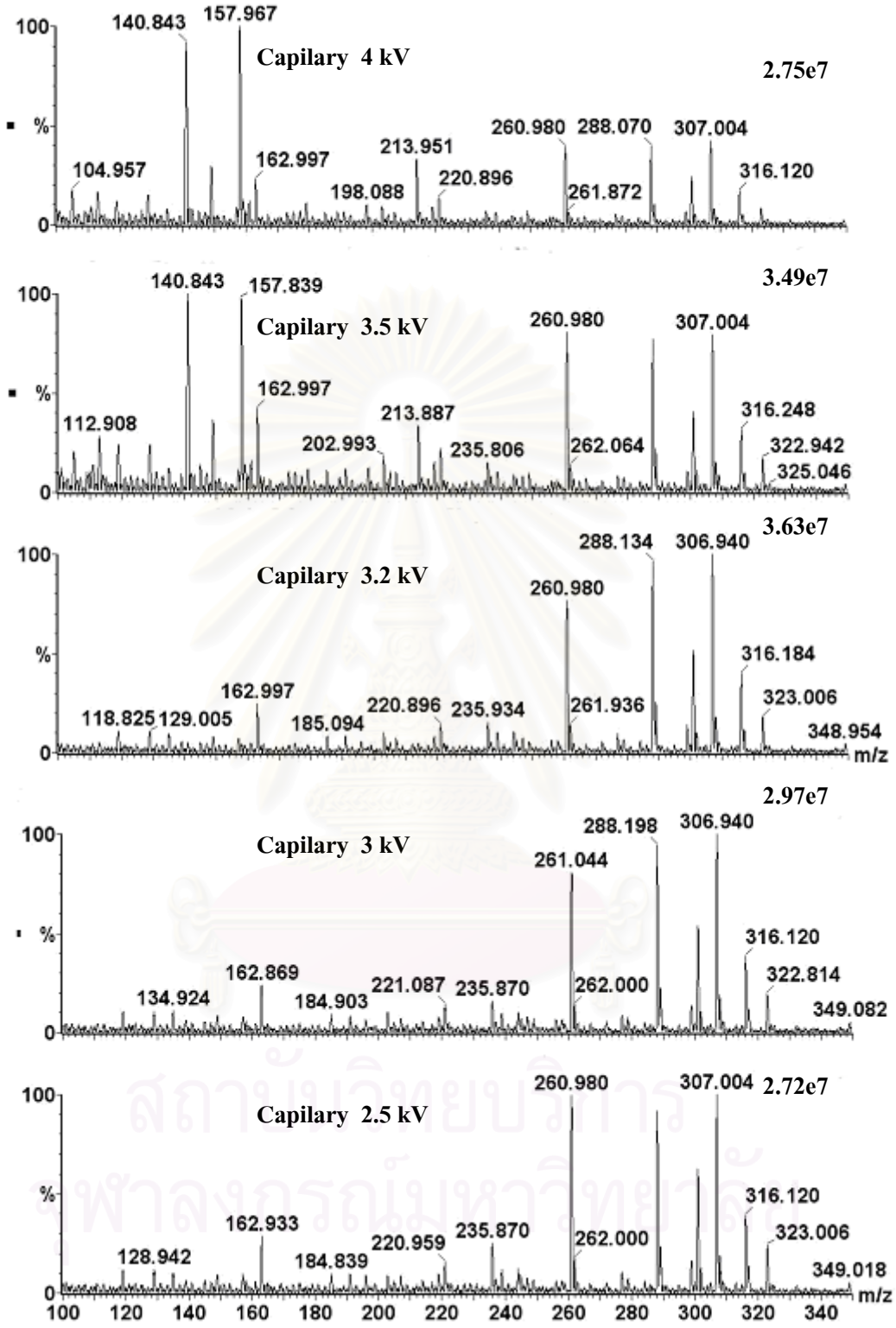

Figure 23. Effect of capillary voltage on internsity of dihydroartemisinin $(M+Na)^+$ at m/z of 307.

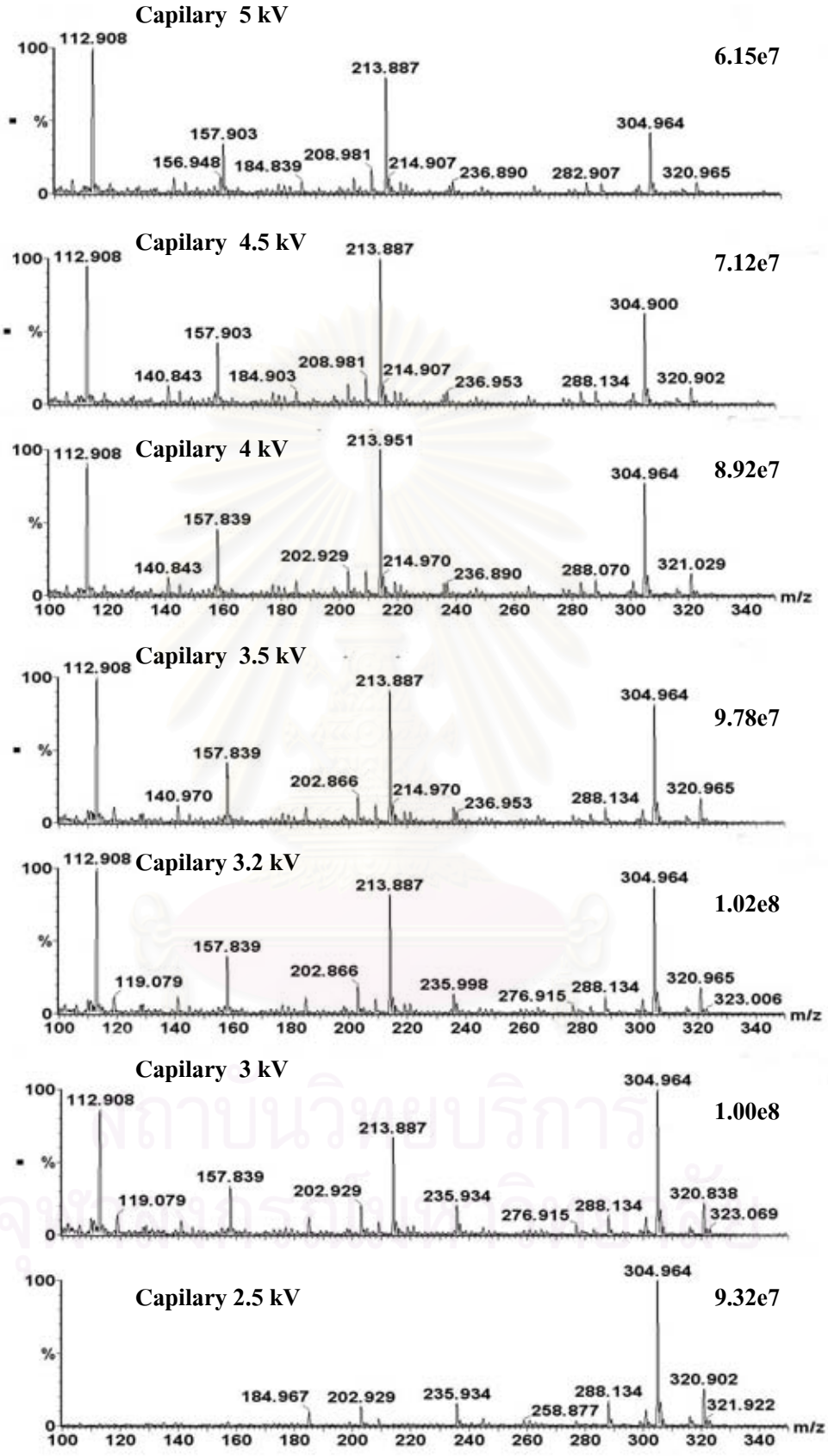

Figure 24. Effect of capillary voltage on internsity of artemisinin $(M+Na)^+$ at m/z of 305 .

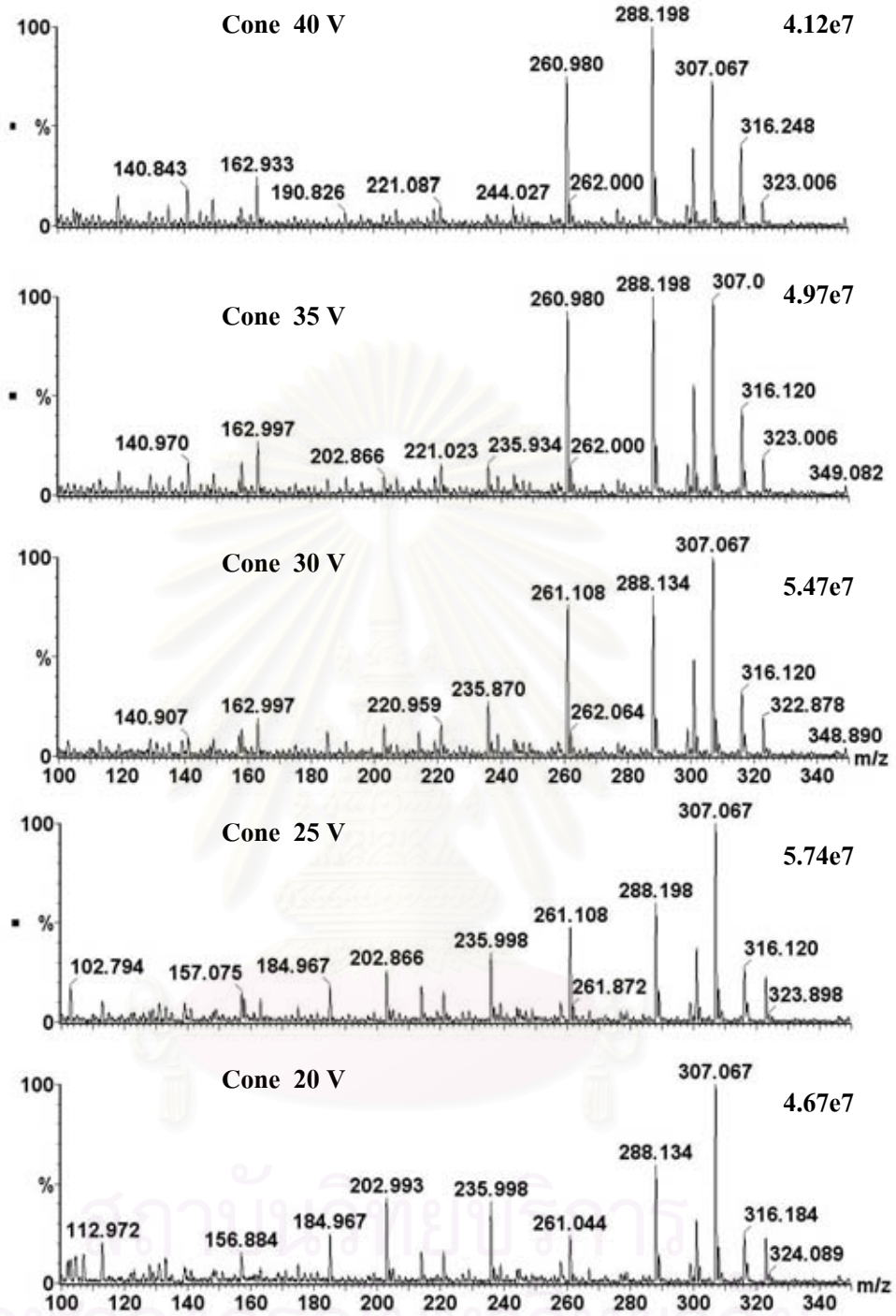

Figure 25. Effect of cone voltage on intensity of dihydroartemisinin $(M+Na)^+$ at m/z of 307.

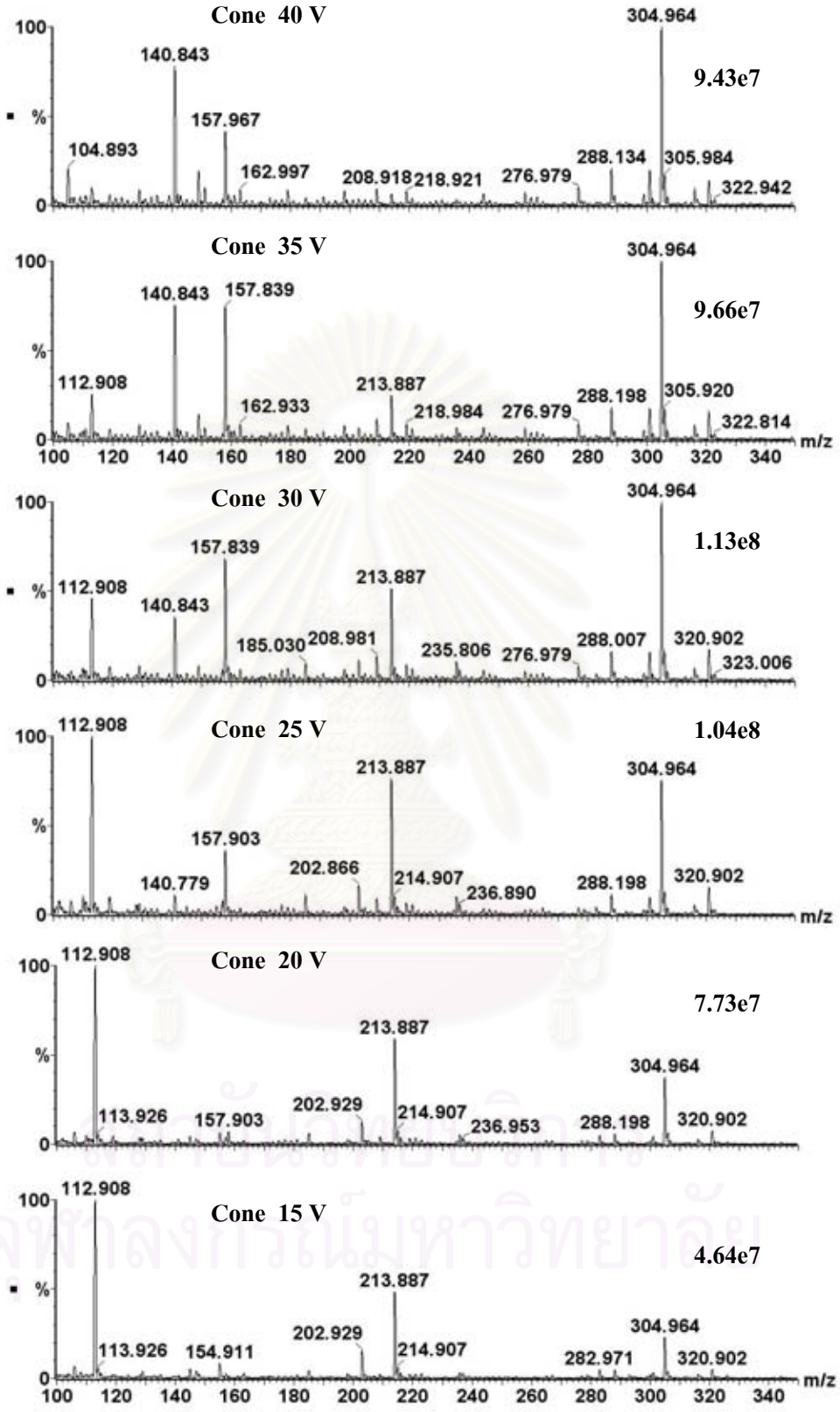

Figure 26. Effect of cone voltage on intensity of artemisinin $(M+Na)^+$ at m/z of 305.

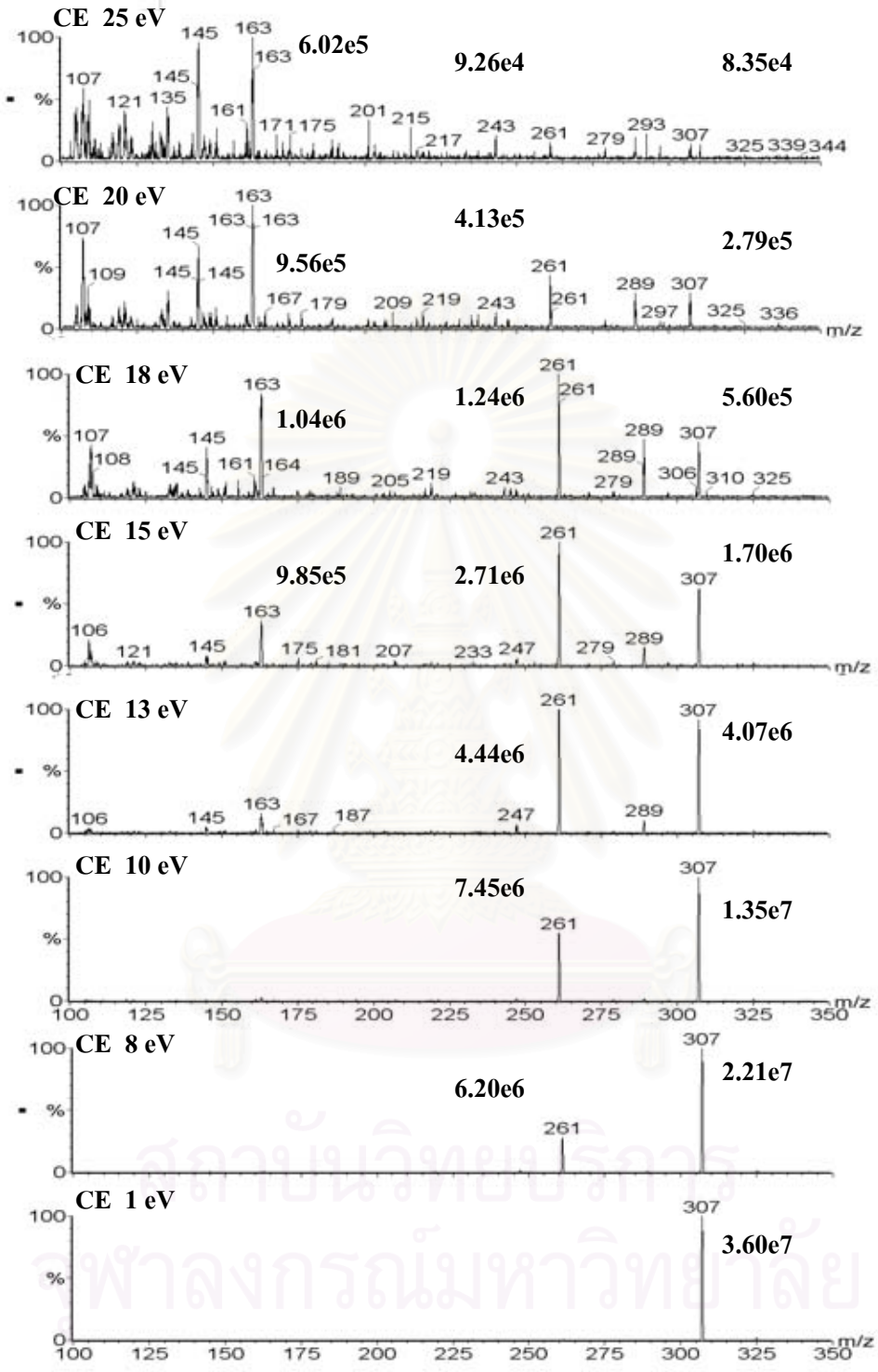

Figure 27. Effect of collision energy (CE) on intensity product ion of dihydroartemisinin at m/z of 261 and 163.

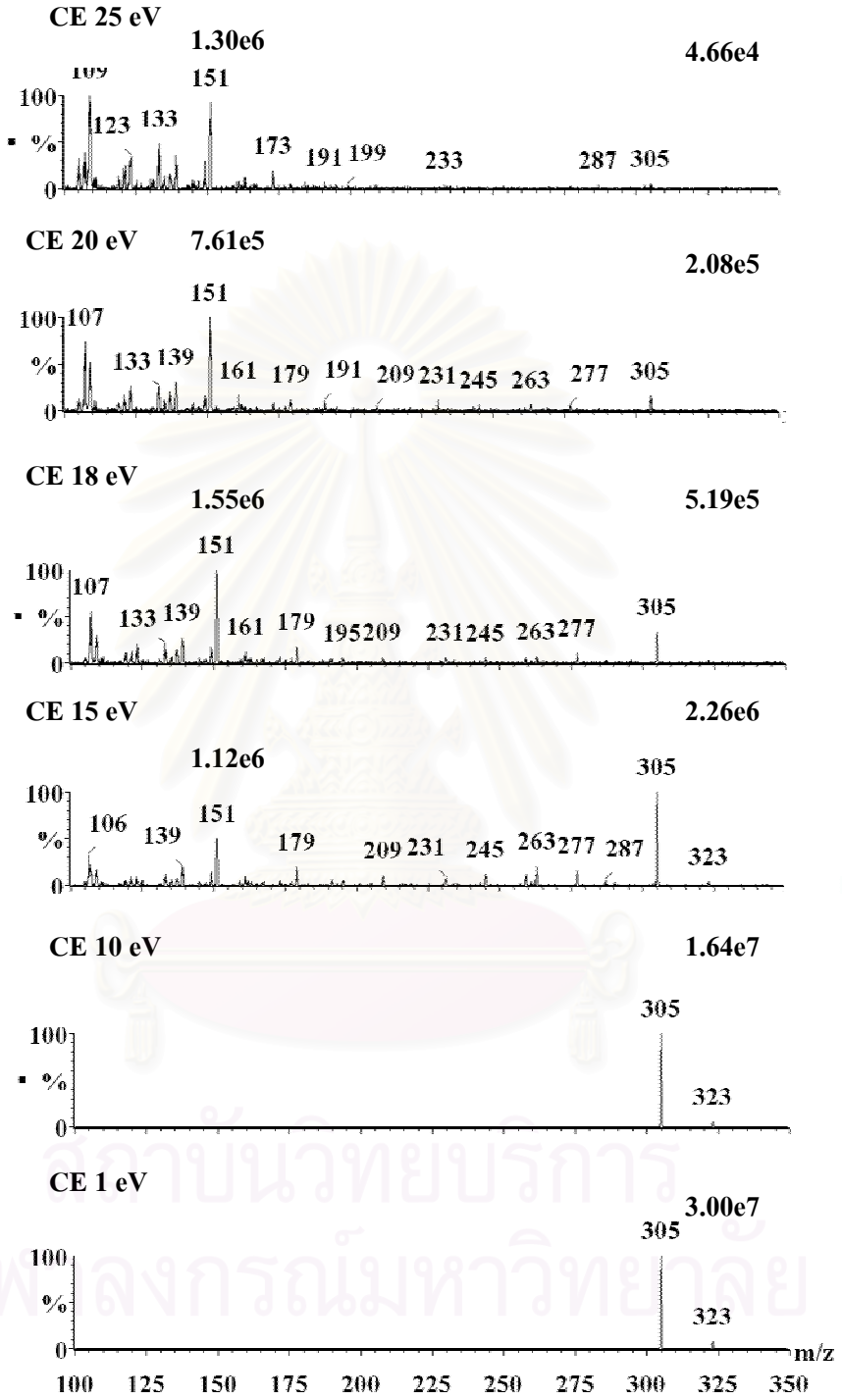

Figure 28. Effect of collision energy (CE) on intensity product ion of artemisinin at m/z of 151.

BIOGRAPHY

Miss Nipa Pradittiemphon was born on November 11, 1980 in Chonburi, Thailand. She graduated with Bachelor Degree of Science from the Department of Biochemistry at Chulalongkorn University in 2001. In 2002, she has studied in Master degree of Science at the department of Biotechnology Program, Chulalongkorn University.