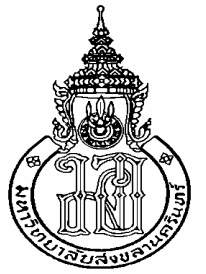


Simultaneous Determination and Method Validation of Methamphetamine and Its Metabolite Amphetamine in Rat Liver Using GC-FID

Wijittra Kaewnam

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Forensic Science
Prince of Songkla University
2011

Thesis Title	Simultaneous Determination and Method Validation of Methamphetamine and Its Metabolite Amphetamine in Rat Liver Using GC-FID
Author	Miss Wijittra Kaewnam
Major Program	Forensic Science

Major Advisor :	**Examining Committee :**
……	……..Chairperson
(Dr. Sathaporn Prutipanlai)	(Asst. Prof. Dr. Orapin Wongsawatkul)
	……..
Co-advisor :	(Dr. Sathaporn Prutipanlai)
……	……..
(Assoc. Prof. Dr. Benjamas Janchawee)	(Assoc. Prof. Dr. Benjamas Janchawee)
	……..
	(Asst. Prof. Dr. Kitja Sawangjaroen)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Science Degree in Forensic Science

……

(Prof. Dr. Amornrat Phongdara)

Dean of Graduate School

ชื่อวิทยานิพนธ์ การตรวจวัดปริมาณของเมทแอมเฟตามีนและเมทาบอไลท์แอมเฟตามีนในเวลาเดียวกันและการตรวจสอบความถูกต้องของวิธีการในตับหนูโดยใช้เทคนิค GC-FID
ผู้เขียน นางสาววิจิตรา แก้วนาม
สาขาวิชา นิติวิทยาศาสตร์
ปีการศึกษา 2553

บทคัดย่อ

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อพัฒนาเทคนิคการตรวจวัดในการวิเคราะห์หาปริมาณของเมทแอมเฟตามีนและเมทาบอไลท์แอมเฟตามีนในตับหนูโดยไม่ทำอนุพันธ์ ด้วยเทคนิคแก๊สโครมาโทกราฟีที่มีตัวตรวจวัดชนิดเฟลมไอออไนเซชัน ใช้คอลัมน์ชนิด vertiBond™ 5 (ความยาว 30 เมตร เส้นผ่านศูนย์กลางภายใน 0.32 มิลลิเมตร และความหนาของฟิล์ม 0.25 ไมโครเมตร) ฮีเลียมเป็นแก๊สพาที่อัตราการไหล 2.5 มิลลิเมตรต่อนาที อัตราการไหลของแก๊สเชื้อเพลิง (แก๊สไฮโดรเจน) และ Make-up แก๊ส (แก๊สไนโตรเจน) เท่ากับ 30 มิลลิเมตรต่อนาที และอัตราการไหลของแก๊สออกซิแดนท์ (แก๊สออกซิเจน) เท่ากับ 300 มิลลิเมตรต่อนาที อุณหภูมิของหัวฉีดและตัวตรวจวัดเท่ากับ 200 และ 300 องศาเซลเซียสตามลำดับ อุณหภูมิของคอลัมน์เริ่มต้นที่ 70 องศาเซลเซียส เป็นเวลา 2 นาที และเพิ่มอุณหภูมิด้วยอัตรา 30 องศาเซลเซียสต่อนาที ถึงอุณหภูมิ 120 องศาเซลเซียส จากนั้นเพิ่มอุณหภูมิด้วยอัตรา 5 องศาเซลเซียสต่อนาที ถึงอุณหภูมิ 150 องศาเซลเซียส และเพิ่มอุณหภูมิสุดท้ายด้วยอัตรา 70 องศาเซลเซียสต่อนาที ถึงอุณหภูมิ 300 องศาเซลเซียส และคงไว้เป็นเวลา 1 นาที ฉีดตัวอย่างจำนวน 1 ไมโครลิตรด้วยโหมด splitless ตัวอย่างตับหนูเตรียมโดยใช้เทคนิคการสกัดด้วยวัฏภาคของเหลว ผลการทดลองพบว่าเมทแอมเฟตามีน และแอมเฟตามีนแยกออกจากกันได้ดีภายใน 12.8 นาที กราฟเทียบมาตรฐานมีความเป็นเส้นตรงโดยมีค่าสัมประสิทธิ์สหสัมพันธ์ที่ดี (r > 0.9990) ค่าความเที่ยงของการวิเคราะห์ภายในวันเดียวกันและระหว่างวัน ซึ่งแสดงในรูปค่าเบี่ยงเบนมาตรฐานสัมพัทธ์ (%RSD) มีค่าอยู่ในช่วง 8.35-10.97% และ 4.79-8.58% สำหรับแอมเฟตามีน และ 7.94-9.58 และ 8.51-12.21% สำหรับเมทแอมเฟตามีน ค่าความถูกต้องของการวิเคราะห์ซึ่งแสดงในรูปค่าการเบี่ยงเบน (%DEV) อยู่ในช่วง (-) 14.77 ถึง (+) 5.41% สำหรับแอมเฟตามีน และอยู่ในช่วง (-) 6.98 ถึง (+) 4.11% สำหรับเมทแอมเฟตามีนค่าร้อยละของการได้กลับคืนอยู่ในช่วง 86-114% สำหรับแอมเฟตามีน และ 88-110% สำหรับเมทแอมเฟตามีน ค่าความเข้มข้นต่ำสุดที่สามารถตรวจวัดได้ของแอมเฟตามีนและเมทแอมเฟตามีนมีค่าเท่ากับ 6.25 ไมโครกรัมต่อกรัม ค่าความเข้มข้นต่ำสุดที่สามารถหาปริมาณได้ของแอมเฟตามีน

และเมทแอมเฟตามีนมีค่าเท่ากับ 9.375 ไมโครกรัมต่อกรัม เทคนิคนี้ได้นำมาใช้ในการวิเคราะห์หาปริมาณของเมทแอมเฟตามีนและเมทาบอไลท์แอมเฟตามีนในตับหนูบริหารยาโดยป้อนเมทแอมเฟตามีนทางปาก 10 มิลลิกรัมต่อกิโลกรัม ผลการทดลองพบว่าความเข้มข้นของเมทแอมเฟตามีนในกลุ่มที่ได้รับครั้งเดียวและหลายครั้งอยู่ในช่วง 10.125 ถึง 86.75 ไมโครกรัมต่อกรัมของเนื้อเยื่อ ความเข้มข้นของแอมเฟตามีนในกลุ่มที่ได้เมทแอมเฟตามีนหลายครั้งมีค่าเท่ากับ 9.375 ไมโครกรัมต่อกรัมของเนื้อเยื่อ สำหรับในกลุ่มที่ได้รับครั้งเดียวไม่สามารถตรวจพบแอมเฟตามีน

สรุปได้ว่า เทคนิคแก๊สโครมาโทกราฟีเป็นเทคนิคที่ง่าย มีความเที่ยงและความถูกต้องของการวิเคราะห์ที่ยอมรับได้ เทคนิคนี้สามารถใช้ได้ทั่วไป มีต้นทุนในการวิเคราะห์ต่ำและช่วยให้งานวิเคราะห์ที่ทำประจำเร็วขึ้น สามารถนำไปวิเคราะห์สาร AM และ MA ในตับได้ วิเคราะห์โดยไม่ทำอนุพันธ์ให้พีกที่สมมาตร และสามารถวิเคราะห์โดยใช้เวลาสั้น ใช้เทคนิคการสกัดด้วยวัฏภาคของเหลวให้ค่าร้อยละของการได้กลับคืนที่สูง เทคนิคนี้น่าจะนำไปใช้ประโยชน์การศึกษาทางด้านเภสัชจลนศาสตร์และงานทางด้านนิติพิษวิทยา

Thesis Title	Simultaneous Determination and Method Validation of Methamphetamine and Its Metabolite Amphetamine in Rat Liver Using GC-FID
Author	Miss Wijittra Kaewnam
Major Program	Forensic Science
Academic Year	2010

Abstract

The present study aimed to develop a method using gas chromatography coupled with a flame ionization detector (GC-FID) for simultaneous determination of methamphetamine (MA) and its metabolite amphetamine (AM) in rat liver without derivatization. A vertiBond^{TM} 5 capillary column (30 m x 0.32 mm i.d., 0.25 μm film thickness) was used for separation. Helium gas was used as the carrier gas with a flow rate of 2.5 mL/min. Fuel gas (H_2) and make-up gas (N_2) flow rate was 30 mL/min. Oxidant gas (O_2) flow rate was 300 mL/min. Injector and detector temperature were 200^{o}C and 300^{o}C, respectively. Column temperature was programmed initially at 70^{o}C (2 min) and increased with a rate of 30^{o}C/min to 120^{o}C. It was further increased to 150 ^{o} C at a rate of 5^{o}C /min and finally increased at a rate of 70 ^{o}C /min to 300 ^{o}C where it was held for 1 min. One μL of sample was injected in a splitless mode. Samples of rat liver were pretreated by liquid-liquid extraction. MA and AM were well separated within 12.8 min. The calibration curves were linear with good correlation coefficient (r > 0.9990). The intra- and inter-day precisions were 8.35-10.97%RSD and 4.79-8.58%RSD for AM and 7.94-9.58%RSD and 8.51-12.21%RSD for MA. The accuracy ranged from (-) 14.77 to (+) 5.41%DEV for AM and (-) 6.98 to (+) 4.11%DEV for MA. The recoveries were 86-114% for AM and 88-110% for MA. The limit of detection and the limit of quantification of AM and MA were 6.25 μg/g of tissue and 9.375 μg/g of tissue, respectively. This method was used to determine the concentration of MA and AM in rat liver received either a single or multiple oral administration of MA (10 mg/kg). The concentration of MA ranged from from 10.125

to 86.75 μg/g for both single and multiple administration. AM concentration of multiple administration was 9.375 μg/g and single administration was not detected.

In conclusion, the present GC-FID method is simple, precise, accurate, universal and cost-saving and facilitates routine analytical work for simultaneously quantifying AM and MA in liver. Without derivertization, peaks of the analytes are symmetry. Additionally, separation is accomplished with a shorter run time. Liquid-liquid extraction of AM and MA from the liver provided a high percentage of recovery. This method may be adopted to a study in pharmacokinetics and forensic toxicology.

Acknowledgements

I would like to express my gratitude and sincere appreciation to Dr. Sathaporn Prutipanlai, my advisor and Assoc. Prof. Dr. Benjamas Janchawee, my co-advisor for their guidance, comments and valuable advices throughout this study.

I would like to thank Police Colonel. Sittipoom Thainchiwattana, Poice major and Policemen at Scientific Crime Detection Division 9, Office of the Police Forensic Science, Thailand for their advices and support involving my research.

I also would like to thank the examination committee members of this thesis for their valuable suggestion.

I wish to thank Assoc. Prof. Dr. Benjamas Janchawee, Head of the Department of Pharmacology and Assist. Prof. Adul Tiengchanya, Head of the Department of Applied Science, Faculty of Science for supporting all Facilities needed for success of this study.

I wish to thank Department of Chemistry, Dr. Thitima Rujiralai, Scientific Equipment Center and Department of Biochemistry, Assoc. Prof. Dr. Nongporn Towatana, Faculty of Science for supporting the Gas Chromatograph and materials for research.

This work was granted by the Department of Applied Science, Faculty of Science and Graduate School, Prince of Songkla University.

Thanks to my family, my relatives and my good friends for their understanding, supporting and encouragement.

Finally, I would like to express my thanks to the Graduate School, Prince of Songkla University for partial financial support to this study.

Wijittra Kaewnam

Contents

List of Tables

List of Figures

List of Figures (cont.)

List of Abbreviations

AM	Amphetamine
ATS	Amphetamine-type stimulant
bp	boiling point
C_{max}	Maximum serum concentration
CNS	Central nervous system
DA	Dopamine
DAT	Dopamine transporter
DEV	Deviation
DW	Distilled water
g	Gram
GC	Gas chromatography
GC-FID	Gas chromatography-flame ionization detector
GC-NPD	Gas chromatography-nitrogen phosphorus detector
GC-MS	Gas chromatography-mass spectrometry
H	Hour
HPLC	High performance liquid chromatography
HS	Headspace
i.e.	Id est
i.p.	Intraperitoneal
i.v.	Intravenous
kg	Kilogram
L	Liter
LC	Liquid chromatography
LC-MS	Liquid chromatography- mass spectrometry
LLE	Liquid- liquid extraction
LD_{50}	Median lethal dose
LOD	Limit of Detection
LOQ	Limit of Quantification
MA	Methamphetamine

List of Abbreviations (cont.)

MAO	Monoamine oxidase
NE	Norepinephrine
mg	Milligram
min	Minute
mL	Milliter
mm	Millimeter
MW.	Molecular weight
N, no	Number
ng	Nanogram
r	Correlation coefficient
RSD	Relative standard deviation
S.D.	Standard deviation
SE	Serotonin
sec	Second
SPE	Solid phase extraction
SPME	Solid phase micro extraction
$T_{1/2\ ab}$	Half-life absorption
$T_{1/2\ el}$	Half-life elimination
TLC	Thin layer chromatography
T_{max}	Time to maximal serum concentration
t_R	Retention time
V_d	Volume of distribution
VMAT-2	Vesicular monoamine transporter 2
v.s.	Versus
v/v	Volume by volume
µg	Microgram
µm	Micrometer

CHAPTER 1

Introduction

1.1 Background and rationale

Methamphetamine (MA) and its metabolite amphetamine (AM) are powerful stimulants of central nervous system and are abused in many countries including Thailand. MA accounts for nearly 70% of all addictions in Thailand. The increase in MA addiction presents a dramatic shift since five years ago. Approximately 80% of the MA tablets in Thai market come from Thai/Myanmar border (Puthaviriyakorn *et al*., 2002; Narongchai *et al*., 2007).

In the past, MA was used among construction workers and truck drivers. Recently, MA use is increasing among school and university students. This causes poor quality of population in the nation. Therefore, in Thailand MA is classified as a category I narcotic substance according to the Narcotic Act of B.E. 2522 (1979) (ONCB, 2007). MA is a dangerous narcotic drug which cause social, economic and health problems. For social problems, MA increase in criminal rate, whereas in health problems MA produce many toxic effects such as, wakefulness, hyperthermia, hypertension and euphoria (Logan, 2002; Yu *et al*., 2003). Short-term abuse of MA result in euphoria, decreased appetite, and increased alertness which related to the release of dopamine (DA), a neurotransmitter that is very abundant in the mammalian striatum, whereas long-term abuse of MA can cause neuropsychiatric complications such as paranoia, hallucination, and even death. The long-term changes that occur in human abusers might be due to neurotoxic or neurodegenerative effects of the drug on monoaminergic terminals (Jayanthi *et al.*, 2005). The death is a hallmark of toxic effect from MA; therefore it is important to measure levels of MA and its metabolite AM in vital organ such as liver.

Ingestion of MA is the most common route of exposure and readily absorbed from the gastrointestinal tract (Schepers *et al*., 2003). After absorption phase, MA and its metabolite AM distribute throughout the body in various organs such as liver, kidney, spleen, brain and heart. The distribution of AM drug in the body was also

studied. The result showed that AM was also measured in kidney spleen, liver, brain and heart (Riviere *et al.*, 2000). In a study using human tissues taken from fatal case of MA user, MA was present in tissues such as liver, spleen, lung, kidney, cerebrum and femoral muscle. Therefore, liver is a tissue that has an highest accumulation of MA and AM (Moriya and Hashimoto, 1999[b]; Raikos *et al.*, 2002; Uemura *et al.*, 2003; Chaturvedi *et al.*, 2004; Hara *et al.*, 2009).

MA and its metabolite AM can be measured in tissues using various method such as high performance liquid chromatography-ultraviolet visible (HPLC-UV) detector (Riviere *et al.*, 2000), gas chromatography-nitrogen phosphorous (GC-NPD) detector with derivatization (Terada, 1985 and Raikos *et al.*, 2002), gas chromatography-flame ionization (GC-FID) detector with derivatization (Moriya and Hashimoto, 1999[a]; Moriya and Hashimoto, 1999[b]; Moriya and Hashimoto, 2002), Liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) (Hendrickson *et al*., 2006) and gas chromatography coupling mass spectrometry (GC-MS) with derivatization (Nagata *et al*., 1990; Kalasinsky *et al*., 2001; Nakagawa *et al*., 2003; Uemura *et al.*, 2003; Chaturvedi *et al*., 2004; Scheidweiler *et al.*, 2008 and Hara *et al*., 2009). Three comprehensive validation methods for the measurement of MA and its metabolite AM in tissues were reported by GC-NPD (Terada, 1985), GC-MS (Scheidweiler *et al*., 2008 and LC-MS/MS (Hendrickson *et al*., 2006).

Investigations of the cause of death at forensic autopsies biological samples were collected and determined. Blood specimens from a victim are usually taken to estimate MA levels. However, blood samples cannot always be obtained at autopsies. When blood can not be obtained, other solid materials such as the liver, kidney, spleen, lung, brain, and muscle should be subjected to drug analysis. For selective technique for determination, mass spectrometry technique which is more sensitive is not widely available, the GC-FID is considered to be more possible for determining MA and AM in the liver. The development and validation method in rat liver from oral route after single and repeated doses by using GC-FID without derivatization was not reported.

Therefore, the development and validation methods of GC-FID for determination of MA and metabolite AM from liver of rat treated with oral route after single and repeated doses are an interesting topic. This result may be useful for

determining MA and metabolite AM as data used to support the investigations of the cause of death.

1.2 Review of literature

1.2.1 History and synthesis of AM and MA

Synthetic AM was discovered in 1887 by Lazar Edeleanu (Rumanian Chemist) in Germany. The compound was named phenylisopropylamine which was one of the compounds related to the plant derivative ephedrine, which had been isolated from Ma-Huang in the same year by Nagayoshi Nagai. The pharmacological use was not found for AM until 1927, when pioneer psychopharmacologist, Gordon Alles, resynthesized and tested it by himself in search of an artificial replacement for ephedrine. AM was introduced commercially in 1932 by the pharmaceutical firm Smith, Kline and French as Benzedrine®, which was the free base administered by inhaler form (http://en.wikipedia.org/wiki/Amphetamine; Sulzer *et al*., 2005).

In 1935, Nathanson, a Los Angeles physician studied the subjective effects of AM in 55 hospital workers who were given 20 mg of Benzedrine®. The results showed that Benzedrine® effects including feeling of exhilaration and lessened fatigue in reaction to work. This is the first report of AM use as a clinical treatment for narcolepsy. During World War II (1939-1945), AM was extensively used to combat fatigue and increase alertness in soldiers. Until 1946, AM was promoted by pharmaceutical industry as a drug for treatments of more than 30 diseases including, schizophrenia, opiate addiction, infantile cerebral palsy, seasickness, radiation sickness, and persistent hiccups. By 1970, pharmaceutical production reached 10 billion tablets, with perhaps 50–90% diverted to the black market. After decades of reported abuse, the Food and Drug Administration (FDA) banned Benzedrine inhalers, and limited amphetamines to prescribe use in 1965. However, non-medical use remained common. AM became a schedule II drug under the Controlled Substances Act in 1971 (http://en.wikipedia.org/wiki/Amphetamine; Rasmussen, 2006; Sulzer *et al*., 2005).

MA, a derivative of AM, has been synthesized by several methods. In 1918, MA was first synthesized from ephedrine and pseudoephedrine by Ogata, a Japanese chemist. Hydroiodic acid, now restricted was used in the synthetic process as a solvent and the reaction was catalyzed by red phosphorus, which obtained from matchbook striker plates or road flares. The other method of MA synthesis involves the reduction of the same ephedrine and pseudoephedrine as a precursor and using

either sodium or lithium metal in condensed liquid ammonia. Lithium can be obtained from lithium batteries whereas sodium was obtained from electrolytic reduction of molten sodium hydroxide. Liquid ammonia can be obtained from agricultural or specialty gas suppliers (Anglin *et al*., 2000; Logan, 2002).

MA was used during World War II when Japan, Germany and United States provided the drug to military personels to increase endurance and performance. MA was also used in Japan to improve productivity of civilian factory workers in military supporting industries. In 1941, MA was firstly sold in Japan. Subsequently, the crimes and homicides were increasing. MA was used widely among construction workers, truck drivers, other blue-collar workers as well as students and office workers (Levine, 2003).

In 1962, illicit MA laboratories emerged in San Francisco. It was synthesized using the phenyl-2-propanone (P2P) and methylamine as precursors. The resulting product (‘crank’) was a mixture of two isomers (levo- and dextro-MA), which yielded a less potent form than the pharmaceutical product. During mid 1960s, Bay Area motorcycle gangs manufactured MA powder (speed) and distributed it in the market of north and south along the Pacific Coast. In 1980s, the population in Hawaii used highly pure form of d-MA hydrochloride (ice) by smoking and ingestion. In the 1980s, MA was imported from Far East sources in the Philippines, Japan, Korea and Taiwan. In 1996, seizures of MA labs increased by 169% over seizures in 1995 and the growing public health threat posed by the use and production of MA. The Comprehensive MA Control Act was enacted in 1996 (Logan, 2002; Rasmussen, 2006).

In 1997 and 1998, the researchers at Texas A&M University claimed that MA and AM were found in the foliage of two Acacia species native to Texas, *A. berlandieri* and *A. rigidula*. Previously, both of these compounds had been thought to be human inventions. These findings have never been duplicated, and the analyses are believed by many biochemists to be the result of experimental error, and as such the validity of the report has come into question. Alexander Shulgin, one of the most experienced biochemical investigators and the discoverer of many new psychotropic substances, has tried to contact the Texas A&M researchers and verify their findings.

The authors of the paper have not responded; natural AM remains most likely a false discovery (Shulgin, 2001).

1.2.2 MA situation in Thailand

AM was first marketed in Thailand in 1955. It is known locally as a street names ya ma, literally translated as horse pill. Initially the pills were imported and imprinted with the picture of a horse head on the one side and London on the other side. AM was orally administered widely among unskilled labors and truck drivers on long cross country routes. The pills were mainly taken for improvement of their work. The government took various administrative actions and designated AM as narcotics in the same schedule of heroin. The import of AM has become under control (Treerat *et al*., 2000).

In 1980-1990, the bogus amphetamines (AMs) are tablets or capsules that were made to look-alike real AMs and roughly imitate their effects. They usually contained varying amount of legal substances such as caffeine, ephedrine, pseudo-ephedrine and phenylpropanolamine. They are sold on the street as speed and purported to be authentic AMs. The law and regulations did not affect until such drugs were spreaded out widely. In 1988, the Psychotropic Substances Act 1975 was amended to strengthen the control over raw materials of look-alike AMs. However, comparing with the AM type, the consumption of look-alike AM produced side-effects more than that of pure AM. Therefore, producers had been searched for the new type of stimulant drugs that had better quality (Treerat *et al.*, 2000).

The new stimulant drug has been brought into the illegal market after the look-alike AMs declined. MA has been synthesized in clandestine laboratories operating in Bangkok in late 1988. The steps of production were divided into 2 steps: producing MA powder and making complete tablet. The base material (precursor) is ephedrine which was smuggled into Thailand through three major routes: the Klong Toey port in Bangkok; the Thai-Burmese border, and the coasts of the Gulf of Thailand (Samut Sakhon) and eastern region. In 1988, Thailand succeeded in destroying 15 clandestine MA laboratories with the seizure of 97.415 kilograms of MA powder, 45 kilograms of ephedrine powder and 240,020 MA tablets. In 1996, the government changed the common name from ya ma to ya ba (meaning mad pill) which ya ba tablets are

predominate in Thailand. Since 1996, the decline of production side inside the country arose from a strong suppression and the Golden Triangle has been the new production side of MA. In 1997 and 1998, 24.25 and 30.86 million tablets of MA were seized in transit from the Golden Triangle to Bangkok. Approximately 70-80 percent of MA used in Thailand is from the Golden-triangle area (overlaps the mountains of four countries of Southeast Asia: Myanmar, Vietnam, Laos and Thailand). Myanmar produces up to 80 percent of the MA tablets that are consumed in Thailand. MA from this source is in orange color and imprinted with wy. The remaining is produced in Cambodia and Thailand but the old producer groups are still producing MA and mainly for pill making (Lotrakul, 2000; Treerat *et al*., 2000).

During 2001-2002, over 100 million tablets of MA were confiscated because of the increase efforts of the government. MA increased from 82.5 million tablets in 2000 to 93.8 million tablets in 2001. In 2002, the number of 187,479 cases with 199,714 offenders related to MA seizures or 81.6% of all drug cases which MA tablets were seized 8,440 kilograms (about 93.8 million tablets) (Narongchai *et al*., 2007). After that, the Royal Thai government declared the War on Drugs in the early 2003, drug situation has positively changed. Since 2003, the volume of MA seizures has significantly and consistently decreased. Approximately, 12.6 millions tablets of ya ba were seized in 2006, a remarkable decrease from 96 million tablets seized in 2002. The estimated number of drug addicts has also dropped by 80 percent from 2,000,000 tablets in 2003 to 400,000 tablets in 2005 - 2006 (Hiruntoe, 2007).

Northern Thailand remains an important route for MA trafficking from Myanmar, with drugs shipped into Thailand for domestic consumption, as well as drugs shipped with intent to reshipping outward to foreign destinations. There have been for some time a flow of reports on increasing number of seizures in Europe, the USA and Australia of MA tablets originating in Thailand and Myanmar (Adam *et al*., 2005). The volume of MA seizures during 2000-2009 and the import area of MA tablets from Myanmar into Thailand are shown in table 1.1 and 1.2, respectively.

The trafficking groups that have historically refined heroin now also produce MA and the laboratories for both substances are often co-located. The main insurgent groups involved in the drug-trafficking business are the United Wa State Army (UWSA), the Shan United Army (SUA), also known as the Mong Tai Army or former

Khun Sa Army and the Myanmar National Defence Alliance Army (Kokang Chinese), also known as the Burma National Democratic Front (Adam *et al*., 2005).

Information from intelligence sources indicates that each manufacturer has a specific logo, for instance, wY is produced by the United Wa State Army (UWSA). WY is connected with the Myanmar National Defence Alliance Army (Kokang Chinese). Logo of wy and Wy associated with the Shan United Army (SUA) or former Khun Sa Army. The UWSA is the largest drug-producing and trafficking group in Southeast Asia, producing heroin and MA. UWSA produces MA tablets embossed with the wY and 99 logos (Adam *et al*., 2005). The study in 2002 showed that most popular logo was WY (in four variants), followed by R, OK, 888 and Ã/99. The four variants of WY logo observed were wY, WY, wy, Wy. wY predominated followed by WY, wy and Wy. In 2005, WY was also found to be the most popular logo (in four variants), followed by R, OK and 888. wY still predominated, followed by wy, WY and Wy (Adam *et al*., 2005).

MA tablets were identified in Thailand during 1983-2000. The MA tablets were classified by their active ingredient as MA only; MA and caffeine; ephedrine and caffeine; MA, ephedrine and caffeine; and caffeine only. In the early period of MA epidemic (1983), 82.8% of MA tablets were ephedrine and caffeine whilst, 13.7% were MA and caffeine. The combination of MA and caffeine was popular in the latter period, 74.0% in 1996 and increasing to 99.4% in 2000 (Palanuvej and Issaravanich, 2007). Impurities in MA tablets seized in Thailand, nine compounds such as 1,2-Dimethyl-3-phenylaziridine, ephedrine, methylehedrine, *N*-formylmethamphetamine, *N*-acetylmethamphetamine, *N*-formylehedrine, *N*-acetylehedrine, *N,O*-diacetylephedrine, methamphetamine dimmer are also identified as impurities with caffeine and ethyl vanillin as diluents or adulterants of the seized package in 200 of MA tablets (Puthaviriyakorn *et al.*, 2002). Additionally, Kuwayama and coworkers (2008) described of impurities in MA tablet. MA tablet usually consists of MA-HCl (20–30%), caffeine (60–70%) and other substances. MA tablet has many kinds of impurities at high levels which caffeine was large impurity peaks.

Table 1.1 The volume of MA seizures in during 2000-2009 years

years	seizures (millions tablets)
2000 (2543)	82.5
2001 (2544)	93.8
2002 (2545)	96
2003 (2546)	64.7
2006 (2549)	12.6
2008 (2551)	20.6
½ 2009 (2552)	12.3

Table 1.2 The import area of MA tablets from Myanmar into Thailand

Regional Thailand	Provinces
north	Chiang Rai, Chiang Mai and Mae Hong Son
northeast	Ubon Ratchatani, Nong khai, Nakhon Phanom, Mukdahan and Amnat Charoen
central	Kanchanaburi and Sa Kaew

1.2.3 Chemical properties of MA and AM

AM (Table 1.3) is a primary amine. Molecular formula is $C_9H_{13}N$ and percentage of molecule consists of carbon 79.95%, hydrogen 9.69% and nitrogen 10.36% with pK_a around 9.77. AM is a weak base and a polar compound with a high partition coefficient between organic solvents and water. Molecular weight is 135.21. Boiling point of AM is 200-203 °C if the pressure is 760 mmHg and 82-85 °C if the pressure is 13 mmHg. Melting point is 156-158.5°C. Specific gravity is 0.913 at 25 °C referred to water at 4 °C (d (25/4) 0.913). It can be dissolved in water, alcohol and diethyl ether and volatiles slowly at room temperature. Structure of AM is enantiomers consisting of d- and l- isomer. It is produced as a racemate, with equal concentrations of both the l-isomer and the d-isomer giving an l/d-isomer ratio of approximately 100%, dependent on manufacturing process (Asatoor *et al.*, 1965; O'Neil *et al*., 2001; Sukkwan, 2006).

MA (Table 1.3) is a secondary amine. MA also exists in two isomeric form, dextrorotatory (d-) and levorotatory (l-). The d-isomer is utilized in the treatment of obesity in single doses of 2.5-15 mg. The l-isomer has a weaker central stimulant activity and a greater peripheral sympathomimetic activity. MA is used in certain non-prescript on inhalers as a decongestant. Molecular formula is $C_{10}H_{15}N$ and percentage of molecule consists of carbon 80.48%, hydrogen 10.13% and nitrogen 9.39%. MA is a weak base and polar compound with pK_a around 9.9. Molecular weight is 149.23. It is liquid at room temperature, so is invariably supplied and used as the hydrochloride salt (MA-HCl). Molecular formula is $C_{10}H_{15}N.HCl$ and molecular weight is 185.70. Melting point and boiling point are 170-175 °C and 214 °C, respectively. It is readily soluble in N-butyl chloride, ethyl acetate, diethyl ether, water, alcohol and chloroform (Basellt, 1982; O'Neil *et al*., 2000; Logan, 2002; Tongroach *et al.*, 2005; Sukkwan, 2006). Chemical properties of MA and AM are shown in table 1.3.

Table 1.3 Chemical properties of MA and AM

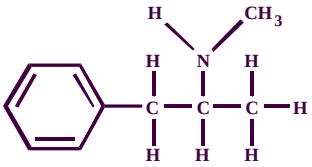
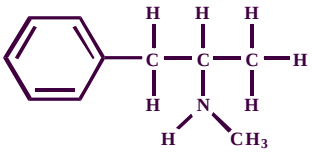
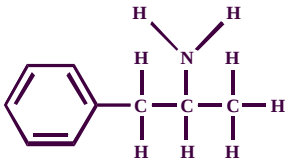
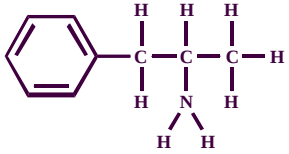
Properties	**Methamphetamine (MA)**	**Amphetamine (AM)**
Synonyms	(α*S*)-N, α-Dimethylbenzeneethanamine; (*S*)-(±)-*N*, α-dimethylphenethylamine; *d*-*N*-methylamphetamine; *d*-deoxyephedrine; *d*-desoxyephedrine; 1-phenyl-2methylaminopropane; *d*-phenylisopropylmethylamine; methyl- β-phenylisopropylamine; norodin	α-Methylbenzeneethanamine; (±)-α-methylphenethylamine; 1-phenyl-2-aminopropane; β-phenylisopropylamine; β-aminopropylbenzene; (±)-desoxynorephedrine
Amine type	secondary amine, weak base	primary amine, weak base
Molecular formula	$C_6H_6CH_2CH(NHCH_3)CH_3$, $C_{10}H_{15}N$	$C_6H_6CH_2CH(NH_2)CH_3$, $C_9H_{13}N$
Structure	 l-methamphetamine  d-methamphetamine	 l-amphetamine  d-amphetamine

Table 1.3 Chemical properties of MA and AM (cont.)

Properties	**Methamphetamine (MA)**	**Amphetamine (AM)**
Molecular weight	149.23	135.21
Boiling point	214 °C	200-203 °C
Melting point	170-175 °C	156-158.5 °C
pK_a	9.90	9.77
Solubility	N-butyl chloride, ethyl acetate, diethyl ether, water, alcohol and chloroform	water, alcohol and diethyl ether

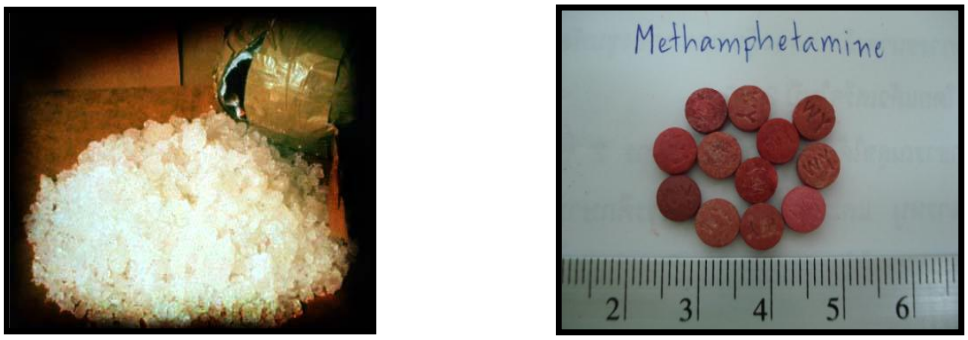
1.2.4 Physical properties of MA and AM

MA and AM represent a class of phenethylamine compounds. The structure is similar to ephedrine (as shown in figure 1.1) which is a sympathomimetic drug. They are synthetic substance of amphetamine-type stimulant (ATS) including, AM, MA and ecstasy-type substances which they are powerful stimulants of the central nervous system (CNS). MA is classified as a category I narcotic drug under the Thai Narcotic Act B.E. 2522 (1979) (Derlet and Heischober, 1990; Treerat *et al*., 2000; Kuwayama *et al*., 2008). MA-HCl is usually found as a yellow or white crystalline powder, odorless and bitter. The purity of it depends on the manufacture process. When compare with structure of AM, the chemical structure of MA has an addition of one methyl group. The methyl group makes the drug even more potent by facilitating its penetration into CNS, also it has higher lipid solubility than the unsubstituted AM (Salocks and Kaley, 2001; Yu *et al*., 2003; Nordahl *et al*., 2003). It is also known as other street names such as speed, crank, go, crystal or meth. This compound is adulterated with a variety of substances such as sugar (usually add of give more volume to the final product) or cheaper stimulants (caffeine, phenylpropanolamine or ephedrine, psuedoephedrine) (Levine, 2003). Generally, the common name of MA in

Thailand is ya ba. The typical form of ya ba widely abused in Thailand is a round shape tablet with WY logo. On it each MA tablet generally contains 10-25 % of MA-HCl and 60 - 70 % of caffeine and others (starch, pigments and flavor compounds) and these diluents and adulterants make it difficult to implement direct comparison of impurity profile of MA itself (Puthaviriyakorn *et al*., 2002). Ya ba tablets are mostly found as orange tablets. Diameter of ya ba tablet is 6 millimeter, thickness 2.5 millimeter and the weight 90 milligram (Hiruntoe, 2007). MA-HCl and ya ba tablets are shown in figure 1.2.

Phenethylamine **ephedrine**

Figure 1.1 Chemical structures of phenethylamine and ephedrine



MA-HCl **Yaba tablets**

Figure 1.2 Methamphetamine hydrochloride (MA-HCl) and Yaba tablets

1.2.5 Mechanism of action of MA

MA is derivative of AM which has similar pharmacological and toxicological effect (Kuwayamaa *et al*., 2008). It acts by altering the levels of certain central nervous system neurotransmitters. MA substitutes monoamines via monoaminergic transporter, entry into monominergic vesicles and displacement of monoamines into the cytoplasm of terminals subsequent stimulates the release and blocks the reuptake of monoamines in the synaptic cleft in several areas of the brain, including nucleus accumbens, prefrontal cortex and striatum (a brain area involved in movement) (Cadet *et al*, 2003). These monoamine included dopamine (DA), norepinephrine (NE) and serotonin (5-HT) (as shown in figure 1.3). These actions result in high concentrations of these neurotransmitters in the synapses and leading to neurodegeneration and neurotoxicity (Rothman *et al*., 2001; Klasser and Epstein, 2005).

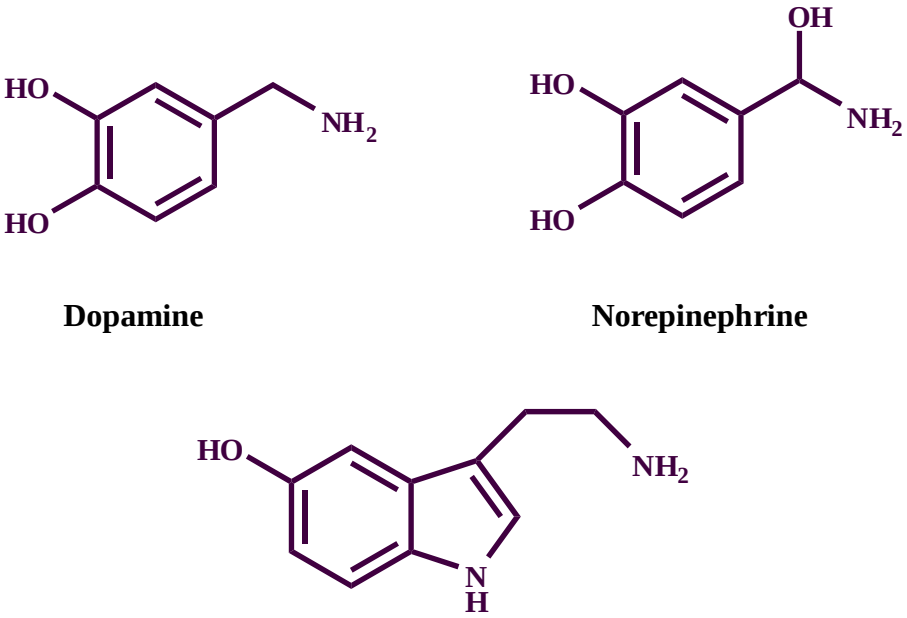


Figure 1.3 Chemical structures of dopamine, norepinephrine and serotonin

Molecular pharmacology of MA involved in MA-induced DA terminal degeneration within striatum (Figure 1.4). MA enters dopaminergic neurons via dopamine transporter (DAT) and passive diffusion. Within these neurons, MA enters

synaptic vesicles through vesicular monoamine transporter 2 (VMAT-2) and causes DA release into the cytoplasm. MA redistributes DA from storage vesicles into the cytosol by reversing the function of VMAT-2 and disrupting the pH gradient that otherwise drives accumulation of DA in the vesicles. The endogenous function of DAT is reversed, resulting in release of dopamine from the cytosol into synapses. Synaptic DA is then available to stimulate postsynaptic DA receptors. MA attenuates DA metabolism by inhibiting monoamine oxidase, the enzyme responsible for the destruction of DA neurotransmitters, further increasing the availability of this neurotransmitters (Cruickshank *et al.*, 2009; Krasnova and Cadet, 2009).

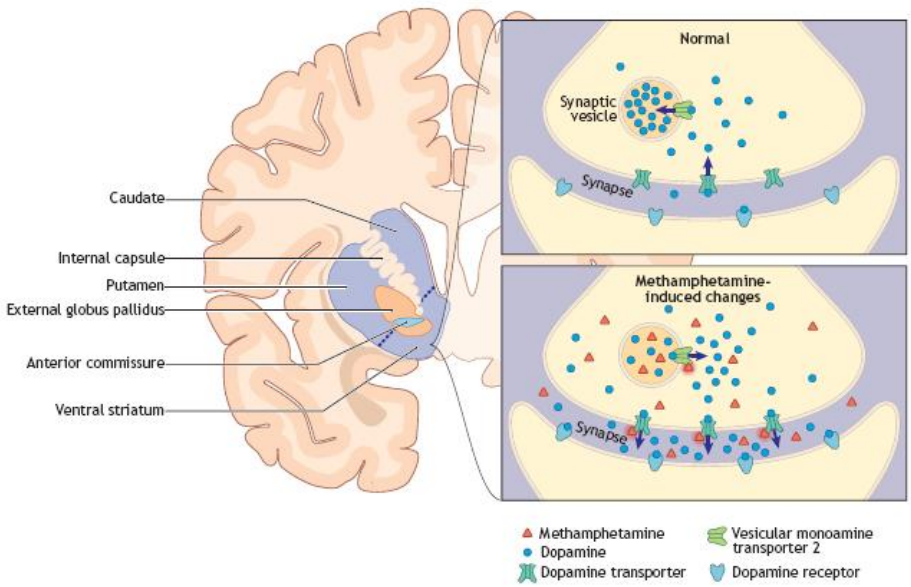


Figure 1.4 Molecular pharmacology of MA involved in MA-induced DA terminal degeneration within striatum (http://www.cmaj.ca/cgi/content/full/178/13/1679)

1.2.6 Pharmacokinetics of MA

1) Route of administration

MA can be administered via a variety of routes, and there is typically a progression following the start of use, from oral ingestion or nasal insufflation, to intravenous. Smoking of the drug achieved popularity in Asia and Hawaii in the 1980s, which was simply larger crystals of MA that were smoked in a pipe, much like crack cocaine. The routes of administration reported in 1998 were smoking (19%),

intranasal (36%), and intravenous (44%) (Logan, 2002). Reported percentages of users who inject MA intravenously range from just over 10% to 67%. High percentages of MA abusers also utilize other routes of administration in addition to intravenous injection, including nasal insufflation or snorting (91%) and smoking (28%). These routes of MA administration are popular because they are associated with rapid input of the drug (Gentry *et al.*, 2004). For in Thailand, the majority of MA administration was smoking (95%) and oral ingestion (5%) of 60 adolescents (18-22 years old) who used MA and were admitted to the Thanyarak Institute in the Patum tanee province (Sampoon, 2008).

2) Absorption

In humans, MA is readily absorbed from the gastrointestinal tract after oral consumption (Schepers *et al.*, 2003). After oral administration, the time to maximum plasma concentration (t_{max}) is 2-3 hours, although the drug's effects can be felt in as little as 20 minutes (Donaldson and Goodchild, 2006). A single oral administration at the dose of 0.125 mg/kg to human male volunteers produced a peak plasma concentration of 0.020 mg/L at 3.6 hour (Levine, 2003). Logan (2002) reported the peak plasma concentration (t_{max}) of 0.020 mg/L and 0.039 mg/L at 2.6 and 3.6 hours following ingestion of 8.75 and 17.5 mg of *d*-MA hydrochloride, respectively. The absorption half-life of MA was 0.67 hour. In addition, the maximum plasma concentrations of metabolite (AM) was absorbed at approximately 12 hour after dosing, with mean concentrations of 0.0016 and 0.004 mg/L after the low and high doses of MA, respectively. This concentration was approximately 15% of the concentration of the parent drug concentration present at that time. Kuwayama and coworkers (2007) studied kinetic parameters of MA in rats and reported that after an oral administration of MA (10 mg/kg), the maximal concentration (C_{max}) and T_{max} were 0.37 μg/mL and 3.5 h, respectively. The area under the plasma concentration–time curve (AUC) was 2.6 μg h/mL.

The kinetic parameters at absorption phase after chronic administration of MA were also studies. Schepers and coworkers (2003) reported in eight participants receive four oral 10 mg of MA within 7 days. Three weeks later, five participants received four oral 20 mg doses. Blood samples were collected for up to 24 h produce

a peak plasma concentration of 14.5-33.8 µg/L (10 mg) at 0.25–2 h and 26.2- 44.3 µg/L (20 mg) within 2-12 hour.

3) Distribution

MA has low plasma protein binding (10-20%) influencing distribution and excretion processes (Huestis and Cone, 2007). Such low binding suggests that almost the total amount of drug in plasma may diffuse to the extravascular component. From the data reported by Halle and coworkers (2005), a distribution volume of 3.7 L/kg was reported after an oral administration of MA in human with a bioavailability of approximately 67%. After absorption, MA distributed to most organs in the body. The results obtained from the study of Terada (1985) using intraperitoneal injection in rats showed that the concentration ranges of MA and AM were 0.4-7.2 µg/g (µg/mL) and 0.05-1.9 µg/g (µg/mL), respectively. The presence of MA from high to low concentrations was kidney, spleen, brain, liver and serum. The presence of AM from high to low concentrations was also the same i.e. kidney, spleen, brain, liver and serum.

Melega and coworkers (1995) reported concentration of MA and AM on the striatal dopamine system and serum after an intravenous administration. The highest MA concentrations in the striatum occur at their first measured time point (5 min). The MA brain-to-serum ratio at this time point was 10:1 and it remained constant for the short duration of their study (1h). The AM concentrations increased in the striatum from the moment of MA administration to peak at 20 to 30 min, after which the AM concentrations remained constant for the duration of sampling in the striatum (60 min). However, these data did not described of the pharmacokinetic processes for MA and AM in the brain and other important tissues such as the heart, liver, and kidney. Thus, Riviere and coworkers (2000) characterized the concentration-time profile of MA and its metabolite AM in the brain and five other tissues of male rat which received MA intravenously and subcutaneously. The rank order of MA tissue accumulation from high to low was kidney, spleen, brain, liver, heart and serum. Kidney was the most significant organ of MA accumulation whereas serum was the least. Terminal elimination half-life values ranged from 53 to 66 min. MA concentrations were the highest at the first measured time point (2 min) in all tissues except the spleen measured peaked at 10 min. The brain-to-serum concentration ratio

rose from 7:1 at 2 min to a peak of 13:1 at 20 min and at ratio 8:1 at equilibrium at 2 h. Following a subcutaneous MA dosing, the MA brain-to-serum concentration ratio was the same as the equilibrated ratio following intravenous dosing. AM concentrations peaked at 20 min in all tissues before decaying with terminal elimination half-life values ranging from 68 to 75 min. This investigation showed that AM distributed extensively into tissues.

Nakagawa and coworkers (2003) reported the MA distribution in the MA sensitized rats after intraperitoneal administration (once daily for 14 consecutive days). After 7 consecutive days of withdrawal, stereotyped behavior was scored using a stereotypy. As a result, the MA distribution in the heart at 10 min when a high accumulation of MA in the MA-sensitized rats brain was significantly higher than the control rats and no significant differences in the liver, kidney, abdominal muscle, femoral muscle and blood. Thus, the high accumulation of MA in the MA-sensitized rat brain may be related to the expression of behavioral sensitization. The MA-sensitized rat heart may be connected with the cardiac toxicity.

Henddrickson and coworkers (2006) measured MA and AM in serum, brain and testis after subcutaneous administration for 14 consecutive days. Concentrations of MA in serum, brain and testis were 65 ng/mL, 388 and 299 ng/g, respectively. The concentrations of AM in serum, brain and testis were 12 ng/mL, 120 and 88 ng/g, respectively.

Scheidweiler and coworkers (2008) measured MA in mice brain regions (cortex, striatum and hippocampus) after single intraperitoneal administration and were euthanized 2 h after dosing. The only concentrations of MA and AM in cortex were 2.2 and 0.5 ng/mg, respectively. The concentrations of MA and AM in striatum and hippocampus were undetectable due to it is small brain regions, making it critical to measure drug concentration.

For distribution of MA and AM in human were reported. Moriya and Hashimoto (1999[b]) carried out human autopsy of three cases during early stages postmortem. The result showed that tissue concentration for MA from high to low was liver, spleen, lungs, kidneys, cerebrum and femoral muscle, respectively. Moriya and Hashimoto (1999[a]) reported concentration of MA in the fifteen autopsy cases. MA was presented in case number 7 and 9. In case number 7, MA concentration

ranges was 0.610-9.55 mg/L or mg/kg in urine, pericardial fluid, cerebrospinal fluid, blood, right cardiac chambers and femoral muscle. They were MA distribution which rank order from high to low, respectively. In case number 9, the MA concentration ranges was 1.18-10.1 mg/L or mg/kg in urine, bile, femoral muscle, pericardial fluid, blood in the right cardiac chambers and cerebrospinal fluid. They were MA distribution which rank order from high to low, respectively. Thus, pericardial fluid is a good sample for quantitative confirmation or a quantitative to blood in exsanguinated victims.

Levisky and coworkers (2001) reported measurement of MA and AM in the adipose tissues with and without livor mortis of cadavers. The result showed that the concentration ranges were 48-64 ng/g for AM and 366-479 ng/g for MA. Therefore, MA determined in postmortem adipose tissues reflects antemortem deposition and are not the result of postmortem redistribution, diffusion or permeation. MA distributes into adipose tissues and storage in skin due to its higher lipid solubility.

Moriya and Hashimoto (2002) reported a case of massive hemorrhage in the cerebral ventricles due to intravenous self administration of MA. The result showed that the concentration ranges was 2.15-9.87 µg/g in brain, including cerebral cortex, diencephalons and cerebellum. Accumulation of MA was the highest in diencephalons. In other biological samples, the concentration of MA in tissue samples from high to low was kidneys, liver, bile, myocardium, stomach content, lungs, urine, spleen, right femoral muscle, right vitreous humor, pericardial fluid and intraventricular hematoma which concentration ranges was 25.6-0.189 µg/g or µg/mL. In blood, including pulmonary arteries, pulmonary veins, left cardiac chambers, right cardiac chambers, aorta Inferior vena cava, and right femoral vein which concentration ranges was 0.347-1.28 µg/mL. AM was not detected in either sample except in bile, urine liver kidneys and stomach content. These results indicated that intraventricular hemorrhage might have occurred shortly after intravenous self administration MA due to cerebral arterial spasm and hypertension.

Raikos and coworkers (2002) reported seven autopsy cases in northern Greece. MA and AM concentration in liver was 6.0 µg/g for case 7 and 0.2 µg/g for case 4. AM and MA were not detected in another cases. Uemura and coworkers (2003) reported on two cases of simultaneous administration of MA. Distribution of

MA in tissues from high to low were liver, kidney and brain for first case and liver, brain and kidney for second case. Concentration ranges of first case and second case were 0.45-1.59 μg/g and 4.63-9.79 μg/g, respectively. Chaturvedi and coworkers (2004) reported evaluation of postmortem biological sample which various tissues collected from pilots. Distribution of MA in tissues from high to low was liver, spleen, brain, heart, muscle and vitreous fluid. Concentration ranges was 0.229-5.534 μg/g or μg/mL. For distribution of AM in tissues from high to low were liver, spleen, brain and heart. Concentration ranges was 0.017-0.133 μg/g. Furthermore, Hara and coworkers (2009) reported distribution of MA in various organs and blood obtained from a cadaver in an autopsy case. Distribution of MA in tissues from high to low was liver, spleen, kidney, brain, lung, blood and muscle. Concentration ranges was 0.272-1.55 μg/g.

Postmortem redistribution

Redistribution of drugs in the body after early postmortem autopsy has been reported. Process affects the concentration of drugs abuse in postmortem cases by diffusion from a higher concentration to a lower concentration following disruption of cellular membranes. This process is particularly significant for drugs with high lipid solubility or high tissue concentrations relative to blood taken from the heart. MA has low extent of redistribution (Drummer, 2004). It is well documented that basic drugs such as tricyclic antidepressants, narcotic analgesics, local anesthetics and antihistamines, which accumulate in the myocardium and lungs in the large amounts can easily be redistributed into the cardiac blood, resulting in enormously elevated drug levels (Moriya and Hashimoto, 1999[b]).

According to the report of the postmortem redistribution of MA, Barnhart and coworkers (1999) compared the concentrations of AM and MA in cardiac blood and peripheral blood in the corpse (20 cases) which the postmortem interval was 12-13 hour. In all cases, the drug concentrations in cardiac blood were higher than these in the peripheral blood. In the cases, myocardium tissue samples were higher than either cardiac blood or peripheral blood. It is apparent that MA diffusion takes place between the heart muscle and the blood within the chambers after death. Therefore, the detection of MA in peripheral blood samples was probably more accurate than in the cardiac blood at a short time after death.

Moriya and Hashimoto (1999[b]) reported redistribution of MA during early stages of postmortem. The results showed that the presence of MA concentration from high to low was lung, pulmonary blood, left cardiac blood, pulmonary arterial blood and right cardiac blood. These results lead to a few conclusions, firstly, drugs deposited in the myocardium in large amounts contribute little to increase in cardiac blood during the early postmortem period. Secondly, drugs in the lungs are redistributed rapidly into pulmonary venous blood and then into the left cardiac chambers if the blood remains liquid after death. As the lungs have the richest supply of blood vessels in the body, drugs can be sequestered in pulmonary tissues at high concentrations antemortem and diffuse postmortem from these tissues into the thin-walled pulmonary veins more rapidly than into thick- walled pulmonary arteries. Thus, the left cardiac blood should not be used for toxicological evaluation, while the and right cardiac blood as well as the peripheral blood be may the specimen of choice.

In addition, MA redistribution also occurs in the different brain region. Kalasinsky and coworkers (2001) measured levels of MA and AM in 15 autopsied brain regions of 14 chronic human MA users. All 15 autopsied brain regions revealed the presence of MA and AM. The results, suggested that during the interval between deaths and freezing of the brain there would have been some redistribution of MA among the different brain areas and between the brain, blood and cerebrospinal fluid components. Therefore, MA might not be retained in dopamine-rich brain areas but distributed into other the brain areas of chronic human users.

4) Metabolism

MA is metabolized largely in the liver and undergoes phase I metabolism by *N*-demethylation to AM and *p*-hydroxymethamphetamine by aromatic hydroxylation via the cytochrome P450 2D6 isoenzyme system. AM itself is extensively metabolized to variety of metabolites, including norephedrine by β-hydroxylation, *p*-hydroxyamphetamine by deamination via the cytochrome P450, both of which are pharmacologically active (Logan, 2002) and phenylacetone; this latter compound is subsequently oxidized to benzoic acid and excreted as glucuronide or glycine (hippuric acid) conjugate. Hydroxylation produces an active metabolite, O-hydroxynorephedrine, which acts as a false neurotransmitter and may account for some drug effect, especially in chronic users (Sukkwan, 2006).

5) Excretion

Caldwell and coworkers (1972) reported that in human orally receiving MA (20 mg/kg), about 90% of MA excreted in the urine in 4 days. Approximately 22% of a MA dose is excreted as unchanged drug from body, 15% as pOH-MA, 2-3% as AM, 5% as hippuric acid, 2% as norephedrine, 1-2% as *p*-hydroxynorephedrine, 1% pOH-AM and a precursor of benzyl methyl ketone (about 1% of the dose in 24 h). In addition, small quantities of an unknown amine (about 1-2 %) and acid (1-3 %) were also detected.

Oyler and coworkers (2002) reported in human, MA is almost entirely (90%) eliminated in urine. The percentage of the dose excreted as parent drug can range from as low as 2% in alkaline (pH $\geq$ 8.0) to 76% in acidic urine (pH $\leq$ 5.0). Which from reported of Schepers and coworkers (2003) in normal urine (pH 6-8) approximately 22% of a MA dose is excreted as unchanged drug, 15% as *p*-hydroxymethamphetamine (pOH-MA), 4-7% as amphetamine (AM), 1% as *p*-hydroxyamphetamine (pOH-AM).

Levine (2003) reported that MA, is mostly excreted in urine as an unchanged drug up to 45% of a dose in 24 hour period with approximately 7% as AM, 15% as pOH-MA, 2-4% as pOH-AM, 2% as norehedrine, 0.3% as hydroxynorephedrine, 0.9% as phenylacetone, benzoic acid and hipuric acid (Figure 1.5).

Terminal elimination half-life of MA was 13 hour. Twenty-three to 43% of the dose is excreted within the first 24 h and total elimination occurs over a period of 3-5 days (Kalasinky *et al*., 2001)

Caldwell and coworkers (1972) reported metabolism of MA in rats given by an intraperitoneal MA injection (45 mg/kg). Some 82% of the dose was excreted in the urine and 2-3% in the faces within 3-4 days. Phenol, pOH-MA (31% of dose), *p*-hydroxynorephedrine (16%) and unchanged drug (11%) were detected in urine within 2 days. Minor metabolites were AM (3%), pOH-AM (6%), benzoic acid out put in small (3.5%) but norephedrine was not detected.

Milesi-Halle and coworkers (2005) reported a short terminal elimination half-life (about 1 h) of MA in male rats. Approximately 53% of MA were eliminated as the 4-hydroxylated metabolite. About 34–48% of MA dose were found as the

pharmacologically active metabolite AM. Furthermore, renal elimination of unchanged MA in male rats is only about 13%.

The metabolism of MA was investigated using freshly isolated rat hepatocytes. Hepatocytes were incubated with MA and their metabolites were extracted from culture fluid. The major metabolite was pOH-MA (47%). Amounts of MA (5.9%), AM (7.9%), pOH-AM (5.5%) were detected, whereas *p*-hydroxynorephedrine, benzoic acid and phenyl acetone were not detected in this experiment (Kanamori *et al*., 2005).

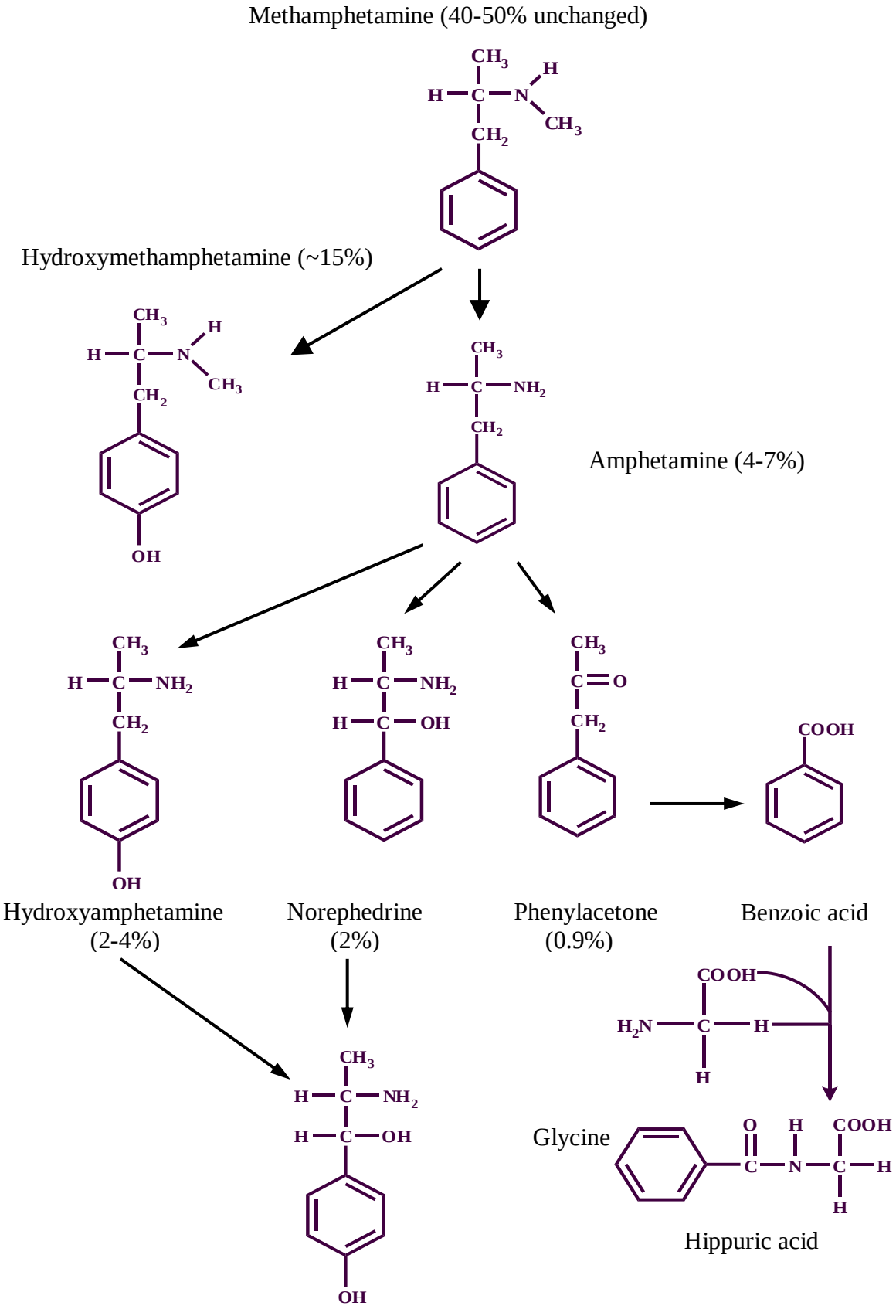


Figure 1.5 Summary of the metabolic pathway of methamphetamine in human (Levine, 2003)

1.2.7 Pharmacodynamics of MA

The high concentrations of dopamine cause feelings of pleasure, euphoria, stereotyped behavior, tremor and psychological effect on central nervous system and whereas MA acts specifically on peripheral nervous system of sympathetic nervous system causing increased release of the neurotransmitter norepinephrine. This increase in norepinephrine accelerates the heart rate, constricts blood vessels, and raises blood pressure, vascular constriction, affect many organ systems and acute kidney failure. Furthermore, norepinephrine may be responsible for the alertness and anti-fatigue effects. Serotonin may cause cognitive impairment and eventual depression (Klasser and Epstein, 2005). Effects of MA include direct effects and adverse effects.

1. Direct effects

MA is associated with both acute and chronic effect. The short-term effects include intensified emotions, euphoria, rush, increased alertness, insomnia, hyperactivity, decreased of appetite and fatigue, increased respiration and hyperthermia. The long-term effects can include psychological (but not physical) addiction and dependence, cardiovascular events (accelerated heartbeat, elevated blood pressure and can cause irreversible damage to blood vessels in the brain) and stroke, immunomodulation, hypertension, weight loss, violent behavior, anxiety, confusion, paranoia, auditory and visual hallucination, mood disturbances, delusions (e.g. formication, the sensation of insects creeping on the skin) and repetitive motor activity, all of which may contribute to homicidal or suicidal thoughts and actions (Yu *et al*., 2003; Klasser and Epstein, 2005).

2. Adverse effects

2.1 MA overdose

The common features of MA overdose include agitation, dilated pupils, tachycardia, hypertension and rapid respiration. Other features include shivering, dyspnoea, chest pain, hyperpyrexia and renal failure.

Case reports indicate that MA associated fatalities arise most commonly from multiple congestion, pulmonary oedema, pulmonary congestion, cerebrovascular haemorrhage (attributed to hypertension), ventricular fibrillation, acute cardiac failure or hyperpyrexia. A number of studies suggest that a significant and possibly greater

proportion of MA related fatalities arise from accidents, suicide and homicides, suggesting severe psychological and behavioural disturbances at toxic doses (Cruickshank and Dyer, 2009).

2.2 Psychosis

MA psychosis refers to paranoid hallucinatory states. The most common signs of MA psychosis are hallucinations, delusions and odd speech. MA induced hallucinations are predominantly auditory (experienced in 85% of cases of MA psychosis), visual (46%) and tactile (21%). MA psychosis may be related to neurotoxicity, increased noradrenergic and dopaminergic sensitivity (Cruickshank and Dyer, 2009).

2.3 Cardiovascular complications

MA-induced hypertension and arrhythmias can lead to acute events such as acute coronary syndrome, acute aortic dissection and sudden cardiac death. Repeated MA insult can also lead to chronic conditions, including coronary heart disease and cardiomyopathy. Coronary heart disease appears to occur more frequently and at a younger age among MA users than others (Cruickshank and Dyer, 2009).

2.4 Neurotoxicity

Repeated exposure to AM leads to damage at dopaminergic and serotonergic axons. The mechanisms of neurotoxicity are not understood completely, but the selectivity of damage may be explained by the oxidation of cytosolic dopamine and serotonin to 6-hydroxydopamine and 5,6-dihydroxytryptamine, which can oxidize proteins and lipids in dopamine and serotonin-rich neurones. Elevated cerebral temperature is also thought to be an important contributing factor. Neuronal damage induced by AMs is localized generally to axons and termini, while cell bodies are typically spared (Cruickshank and Dyer, 2009).

2.5 Parkinson's disease

Parkinson's disease is the long-term effect of using MA. It is a neurodegenerative disorder affecting dopamine neurones in the nigrostriatal pathway. The decrease in dopamine levels is the result of degeneration of dopamine-containning neurons in the substantia nigra (striatum) and leads to emergence of motor impairments. Parkinson's disease patients exhibit rhythmic tremor at rest, increase muscle tone or rigidity, slowness of movement (akinesia) and excecution of

movement (bradykinesia) (Guilarte, 2001).

2.5 Sexual behaviour

MA use has been reported to enhance sexual pleasure among a sample of dependent heterosexual female users. Some users reported that MA delays orgasm, facilitating prolonged sexual activity and a particularly intense orgasm. There are some indications that the prevalence of MA use is particularly high among urban homosexual men. Intravenous administration of the substituted AM, methylphenidate, leads to increased in sexual desire and pathological hypersexuality has been associated with dopamine agonist therapy. Therefore, sexual effects may be mediated by excessive dopaminergic activation (Cruickshank and Dyer, 2009).

2.6 Other adverse effects

MA intoxication is associated with dry mouth, which may lead to dental caries, and activation of mandibular muscles, which may lead to bruxism. Recent MA use appears to be a risk factor for methicillin resistant *Staphylococcus aureus* skin infections. Skin infections may be associated with formication (a sensation of something crawling on the skin) and skin-picking (Cruickshank and Dyer, 2009).

1.2.8 Samples preparation for of MA and AM in tissues

Sample preparation and clean-up procedures are an essential step in analysis for reliable and accurate results, particularly in biological samples such as urine, hair, blood and tissues. These samples must usually be processed in order to isolate by using a suitable sample extractant. From reporting of MA extraction, it is readily extracted from a biological material into organic solvents at alkaline pH due to its prototypical basic property (pKa 9.9). MA is also readily back-extracts into acid, and back into organic solvents without significant loss. Because of its volatility, however, it can be lost during a dry-down or evaporation step if that is a part of the procedure. This loss can be avoided by the addition of a small amount of hydrochloric acid during the evaporation step, or the addition of a less volatile keeper solvent such as dimethylformamide (DMF) (Logan, 2002). Sample preparation techniques of MA and AM in tissues reported include protein precipitation (Hendrickson *et al.*, 2006), liquid- liquid extraction (LLE) (Terada, 1985; Nagata *et al.*, 1990; Moriya and Hashimoto, 1999[a]; Moriya and Hashimoto, 1999[b]; Riviere *et al.*, 2000; Kalasinsky *et*

al., 2001; Moriya and Hashimoto, 2002), solid phase extraction (SPE) (Raikos *et al*., 2002; Nakagawa *et al*., 2003; Uemura *et al.*, 2003; Chaturvedi *et al*., 2004; Scheidweiler *et al.*, 2008) and headspace-solid phase microextraction (headspace-SPME) (Hara *et al*., 2009).

1.2.8.1 Protein precipitation

The protein content of human body fluids and tissues is considerable, from 6% by weight in plasma to greater than 50% by weight in liver and other organs. Once proteins have been precipitated, separation of aqueous and solid protein must occur by filtering or centrifugation. The purpose of protein precipitation is to obtain a cleaner preparation. Some drugs are occluded in the precipitate but can be at least partially recovered by washing the precipitate with hot water or hot dilute hydrochloric acid (Levine, 2003). In order to extract MA and metabolite AM from biological samples, three reagents, i.e. zinc sulfate heptahydrate ($ZnSO_4$), acetonitrile (ACN) and trichloroacetic acid (TCA) were used to precipitate samples such as serum, brain and testis. Separate aliquots of serum or tissue were treated with either a 1:1 or 2:1 ratio (agent: sample) of precipitating agent and mixed on a rotary mixer at 4°C (15 minutes). Mixtures were centrifuged for 5 minutes and the supernatant was filtered using 0.2-µm nylon centrifugal filters (Millipore). The ranges of recovery in serum, brain and testis were 17-56% (3.3-6.2 %CV) with $ZnSO_4$, 17-95 % (1.1-17 %CV) with ACN, 82-94 % (1.2-4.2 %CV) with 10 % TCA and 86-97 % (0.2-5.8 %CV) with 20 % TCA (Hendrickson *et al*., 2006).

1.2.8.2 Liquid- liquid extraction (LLE)

LLE or solvent extraction is a method to separate compounds based on the partition of organic compounds between the aqueous sample and immisible organic solvent. It is one of the most techniques that are being used for select extraction of MA and metabolite AM (Fifield and Kealet, 2000). Body fluids (blood, urine, bile) or an aqueous tissues homogenate can be extracted directly with an organic solvent to obtain organic substance (Levine, 2003). Extraction of MA and AM in tissues by LLE has been reported by using of wide variety of solvents, including haxane (Terada, 1985; Riviere *et al.*, 2000), diethyl ether (Nagata *et al*., 1990; Moriya and Hashimoto,

1999[a]; Moriya and Hashimoto, 1999[b]; Moriya and Hashimoto, 2002) and n-butyl chloride (Kalasinsky *et al*., 2001). Most extraction are readily back-extraction into acid by adding a small volume of HCl which are used for prevention of extractant loss during a dry-down or evaporation step (Terada, 1985; Nagata *et al*., 1990; Moriya and Hashimoto, 1999[a]; Moriya and Hashimoto, 1999[b]; Riviere *et al.*, 2000; Moriya and Hashimoto, 2002). The recovery ranges in tissues were 94-115 % (Terada, 1985).

However, LLE methods have major disadvantages, including the need of large volumes of organic solvents, toxicity of solvents, requiring expensive glasswares, the need of clean-up step before instrumental analysis and ease to emulsion formation (Fifield and Kealet, 2000).

1.2.8.3 Solid-phase extraction (SPE)

SPE is one of various techniques available to analysts to bridge the gap that exists between the sample collection and the analysis step. The principles of SPE is selective transfer of material between a solid sorbent and a liquid phase; separations depend on different relative affinities for the two phases based on adsorption, size or charge; selectivity achieved by pH control, solvent composition and surface chemistry of the sorbent. It is a techniques that is increasingly used for sample clean-up prior to chromatographic analysis by without using large volume of a solvent in extraction and other sample preparation steps, such as dilution or pH adjustment (Fifield and Kealet, 2000; Simpson, 2000).

A typical SPE procedure consists of four distinct steps (Figure 1.6); 1) sorbent conditioning, the cartridge is flushed through with sample solvent to remove impurities and to ensure that the sample solution will properly wet the surface of the sorbent particles; 2) sample loading, the sample solution is passed through the cartridge; 3) rinsing, This is necessary to remove the matrix components; and 4) elution, this final step is to recover retained analytes (Fifield and Kealet, 2000). There are four types of sorbent; reversed phase, normal phase, ion exchange and adsorption. Reversed phase such as C8, C18 and phenyl interact via Van der Waals forces and extracting relatively non-polar compounds. Normal phases, such as silica, florisil and alumina interact by H-bond and extracting relatively polar compounds. Ion exchange phases, such as weak anion exchange (WAX) and strong cation exchange (SCX) bind

compounds through ionic interactions and extracting relatively as either anionic or cationic which it retain negatively charged and positively charged compounds, respectively. Adsorption phase, sorbents interact via polar compounds (Simpson, 2000).

Several research works used SPE technique for exracting MA and AM from tissues. The SPE cartridges included Bond Elute Certify® (Nakagawa *et al.*, 2003; Chaturvedi *et al*., 2004; Uemura *et al.*, 2003), abselut NEXUS 60 mg LRC® columns (Raikos *et al*., 2002) and SPEC MP1, 10 mL reservoir/70 mg bed mass (Schidweiler *et al*., 2008).

Bond Elute Certify® is popular cartridge for extracting MA and AM from tissues. This cartridge utilizes a packed bed consisting of a special, non-polar C8 sorbent and a strong cation exchanger (SCX). It is most commonly used to extract basic (cationic) drugs from urine, serum and blood, but it is also very effective for extraction of a wide range of compounds from all aqueous matrices. Analytes are strongly retained to the sorbent by the combination of coulombic and non-polar mechanisms, allowing a relatively strong series of aqueous and organic washes to rinse away non polar, polar, and anionic matrix contaminants. This results in outstanding sample clean-up and concentration of samples prior to screening or confirmation by TLC, HPLC, GC and GC/MS.

Many reports of extracting MA and AM from tissues were investigated by using this cartridge. Nakagawa and coworker (2003) measured MA in tissues .The sample is first mixed with pH 6.0 buffers. The samples are then added to Bond Elute Certify® cartridges preconditioned with methanol and 0.1 M phosphate buffer (pH 6.0). The cartridges are then rinsed with 1.0 M acetic acid and MA is eluted with ethyl acetate containning 2% ammonium hydroxide. The extracted MA are then derivatized with heptafluorobutyric anhydride (HFB) and injected to GC-MS. Uemura and coworker (2003) determined MA in tissues of two fatal case using Bond Elute Certify® column. The columns are preconditioned with 2 mL water and methanol. The samples are added to columns and wash with 2 mL water and methanol. All analytes were eluted with 2 mL of methanol/chloroform (98:2) and derivatized with trifluoroacetic anhyride (TFA)/ethyl acetate (1:1). The LOQ of MA of this method by

determination with GC-MS in first case was 0.45 µg/g and the second case was 2.64 µg/g.

In another work, Chaturvedi and coworker (2003) reported toxicological evaluation of postmortem biological samples collected from pilots involved in fatal civil aircraft accidents and sample preparation in tissues by using Bond Elute Certify® columns, first conditioned with 2.0 mL of methanol, followed by 2.0 mL of the phosphate buffer. The samples are added to columns before it was rinsed with 1.0 mL of 1.0 M acetic acid and dried. Analytes were eluted from the columns by using 4.0 mL of 2% NH_4OH in ethyl acetate. The LOQ of MA and AM of this method after determined with GC-MS was 0.229 µg/mL and 0.017 µg/mL, respectively.

Furthermore, the report of extraction by another SPE, Raikos and coworker (2002) measured MA and AM in tissues of autopsy cases by using abselut NEXUS 60 mg LRC® columns. Property of Abselut NEXUS® is a highly crosslinked, spherical polymeric sorbent with a unique combination of hydrophilic and lipophilic moieties which have the ability to bind both polar and non-polar analytes present in an aqueous sample. It was designed to extract a wide range of pharmaceutical compounds from biological fluids while providing high, reproducible recoveries. Extraction of this SPE, the columns were rinsed with water, eluted from column by using methanol and derivatized with ethyl acetate (100 µL) and heptafluorobutyric acid (HFBA) (50 ul) prior to GC-NPD. The LOQ of MA and AM in tissue were180 ng/mL and 130 ng/mL, respectively.

In addition, Schidweiler and coworker (2008) developed and validation of a gas chromatographic electron impact ionization mass spectrometric (GC-MS-EI) method of MA and metabolites AM in brain of mouse. Sample preparation by using SPEC MP1 column, is mixed mode monolithic disc solid phase extraction for analyzing drugs with polar functional groups. SPEC MP1 combines two different mechanisms of retention: the hydrocarbon chain which provides the hydrophobic interaction and the benzenesulfonyl group which provides the ionic interaction. During the extraction, polar basic drugs are strongly held by the benzenesulfonyl group while rigorous wash steps can be applied to remove the matrix effects which cause ion suppression. Extractions of this method, the samples are then added to the column preconditioned with 1 mL of methanol, 1 mL distilled water and 1 mL 0.2 M

sodium acetate buffer (pH 4.5). Following, the columns were washed with 1mL of 0.1 M acetic acid, 1 mL of tetrahydrofuran and 2 mL of methanol. All analytes were eluted using 1.5 mL of ethyl acetate: methanol: ammonium hydroxide (77: 20: 3; v/v/v). The recovery ranges of this method in brain were 99.6 to 103.5%.

SPE technique has advantages including, increased speed and simplicity, reduced solvent usage and disposal costs, improved selectivity and sample matrix clean up but SPE disadvantages are analyte breakthrough occurs when solvent have not been properly selected, pores become blocked and flow through the column is too fast. Flow rates through the SPE cartridge require careful optimization to ensure efficient analyte adsorption (Shibamoto, 1994).

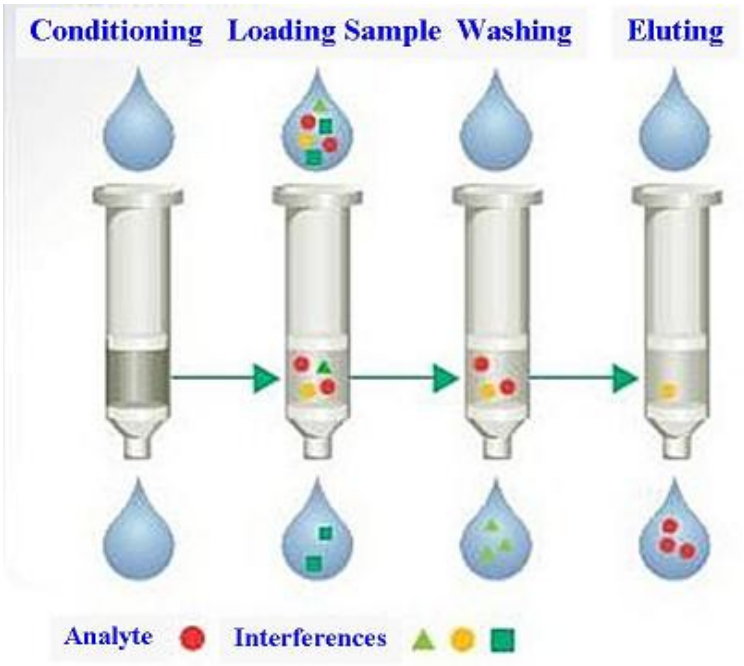


Figure 1.6 Process of SPE**;** conditioning, loading sample, washing and eluting (http://www.pretech.nu/products/Gil_SPE.htm)

1.2.8.4 Solid-phase microextraction (SPME)

SPME is method of sample preparation that has been widely used for the extraction and pre-concentration of an extensive range of analytes in a variety of samples and it was used for qualitative analysis of the volatile components

(Susawaengsup *et al*., 2005). In Forensic Science, SPME has many advantages, for example, preservation of the sample while minimizing the risk of sample contamination, cost saving of solvent and reducing the risk of analysts being exposed to toxic substances. SPME utilizes a short, thin, solid rod of fused silica, coated with an absorbent polymer (Figure 1.7). The fiber is the same type of chemically inert fused silica use to make capillary GC column and it is vary stable even at high temperatures. There are three types of extraction that can be utilized with an SPME fiber; direct extraction SPME (DI-SPME), headspace SPME (HS-SPME) and membrane protected SPME. The SPME extraction technique consists of two processes (Figure 1.8); 1) analytes partition between the sample; 2) fiber coating and the concentrated analytes desorb from coated fiber to an analytical instrument (Wercinski, 1999). Therefore, SPME relies upon the extraction of solutes from a sample into the SPME absorptive layer. After a sampling period during, extraction has ideally reached equilibrium. The absorbed solutes are transferred with the SPME layer into an inlet system that desorbs the solutes into a gas (for GC) or liquid (for LC) mobile phase (Hinshaw, 2003).

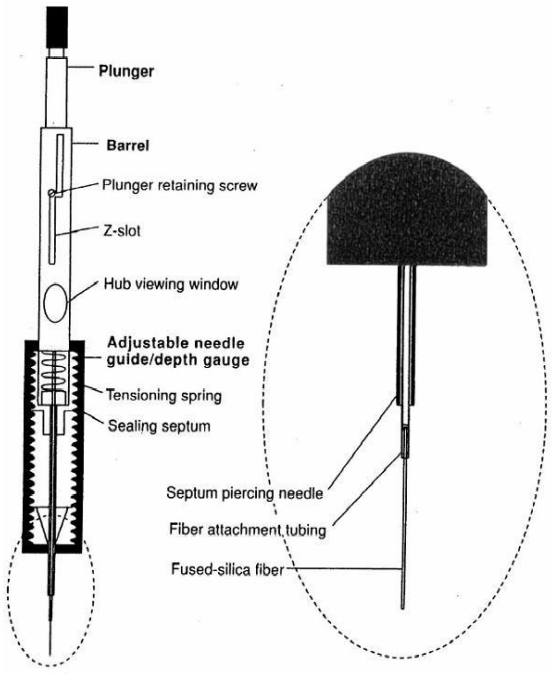
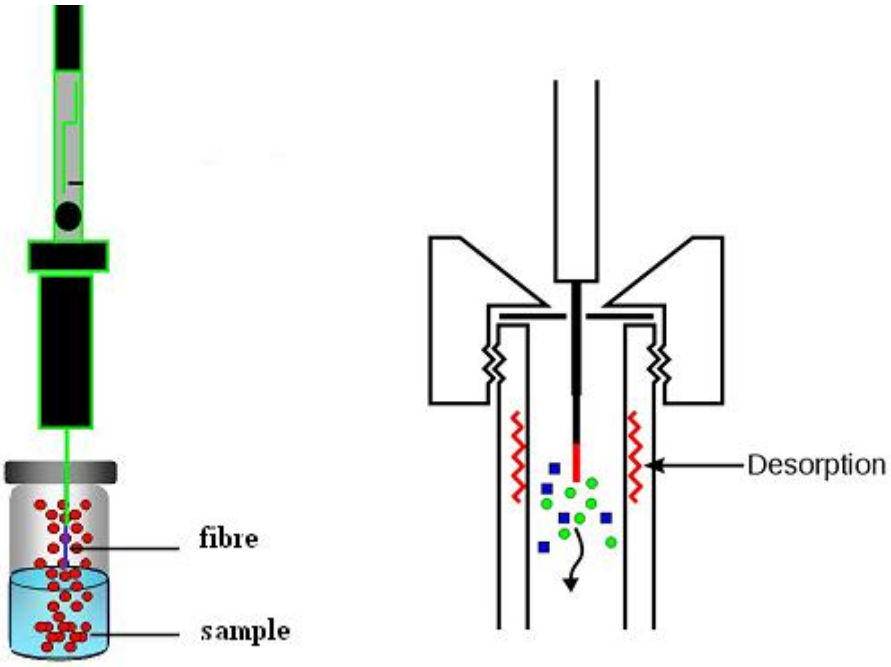


Figure 1.7 Composition of SPME (http://share.psu.ac.th/blog/sci-discus/17227)



(a) **(b)**

Figure 1.8 Extraction of solutes from a sample into the SPME absorptive layer (a); desorption of absorbed solutes by using heat from inlet system of GC (b). (http://share.psu.ac.th/blog/sci-discus/17227)

Hara and coworker (2009) reported using HS-SPME for analyzing MA and AM in solid tissues (1.5 g). Tissues collected from a cadaver of 29-year-old woman was incubated at 70°C for 2 h after adding 100 ng each of the two ISs (L-AMP-d_3 and AMP-d_6) and 5 ml of 0.1 M hydrochloric acid. Incubation under acidic condition softened the tissue and made it more suitable for homogenization. The samples were homogenized with a Polytron® homogenizer. The crude supernatant fraction was obtained after centrifugation at 3000 rpm for 25 min. Two milliliters of the fraction were washed with 10 ml of diethyl ether by shaking vigorously in a test tube. After standing for a few minutes, 1.0 ml of the aqueous phase was placed in a headspace vial, to which 0.5 g of sodium chloride, 0.2 ml of 1 M sodium hydroxide solution, and 0.01 ml of heptafluorobutyric chloride (HFBCl) were added. The septum-capped vial was shaken gently and set on a tray of an automated sampler for headspace-SPME followed by GC-MS analysis. The coefficient of variation obtained from this method

ranged between 2.3% and 12.1%. The relationship between detection response and concentration showed good linearity, r^2 = 0.9995 for AM and 0.9998 for MA.

However, SPME technique has some disadvantages. Fibers are expensive, short in life usability, fragile and low resistant to high temperature and organic solvents (Jager and Andrews, 2002).

1.2.9 Techniques for determination of MA and AM in tissues

In forensic science, detection of drugs in biological samples is usually done by an initial screening and then a confirmation of the compounds, which may include a quantitation of the compounds.

Screening tests are short tests and indicate the possible presence of certain types of compound in biological samples. Screening test sometimes use test kits which it is available commercially for a variety of types of substance including aspirin, paracetamol, drugs of abuse, heavy metals and many other groups of compound. The test results are not conclusion must then be applied to confirm the results and identify the specific compounds involved. In addition, screening test by using chemical screening test is thin layer chromatography (TLC). This allows separation of the components of a mixture in solution plus the possibility of applying colorimetric identification of the separated components. Drugs can also be screened using immunoassay techniques including radioimmunoassay (RIA), enzyme multiplied immunoassay technique (EMIT), enzyme linked immunosorbent assay (ELISA). These immunoassay techniques are only presumptive and indicate the possible need for more specific forms of analysis. For confirmation testing is the second step of testing following the detection of a positive result on the preliminary or screening test. The purpose of confirmation is to eliminate any false positive results that may have originated from an initial screening process. A confirmation test should be used highly specific and possibly more sensitive method. More specific confirmation tests used to identify drugs of abuse are gas liquid chromatography (GC), high performance liquid chromatography (HPLC) and gas chromatography with mass spectrometry (GC/MS). All three of these methods are both highly specific and sensitive to small quantities of the particular drug being tested (SOFT/ AAFS, 2006).

The most screening test was used to detected AM and MA in urine and blood sample such as Triage^{TM} screening, this screening is an immunoassay test for phencyclidine, benzodiazepines, cocaine, amphetamines, tetrahydrocannabinol, opiates, barbiturates and tricyclic antidepressants which it was used for drugs screening with urinary samples (Moriya and Hashimoto, 2002; Uemura *et al*., 2003) and florescence polarization immunoassay (FPIA), was performed preliminary immunoassay in urine and blood samples by using Abbott TDx system for opiates, cannabinoids, cocaine, benzodiazepines, alcohol and amphetamines (Raikos *et al.*, 2002). Moriya and Hashimoto (2002) and Uemura and coworker (2003) reported screening test in urine and blood from autopsy case by using Triage^{TM} screening kits. The result showed that both urine and blood samples were identified positive results with amphetamine type. Raikos and coworkers (2002) reported screening test in urine and blood by using FPIA test and in tissues by TLC technique from autopsy cases. The result showed that blood and tissues samples were identified positive screening results for AM. The limit of detection (LOD) for amphetamines by TLC was 0.1 µg.

However, FPIA screening test have limitation, are fluorescent salts in bile occasionally give false-positive results and the assays are generally more expensive than comparable enzyme-linked assays (Levine, 2003).

Several quantification methods of MA and metabolite AM in biological samples are reported by using thin-layer chromatography (TLC) (Raikos *et al*., 2002), capillary electrophoresis (CE) (Znaleziona *et al.*, 2007), high performance liquid chromatography (HPLC) (Riviere *et al.*, 2000), gas chromatography (GC) (Terada, 1985; Moriya and Hashimoto, 1999[a]; Moriya and Hashimoto, 1999[b]; Moriya and Hashimoto, 2002), Liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) (Hendrickson *et al*., 2006), and gas chromatography coupling mass spectrometry (Nagata *et al*., 1990; Kalasinsky *et al*., 2001; Nakagawa *et al*., 2003; Uemura *et al.*, 2003; Scheidweiler *et al.*, 2008 and Hara *et al*., 2009). However, poor UV absorption properties of MA make it an unsuitable candidate for high performance liquid chromatography (HPLC) with ultraviolet (UV) detection. In addition, MA also has no native fluorescence and no significant oxidative electrochemical properties at low voltages (Logan, 2002).

1.2.9.1 Thin-layer chromatography (TLC)

TLC is the most useful tool in assaying the purity of organic compounds that it is use to determine the proper solvent system for performing separations using column chromatography. TLC takes advantage of the different affinity of the analyte with the mobile and stationary phases to achieve separation of complex mixtures of organic molecules. Stationary phase is usually alumina or silica (polar or non-polar) and the mobile phase is an organic solvent or mixture of organic solvents (Jork *et al*, 1990). In forensic toxicology, TLC is useful in detecting chemicals, such as chemical weapons, explosives and illicit drugs (Lee and Wilmoth, 2006). For detection of AM derivative in tissues of autopsy by using TLC, the LOD for amphetamine by TLC was 0.1 µg (Raikos *et al*., 2002)

However, disadvantages of TLC are migration characteristics vary sensitive to conditions. Thin layer is easy damage. Quantitative precision is only moderate 5-10% (Fifield and Kealey, 2000). The reversed phases of TLC are not wetted by solvent mixtures having high water contents and consequently, plate development becomes difficult if not impossible (Scott, 2008).

1.2.9.2 Capillary electrophoresis (CE)

CE is a technique that uses a narrow-bore (20-200 mm i.d.) fused-silica capillary to separate a complex array of large and small molecules. The separations use high voltages (10-30 kV) (Tagliaro and Smith, 1996). The capillary was filled with an electrolyte buffer solution, links a source vial and applying pressure. High electric field strengths are used to separate molecules based on differences in charge, size and hydrophobicity (Currell, 2000). CE is applied to quanitative and quantitative characterization of biological active materials, especially, use for clinical and forensic works (Fifield and Kealey, 2000).

In forensic work, CE can be utilized for separation of complicated mixtures of target analytes (e.g. abused drugs, diagnostic markers, etc.) from matrix without multi step sample preparation. CE used methods to screen various types of abused drug in biofluids such as urine, blood, plasma and serum (Thormann *et al.*, 1998). Znaleziona and coworkers (2007) have developed a simple capillary electrophoretic method as the verification and confirmation tool for screening analysis of amphetamines,

opiates, benzodiazepines, cocaine and their metabolites in urine and saliva, for toxicological application. The separations were performed on the capillary electrophoresis Spectra PHORESIS 100 with fast-scanning UV/VIS detector Spectra FOCUS. These experiments uncoated fused silica capillaries of total and effective lengths of 75 cm and 45 cm, respectively, 75 μm I.D. x 365 μm O.D. The experimental conditions of CE were partially optimized (mainly the influence of concentration and types of additives, e.g. cyclodextrines, organic solvents) and validated the method was used for analyzing samples from drug abusers. The advantages of this CE screening toxicological method include its simplicity with relatively high selectivity for separation and identification of abused drugs.

However, CE have disadvantages, mobility of CE are vary sensitive to supporting medium and precision poor for quantitative work (5-20%) (Fifield and Kealey, 2000).

1.2.9.3 High performance liquid chromatography (HPLC) and liquid chromatography coupling mass spectrometry (LC-MS)

HPLC system is based on chromatographic separate in column with liquid solvent as the carrier gas medium. Column of HPLC is packing with small particle size in order to achieve sufficient column efficiency which produces a high resistance to fluid flow and it is essential to use high pressure pumps in order to obtain a sufficient flow of the mobile phase (Currell, 2000). HPLC use to separate non-volatile substances including ionic and polymeric samples by the sample is introduce into a liquid mobile phase which flow through a column of relatively coarse partical of a stationary phase, usually silica or aluminar , under the influence of gravity (Fifield and Kealey, 2000). The column packings used in HPLC include microparticulate silicas; spherical or irregular particles; mean particle size 3 µm, 5 µm, 10 µm. type of HPLC is liquid solid chromatography mode and application relatively non-polar to moderately polar compounds (polyaromatic, fats, oils and mixure of isomer), octadecyl (ODS or C_{18}) is bond-phase chromatography and ion pair chromatography HPLC mode and application relatively moderately polarmixtures (pharmaceuticals, drugs and amino acid) and octyl (C_8) is bond-phase chromatography and ion pair chromatography HPLC mode by application relatively more polar matrix (pesticides,

herbicides, peptides and metabolites in body fluids). In addition, ability of these techniques to detect compounds depends on the type of detector used which it have very important for selectivity and sensitivity include ultraviolet-visible (UV) detector, electrochemical (EC) detector and mass spectrometry (MS) detector diode array (DAD) detector and fluorescence (FL) detector (Neue, 1997).

The HPLC technique was used to determine MA and AM in tissues by using µ-Bondapak C_{18} column (300 x 3.9 mm i.d). Separation was performed by using a convex gradient of acetonitrile and water with 0.1% trifluoroacetic acid (from 10 to 20% acetonitrile over a 10-min period). The flow rate was 1 ml/min and the UV detector absorbance was at 215 nm (Riviere *et al.*, 2000).

The advantage of HPLC is a shorter time and achieves a higher degree of resolution, that is, the separation of constituents is more complete (Prema, 2003). However, HPLC have disadvantages, detection system is not available due to column performance is vary sensitive to settling of pack bed or accumulation of strongly adsorbed materials (Fifield and Kealey, 2000).

LC-MS is a powerful technique for many applications and it has very specificity and high sensitivity. Generally, in forensic or clinical toxicology is popular technique because drug detection in organs of the body gives rapid result. MS is high sensitivity detector and exceptional specificity compared to UV and short analysis time. The major advantage of MS is using of tandem MS-MS. Advantage of MS-MS is analysis times of 1 minute compared to over 10 mins with UV detection and simple sample preparation procedures (Sasaki, 2008)

Recently, the researcher reported the determination by LC-MS/MS technique for MA and 3 active metabolites (AM, 4-OH-MA, and 4-OH-AM) in serum, brain, and testis. The LC system was a Waters Alliance 2695, coupled to a Waters/Micromass Quattro LC triple quadrupole mass spectrometer with an electrospray interface and equipped with a Mark II source. The column was a BDS Hypersil C_8 column, 100 × 2.1 mm (3 mm). The guard-column was a BDS Hypersil C_8, 10 × 2.1 mm (3 mm). A binary linear gradient was used for analytical separation. Solvent A was 5 mM ammonium acetate buffer (pH 3.7) with 5% (vol/vol) acetonitrile. Solvent B was 5 mM ammonium acetate buffer (pH 3.7) with 95% (vol/vol) acetonitrile. The flow rate was 0.3 mL/min. The method was linear over a

wide dynamic range (0.3-1000 ng/mL). Lower limits-of-quantitation (LOQ) for MA and AM were obtained 1 ng/mL (Hendrickson *et al*., 2006).

1.2.9.4 Gas chromatography (GC)

GC is separation technique for identification and quantification of separating chemicals in a complex sample in a volatile state without decomposing. The compounds are partition between a stationary phase and a mobile phase (gas). A carrier gas is helium (He), nitrogen (N_2) or hydrogen (H_2) which these gases are the mobile phase and it must be very high purity, because traces of water or oxygen may decompose the stationary phase, which leads to column bleeding and finally destruction of the column. Helium is the most frequently used carrier gas. It has high separation efficiency and is inert, making it safe to work with and suitable for a wide range of detectors. The stationary phase can be either solid or liquid which it is held a solid support material and coated on inside of a capillary column or held on particulate packing with column (Rood, 2007; Currell, 2000).

The detector of GC interacts with the compounds based on some physical or chemical property. Some detectors respond to every compound while others respond only to a select group of compounds. GC has been used to analyze compounds for many years using several different detectors including, Flame ionization detector (FID), Thermal conductivity detector (TCD) and Nitrogen phosphorus detector (NPD).

ECD is selective for compound that absorb electron which then ionizes some of the carrier gas to produce electron. TCD records the temperature of the heated filament in the gas flow. The heat is conducted away from the filament. When the conductivity decreases, filament temperature increase, thus leading to the change of resistance and electron was measured. NPD is a highly sensitive. The group of ionization detectors is used as source for ionization (Scott, 1998; Kenndler, 2004; Rood, 2007). In a part of FID is one of the most widely used for hydrocarbons. It is based on the measurement of the electric charges (Fifield and Kealey, 2000; Kenndler, 2004). The eluent exits the GC column (A) and enters the FID detector's oven (B) (Figure 1.9). As the eluent travels up the FID, it is first mixed with the hydrogen fuel (C) and then with the oxidant (D). The effluent/fuel/oxidant mixture

continues to travel up to the nozzle head where a positive bias voltage exists (E). This positive bias helps to repel the reduced carbon ions created by the flame (F) pyrolyzing the eluent. The ions are repelled up toward the collector plates (G) which are connected to a very sensitive ammeter, which detects the ions hitting the plates and then feeds that signal (H) to an amplifier, integrator, and display system. The products of the flame are finally vented out of the detector through the exhaust port (J).

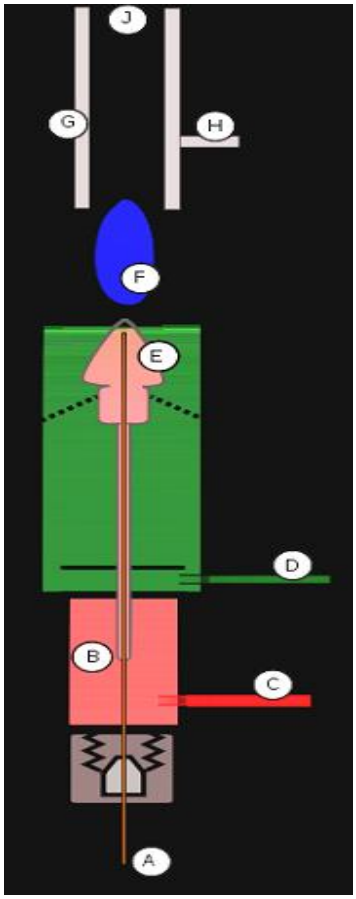


Figure 1.9 Principle of GC-FID
(http://en.wikipedia.org/wiki/Flame_ionization_detector)

Forensic application, MA is readily analyzed by GC. GC is the most popular method for analysis of MA in biological material including, blood, urine, sweat, biological fluids, hair, nails and other tissues. MA and AM can be detected with most

GC detectors including, FID, NPD and when derivertized properly, ECD. They can also readily be derivertized which advantages of the derivatization prior GC analysis. They are more stable, less polar, better separation, more symmetrical peaks and greater sensitivity. If analysis by without derivatization, MA is readily eluted from most stationary phases at low temperatures (~50 ^{O}C) due to its low molecular weight, but its basicity results in peak-tailing on some phases but identification are readily accomplished. Because of its early elution time, care should be taken in underivatized GC analysis where the detector is initially turned off to allow elution of the solvent front because the drug may elute before the detector turns on (Logan, 2002; Levine, 2003; Mitrevski and Zdravkovski, 2005).

The research works for determining MA and metabolite AM in tissues by using GC were reported. Moriya and Hashimoto (1999[a]), Moriya and Hashimoto(1999[b]) and Moriya and Hashimoto (2002) measured MA from several tissues of autopsy cases and experimental on rabbits by using TC-1 capillary column (15 m x 0.53 mm I.D.x 1.5 µm film thickness), and a TC-17 capillary column (15 m x 0.53 mm I.D., 1 µm film thickness). The temperature of both injection port and detector was 260°C. The column temperatures were programmed as follows: the initial temperature of 100 or 150°C was maintained for 0 or 2 min for TC-1 and TC-17 capillary column, respectively, then increased to 260°C at a rate of 10°C/min and the final temperature was maintained for 27 min. The carrier gas was nitrogen at a flow pressure of 15 kPa. The detector is FID and *N*-methylbenzylamine as internal standard.

Recently, mass spectrometry (MS) detector is widely use as a gold standard and an indispensable tool for forensic substance identification. It used to perform as specific test and can identify a very large number of substances (Scott, 1998). MS offers a significant advantage over other the detectors. The ability of the GC to separate compounds chromatographically is the GC can provide a pure compound to the mass spectrometer to facilitate spectral analysis. Because the mass spectrometer operates at low pressure, the amount of carrier gas introduced in to the mass spectrometer must be limited so that the pumping system can keep up the volume of coming gas (Levine, 2003).

For quantitative analysis of GC-MS, the internal standard method is the most

often used and typically the most accurate of available techniques. Internal standard was used for analysis of MA and AM in tissues, is deuterium such as methamphetamine-d_2 (Nagata et *al.*, 1990) methamphetamine-d_3 (Nakagawa et *al.*, 2003), methamphetamine-$d_{14,}$ amphetamine-d_{11} (Scheidweiler et *al.*, 2008), L-amphetamine-d_3 and L-methamphetamine-d_6 (Hara et *al.*, 2009). In addition, the other internal standard of GC-MS method is *N*-methylbenzylamine hydrochloride (Uemura et *al.*, 2003).

Many drugs must be derivatized prior to GC/MS analysis including MA and AM. Analysis of underivatized amphetamine produces irregular and asymmetric peaks which are difficult to integrate and lead to irreproducible results. Derivatization amphetamines result in symmetric peaks but derivertizing agent can contaminate the inlet or column. This contamination can shorten column lifetime and cause noise. So, a clean up step are then performed to remove acidic byproducts of derivatization (Seller and Rigdon, 2008). Common derivatizing agents for analyzing MA and AM in tissues are heptafluorobutyric anhydride (HFB) (Terada, 1985; Nakagawa et *al.*, 2003), trifluoroacetic anhydride (TFAA) (Nagata *et al.*, 1990), chlorodifluoroacetic anhydride (Kalassinsky *et al.*, 2001), bis(trimethylsily) trifluroacetamide (BSTFA) (Uemura *et al.*, 2003), heptafluorobutyryl chloride (HFB-Cl) (Hara et *al.*, 2009) and heptafluorobutyric acid anhydride (HFAA) and followed by a clean up (Scheidweiler *et al.*, 2008).

Analyzing MA and AM in tissues by GC-MS can be carried out by type of columns. The most columns were used rest polar and midpolarity types. The column were used for MA and AM analysis in tissues including, glass tube packed with 2% Thermon-3000 on chromosorb WAW DMCS (80-100 mesh) (1 m x 3 mm I.D.), glass tube packed with 3% OV-17 on gas-Chrom Q (80-100 mesh) (2 m x 3 mm I.D.) and glass tube packed with 1% OV-17 on gas-Chrom Q (80-100 mesh) (0.5 m x 3 mm I.D.). The carrier gas was helium at flow rate 30 mL/min. LOD was obtained 0.15 ng/g (Terada, 1985), glass tube packed with 5% OV 17 on chromosorb W, HP (80-100 mesh) (2 m x 3 mm I.D) and helium as a carrier gas at flow rate 40 mL/min) (Nagata *et al.*, 1990), W Scientific DB5-MS capillary column (20 m,0.18 mm i.d., 0.18 µm film thickness) by using helium as the carrier gas at a flow rate of 1 ml/min. with LOD was 0.01 ng/g (Kalassinsky *et al.*, 2001)., The fused silica capillary column

(Shimadzu CBJ1-S30-100; 30 m, 0.32 i.d., 1 µm film thickness) with LOQ was obtained 0.45 µg/g (Uemura *et al.*, 2003)., fused silica capillary cross-linked column with (5%-phenyl)-methylpolysiloxane (25 m x 0.25 mm I.D.). The carrier gas was helium (Nakagawa *et al.*, 2003), Agilent DB-17ms capillary column (30 m, 0.32 mm i.d., 0.25 µm film thickness). High-purity helium (99.999%) was the carrier gas with a flow rate of 1.5 mL/min. LOD were obtained 2.5 and 5.0 ng/mL for MA and AM, respectively (Scheidweiler *et al.*, 2008). and Rtx-5 capillary column (10 m, 0.18 mm i.d., 0.20 µm film thickness) Helium was used as carrier gas at 100 kPa. The range of linearity was obtained 0.010 to 2.0 µg/g ($y = 12.6 x + 0.0319$, $r^2 = 0.9998$ for AM; $y = 12.5 x + 0.0093$, $r^2 = 0.9995$ for MA) (Hara et *al.*, 2009).

GC-MS is more sensitive technique for analyzing MA and AM in biological sample. However, limitation for this technique, analyzing compounds that can be vaporized without decomposition and several countries cannot afford such as an expensive instrument (Fifield and Kealey, 2000).

1.3 Objectives

The objectives of this work are the development and validation method of GC-FID for determining of MA and AM in rat liver without derivertization. This method was investigated by measurement in real sample after single and repeated doses by oral administration of rat liver.

CHAPTER 2

Methodology

2.1 Chemicals and Reagents for MA and metabolites AM analysis

Chemicals and reagents employed in this study are summarized in Table 2.1.

Table 2.1 List of chemicals and reagents used in this study

Chemicals	Grade	(MW/FW/g/mol)	Supplier
1) Acetic acid (CH_3COOH) (Lot no. B44806)	AR	60.05	J.T. Baker Neutrasorb®, NG, U.S.A.
2) Amphetamine hydrochloride	Purity 99.99%	135.21	It was obtained from Department of Pharmacology, Faculty of Science, Prince of Songkla University.
3) Diethyl ether (CH_3OCH_3) (Lot no. H15B16)	AR	74.12	J.T. Baker Solusorb®, NG U.S.A.
4) Hydrochloric acid (HCl)	AR	36.46	J.T. Baker Neutrasorb®, NG U.S.A.
5) Methamphetamine hydrochloride (Lot no. 367)	purity 99.99%	149.23	Alltech-Applied from Science (It was obtained Regional Forensic Science Division 4, Songkhla, Thailand)

Table 2.1 List of chemicals and reagents used in this study (cont.)

Chemicals	Grade	(MW/FW/g/mol)	Supplier
6) Methanol (CH_3OH) (Lot no. H32E03)	HPLC	32.04	Mallinckrodt Baker Inc., Phillipsburg, NJ, U.S.A.
7) Sodium carbonate (Na_2CO_3)	AR	105.99	Fisher Scientific, Leicester shire LE 11 5RG, UK
8) Sodium chloride (NaCl) (Lot K37160333 730)	AR	58.44	VWR International Ltd., Poole, England
9) gas			
Air, Zero gas	Purity >99.99%	15.9994	TIG, Thailand
Helium gas (He)	Purity > 99.99%	4.002602	TIG, Thailand
Hydrogen gas (H_2)	Purity > 99.99%	1.00794	TIG, Thailand
Nitrogen gas (N_2)	Purity > 99.99%	14.0067	TIG, Thailand

2.3 Materials and Instruments

Materials and instruments employed in this study are summarized in Table 2.2.

Table 2.2 List of materials and instruments for analytical method

Materials and instruments	Sources
1) Gas Chromatograph automatic system Hewlett-Packard (HP 6890) Series GC equipped with flame ionization detector (GC-FID)	Agilient, U.S.A.
2) Computer system model intel inside Pentium digital venturis, Chemstation software	Agilient, U.S.A.
3) Capillary Column: VertiBond™ 5, 30 m x 0.32 mm i.d. x 0.25 µm film thickness of 5% diphenyl and 95% dimethypolysiloxane	Vertical®, Bangkok, Thailand
4) High intensity ultrasonic processor	Sonics Vibra Cell, Bangkok, Thailand
5) Tissue homoginizer	Kinematica CH-6010 Kriens-LU, Polytron®, Switzerland
6) Refrigerated centrifuge	Sorvall RC-3B plus, California, U.S.A.
7) CentriVap Concentrator	LABCONCO, MO, U.S.
8) Ultrasonic bath model JAC-2010	Kodo, Hwaseong, South Korea

Table 2.2 List of materials and instruments for analytical method (cont.)

Materials and instruments	Sources
9) Vortex Genie-2	Scientific Industries, Inc., Bohemia, N.Y., U.S.A.
10) Automatic pipettes 100, 1000 µL	Eppendorf, Germany
11) PH meter model Seven Esy	MEITLER TOLEDO, Greifensee, Switzerland
12) Analytical balances model	Precisa Instruments Ltd, Switzerland
13) rotator	GLAS-COL®, Terre havte, Indiana

2.4 Procedure of sample preparation

2.4.1 Liver sample collection

Rats were anesthetized with diethyl ether. The abdomen of rat was cut with scissors to expose the thorax. A thoracectomy was performed and the heart was rapidly perfused with 0.9% NaCl. At the end of the perfusion, liver was carefully removed and placed on an ice-cooled plate. Liver was wrapped in foil and stored at -70°C in a freezer until analysis.

2.4.2 Liver sample preparation

Prior to extraction procedure, liver tissue was homogenized using a homogenizer (Kinematica CH-6010 Kriens-LU, Polytron®, Switzerland) and sonicated with ultrasonic processor (Sonics Vibra Cell®, Thailand) in an ice-chilled tube. Homogenized tissue was divided into 2 portions for analysis of validation method. One was used as a blank and another was used for preparation of AM and MA spiked samples.

2.4.3 Extraction method

Extraction procedure was modified from Moriya and Hashimoto (1999[a]). One milliliter of homogenated tissue was mixed with 2 mL carbonate buffer (pH 9.7) for 30 s using the vortex mixer. The mixture was extracted with 8 mL diethyl ether for 40 min using rotator. The solvent-sample mixture was centrifuged at 2200 g for 5 min. The upper organic phase was back-extraction with 1 mL 0.1 N HCl for 40 min using rotator and then centrifuged at 2200 g for 5 min. The lower aqueous phase was mixed with 1 mL carbonate buffer (pH 9.7). The mixture was re-extracted with 4 mL diethyl ether for 30 s using the vortex mixer and then centrifuged at 2200 g for 5 min. This upper phase was mixed with lower phase in back-extraction. Add 20 µL of acetic acid and evaporated under speed vacuum (LABCONCO, MO, U.S.) to dryness at 60°C.

2.5 Analytical procedure

2.5.1 Instrument system

A gas chromatographic system consisted of model GC 6890 (Agilent, U.S.A.) auto System with a flame ionization detector (Agilent, U.S.A.). Data were collected and processed using the Chemstation Software System (Agilent, U.S.A.). The analytes were concentrated to dryness using speed vacuum (LABCONCO, MO, U.S.) and extracted by liquid-liquid extraction (Moriya and Hashimoto, 1999[a]).

2.5.2 Chromatographic conditions

A gas chromatography model GC 6890 equipped with a flame ionization detector and capillary column VertiBond^{TM} 5 (30 m x 0.32 mm i.d., 0.25 μm film thickness) was used. Determination of AM and MA were modified from Cheung *et al* (1997). Column temperature was programmed initially at 70 °C (2 min) and increased with a rate of 30 °C/min to 120 °C. It was further increased to 150 °C at a rate of 5° C/min and finally increased at a rate of 70 °C/min to 300 °C where it was held for 1 min. One μL of sample was injected in a splitless mode (splitless injection with inlet insert purge time of 0.3 min and split vent flow rate of 80 mL/min). Helium gas was used as the carrier gas with a flow rate of 2.5 mL/min. Fuel gas (H_2) and make-up gas (N_2) flow rate was 30 mL/min. Oxidant gas (O_2) flow rate was 300 mL/min. Injector and detector temperature were 200 °C and 300 °C, respectively.

2.5.3 Preparation of standard solutions

1) Stock solutions

For analysis of MA and AM in liver, stock solutions of 1000 μg/mL of MA and AM were prepared in pure methanol and stored at -20ºC.

2) Working standard solutions

Working standard solutions were prepared by diluting the stock solutions to concentrations ranging from 2.5-50 μg/mL. The standard solutions were prepared as a mixture of the standard MA and AM.

3) Calibration curves

For analysis of in liver, calibration curves prepared by adding working standard MA and AM solutions 200 µL to liver homogenate 800 µL (final volumn 1 mL) so that final concentrations were 6.25, 9.375, 18.75, 31.25, 62.5 and 125 µg/g of tissue.

2.5.4 Validation method in rat liver

Measurements of MA and AM in liver sample was validated. The method consists of linearity, accuracy, intra-day and inter-day precisions, recovery, limit of detection and limit of quantification. Validation of the analytical method was performed in accordance with the U.S. Food and Drug Administration (FDA) (FDA, 2001). The rat liver homogenate spiked with MA and AM was used throughout the method validation.

1) Linearity and range

Linearity was evaluated by preparing five standard concentrations (6.25-125 µg/g of tissue; 5 replicates each concentration) of MA and AM in liver rat. The calibration curve was constructed by plotting the peak area of the analyte (Y) versus its concentrations (X). Regression analysis for each calibration curve was performed to obtain the calibration equation and correlation coefficient (*r*). The response from chromatogram of unknown samples was used to calculate the concentration from calibration curve.

2) Precision

Precision was evaluated from intra-day and inter-day precision. Both intra-day and inter-day precision were determined using three quality control (QC) samples (18.75, 31.25 and 125 µg/g of tissue; 5 replicates for each concentration) into rat liver. Intra-day precision was determined by assaying sample of each concentration during the same day under the same experimental condition. Inter-day precision was determined by daily assay of the sample for consecutive days. Precision is expressed as the percentage of the relative standard deviation (%RSD). The precision determined at each concentration level should not exceed 15% of %RSD.

$$\%RSD = \frac{\text{standard deviation (S.D.)}}{\text{mean}} \times 100$$

3) Accuracy

Accuracy was evaluated to determine the closeness of mean of the test results to the true value (concentration) of the analyte. Accuracy was also determined using QC with five replicates for each concentration. Accuracy was expressed as the deviation (DEV) and is acceptable when the DEV is within the range ±15%.

$$DEV\ (\%) = \frac{(\text{measured concentration - nominal concentration})}{\text{nominal concentration}} \times 100$$

4) Recovery

Recovery was determined by comparing the GC responses of the extracted MA and AM from liver with response after direct injection of standard MA and AM in methanol. Concentrations were determined, low, medium and high (15.625, 31.25, 125 μg/g of tissue; 5 replicates of each concentration) by spiking the standard MA and AM into rat liver homogenate. The percent recovery is acceptable when the value falls within 80-120%. The percent recovery was calculated as follows.

$$Recovery\ (\%) = \frac{\text{response after extraction}}{\text{response after direct injection}} \times 100$$

5) The limit of detection (LOD) and the limit of quantification (LOQ)

LOD and LOQ was determined base on the signal to noise ratio method. Signal from liver sample with low known concentrations of MA and AM (6.25, 9.375, 12.5 μg/g of tissue; 5 replicates of each concentration) were compared with those from blank liver samples. The concentration values in signal to noise ratio are usually required to be equal to or greater than 3 and 10 of LOD and LOQ, respectively.

2.6 Procedure of animal study

2.6.1 Sample preparation of seized Ya-Ba tablets

Extraction method of Ya-Ba tablets analysis (Figure 2.1). and condition for determination using GC-FID was reference from Phonchai (2009). After each Ya-Ba tablet was finely crushed, five milligrams of Ya-Ba powder were dissolved in 2.5 mL of methanol and mixed on the vortex mixer for 30 s. The mixture was filtered using filter paper no.1 (Whatman). A 500 μL aliquot of the filtrate was then mixed with 200 μL of 50 μg/mL diphenylamine (DPA) (final concentration of 10 μg/mL) and 300 μL of methanol. One microliter of this mixture was injected into the GC-FID (three replicates for each tablet).

The calibration curve standard solutions of Ya-Ba tablets analysis were prepared by adding working standard mixture of MA and 20 μL aliquot of 50 μg/mL DPA (final concentration of 10 μg/mL). Sample final volumes were made up to 100 μL with pure methanol to obtain the final concentrations of 6.25, 25, 100 and 200 μg/mL.

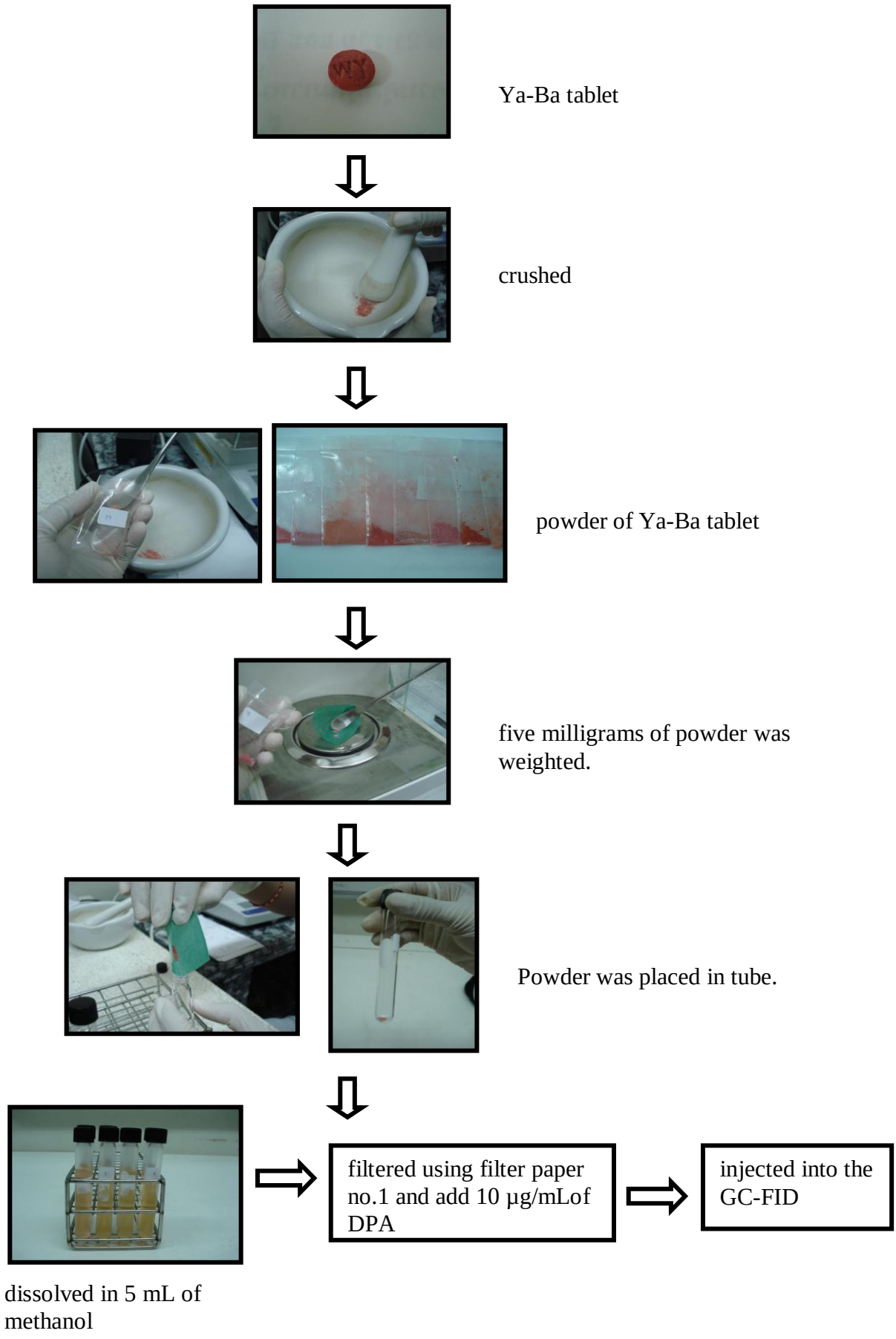


Figure 2.1 Sample preparations for determination Ya-Ba tablets

2.6.2 Preparation and treatment in animal

Male Wistar rats, weighing 190-200 g are used in this study from the southern laboratory animal facility, Faculty of Science, Prince of Songkla University were used. During experimental period, the rats were housed in an experimental room maintained at temperature (23 ± 2°C) and a relative humidity of 50 ± 5% under a 12 h/light/dark cycle. They were freely accessed to normal food and water *ad libitum*. The experiment protocol was approved by the Ethics Committee for Experimental Animals (no. Ref 25/51), Prince of Songkla University.

In each experiment, the animals were divided into two groups. Two animals per group:

Group I: the rats were given a single dose of MA (10 mg/kg) orally and were sacrificed after oral administration 2 h.

Group II: the rats were obtained MA with 10 mg/kg of oral administration once daily for five consecutive days and were sacrificed after oral administration 2 h on the fifth day.

CHAPTER 3

Results and discussion

3.1 Analytical study

3.1.1 Chromatographic profile of amphetamine (AM) and methamphetamine (MA) standards in methanol

The condition used for in analysis of AM and MA was modified from that of Cheung and coworkers (1997). The separation times of AM and MA obtained from this work were shorter than previous reported by Cheung and coworkers (1997) i.e 5 vs. 9 min due to the different of the film thickness of stationary phase (i.e. 0.25 vs. 0.52 μm).

The chromatographic separation of standard mixture of AM and MA in methanol using GC-FID technique is shown in Figure 3.1. The retention time of AM and MA were 4.67 and 5.18, respectively, within a run time of 12.8 min. The analytes were well separated with no interference, symmetry and the run time was shorter than previously reported by Cheung and cowerkers (1997). Symmetrical peaks were achieved despite of no derivertization. Additionlly, without derivatization, AM and MA analyzing results were not the presence in peak doublets of both the salt (hydrochloride) and free base forms when compared with reported by Sellers and Rigdon (2008).

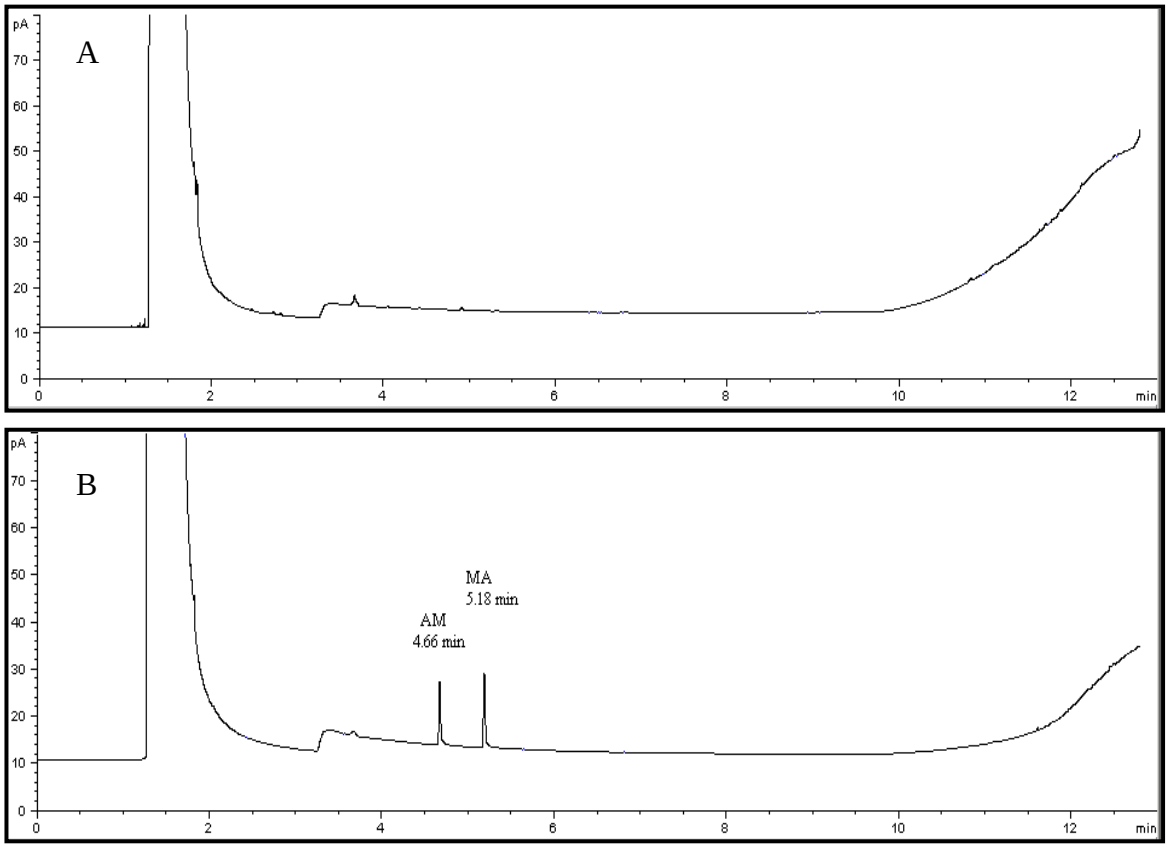


Figure 3.1 Representative chromatograms of standard AM and MA in methanol; (A) methanol blank; (B) standard mixture of AM and MA (25 µg/mL) in methanol.

3.1.2 Chromatographic profile of standard AM and MA in rat liver samples

The chromatographic separation of standard mixture of AM and MA in spiked rat liver using liquid-liquid extraction (LLE) method and GC-FID technique is shown in Figure 3.2. The chromatogram of AM and MA in spiked rat liver samples shows high peak resolution, good shape, well separation (Figure 3.2B) and such a peak was not found in the blank rat liver (Figure 3.2A). Retention time of AM and MA were 4.66 and 5.18, respectively, within a run time of 12.8 min.

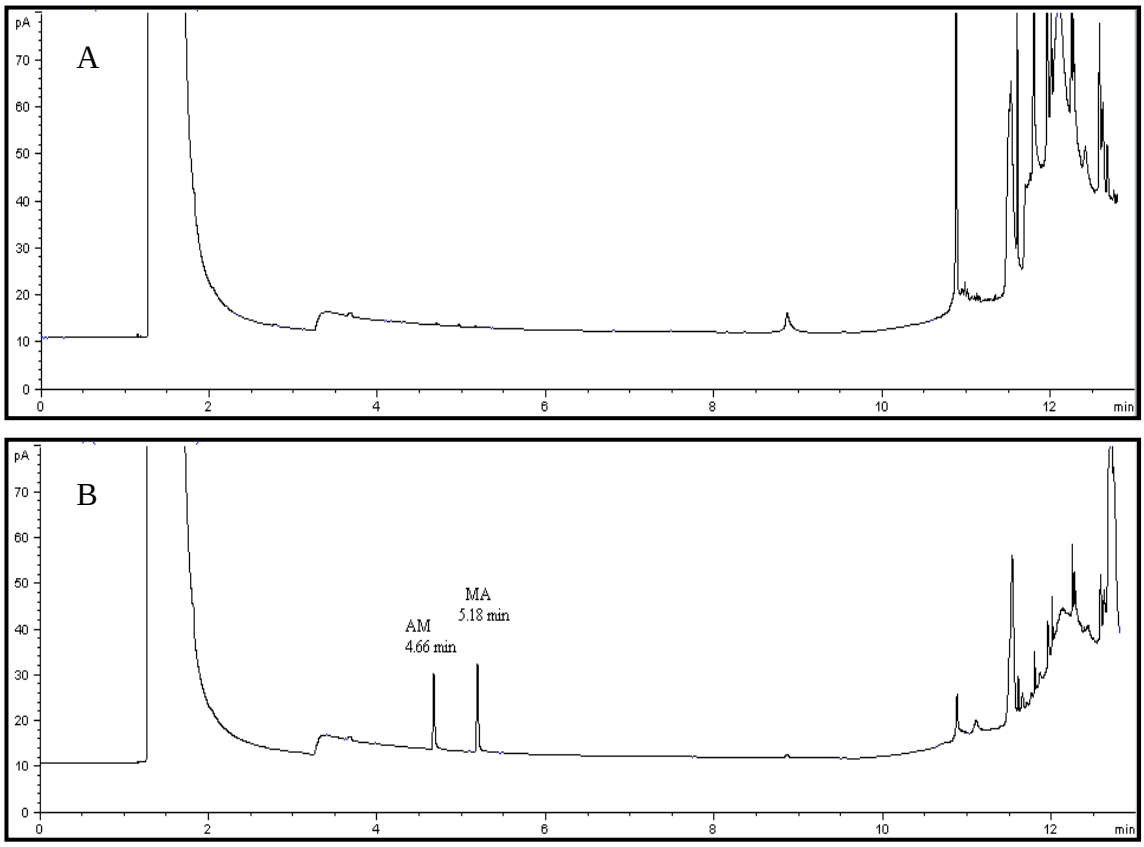


Figure 3.2 Representative chromatograms of AM and MA in rat liver; (A) blank liver; (B) liver homogenates spiked with standard mixture of AM and MA (31.25 μg/g of tissue).

AM and MA were well separated with no interference with the analytes. The chromatograms are symmetry and the run time was shorter than previously reported (Cheung *et al.*, 1997). For quantitative analysis, the internal standard method is the most often used and typically the most accurate of available techniques (Levine, 2003). However, the present study was performed without the internal standard. An internal standard was spiked a sample, not extracted. However, without the use of internal standard, this work provided good precision and available extraction method.

The most undesirable effect in direct amphetamine separation appears as peak tailing chromatogram which makes the acquisition of chromatographic signal information more difficult, affecting retention time, peak area, peak width at half peak height and peak overlapping. AM are polar compounds, thus their direct

determination is often followed with absorption in the injector system and column. These effects cause peak tailing of chromatograms or even unsuccessful baseline separation. To avoid these undesirable effects the polar amino groups are being derivatization (Mitrevski and Zdravkovski, 2005). Additionally, Nagata and coworkers (1990) used the derivatizing agent to form a derivative in determination which is more stable, less polar, better separation, more symmetrical peaks and greater sensitivity. The method described in this work provided good separation, symmetrical peaks of the analytes by without derivertization and short analysis time. Analysis without derivatization, MA is readily eluted from most stationary phases at low temperatures (~50 ^{O}C) due to its low molecular weight, but its basicity results in peak-tailing on some phases (Levine, 2003).

Additionally, the analyzing using the derivatization of AM and MA analysis have disadvantage: derivatizing agents can contaminate the inlet or column. The contamination can shorten column lifetime and cause noisy, elevated baselines that interfere with the analysis of target compounds (Sellers and Rigdon, 2008). Analysis without derivatization is necessary in the study.

In this work, the capillary column coated with 5% phenyl/95% dimethylpolysiloxane was shown to be better choice for the separation of AM and MA than capillary column coated with 50% phenyl/50% dimethylpolysiloxane (Mitrevski and Zdravkovski, 2005; Phonchai, 2009). Using this type of capillary column resulted in higher peak resolution and more symmetrical than capillary column coated with 50% phenyl/50% dimethylpolysiloxane.

3.1.3 Method validation for MA and AM in liver

1) Linearity and range

Regression analysis showed that the calibration curves were linear over the concentration ranges 9.375-125 μg/g of tissue for MA and AM (Figure 3.3) (five replicates of each concentration). The regression equation was y = (8.23±0.69) x – (2.03±0.95), r = 0.9996 for AM and y = (10.25±0.80) x – (2.30±0.79), r = 0.9997 for MA (Table 3.1). When x are the AM and MA concentrations and y is the peak area.

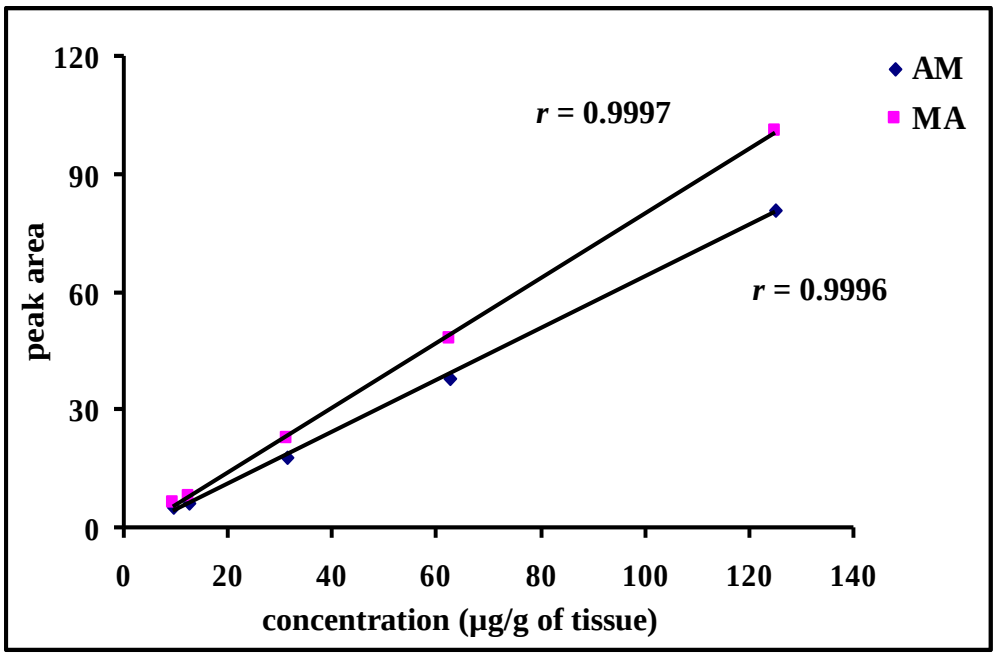


Figure 3.3 Linearity plot of mean peak area ± SD against different concentrations of AM and MA spiked in rat liver; correlation coefficient (*r*) = 0.9996 for AM, 0.9997 for MA (n=5)

Table 3.1 Linear regression analysis of AM and MA (n=5)

Analyte	Range of linearity (µg/g of tissue)	Calibration equation [a]	Correlation coefficient (*r*)
AM	9.375-125	(8.23±0.69) *x* – (2.03±0.95)	0.9996
MA	9.375-125	(10.25±0.80) *x* – (2.30±0.79)	0.9997

[a]Mean ± SD

2) Precision

Both intra-day and inter-day precisions were determined using three QC samples (18.75, 31.25 and 125 µg/g of tissue) by spiking of AM and MA in blank liver. Intra- and inter-day precisions of AM and MA are shown in Table 3.2. Intra-day precision was determined by analysis five samples of each concentration during the same day under the same instrumental operating condition. Inter-day precision were determined by analysis of the samples for five consecutive days. The intra- and inter-

day precisions of the method were 8.35-10.97%RSD and 4.79-8.58%RSD for AM, 7.94-9.58%RSD and 8.51-12.21%RSD for MA. Both intra- and inter-day precisions determined at each concentration level of acceptance for precision is 15% RSD value.

3) Accuracy

The accuracy was determined using three QC samples (18.75, 31.25 and 125 µg/g tissue) by spiking of AM and MA standards in blank liver with five replicates for each concentration. The results are shown in Table 3.2. The accuracy ranged from (-) 14.77 to (+) 5.41%DEV for AM and (-) 6.98 to (+) 4.11%DEV for MA. The accuracy was expressed as the deviation (DEV) and is acceptable when the DEV is within ±15% above LOQ.

Table 3.2 Precision and accuracy of the method for determination AM and MA in rat liver samples (n=5)

Analyte	Concentration (µg/g of tissue)	Precision[a] (%RSD)		Accuracy[a] (%DEV)	
		Intra-day	Inter-day	Intra-day	Inter-day
AM	18.75	10.975	4.79	-13.80	-14.77
	31.25	8.47	8.58	-2.88	-10.51
	125	8.35	8.55	+5.41	-6.23
MA	18.75	9.39	12.21	-6.98	+0.37
	31.25	9.58	8.51	-2.46	-3.40
	125	7.94	8.51	+4.11	+1.26

[a] Data are expressed as mean values.

4) Recovery

The extraction recovery in this study was performed using LLE method by spiking the standard of AM and MA in liver blanks at the concentrations of 15.625, 31.25, 125 µg/g of tissue (5 replicates of each concentration). The results of extraction recovery for determining of AM and MA in rat liver are shown in Table 3.3. The results show that the mean percentages of recovery were 86.25-113.98% for AM and 88.17-110.15% for MA. This extraction method of AM and MA from the liver

provided a high percentage of recovery. Result of recovery ranges were the same as the LLE method and solid phase extraction (SPE) method of Terada (1985) and Scheidweiler and coworkers (2008), were 94-115% and 99.6-103%, respectively. This study provided a good recovery and cost saving apparatus more than the SPE method.

Table 3.3 Recovery of AM and MA in rat liver samples (n=5)

Analyte	Concentration (μg/g of tissue)	Mean peak area		% Recovery [a]
		Direct injection	After extraction	
AM	15.625	6.45	7.35	113.98 (7.90)
	31.25	18.22	17.96	98.46 (9.62)
	125	93.81	80.91	86.25 (7.08)
MA	15.625	8.82	9.72	110.22 (7.13)
	31.25	22.53	22.59	100.37 (10.30)
	125	114.30	100.83	88.17 (6.75)

[a]Mean (SD)

5) The limit of detection (LOD) and the limit of quantification (LOQ)

The limit of detection (LOD) and the limit of quantification (LOQ) were determined base on the signal to noise ratio method. Signal from liver sample with low known concentrations of MA and AM (6.25, 9.375, 12.5 μg/g of tissue; 5 replicates of each concentration) were compared with those from blank liver samples. The concentration values resulting in signal to noise ratio of equal to or greater than 3 and 10 are defined as LOD and LOQ, respectively. The results showed that the LOD (signal to noise ratio ≥ 3) and LOQ (signal to noise ratio ≥ 10) of AM and MA were 6.25 μg/g of tissue and 9.375 μg/g of tissue, respectively. When comparing with those reported by Raikos and coworkers (2002) using the nitrogen phosphorus detector (NPD) detector, the NPD provided lower LOD and LOQ value than our results (AM and MA; 40 ng/mL and 55 ng/mL for LOD, 130 ng/mL and 180 ng/mL for LOQ). This may be due to the instrument was a good sensitivity than the present work. However, the sensitivity of the present method was sufficiently high for determining AM and MA in rat liver of fatal case from MA overdoses.

3.2 Animal study for determining MA and metabolite AM in liver

3.2.1 Determination of seized Ya-Ba tablets for administration to rats

Extraction method of Ya-Ba tablets analysis and condition for the determination of MA and AM using GC-FID in this study were performed as previously described (Phonchai, 2009). The calibration curve using standard solutions (6.25, 25, 100 and 200 μg/mL) of MA is shown in Figure 3.4. The regression equation was y = 0.1115 *x* - 0.2208, *r* = 0.9996. When *x* are Ya-Ba tablets concentrations and y is the peak area ratio.

Physical characteristics and amount of MA from analysis of each Ya-Ba tablets is shown in Table 3.4. The result showed that the amount ranges of 22 Ya-Ba tablets were 1.80 - 8.36 mg (1.92-9.05%) as MA hydrochloride. The tablets weighed between 84.2 and 102.6 mg. Color of Ya-ba tablets includes one violet and the most found orange tablets. The average diameter of tablet was 0.6 cm and thickness 0.2 cm. Ya-Ba tablets of all samples were a round shape and WY logo (Figure 3.5). However, the report of Ya-Ba tablets were different from these results, included generally contains 10-25% of MA hydrochloride and 60-70% of caffeine and others (starch, pigments and flavor compounds). Ya ba tablets were mostly found as orange tablets and the average weight 90 milligram. The average diameter of Ya-Ba tablets was 6 millimeter and thickness 2.5 millimeter (Hiruntoe, 2007). Puthaviriyakorn and coworkers (2002) reported that Ya-Ba tablet seized in Thailand. MA tablet was usually orange color, but occasionally green, yellow, pink, red, brown colored tablets were founded. Size of tablet was 6 mm of diameter and 3 mm of thickness. Average weights were 90 mg. The most popular logo mark were WY or Wy with R and SY logos also present which logos of these results in Songkhla province was not found R and SY logos.

In during 2001 to 2002, the Regional Medical Sciences Center Chiang Rai reported the physical characteristics of Ya-Ba tablets seized in Thailand (133 samples). The average purity of Ya-Ba tablets 19.81% as MA hydrochloride. The average weight was 0.1076 gram. The most common Ya-Ba tablets were orange or green flat round tablet with WY or R logo and flavored with vanilla flavor (Kondee *et al.*, 2003). During October 2003-May 2004, two hundred and ninety eight MA tablet samples which were seized by the police in the northern region of Thailand were

reported. The samples display 7 kinds of logos. Regarding logos, wY predominated followed by wy, WY, R, Wy, OK and 888 in decreasing prevalence. The average weight of all samples was 0.0914 ± 0.007 gm. The average diameter was 6.019 ± 0.004 mm. The average thickness was 3.027 ± 0.136 mm. Average MA and caffeine content were 21.76 ± 6.39 mg % and 62.43 ± 9.15 mg %, respectively (Adam *et al*., 2005).

Information from intelligence sources indicates that each manufacturer has a specific logo, for instance, wY is produced by the United Wa State Army (UWSA). WY is connected with the Myanmar National Defence Alliance Army (Kokang Chinese). wy, Wy is the mark associated with the Shan United Army (SUA) or former Khun Sa Army. The UWSA is the largest drug-producing and-trafficking group in Southeast Asia, producing heroin and MA. UWSA produces MA tablets embossed with the wY and 99 logos (Adam *et al*., 2005). So, WY logos of Ya-Ba tablets seized in Songkhla province were imported from Myanmar and produced by Myanmar National Defence Alliance Army.

.

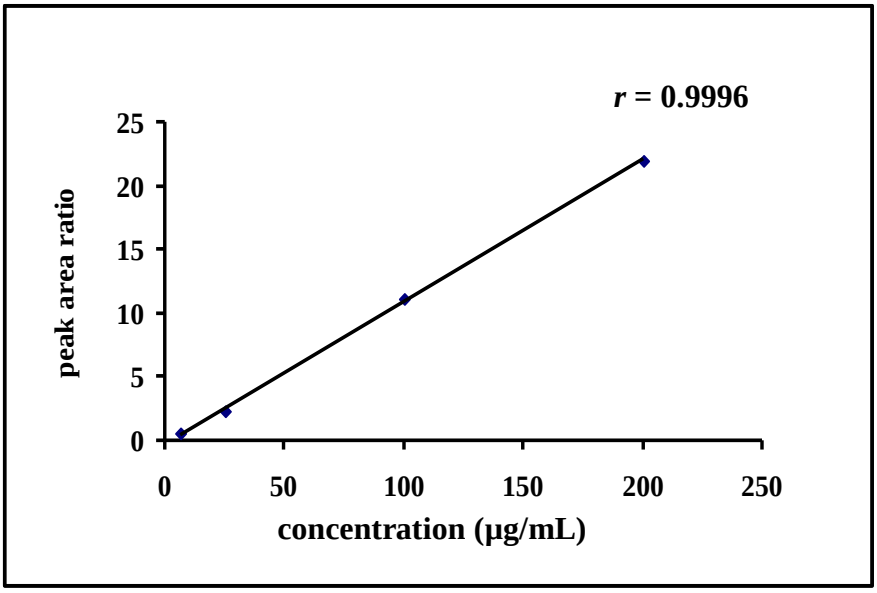


Figure 3.4 Calibration curve in standard solution of MA, correlation coefficient (*r*) = 0.9996 (n=1)

Table 3.4 Physical characteristics and amount of MA of 22 Ya-Ba tablets

Sample no.	Color	Logo	Weigh (mg)	Amount of MA (mg)	MA (%)
1	orange	WY	93.5	4.78	5.11
2	orange	WY	90.6	4.29	4.73
3	violet	WY	95.6	3.69	3.86
4	orange	WY	93.9	1.80	1.92
5	orange	WY	93.9	2.47	2.63
6	orange	WY	89.9	4.99	5.55
7	orange	WY	88.5	7.35	8.31
8	orange	WY	89.0	6.97	7.83
9	orange	WY	84.2	5.97	7.09
10	orange	WY	89.0	7.76	8.72
11	orange	WY	87.2	6.65	7.62
12	orange	WY	88.8	4.88	5.50
13	orange	WY	86.9	6.92	7.96
14	orange	WY	93.4	5.40	5.79
15	red orange	WY	90.0	5.85	6.50
16	red orange	WY	93.7	6.90	7.36
17	red orange	WY	89.7	6.66	7.42
18	red orange	WY	92.4	8.36	9.05
19	red orange	WY	102.6	2.58	2.51
20	red orange	WY	91.8	7.68	8.36
21	red orange	WY	93.5	6.11	6.53
22	red orange	WY	87.7	4.96	5.66

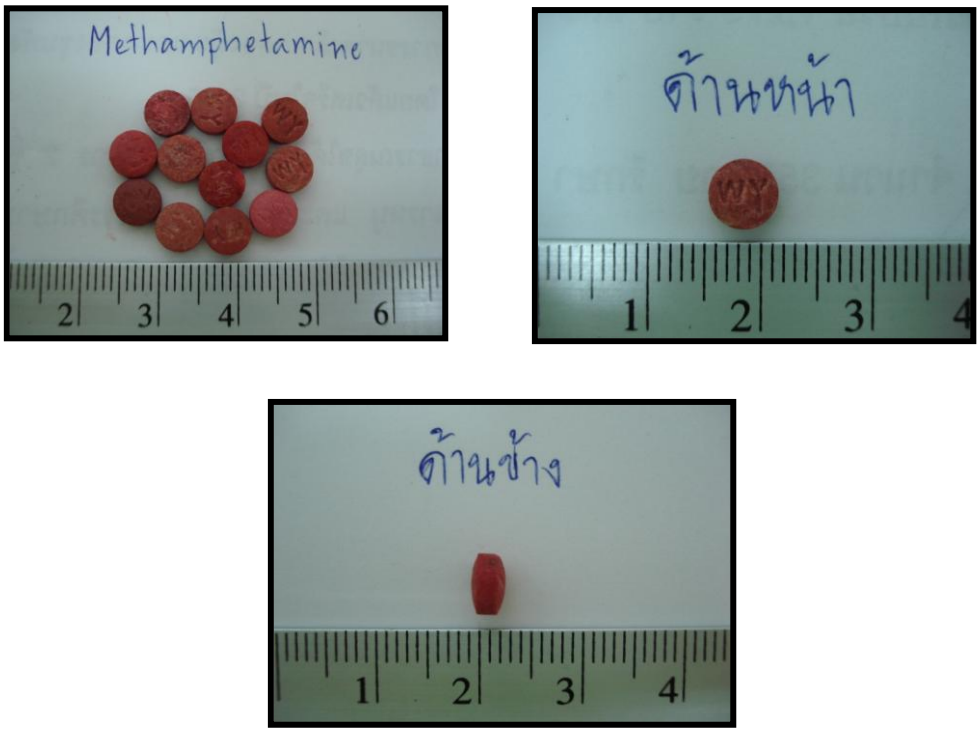


Figure 3.5 Picture of Ya-ba tablets obtain from Regional Forensic Science Division 4 in Songkhla province for administration to rat

3.2.2 Dose of MA administration

An optimum dose of MA should be lower than LD_{50}. The LD_{50} of MA after subcutaneous administration in mice and oral administration in mouse was 95 mg/kg body wt. (Funahashi *et al.*, 1988) and 143 mg/kg, respectively. Prior to experiment, a preliminary study was examined by reference of dose for oral administration in rat from Kuwayama and coworkers (2007). Rat was given a single dose of 10 mg/kg (n=1) and clinical effects were observed. Clinical effects were observed stereotyped behavior such as licking a cage, biting the teeth, restlessness, walk backwards which these effects related with report of Sutisrisung and makmanee (1999). Therefore, this dose was chosen.

3.2.3 Chromatographic analysis and proof of method for determining MA metabolite AM in rat liver

The chromatographic separation of MA and AM in liver homogenates spiked with 12.5 and 31.25 µg/g of tissue and in the liver of rat orally treated with MA 10 mg/kg of both single and multiple doses (five consecutive days) using GC-FID technique are shown in Figure 3.6. Analyte concentrations in the rat liver collected 2 h after oral administration of 10 mg/kg MA of both single and multiple doses are shown in Table 3.5. The result showed that after single dose administration, AM concentration was not detected in rat liver. MA concentration in rat liver was less than LOQ (9.375 µg/g of tissue) but more than LOD (6.25 µg/g of tissue) for rat no.1. MA concentration in rat liver was 10.125 µg/g of tissue for rat no.2. After multiple dose administration, AM and MA concentration in rat liver were 9.375 µg/g of tissue and 86.75 µg/g of tissue rat no.1, respectively. AM concentration in rat liver was less than LOQ but more than LOD for rat no.1. MA concentration in rat liver was 44 µg/g of tissue for rat no.2.

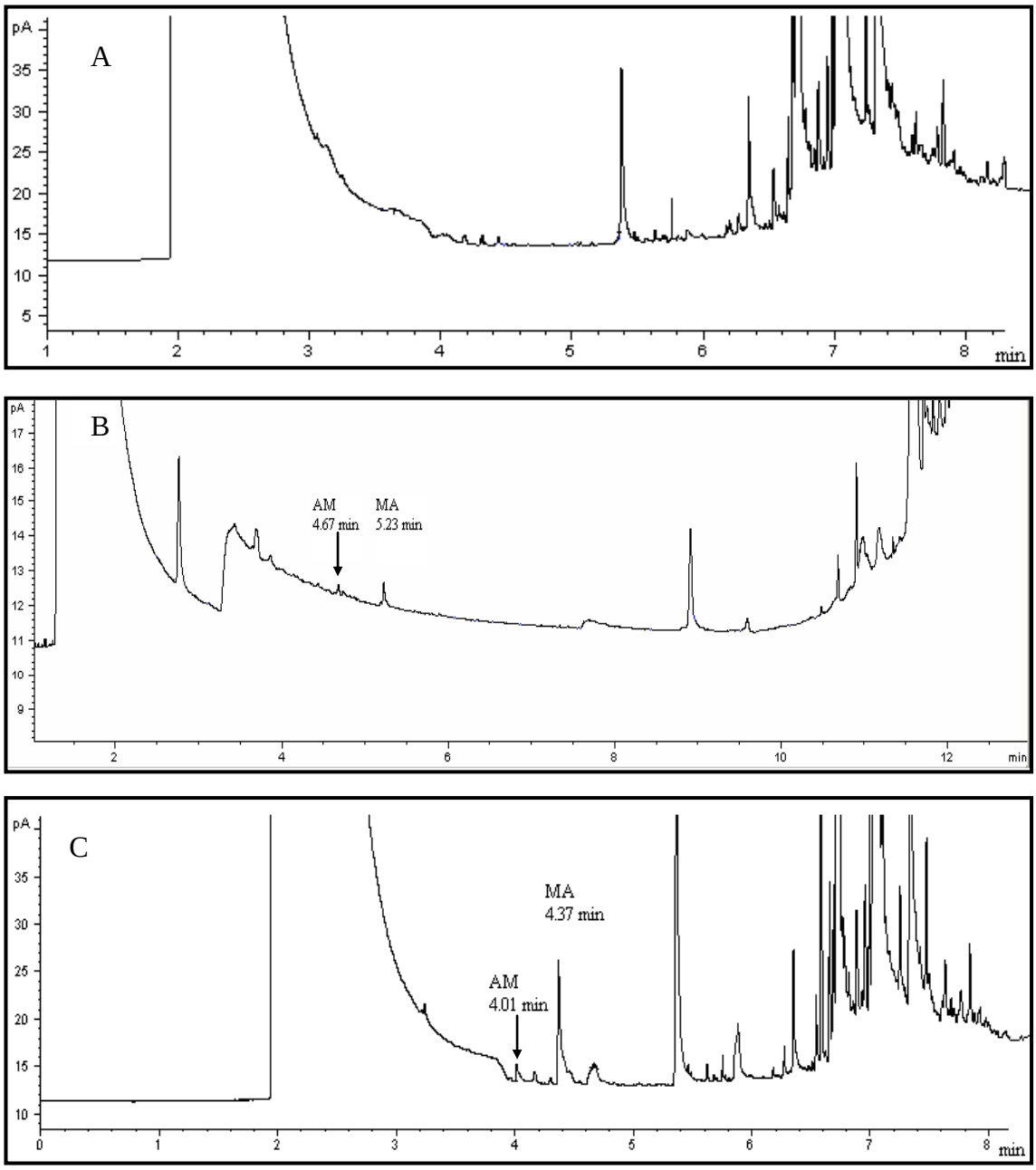


Figure 3.6 Representative chromatograms of liver blank (A), MA and metabolite AM in the liver of rat orally treated with MA 10 mg/kg of single dose (B) and in the liver of rat orally treated with MA 10 mg/kg of multiple doses (C) (five consecutive days) (retention time changed in each chromatogram due to a sample was detected different GC instrument).

Table 3.5 Analyte concentrations in the rat liver collected 2 h after oral administration of 10 mg/kg MA of both single and multiple doses

Dose 10 mg/kg	N	Measured concentration (µg/g of tissue)	
		AM	MA
single	1	ND	9.25[a]
	2	ND	10.125
multiple	1	9.375	86.75
	2	7.75[a]	44

Abbreviation: amphetamine (AM), methamphetamine (MA)

ND = not detectable

[a] data was less than lower limit of quantification (LOQ)

In this method, Intra- and inter-day precisions were found to be within the acceptable range, i.e. 15%RSD. The accuracy (%DEV) was of ±15%. The mean percentages of recovery were 86-114% for AM and 88-110 % for MA. The regression equations were y = (8.23 ± 0.69) x – (2.03 ± 0.95), r = 0.9996 for AM and y = (10.25 ± 0.80) x – (2.30 ± 0.79), r = 0.9997 for MA. The LOD and LOQ for both AM and MA were 6.25 µg/g of tissue and 9.375 µg/g of tissue, respectively. The concentration ranges of AM and MA in rat livers were 9.375 µg/g of tissue to 86.75 µg/g of tissue for both single and multiple dose administration.

MA concentration of one individual of multiple doses administration was detected higher value than two individual due to limit of perfusion that rat was not perfused in equivalent amount. Time period of one individual was perfused with 0.9% normal saline on a liver less than two individual. Therefore, MA concentration of one individual was measured combination for both blood and liver sample. AM in rat liver was not detected in single dose. This experiment, Ya-Ba tablets was given in rat by

oral administration which Ya-Ba tablets had low purities (1.92-9.05%). From previously report, MA tablet usually consists of MA-HCl (20–30%), caffeine (60–70%) and other substances. MA tablet has many kinds of impurities at high levels which caffeine was large impurity peaks. Additionally, Ya-Ba tablets seized in Thailand found nine compounds (1,2-Dimethyl-3-phenylaziridine, ephedrine, methylehedrine, *N*-formylmethamphetamine, *N*-acetylmethamphetamine, *N*-formylehedrine, *N*-acetylehedrine, *N,O*-diacetylephedrine, methamphetamine dimmer) as impurities with caffeine and ethyl vanillin as diluents (Puthaviriyakorn *et al.*, 2002; Kuwayama *et al.*, 2008). Ya-Ba tablets had low purities and bioavailability after oral administration was approximately 67%. Therefore, the level of MA in blood was low. In addition, the metabolite of MA was small amount also. Metabolism of MA in rats after given by an intraperitoneal injection (45 mg/kg) was reported. Unchange from MA (82%) was excreted in the urine within 3-4 days and 3% as metabolite AM (Caldwell *et al.*, 1972). So, the possibility of metabolites AM in rat liver was not detected should be occurred.

Comprehensive validation methods for measurement MA and its metabolite AM in rats using GC-NPD with derivatization were reported by Terada (1985), GC-MS with derivatization (Scheidweiler *et al.*, 2008) and LC-MS/MS (Hendrickson *et al.*, 2006). Scheidweiler and coworkers (2008) reported that concentration of MA and AM in cortex (20 mg/kg of MA; intraperitoneal injection) in tissues was 2.2 ng/mg and 0.5 ng/mg, respectively. Hendrickson and coworkers (2006) reported that the concentration ranges of MA and AM in brain and testis (5.6 mg/kg/day of MA; subcutaneous injection (14 consecutive days)) were 299-388 ng/g and 88-120 ng/g, respectively. These concentrations were provided lower value than this method. Mass spectrometry is more sensitive technique. However, mass spectrometry is not widely available technique of analysis biological sample in forensic science work. Validation methods for measurement MA and its metabolite AM in rats were also reported by GC-NPD with derivatization. Terada (1985) reported the concentrations of MA and metabolites AM in rat liver determined 2 h after intraperitoneal injection of MA 10 mg/kg, were 2.0 µg/g and 0.3 µg/g, respectively. From these concentrations showed that LOQ (10 ng/g) of GC-NPD were provided lower value than GC-FID (9.375 µg/g of tissue) in this result. Determination by derivatization provided more stable, less

polar, better separation, more symmetrical peaks and greater sensitivity. Possibility of sensitivity of previously report by without derivatization provided low sensitivity. However, derivatizing agents can contaminate the inlet or column.

In cases of death caused of MA, liver sample was measured GC-FID technique. Moriya and Hashimoto (1999[b]) reported that MA concentration of three autopsy cases in livers with derivatizations were 10.8, 8.20 and 1.37 µg/g for case 1 to 3, respectively. Moriya and Hashimoto (2002) reported that MA and AM concentration of autopsy cases in liver with derivatizations was 16.3 and 0.271 µg/g, respectively. From concentration was showed of some case, possibility of sensitivity in the present this method was sufficiently high for determining in liver samples of MA overdose by using GC-FID technique (LOQ of this method was 9.375 µg/g of tissue).

In addition, liver sample of autopsy cases were measured by also using GC-NPD with derivatization (Raikos *et al.*, 2002) and GC-MS with derivatization (Uemura *et al.*, 2003; Chaturvedi *et al.*, 2004; Hara *et al.*, 2009). Raikos and coworkers (2002) reported that MA and AM concentration of seven fatal cases in liver was 6.0 µg/g for case 7 and 0.2 µg/g for case 4. AM and MA were not detected in another cases. Uemura and coworkers (2003) reported that MA concentration of two fatal cases in livers were 1.59 µg/g for case 1 and 9.79 µg/g for case 2. Chaturvedi and coworkers (2002) reported that AM and MA concentrations in liver from the Pilot Fatality were 0.133 µg/g and 5.534 µg/g, respectively. Hara and coworkers (2009) reported that MA concentrations in liver from a cadaver in an autopsy case was 1.55 µg/g. MS with derivatizatiion is more sensitive technique than other technique but it is not widely available technique. Some concentration was reported from fatal case, sensitivity of this method was sufficiently high for determining MA in liver.

CHAPTER 4

Conclusions

GC-FID method is simple, precise, accurate and cost saving technique for simultaneously quantifying AM and MA in liver. Without derivertization, peaks of the analytes are symmetry. Additionally, separation is accomplished with a short run time and this method is appropriate for routine work which the samples are routinely sent for analysis. Liquid-liquid extraction of AM and MA from the liver provided a high percentage of recovery. This technique is simple and cost saving without derivatization which limit of derivatization is expensive derivatizing agent, budget of research is not sufficiency. This method may be adopted to a study of pharmacokinetics and forensic toxicology.

Investigations the cause of death at forensic autopsies, biological samples were collected for determination. Blood and urine specimens are usually taken to estimate MA levels of a victim. However, blood and urine cannot always be obtained at autopsies. Liver or other organ should be subjected to drug analysis. Sensitivity of this method was sufficiently high for determining amphetamine and methamphetamine in liver of fatal case from MA overdoses as data used to support the investigations the cause of death.

Future works are to modify this method to determine MA and AM in other tissues, for example, brain, kidney, muscle and lipid. In Forensic Science, the levels of these drugs in these biological samples are useful for investigation the cause of death also. This method should be develop to increase the sensitivity of detection for example by derivatization.

References

Adam, P., Natakankitkul, S., Sirithunyalug, J. and Aramrattana, A. 2005. Physico-chemical profiles of methamphetamine tablets. *Chiang mai University Journal* **4** (1): 65-70.

Anglin, M. D., Burke, C., Perrochet, B., Stamper, E. and Dawud-Noursi, S. 2000. History of the methamphetamine problem. *Journal of Psychoactive Drugs* **32**: 137-141.

Asatoor, A. M., Galman, B. R., Johnson, J. R. and Miline, M. D. 1965. The excretion of dexamphetamine and its derivatives. *British Journal Pharmacology* **24**: 293-300.

Barnhart, F. E., Fogacci, J. R. and Reed, D. W. 1999. Methamphetamine-a study of postmortem redistribution. *Journal of Analytical Toxicology* **23**: 69-70.

Basellt, R. C. 1982. Disposition of toxic drugs and chemicals in man. 2nd Edition USA. California.

Cadet, J. L., Jayanthi, S. and Deng, X. 2003. Speed kills: cellular and molecular bases of methamphetamine-induced nerve terminal degeneration and neuronal apoptosis. *FASEB Journal* **17**: 1775–1788.

Caldwell, J., Dring, L. G. and Williams, R. T. 1972. Metabolism of [IC] Methamphetamine in man, the guinea pig and the rat. *Biochemical Journal* **129**: 11-22.

Chaturvedi, A. K., Cardona, P. S., Soper, J. W. and Dennis V. Canfield. 2004. Distribution and optical purity of methamphetamine found in toxic concentration in a civil aviation accident pilot fatality. *Journal Forensic Science* **49**(4): 1-5.

Cruickshank, C. C. and Dyer, K. R. 2009. A review of the clinical pharmacology of methamphetamine. *Addiction* **104**: 1085–1099.

Currell, G. 2000. Analytical instrumentation performance characteristic and quality. England.

Derlet, R. W. and Heischober, B. 1990. Methamphetamine stimulant of the 1990s?. *The Western Journal of Medicine* **153**: 625-628.

Donaldson, M. and Goodchild, J. H. 2006. Oral health of the methamphetamine abuser. *American Journal Health-System Pharmacy* **63**: 2078-2082.

Drummer, O. H. 2004. Postmortem toxicology of drugs of abuse. *Forensic Science International* **142**: 101-113.

Fifield, F. W. and Kealey, D. 2000. Principle and practice analytical chemistry. University Press, Cambridge, United Kindom.

Funahashi, M., Kohda, H., Shikata, I. and Kimura, H. 1988. Potentiation of lethality and increase in body temperature by combined use of d-methamphetamine and morphine in mice. *Forensic Science International* **37** (1): 19-26.

Gentry, W. B., Ghafoorb, A. U., Wessingera, W. D., Laurenzanaa, E. M., Hendricksona, H. P. and Owensa, S. M. 2004. (+)-Methamphetamine-induced spontaneous behavior in rats depends on route of (+)METH administration. *Pharmacology, Biochemistry and Behavior* **79**: 751-760.

Guilarte, T. R. 2001. Is Methamphetamine Abuse a Risk Factor in Parkinsonism?. *Neuro Toxicology* **22**: 725-731.

Hara, K., Kashiwagi, M., Kageura, M., Matsusue, A. and Kubo, S. I. 2009. Solid-phase microextraction for amphetamines in solid tissues: washing the homogenates with ethyl ether enables their measurements by GC-MS after heptafluorobutyryl derivatization. *Forensic Toxicology* **27**: 52–53.

Hendrickson, H., Laurenzana, E. and Owens, S. M. 2006. Quantitative Determination of Total Methamphetamine and Active Metabolites in Rat Tissue by Liquid Chromatography With Tandem Mass Spectrometric Detection. *The AAPS Journal* **8** (4): E709-E717.

Hinshaw, J. V. 2003. Solid-Phase Microextraction. http://chromatographyonline.findanalytichem.com/lcgc/data/articlestandard//lcgceurope/462003/75775/article.pdf (accessed 13/08/10).

Hiruntoe, K. 2007. Trafficking in drugs: prevention and control strategies, strengthening of international cooperation in combating trafficking in drugs, *Illicit Drug Trafficking in Thailand*. The asean crime prevention conference, Jakarta: Inspector, Interpol Bangkok, Royal Thai Police.

http://www.wikimediafoundation.org/.Amphetamine (accessed 18/08/10).

Huestis, M. A., and Cone, E. J. 2007. Methamphetamine Disposition in Oral Fluid, Plasma, and Urine. *Annals of the New York Academy of Sciences* **1098**: 104–121.

Jager, L. d. and Andrews, A. R. J. 2002. Development of a screening method for cocaine and cocaine metabolites in saliva using hollow fiber membrane solvent microextraction. *Analytica Chimica Acta* **458** (2): 311-320.

Jayanthi, S., Deng, X., Ladenheim, B., McCoy, M. T., Cluster, A., Cai, N-S. and Cadet, J.L. 2005. Calcineurin/NFAT-induced up-regulation of the Fas ligand/Fas death pathway is involved in methamphetamine-induced neuronal apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* **102** (3): 868-873.

Jork, H., Funk, W., Fischer, W. and Wimmer, H. 1990. *Thin-layer chromatography*: reagents, and detection method. Federal Republic of Germany.

Kalasinsky, K. S., Bosya, T. Z., Schmunk, G. A., Reiber, G., Anthony, R. M., Furukawa, Y., Guttman, M. and Kish, S. J. 2001. Regional distribution of methamphetamine in autopsied brain of chronic human methamphetamine users. *Forensic Science International* **116**: 163-169.

Kanamori, T., Tsujikawa, K., Ohmae, Y., Iwata, Y. T., Inoue, H., Kishi, T., Nakahama, T. and Inouye Y. 2005. A study of the metabolism of methamphetamine and 4-bromo-2,5-dimethoxyphenethylamine (2C-B) in isolated rat hepatocytes. *Forensic Science International* **148**: 131-137.

Kenndler, E. 2004. Gas chromatography. http://anchem.univie.ac.at/fileadmin/user_upload/anchem/Gas chromatography_in capillaries.pdf. (accessed 13/10/09).

Klasser, G. D. and Epstein, J. 2005. Methamphetamine and its impact on dental care. *Journal of the Canadian Dental Association* **71**(10): 759-762.

Krasnova, I. N. and Cadet, J. L. 2009. Methamphetamine toxicity and messengers of death. *Brain research reviews* **60**: 379-407.

Kuwayama, K., Inoue, H., Kanamori, T., Tsujikawa, K., Miyaguchi, H., Iwata, Y. Miyauchi, S., Kamo, N. and Kishi, T. 2007. Interactions between 3,4-methylenedioxymethamphetamine, methamphetamine, ketamine, and caffeine in human intestinal Caco-2 cells and in oral administration to rats. *Forensic Science International* **170**: 183-188.

Kuwayamaa, K., Inouea, H., Kanamoria, T., Tsujikawaa, K., Miyaguchia, H., Iwataa, Y. T., Miyauchi, S. and Kamo, N. 2008. Analysis of amphetamine-type stimulants and their metabolites in plasma, urine and bile by liquid chromatography with a strong cation-exchange column-tandem mass spectrometry. *Journal of Chromatography B* **867**: 78-83.

Kuwayama, K., Inoue, H. Phorachata, J., Kongpatnitiroj, K. Puthaviriyakorn, V., Tsujikawa, K. Miyaguchi, H., Kanamori, T. Iwata, Y.T., Kamo, N. and Kishi, T. 2008. Comparison and classification of methamphetamine seized in Japan and Thailand using gas chromatography with liquid–liquid extraction and solid-phase microextraction. *Forensic Science International* **175**: 85-92.

Lee, E. K. and Wilmoth, B. 2006. Thin layer chromatoghraphy. http://www.enotes.com/forensic-science/thin-layer-chromatography (accessed 13/08/10).

Levine, B. 2003. Principles of Forensic Toxicology. United States of America.

Levisky, J. A., Bowerman, D. L., Jenkins, W. W., Johnson, D. G. and Karch, S. B. 2001. Drugs in postmortem adipose tissues: evidence of antemortem deposition. *Forensic Science International* **121** (3): 157-160.

Logan, B. K. 2002. Methamphetamine-effects on human performance and behavior *Forensic Science Review* **14** (133): 133-151.

Lotrakul, M. 2000. Patients with Amphetamine Psychosis Admitted at Somdet Chaopraya Hospital. *Journal of the Psychiatric Association of Thailand* **45** (1): 17-31.

Melega, W. P., Williams, A. E., Schmitz, D. A., Distefano, E. W. and Cho, A. K. 1995. Pharmacokinetic and Pharmacodynamic Analysis of the Actions of D-Amphetamine and D-Methamphetamine on the Dopamine Terminal. *The Journal of Pharmacology and Experimental Therapeutics* **274** (1): 90-96.

Milesi-Halle, A., Hendricksona, H. P., Laurenzanaa, E. M., Gentrya, W. B. and Owens, S. M. 2005. Sex- and dose-dependency in the pharmacokinetics and pharmacodynamics of (+)-methamphetamine and its metabolite (+)-amphetamine in rats. *Toxicology and Applied Pharmacology* **209**: 203-213.

Mitrevski, B. and Zdravkovski, Z. 2005. Rapid and simple method for direct determination of several amphetamines in seized tablets by GC-FID. *Forensic Science International* **152**: 199-203.

Moriya, F., and Hashimoto, Y. 1999[a]. Pericardial fluid as an alternative specimen to blood for postmortem toxicological analyses. *Legal Medicine* **1**: 86-94.

Moriya, F., and Hashimoto, Y. 1999[b]. Redistribution of basic Drugs into Cardiac Blood from Surrounding Tissues during Early-Stages Postmortem. *Journal Forensic Science* **44** (1): 10-16.

Moriya, F., and Hashimoto, Y. 2002. A case of fatal hemorrhage in the cerebral ventricles following intravenous use of methamphetamine. *Forensic Science International* **129**: 104–109.

Nagata, T., Kimura, K., Hara, K. and Kudo, K. 1990. Methamphetamine and amphetamine concentrations in postmortem rabbit tissues. *Forensic Science International* **48**: 39-47.

Nakagawa, N., Hishinuma, T., Nakamura, H., Yamazaki, T., Tsukamoto, H., Hiratsuka, M., Ido, T., Mizugaki, M., Terasaki, T. and Goto, J. 2003. Brain and heart specific alteration of methamphetamine (MAP) distribution in MAP-Sensitized Rat. *Biological & Pharmaceutical Bulletin* **26** (4): 506-509.

Narongchai, P., Narongchai, S. and Thampituk, S. 2007. The incidence of drug abuse in unnatural deaths in northern Thailand. *Journal of The Medical Association of Thailand* **90** (1): 137-142.

Neue, U. D. 1997. HPLC columns: theory, technology and pracetice. United States of America.

Nordahl, T. E., Salo, R. And Leamon, M. 2003. Neuropsychological Effects of Chronic Methamphetamine Use on Neurotransmitters and Cognition: A Review. *The Journal of Neuropsychiatry and Clinical Neurosciences* **15**: 317-325.

Office of the Narcotics Control Board (ONCB). 2007. Narcotics Act of B.E. 2522. http://www1.oncb.go.th/document/Narcotics%20Act%20B.E.2522%20p10-40.pdf (accessed 6/10/09).

O'Neil, M. J., Smith, A., Heckelman, P. E., Jr., J. R. O., Gallipeau, J. A. R., Mary Ann D, A. and Budavari, S. 2001. *The merck in dex* (Thirteenth ed.). Merck & CO., INC.: Whitehouse Station, NJ.

Oyler, J. M., Cone, E. J., Robert E. Joseph, J., Moolchan, E. T. and Huestis, M. A. 2002. Duration of detectable methamphetamine and amphetamine excretion in urine after controlled oral administration of methamphetamine to humans. *Clinical Chemistry* **48** (10): 1703-1714.

Palanuvej, C. and Issaravanich, S. 2007. The enantiomeric study of methamphetamine tablets in Thailand. *Journal Health Research* **21** (2): 109-112.

Phonchai, A. 2009. Determination of MDMA, MDA and MA in ecstasy tablets and urine samples of suspects seized in Songkhla province using GC-FID technique. M.Sc. Thesis, Department of Applied Science, University of Prince of Songkla, Thailand.

Prema, R. 2003. HPLC: High-Performance Liquid Chromatography. http://www.encyclopedia.com. (accessed 15/08/09).

Puthaviriyakorn, V., Siriviriyasomboon, N., Phorachata, J., Pan-ox, W., Sasaki, T. and Tanaka, K. 2002. Identification of impurities and statistical classification of methamphetamine tablets (Ya-Ba) seized in Thailand. *Forensic Sience International* **126**: 105-113.

Raikos, N., Tsoukali, H., Psaroulis, D., Vassiliadis, N., Tsoungas, M. and Njau, S. N. 2002. Amphetamine derivative related deaths in northern Greece. *Forensic Science International* **128**: 31-34.

Rasmussen, N. 2006. Making the First Anti-Depressant: Amphetamine in American Medicine,1929–1950. *Journal of the history of medicine and allied sciences* 61(3): 288-323.

Riviere, G. J., Gentry, W. B., and Owens, S. M. 2000. Disposition of methamphetamine and its metabolite amphetamine in brain and other tissues in rats after intravenous administration. *The Journal of Pharmacology and Experimental Therapeutics* **292** (3): 1042-1047.

Rood, D. 2007. The troubleshooting and maintenance guide for Gas Chromatographers. Weinheim.

Rothman, R. B., Baumann, M. H., Dersch, C. M., Romero, D. V., Rice, K. C., Carroll, F. I. and Partilla, J. S. 2001. Amphetamine-type central nervous system stimulants release norepinephrine more potently than they release dopamine and serotonin. *SYNAPSE* **39**: 32-41.

Salocks, C., and Kaley, K. B. 2001. Technical Support Document: Toxicology Clandestine Drug Labs: Methamphetamine (Vol. 1, pp. 1-11): Cal/EPA, Office of Environmental Health Hazard Assessment.

Sampoon, K. 2000. Personallity traits of methamphetamine abuser. M.Sc. Thesis, Department of Human Development, University of Mahidol, Thailand.

Sasaki, T. A. 2008. Drug Screening and Confirmation: LC-MS-MS emerges on the forensic science. http://www.laboratoryequipment.com/uploadedFiles/CT88_appliedbio.pdf (accessed 20/08/09).

Scheidweiler, K. B., Barne, A. J., and Huestis, M. A. 2008. A validated gas chromatographic–electron impact ionization mass spectrometric method for methamphetamine, methylenedioxymethamphetamine (MDMA), and metabolites in mouse plasma and brain. *Journal of Chromatography B* **876**: 266-276.

Schepers, R. J. F., Oyler, J. M., Joseph, R. E., Cone, E. J., Moolchan, E. T., and Huestis, M. A. 2003. Methamphetamine and amphetamine pharmacokinetics in oral fluid and plasma after controlled oral methamphetamine administration to human volunteers. *Clinical Chemistry* **49** (1): 121-132.

Scott, R. 2008. Thin-layer chromatography. http://www.chromatography-online.org/TLC/Introduction.html. (accessed 13/08/10).

Scott, R. P. W. 1998. Introduction to analytical gas chromatography. United Stated of America.

Sellers, K. and Rigdon, A. 2008. Accurate, reproducible amphetamines analysis: cean up procedure improves chromatography and reduces maintenance. *Clinical/Forensic* **1**: 22-23.

Shibamoto, T. 1994. Liquid chromatography analysis (Chromatographic science series: volume 65). United stated of America.

Shulgin, A. 2001. Acacias and Natural Amphetamine. www.cognitiveliberty.org/shulgin/adsarchive/acacia.htm (accessed 17/10/09).

Simpson, N. J. K. 2000. Solid-phase extraction: principle, technique, and application. United States of America.

SOFT / AAFS. Forensic Laboratory Guidelines. 2006. www.soft-tox.org/docs/Guidelines%202006%20Final.pdf. (accessed 13/08/10).

Sukkwan, J. 2006. Separation and detection of stereoisomers of methamphetamine and amphetamine in Forensic samples by GC/MS. M.Sc. Thesis, Department of Forensic Science, University of Mahidol, Thailand.

Sulzer, D., Sonders, M. S., Poulsen, N. W. and Galli, A. 2005. Mechanisms of neurotransmitter release by amphetamines: A review. *Progress in Neurobiology* **75**: 406-433.

Susawaengsup, C., Rayanakorn, M. and Wangkarn, S. 2005. Gas Chromatography-mass spectrometry of volatile components of some local fruits in northern Thailand. M.Sc. Thesis, Department of Chemistry, Faculty of Science, Chiang Mai University, Thailand.

Suthisrisung, J., and makmanee, R.1999. pharmacology1. Pharmacology department, Faculty of pharmacology, Mahidol university.

Tagliaro, F. and Smith, F. P. 1996. Forensic capillary electrophoresis. *trends in analytical chemistry* **75** (70): 513-525.

Terada, M. 1985. Determination of methamphetamine and its metabolite in rat tissues by Gas Chromatography with a nitrogen-phosphorus detector. *Journal of Chromatography* **318**: 307-318.

Thormann, W., Aebi, Y., Lanz, M., and slavska, J. 1998. Capillary electrophoresis in clinical toxicology. *Forensic Science International* 92: 57-183.

Tongroach, C., Samee, W. and Kamkaen, N. 2005. National Reference Standard of Methamphetamine. *Thai pharmaceutical and health science journal* **10** (1): 1-9.

Treerat, N., Wannathepsakul, N. and Lewis, D. R. 2000. Global Study on Illegal Drugs:The Case of Bangkok, Thailand (pp. 1-131).

Uemura, K., Sorimachi, Y., Yashiki, M. and Yoshida, K.I. 2003. Two fatal cases involving concurrent use of methamphetamine and morphine. *Journal Forensic Science* **48** (5): 1-3.

Wercinki, S. A. S. 1999. Solid phase microextraction: a practical guide. United States of America.

Yu, Q., Larsonb, D. F., and Watson, R. R. 2003. Heart disease, methamphetamine and AIDS. *Life Sciences* **73**: 129–140.

Znalezionaa, J., Petra, J., Maiera, V., Knoba, R., Horakova J., Smetanova D. and Sevcik J. 2007. Capillary electrophoresis as verification tool for immunochemical drug screening. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* **151** (1): 31-36.

.

Appendix-1

Method validation for analysis of amphetamine and methamphetamine in rat liver

Table A1 Raw data for determination of linearity for analyzing amphetamine and methamphetamine in liver

Concentration (µg/mL or µg/g of tissue)	N	t_R (min)	Peak area of amphetamine (P_A)				N	t_R (min)	Peak area of methamphetamine (P_A)			
			Individual	Mean	S.D.	%RSD			Individual	Mean	S.D.	%RSD
0.75 (9.375)	1	4.673	5.913	5.172	0.690	13.332	1	5.190	7.249	6.384	0.718	11.248
	2	4.672	4.979				2	5.189	6.199			
	3	4.674	4.495				3	5.190	5.514			
	4	4.673	5.889				4	5.189	6.981			
	5	4.674	4.586				5	5.189	5.976			
1(12.5)	1	4.671	7.818	6.230	0.932	14.964	1	5.189	9.714	8.074	0.981	12.148
	2	4.678	6.302				2	5.197	8.138			
	3	4.672	5.544				3	5.188	7.160			
	4	4.672	5.690				4	5.187	7.733			
	5	4.673	5.797				5	5.188	7.624			
2.5 (31.25)	1	4.668	20.591	17.906	1.521	8.495	1	5.186	26.458	22.591	2.164	9.580
	2	4.669	17.635				2	5.187	21.684			
	3	4.671	17.145				3	5.189	21.728			
	4	4.669	17.187				4	5.188	21.461			
	5	4.669	16.970				5	5.187	21.623			

Table A1 Raw data for determination of linearity for analysing amphetamine and methamphetamine in liver (cont.)

Concentration (µg/mL or µg/g of tissue)	N	t_R (min)	Peak area of amphetamine (P_A)				N	t_R (min)	Peak area of methamphetamine (P_A)			
			Individual	Mean	S.D.	%RSD			Individual	Mean	S.D.	%RSD
5 (62.5)	1	4.668	20.591	17.906	1.521	8.495	1	5.186	26.458	22.591	2.164	9.580
	2	4.669	17.635				2	5.187	21.684			
	3	4.671	17.145				3	5.189	21.728			
	4	4.669	17.187				4	5.188	21.461			
	5	4.669	16.970				5	5.187	21.623			
10 (125)	1	4.665	89.869	80.906	6.752	8.346	1	5.185	112.940	100.783	8.006	7.944
	2	4.667	71.032				2	5.187	90.517			
	3	4.666	80.127				3	5.186	100.139			
	4	4.666	80.634				4	5.185	99.162			
	5	4.666	82.868				5	5.186	101.157			

Table A2 Raw data for determination of limit of detection and limit of quantification for analyzing amphetamine in liver

Concentration (µg/mL) or (µg/g of tissue)	N	response of amphetamine						
		noise (N) of blank		mean	signal (S)		mean	mean of S/N ratio
		t_R (min)	peak area		t_R (min)	peak area		
0.5 (6.25)	1	4.664	0.452	0.372	4.678	3.725	3.416	9.183
	2	4.694	0.451		4.673	4.548		
	3	4.646	0.254		4.676	2.864		
	4	4.626	0.453		4.676	2.877		
	5	4.623	0.250		4.675	3.067		
0.75 (9.375)	1	4.664	0.452	0.372	4.673	5.913	5.172	13.903
	2	4.694	0.451		4.672	4.979		
	3	4.646	0.254		4.674	4.495		
	4	4.626	0.453		4.673	5.889		
	5	4.623	0.250		4.674	4.586		
1(12.5)	1	4.664	0.452	0.372	4.671	7.818	6.230	16.984
	2	4.694	0.451		4.678	6.302		
	3	4.646	0.254		4.672	5.544		
	4	4.626	0.453		4.672	5.690		
	5	4.623	0.250		4.673	5.797		

Table A3 Raw data for determination of limit of detection and limit of quantification for analyzing methamphetamine in liver

Concentration (μg/mL) or (μg/g of tissue)	N	response of amphetamine						
		noise (N) of blank		mean	signal (S)		mean	mean of S/N ratio
		t_R (min)	peak area		t_R (min)	peak area		
0.5 (6.25)	1	5.196	0.735	0.533	5.195	4.55	4.227	7.931
	2	5.160	0.457		5.189	6.162		
	3	5.199	0.514		5.190	3.438		
	4	5.182	0.390		5.189	3.426		
	5	5.223	0.570		5.190	3.557		
0.75 (9.375)	1	5.196	0.735	0.533	5.190	7.249	6.384	11.977
	2	5.160	0.457		5.189	6.199		
	3	5.199	0.514		5.190	5.514		
	4	5.182	0.390		5.189	6.981		
	5	5.223	0.570		5.189	5.976		
1(12.5)	1	5.196	0.735	0.533	5.189	9.714	8.074	15.148
	2	5.160	0.457		5.197	8.138		
	3	5.199	0.514		5.188	7.160		
	4	5.182	0.390		5.187	7.733		
	5	5.223	0.570		5.188	7.624		

Table A4 Raw data for determination of inter-day precision for analyzing amphetamine and methamphetamine in liver

Concentration (µg/mL or µg/g of tissue)	Day	N	t_R (min)	Peak area of amphetamine (P_A)				t_R (min)	Peak area of methamphetamine (P_A)			
				Individual	Mean	S.D.	%RSD		Individual	Mean	S.D.	%RSD
1.5 (18.75)	1	1	4.671	8.628	8.979	0.430	4.791	5.188	11.714	13.439	1.641	12.208
	2	2	4.686	9.558				5.203	12.761			
	3	3	4.687	8.501				5.202	15.511			
	4	4	4.686	9.209				5.203	14.827			
	5	5	4.671	8.997				5.189	12.384			
2.5 (31.25)	1	1	4.669	17.635	16.424	1.409	8.579	5.187	21.684	22.361	1.902	8.508
	2	2	4.683	15.119				5.202	21.400			
	3	3	4.684	14.687				5.201	21.246			
	4	4	4.683	17.535				5.202	25.745			
	5	5	4.671	17.145				5.189	21.728			
10 (125)	1	1	4.667	71.032	71.866	6.141	8.545	5.187	90.517	97.984	8.341	8.513
	2	2	4.681	75.23				5.201	110.719			
	3	3	4.682	63.986				5.203	98.026			
	4	4	4.684	68.953				5.204	90.52			
	5	5	4.666	80.127				5.186	100.139			

Table A5 Raw data for determination of inter-day accuracy for analyzing amphetamine and methamphetamine in liver

Concentration (µg/mL or µg/g of tissue)	Day	N	Measured concentration of amphetamine (µg/mL) (accuracy)					Measured concentration of methamphetamine (µg/mL) (accuracy)				
			t_R (min)	Individual	mean	S.D.	%DEV	t_R (min)	Individual	mean	S.D.	%DEV
1.5 (18.75)	1	1	4.671	1.233	1.278	0.055	-14.774	5.188	1.330	1.506	0.167	0.367
	2	2	4.686	1.353				5.203	1.436			
	3	3	4.687	1.217				5.202	1.717			
	4	4	4.686	1.308				5.203	1.647			
	5	5	4.671	1.281				5.189	1.398			
2.5 (31.25)	1	1	4.669	2.393	2.237	0.181	-10.513	5.187	2.346	2.415	0.194	-3.395
	2	2	4.683	2.069				5.202	2.317			
	3	3	4.684	2.013				5.201	2.301			
	4	4	4.683	2.380				5.202	2.760			
	5	5	4.671	2.330				5.189	2.351			
10 (125)	1	1	4.667	9.269	9.377	0.791	-6.235	5.187	9.364	10.126	0.850	1.258
	2	2	4.681	9.810				5.201	11.424			
	3	3	4.682	8.362				5.203	10.130			
	4	4	4.684	9.001				5.204	9.365			
	5	5	5.186	10.440				5.186	10.346			

Table A6 Raw data for determination of intra-day precision for analyzing amphetamine and methamphetamine in liver

Concentration (µg/mL or µg/g of tissue)	N	t_R (min)	Peak area of amphetamine (P_A)				t_R (min)	Peak area of methamphetamine (P_A)			
			Individual	Mean	S.D.	%RSD		Individual	Mean	S.D.	%RSD
1.5 (18.75)	1	4.670	10.819	9.092	0.998	10.973	5.188	14.288	12.359	1.161	9.390
	2	4.671	8.628				5.188	11.714			
	3	4.671	8.997				5.189	12.384			
	4	4.672	8.294				5.189	11.261			
	5	4.672	8.721				5.188	12.149			
2.5 (31.25)	1	4.668	20.591	17.906	1.521	8.495	5.186	26.458	22.591	2.164	9.580
	2	4.669	17.635				5.187	21.684			
	3	4.671	17.145				5.189	21.728			
	4	4.669	17.187				5.188	21.461			
	5	4.669	16.970				5.187	21.623			
10 (125)	1	4.665	89.869	80.906	6.752	8.346	5.185	112.940	100.783	8.006	7.944
	2	4.667	71.032				5.187	90.517			
	3	4.666	80.127				5.186	100.139			
	4	4.666	80.634				5.185	99.162			
	5	4.666	82.868				5.186	101.157			

Table A7 Raw data for determination of intra-day accuracy for analyzing amphetamine and methamphetamine in liver

Concentration (µg/mL or µg/g of tissue)	N	Measured concentration of amphetamine (µg/mL) (accuracy)					Measured concentration of methamphetamine (µg/mL) (accuracy)				
		t_R (min)	Individual	mean	S.D.	%DEV	t_R (min)	Individual	mean	S.D.	%DEV
1.5 (18.75)	1	4.670	1.515	1.293	0.869	-13.802	5.188	1.592	1.395	0.118	-6.976
	2	4.671	1.233				5.188	1.330			
	3	4.671	1.281				5.189	1.398			
	4	4.672	1.190				5.189	1.283			
	5	4.672	1.245				5.188	1.374			
2.5 (31.25)	1	4.668	2.774	2.428	0.196	-2.882	5.186	2.833	2.439	0.221	-2.456
	2	4.669	2.393				5.187	2.346			
	3	4.671	2.330				5.189	2.351			
	4	4.669	2.335				5.188	2.323			
	5	4.669	2.307				5.187	2.340			
10 (125)	1	4.665	11.695	10.541	0.128	5.407	5.185	11.651	10.411	0.816	4.112
	2	4.667	9.269				5.187	9.364			
	3	4.666	10.440				5.186	10.346			
	4	4.666	10.506				5.185	10.246			
	5	4.666	10.793				5.186	10.449			

Table A8 Raw data for determination of recovery for analyzing amphetamine in liver

Concentration (µg/mL or µg/g of tissue)	N	Direct injection		After extraction		Recovery		
		t_R (min)	peak area(PA)	t_R (min)	peak area(PA)	Individual	Mean	S.D.
1.25 (15.625)	1	4.680	6.350	4.672	7.478	117.76	114.07	7.90
	2	4.678	6.456	4.670	8.122	125.81		
	3	4.676	6.251	4.672	7.024	112.37		
	4	4.674	6.500	4.672	7.010	107.85		
	5	4.674	6.658	4.672	7.095	106.56		
2.5 (31.25)	1	4.667	18.249	4.668	20.591	112.83	98.46	9.62
	2	4.667	17.599	4.669	17.635	100.20		
	3	4.667	17.811	4.671	17.145	96.26		
	4	4.668	17.736	4.669	17.187	96.90		
	5	4.666	19.715	4.669	16.970	86.08		
10 (125)	1	4.663	92.492	4.665	89.869	97.16	86.25	7.08
	2	4.663	91.033	4.667	71.032	78.03		
	3	4.663	96.419	4.666	80.127	83.10		
	4	4.663	94.704	4.666	80.634	85.14		
	5	4.663	94.383	4.666	82.868	87.80		

Table A9 Raw data for determination of recovery for analyzing methamphetamine in liver

Concentration (µg/mL or µg/g of tissue)	N	Direct injection		After extraction		Recovery		
		t_R (min)	peak area (PA)	t_R (min)	peak area (PA)	Individual	Mean	S.D.
1.25 (15.625)	1	5.1950	8.641	5.190	10.298	119.18	110.22	7.13
	2	5.1940	8.741	5.188	10.199	116.68		
	3	5.1910	8.823	5.189	9.345	105.92		
	4	5.1910	9.001	5.188	9.351	103.89		
	5	5.1900	8.901	5.189	9.387	105.46		
2.5 (31.25)	1	5.185	22.438	5.186	26.458	117.92	100.37	10.30
	2	5.186	21.898	5.187	21.684	99.02		
	3	5.186	22.457	5.189	21.728	96.75		
	4	5.185	22.029	5.188	21.461	97.42		
	5	5.185	23.832	5.187	21.623	90.73		
10 (125)	1	5.182	113.406	5.185	112.940	99.59	88.17	6.75
	2	5.182	110.127	5.187	90.517	82.19		
	3	5.183	117.243	5.186	100.139	85.41		
	4	5.183	116.359	5.185	99.162	85.22		
	5	5.183	114.385	5.186	101.157	88.44		

Appendix-2

Determination of seized Ya-Ba tablets

Table A10 Raw data for MA calibration curve of determination of seized Ya-Ba tablets

Standard	concentration (µg/mL)	MA		IS		peak area ratio
		$t_{R\ (min)}$	peak area (PA)	$t_{R\ (min)}$	peak area (PA)	
MA	200	4.258	164160.2	9.183	7471.2	22
MA	100	4.222	104292.3	9.138	9332	11.17
MA	25	4.234	30280.2	9.135	13478.1	2.25
MA	6.25	4.259	5086.4	9.135	7997.5	0.64

Table A11 Raw data for determination of seized Ya-Ba tablets

Sample	MA		IS		peak area ratio
	$t_{R\ (min)}$	peak area (PA)	$t_{R\ (min)}$	peak area (PA)	
1	4.157	142638.7	8.970	31828.2	4.48
	4.172	196559.1	8.968	41479.7	4.74
	4.153	141361.5	8.968	37152.7	3.80
mean	4.161	160186.43	8.969	36820.20	4.34
2	4.224	80475.8	8.938	32746.4	2.46
	4.153	128387.7	8.936	26076.9	4.92
	4.147	150658	8.938	32644.9	4.62
mean	4.175	119840.50	8.937	30489.40	4.00
3	4.196	122655.5	8.938	37661.6	3.26
	4.189	101709.5	8.943	31649.5	3.21
	4.183	128659.9	8.944	35543.1	3.62
mean	4.189	117674.97	8.942	34951.40	3.36
4	4.168	70349.1	8.945	31387.8	2.24
	4.177	62970.4	8.945	33020.4	1.91
	4.197	56840.5	8.946	34205.5	1.66
mean	4.181	63386.67	8.945	32871.23	1.94

Table A11 Raw data for determination of seized Ya-Ba tablets (cont.)

Sample	MA		IS		peak area ratio
	$t_{R\ (min)}$	peak area (PA)	$t_{R\ (min)}$	peak area (PA)	
5	4.163	71346.1	8.947	27742.6	2.57
	4.219	70159.4	8.948	36268.1	1.93
	4.189	62165.4	8.95	31139.6	2.00
mean	4.190	67890.30	8.948	31716.77	2.17
6	4.191	134133.5	8.954	27156.2	4.94
	4.201	115837.1	8.957	27066.1	4.28
	4.182	137393.5	8.959	26098	5.26
mean	4.191	129121.37	8.957	26773.43	4.83
7	4.151	266323.3	8.960	36768.5	7.24
	4.156	260829	8.958	37641.6	6.93
	4.173	313702.2	8.955	40047.6	7.83
mean	4.160	280284.83	8.958	38152.57	7.34
8	4.164	281358.7	8.968	38822.3	7.25
	4.18	313344.1	8.97	45457.2	6.89
	4.179	332875.3	8.969	47635.5	6.99
mean	4.174	309192.70	8.969	43971.67	7.04

Table A11 Raw data for determination of seized Ya-Ba tablets (cont.)

Sample	MA		IS		peak area ratio
	$t_{R\ (min)}$	peak area (PA)	$t_{R\ (min)}$	peak area (PA)	
9	4.17	255283.2	8.968	41046.8	6.22
	4.148	221639.5	8.966	38649.9	5.73
	4.162	260014.6	8.971	40895.2	6.36
mean	4.160	245645.77	8.968	40197.30	6.10
10	4.16	318509.9	8.952	40232.6	7.92
	4.157	289590.4	8.957	38156.7	7.59
	4.153	240642.2	8.955	33583.4	7.17
mean	4.157	282914.17	8.955	37324.23	7.56
11	4.159	283597.1	8.954	42372.1	6.69
	4.176	310494.6	8.954	42324.6	7.34
	4.146	251499.5	8.955	38643.6	6.51
mean	4.160	281863.73	8.954	41113.43	6.85
12	4.171	243042.5	8.957	42943.7	5.66
	4.157	162217.7	8.958	35067.8	4.63
	4.180	153411	8.955	37909.2	4.05
mean	4.169	186223.73	8.957	38640.23	4.78

Table A11 Raw data for determination of seized Ya-Ba tablets (cont.)

Sample	MA		IS		peak area ratio
	$t_{R\ (min)}$	peak area (PA)	$t_{R\ (min)}$	peak area (PA)	
13	4.175	304731.6	8.955	38885.9	7.84
	4.148	261241.8	8.954	38178.4	6.84
	4.149	227138.5	8.953	38018.7	5.97
mean	4.157	264370.63	8.954	38361.00	6.88
14	4.152	164902.9	8.952	37709.1	4.37
	4.146	194814.1	8.951	37157.2	5.24
	4.146	196009.4	8.95	37597.3	5.21
mean	4.148	185242.13	8.951	37487.87	4.94
15	4.164	246372.6	8.949	39714.7	6.20
	4.156	236275.5	8.949	42968.2	5.50
	4.149	201932.4	8.948	40154.6	5.03
mean	4.156	228193.50	8.949	40945.83	5.58
16	4.146	146053.7	8.971	24756.3	5.90
	4.169	325444.9	8.968	44512.6	7.31
	4.146	225841	8.968	36312	6.22
mean	4.154	232446.53	8.969	35193.63	6.48

Table A11 Raw data for determination of seized Ya-Ba tablets (cont.)

Sample	MA		IS		peak area ratio
	$t_{R\ (min)}$	peak area (PA)	$t_{R\ (min)}$	peak area (PA)	
17	4.151	219223.2	8.969	35676.3	6.14
	4.149	209025.3	8.968	34444.9	6.07
	4.168	293236.5	8.967	42018.9	6.98
mean	4.156	240495.00	8.968	37380.03	6.40
18	4.163	319550.3	8.968	39207.2	8.15
	4.156	322055.7	8.968	39213.4	8.21
	4.144	259063.4	8.969	36054.2	7.19
mean	4.154	300223.13	8.968	38158.27	7.85
19	4.174	88923.1	8.968	38248.6	2.32
	4.163	53477.7	8.97	31401.4	1.70
	4.157	67723.7	8.97	33531.9	2.02
mean	4.165	70041.50	8.969	34393.97	2.02
20	4.148	217171.9	8.969	30768.9	7.06
	4.148	274264	8.969	35800.5	7.66
	4.150	252622.1	8.969	33948.7	7.44
mean	4.149	248019.33	8.969	33506.03	7.39

Table A11 Raw data for determination of seized Ya-Ba tablets (cont.)

Sample	MA		IS		peak area ratio
	$t_{R\ (min)}$	peak area (PA)	$t_{R\ (min)}$	peak area (PA)	
21	4.137	201089	8.970	36628.4	5.49
	4.137	179681.9	8.969	34441.4	5.22
	4.149	262333.7	8.969	40604	6.46
mean	4.141	214368.20	8.969	37224.60	5.72
22	4.150	176813.8	8.970	36462.5	4.85
	4.154	224789.4	8.971	37377.1	6.01
	4.143	151822.2	8.970	35877.6	4.23
mean	4.149	184475.13	8.970	36572.40	5.03

Appendix-3

Determination of methamphetamine and metabolite amphetamine in rat liver

Table A12 Raw data of experiment of in rats collected 2 h after oral administration of 10 mg/kg/5mL MA tablet of both single and multiple doses

Single dose

Date	Rats (weight (g))	Time of feeding (a.m.)	Time of tissues collection (a.m.)
16-03-53	1 (195)	8.36	10.36
16-03-53	2 (196)	9.36	11.36

Multiple dose

Rat / Day	Day 1 (16-03-53)		Day 2 (17-03-53)		Day3 (18-03-53)		Day4 (19-03-53)		Day5 (20-03-53)		
	Time of feeding (a.m.)	Weight (g)	Time of feeding (a.m.)	Weight (g)	Time of feeding (a.m.)	Weight (g)	Time of feeding (a.m.)	Weight (g)	Time of feeding (a.m.)	Weight (g)	Time of tissues collection (a.m.)
1	8.45	195	8.45	185	8.45	175	8.45	180	8.45	175	10.45
2	9.45	196	9.45	190	9.45	190	9.45	195	9.45	190	11.45

Table A13 Raw data for determination MA and AM spiked in liver homogenates for demonstration in real sample

Concentration (µg/mL or µg/g of tissue)	N	Peak area of AM (P_A)		Peak area of MA (P_A)	
		t_R (min)	Individual	t_R (min)	Individual
1 (12.5)	1	4.672	5.54	5.188	7.16
1 (12.5)	2	4.672	5.7	5.187	7.73
2.0 (25)	1	3.507	12.12	3.813	16.72
2.5 (31.25)	2	4.000	11.52	4.358	11.8

Table A14 Raw data analyte concentrations in the rat liver collected 2 h after oral administration of 10 mg/kg MA of both single and multiple doses

Dose 10 mg/kg	N	Peak area of AM (P_A)		Peak area of MA (P_A)		Measured concentration (µg/g of tissue)	
		t_R (min)	Individual	t_R (min)	Individual	AM	MA
single	1	4.671	ND	5.221	5.32	ND	9.25[a]
	2	4.675	ND	5.228	6.21	ND	10.125
multiple	1	3.51	4.54	3.819	58	9.375	86.75
	2	4.014	2.85	4.37	16.62	7.75[a]	44

Abbreviation: amphetamine (AM), methamphetamine (MA)

ND = not detectable

[a] lower than lower limit of quantification (LOQ)

Appendix-4

PRINCE OF SONGKLA UNIVERSITY
15 Karnjanawanij Road, Hat Yai, Songkhla 90110, Thailand
Tel (66-74) 286957-8 Fax (66-74) 212839
Website : www.psu.ac.th

MOE 0521.11/978 Ref. 25/51

September 10, 2008

This is to certify that the research project entitled "Bioaccumulation of Methamphetamine and its Metabolite Amphetamine in Rat Tissues after Single and Repeated Dose(s)." which was conducted by Dr. Sataporn Prutipanlai, Faculty of Sciences, Prince of Songkla University, has been approved by The Animal Ethic Committee, Prince of Songkla University.

K. Sawa.

Kitja Sawangjaroen, Ph.D.
Chairman,
The Animal Ethic Committee, Prince of Songkla University

VITAE

Name Miss Wijitra Kaewnam

Student ID 4910220009

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Biology)	Prince of Songkla University	2006

List of Publication and Proceedings

Poster presentation

Kaewnam, W., Prutipanlai, S., Janchawee, B. and Thainchaiwattana, S. 2010. Method Validation for Simultaneous Determination of Methamphetamine and Its Metabolite Amphetamine in Rat Liver Using GC-FID. Proceeding of the 32th Chemotherapy 2010: Discovery and Development, Bangkok, Thailand, 25-26 March 2010.