ความสัมพันธ์ระหว่างความเข้มข้นของเมทแอมเฟตามีนในปัสสาวะ เลือด และน้ำลูกตาภายหลังเสียชีวิต

นางสาวรุ่งทิพย์ นราปัญญากุล

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THE RELATIONSHIP BETWEEN POSTMORTEM METHAMPHETAMINE CONCENTRATIONS IN URINE, BLOOD AND VITREOUS HUMOR

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A Thesis Submitted in Partial Fulfillment of the Requirements

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เมทแอมเฟตามีนเป็นยาเสพติดที่แพร่ระบาดที่สุดในประเทศไทย ปัสสาวะเป็นตัวอย่างที่ใช้ในการตรวจวิเคราะห์เมทแอมเฟตามีนเพื่อการแปลตามกฎหมายยาเสพติดของประเทศไทย ในขณะที่เลือดเป็นตัวอย่างที่ใช้ในการตรวจวิเคราะห์เพื่อการแปลผลความเป็นพิษจากเมทแอมเฟตามีน อย่างไรก็ตาม ในบางครั้งที่เก็บตัวอย่างปัสสาวะหรือเลือดไม่ได้ หรือมีการปนเปื้อนในตัวอย่างดังกล่าว น้ำลูกตาเป็นชีววัตถุที่มีการปนเปื้อนน้อยกว่าและสามารถวิเคราะห์ได้ง่าย การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาความสัมพันธ์ระหว่างความเข้มข้นของเมทแอมเฟตามีนในปัสสาวะ เลือด และน้ำลูกตา โดยเก็บตัวอย่างปัสสาวะ ตัวอย่างเลือด และตัวอย่างน้ำลูกตาจากศพผู้เสียชีวิตชาวไทย 40 ราย วิเคราะห์ความเข้มข้นของเมทแอมเฟตามีนในตัวอย่างทั้งสามด้วยเทคนิคเฮดสเปซโซลิดเฟสไมโครเอ็กซ์แทรคชัน/แกสโครมาโทกราฟี-แมสสเปกโทรเมตรี ผลการศึกษาแสดงให้เห็นว่าความสัมพันธ์ระหว่างความเข้มข้นของเมทแอมเฟตามีนในปัสสาวะและเลือด ปัสสาวะและน้ำลูกตา และน้ำลูกตาและเลือด เท่ากับ 0.89, 0.99 และ 0.88 ตามลำดับ สมการถดถอยเชิงเส้นแสดงความสัมพันธ์ของความเข้มข้นของเมทแอมเฟตามีนในปัสสาวะและเลือด ปัสสาวะและน้ำลูกตา และน้ำลูกตาและเลือด คือ $y = 0.001x + 8.08$, $y = 0.056x - 262.86$ และ $y = 0.027x + 16.20$ ตามลำดับ การศึกษานี้ยังแสดงให้เห็นว่า น้ำลูกตาเป็นชีววัตถุทางเลือกหนึ่งสำหรับการตรวจวิเคราะห์ความเข้มข้นของเมทแอมเฟตามีนได้

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สาขาวิชา เภสัชวิทยา ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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KEYWORDS : METHAMPHETAMINE / URINE / BLOOD / VITREOUS HUMOR

RUNGTIP NARAPANYAKUL : THE RELATIONSHIP BETWEEN POSTMORTEM METHAMPHETAMINE CONCENTRATIONS IN URINE, BLOOD AND VITREOUS HUMOR. ADVISOR : ASSOC. PROF. POL. LT. COL.SOMSONG LAWANPRASERT, Ph.D., CO-ADVISOR : POL. LT. COL.WICHIAN TUNGTANANUWAT, 67 pp.

Methamphetamine (MA) is the most widespread narcotic in Thailand. Urine samples are used as evidences of MA abuse under the Narcotics Law of Thailand, whereas blood samples are specimens for investigation of MA poisoning. However, both specimens are occasionally not available or contaminated. Vitreous humor is a specimen which is less contaminated and easy to work with analytically. The aim of this study is to examine the relationships between MA concentrations in urine, blood and vitreous humor. Those three specimens were collected from 40 Thai deceased and their MA concentrations were quantitatively analyzed by headspace-solid phase microextraction/gas chromatography-mass spectrometry technique. The results showed that the relationships between MA concentrations in urine *vs* blood, urine *vs* vitreous humor, and vitreous humor *vs* blood were linearly correlated with a correlation coefficient (r) of 0.89, 0.99, and 0.88, respectively. Linear regression equations of those relationships between urine *vs* blood, urine *vs* vitreous humor, and vitreous humor *vs* blood were $y = 0.001x + 8.08$, $y = 0.056x - 262.86$, and $y = 0.027x + 16.20$, respectively. This study suggests vitreous humor as an alternative specimen for MA investigation.

Department : Pharmacology and Physiology Student's Signature .....

Field of Study : Pharmacology Advisor's Signature .....

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	= percent
°C	= degree Celcius
μg/l	= microgram per liter
μg/ml	= microgram per milliliter
μl	= microliter
4-MTA	= 4-methylthioamphetamine
AA	= African Americans
ADHD	= attention deficit hyperactivity disorder
AM	= amphetamine
AUC	= area under the curve
B.E.	= Buddist Era
C	= Caucasians
CB	= central blood
CSA	= Controlled Substances Act
CYP 2D6	= Cytochrome P450 2D6
e.g.	= for example, for instance
et al.	= et alii (and others)
etc.	= etcetera (and so forth, and so on)
eV	= electron volt
g	= gram
GC-FID	= gas chromatography-flame ionization detection
GC-NPD	= gas chromatography-nitrogen phosphorous detection
GC-MS	= gas chromatography-mass spectrometry
g/kg	= gram per kilogram
HPLC	= high performance liquid chromatography
HS-SPME	= head space-solid phase microextraction (HS-SPME)
ICH	= The International Conference on Harmonization
IV	= intravenous

LC-MS	= liquid chromatography-mass spectrometry
LC-MS-MS	= liquid chromatography-mass spectrometry-mass spectrometry
LLOQ	= lower limit of quantification
LOD	= limit of detection
LOQ	= limit of quantification
mg	= milligram
ml	= milliliter
mg/kg	= milligram per kilogram
MA	= methamphetamine
MCA	= Methamphetamine Control Act
MDA	= 3,4-methylenedioxyamphetamine
MDEA	= 3,4-methylenedioxyethylamphetamine
MDMA	= 3,4-methylenedioxymethamphetamine
mOsm/kg	= milliosmole per kilogram
NA	= not available
NaCl	= sodium chloride
NaOH	= sodium hydroxide
ND	= not detectable
ng/g	= nanogram per gram
ng/ml	= nanogram per milliliter
PB	= peripheral blood
PET	= Positron Emission Tomography
PO	= per oral, orally
rpm	= revolutions per minute
SAMHSA	= Substance Abuse and Mental Health Services Administration
SOFT/AAFS	= The Society of Forensic Toxicologists/The Toxicology Section of the American Academy of Forensic Sciences
S.D.	= standard deviation
S.E.M.	= standard error of mean

SPME	= solid phase microextraction
vs	= versus
U.S. FDA	= The United States Food and Drug Administration

CHAPTER I

INTRODUCTION

Methamphetamine (MA) is an amphetamine-type central nervous system stimulant. Even though MA is the active compound of medication prescribed for narcolepsy, attention deficit hyperactivity disorder (ADHD) and appetite suppression, it is commonly used as illicit drug. In the United States, MA is classified as a Schedule II controlled substance under the Controlled Substances Act (CSA) 1970 which has a high potential for abuse and its abuse may lead to severe psychological or physical dependence. Substances or chemicals used for the production of MA are also controlled under the Comprehensive Methamphetamine Control Act (MCA) 1996 (Franco, 2007). In Thailand, MA is classified as a narcotic of category I according to the Narcotics Act B.E. 2522. Its widespread and addictive uses are currently a national issue needed to be urgently solved. Drug testing in biological samples is used as a deterrent to illicit drug uses and as an information for forensic or clinical purposes. The cutoff concentrations in urines mandated by Federal Drug Testing Programs are 1000 ng/ml of amphetamines using immunoassay and 500 ng/ml of amphetamine or MA using GC-MS (Substance Abuse and Mental Health Services Administration, SAMHSA, 2004). In Thailand, any persons with urine MA concentrations of $\geq$ 1000 ng/ml is accused as illegal MA consumption according to the Notification of Narcotics Control Board announced in B.E. 2543 under the Narcotic Act B.E. 2522.

While MA concentrations in urine samples are used as evidences of MA abuser under the law, its concentrations in urines are not related to its effects. MA concentrations in blood samples represent MA physiological effects. Thus, blood samples are also collected for forensic investigation if drugs or illicit substances are suspected to be a cause of death. MA use or toxicity is implicated as either a direct/an antecedent cause of death or even a significant contributing factor. However, the study of MA-related fatalities of Thai MA abusers is limited (Sribanditmongkol, Chokjamsai, and Thampitak, 2000; Narongchai, Narongchai, and Thampituk, 2007). Most studies

were performed based on small case reports and the relationship between urine and blood MA concentrations were not shown in those previous studies. Thus, the first objective of this study was to investigate the relationship between MA concentrations in urine and blood samples of Thai MA abusers and the correlation equation was constructed so as for predicting MA concentration in blood from urine samples or vice versa.

Vitreous humor is one of the specimens used in forensic toxicology. Its value for postmortem analysis has been reported for many compounds such as alcohol (Mackey-Bojack, Kloss, and Apple, 2000); opiates: morphine, heroin, methadone (Sturner and Garriott, 1975; Ziminski et al., 1984; Bermeo et al., 1992; Bogusz, 1994; Pragst et al., 1995; Bogusz, Maier, and Driessen, 1997; Gerostamoulos and Drummer, 1997; Lin et al., 1997; Wyman and Bultman, 2004; Jennings, 2005); cocaine and its metabolites (Sturner and Garriott, 1975; Poklis, Mackell, and Graham, 1985; Logan and Stafford, 1990; Mackey-Bojack et al., 2000; Chronister, Walrath, and Goldberger, 2001; Furnari et al., 2002); cannabinoids (Lin and Lin, 2005); as well as amphetamines or hallucinogenic amines (Crifasi and Long, 1996; Decaestecker et al., 2001; De Letter et al., 2002; 2004). Amphetamine-type stimulants that had been determined in vitreous humor in previous studies were mostly 3,4-methylenedioxymethamphetamine (MDMA; Ecstasy), 3,4-methylenedioxyamphetamine (MDA; love pill) using gas chromatography-mass spectrometry (GC-MS) (Crifasi and Long, 1996), high performance liquid chromatography (HPLC) (Clauwaert et al., 2000; De Letter et al., 2000; 2002; 2004) and liquid chromatography-mass spectrometry-mass spectrometry (LC-MS-MS) (Decaestecker et al., 2001). Regarding MA, there is a study investigating MA in blood and vitreous humor of 18 deceased using GC-MS following liquid-liquid extraction. They found that the ratio of MA concentrations in vitreous humor to peripheral blood was shown by the mean ± S.D. of 1.63 ± 0.75 (McIntyre, Hamm, and Bader, 2011).

Vitreous humor possesses a number of advantages to analysis of drugs for forensic purpose. It is easily collected even though autopsy is not completely

performed. Vitreous humor is clear and contains 99% of water. Besides collagen, hyaluronic acid and other non-collagenous proteins, vitreous humor comprises several substances comparable to the serum such as sodium ion, chloride ion, calcium ion, glucose, urea and creatinine, etc. This specimen is easy to be used in the analytical procedure without complicated sample preparation. The outstanding property of vitreous humor over blood, urine and other tissue specimens is an anatomically isolated location of this specimen resulting in more protection from putrefaction, charring and trauma (Levine and Jufer, 2008). These advantages of vitreous humor as well as the previous report of distribution of MA into vitreous humor (McIntyre et al., 2011), indicate that vitreous humor may be a useful alternative specimen, while blood, urine or other specimens are not satisfactory or not available. Thus, the second aim of this study was to investigate the relationship between MA concentrations in vitreous humor and blood or urine samples of Thai abusers and the correlation equations were constructed for the prediction of MA concentrations in blood or urine from vitreous humor. The findings of this study may contribute beneficial information of alternative specimens for postmortem MA investigation in the cases that either urine or blood samples is not suitable or not available.

Hypothesis

There are relationships among MA concentrations in urine, blood and vitreous humor samples.

Objectives

To investigate the relationships of MA concentrations among three specimens: urine and blood, urine and vitreous humor, as well as blood and vitreous humor.

Benefit gained from the study

This study will provide an information of alternative specimens for postmortem MA investigation in the cases that either urine or blood is not satisfactory or not available.

CHAPTER II

LITERATURE REVIEWS

Methamphetamine (MA) or R,S-N-methyl-1-phenyl-propanamine is an amphetamine compound drug with the structure of secondary amine (Figure 1). Its molecular formula and weight are $C_{10}H_{15}N$ and 149.2, respectively. MA is stipulated in the Narcotics Act B.E. 2522 as a Schedule I narcotic drug. Besides oral form, MA are also found in various forms, e.g. smoked, snorted, and injected forms (Kim et al., 2004). Its smokable form is called 'Crank, Crystal, Crystal meth, Ice, meth, Speed', etc.

MA is rapidly absorbed both in gastrointestinal and respiratory tract. After penetrating into bloodstream, it distributes to other parts of the body. Due to its highly lipid solubility, it can penetrate into the brain. Also, it can penetrate into the heart, lungs, kidneys, and liver (Volkow et al., 2010).

MA is mainly metabolized in the liver via 3 reactions: (1) *N*-Demethylation to yield amphetamine, an active metabolite, (2) aromatic hydroxylation to yield 4-hydroxymethamphetamine, and (3) β-hydroxylation to yield norephedrine (Caldwell, Dring, and Williams, 1972; Lin et al., 1997; Cruickshank and Dyer, 2009). *N*-Demethylation and aromatic hydroxylation are major reactions occurred in human, whereas a minor reaction is deamination (Caldwell et al., 1972; Musshoff, 2000; Kraemer and Maurer, 2002; Kim et al., 2004). Cytochrome P450 2D6 (CYP 2D6) significantly plays a role in MA metabolism by participating in aromatic hydroxylation and *N*-Demethylation (Kraemer and Maurer, 2002; Cruickshank and Dyer, 2009). Thus, CYP 2D6 polymorphism may be one of the factors contributing to the metabolic variability of each individual (Lin et al., 1997; Cruickshank and Dyer, 2009).

MA is mainly excreted by urination with approximately 70% of the dose is excreted in urine within 24 hours after administration. In normal condition, it is excreted 40–50% as an unchanged form, up to 15% as 4-hydroxymethamphetamine and 4-7% as amphetamine within 24 hours (Baselt, 1978; Moore, 2003; Kim et al., 2004) (Figure 1).

However, urinary pH may interfere renal MA excretion. Its excretion may be enhanced with acidified urine (up to 76% of administered dose excreted as unchanged form, and 7% as amphetamine) whereas its excretion may be decreased with alkaline urine (up to 2% of administered dose excreted as unchanged and 0.1% as amphetamine) (Musshoff, 2000; Kim et al., 2004). Repeated dose may increase its half-life to 25 hours (Cruickshank and Dyer, 2009). Pharmacokinetics and pharmacodynamics profiles of MA are presented in Table 1.

Not only increasing neurotransmitter such as dopamine, serotonin, and norepinephrine levels in the brain, MA also behaves like α- and β-adrenergic agonists which induce both central and peripheral sympathetic nervous systems (Cruickshank and Dyer, 2009). It is believed that this circumstance can lead to organ damage, e.g. cerebral stroke, psychosis, myocardial infarction, cardiac arrhythmia, hypertension, pulmonary edema, and acute renal failure, etc. (Volkow et al., 2010).

There are some studies investigated the disposition or distribution of MA in healthy volunteers and the deceased (Kojima et al., 1984; Cook et al., 1992; 1993; Oyler et al., 2002; Harris et al., 2003; Moore et al., 2003; Schepers et al., 2003; Verstraete et al., 2004; Mendelson et al., 2006; Cruickshank and Dyer, 2009; Volkow et al., 2010; McIntyre et al., 2011). The findings of these studies revealed that MA can distribute to various organs or tissues with different MA concentrations. Cruickshank and Dyer (2009) examined the distribution of MA in various matrices by reviewing the relevant literature through a PubMed search. The summarized data are shown in Table 2.

Volkow et al. (2010) reviewed the distribution of MA in human body using Positron Emission Tomography (PET) in conjunction with [^{11}C]d-methamphetamine technique. Peak uptake (% dose/ml), rate of clearance (time to reach 50% peak-clearance) and accumulation (area under the curve; AUC) was assessed in healthy participants (9 Caucasians and 10 African Americans). The results showed that MA can

distribute throughout the whole body, but its uptake amount and rate differs among organs as presented in Figure 2 and Table 3.

According to the Volkow (2010) study, the highest [^{11}C]d-methamphetamine uptake and peak concentration (% dose/ml) occurred in kidneys and lungs, lowest in heart and brain. Uptake of MA was fastest in lung and heart (55–60 seconds), slowest in stomach and liver (30 minutes) and its clearance (half-peak clearance) was fastest in heart and lungs (7–16 minutes), slowest in brain, liver and stomach (>75 minutes). Furthermore, lung uptake (% dose per ml/tissue) and accumulation (AUC) of [^{11}C]d-methamphetamine was higher for African Americans than Caucasians but did not differ in other organs. The researchers discussed that high MA accumulation in kidneys could reflect its high urine excretion, which is likely to reflect both its active secretion by renal tubule cells as well as its partition into acidic urine. It is estimated that 37–45% of an intravenous (IV) or smoked MA dose is excreted in the urine as the parent form and 6–7% as AM within 3 days of dosing (most of the excretion occurring within the first 20 hours). Their assumption is supported with the evidence which showed that 10% of the injected [^{11}C]d-methamphetamine dose was present in urine 90 minutes after injection.

Determination of MA in biological specimens

Since MA is world-wide used illegally, determination of MA in biological specimens is required for forensic purpose as well as an objective means to document drug abuse among patients, job applicants, athletes, students, etc. (Kwong, 2008). MA investigation consists of two tests: screening and confirmation tests. The screening test is primarily based on an immunoassay. If a positive result presents in this step, the confirmation test, chromatographic technique, is required (Broussard, 2008; Kwong, 2008). Various techniques are used to determine MA concentration in various specimens including urine, blood, serum, bile, hair, liver, saliva, sweat, and vitreous humor (Suzuki, Hattori, and Asano, 1984; Suzuki et al., 1989; Moriya, Miyaishi, and Ishizu, 1992; Cairns, Kippenberger, and Gordon, 1996; Nakashima et al., 2000; McIntyre

et al., 2011). Those techniques are HPLC, LC-MS, GC-MS, GC-NPD, GC-FID. In this present study, GC-MS was used. Lists of studies determined MA concentrations in various specimens are summarized in Table 4.

In addition, other specimens such as bile (Moore et al.,1996), gastric contents (Moore et al., 1996), brain (Kojima et al., 1984; Moore, et al., 1996), nail (Suzuki et al., 1984; 1989; Moriya et al., 1992) and kidneys (Kojima et al., 1984) were also determined for MA concentrations.

GC-MS (Figure 3) is a gold standard analytical method for forensic science investigation (Broussard, 2008). Analyte in gaseous form moves through the GC column for separating before entering to the mass spectrometer. Within this detector, analyte molecule is induced to ionize at ion source area under vacuum and then is bombarded with an electron beam. After losing its electron, positive molecular ion obtains high energy (70 eV) electron to ionize. Each substance or analyte has its unique fragmentation pattern or mass spectrum that can be used to identify the substance (Kwong, 2008).

Head space-solid phase microextraction (HS-SPME) is a sample extraction technique for volatile substances or analytes which are easy to be vaporized (Figure 4). Compared to other techniques, e.g. liquid-liquid extraction this technique is a solventless technique. Therefore, contamination in a sample preparation step and harmful effect to analyst's health or to environment would less occur. It is also beneficial to column lifetime because SPME fiber absorbs only small vaporized molecule before injection to column. Therefore, there is less chance of water coming to the column and damaging it. Furthermore, this technique is suitable for analyzing a substance which is complicated or causes difficulties in sample preparation. Its separation mechanism is heating a well-closed glass container in which a sample is filled. When achieving an equilibrium, an analyte is both in gaseous form at headspace phase and in liquid form at sample phase. The fiber absorbs analyte's vapor at headspace phase and then desorbs

it into the GC column for separating further by chromatography, e.g. gas chromatography (Plutowska and Wardencki, 2007) (Figure 4).

Vitreous humor (Levine and Jufer, 2008)

Vitreous humor is fluid inside the eyes (Figure 5). It is a clear fluid which mainly composes of water (99%) and weighs approximately 4 g. Its specific gravity is 1.0050–1.0089 with the viscosity of approximately two times of water. The pH of it is 7.5 and the osmolality of it is higher than that of serum, ranges from 288 to 323 mOsm/kg.

Many substances and ion concentrations in vitreous humor at death, e.g. sodium, chloride, calcium, glucose, urea, and creatinine are close to those in serum. Therefore, those concentrations in vitreous humor provide as indicators for those of serum at death.

There is blood-vitreous barrier at the junction of blood vessel circulation supply and eyeball. Because of forming from vascular endothelium and its basement membrane, stroma, and ciliary, an equilibrium between blood and vitreous is slower than that of blood and other extracellular fluids. The movement of molecules across the vitreous may be a number of mechanisms. For example, diffusion, hydrostatic pressure, osmotic pressure, convection, active transport. Diffusion is a main mechanism of small molecules and only free or low protein bound drugs can diffuse across this area. Therefore, not highly protein bound drugs are able to be detected in vitreous humor.

The studies using vitreous humor to determine amphetamine-type stimulants are as following: De Letter et al. (2000) investigated MDMA in vitreous humor of rabbits using HPLC-fluorescence detection. Clauwaert et al. (2000) examined 3,4-methylenedioxyethylamphetamine (MDEA), MDMA, and MDA in blood, serum, vitreous humor, and urine samples of rabbits with the same technique as the De Letter et al. (2000) study. Regarding the studies in humans, most of them are case reports. Crifasi and Long (1996) studied MDMA related traffic fatality in a 29-year-old white male with the technique of GC-MS. They found MDMA and MDA in urine, blood, vitreous humor of

their subject. Decaestecker et al. (2001) examined MDMA in urine sample, vitreous humor, bile, liver, spleen, frontal lobe, and blood samples collected from femoral and subclavian veins of the deceased whose overdose fatality involving 4-methylthioamphetamine (4-MTA, Flatliner, serotonin releaser) and MDMA using LC-MS-MS. De Letter et al. (2002) investigated MDMA and MDA distribution in a fatal overdose using HPLC-Fluorescence detection. Many specimens, for example, urine, vitreous humor, bile, stomach content, cardiac muscle, lungs, kidneys, spleen, iliopsoas muscle, abdominal adipose tissue, serum, brain, and blood samples were collected to measured MDMA and MDA levels. In that study, blood samples and brain tissues were collected from different sites. They found that various sites of blood sampling influenced the concentrations of MDMA and MDA. De Letter et al. (2004) measured MDMA and MDA concentrations in cardiac muscle, lungs, liver, kidneys, brain lobes, stomach content, peripheral (femoral and subclavian) and cardiac blood samples, vitreous humor, and iliopsoas muscle of two MDMA-related fatalities using the same technique as Clauwaert et al. (2000) and De Letter et al. (2002). The results showed that MDMA and MDA concentrations detected in cardiac blood samples were higher than those of peripheral blood samples. Furthermore, the researchers suggested that vitreous humor was an alternative sample for toxicological analysis when a suitable blood sample is not available.

There is a study investigating MA in blood and vitreous humor of 18 deceased using GC-MS following liquid-liquid extraction. McIntyre et al. (2011) found that the ratio of MA concentrations in vitreous humor to peripheral blood was shown by the mean ± S.D. of 1.63 ± 0.75. Liver tissues were also investigated for MA distribution. Besides MA, AM was examined in their study as well. In that study, peripheral blood (PB) samples were collected distally from the femoral vein while central blood (CB) samples were collected from heart cavity after removing the heart. Liver tissues were sampling from the upper right lobe of the liver. The results were presented in Table 5. They concluded that MA concentrations in vitreous humor of most of the deceased were higher than those in PB samples except for three cases whose vitreous MA

concentrations were lower than PB. They suggested vitreous humor as an alternative specimen when blood sample is not available or contaminated. In that study, urine samples were not used. Therefore, a conclusion cannot be drawn about the relationship between postmortem MA concentrations in urine, blood, and vitreous humor.

Compared to other fluids (e.g. urine, blood), vitreous humor has a small volume. Although this may affect an assay sensitivity, its advantages which are suitable for analysis are:

1. Vitreous sample collection is easy even if an autopsy is not completely performed

2. Due to clear and mainly consists of water (99%), vitreous humor is easy to analyze with reduced time and less requirement of sample preparation.

3. Drug and substance stability in vitreous humor is higher as compared to other fluids.

4. Analytical method which is developed for urine or blood can be adapted to vitreous humor.

5. Putrefaction, charring, and trauma may affect sample quality. Tyramine and phenethylamine, decomposition products, may interfere both blood and tissues extraction and analysis as well. These situations less occur with vitreous humor due to its anatomically isolated location. Even though trauma and severe major organ damage occur, an available specimen such as cavity blood, is potentially contaminated from tissues or stomach contents. In this situation, vitreous humor may be useful as a promising specimen.

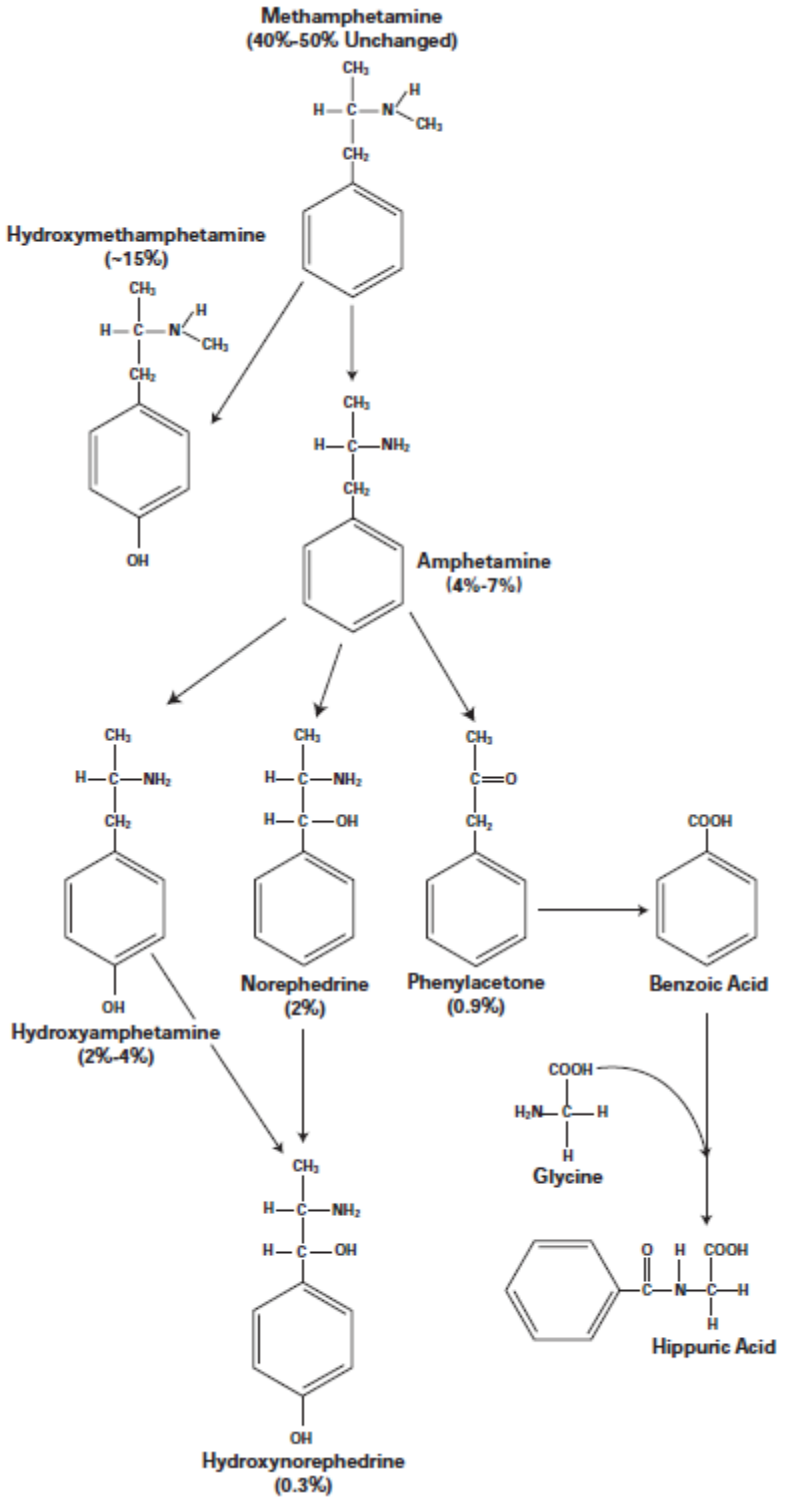

Figure 1 Chemical structures and metabolic pathway of MA and its metabolites (Moore, 2003)

Table 1 Pharmacokinetics and pharmacodynamics profiles of MA (Adapted from Greene et al., 2008)

Pharmacodynamics	Predominant sympathomimetic actions, most potent cardiovascular effects of all amphetamine, greater central nervous system effects than amphetamine
Pharmacokinetics	Volume of distribution 3-7 L/kg, hepatic metabolism, 40-50% renal elimination, half-life 8-12 hour, active metabolites-amphetamine, norephedrine
Methods of exposure	Powder, tablet, crystal, liquid, ingested, vaporized (smoked), insufflated (snorted), taken sublingually or rectal
Desired effects	Euphoria, increased stamina, energy, concentration and sexual drive, decreased appetite
Clinical associations	Bruxism, agitation, paranoia, formication, violent behaviour, high-risk sexual activity

Table 2 Distribution and detection times of MA in various matrices. (Adapted from Cruickshank and Dyer, 2009)

Matrices	Dose	LLOQ/ cut-off (μg/l)	Typical detection time after single dose administration (hours)	Maximum detection time after repeated dosing (days)
Plasma	10 mg IV	1	36-48	Not reported
	35 mg* IV	1	36-48	Not reported
	10 mg PO, slow-release	2.5	24	Not reported
Oral fluid	10 mg PO, slow-release	2.5	24	3
Urine	10 mg PO, slow-release	2.5	87	7
	22 mg smoking	300	60	Not reported

LLOQ = lower limit of quantification; IV = Intravenous; PO = per oral

* The administered dose was 0.5 mg/kg, equivalent to 35 mg/70 kg

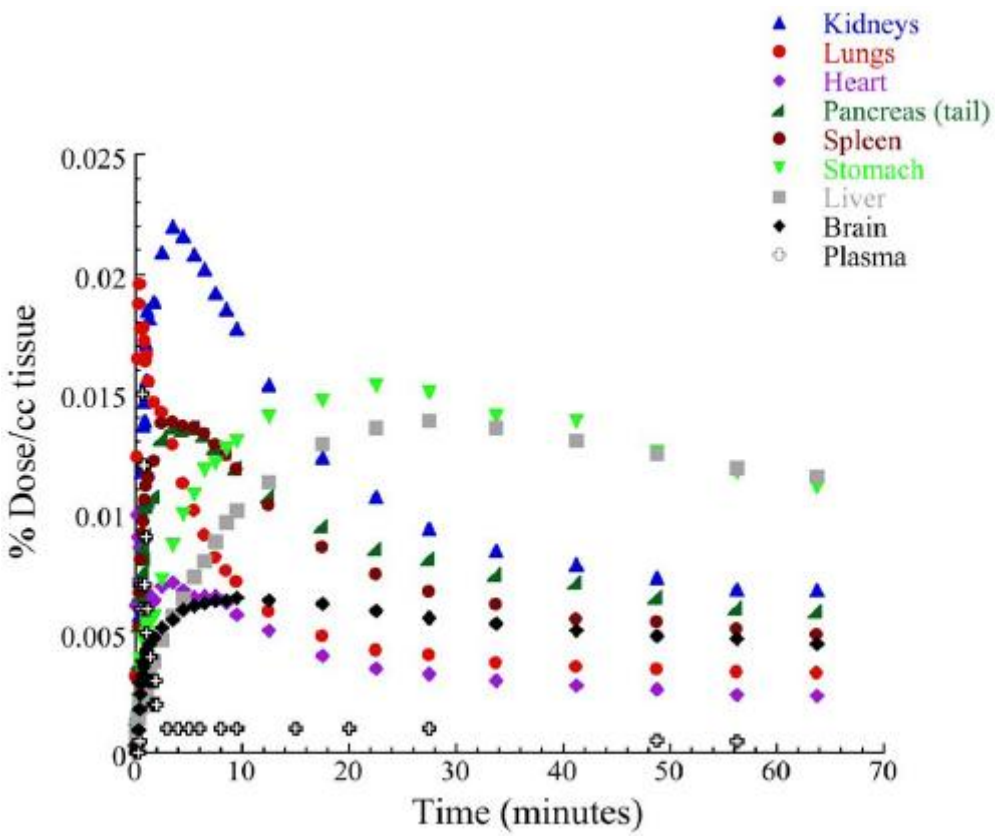

Figure 2 Distribution and accumulation of [^{11}C]d-methamphetamine in the human body represented as averaged time-activity curves of [^{11}C]d-methamphetamine. The values correspond to the average for all subjects (African Americans and Caucasians). (Volkow et al., 2010)

Table 3 Uptake and pharmacokinetics of [^{11}C] methamphetamine in various organs (Adapted from Volkow et al., 2010)

Organ	Ethnicities	Peak time	Half-peak clearance (minutes)	Peak %Dose/ml	Organ weight	%Dose organ	AUC
Plasma	African Americans	50 sec	1.5	0.014 ± 0.002	4500 g[a]	63%	0.029 ± 0.004
	Caucasians	50 sec	1.5	0.014 ± 0.003	4500 g[a]	63%	0.030 ± 0.003
Lung	African Americans	55 sec	7	0.025 ± 0.005*	1246 g[b]	31.2%	0.393 ± 0.069*
	Caucasians	55 sec	7	0.019 ± 0.007	1246 g[b]	23.7%	0.294 ± 0.082
Heart	African Americans	1 min	16	0.007 ± 0.003	365 g	2.55%	0.259 ± 0.072
	Caucasians	1 min	16	0.007 ± 0.001	365 g	2.55%	0.205 ± 0.087
Kidneys	African Americans	3 min	22	0.022 ± 0.004	305 g[b]	6.7%	0.732 ± 0.125
	Caucasians	3 min	22	0.022 ± 0.005	305 g[b]	6.7%	0.720 ± 0.167

Table 3 Uptake and pharmacokinetics of [^{11}C] methamphetamine in various organs (Adapted from Volkow et al., 2010) (cont.)

Organ	Ethnicities	Peak time	Half-peak clearance (minutes)	Peak %Dose/ml	Organ weight	%Dose organ	AUC
Pancreas (tail)	African Americans	5 min	50	0.013 ± 0.002	144 g	1.9%	0.518 ± 0.225
	Caucasians	5 min	50	0.015 ± 0.002	144 g	2.2%	0.655 ± 0.239
Spleen	African Americans	3.5 min	30	0.014 ± 0.002	156 g	2.2%	0.513 ± 0.101
	Caucasians	3.5 min	30	0.013 ± 0.002	156 g	2.0%	0.503 ± 0.064
Liver	African Americans	30 min	>75	0.014 ± 0.003	1677 g	23.5%	0.871 ± 0.215
	Caucasians	30 min	>75	0.013 ± 0.002	1677 g	21.8%	0.778 ± 0.138
Stomach	African Americans	30 min	>75	0.014 ± 0.007	330 g	4.6%	0.855 ± 0.215
	Caucasians	30 min	>75	0.017 ± 0.005	330 g	5.6%	0.967 ± 0.457

Table 3 Uptake and pharmacokinetics of [^{11}C] methamphetamine in various organs (Adapted from Volkow et al., 2010) (cont.)

Organ	Ethnicities	Peak time	Half-peak clearance (minutes)	Peak %Dose/ml	Organ weight	%Dose organ	AUC
Brain	African Americans	9 min	>75	0.006 ± 0.001	1600 g	9.6%	0.371 ± 0.043
	Caucasians	9 min	>75	0.006 ± 0.001	1600 g	9.6%	0.380 ± 0.041

Measured correspond to: time to peak concentration (Peak time), time to half-peak clearance averaged across both groups and peak concentration (expressed as %Dose/ml) and AUC for the time activity curves for the African Americans and for the Caucasians

*Unpaired Student t test (2-tail) $p < 0.05$

[a]The plasma value was extrapolated to whole blood assuming a 55% plasma volume

[b]Reflects the total weight of both left and right organs. Note that the total percent of organ accumulation is greater than 100%; this is because the times at which the peak uptake and the clearance occurs differs among the organs. The weight of the organs corresponds to the average weights recorded from male autopsies; except in brain, which corresponds to weights obtained with MRI

Table 4 The studies of MA concentration detected in various specimens using GC-MS technique (Adapted from Moffat, Osselton, and Widdop, 2004)

Matrices	Researchers	LOD
Urine	Meatherall (1995)	5 ng/ml
	Dallakian et al. (1996)	90 ng/ml
	Dasgupta and Spies (1998)	100 ng/ml
	Myung et al. (1998)	< 1 - 10 ng/ml
	Hensley and Cody (1999)	10 ng/ml
	Jurado et al. (2000)	NA
	Stout et al. (2002)	16 ng/ml
	Yamada et al. (2002)	NA
	Namera et al. (2002)	NA
Blood	Nagasawa et al. (1996)	NA
	Sato and Mitsui (1997)	NA
	Namera et al. (2000)	5 ng/g
	Okajima et al. (2001)	NA
	Nishida et al. (2002)	NA
	Peters et al. (2002)	NA
Hair	Kintz et al. (1995)	0.05 ng/g
	Skender et al. (2002)	0.05 - 3 ng/g
Sweat	Fay et al. (1996)	NA
Serum	Lee et al. (2000)	NA
	Weinmann et al. (2000)	NA
Oral fluid, plasma, and urine	Huestis and Cone (2007)	2.5 ng/mL (LOQ)
Blood, liver, and vitreous humor	McIntyre et al. (2011)	NA

LOD = Limit of detection; LOQ = Limit of quantification; NA = not available

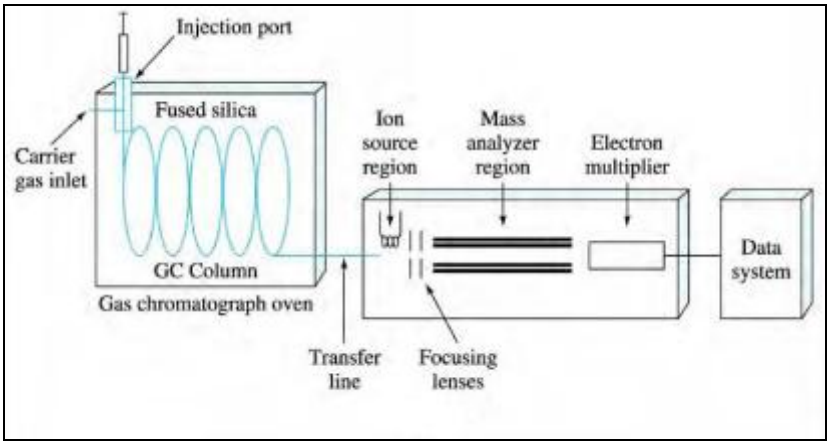

Figure 3 Components diagram of gas chromatography-mass spectrometer (Food and Agriculture Organization, 2010 : online)

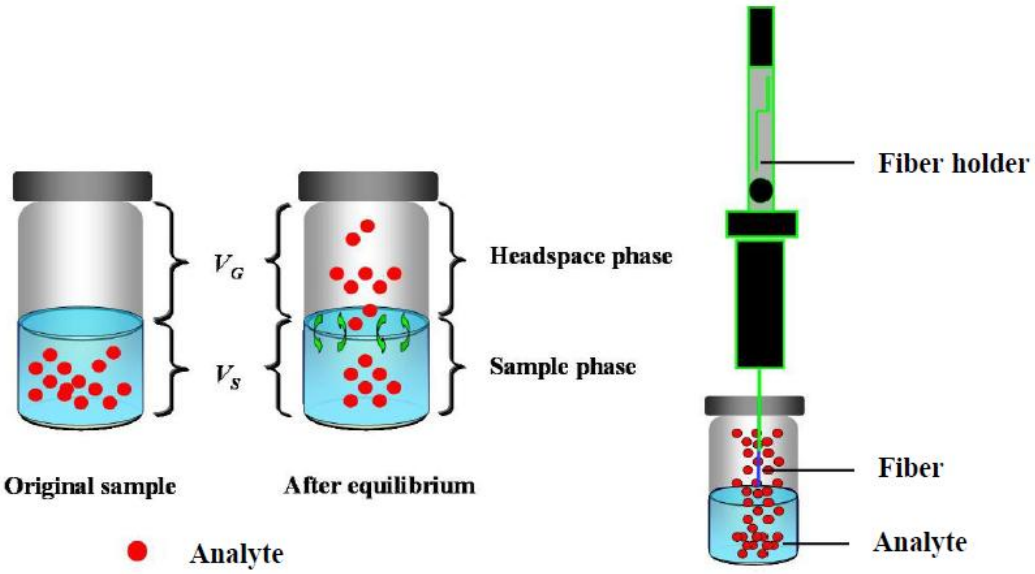

Figure 4 A headspace-solid phase microextraction technique (Scientific Equipment Center Prince of Songkla University, 2010 : online)

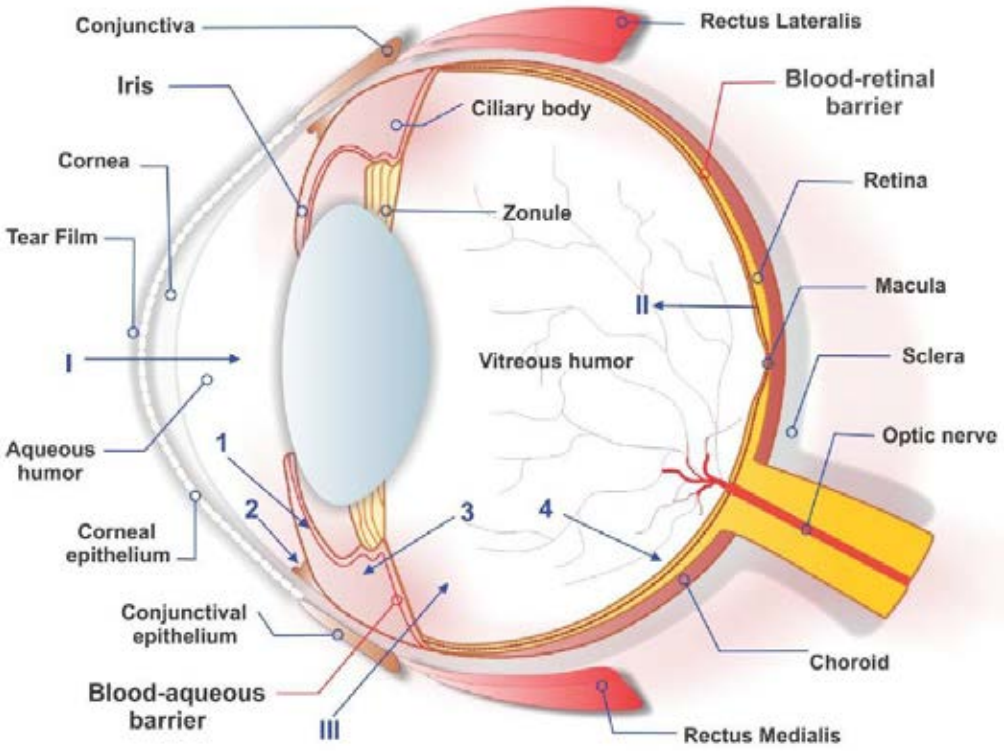

Figure 5 Locations of vitreous humor and blood-ocular barrier (Nakhlband and Barar, 2011)

Table 5 MA concentrations (μg/ml) detected in various specimens (Adapted from McIntyre et al., 2011)

Case #	Peripheral Blood	Central Blood	Vitreous humor
1	0.61	1.40	0.84
2	0.24	0.48	0.21
3	0.25	0.48	0.64
4	0.38	0.42	0.53
5	0.26	0.25	0.29
6	0.40	0.78	0.57
7	0.26	0.29	0.42
8	0.33	0.53	0.89
9	0.35	0.75	1.10
10	0.31	0.73	0.85
11	1.60	2.40	1.10
12	0.42	0.91	0.45
13	1.10	1.80	2.10
14	0.25	0.30	0.52
15	1.60	2.00	2.00
16	0.60	1.00	1.00
17	1.70	1.60	1.90
18	0.69	0.78	0.40

CHAPTER III

METHODOLGY

Chemicals

1. Diphenhydramine hydrochloride (Sigma Chemical Ltd., U.S.A)
2. Helium carrier gas 99.99%
3. Methamphetamine hydrochloride (Lipomed, U.S.A.)
4. Methanol, HPLC grade (Burdick & Jackson, Muskegon, U.S.A)
5. Sodium chloride, AR grade (Merck, Darmstadt, Germany)
6. Sodium hydroxide, AR grade (Merck, Darmstadt, Germany)

Instruments

1. Extraction tube, 2 ml (Eppendorf)
2. SPME glass vial 20 ml
3. Gray-top vacutainer tube, 3 ml containing sodium fluoride (NaF) for collecting blood and vitreous humor samples
4. Plastic container, 60 ml with lid for collecting urine samples
5. Analytical balance
6. Centrifuge apparatus
7. Crossband 100% dimethyl polysiloxane 0.25 mm i.d. x 0.5 μm thickness x 30 m length Rtx-1MS column (Restex, U.S.A)
8. Gas chromatography-mass spectrometry (GC-MS) QP2010 Plus Shimadzu equipped with an AOC-5000 auto injector (Shimadzu, Kyoto, Japan)

9. Micropipette, 10, 100, 1000, and 5000 μl and pipette tips

10. Orange Test Methamphetamine Strip® (True line Med. Co. Ltd., Switzerland)

11. Silicone rubber lid with steel ring cap set for capping the 20 ml SPME glass vial

12. Solid phase microextraction (SPME) fiber assembly 100 μm polydimethylsiloxane (PDMS) 23 gauge needle Auto Holder Red Fused Silica/SS 57341-U (Supelco, Bellefonte, U.S.A)

13. Volumetric flasks, 10, 25, 50, and 100 ml

Subjects

Thai male and female deceased whose bodies were performed autopsies at the Institute of Forensic Medicine, Police General Hospital, The Royal Thai Police Headquarter, during May 2011 - January 2012. A total number of subjects were not less than 40.

Sample size calculation (Zou et al., 2003)

$$n = \left(\frac{Z_{\alpha} + Z_{\beta}}{Z_{(r)}} \right)^2 + 3$$

Represent: n = sample size

α = type I error and β = type II error

r = correlation coefficient

$$Z_{(r)} = \frac{1}{2} \ln \frac{1+r}{1-r}$$

Prior to perform the experiment, a pilot study was performed regarding the relationship between postmortem MA concentrations in urine, blood, and vitreous humor. The result showed that the relationship between MA concentrations in urine and blood demonstrated with the r = 0.5, whereas the relationship between MA concentrations in urine and vitreous humor demonstrated with the r = 0.5. Thus, the value of r of approximately 0.5 was substituted in the above equation: $Z_{(r)} = \frac{1}{2}\ln\ \frac{1+0.5}{1-0.5}\ = 0.55$. If the hypothesis tested was two-tailed (α = 0.05), $Z_{\alpha/2}$ was 1.96 and the power was 0.80. Then Z_{β} was 0.84.

$$n = \frac{Z_{\alpha} + Z_{\beta}}{Z_{(r)}}^{2} + 3$$

$$n = \frac{1.96 + 0.84}{0.55}^{2} + 3$$

$$n = 29$$

In this study, n = 40 cases were used to test the correlations.

Inclusion criteria

Thai male and female deceased whose bodies were performed autopsies at the Institute of Forensic Medicine, General Police Hospital, The Royal Thai Police Headquarter, during May 2011 - January 2012 were recruited. Orange Test Methamphetamine Strip® was used to exclude the deceased whose urine samples were negative (figure 6). The deceased whose urine samples were positive by the strip test (figure 6), their urine, blood, and vitreous humor samples were collected for further analysis using headspace-solid phase microextraction/gas chromatography-mass spectrometry (HS-SPME/GC-MS) technique.

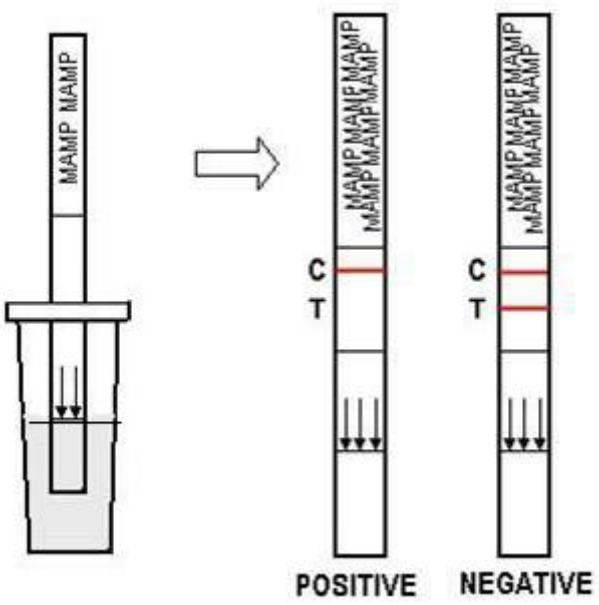

Figure 6 Interpretation of positive and negative results using Orange Test Methamphetamine Strip®

Exclusion criteria

1. Any deceased whose their bodies were putrefactive or the ones who had died for more than 24 hours. Also, any deceased whose their urinary bladder or eyes or major organs were severely damaged and their urine, blood, and vitreous humor samples were contaminated.

2. Any deceased who died from poisonous substances/drug related causes or had selegiline used history.

3. Any deceased whose their urine samples were negative to MA strip test.

4. Any deceased whose their urine samples were negative to MA confirmatory test with HS-SPME/GC-MS technique.

Urine, blood, and vitreous humor samples collections

Urine samples were collected from urinary bladder and then were kept in a well closed plastic container. Blood samples and vitreous humor samples were collected from basal artery and eyes respectively before being kept in a sodium fluoride tube. All samples were stored at 4°C until analysis.

Procedure

1. Recruitment

Thai male and female deceased were recruited into the study using Orange Test Methamphetamine Strip®. The deceased with negative urine samples were excluded whereas the ones with urine positive results, their blood, and vitreous humor samples were collected. Those positive urine samples from MA strip test were then performed the confirmatory test with a HS-SPME/GC-MS technique to exclude the deceased whose urine samples demonstrating false MA positive using MA strip test. Urine, blood, and vitreous humor samples of the deceased with positive confirmatory test were further quantified for MA concentrations. Urine and vitreous humor samples could be directly analyzed by HS-SPME/GC-MS technique while blood samples needed to be deprotenized with strong alkali before analyzing as described in the sample preparations.

2. Sample preparations

2.1 Blood sample preparation was modified from Namera et al. (2000). One milliliter of blood sample was pipetted to 2 ml extraction tube and then 1 ml of 5 M sodium hydroxide was added. The mixture was vortex-mixed for 10 minutes before centrifugation at 14,000 rpm for 20 min. Each blood sample was performed protein precipitation as mentioned above for 4 tubes. The supernatants from 4 tubes were pooled and transferred to a 20 ml SPME glass vial and 200 μl of diphenhydramine in methanol (20 μg/ml) and 0.5 g of sodium chloride were added. Finally, a silicone rubber

lid and a steel ring cap were capped on the top of glass vial before analyzing by HS-SPME/GC-MS technique. Each sample was performed in duplicate.

2.2 Urine/vitreous humor sample preparations were modified from Myung et al. (1998). One milliliter of urine/vitreous humor sample was transferred to a 20 ml SPME glass vial. Then, 200 μl of diphenhydramine in 1 M sodium hydroxide (1 μg /ml) was added. Next, 0.3 g of sodium chloride was added. After that, a silicone rubber lid and a steel ring cap were capped on the top of glass vial before analyzing by HS-SPME/GC-MS. Each sample was performed in duplicate.

3. Head space-solid phase microextraction/gas chromatography - mass spectrometry (HS-SPME/GC-MS) technique

HS-SPME is a sample extraction technique for volatile substances or analytes which are capable to be vaporized. Compared to other techniques, e.g. liquid-liquid extraction, this technique is a solventless technique. Therefore, contamination in a sample preparation step and harmful effect to analyst's health or to environment would be less. It is also beneficial to column lifetime because SPME fiber adsorbs only small vaporized molecule before injection to column. Therefore, there is less chance of water coming to the column and damaging it. This technique is suitable for analyzing a substance of which the sample preparation and extraction are complicated because they are not required. Regarding this technique, a substance is vaporized in a well-closed glass container. When an equilibrium is achieved, an analyte is in both gaseous form in the space above the liquid and dissolved in the liquid in the glass container. The fiber adsorbs analyte's vapor at the headspace and then desorbs it into the GC column for further separation (Plutowska and Wardencki, 2007).

Samples were separated on a crossband 100% dimethyl polysiloxane 0.25 mm i.d. x 0.5 μm thickness x 30 m length Rtx-1MS column. Initial oven temperature of 100 °C was held for 5 min, then increased at the rate of 15 °C/min to 150 °C for 1 min, and finally increased to 250°C at 15°c/min for 3 min. The injection port and

interface temperature were set at 240 °C and 220 °C, respectively. The split injection mode and helium carrier gas was used. MS detection was operated in selective ion monitoring method and characteristic ion for MA quantification was m/z = 58.

4. MA stock standard solution preparations

MA of 0.3 g was dissolved in 100 ml of methanol to obtain the MA concentration of 3 mg/ml MA. Then, 0.09 ml of 3 mg/ml MA was diluted with 8.91 ml of deionized water (DW) to obtain 9 ml of 0.03 mg/ml (30,000 ng/ml). After that, 9 ml of 0.03 mg/ml MA which further diluted with 18 ml of DW to obtain 27 ml of 0.01 mg/ml MA. Finally, 27 ml of 0.01 mg/ml MA in was diluted with 27 ml of DW to obtain 54 ml of 0.005 mg/ml MA.

4.1 MA standard curves

4.1.1 MA standard curves of urine and vitreous humor samples

Working MA standard solutions of 250, 500, 1000, 1250, 2000, 2500 and 4000 ng/ml were prepared by serial dilution from the stock standard solution (0.005 mg/ml; 5,000 ng/ml). The working MA standard solutions of urine/vitreous humor samples were prepared as following:

1. 6.9 ml of 5,000 ng/ml MA was diluted with 1.725 ml of blank urine/vitreous humor sample to obtain 8.625 ml of 4,000 ng/ml MA.

2. 5.625 ml of 4,000 ng/ml MA was diluted with 3.375 ml of blank urine/vitreous humor sample to obtain 9 ml of 2,500 ng/ml MA.

3. 6 ml of 2,500 ng/ml MA was diluted with 1.5 ml of blank urine/vitreous humor sample to obtain 7.5 ml of 2,000 ng/ml MA.

4. 4.5 ml of 2,000 ng/ml MA was diluted with 2.7 ml of blank urine/vitreous humor sample to obtain 7.2 ml of 1,250 ng/ml MA.

5. 4.2 ml of 1,250 ng/ml MA was diluted with 1.05 ml of blank urine/vitreous humor sample to obtain 5.25 ml of 1,000 ng/ml MA.

6. 2.25 ml of 1,000 ng/ml MA was diluted with 1.05 ml of blank urine/vitreous humor sample to obtain 2.25 ml of 500 ng/ml MA.

7. 1.5 ml of 500 ng/ml MA was diluted with 1.5 ml of blank urine/vitreous humor sample to obtain 3 ml of 250 ng/ml MA.

Each concentration of MA standard solutions was analyzed by HS-SPME/GC-MS in triplicate.

4.1.2 MA standard curves of blood samples

Working MA standard solutions of 100, 200, 400, 800 and 1000 ng/ml were prepared by serial dilution from the stock standard solutions (0.005 mg/mL; 5000 ng/ml). The working MA standard solutions of blood samples were prepared as following:

1. 24 ml of 5,000 ng/ml MA was diluted with 6 ml of blank blood sample to obtain 30 ml of 4,000 ng/4 ml MA.

2. 18 ml of 4,000 ng/4 ml MA was diluted with 6.75 ml of blank blood sample to obtain 22.5 ml of 3,200 ng/4 ml MA.

3. 10.5 ml of 3,200 ng/4 ml MA was diluted with 10.5 ml of blank blood sample to obtain 21 ml of 1,600 ng/4 ml MA.

4. 9 ml of 1,600 ng/4 ml MA was diluted with 9 ml of blank blood sample to obtain 18 ml of 800 ng/4 ml MA.

5. 6 ml of 800 ng/4 ml MA was diluted with 6 ml of blank blood sample to obtain 12 ml of 400 ng/4 ml MA.

Each concentration of MA standard solutions was analyzed by HS-SPME/GC-MS in triplicate.

5. Method validation

Analytical method validation performed in this study was modified from the guideline described by Guidance for industry: Bioanalytical method validation (The United State Department of Health and Human Services Food and Drug Administration, U.S. FDA, 2001). The validation of analytical method for determining MA concentrations in urine, blood, and vitreous humor samples included linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy.

Linearity

Linearity was evaluated using various MA concentrations (250, 500, 1000, 1250, 2000, 2500 and 4000 ng/ml) in blank urine/vitreous humor samples or various MA concentrations (100, 200, 400, 500, 800, 1000 ng/ml) in blank blood samples. Each sample was analyzed for MA concentrations using HS-SPME/GC-MS for 5 times. Relationship between actual and measured MA concentrations was assessed by Pearson's correlation and simple linear regression. Linearity should be achieved with coefficient of determination (R^2) of 0.99 (The International Conference on Harmonization, ICH, 1996; U.S. FDA, 2001; The Society of Forensic Toxicologists/The Toxicology Section of the American Academy of Forensic Sciences, SOFT/AAFS, 2006).

LOD and LOQ

LOD is the lowest concentration of an analyte in a sample that can be detected by the peak with a signal-to-noise ratio of at least 3:1. LOQ is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy. Signal-to-noise ratio of ten is generally used for estimating LOQ (ICH, 1996; SOFT/AAFS, 2006). Determination of signal to noise was illustrated in Figure 7.

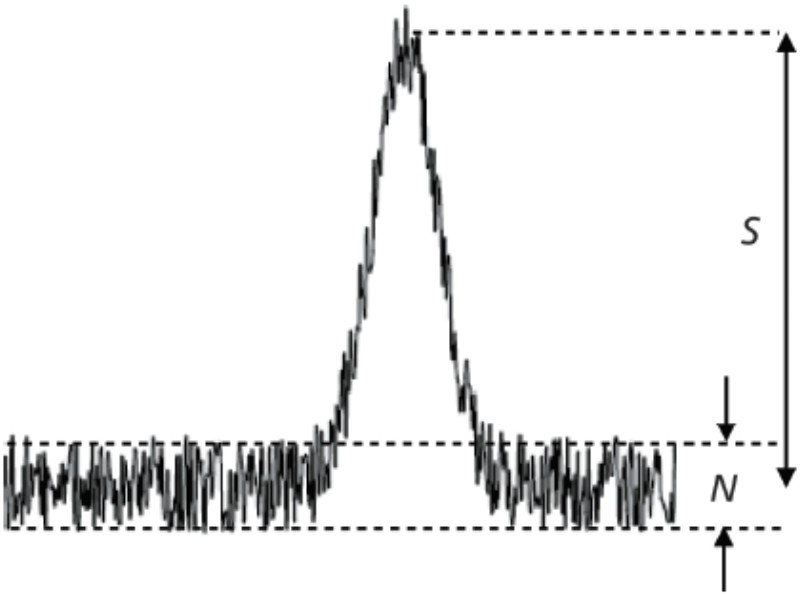

Figure 7 Chromatogram demonstrating measurement of the signal and noise

Precision

Precision of the assays was evaluated both within- and between-day by assessing from the percentage of coefficient of variation (%CV). To evaluate within-day precision, urine/vitreous humor samples containing 1000, 2000, and 4000 ng/ml of MA in blank urine/vitreous humor samples were analyzed according to the sample preparations mentioned above followed by HS-SPME/GC-MS technique. Each concentration was performed 5 times. Blank blood samples containing 200, 400, 800 ng/ml of MA were also performed in the same manner 5 times for each concentration within 24 hours. Between-day precision was evaluated by analyzing blank urine/vitreous humor samples containing 1000 ng/ml of MA with 3 replicate analyses and blank blood sample containing 400 ng/ml of MA with 3 replicate analyses. The experiments were performed for four consecutive days. Percentage of CV was calculated as following:

$$\%CV = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

The %CV should not exceed 15% (U.S. FDA, 2001).

Accuracy

To evaluate accuracy, urine/vitreous humor samples containing 1000, 2000, and 4000 ng/ml of MA in blank urine/vitreous humor samples were analyzed according to the sample preparations mentioned above followed by HS-SPME/GC-MS technique. Each concentration was performed 5 times. Blank blood samples containing 200, 400, 800 ng/ml of MA were also performed in the same manner 5 times for each concentration. Accuracy of the assay was evaluated by the percentage of recovery by the following equation:

$$\%\text{Recovery} = \frac{\text{Measured MA concentration}}{\text{Actual MA concentration}} \times 100$$

The mean value of %recovery should be within 15% (U.S. FDA, 2001).

Data analysis

All results were expressed as mean ($\bar{X}$) $\pm$ standard deviation (S.D.) or standard error of mean (S.E.M. or S.E.). Relationship between urine, blood, and vitreous MA concentrations was tested by Pearson's correlation and simple linear regression using SPSS for Windows, version 16.0. A *p*-value of less than 0.05 was considered statistically significant.

CHAPTER IV

RESULTS

Method validation

The standard curves plotting from peak area ratio of MA to internal standard (diphenhydramine) and MA concentrations in blank urine, blood and vitreous humor samples were shown in Figure 8, 9, and 10, respectively. These standard curves were used for determinations of MA concentrations in the corresponding specimens in the method validation and in the samples of the subjects.

The method validation of this study was reported by linearity, LOD, LOQ, precision, and accuracy. Linearity was shown by the closely linear relationship between measured MA concentrations and actual MA concentrations in urine samples (R^2 = 0.99, p = 0.000, Figure 11), blood samples (R^2 = 0.99, p = 0.000, Figure 12) and vitreous humor samples (R^2 = 0.99, p = 0.000, Figure 13). LOD of the method was shown to be 25, 2.5, and 25 ng/ml for urine, blood, and vitreous humor samples, respectively (Figure 14-25). LOQ of the method was shown to be 100, 100, and 100 ng/ml for urine, blood, and vitreous humor, respectively (Figure 26-28). Accuracy as well as within-day and between-day precision of the method for determination of MA concentrations in urine, blood, and vitreous humor samples were shown in Table 6.

Demographic profile of subjects

Forty Thai male and female deceased were recruited into the study. Most of them were male (n = 38, 95%) whereas the remaining (n = 2, 5%) was female. Mean $\pm$ S.E. of their ages was 30.84 ± 1.54 years (range of 16 - 60 years). Majority of causes of death was unknown (35%) (Table 7).

MA concentrations in urine, blood, and vitreous humor samples of the deceased

Urine MA concentrations of all deceased were higher than 1 μg/ml (1000 ng/ml). MA concentrations detected in urine samples were far higher than those of in blood and vitreous humor. Also, MA concentrations detected in vitreous humor were higher than those of in blood. There are 7 deceased who MA could not be detected in their blood and vitreous humor samples using the technique of this study. These 7 deceased had urine MA concentrations in the range of approximately 1000 - 3000 ng/ml (Table 8).

Relationships between MA concentrations in urine, blood, and vitreous humor samples

To determine the relationships between MA concentrations in these three biological samples, the data of 33 deceased (82.5 %) from the total of 40 deceased were used because MA concentrations of 7 cases (17.5%) could not be detected due to limitation of the sensitivity of the method used in this study. The relationships between MA concentrations between in urine, blood, and vitreous humor samples were presented by correlation coefficient (r), coefficient of determination (R^2), and linear regression equation (Figure 20-22). The average ratios of MA concentrations between specimens were also presented (Table 4).

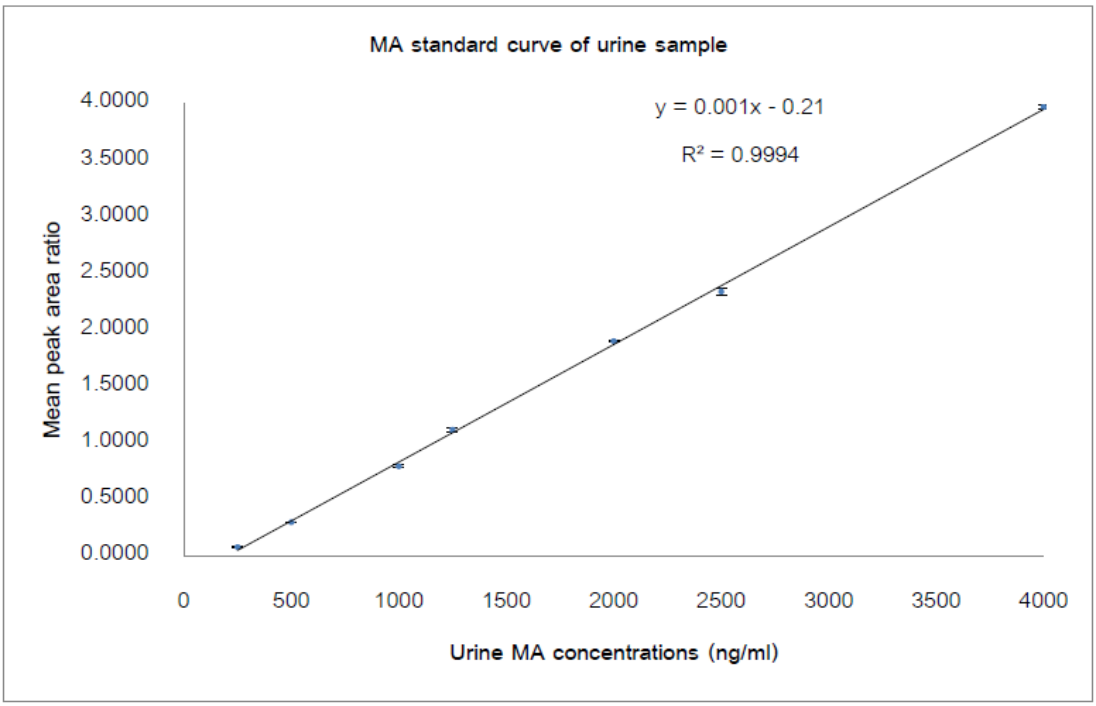

Figure 8 Standard curve plotting from peak area ratio of MA to internal standard and MA concentrations in blank urine sample. The data shown were mean ± S.D. of n = 3.

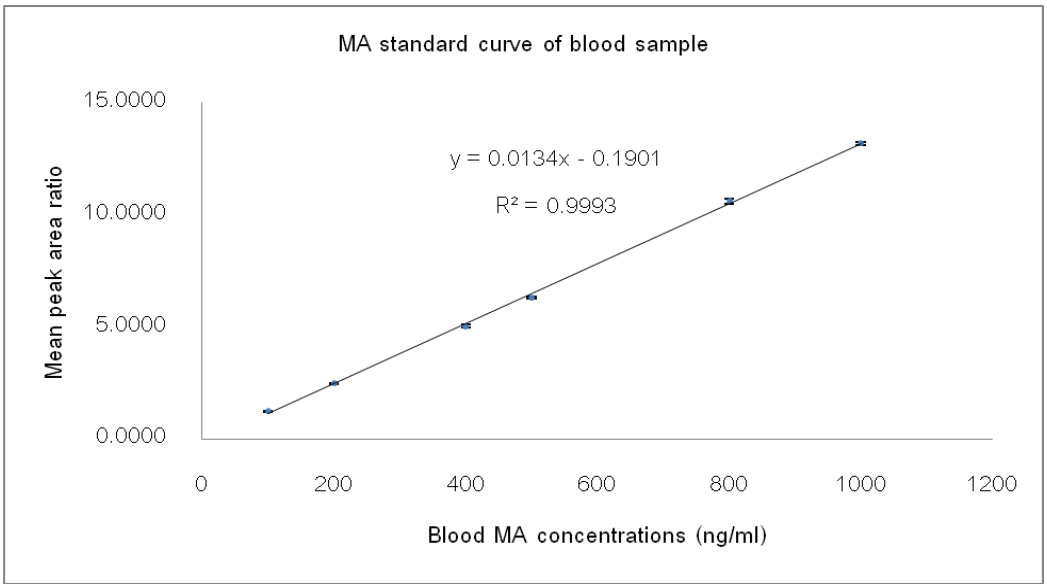

Figure 9 Standard curve plotting from peak area ratio of MA to internal standard and MA concentrations in blank blood sample. The data shown were mean ± S.D. of n = 3.

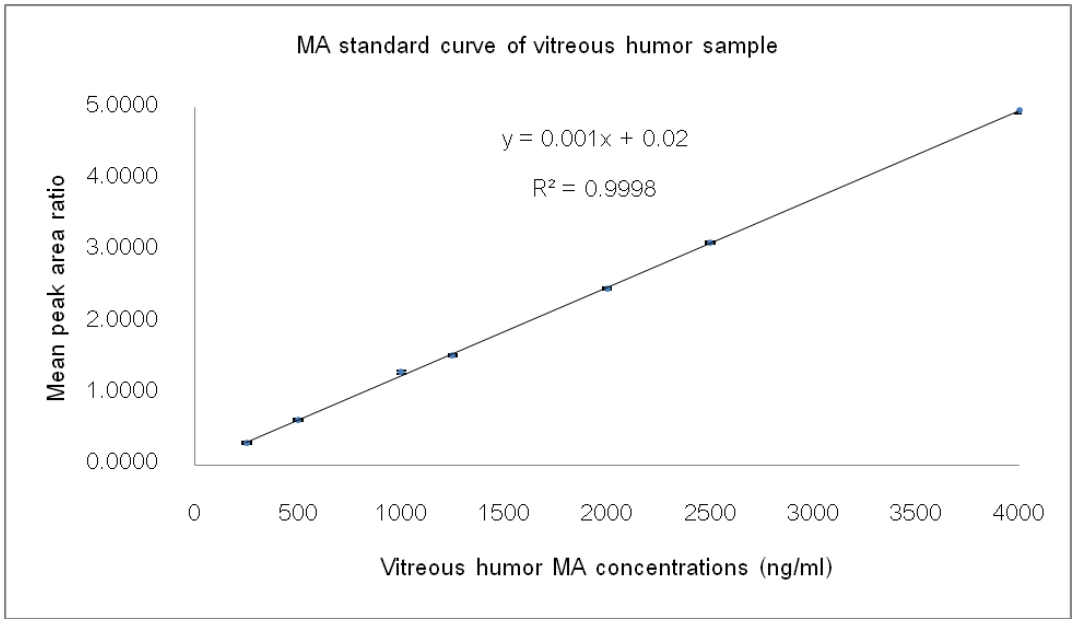

Figure 10 Standard curve plotting from peak area ratio of MA to internal standard and MA concentrations in blank urine sample. The data shown were mean ± S.D. of n = 3.

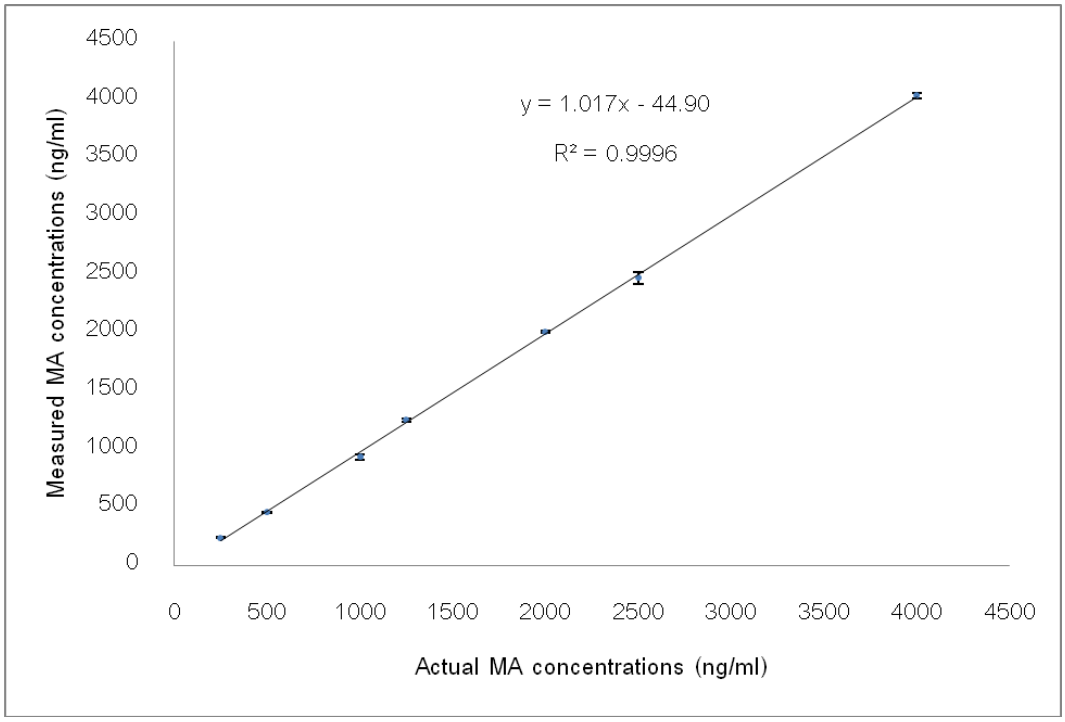

Figure 11 Linearity of the method for determination of MA in urine samples

The data shown were mean ± S.D. of n = 5.

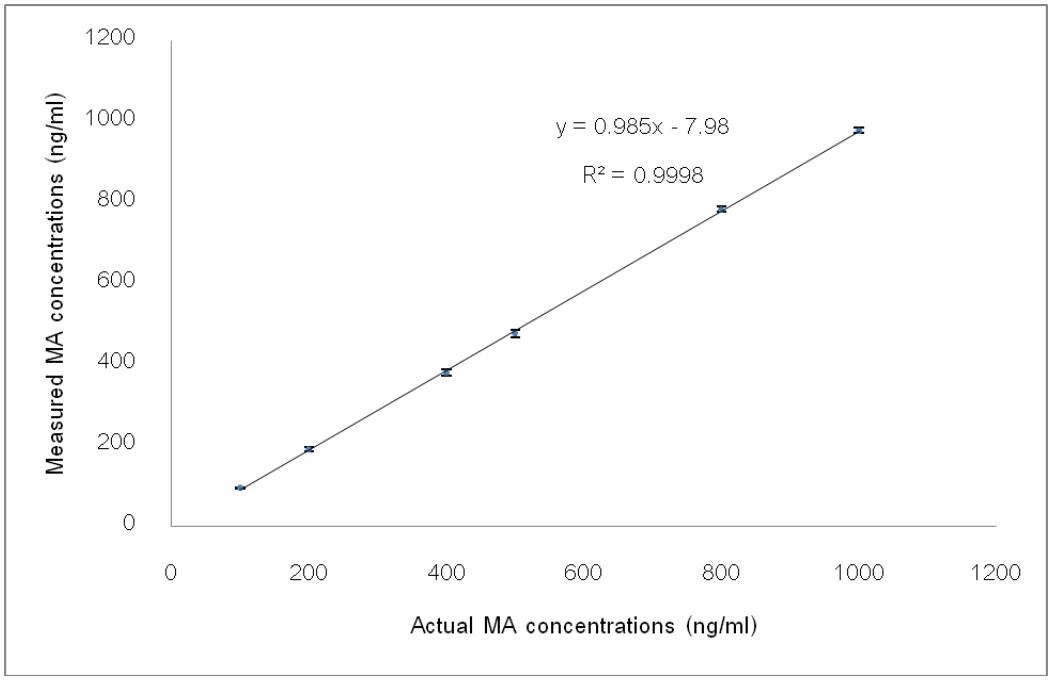

Figure 12 Linearity of the method for determination of MA in blood samples

The data shown were mean ± S.D. of n = 5.

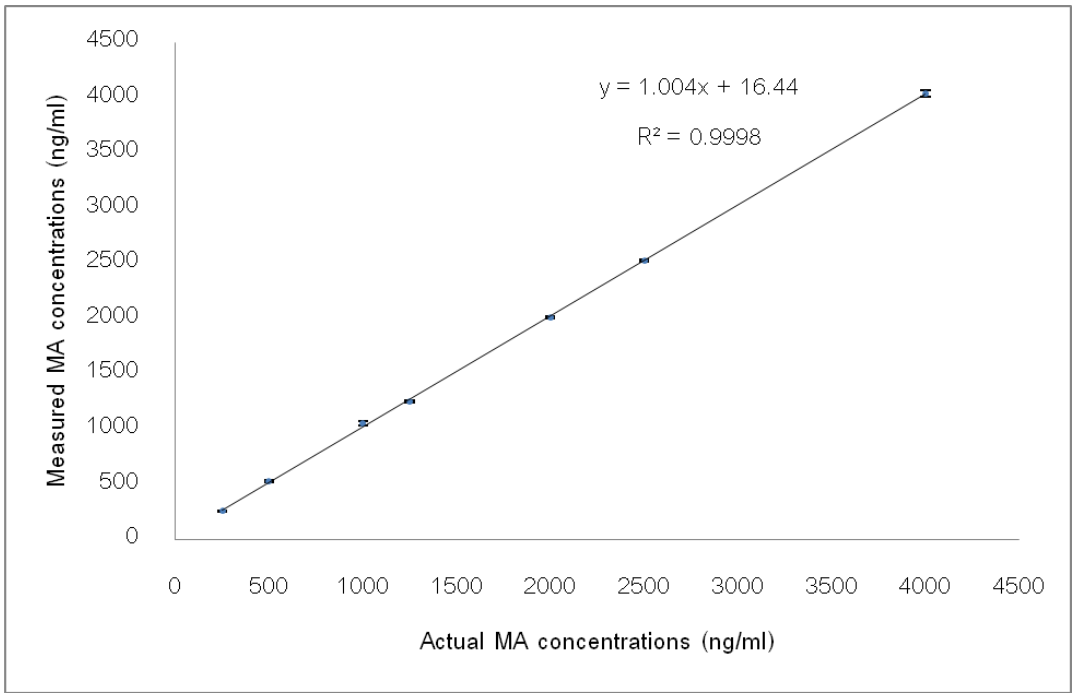

Figure 13 Linearity of the method for determination of MA in vitreous humor samples

The data shown were mean ± S.D. of n = 5.

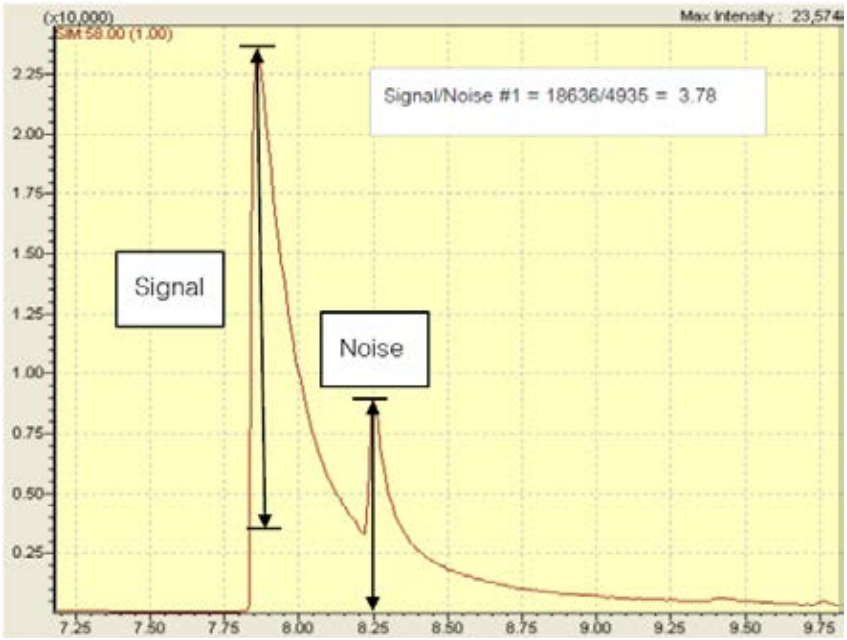

Figure 14 Chromatogram demonstrating LOD#1 of the method for determination of MA in urine samples (MA concentration = 25 ng/ml)

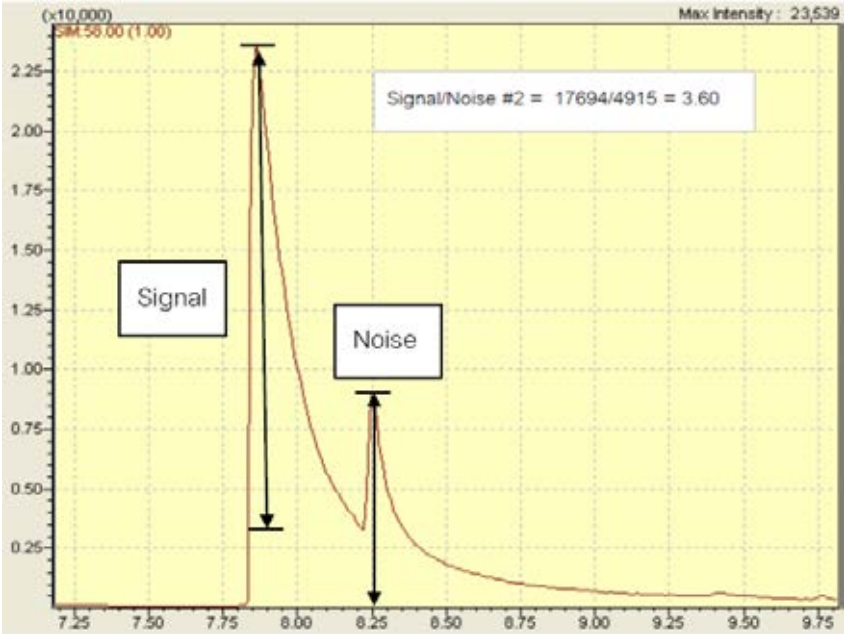

Figure 15 Chromatogram demonstrating LOD#2 of the method for determination of MA in urine samples (MA concentration = 25 ng/ml)

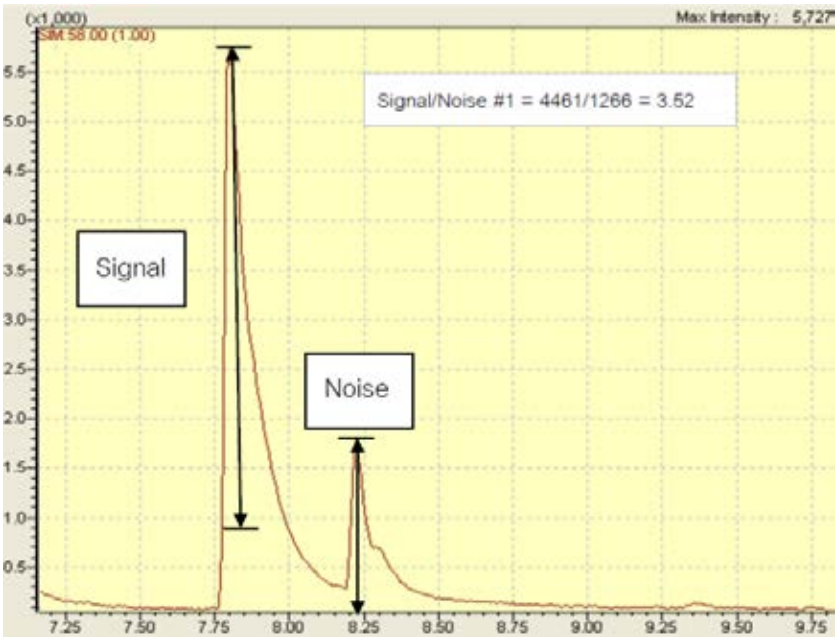

Figure 16 Chromatogram demonstrating LOD#1 of the method for determination of MA in blood samples (MA concentration = 2.5 ng/ml)

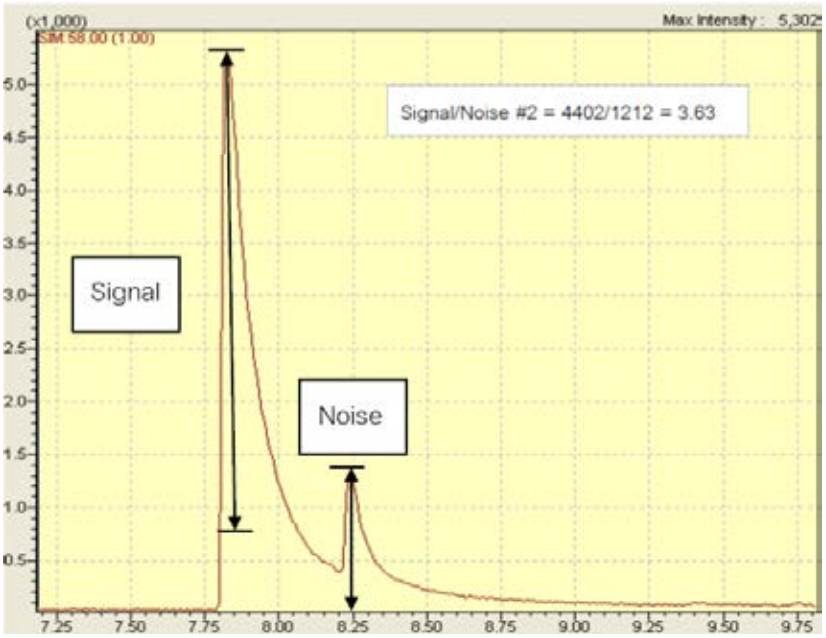

Figure 17 Chromatogram demonstrating LOD#2 of the method for determination of MA in blood samples (MA concentration = 2.5 ng/ml)

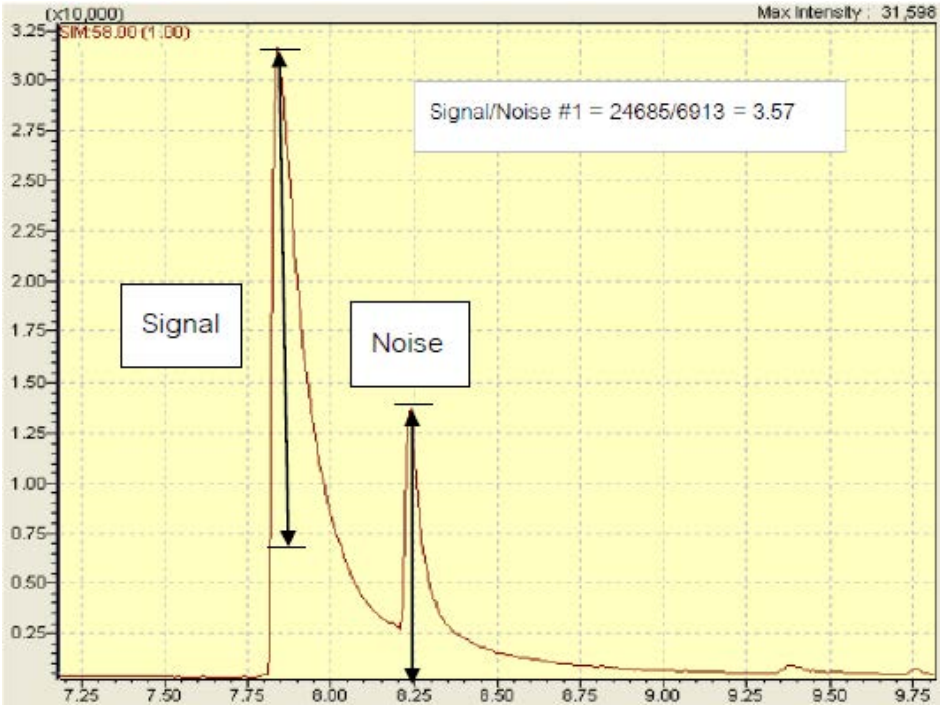

Figure 18 Chromatogram demonstrating LOD#1 of the method for determination of MA in vitreous humor samples (MA concentration = 25 ng/ml)

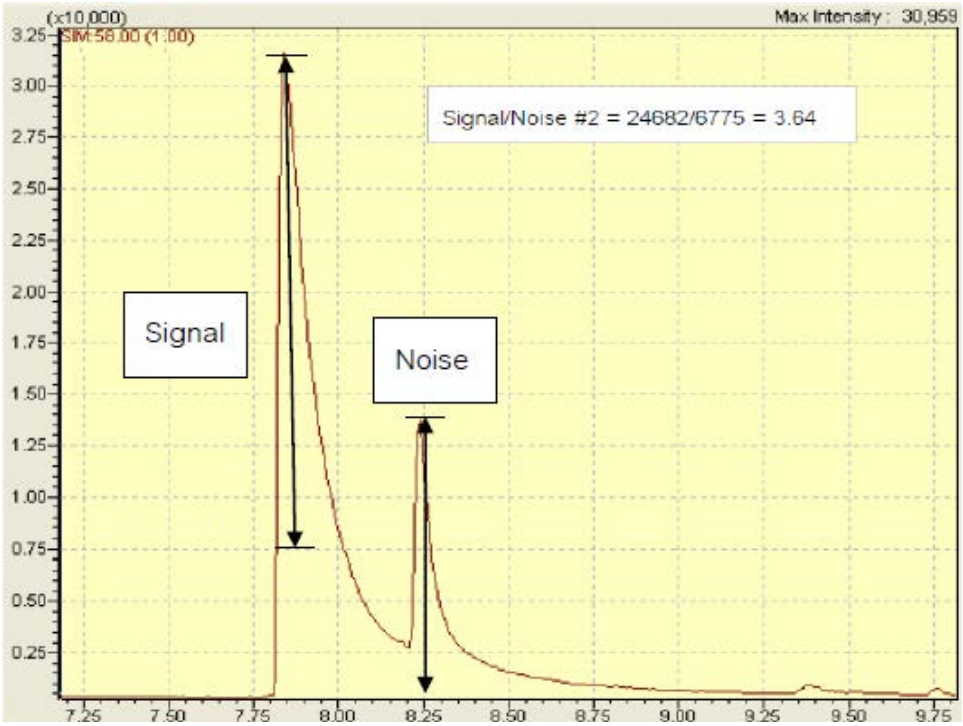

Figure 19 Chromatogram demonstrating LOD#2 of the method for determination of MA in vitreous humor samples (MA concentration = 25 ng/ml)

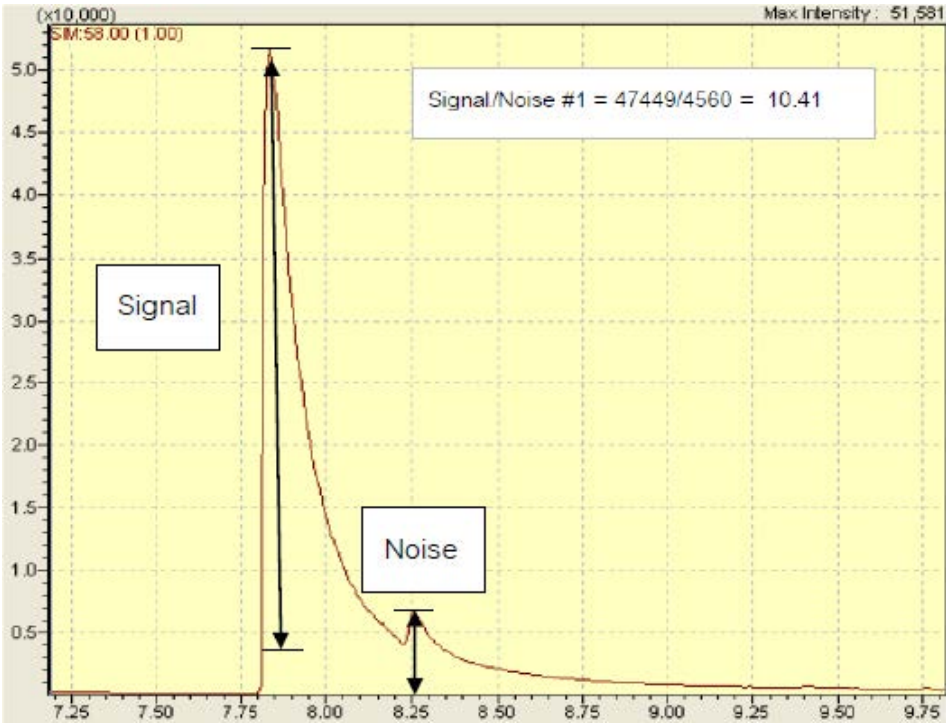

Figure 20 Chromatogram demonstrating LOQ#1 of the method for determination of MA in urine samples (MA concentration = 100 ng/ml)

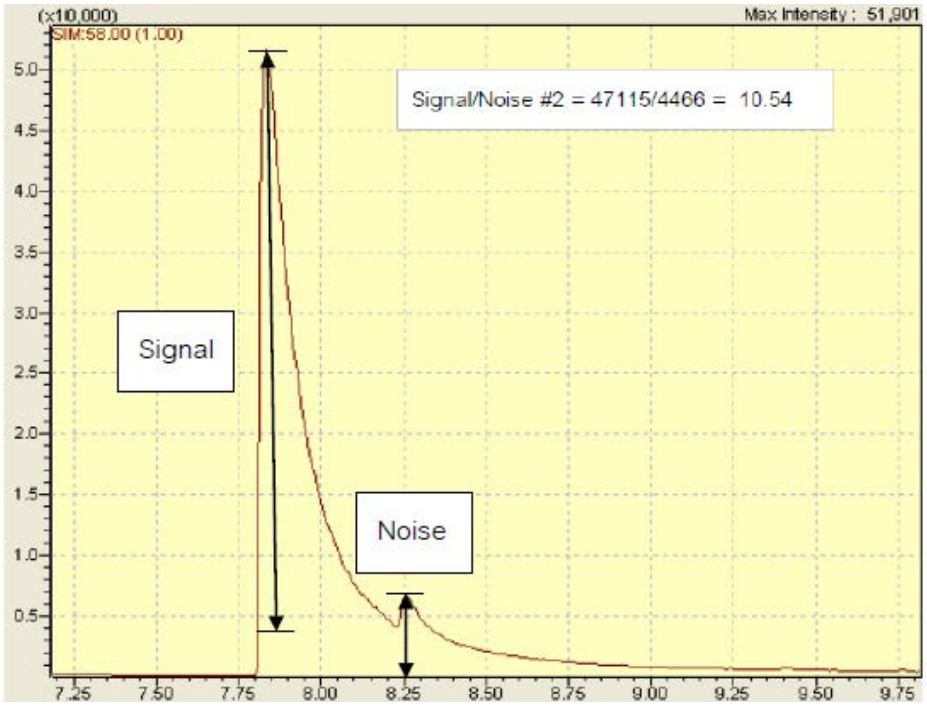

Figure 21 Chromatogram demonstrating LOQ#2 of the method for determination of MA in urine samples (MA concentration = 100 ng/ml)

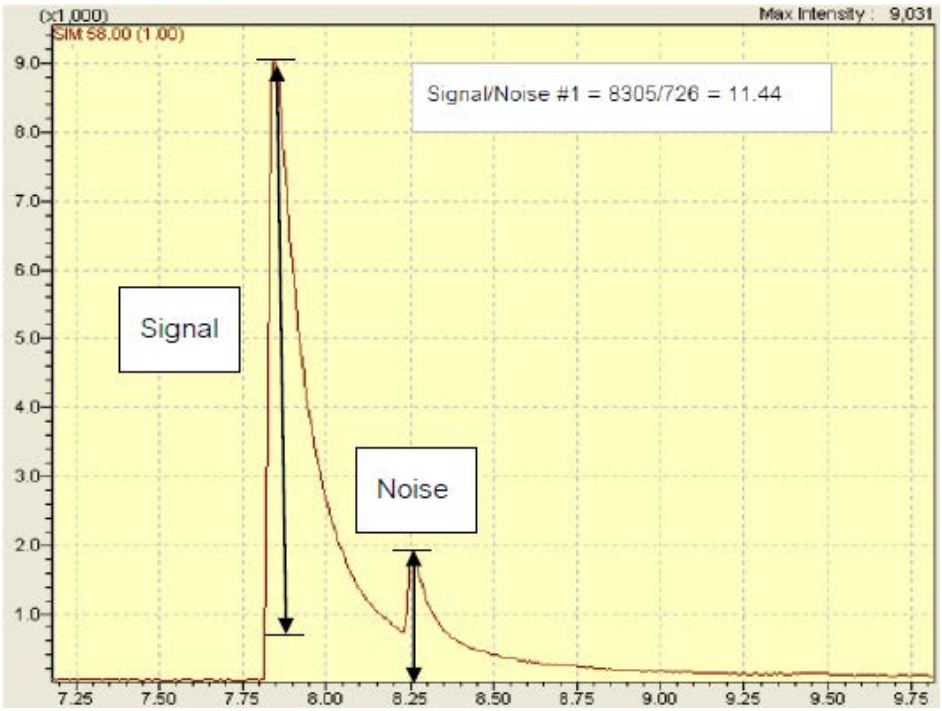

Figure 22 Chromatogram demonstrating LOQ#1 of the method for determination of MA in blood samples (MA concentration = 100 ng/ml)

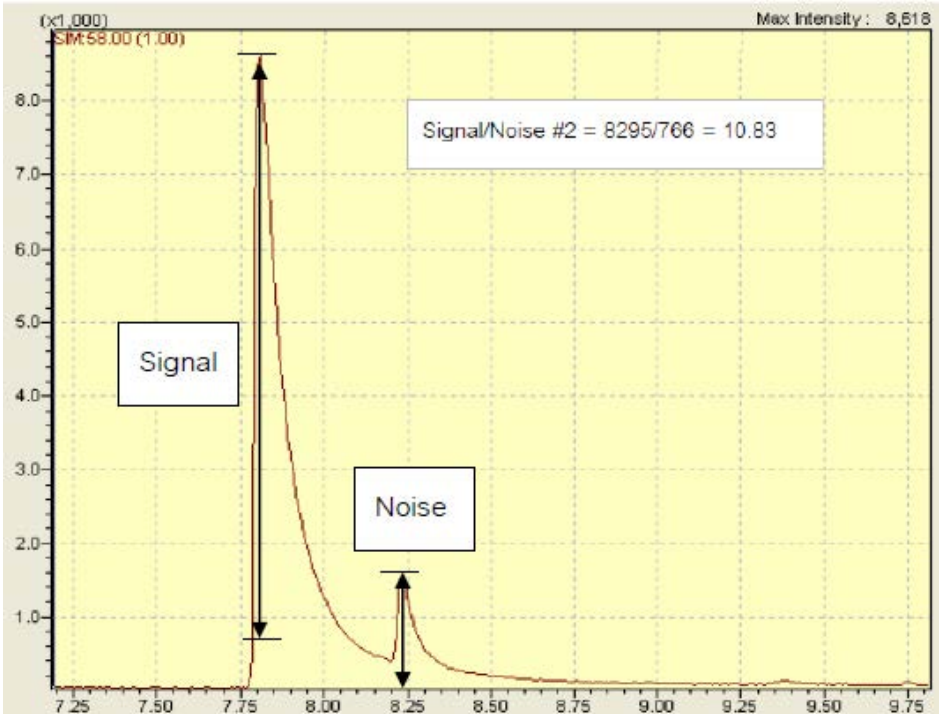

Figure 23 Chromatogram demonstrating LOQ#2 of the method for determination of MA in blood samples (MA concentration = 100 ng/ml)

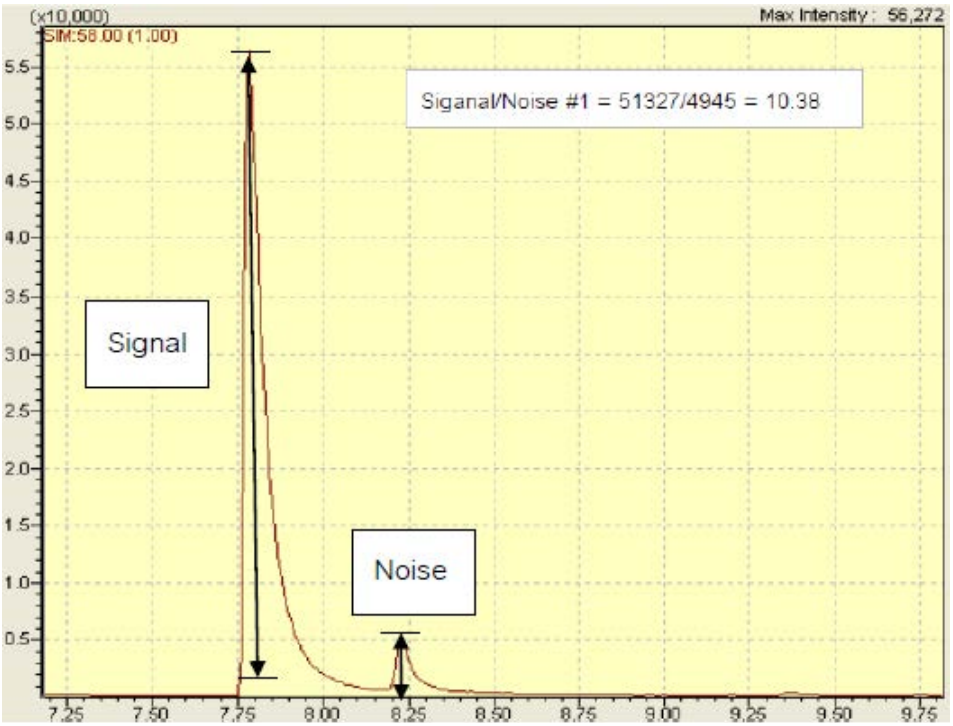

Figure 24 Chromatogram demonstrating LOQ#1 of the method for determination of MA in vitreous humor samples (MA concentration = 100 ng/ml)

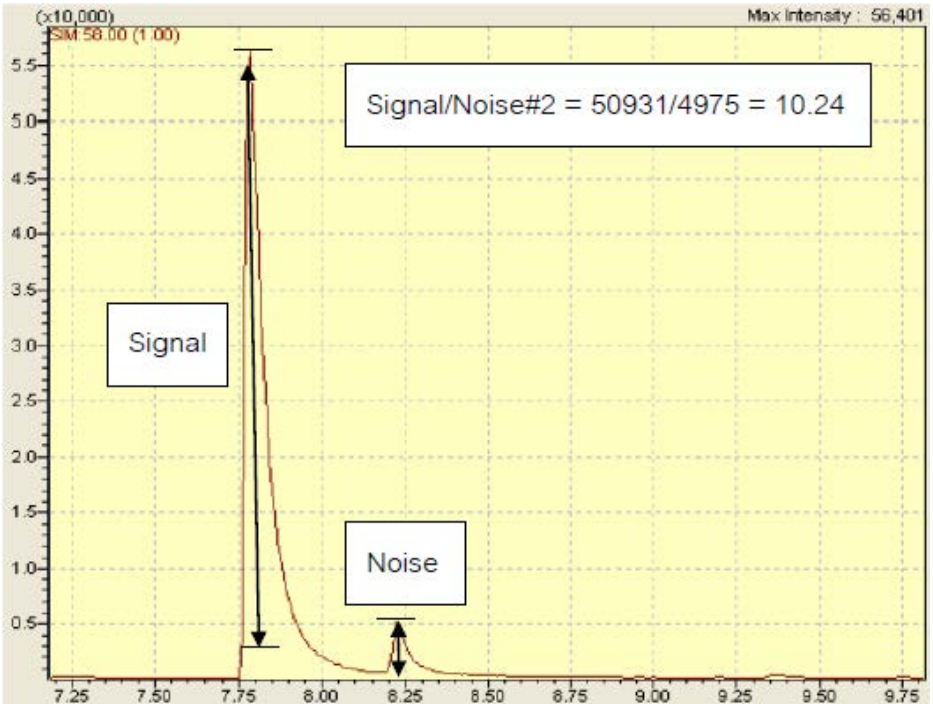

Figure 25 Chromatogram demonstrating LOQ#2 of the method for determination of MA in vitreous humor samples (MA concentration = 100 ng/ml)

Table 6 Accuracy, within- and between- day precision of the method using for determination of MA concentrations in urine, blood, and vitreous humor samples

Specimens	MA Concentrations (ng/ml)	Accuracy (%Recovery)[a]	Precision (%CV)	
			Within-day[b]	Between-day[c]
Urine	1000	99.63 ± 2.57	2.58	2.98 ± 0.50
	2000	98.68 ± 2.72	2.75	-
	4000	98.56 ± 1.89	1.91	-
Blood	200	90.04 ± 1.98	2.19	2.30 ± 0.50
	400	90.68 ± 1.95	2.15	-
	800	91.13 ± 1.43	1.57	-
Vitreous	1000	100.39 ± 8.00	7.96	6.25 ± 1.59
	2000	98.86 ± 3.26	3.29	-
	4000	98.43 ± 3.64	3.70	-

[a] The data shown were mean ± S.D. of n = 5.

[b] The data shown were calculated from mean and S.D. of n =5 within one day.

[c] The data shown were mean ± S.D. of n = 4 (4 days). The experiments were performed in triplicate in each day.

Table 7 Demographic profile of the subjects (n = 40)

No.	Sex	Ages (years)	Causes of death
1	M	37	Unknown
2	M	24	Hanging
3	M	NA	Unknown
4	M	NA	Unknown
5	M	20	Fall
6	F	31	Physical injury
7	M	34	Accident
8	M	30	Accident
9	M	NA	Drowning
10	M	37	Unknown
11	M	NA	Gunshot Wound
12	F	26	Gunshot Wound
13	M	30	Accident
14	M	NA	Electrocution
15	M	NA	Unknown
16	M	29	Accident
17	M	28	Unknown
18	M	29	Electrocution
19	M	16	Fall
20	M	31	Stab wound

No.	Sex	Age (years)	Causes of death
21	M	NA	Unknown
22	M	33	Hanging
23	M	45	Unknown
24	M	30	Accident
25	M	35	Hanging
26	M	37	Unknown
27	M	30	Unknown
28	M	NA	Unknown
29	M	27	Accident
30	M	20	Gunshot Wound
31	M	40	Unknown
32	M	25	Unknown
33	M	21	Electrocution
34	M	60	Unknown
35	M	26	Accident
36	M	24	Fall
37	M	30	Accident
38	M	28	Accident
39	M	NA	Accident
40	M	43	Accident

M = male

F = female

NA = not available

Table 8 Average MA concentrations in urine, blood, and vitreous humor samples (Data shown were in mean ± S.D. of duplicated experiments)

No.	Urine MA (ng/ml)	Blood MA (ng/ml)	Vitreous humor MA (ng/ml)
1	25770.29 ± 402.84	53.38 ± 0.81	877.48 ± 14.32
2	2301.26 ± 40.55	ND	ND
3	21264.43 ± 314.08	44.48 ± 0.76	673.93 ± 11.34
4	3604.91 ± 55.15	7.74 ± 0.13	65.69 ± 1.11
5	5157.54 ± 86.68	22.4 ± 0.35	123.69 ± 1.97
6	10128.80 ± 168.30	32.76 ± 0.50	417.14 ± 7.00
7	11430.96 ± 183.87	38.00 ± 0.62	419.14 ± 6.24
8	144715.99 ± 2440.13	316.42 ± 4.50	7961.95 ± 137.73
9	7391.41 ± 126.39	25.78 ± 0.40	189.95 ± 3.39
10	8996.30 ± 141.04	32.40 ± 0.56	403.65 ± 7.07
11	4709.12 ± 83.30	22.06 ± 0.34	116.26 ± 1.84
12	8953.27 ± 142.06	30.02 ± 0.48	380.82 ± 6.22
13	4265.14 ± 70.58	12.72 ± 0.22	104.44 ± 1.70
14	3267.46 ± 49.77	5.98 ± 0.09	45.60 ± 0.76
15	38492.33 ± 619.84	60.04 ± 1.13	1293.06 ± 21.21
16	2458.29 ± 34.73	ND	ND
17	21914.63 ± 381.83	50.04 ± 0.83	724.79 ± 14.14
18	7271.43 ± 127.27	24.56 ± 0.42	182.64 ± 3.17
19	8467.83 ± 135.01	29.88 ± 0.50	272.89 ± 4.80
20	26557.74 ± 452.43	53.80 ± 0.97	960.99 ± 11.58
21	57645.00 ± 856.72	75.02 ± 1.28	3701.21 ± 59.55
22	34742.35 ± 512.86	56.92 ± 0.98	1044.88 ± 18.52
23	15040.72 ± 241.02	39.46 ± 0.71	522.44 ± 9.33

Table 8 Average MA concentrations in urine, blood, and vitreous humor samples (Data shown were in mean ± S.D. of duplicated experiments) (cont.)

No.	Urine MA (ng/ml)	Blood MA (ng/ml)	Vitreous humor MA (ng/ml)
24	2104.31 ± 29.73	ND	ND
25	19298.95 ± 310.35	40.32 ± 0.67	646.53 ± 11.63
26	4229.87 ± 81.95	10.46 ± 0.17	102.45 ± 1.76
27	21903.19 ± 389.04	48.62 ± 0.84	705.34 ± 10.35
28	3053.94 ± 50.76	2.52 ± 0.04	42.62 ± 0.75
29	3357.80 ± 59.39	6.78 ± 0.10	50.86 ± 0.91
30	4041.26 ± 61.80	10.24 ± 0.16	85.87 ± 1.51
31	4349.21 ± 75.50	14.88 ± 0.23	107.12 ± 1.71
32	7425.29 ±112.27	28.00 ± 0.48	260.66 ± 4.53
33	40114.00 ± 567.09	60.16 ± 1.02	1843.02 ± 31.35
34	47264.93 ± 814.66	63.94 ± 0.99	1940.11 ± 32.38
35	49124.80 ± 950.38	73.78 ± 1.26	3009.93 ± 50.91
36	108903.19 ± 1627.97	82.20 ± 1.24	5991.61 ± 97.73
37	2179.71 ± 32.33	ND	ND
38	1132.02 ± 18.36	ND	ND
39	1459.99 ± 22.76	ND	ND
40	2079.31 ± 30.12	ND	ND
$\bar{X}$ ± S.E.	19914.22 ± 4627.70	44.70 ± 9.31	1068.76 ± 306.32
Range	1132.02 - 144715.99	2.52 - 316.42	42.62- 7691.95
n	40	33	33

ND = not detectable

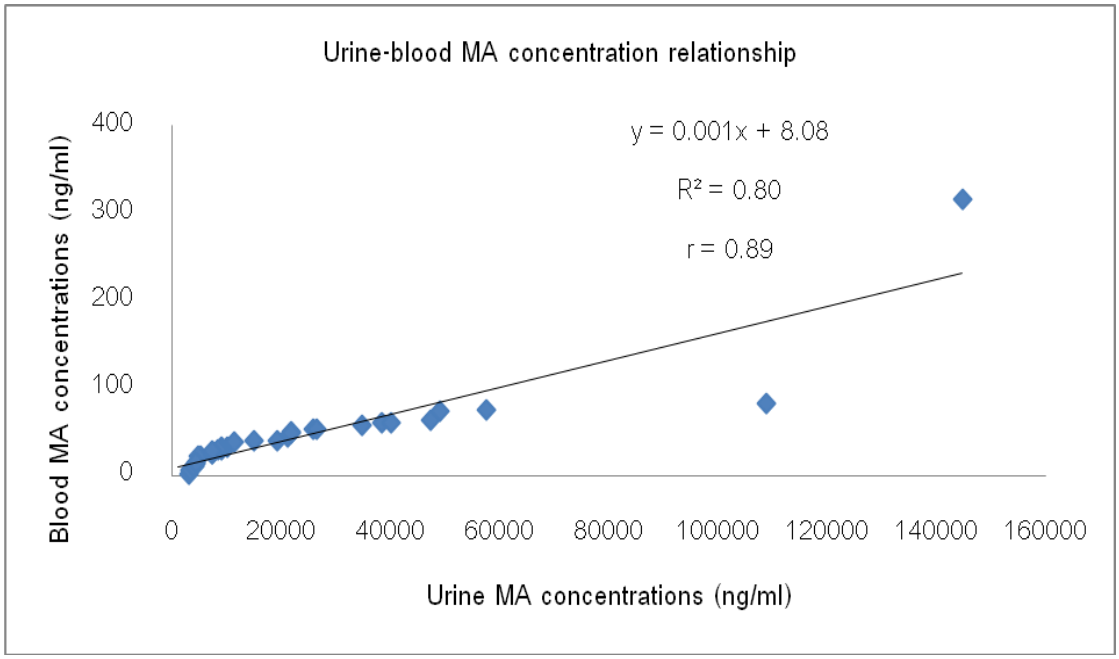

Figure 26 Relationship between MA concentrations in urine and blood samples (n = 33)

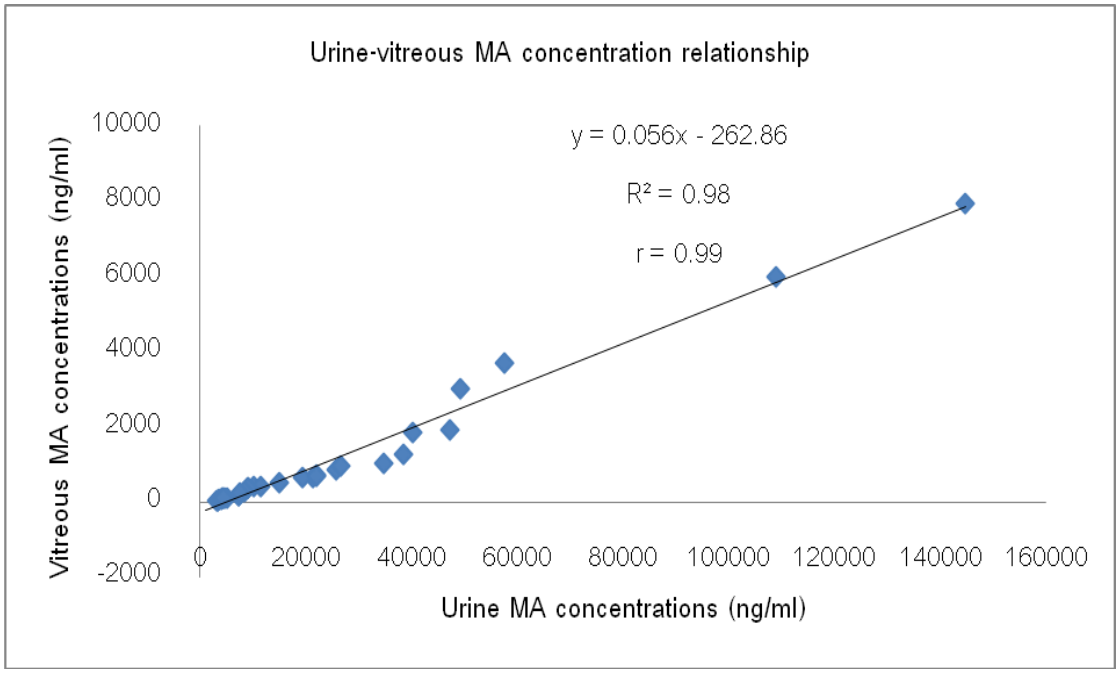

Figure 27 Relationship between MA concentrations in urine and vitreous humor samples (n = 33)

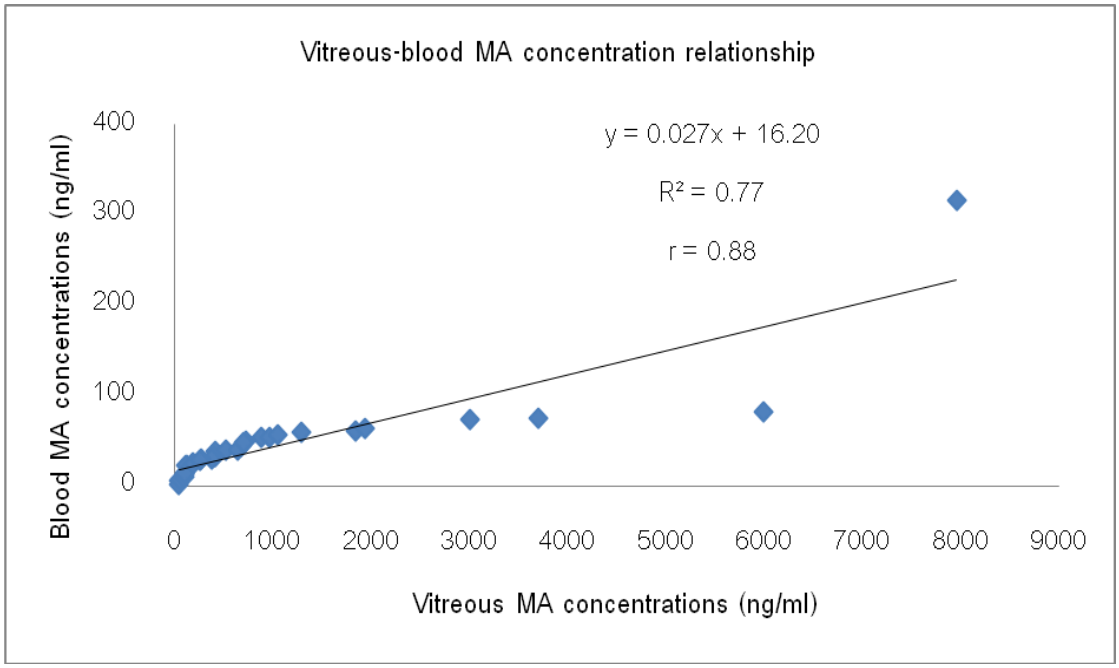

Figure 28 Relationship between MA concentrations in vitreous humor and blood samples (n = 33)

Table 9 Relationships between MA concentrations in urine, blood, and vitreous humor samples

Specimens	Equation	r	R^2	*p*-value*	Average MA concentration ratio
Urine-Blood	y = 0.001x + 8.08	0.89	0.80	0.000	493.44
Urine-Vitreous	y = 0.056x - 262.86	0.99	0.98	0.000	37.47
Vitreous-Blood	y = 0.027x + 16.20	0.88	0.77	0.000	17.21

*Correlation significant at 0.01 levels (2-tailed).

CHAPTER V

DISCUSSION AND CONCLUSION

Method validation

Before using the method for determination of MA concentrations in urine, blood and vitreous humor samples of the deceased, method validation was performed according to the guidelines (ICH, 1996; U.S. FDA, 2001; SOFT/AAFS, 2006). Linearity, LOD, LOQ, accuracy and precision both within- and between-day were tested. The results showed that linearity of the procedure shown by the correlation coefficient of 0.99 for all specimens which is acceptable according to the criteria suggested by the guidelines (ICH, 1996; U.S. FDA, 2001; SOFT/AAFS, 2006). LODs of the procedure for determination of MA in urine and vitreous humor were 25 ng/ml while that for blood sample was 2.5 ng/ml. This could be explained by the higher volume of blood sample used in the method. Four milliliters of blood sample were used in each SPME glass vial while 1 ml of urine or vitreous humor was used. Actually, 4 ml of urine sample can be used so as to lower the limit of detection as the blood sample. However, total volume of vitreous humor in both eyes of each person is approximately 4 ml (Levine and Jufer, 2008). In this study, vitreous humor could be practically collected up to 2 ml. Thus, vitreous humor could be used at the maximum volume of 1 ml for each of the experiment which was needed to perform in duplicate. As mentioned in the MATERIALS AND METHODS, the procedure used to determine MA concentrations in urine and vitreous humor samples in this study was modified from the method of Myung et al. (1998). In that study, they demonstrated the LOD of the method was 10 ng/ml whereas LOD of the method shown in this study was 25 ng/ml. This somewhat difference of LOD between studies could contribute from any of these contributing factors: volumes of the specimens (3 ml *vs* 1 ml); differences of extraction process by SPME (direct immersion-SPME *vs* head space-SPME); GC-MS conditions (e.g. length of column, oven temperature, etc); interlaboratory variation, etc. Those two extraction processes provided different advantages and disadvantages. Regarding the direct immersion-

SPME technique, the fiber directly contacted with the sample in sample phase and all kinds of molecule dissolved in the sample phase were adsorbed. Thus, more concentrated analyte molecules were likely to be adsorbed and injected to the column resulting in lower LOD. However, undesired molecules such as water, impurities which could damage the column could also be adsorbed. For HS-SPME technique, only small vaporized molecules could be adsorbed, less concentrated analyte molecules could be injected to the column resulting in higher LOD. On the other hand, this technique more prolonged the column lifetime because of reducing chance of undesired molecules exposure to damage the column. Determination of MA concentrations in blood samples was modified from the method of Namera et al. (2000). In that study, LOD of the method was shown to be 5 ng/g. Because an average density of whole blood is approximately 1.060 g/ml (Cutnell and Kenneth, 1998: 308), thus, LOD of the method reported by Namera et al. (2000) was approximately 5 ng/ml. LOD of the method demonstrated in this study was 2.5 ng/ml which was quite comparable to the value reported by Namera et al. (2000). Somewhat difference of the LOD between studies could be explained by some differences between these 2 studies such as volume of the whole blood used (0.5 g or 0.5 ml *vs* 4 ml); utilization of derivatizing agent *vs* non-derivatizing agent method; as well as interlaboratory variation, etc. Regarding the accuracy and precision test, the results showed that accuracy of the procedure as presented by the % recovery as well as % C.V. of both within- and between-day precision were within 15% which are suggested by the guidelines (ICH, 1996; U.S. FDA, 2001; SOFT/AAFS, 2006).

Relationships between MA concentrations in urine, blood, and vitreous humor samples

In this study, urine, blood and vitreous humor samples were mostly collected from male deceased (95%) whereas the remaining (5%) was female. Mean ± S.D. of the age of all deceased was 30.84 ± 8.56 years (range of 16 - 60 years). Majority of the subjects' cause of death was unknown (35%). Urine MA concentrations of all deceased were higher than 1 μg/ml (1,000 ng/ml). MA concentrations in urine were far higher than the corresponding MA concentrations in blood. Mean ± S.E. of MA

concentrations in urine, blood and vitreous humor samples were 19914.22 ± 4627.70 ng/ml (range = 1132.02 - 144715.99 ng/ml), 44.70 ± 9.31 ng /ml (range = 2.52 - 316.42 ng/ml), and 1068.76 ± 306.32 ng/ml (range = 42.62 - 7691.95 ng/ml), respectively. To determine the correlations between MA concentrations in urine, blood and vitreous humor, the data of 33 from the total of 40 deceased were used because blood MA concentrations of 7 cases were lower than the limit of detection of the method used in this study. The results showed that MA concentrations in urine, blood and vitreous humor samples were linearly correlated with a correlation coefficient (r) of 0.89 (urine *vs* blood, p-value < 0.05), 0.99 (urine *vs* vitreous humor, p-value < 0.05) and 0.88 (vitreous humor *vs* blood, p-value < 0.05). The corresponding linear regression equations were y = 0.001x + 8.08, y = 0.056x - 262.86, and y = 0.027x + 16.20, respectively.

The results showed that MA concentrations in urine were far higher than the corresponding MA concentrations in blood. This is consistent to a previous study of Lebish, Finkle and Brackett (1970). Actually, peak plasma MA concentration was shown to occur after 4 hours and 2.5 hours via intranasal administration and smoking, respectively (Hart et al., 2008, Perez-Reyes et al., 1991) and approximately 70% of MA dose was excreted in urine within 24 hours (Cruickshank and Dyer, 2009). In addition, MA could be detected in urine several days (7 days) after repeated MA doses (Oyler et al., 2002; Connell et al., 1958). In this study, if MA concentrations in blood were not the toxic level or the cause of death, urine sample collections which were performed after death, were not supposed to be performed at the time close to the time after MA use. Thus, MA concentrations in urines were found with higher concentrations than in blood samples. The reports regarding toxic/fatal MA concentrations in blood vary among studies. Toxic concentrations of blood MA were ranged from 0.2 - 5.0 mg/ml (200,000 ng/ml – 5,000,000 ng/ml) (Nagata, 1983; Winek, Wahba, and Winek Jr, 2001; Schulz and Schmoldt, 2003). Fatal blood MA concentrations were reported as > 10 mg/ml (10,000,000 ng/ml) (Nagata, 1983; Winek et al., 2001; Schulz and Schmoldt, 2003) or > 0.5 mg/L (500 ng/ml) (Logan, Fligner, and Haddix, 1998). These variations could be due to differences of route of administration, amount and purity of the substance, co-

administrated drugs/substances, physiological condition of the individual as well as ethnicity which can influence CYP 2D6 polymorphism. (He et al., 1996; Logan et al., 1998; Matoba, 2001; Ago M, Ago K, and Ogata, 2006; Inoue et al., 2006). However, blood MA concentrations of all the subjects in this study (Mean ± S.E. of blood MA concentrations = 44.70 ± 9.31 ng /ml) were lower than the reported toxic/fatal concentrations. Thus, MA exposures of the subjects in this study were possibly a contributing factor not the direct cause of death.

In this study, MA concentrations in vitreous humor were higher (17.21 fold) than in blood. Distribution of MA in postmortem was assessed by McIntyre et al. (2011). They found that the mean ratio of MA concentrations in vitreous humor to peripheral blood was 1.63 while this ratio was shown to be 17.21 in this study using blood collected from basilar artery not peripheral blood. Actually, the mean of MA concentrations in vitreous humor in this study was comparable to that found in the study of McIntyre et al. (2011). The difference between these 2 studies was the difference of MA concentrations in blood as shown by the mean ± S.E. of 630.55 ± 119.87 ng/ml (using peripheral blood in the study of McIntyre et al., 2011) and 44.70 ± 9.31 ng /ml (using blood collected from basilar artery). Thus, the ratio of MA concentrations in vitreous humor to blood in this study was far higher than that reported by McIntyre et al. (2011). MA concentrations in peripheral blood and vitreous humor are not markedly different (McIntyre et al., 2011). The markedly lower concentrations of MA in blood collected from basilar artery as compared to those in vitreous humor found in this study could be probably due to the difference types of blood vessels (vein *vs* artery). Thus, MA concentrations in blood in basilar artery were much lower than in peripheral artery. The closely linear relationships between MA concentrations in vitreous humor and other specimens of particular purpose: urine for forensic purpose and blood samples for physiological interpretation, suggest that vitreous humor can be used as an alternative to urine or blood samples in the situation that both samples are not available or contaminated.

Vitreous humor possesses several advantages. Collection of this specimen is easy even if an autopsy is not completely performed. Due to clear and mainly consists of water (99%), vitreous humor is easy to analyze with reduced time and less requirement of sample preparation. Analytical method which is developed for urine or blood can be adapted to vitreous humor. Drug and substance stability in vitreous humor is higher as compared to other fluids. Putrefaction, charring, and trauma may affect sample quality. Tyramine and phenethylamine, decomposition products, may interfere both blood and tissues extraction and analysis. These situations less occur with vitreous humor due to its anatomically isolated location. Even though trauma and severe major organ damage occur, an available specimen such as cavity blood, is potentially contaminated from tissues or stomach contents. In this situation, vitreous humor may be useful as a promising specimen.

Further study to verify the linear regression equations obtained from this study is suggested. This could be simply performed by using the specimens (urine, blood and vitreous humor samples) collected from other unrelated deceased. All specimens are analyzed for MA concentrations by the same procedure as in this study. Calculated MA concentrations of each sample can be obtained by calculation using the linear regression equations. Then, the calculated MA concentrations are statistically analyzed compared to the actual MA concentrations.

In conclusion, MA concentrations in urine, blood and vitreous samples collected from 33 Thai deceased were linear correlated with a correlation coefficient (r) of 0.89 (urine vs blood), 0.99 (urine vs vitreous humor) and 0.88 (vitreous humor vs blood). The corresponding linear regression equations were $y = 0.001x + 8.08$, $y = 0.056x - 262.86$, and $y = 0.027x + 16.20$, respectively. This relationship is preliminarily advantageous for prediction of MA concentrations in urine from MA concentration in blood sample while urine sample is not available or vice versa. However, application of this study should be under inclusion and exclusion criteria of subjects as described in Chapter III. Also, vitreous humor can be used as an alternative to blood and urine

samples for determination of MA concentrations in case that both samples are not available or contaminated.

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