STABILITY OF METHAMPHETAMINE CONCENTRATIONS IN BILE SAMPLES UNDER EXPERIMENTAL CONDITION

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STABILITY OF METHAMPHETAMINE CONCENTRATIONS IN BILE SAMPLES UNDER EXPERIMENTAL CONDITIONS

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ABSTRACT

In forensic laboratories, bile is an alternative specimen that can be used in a comprehensive toxicological analysis. In this study, bile samples were prepared for a stability study and for optimization of extraction conditions. Methamphetamine was spiked into bile to make five concentrations: 2, 5, 10, 15 and 20 µg/ml, respectively. These samples were stored at 4 ºC and RT for different fixation periods (0, 1, 3, 5, 7, 14, 28, 60 and 180 day) for stability study. Then, these samples were extracted with dichloromethane, before being analyzed by gas chromatography-nitrogen phosphorus detector (GC-NPD).

The study reveals that the methamphetamine concentration showed no significant difference in either condition (4ºC and RT), when compared with the initial day (p-value≥0.05). The same result of percent concentration showed no significant loss when compared between 4ºC and RT. These results demonstrate no significant difference for methamphetamine analysis in bile during storage for 6 months. There are no different analytical results on methamphetamine when the storage condition studied between at 4ºC and RT was compared. Therefore, bile samples containing methamphetamine can be stored for analysis at 4ºC and RT condition for 6 months (180 days).

KEY WORDS: METAMPHETHAMINE/ BILE / STABILITY

58 pages

ความคงตัวของความเข้มข้นเมทแอมเฟตามีนในตัวอย่างน้ำดีภายใต้สภาวะการทดลอง

THE STABILITY OF METHAMPHETAMINE CONCENTRATION IN BILE SAMPLE

UNDER EXPERIMENTAL CONDITION

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บทคัดย่อ

ในห้องปฏิบัติการทางนิติเวช น้ำดีเป็นสิ่งส่งตรวจที่สามารถนำมาใช้ในการตรวจสาร
เสพติด ซึ่งการศึกษาครั้งนี้ได้ทำการตรวจวิเคราะห์ เมทแอมเฟตามีน เพื่อศึกษาเสถียรภาพของความ
เข้มข้นเมทแอมเฟตามีนในน้ำดี รวมทั้งหาวิธีการสกัดที่เหมาะสมสำหรับเมทแอมเฟตามีนในน้ำดี
ซึ่งพบว่าการสกัดด้วย Dichloromethane จากน้ำดีที่ถูกเจือจางด้วยน้ำเกลือในสภาวะด่างนั้น เป็นวิธีที่
เหมาะสมที่สุด ในส่วนของการศึกษาเสถียรภาพนั้น ทำโดยการเติมเมทแอมเฟตามีนลงในน้ำดี ให้
ได้ความเข้มข้นต่างๆ (2, 5, 10, 15 และ 20 μg/ml) และเก็บรักษาไว้ที่ 4 ℃ และอุณหภูมิห้อง ใน
ระยะเวลาต่างๆ (0, 1, 3, 5, 7, 14, 28, 60 และ 180 วัน) จากนั้นทำการตรวจวิเคราะห์ด้วยเครื่องแก๊ส
โครมาโทกราฟี-ไนโตรเจนฟอสฟอรัสดีเท็คเตอร์ ผลการทดลองพบว่าระดับของแมทแอมเฟตามีน
ไม่มีความแตกต่างกันอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับวันแรกของการทดลอง (p-value $\geq$ 0.05)
ของทั้งสองสภาวะการทดลอง และเมื่อเปรียบเทียบเปอร์เซ็นต์ความเข้มข้นของเมทแอมเฟตามีนที่
ได้เก็บรักษาไว้ระหว่างสองอุณหภูมิ ผลการศึกษาพบว่าไม่มีความแตกต่างกันอย่างมีนัยสำคัญ (p-
value $\geq$ 0.05) ของความเข้มข้นเมทแอมเฟตามีนเช่นเดียวกัน ซึ่งแสดงให้เห็นว่าเมทแอมเฟตามีน
สามารถคงตัวอยู่ในน้ำดีที่เป็นตัวอย่างส่งตรวจ ได้ในช่วงระยะเวลาประมาณ 180 วัน (6 เดือน)

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LIST OF ABBREVIATIONS

%	=	percent
˚C	=	degree celsius (centigrade)
RT	=	room temperature
mg.	=	milligram
ml.	=	milliliter(s)
min	=	minutes
μg	=	microgram
μm	=	micrometer(s)
LLE	=	liquid-liquid extraction
SPE	=	solid-phase extraction
pH	=	potential of hydrogen ion

CHAPTER I
INTRODUCTION

Methamphetamine or "meth" is a compound with strong activity on central nervous system. Meth is the most common drug of abuse throughout the world, including in Thailand. The drug is produced easily in illegal clandestine laboratories. Due to this property it became the cause of serious social and law enforcement problems (1). That is the reason why there is an increasing interest in analytical methods for the determination of this drug, especially in biological matrices. Most forensic science laboratories analyse methamphetamine in urine and blood because the sample collection and extraction is simple (2). However, the analysis in urine and blood has limitation such as in case of empty bladder before dying that cause inadequate specimen for analysis and in case of the absence of drugs from analysis in blood and urine but presence of drugs found in bile depending on pharmacokinetic and rate of metabolism in each drug. Moreover, the concentration of drugs or their metabolites in bile are generally several folds higher than those in blood concentrations (3). Therefore, bile is alternative biological sample for drug abuse analysis because most drug abuse and their metabolites excreted in bile. Biliary excretion is an important route for the elimination of some drugs and drug metabolites in humans which occurs along a pathway from the liver to the duodenum with storage in gall bladder. In addition to excretion of bile all the time and remains for a while after dead, Therefore, the sample of bile can be used in the comprehensive toxicological analysis and complementary to the other fluids or matrices.

At present, analysis of drug abuse and poisonous substances in forensic science laboratories must have procedure for biological specimen preservation to keep them stable. i.e., in case of long weekend storage or when there are questions about the analysis report and more substance analysis is required. Therefore, the suitable method of sample storage for analysis and retesting is required. The storage time is about 180 days or 6 months after the crime or after performing autopsy until prosecutor file to

court as mentioned in procedure criminal law code 150. Long-term biological specimen preservation may cause degeneration of drug abuse and all poisonous substances that affect the proper interpretation of analytical results. Loses of analytes due to thermal or chemical degradation, enzymatic metabolism, hydrolysis, or the presence of interfering compounds due to severe matrix degradation, may take place due to improper transport, handling or sample storage conditions(4). As a consequence of analytes instability, reliability of analytical results is severely compromised. Stability testing can be used to explain discrepancies between reanalysis long after initial analyses and may help to determine time limits that must be imposed between the collection and identify the optimal storage conditions for specimens retained in forensic laboratories. For these reasons, stability assessment is considered a fundamental parameter for the validation of bioanalytical methods. So the stability study of methamphetamine in bile has a very important role in forensic science laboratories.

CHAPTER II
OBJECTIVE

Objective

The objective of this work is to study the stability of methamphetamine concentration in bile sample under experimental condition.

Specific objective

1. To study methamphetamine concentration in bile during storage time
2. To examine the stability of methamphetamine in bile stored in different temperatures at $4^{o}C$ and room temperature.

CHAPTER III
LITERATURE REVIEW

Part I. Methamphetamine

1. Introduction (5, 6)

Methamphetamine is one in group of drugs stimulating the central nervous system. It was developed in the last century from its parent drug amphetamine and was originally used in nasal decongestants, bronchial inhalers, and in the treatment of narcolepsy and obesity. At present the drug is made easily in clandestine laboratories with relatively inexpensive over-the-counter ingredients and synthesized via simple reactions using readily available chemicals and over-the-counter cold medicines such as ephedrine and pseudoephedrine. These factors combine to make methamphetamine a drug with high potential for widespread abuse.

The recent methamphetamine abuse is characteristically involving young generation and still the main problem in Thai society. The drug is being smuggled from China, Korea Peninsula and the so-called "Golden Triangular Zone", where borders of Myanmar, Thailand and Laos are intercrossing (7).

2. Chemistry (8, 9)

2.1 Nomenclature

Methamphetamine is the common name for N-dimethylphenethylamine, also referred to as desoxyephedrine, methyl-amphetamine, phenyl-isopropyl methylamine, and a variety of other similar systematic names. Moreover, there are a variety of popular terms including "speed", "meth" and "chalk". It usually comes in the form of a white crystalline powder and is snorted, injected, smoked or taken orally. It also comes in solid crystal form, known as "crystal meth", "ice", "crystal" and "glass" which are usually smoked, though it can also be injected.

2.2 Physico -Chemical Properties

2.2.1 Empirical Formula $C_{10}H_{15}N$
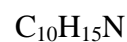
2.2.2 Chemical Structure

Methamphetamine's chemical structure is similar to that of amphetamine (Shown in figure 1), but it has more pronounced effects on the central nervous system. Like amphetamine, it causes increased activity, decreased appetite, and a general sense of well-being.

Figure 1 Structures of the synthetic sympathomimetics amphetamine and methamphetamine (10).

2.2.3 Chemical Properties

Methamphetamine exists in two isomeric forms, dextro (*d-*), and levo (*l-*), and these may appear as prefixes to any of the terms discussed above to denote the particular isomer. Methamphetamine being abused is largely its *d-*form, but the *l*-form has occasionally become detectable from specimens of abusers and *d-*isomer of this optically active amine shows greater physiological effect than the *l*-form.

The free base (pKa 9.9) has a molecular weight of 149.24 a.m.u., and is a liquid at room temperature, so is invariably supplied and used as the hydrochloride salt ($C_{10}H_{16}C_lN$, 185.74 a.m.u.), which has a melting point of 170-175°C.

2.2.4 Physical Properties

Methamphetamine is easily dissolved in water (1gm: 2ml), in alcohol (1gm: 3ml), in chloroform (1gm: 5ml) and very slightly soluble in absolute ether. It is a white, odorless, bitter-tasting, crystalline powder and it is emerging in the

form of brightly colored pills, reminiscent of another popular and dangerous club drug, Ecstasy. These pills, known as Ya Ba (Thai for "crazy drug"), are a powerful and very pure form of methamphetamine

3. Pharmacology and Toxicology (9)

3.1 Pharmacodynamics

Methamphetamine is a sympathomimetic drug, meaning that it mimics endogenous transmitters in the sympathetic nervous system by interaction with their receptors and increases synaptic levels of the neurotransmitters. The structural neurotransmitters similarities of methamphetamine are the dopamine, serotonin (5-HT) and norepinephrine. Example, possible mechanism for the neurotoxicity produced by methamphetamine (Shown in figure 2). This amine enters dopamine neurones/terminals *via* the dopamine transporter (DAT) and then releases dopamine from vesicles and prevents storage there by blocking the vesicular transporter (VMAT2). Methamphetamine also inhibits the activity of mitochondrial MAO involved in the catabolism of dopamine while increasing its synthesis by stimulating tyrosine hydroxylase. The net result is an increase in cytostolic dopamine, which may be metabolised to reactive dopamine metabolites, resulting in neuronal damage and degeneration.

Specifically, methamphetamine interacts with presynaptic receptors by competitive antagonism, and has minimal, if any, effect as an agonist at postsynaptic receptors. Norepinephrine is responsible for methamphetamine's alerting, anorectic, locomotor and sympathomimetic effects; dopamine stimulates locomotor effects, psychosis, and perception disturbances; and 5HT is responsible for delusions and psychosis. Methamphetamine's effects are similar to cocaine but its onset is slower and the duration is longer (11).
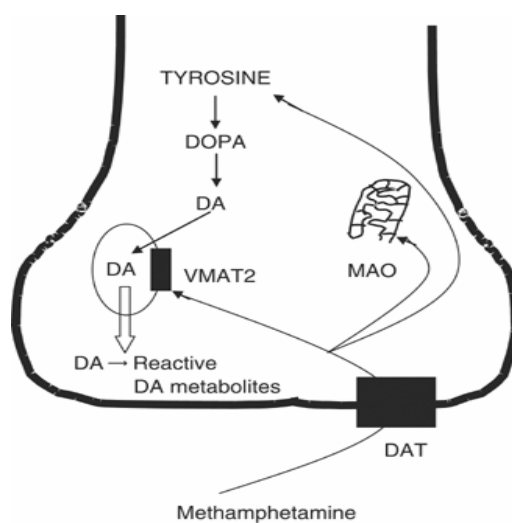
Figure 2 Mechanism for the neurotoxicity produced by methamphetamine (12).

3.2 Toxicity (13)

The toxic dose varies considerably due to individual variations and the development of tolerance. Fatalities have been reported following ingestion of doses as low as 1.3 mg/kg, while tolerance has been developed to1000 mg at a time and up to 5 g in a day.

Human data

Adults

1 to 4 grams of street methamphetamine was ingested by a 27 years old man; blood pressure was 180/100 mmHg, pulse rate 146/min, respirations 26/min and oral temperature 37°C, pupils widely dilated and reactive to light. (14) After intravenous injection of an unknown amount of methamphetamine by an adult patient, he was lethargic with restless movements of extremities and hyperresponsive to stimuli. The blood pressure was 120/80 mm/Hg and the pulse rate was 150 beats/min with loud heart sounds. Deep-tendon-reflexes were hyperactive (15).

Children

Children appear to be more susceptible than adults and are less likely to have developed tolerance.

Relevant animal data

  Monkey

    Adult monkeys have an LD_{50} of 15 to 20 mg/kg, whereas for young monkeys the LD_{50} is only 5 mg/kg.

  Mice

    The lethality was measured 24 hours after intraperitoneal injection of methamphetamine at dose from 10 to 100 mg/kg. Confinement and aggregation markedly increase lethality of methamphetamine in mice (16).

  Pregnant Sheep

    Received 1.2 mg/kg appeared anxious,with quick side to side movements of the head and feet (17).

3.3 Potency, Purity and Dose (18)

Purity of methamphetamine is currently very high, at 60-90%, and is predominantly *d*-methamphetamine which has greater CNS potency than the *l*-isomer or the racemic mixture. Common abused doses are 100-1000 mg/day, and up to 5000 mg/day in chronic binge use. Therapeutic doses of Desoxyn® are 2.5-10 mg daily, with dosing not exceed 60 mg/day. To treat narcolepsy, 5-60 mg/day of amphetamine is ingested in divided doses; and in ADD and ADHD doses of 2.5-10 mg/day is administered, depending on age.

3.4 Effect of use (8)

3.4.1 Short term effect

As powerful stimulant, methamphetamine, even in small doses, can increase wakefulness, physical activity and respiration, along with decrease fatigue and appetite. A brief, intense sensation, or rush, is reported by those who smoke or inject methamphetamine. Oral ingestion or snorting produces a long- lasting high instead of a rush, which reportedly can continue for as long as half a day.

3.4.2 Long term effect (19)

Long-term methamphetamine abuse results in many damaging effects, including addiction. Addiction is a chronic, relapsing disease, characterized by

compulsive drug seeking and drug use, which is accompanied by functional and molecular changes in the brain. In addition to being addicted to methamphetamine, chronic methamphetamine abusers exhibit symptoms that can include violent behavior, anxiety, confusion, and insomnia. They also can display a number of psychotic features, including paranoia, auditory hallucinations, mood disturbances, and delusions (for example, the sensation of insects creeping on the skin, called "formication"). The paranoia can result in homicidal as well as suicidal thoughts.

Additional problems may also arise, such as rapid, irregular heartbeat, damage to small blood vessels in the brain causing an elevated risk for stroke and extreme weight loss. An overdose of meth can produce extreme hyperthermia, convulsions and death.

4. Metabolism (9)

Methamphetamine undergoes phase I metabolism by N-demethylation to amphetamine via the cytochrome P4502D6 isoenzyme system. Amphetamine itself is extensively metabolized to a variety of metabolites, including norephedrine and p-hydroxyamphetamine, both of which are pharmacologically active, and may be glucuronidated prior to excretion.

Several other drugs are metabolized to amphetamines. Example, Benzphetamine (Didrex [®]), is metabolized to desmethylbenzphetamine, but also to d-methamphetamine and amphetamine, making enantiomeric resolution of limited value in determining the origin of the amphetamines in these cases. Selegeline (Deprenyl[®]), a drug given in the treatment of Parkinsonis disease, is unusual in that it is rapidly metabolized to *l*-amphetamine and *l*-methamphetamine, generally in equivalent amounts. Fenethylline (Catpagon [®]), used in the treatment of attention deficit disorder, is metabolized to *d,l*-amphetamine. Other drugs metabolized to amphetamine include furfenorex , mefenorex ,fenproprex , and prenylamine ,etc (20).

In summary, there are many drugs that metabolize to amphetamine or methamphetamine. Analysis of the specific enantiomer present, together with tests for the specific metabolite of the ingested drug and a consideration of the concentration of methamphetamine or amphetamine present, will all provide clues as to the origin of themeasured drug (20, 21).

5. Pharmacokinetics (9, 22)

5.1 Absorption by route of exposure

Amphetamine is rapidly absorbed after oral ingestion. Peak plasma levels occur within 1 to 3 hours, varying with the degree of physical activity and the amount of food in the stomach. Absorption is usually complete by 4 to 6 hours. Methylamphetamine is readily absorbed from the GI tract and effects persist for 6 to 12 hours and may persist up to 24 hours after large doses (23).

5.2 Distribution by route of exposure

Amphetamines are concentrated in the kidney, lungs, cerebrospinal fluid and brain. They are highly lipid soluble and readily cross the blood-brain barrier. Protein binding and volume of distribution varies widely, but the average volume of distribution is 5 L/kg body weight.

5.3 Elimination and excretion

Normally 5 to 30 % of a therapeutic dose of amphetamine is excreted unchanged in the urine by 24 hours, but the actual amount of urinary excretion and metabolism is highly pH dependent (24). Alkaline urine will significantly increase the drug half-life.

Part II. Forensic specimens

1. Introduction

Many biological specimens have been utilized for analysis for the presence of drugs of abuse. The forensic toxicologist provides for the isolation of chemicals from biological specimens and the subsequent analysis to determine whether a chemical agent played a role in the cause of death. At autopsy, any body fluid and tissue can be used for analysis; blood, urine, bile, stomach contents and the liver are being well used (25, 26). For assessment of intoxication degree, the blood levels of drugs and poisons are usually used. Urine has been and remains the most widely used body fluid specimen for routine testing for drugs of abuse, contains large amounts of metabolites and/or an unchanged form of a toxin; it contains low levels of proteins, which usually interfere with analysis, and thus is suitable for screening tests using

immunoassays without tedious pretreatments. Stomach contents can be a useful specimen for identification of a toxin, only when the time after ingestion is short; it contains a large amount of an unchanged form of a compound ingested (26-28).

However, there are many cases, in which neither blood, urine nor stomach contents can be obtained, because of various reasons. Even with urine, illegal drugs become undetectable several days after their administration. In recent years, remarkable advances in sensitive analytical techniques have encouraged the analysis of drugs in unconventional biological samples such as hair, saliva and sweat (26, 28, 29) .Nails and hair are good specimens for long-term detection of drugs and poisons or chronic use; it is possible to analyze a compound many days later, while the analysis of sweat or oral fluid has an advantage of noninvasive collection (26, 27, 30-32). These samples are proving to be useful for toxin analysis, because many toxins are excreted into these specimens and easily collected, although drug levels are often lower than the corresponding levels in urine or blood. Each specimen provides different information about time and extent of use and likelihood of impairment. All of these specimens lend themselves to accurate analysis of drug and/or metabolite levels through conventional scientific techniques (26, 29-30).

2. Bile

2.1 Physiology

Bile mainly consists of water, in which there are organic and inorganic substances in suspension, dissolved, or in equilibrium between both states. In bile samples, collected from the human common bile duct, the concentrations of the inorganic electrolytes sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and bicarbonate (HCO^{3-}) are slightly higher than their plasmatic concentrations, whereas biliary chloride (Cl^-) concentrations are slightly lower than these found in plasma. In addition, bile contains significant amounts of bile pigments, cholesterol, phospholipids, and protein. In humans the pH of bile as it enters the duodenum is around 7.6 (33, 34).

Production of bile by hepatocytes is a major. Bile formed at the canaliculi is modified in the ductules and ducts by processes of reabsortion or secretion of electrolytes and water. Bile is obtained from the gall bladder in autopsy

cases. The mean basal flow of bile in humans is approximately 620 ml/day and the bile pool circulates 5 to 10 times per day (i.e., 2.5 hours recirculation) (33, 35).

2.2 Analytical procedures

Bile can be extracted and analyzed as other biological fluids such as blood and urine. In general, there have many drugs and drug metabolites demonstrated to accumulate in this specimen. The qualitative finding of the presence of drug and/or metabolites in this specimen is important, for documentation of historic exposures to specific agents and chronic drug-use history. Historically, bile has most often been used in the determination of opiates in general and morphine in particular. More recently, Bile reviews have been written regarding that many drugs or their metabolites are found to accumulate at concentrations significantly higher then those in blood (36, 37). The data several antidepressant drugs showed several-fold higher concentrations in bile than in blood. And drugs such as doxepin, imipramine, trimipramine, sertraline and norsertraline showed higher concentration in bile than in liver. An average of 50 case indicated that cocaine was present in bile in amounts approximately five times higher than in blood (38). Moreover, in certain cases (16over44), although the drug or its metabolite was not detected in blood from different sites, it was detected in bile depending on pharmacokinetic and rate of metabolism in each drug. And the mean bile to blood ratios vary from about 1(for acetaminophen, amphetamine) to about 2000 (for desmethylclobazam) (3). Furthermore, detection of morphine and many other drugs (e.g. benzodiazepines, colchicine and buprenorphine) is therefore more likely in the bile than in the blood, in which concentrations may be as much as 1000 times lower. The possibility that drugs in the bile may undergo enterohepatic re-circulation should not be overlooked (39). In the analysis in urine has limitation such as in case of empty bladder before dying that cause inadequate specimen for analysis. Bile has also been useful as alternative specimen detection of substances may be owing to acute or chronic use (40, 41). However, Bile contains many constituents which can cause interference in the analytical methods; Interpretation of drug levels in bile can be difficult due to lack of reference data (37).

Part III. Stability studies

1. Introduction

At present, analysis of drug abuse and poisonous substances in forensic science laboratories must have procedure for biological specimen preservation to keep them stable. i.e., in case of long weekend storage or when there're questions about the analysis report and more substance analysis is required, therefore we need the storage sample for analysis and retesting. The storage time is about 180 days or 6 months after the crime or after performing autopsy until prosecutor file to court as a mention in procedure criminal law code 150. Therefore, the knowledge of the stability of drugs in biological specimens is important for the interpretation of toxicological findings. Stability testing can be used to explain discrepancies between re-analyses long after initial analyses and may help to determine time limits that must be imposed between the collection and analysis of samples for pharmacokinetic studies, or to identify the optimal storage conditions for specimens retained in drug-testing and forensic laboratories(42).

The purpose of the tests is to study how the quality of the extract varies in time under the influence of a variety of environmental factors such as temperature, humidity and light. The result enables to establish recommended storage conditions and the shelf life of the raw material.

2. Stability testing data

Stability test are usually performed on different batches of extract stored in the original container at $25^{o}C$. The length of the study should be sufficient to cover storage, shipment and subsequent use of the plant extract. There are many studies in the literature on the issue of sample storage and putrefaction on substances of forensic interest. By far most data on drug stability in biological matrices are available for the classic drugs with abuse potential, i.e., amphetamines, methamphetamine, barbiturates, benzodiazepines, cocaine, ephedrine, lysergide (LSD), 6-acetylmorphine (6AM), estazolam (EZ), nitrazepam (NZ), opiates, and phencyclidine (PCP) (42-44). Stability data for these compounds in blood, plasma, serum, urine, oral fluid, and tissue samples were first reviewed by Levine and Smith in1990. Since then, numerous systematic

studies have been dedicated to the stability of the same drugs or drug classes under various storage conditions. For example, a review on optimization of storage conditions for cocaine in urine was provided by Hippenstiel and Gerson in1994 and recently another by Skoppand Potsch updating stability data for drugs of abuse in blood, plasma, or serum. Aspects of drug stability have further been covered in review articles on oral fluid analysis, hair analysis and postmortem toxicology (42).

Stability studies of methamphetamine (MA) in biological specimens. The result in long-term storage study of Giorgi and Meeker, the mean differences of amphetamine (AP) and methamphetamine (MA) changed erratically during the 5-year study, with maximum deviations of -38% (3years) and -77%(1year) for methamphetamine and amphetamine, respectively. Furthermore, Peters et al, studied the stability of drug abuse such as amphetamine, methamphetamine, ethylamphetamine, 3,4-methylenedioxy-amphetamine(MDA), 3,4-methylene-dioxy-methamphetamine(MDMA). Stability in spiked plasma was tested over three freeze/thaw cycles, each cycle consisting of 21-h freezing at -20°C and thawing and keeping the sample at RT for 3h. No instability was observed for any of the compounds in processed samples kept on the autosampler at RT for 32 h. Moreover, Peters et al. studied the stability of enantiomers of amphetamine and methamphetamine. These drugs were stable in plasma stored at -20°C for 6months and after three freeze/thaw cycles. Amphetamine and methamphetamine enantiomer derivatives were further stable in processed samples for 17 h (42). And In work of C. Jimenez et al (43), the stability of ephedrine derivatives and amphetamine derivatives (amphetamine, methamphetamine, MDA and MDMA) in urine has been studied. Spiked urine samples were prepared for stability testing. In summary, data obtained in the different stability studies carried out in this work demonstrates the stability of ephedrine and amphetamine derivatives in preserved sterile and non-sterile (only MDMA and methamphetamine were studied) urine samples in all the conditions of time and temperature evaluated. According to our observations, urine samples containing ephedrine and amphetamine derivatives can be stored at the least demanding conditions studied, i.e.4 ^{o}C, for up to 24 months for sterile samples, and for up to 6 months for non-sterile sample. Kei Zaitsu et al (44) reported similar results for the stability of various drugs and metabolites in urine and preventive measures against

their decomposition with special attention to filtration sterilization. Results for the slightly contaminated urine, there were no noticeable changes of AP and MA over 150 days, even when stored at $25^{o}C$, without any preservative measures. Decreases of MA and AP by 32 and 44%, respectively, were observed in the severely contaminated urine after a storage period of 150days under the same conditions. A comprehensive study by Clauwaert et al (45) reported results for the stability of 3, 4-methylenedioxy derivatives of MA and AP (i.e., MDMA and MDA) in water, serum, whole blood, and urine under various storage temperatures. Spiked samples of each matrix were stored at -20, 4, and $20^{o}C$ for up to 21 weeks. No instability was observed for any of the analytes under the given conditions.

Part IV. Analytical method: Gas chromatography (GC)

1. Introduction

Gas chromatography (GC) is a powerful and one the most widely used analytical tools. GC is a technique for separation, detection, quantitation and identification of components in a mixture. It offers rapid and very high resolution separations of a very wide rang of compounds (organic and inorganic; MW 2 to >1000 Daltons) (46), with the only restriction that analytes should have sufficient volatility. Most compounds with boiling points less than about 250°C (480°F) can be readily analyzed by this technique (47). In chromatography the compounds are distributed between two phase: the fixed stationary phase, which can be either solid or liquid and the mobile phase (gas). The compounds are continuously absorbed and desorbed from the stationary phase the resulting of each particular component in the pass through at a different velocities and thus different retention times. Retention time is defined as the time from injection of the sample to the time a specific sample component is detected. That the coating into the stationary phase (solid or liquid) has different affinities for separated of each component in the mixture. And while polarity is usually the major factor governing separation, the boiling points of components of the sample also play a role in determining retention time. Components with higher volatility (lower boiling points) tend to spend more time in the moving gas phase and therefore tend to have

shorter retention times. After as each component emerges from the column it is detected by sensitive detection device and recorded (48-50).

2. Principle and basic components of GC (51, 52)

In gas chromatography (GC), the sample is injected into the chromatograph, and it is immediately vaporized in a heated injection chamber and introduced into a moving stream of gas, called the carrier gas. The vaporized sample is transported through the column that contained in a temperature-controlled oven. The column contains the stationary phase, which is either a liquid or solid. As the sample passes through the column, it is subjected to many gas-liquid partitioning processes, and the components are separated. The rate at which the molecules progress along the column depends on the strength of adsorption or distribution, which in turn depends on the type of molecule and on the column chosen. As each component leaves the column, it is detected and identified by an electrical detector that generates a signal that is recorded on a strip chart recorder. They are schematically shown a gas chromatographic system in a block diagram in Figure 3.
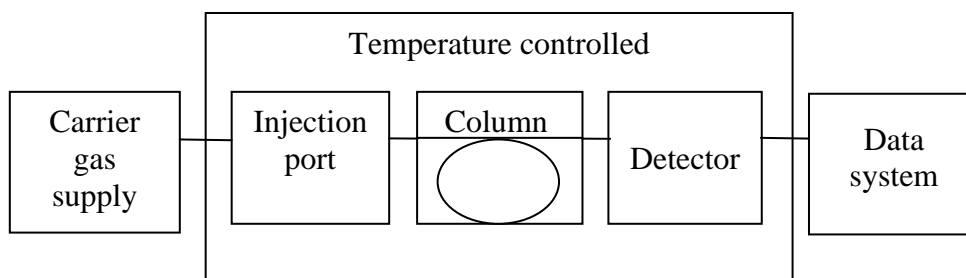
Figure 3 Basic block diagram of a gas chromatograph (53, 54)

3. Basic components of GC

3.1 Gas supply or Carrier Gas (53, 55-58)

The carrier gas or mobile phase in GC must be chemically inert. The most common carrier gases are H_2, N_2, He. This serves as the mobile phase that moves the sample through the column. The carrier gas must be of 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. Although of the carrier

gas in GC is an essential, but limiting, facet in separations. Carrier gas is the means to move constituents of a sample through the column and yet the choice of possible gases is restricted. Moreover, the carrier gas has properties that sometimes can complicate an analysis. Unlike liquid chromatography (where a wide selection of mobile phase compositions may be possible), very little can be gained in separations through altering the mobile phase composition to influence the partition coefficient (k) or separation factor(a) in GC.

3.2 Injector (53, 59-60)

The chromatographic process begins when sample is introduced into the column, ideally without disrupting flows in the column. Therefore, the optimum column efficiency, the sample should not be too large, and should be introduced onto the column as a "plug" of vapour - slow injection of large samples causes band broadening and loss of resolution. Moreover, the chromatographic results will be reproducible inasmuch as this is accomplished with a minimum of change in pressure or flow of the carrier gas or mobile phase. Also, the injection step establishes the initial (and best possible) peak width for the GC measurement. Thus, delivery of sample in to the column should be controlled, reproducible, and rapid.

The most common injection method is where a microsyringe is used to inject sample through a rubber septum into a flash vapouriser port at the head of the column. The temperature of the sample port is usually about 50°C higher than the boiling point of the least volatile component of the sample. For packed columns, sample size ranges from tenths of a microliter up to 20 microliters. Capillary columns, on the other hand, need much less sample, typically around 10^{-3} mL. For capillary GC, split/splitless injection is used. The injector can be used in one of two modes; split or splitless. The injector contains a heated chamber containing a glass liner into which the sample is injected through the septum. The carrier gas enters the chamber and can leave by three routes (when the injector is in split mode). The sample vapourises to form a mixture of carrier gas, vapourised solvent and vapourised solutes. A proportion of this mixture passes onto the column, but most exits through the split outlet. The septum purge outlet prevents septum bleed components from entering the column.

3.3 Column (54, 61-64)

The GC column is the heart of the system. It is coated with a stationary phase which greatly influences the separation of the compounds. The structure of the stationary phase affects the amount of time the compounds take to move through the column.

There are two general types of column, i.e., packed and capillary (also known as open tubular). Packed columns contain a finely divided, inert, solid support material (commonly based on diatomaceous earth) coated with liquid stationary phase. Most packed columns are tubes made of glass or metal with 2-4mm I.D. and 1-6 m length. Capillary columns are open tubes with 0.1 to 0.5 mm I.D. and 5 to 100 m lengths. Originally the capillaries were made from metal or glass; in the last decade fused silica replaced all other materials. Fused silica has the advantage of a very inactive inner surface, which avoids adsorptive interactions between analytes (especially when they are polar) and adsorption centers, leading otherwise to tailing peaks or even loss of material due to irreversible adsorption. Moreover, the packed column can accept a larger injection volume, but the capillary column has better separating power. Therefore, the choice of a packed or capillary system depends on the complexity of the atmosphere to be sampled and the desired detection limit.

The interaction between the injected sample containing one or more compounds in the vapor phase and a liquid phase present, e.g., on the walls of a capillary column or coated on a diatomaceous earth (solid) in a packed column results in a separation. The stronger the interaction between the component in the vapor phase and the liquid phase, the move strongly the flow of the component will be retarded by the column, i.e., the longer the retention time.

3.4 Oven (53, 64-65)

The column is placed in an oven where the temperature can be controlled very accurately over a wide range of temperatures. The optimum column temperature is dependant upon the boiling point of the sample. Typically, GC ovens usually require an operating range from about 5°C to about 400°C although the majority of GC analyses are carried out between temperatures of 75°C to 200°C.

As a rule of thumb, a temperature slightly above the average boiling point of the sample results in an elution time of 2 - 30 minutes. Minimal temperatures give good resolution, but increase elution times. Therefore, increasing the column temperature increases the speed of solute migration through the column. Thus, to shorten the analysis time for mixtures that contain solutes that extend over a wide range of molecular weight or polarity, the temperature of the column must be continuously increased during chromatographic development in a carefully controlled manner. The necessary temperature/time profile is established by a temperature programmer. This device can increase the temperature of the oven at a chosen rate and holds the oven at a specified temperature for a defined time before returning to a preset starting temperature. Temperature programming is useful and essential feature of all GC column ovens.

3.5 Detectors (59, 66-68)

As each compound exits the column, it enters the detector. The detector interacts with the compounds based on some physical or chemical property. In general, GC detectors vary according to their selectivity, sensitivity, and linearity. There are several different types of detectors common to gas chromatography instruments. Some of these detectors are listed in Table 1. Some detectors respond to every compound while others respond only to a select group of compounds. Therefore, the choice of detector is determined by the general class of compounds being analyzed and the sensitivity required. The interaction generates an electrical signal whose size corresponds to the amount of the compound. The detector signal is then sent to a recording device for plotting.

Table 1 Applications of Commercially Available detectors for GC

Detector	Applications
Thermal conductivity (TCD)	Universal
Flame ionization (FID)	Most carbon compounds
Electron capture (ECD)	Halogenated compounds
Photoionization (PID)	Aromatic compounds
Nitrogen/phosphorus (NPD)	N-,P-, and halogen-containing compounds
Flame photometric(FPD)	S- and P-containing compounds
Atomic emission (AED)	Metals; halogens,C- and O-containing compound
Electroconductivity(ECD)	S-, N-, and halogen-containing compounds
Mass spectrometer (MSD)	S-containing compounds
Radioactivity	3H- and 14C-containing compounds
Chemiluminescent	Variety of compounds

3.6 Data recorder system (57, 69-70)

The output from a GC detector is usually an electrical signal, and it was passed to a potentiometric recorder. The recording device plots the size of the detector signal versus the time elapsed since sample introduction into the injector. This plot is called a chromatogram. Each of the peaks in the chromatogram must be identify type of compound on the basis of retention time and able to measure a suitable parameter (the area under or height of the peak) of each peak which can be related to the concentration of that component of the sample.

The most common data recording devices are computer (PC) based. Older GC systems may use an integrator or a strip chart recorder which produce printed versions of the chromatogram and report with little or no data storage and recall capability. The modern gas chromatograph includes a computer that acquires the data, processes it using standard software, and then reports the results on a printer. PC based data system are extremely powerful and offer numerous data plotting, reporting and storage options, thus their popularity. Most computer data system can also control and automate the operation of the GC.

CHAPTER IV
MATERIALS AND METHODS

Materials

1. **Sample**

    Bile collected at Department of Forensic Medicine Siriraj Hospital.

2. **Chemicals and reagents**

    - Acetonitril (CH_3CN, Merck, Germany)
    - Dichloromethane (CH_2Cl_2, LAB-SCAN analytical science, Thailand)
    - Heptane (Merck, Germany)
    - Hydrochloric acid (HCl, Merck, Germany)
    - Methanol (MeOH, LAB-SCAN analytical science, Thailand)
    - Normal saline (NSS, Thai Otsuka Pharmaceutical, Thailand)
    - 2-Propanol (Merck, Germany)
    - Sodium hydroxide (NaOH, Merck, Germany)
    - Standard Methamphetamine (Lipomed services to medicine, Switzerland)
    - Zinc sulfate ($ZnSO_4$, Asia pacific specialty chemical, Australia)

3. **Instrumentation**

    - Centrifuge (Kendro Laboratory Products, Germany)
    - Gas chromatography

        Gas chromatograph: Agilent 6890

        Software: HP Chemstation

        Hardware: HP d530 CMT (PC571PA)

        Column: PERKIN, Length 30 m, Diameter 250μm,

        Thickness1μm nominal

    - High performance liquid chromatography (HPLC, Alliance water2695 separations module)
    - Shaker (GFL shaker 3018, GFL, Germany)

- Vortex-2 Genie, model G-560E (Scientific Industries Bohemia, N.Y.,USA)

4. **Other materials**

- Cap seal PTFE rubber 11 mm
- Clear target crimp top vials 2ml
- Glass ware (Pyrex Lab ware, and Corning Laboratory Science Company)
- Phase separator (1PS, silicone treated filter paper, circles 110mm, Whatman International Limited, England)
- Separating funnel 100ml
- Solid-phase extraction MCX 3 CC (SPE, Water Oasis®)
- Syringe filter membrane (13 mm diameter, 0.2 μm pore size)
- Syringe (single use 3 cc/ml, TERUMO)
- Universal-indicator paper (Art. 9562, Merck, Germany)

Methods

Part I. Study the optimal condition extraction method for methamphetamine in bile

1. Sample preparation

Bile samples were prepared by collecting from gall bladder of victims with expected cause of death from poisoning or drug abuse overdose. These samples were analyzed by screening and confirming test. Bile samples with negative methamphetamine were used for this study work. Each of these samples were pooled and homogenate to prepare for the optimize condition extraction.

2. Trial condition extraction procedure

2.1 Trial condition for comparison between hydrolysis and non hydrolysis at the same extraction solvent

A stock solution of methamphetamine (100μg/ml) was made by dissolving

the compound in MeOH. Reaction was initiated by adding a stock solution to bile sample to obtain 5µg/ml. After that these samples were divided into two groups.

Extraction group 1: One milliliter of a bile sample was added to a 10-ml tube, 1000 µl of 3M hydrochloric acid was added, and the tube was placed in a water bath at 120 ºC for 30 min for hydrolysis of the conjugated compounds. The sample was extracted with acetonitrile 5ml (71). After shaking for 10 min and centrifuging at 6,000 rpm for 10 min, the supernatant was transferred to beaker and was dried by air flow.

Extraction group 2: One milliliter of a bile sample was added to a 10-ml tube (no hydrolysis of bile was performed in this group). Then, added 5ml of acetonitrile for extraction. After shaking for 10 min and centrifuging at 6,000 rpm for 10 min, the mixtures took to freeze for 10 min. The supernatant was transferred into beaker and dried by air flow.

The residues of each group were redissolved in 1 ml of MeOH and analyzed by HPLC apparatus.

2.2 Trial condition for comparison of extraction solvent between acetonitrile and mixture contain dichloromethane: heptane: 2-propanol

A stock solution of methamphetamine (100µg/ml) was made by dissolving the compound in MeOH. Reaction was initiated by adding a stock solution to bile sample to obtain 3µg/ml. After that these samples were divided into two groups.

Extraction group 1: Five hundred microliters of a bile sample was added to a 10-ml tube and no hydrolysis of the conjugated compounds because of its time-consuming procedure and no need. The sample was extracted with acetonitrile 5ml (71). After shaking for 10 min and centrifuging at 6,000 rpm for 10 min, the supernatant was transferred to beaker and was dried by air flow.

Extraction group 2: The samples were prepared and non-hydrolyzed the same way with extraction group1, but samples were divided into three groups by extracting the different matrices in acid (pH 1), neutral (pH 5-6) and alkaline (pH 9.2) media. Then, the sample was extracted with dichloromethane: heptane: 2-propanol (14.8: 13.2: 2 v/v/v) 30 ml (3), add into 50-ml tube together with each samples. Then, each mixture

was shaken on a mechanical shaker for 5min and the lower layer was removed to beaker for evaporated to dryness.

The residues of each group were redissolved in 1 ml of MeOH and analyzed by GC apparatus.

2.3 Trial condition for comparison of extraction solvent between acetonitrile and mixture contain dichloromethane: heptane: 2-propanol under protein precipitation condition

A stock solution of methamphetamine (100μg/ml) was made by dissolving the compound in MeOH. Reaction was initiated by adding a stock solution to bile sample to obtain 20μg/ml. After that these samples were divided into two groups.

Extraction group 1: Two milliliters of a bile sample was added to a 10-ml tube, and then added $ZnSO_4$ 2 ml (1:1) for precipitation of protein. The mixture was vortexed for 1 min and centrifuged at 4,000 rpm for 15 min. After the supernatant was removed to another tube for extraction with acetonitrile 10 ml and shaking for 15 min, the mixtures took to freeze for 10 min. The supernatant was transferred into beaker and dried by air flow.

Extraction group 2: The samples were prepared and precipitated protein the same way with extraction group 1. Samples were divided into three groups by extracting the different matrices in acid (pH 1), neutral (pH 5-6) and alkaline (pH 8-9) media. Extract with dichloromethane: heptane: 2-propanol (14.8: 13.2: 2 v/v/v) 10 ml and then shake for 15 min before transfer the lower layer into beaker for evaporate to dryness.

The residues of each group were redissolved in 1 ml of MeOH and analyzed by GC apparatus.

2.4 Trial condition for comparison at different concentration of methamphetamine in bile under protein precipitation condition

A stock solution of methamphetamine (100μg/ml) was made by dissolving the compound in MeOH. Reaction was initiated individually by adding a stock solution to bile samples to obtain three different concentration (5, 10, 20 μg/ml). One milliliters of each bile sample was added to a 10-ml tube then added $ZnSO_4$ 1 ml (1:1)

for precipitation of protein. The mixture was vortexed for 1 min and centrifuged at 4,000 rpm for 15 min. After the supernatant was removed to another tube and adding NaOH to adjust pH to 8-9, the mixture was extracted with dichloromethane: heptane: 2-propanol (14.8: 13.2: 2 v/v/v) 10 ml. Then, the mixture was shaken for 15 min and transferred the lower layer into beaker for evaporate to dryness.

The residues of each group were redissolved in 1 ml of MeOH and analyzed by GC apparatus.

2.5 Trial condition for comparison between diluted and non-diluted sample under protein precipitation condition

A stock solution of methamphetamine (100μg/ml) was made by dissolving the compound in MeOH. Reaction was initiated by adding a stock solution to sample bile to obtain 20μg/ml. After that these samples were divided into two groups.

Extraction group 1: These samples were divided into two groups for diluted sample group and non-diluted sample group. For diluted sample group, bile was diluted 1:3 with normal saline. Aliquots of 2.0 and 4.0 ml of each sample groups and precipitated the protein with $ZnSO_4$ (1:1). After vortexing for 1min and centrifuging at 4,000 rpm for 15 min, the supernatant was transferred to another tube. Before extraction with dichloromethane (1:5), the supernatant was adjusting pH to 8-9 with 0.5ml NaOH (0.1N). Then, the mixture was shaking for 15 min and removed lower layer into beaker for evaporate to dryness.

Extraction group 2: These samples were divided into two groups for diluted sample group and non-diluted sample group. For diluted sample group, bile was diluted 1:3 with normal saline (72). Aliquots of 2.0 and 4.0 ml of each sample groups and precipitated protein with $ZnSO_4$ (1:1). After vortexing for 1min and centrifuging at 4,000 rpm for 15 min, the supernatant was transferred to another tube. Before extraction with dichloromethane: heptane: 2-propanol (14.8: 13.2: 2 v/v/v) (1:5), the supernatant was adjusting pH to 8-9 with 0.5ml NaOH (0.1N).Then, the mixture was shaken for 15 min and transferred the upper layer into beaker for evaporate to dryness.

The residues of each group were redissolved in 1 ml of MeOH and analyzed by GC apparatus.

2.6 Trial condition for comparison between protein precipitation and non-protein precipitation in diluted sample

A stock solution of methamphetamine (100μg/ml) was made by dissolving the compound in MeOH. Reaction was initiated by adding a stock solution to bile sample to obtain 20μg/ml. After that these samples were divided into three groups and each sample group was diluted 1:3 with normal saline. The first group was divided into two subgroups, 2ml of diluted sample for each experiment, one sub group freeze for 15min and the other non freeze. The samples in these groups protein precipitation were not performed. The second group was divided into two sub groups, 2ml of diluted sample for each experiment, one freeze for 15min and the other non freeze like the first group. But the samples in these groups protein precipitation was performed with $ZnSO_4$ (1:1). For the third group, protein precipitation was performed with 10 ml acetonitrile for 2 ml diluted sample and freezing for 30 min. These samples were divided into two groups for extracted with dichloromethane in one group and not extracted in other group. The sample of each group was made alkaline with 0.5 ml NaOH (0.1N) and extracted with 10 ml dichloromethane. Then, the mixture was shaken for 15 min and the dichloromethane layer was transferred into beaker for evaporate to dryness. The residues of each group were redissolved in 1 ml of MeOH and analyzed by GC apparatus.

3. The optimal condition of extraction method for methamphetamine in bile

The optimal extraction procedures from trial condition study were as follows: aliquot of 0.5 ml of bile sample with spiked standard methamphetamine of each concentration was diluted 4 folds with normal saline (1.5 ml). After vortexed for 1 min and add 0.1 N NaOH 0.5 ml to adjust pH (pH $\approx$ 9). The mixture was vortexed for 1 min and placed into separating funnel. After addition of 10 ml of dichloromethane the funnel was shaken on a mechanical shaker for 15 min. The under layer (aqueous dichloromethane, organic phase) was removed by filtration with phase separator paper Whatman No. 1 PS and then evaporated to dryness. The residue was dissolved in 1 ml of MeOH and 2 μl were injected into the GC apparatus for analysis.

Part II. Method validation

1. Preparation of solutions

1.1 Preparation of Standard Curve

The solutions of 0.1, 0.5, 1, 2, 5, 10, 25, and 50 µg/mL of standard methamphetamine were prepared by serial diluted from stock standard 1 mg/ml. The concentration of test solution was 1-10 µg/mL, so the Analytical Validation Standard Solution (AVSS) covered from 10% to 500% of assay concentration.

1.2 Standard Solution and Sample preparation

The stock standard solution of methamphetamine (1mg/ml) was appropriately diluted with MeOH to prepare working standards solution at concentration of 100µg/ml and 500µg/ml. The working standards were then used to spike bile samples to the desired concentration. Drug-free bile samples were homogenized and spiked working standards at 1, 5, 10, 20 µg/ml (100% recovery). Shown in Table 2.

Table 2 Show the preparation of spiked working standard methamphetamine in bile

Spiked Std Meth	Bile sample	Working std 100ug/ml	Working std 500ug/ml
2 µg/ml	2 ml	40 µl	-
10 µg/ml	2 ml	200 µl	-
20 µg/ml	6 ml	-	240 µl
40 µg/ml	2 ml	-	160 µl

2. Validation method

2.1 Selectivity and Specificity

In many publications, selectivity and specificity are often used interchangeably. In this study, each stock standard (amphetamine, methamphetamine, ephedrine, pseudoephedrine and MDMA) were appropriately diluted with MeOH to

prepare standards concentration at 5μg/ml. These standards were injected into GC apparatus to analyze for retention time of each. Then, a mixed standard was prepared by combining aliquots of each standard and diluted with MeOH (10.0 μg/ml) to prepare for analyzing the resolution between the peaks of each standard.

2.2 Accuracy

Accuracy was calculated as the percentage of recovery by the assay from a known added amount; hence the recovery of methamphetamine from bile was studied by assaying the sample preparation spiked with standard methamphetamine, corresponding to a final concentration of 1, 5, 10 and 20 μg/ml for which the processing preparation was shown in second part of step1. At each level, samples were injected in triplicate by three time extractions following the optimal extraction procedures performed in the first part of study. Then, the result of analysis by GC apparatus was calculated as the percentage of recovery by comprise with standard curve from part 2 in step1.

2.3 Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between series of measurements obtained from multiple samples of the same homogeneous sample under prescribed conditions. In this study, the intra-assay precision was determined for bile samples spiked standard methamphetamine at 10 μg /mL shown in Table 2. The intra-assay precision was determined by extracted in ten times of methamphetamine in bile which was prepared. The extraction follows into the optimal extraction procedures from the first part of study and analyzed by GC apparatus. The precision was expressed as relative standard deviation (RSD) or coefficient of variation (CV) and evaluate acceptation of precision by comparison with calculated results from Horwitz's equation.

2.4 Linearity and Range

The linear range of this method was determined by spiked standard methamphetamine in bile sample at 4 levels: 1, 5, 10 and 20μg/ml that prepared as shown in Table 2 which covered the working range. The mixtures were collected into the optimal extraction procedures from the first part of study for which each level extracted in triplicate. And then, each level sample was injected and analyzed by GC apparatus. The average results of each level were determined by plotting method

calibration curve for assessing the linearity; the regression equation and the determination coefficient (r^2) were calculated.

2.5 Limit of detection(LOD) and Limit of quantitation (LOQ)

The limit of detection (LOD) is a characteristic for lowest concentration of an analyte in a sample matrix that can be detected. In part of the limit of quantitation (LOQ) is a characteristic of quantitative assays for low levels of compounds in sample matrices. The detection and quantitation limits of methamphetamine in this study were determined the signal - to - noise ratio by comparing measured signals from samples with known low concentrations of analayte with those of blank samples. Ratios of 3:1 and 10:1 signal-to-noise were considered acceptable for estimation of the LOD and LOQ, respectively.

3. Analytical condition

The instrument used was an Agilant 6890N Gas chromatography equipped with a nitrogen phosphorus detector and 30 m × 250 µm × 1 µm PERKIN capillary column. Temperature program, Initial temperature: 150 ºC; Final temperature: 200 ºC; rate 10 ºC/min. Run time 5 min. Capillary column had a nominal initial flow: 1.9 ml/min; Average velocity: 45 cm/sec. Injector used splitless mode and NPD-detector adjust offset at 26. Helium used as the carrier gas a flow at 1.9 ml/min. Make up gas is nitrogen flow at 30.0 ml/min. Detector gas is air and hydrogen gas flow at 60.0 and 3.0 ml/min, respectively. Each type of gas has purity of 99.999%.

4. Statistical analysis

Data were statistically analyzed using Microsoft office Excel 2007 program, and analysis undertaken using mean, standard deviation (SD) to calculate for precision, accuracy, including limit of detection (LOD) and limit of quantitation (LOQ) which reflects the results of method validation.

Part III. The stability of methamphetamine in bile

1. Sample preparation

Bile samples were prepared by collecting from gall bladder of victims with expected cause of death due to poisoning or drug abuse overdose. These samples were analyzed by screening and confirming test. Bile samples with negative methamphetamine were used for this study work. Each of these samples were pooled and homogenate to prepare for stability study. These samples were divided into five batches for five different concentrations of standard methamphetamine (2, 5, 10, 15 and 20µg/ml).

A stock standard methamphetamine solution was made by dissolving the compound in MeOH. Reactions were initiated individually by adding a stock solution to bile samples to obtain five different concentrations (2, 5, 10, 15 and 20 µg/ml). Aliquot the mixtures from each batch for storage at 4ºC and room temperature and each storage condition were prepared in triplicate for stability testing at different time intervals (0, 1, 3, 5, 7, 14, 28, 60, 90 and 180 day).

2. Methamphetamine analysis

Methamphetamine in bile

The reaction mixture was divided into three parts for three replicate extractions.

Liquid-liquid extraction method

0.5 ml of bile sample that spiked standard methamphetamine of each concentration and then the bile was diluted 4 folds with normal saline (1.5 ml). After vortexed for 1 min and add 0.1 N NaOH 0.5 ml to adjust pH (pH ≈ 9). The mixture was vortexed for 1 min and placed into separating funnel. After addition of 10 ml of dichloromethane the funnel was shaken on a mechanical shaker for 15 min. The lower layer (aqueous dichloromethane, organic phase) was removed by filtration with filter paper Whatman No. 1 PS and then evaporated to dryness. The residue was dissolved in 1 ml of MeOH and the solution of 2 µl were injected into the GC apparatus for analysis.

3. Analytical conditions

The instrument used was an Agilant 6890 Gas chromatography equipped with a nitrogen phosphorus detector and 30 m × 250 μm × 1 μm PERKIN capillary column. Temperature program have an initial temperature at 150 ºC and final temperature at 200 ºC with rate 10 ºC/min. Capillary column had a nominal initial flow: 1.9 ml/min; average velocity: 45 cm/sec and run time at 5 min for methamphetamine analysis. Injector was used splitless mode and NPD-detector adjust offset at 26. Helium was used as the carrier gas with a flow at 1.9 ml/min. Make up gas is nitrogen flow at 30.0 ml/min. Detector gas is air and hydrogen gas flow at 60.0 and 3.0 ml/min, respectively. Each type of gas has purity of 99.999%.

4. Statistical analysis

Data were statistically analyzed using SPSS for MS windows programs, and analysis undertaken using analysis by repeated measurement analysis test to compare for means of concentration of methamphetamine at storage time interval (0, 1, 3, 7, 14, 28, 60 and180 days) and one sample pair t-test to evaluate significant difference between 4ºC and room temperature groups. Statistically significant difference was set at p ≤ 0.05.

CHAPTER V
RESULTS

Part I. Evaluation of the extraction methods

1. Evaluation of trial condition methamphetamine extraction in bile

1.1 Comparisons between hydrolysis and non-hydrolysis at the same extraction solvent

The results of the methamphetamine concentration in bile by experiments extracted with acetonitrile solvent at different condition between hydrolysis and non-hydrolysis. Methamphetamine cannot be detected in each group of samples when analyzed by HPLC apparatus. The chromatogram of methamphetamine that extracted from bile sample compared with standard methamphetamine was shown in figure 4.
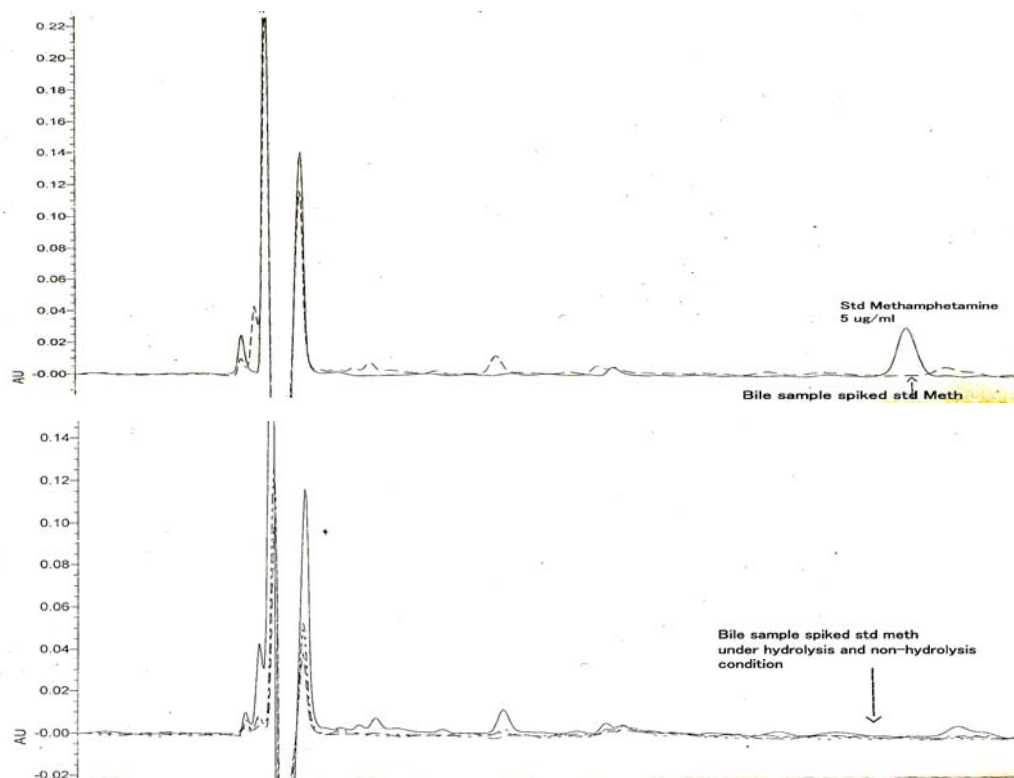

Figure4. **Chromatogram of standard methamphetamine compare with methamphetamine in bile by HPLC analysis**

1.2 Comparison of solvent used in extraction between acetonitrile and mixture containing dichloromethane: heptane: 2-propanol

The extraction of sample with mixed solvent under alkaline and natural condition can be detected for methamphetamine by GC apparatus, but in acid condition low value of methamphetamine was detected with high contamination similar to sample extracted with acetonitrile. The results of these samples have a percent of recovery less than 20 approximately (Table 3). Therefore, in each condition the extracts of methamphetamine in bile can be used to trial in next step.

Table 3 Methamphetamine concentration at different extraction condition

	Extraction solvent & Condition	Peak Height NPD-signal (mpA)	Concentration (μg/ml)	% Recovery
Spiked Std MA 3 μg/ml	Mixed solvent under acid condition	75.33	0.53	17.67
	Mixed solvent under nature condition	77.47	0.55	18.33
	Mixed solvent under alkaline condition	80.26	0.57	19.00
	Acetonitrile solvent	58.22	0.28	9.33

Each value is the mean of two determinations

1.3 Comparison of extraction solvent between acetonitrile and mixture containing dichloromethane: heptane: 2-propanol under protein precipitation condition

In previous experiment, the problem was that many contaminated interference from substance were found in samples. Thus, the protein precipitation which has an advantage to remove and reduce interference was used to trial in this extraction. When the sample extracted under protein precipitation condition, methamphetamine was not detected in both extraction solvent except in alkaline condition that extracted with mixed solvent (dichloromethane: heptane: 2-propanol).The results of these samples have lower interference but a percent recovery

was less than 10 approximately as shown in table 4. Therefore, extraction of methamphetamine in bile with mixed solvent under alkaline condition was used for trial in next step.

Table 4 Methamphetamine concentration under protein precipitation condition

	Extraction solvent & Condition	Peak Height NPD-signal (pA)	Concentration (µg/ml)	% Recovery
Spiked Std MA 20 µg/ml	Mixed solvent under acid condition	-*	-*	-*
	Mixed solvent under nature condition	-*	-*	-*
	Mixed solvent under alkaline condition	6.57	3.12	7.81
	Acetonitrile solvent	-*	-*	-*

Each value is the mean of two determinations

-* Not detected

1.4 Comparison of methamphetamine at different concentration in bile under protein precipitation condition

After the sample extracted with mixed solvent at varied methamphetamine concentration (5, 10, 20 µg/ml) under alkaline and protein precipitation condition, methamphetamine can be detected at each concentration but these results have a percent of recovery remain less than 10 approximately (Table 5). Therefore, the conditions of methamphetamines extractions must be improved to have a better recovery result in trial of next step.

Table 5 Methamphetamine in bile at different concentration

Spiked Std MA (μg/ml)	Peak Height NPD-signal (pA)	Concentration (μg/ml)	% Recovery
5	1.15	0.59	5.40
10	2.33	1.09	5.46
20	4.93	2.30	5.76

Each value is the mean of two determinations

1.5 Comparison between diluted and non-diluted samples under protein precipitation condition

The extraction condition was improved by diluted the sample for comparison with non-diluted for which each group was divided into two groups and extracted with dichloromethane and mixed solvent under protein precipitation condition, respectively. Methamphetamine was detected in all groups which were extracted with dichloromethane. The results of diluted sample have higher percent of recovery than non-diluted samples (Table 6). Diluted samples have a percent recovery result more than 10. Therefore, these conditions were used to trial in next step.

1.6 Comparison between protein precipitation and non-protein precipitation in diluted samples

The result of diluted sample in each condition groups was shown in table 7. In non-protein precipitation conditions, percent of recovery was about 40 approximately, which was higher than in other groups. The precipitation groups have a percent of recovery less than 30. Therefore, the optimal condition for methamphetamine extraction in bile was to dilute sample with normal saline and extracted with dichloromethane.

Table 6 Percent of recovery of methamphetamine in diluted and non-dilute sample

	Extraction Solvent	Sample Volume		Peak Height NPD-signal (pA)	Concentra-tion (µg/ml)	% Recovery
		Dilute (1:3)	Non-dilute			
Spiked Std MA 20 µg/ml	Dichloromethane	2ml	-	4.56	2.13	15.97
		4ml	-	6.11	3.18	11.93
		-	2ml	4.85	2.26	5.67
		-	4ml	13.17	6.13	7.66
	Mixed Solvent (dichloromethane: heptanes: 2-propanol)	2ml	-	-*	-*	-*
		4ml	-	-*	-*	-*
		-	2ml	-*	-*	-*
		-	4ml	-*	-*	-*

Each value is the mean of two determinations, -* Not detected

Table 7 Percent of recovery of methamphetamine in diluted sample

	Extraction condition	Peak Height NPD-signal (pA)	Concentration (µg/ml)	% Recovery
Spiked Std MA 20 µg/ml	Non-protein precipitation			
	- Freeze	8.77	4.08	40.80
	- Non-Freeze	9.97	4.64	46.40
	Protein precipitation*			
	- Freeze	3.03	1.42	14.20
	- Non-Freeze	5.06	2.36	23.60
	Protein precipitation **			
	- Freeze	2.89	1.36	13.60
	- Freeze***	4.49	2.10	21.00

Each value is the mean of two determinations

  * Protein precipitation with $ZnSO_4$ (1:1)

 ** Protein precipitation with acetronitrile

*** Not extracted with dichloromethane

2. The conclusion for optimal condition of extraction method for methamphetamine in bile

All in all, in this study the optimal condition of methamphetamine extraction is to extract with dichloromethane solvent under alkaline condition from diluted bile sample for which the procedure was explained in chapter 3. These methods have a result of the recovery more than 40% which was higher than in other conditions. When compare recovery result between solid-phase extraction (SPE) and liquid-liquid extraction (LLE). The result of recovery in SPE showed no different value to these obtained in optimal condition LLE. Even though, solid-phase extraction is a more convenient, rapid, sensitive and efficient than liquid-liquid extraction but this extraction method is cost expensive per case, therefore, unsuitable for routines.

Part II. Evaluation of the method validation

1. Resolution

The GC separation of the analyses assure resolution R >1 for all pairs of chromatographic peaks. Each standard (Amphetamine, Methamphetamine, Ephedrine, Pseudoephedrine and MDMA) have retention time at 3.021, 3.412, 5.035, 5.093 and 6.554 min, respectively (Figure 5). All fifth solvent components used for method development are well separated. The specificity of the method was clearly demonstrated in Figure 5 that illustrates the complete separation of the main solvents considered in this work and their corresponding contaminants.
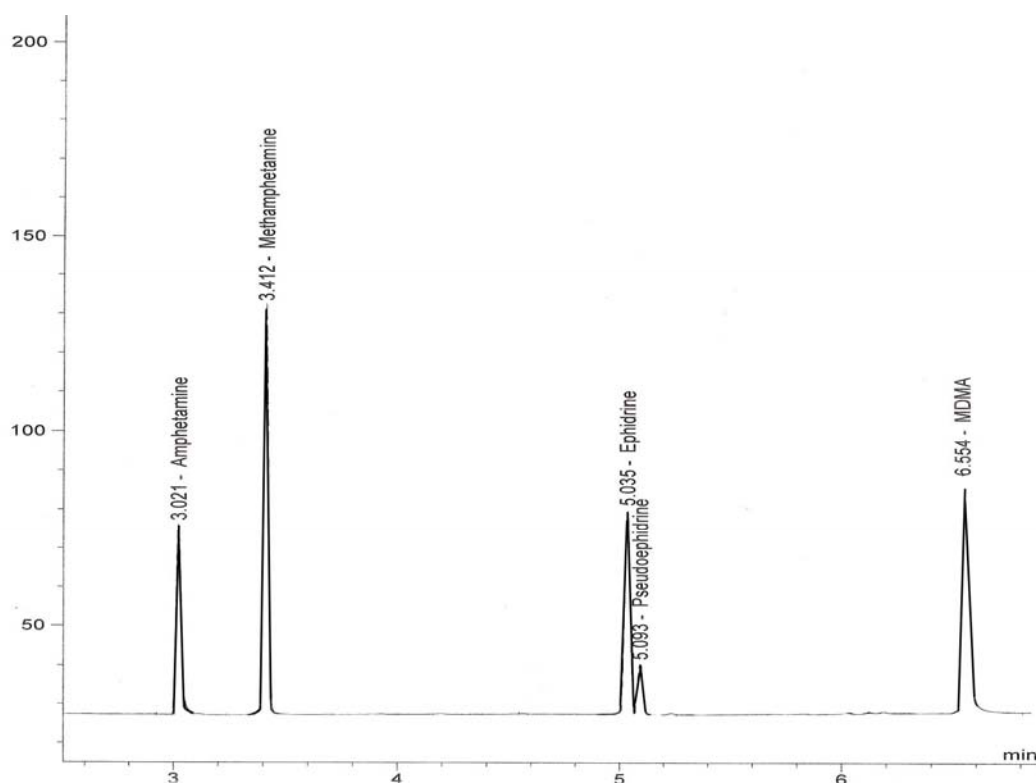
Figure 5 Chromatogram of each standard analysis by GC

2. Accuracy

The recovery values, calculated for spiked standard methamphetamine 10µg/ml in bile, range from 8.00 to 9.97 µg/ml. These calculated recovery results were about 80.0-99.7 % which showed that the accuracy of method was very good.

3. Precision

For Methamphetamine, the relative standard deviation (RSD) was calculated from mean and standard deviation which RSD values were 2.427 (< 5%). When calculated from Horwitz's equation, the expect RSD have an estimated value of 7.4. Then, evaluate acception of precision with Horwitz ratio calculation by comparison between analyzed RSD and expected RSD. The value of Horwitz ratio is less than 2 which is in acceptable range.

4. Linearity Range

Methamphetamine concentration was expressed in ug/ml. The response was linear over the whole range: 1.0 – 20ug/ml. The calibration graphs were prepared using four concentration levels: 1, 5, 10 and 20 ug/ml of methamphetamine standard which cover the working range. The correlation coefficients are 0.99985 for signal detectors NPD.

5. Limit of detection(LOD) and Limit of quantitation(LOQ)

In order, determination of the limit of detection (LOD) and limit of quantitation (LOQ) for methamphetamine was done before analysis. The LODs of methamphetamine was determined at the lowest range of working ranges. The procedure according to standard operation was applied in calculation of LODs and LOQs with signal to noise ratio 3 and 10, respectively. The LODs and LOQs for methamphetamine in bile were 0.3 and 1.0 ug/ml, respectively.

Part III. Stability of methamphetamine in bile

1. Stability of methamphetamine in bile at 4ºC condition

In this study, methamphetamine analyses were performed on bile samples from spiked standard at five concentrations and the optimal extraction procedures following at method mentioned in chapter 3 which was considered from part 1 results. Bile samples were stored at 4ºC condition for stability testing at different time intervals (0, 1, 3, 5, 7, 14, 28, 60, 90 and 180 day),respectively.

In Figure 6 and Table 8 the available data at 4ºC on the stability of methamphetamine concentrations are presented. During the 6-month experiment different concentrations of methamphetamine in bile (2, 5, 10, 15, 20μg/ml) were investigated in each period of times. The concentrations of methamphetamine were not significantly different when compared with those of the initial day (p>0.05) and was evaluated by using repeated measurement ANOVA.
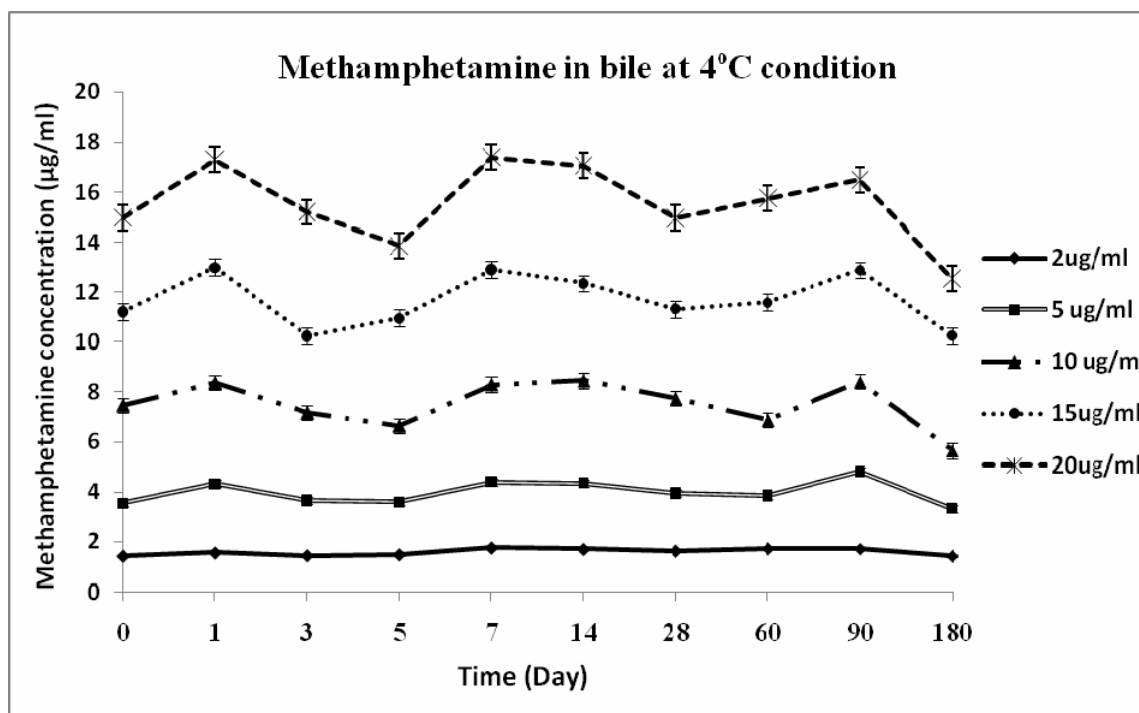
Figure 6 The methamphetamine stability in bile at 4°C condition

The percent of recovery for concentrations of methamphetamine were not significantly different at each time when compared with initial day (p-value≥ 0.05 at all times and each concentration levels; p-value =1.00 at day3, day5, day28, month2 and month6, p-value=0.089, 0.083, 0.090, 0.627 at day1, day7, day14 and month3, respectively). After one day the concentration change to 109.43-115.94 % and 91.29-102.85%, 88.83-104.20%, 111.22-123.89%, 110.21-122.42%, 99.93-114.54%, 91.90-121.17%, 110.11-135.27%, 75.51-99.69% after a 3-day, 5-day , 7-day, 14-day, 28-day, 60-day, 90-day and 180-day storage, respectively. These results were shown in Table 9, Figure 8.

Table 8 The concentration of methamphetamine in bile at 4ºC condition at different storage time

MA spiked (µg/ml) / Day of Analysis	Concentration of methamphetamine at 4ºC (µg/ml)				
	2	5	10	15	20
Day 0	1.429 ± 0.087	3.562 ± 0.267	7.472 ± 0.662	11.207 ± 0.660	14.974 ± 1.014
1	1.564 ± 0.070	4.337 ± 0.247	8.389 ± 0.017	12.993 ± 0.322	17.307 ± 0.190
3	1.450 ± 0.028	3.664 ± 0.154	7.169 ± 0.046	10.230 ± 0.706	15.196 ± 0.094
5	1.489 ± 0.035	3.611 ± 0.218	6.638 ± 0.204	10.950 ± 0.924	13.858 ± 0.424
7	1.764 ± 0.023	4.413 ± 0.109	8.311 ± 0.149	12.911 ± 0.156	17.403 ± 0.128
14	1.705 ± 0.061	4.361 ± 0.433	8.463 ± 0.053	12.351 ± 0.416	17.057 ± 0.503
28	1.637 ± 0.029	3.963 ± 0.150	7.740 ± 0.587	11.319 ± 0.155	14.964 ± 0.442
60	1.732 ± 0.056	3.872 ± 0.193	6.867 ± 0.769	11.581 ± 0.251	15.753 ± 0.996
90	1.722 ± 0.133	4.818 ± 0.435	8.424 ± 0.937	12.884 ± 0.568	16.488 ± 0.796
180	1.425 ± 0.094	3.344 ± 0.019	5.642 ± 1.189	10.252 ± 0.738	12.540 ± 0.599

Each value is the mean of three determinations with standard deviation

Table 9 Mean percentages of methamphetamine in bile samples at 4ºC condition

MA spiked (µg/ml) / Day of Analysis	Analytical Recovery at 4ºC (%)					
	2	5	10	15	20	mean
Day 0	100.00	100.00	100.00	100.00	100.00	100.00
1	109.43	121.76	112.27	115.94	115.58	115.00
3	101.46	102.85	95.94	91.29	101.48	98.60
5	104.20	101.38	88.83	97.71	92.55	96.93
7	123.38	123.90	111.22	115.22	116.22	117.99
14	119.27	122.42	113.26	110.21	113.91	115.81
28	114.54	111.24	103.59	101.00	99.93	106.06
60	121.17	108.71	91.90	103.34	105.20	106.07
90	120.47	135.27	112.73	114.97	110.11	118.71
180	99.69	93.87	75.51	91.48	83.74	88.86

2. Stability of methamphetamine in bile at room temperature (RT) condition

Methamphetamine analyses were performing in bile samples from storage preparation at room temperature (RT) condition. These bile samples were storage for stability testing at different time intervals (0, 1, 3, 5, 7, 14, 28, 60, 90 and 180 day), respectively. For each time period, bile samples were extracted by optimal extraction method mentioned in chapter 3 and considered from part 1 results. The results of methamphetamine concentrations in bile samples at RT are shown in Figure 7, Table 10.

During the 6-month experiment the different concentrations of methamphetamine in bile at RT condition (2, 5, 10, 15, 20µg/ml) were investigated at each period of times (Figure 7). The concentrations of methamphetamine were not significantly different when compared with those of the initial day ($p>0.05$).
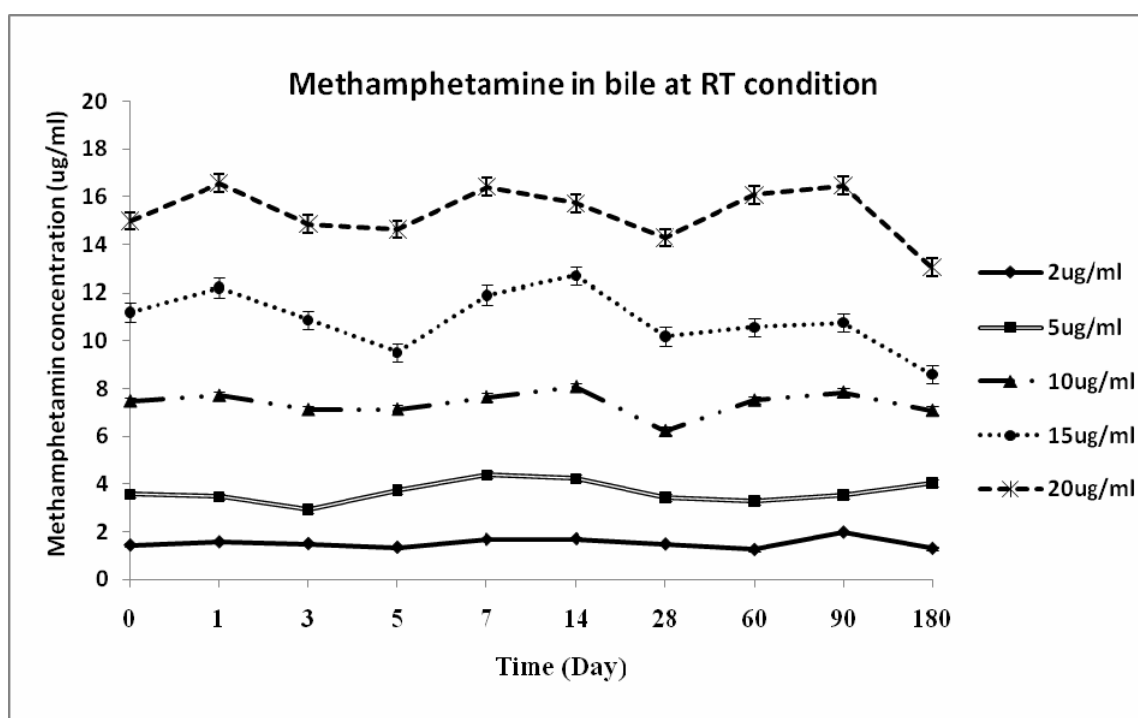

Figure 7 The methamphetamine stability in bile at RT condition

Table 10 The concentration of methamphetamine in bile at RT condition at different storage time

MA spiked (μg/ml) \ Day of Analysis	Concentration of methamphetamine at RT (μg/ml)				
	2	5	10	15	20
Day 0	1.429 ± 0.087	3.562 ± 0.267	7.472 ± 0.662	11.207 ± 0.660	14.974 ± 1.014
1	1.564 ± 0.062	3.470 ± 0.374	7.704 ± 0.368	12.205 ± 1.544	16.561 ± 1.176
3	1.491 ± 0.054	2.937 ± 1.119	7.112 ± 0.264	10.881 ± 0.404	14.849 ± 0.293
5	1.339 ± 0.150	3.745 ± 0.107	7.131 ± 0.189	9.520 ± 0.442	14.637 ± 0.465
7	1.668 ± 0.008	4.384 ± 0.111	7.644 ± 0.482	11.899 ± 0.436	16.416 ± 0.837
14	1.695 ± 0.057	4.232 ± 0.402	8.072 ± 0.283	12.691 ± 0.237	15.721 ± 0.992
28	1.472 ± 0.082	3.429 ± 0.143	6.229 ± 0.277	10.194 ± 0.641	14.264 ± 0.728
60	1.245 ± 0.057	3.292 ± 0.362	7.511 ± 0.270	10.576 ± 0.885	16.067 ± 0.752
90	1.967 ± 0.217	3.533 ± 0.340	7.843 ± 0.632	10.764 ± 0.461	16.466 ± 1.797
180	1.293 ± 0.303	4.041 ± 0.560	7.081 ± 0.057	8.592 ± 1.467	13.030 ± 1.367

Each value is the mean of three determinations with standard deviation

The percent of recovery for concentrations of methamphetamine were not significantly different at each time when compared with initial day (p-value≥ 0.05 at all time and each concentration level; p-value =0.451 at day14, p-value =1.00 in other day). After one day the concentration change to 97.43-110.60 % and 82.45-104.33 %, 84.95-105.14 %, 102.30-116.69 %, 118.80-104.99 %, 83.36-102.95 %, 87.02-107.30 %, 96.05-137.67 %, 76.67-113.45 % after a 3-day, 5-day ,7-day, 14-day, 28-day, 60-day, 90-day and 180-day storage, respectively. These results were presented in Table 11, Figure 8.

Table 11 Mean percentages of methamphetamine in bile samples at RT condition

MA spiked (μg/ml) / Day of Analysis	Analytical Recovery at RT (%)					
	2	5	10	15	20	mean
Day 0	100.00	100.00	100.00	100.00	100.00	100.00
1	109.44	97.43	103.11	108.91	110.60	105.90
3	104.33	82.45	95.18	97.09	99.16	95.64
5	93.65	105.14	95.43	84.95	97.75	95.38
7	116.69	123.06	102.30	106.18	109.63	111.57
14	118.55	118.80	108.03	113.25	104.99	112.72
28	102.95	96.23	83.36	90.97	95.26	93.75
60	87.02	92.41	100.53	94.37	107.30	96.33
90	137.67	99.21	104.95	96.05	109.96	109.57
180	90.48	113.45	94.76	76.67	87.02	92.48

3. Comparison of stability of methamphetamine in bile between 4ºC and RT conditions

The data of each methamphetamine concentration have shown stable trend with no different value when compared between 4ºC and RT through out the storage time. Therefore, the concentrations are changed into percentage of methamphetamine by calculated compare with those of the initial day to illustrate an overview of data. Mean percentage of methamphetamine in bile at both conditions were shown in Table 9 and 11, respectively. These data revealed the percentage change to 88.86-118.71 % at 4ºC and 92.48-112.72 % at RT which was plotted graph and demonstrated in figure 8 to compare between 4ºC and RT conditions.

The mean percentage of methamphetamine in bile storage comparison between at 4ºC and RT condition were evaluated by using independent sample t-test. The result showed significant difference at day 1 and day 28 (p-value = 0.023 and 0.022, respectively) that demonstrated in table 12 and figure 8. However, the overall picture of the graph percentages showed no significant difference in either condition as demonstrated in figure 8.

Table12. Independent sample t-test comparison between 4ºC and RT

DAY	Mean percentage		t	Sig (2-tailed)
	4ºC	RT		
1	115.00	105.90	2.817	.023*
3	98.60	95.64	0.698	.505
5	96.93	95.38	0.360	.728
7	117.99	111.57	1.439	.188
14	115.81	112.72	0.873	.408
28	106.06	93.75	2.835	.022*
60	106.07	96.33	1.660	.135
90	118.71	109.57	1.055	.322
180	88.86	92.48	-0.492	.636

* Significant difference between 4ºC and RT (p-value≤0.05, independent pair t-test)
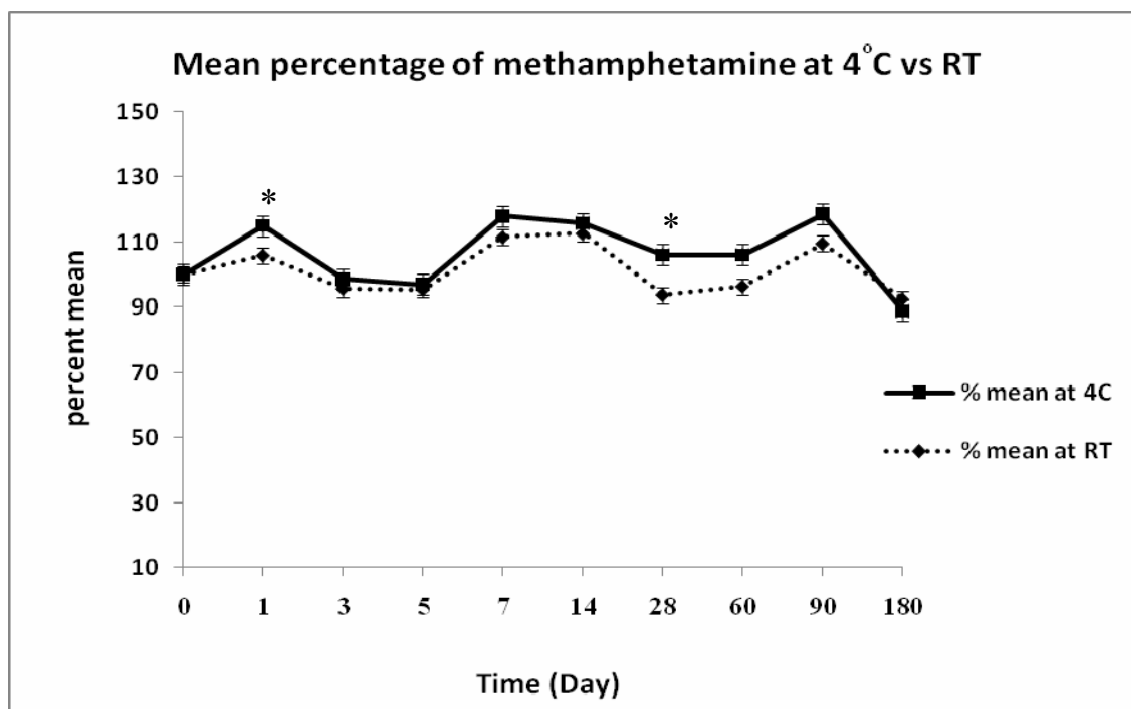
* Significant difference between 4ºC and RT (p-value≤0.05, independent pair T-test)

Figure8. The mean percentage of methamphetamine in bile at 4ºC and RT condition.

CHAPTER VI
DISCUSSION

Methamphetamine analysis is generally performed in biological specimens (urine, blood, etc). However, in some cases these specimens might not be available. The postmortem analysis of methamphetamine from bile is the most advantage and useful because the concentration of drugs or their metabolites in bile are generally several folds higher than those in blood concentrations (3). Moreover, in case of empty bladder before dying it may cause inadequate urine specimen for analysis. Therefore, toxicological analyses of methamphetamine using bile are necessary and it can be used in the comprehensive toxicological analysis. Previously, the analysis of methamphetamine in bile has been reported less than the study in other specimens. In this study, the analysis of methamphetamine concentration in bile sample has been focused stability during storage time. The study was divided into two parts: part I extraction and analytical method, part II stability study for which results can be applied to use in the laboratories.

Part I Extraction and analytical method

At present, many extraction methods for drug abuse analysis used in toxicological laboratories. SPE is an increasingly used to isolate substance and very effectively clean up of biological samples in all types of drug testing in laboratories. However, LLE is the technique mostly used in routine laboratories due to low cost per case. In this study, LLE technique used to trial optimize condition for isolating methamphetamine in bile. The trial condition was to extract under hydrolysis condition for disintegrate conjugated compound. These conditions were unsuitable because of its time-consuming procedure and this study used spiked standard under experiment condition, therefore, there is no need to perform hydrolysis. Moreover, it has more interference, thus trial in next step was to optimize condition by protein

precipitation with zinc sulphate ($ZnSO_4$) to remove proteins and other particular materials which disclosed less contamination, however, percent of recovery still was not good enough (< 20%). When tried to dilute samples and un-precipitate protein, the result revealed increased percent of recovery to an average about 35-40% and revealed less interference similar to the extraction with protein precipitation condition. In part of the method validation result has a percent recovery to 80-90 % as shown in part 2 of chapter 5. The better result of method validation because the procedures had a new by prepared standard curve and number of repeated extraction more than trial condition procedure. Therefore, the percent recovery result was better and more accurate so this condition is suitable for extraction of methamphetamine in bile.

In this study, the extraction procedure is based on LLE techniques. This method carried out in separating funnel and extracted with dichloromethane from diluted bile samples under alkaline condition. This condition can be used to extract methamphetamine in bile but sometime has the possibility of emulsion formation because component of bile also includes bile salt, cholesterol and protein. These may be the cause of low recoveries. Although LLE is laborious and time-consuming extraction steps, the steps are inherently more complicated than those in SPE. LLE is still remaining widely used for methamphetamine extraction.

The quantitative measurements of methamphetamine may have analytical methods such as high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), GC-nitrogen-phosphorus detection (GC-NPD), GC-flame ionization detection (GC-FID), etc. GC-NPD is the instrument for selective detection of compounds containing nitrogen and phosphorus atoms. These are suitable and interesting analytical tools to measure methamphetamine because of methamphetamine contains a basic nitrogen group. Moreover, GC-NPD is instrument for confirming test which is widely used for methamphetamine analysis at present.

As for the analytical methods used in this study to quantify and qualify methamphetamine in bile have been assayed to accepted validation criteria by process of method validation is confirm about method performance characteristic and evaluated with statistic analysis. In this study, the following parameters were evaluated: specificity or selectivity, accuracy, precision, working range, limit of

detection and limit of quantification. The results of these parameters show in chapter 5 that the data indicated that analytical methods exhibit good reproducibility.

In conclusion, LLE before GC-NPD represent a convenient method that obtained extraction with optimum recoveries and could be an accurate analytical tool for methamphetamine analysis in bile.

Part II Stability study

Knowledge of the stability of drugs in biological specimens is important for interpretation of analytical result. There have been reviews of data on the stability of drug abuse in blood, plasma, or serum. Some reports on the stability study in urine, oral fluid and tissue samples were first reviewed by Levine and Smith (42) in 1990. But the report had not been studied on the stability of methamphetamine in bile including a concern about the detection of methamphetamine in bile was very few. Therefore, this thesis has interested in studying about stability of methamphetamine in bile. The protocol used for stability testing was mainly focused on the evaluation of the suitable storage condition of biological samples. In part of the conditions studied in this thesis is to store at 4ºC and room temperature (RT), a similar protocol in previous studies in other type of specimens.

There had been a study in bile that spiked methamphetamine at five concentrations (2, 5, 10, 15 and 20 µg/ml) and storage at two conditions (4ºC and RT). The effects of storage time and temperature on the methamphetamine concentration in bile were determined. The statistical analysis showed that the storage temperature at 4 ºC and RT for 6 months did not significantly affect in all the methamphetamine concentrations in bile when analyzed by GC-NPD.

In the previous study, the degradation of methamphetamine may be defined as the progressive removal of amine groups and involves the breaking of the hydrogen bound in methamphetamine. These may influence and affect the changes of drug concentration that arise from many factors i.e. ultra-violet irradiation, bacterial degradation, residual tissue enzymatic activity and temperature, etc. Moreover, not only drug levels can change during the post sampling period while samples are in

storage but they can also alter during the post-mortem interval for which this issue was not studied(73).

For part of storage period, temperature is an important factor for stability of drugs. The general drugs are accelerated of chemical reaction by a raise in temperature. When temperature increased, the molecules of drug move faster with increased kinetic energy and rate of collision increases. It's said that typically a 10 ℃ increase in temperature produces a 2-5 fold increase in decomposition (74).

As suggested in the literature, there are many reports about the relationship between temperature and storage time of methamphetamine concentration that stability of drug were stable for longer period when stored at 4℃ and lower temperature e.g. Jimenez C. et al (43) studied that spiked urine sample can be stored for up to 24 months at 4 ℃ for sterile samples, and for up to 6 months for non-sterile samples which is similar to study of K. Zaitsu et al (44). Peters et al. (42) studied the stability of enantiomers of amphetamine and methamphetamines that these drugs analyze were stable in plasma storage at -20 ℃ for 6 months. And the Clauwaert et al (45), who demonstrated the stability of 3, 4-methylenedioxy derivatives of MA and AP (i.e., MDMA and MDA) in non-preserved urine, whole blood and serum sample which are stable when stored at -20 ℃ for 21 weeks, etc. In this study, the results of bile samples containing methamphetamine can be analyzed for 6 months when stored at 4℃ which agreed with previous investigation.

From previous studies, many have results about stability of methamphetamine in biological samples when stored at RT., e.g. K. Zaitsu et al. (44) studied the stability of various drugs and metabolites in urine. Methamphetamine and amphetamine in slightly contaminated urine, there were no noticeable changes over 150 days when stored at RT (25℃). The same result was reported by Clauwaert et al. (45) that no significant loss of derivatives of MA and AP (i.e., MDMA and MDA) in water, serum, whole blood, and urine samples when stored at RT for long time periods (21 weeks for water and urine, 5 weeks for whole blood and 17 weeks for serum)., etc. In this study revealed that methamphetamine concentration showed no significant different when bile samples stored at RT for 6 months. This result was consistent with previous study. Therefore, methamphetamine in bile probably can be analysed for 6 months when stored at RT. Several reasons may explain why methamphetamine not

degraded in bile sample when stored at RT. The methamphetamine include many compounds themselves are very stable chemical entities and requiring no particular attention in a great variety of biochemical condition (45). In part of biochemical condition, pH is one of factor influence the rate of decomposition of most drugs. If the pH of drugs solution has to be changed, it will be lead to instability. The pH of bile is 7.0-7.7 with no change during stored.

Moreover, the previous study had detected methamphetamine in urine stains on gauze and filter paper. It was found significantly decrease of methamphetamine in urine stains throughout 24 week when stored at RT. These were study in open condition so the drug may be degraded by higher temperature or humid environment. As this study, Bile were sampled and stored in sealed containers which resulted in no significant difference of concentration at RT. Similarly, in an earlier study methamphetamines in rabbit's blood were stored in sealed containers at 25ºC over 24 months (75). There was no significant change in concentration for methamphetamine have been demonstrated.

Furthermore, the degradation may be depending on physical property of each biological sample. Bile sample is a viscous fluid and consists of bile acids (BAs), electrolytes, cholesterol, fatty-acids, and bilirubin which may cause difficult to be multiplying of bacteria. Thus, methamphetamines in bile seem to be relatively stable against bacteria propagation and decomposition slow down.

All of the above, methamphetamines in bile seem to be relatively stable in sealed containers at 4ºC and RT for 6 months. There was no significant change in concentration for methamphetamine. As for comparison methamphetamine concentration between storage at 4ºC and RT, the result showed no significant difference of the analysis under study was observed between both conditions. But in part of RT storage condition, bile sample has a physiological change such as more viscous and dried. This problem can affect the analysis to be more difficult. Therefore, the most optimal condition for storage of methamphetamine in bile is to store bile specimen at 4ºC.

CHAPTER VII
CONCLUSION

In this study, the liquid-liquid extraction is used as optimal method for methamphetamine in bile before analyzing by GC-NPD apparatus. These methods have been validation and accepted in all characteristics of criteria, therefore, these techniques is highly suitable for extraction of methamphetamine in bile. Moreover, the liquid-liquid extraction (LLE) is the mostly used technique in routine laboratory due to its low cost per case

The study revealed that methamphetamine concentration showed no significant difference of both conditions when compared with the initial day (p-value$\geq$0.05). The same result of percent concentration showed no significant loss when compared between 4ºC and RT. These demonstrated that methamphetamine is stable in bile and can be kept for analysis for 6 months. This result was similar to previous study in the other sample such as urine, blood, plasma and serum. The information can be available to help guide and assume the fate of drugs in bile sample. Thus, the period of storage time had no effect on methamphetamine analysis in bile.

In conclusion concerning storage condition, the storage at 4ºC is better condition for storage methamphetamines in bile than at RT because bile samples have a physiological change such as more viscous and dried when stored at RT. Furthermore, this methodology can be used as a reference for positive bile samples

For further study similar techniques for positive methamphetamine sample should be studied because real sample may be degraded differently from spiked samples and the extraction technique should be applied for other drug abuse analyses.

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