SEPARATION AND DETECTION OF STEREOISOMERS OF METHAMPHETAMINE AND AMPHETAMINE IN FORENSIC SAMPLES BY GC/MS

JUMPOL SUKKWAN

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Thesis

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SEPARATION AND DETECTION OF STEREOISOMERS OF METHAMPHETAMINE AND AMPHETAMINE IN FORENSIC SAMPLES BY GC/MS

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ABSTRACT

Separation of enantiomers is of great use in forensic science. It can help to identify original source of drug detected in forensic samples. This work studied the extraction, detection and quantification of the stereoisomers of amphetamine and methamphetamine in urine samples by GC-MS (Gas chromatograph-Mass spectrometer). The chiral derivatizing reagent, (*S*)-(-)-*N*-trifluoroacetyl-prolyl chloride (*l*-TPC) was employed to separate AP and MA enantiomeric pairs using a normal GC capillary column, Rtx5MS. The method was used to analyze 28 human urine samples, 26 samples which had already tested positive for methamphetamine/amphetamine, 1 excretion of urine from a user of the drug selegiline and one sample from a person who had inhaled Vicks® Vapor Inhaler. A method for extraction, separation and quantification of the nonderivatized drugs was also carried out.

The results of derivatization step in urine gave good separation of the *d* and *l* forms of AP and MA. Quantitation employed m/z 237 and 251 as the quantifier ions for AP-*l*-TPC and MA-*l*-TPC, respectively. The calibration curves for the pairs of analytes gave good linearity with limits of detection for *l*-AP, *d*-MA, *l*-AP and *d*-MA of 0.032, 0.110, 0.059, 0.101 µg/ml, respectively. For the nonderivatized drugs the quantifier ions are m/z 44 and 58 for AP and MA, respectively.

KEY WORDS: STEREOISOMERS / ENANTIOMERS / METHAMPH ETAMINE / AMPHETAMINE / GAS CHROMATOGRAPHY-MASS SPECTOMETRY

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การวิเคราะห์และแยกโครงสร้างสเตอริโอไอโซเมอร์ของเมทแอมเฟตามีนและแอมเฟตามีนในตัวอย่างทางนิติวิทยาศาสตร์โดยวิธีแก๊สโครมาโทกราฟฟีและแมสสเปกโทรเมตรี
(SEPARATION AND DETECTION OF STEREOISOMERS OF METHAMPHETAMINE AND AMPHETAMINE IN FORENSIC SAMPLES BY GC/MS)

จุมพล สุขขวัญ 4837485 SCFS/M

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บทคัดย่อ

การศึกษาเกี่ยวกับการวิเคราะห์และแยกแยะโครงสร้างของ enantiomers ของสาร มีความสำคัญอย่างมากในงานด้านนิติวิทยาศาสตร์ เพื่อช่วยในการติดตามหาต้นตอของส่วนประกอบของสารนั้นๆว่าสารนั้นคือ สารเสพติดหรือไม่ ในงานวิจัยนี้ได้พัฒนาการแยก การหาปริมาณโครงสร้างรูปแบบ *d* และ *l* ของเมทแอมเฟตามีน และแอมเฟตามีนในปัสสาวะของผู้เสพยาบ้า 26 คน ผู้ทานยารักษาโรคและผู้ที่สูดดมสารที่มีเมทตาบอไลท์ของเมทแอมเฟตามีนและหรือแอมเฟตามีน ของยา Selegiline และ Vicks® Vapor Inhaler อย่างละหนึ่งคน ตามลำดับ ซึ่งทดลองโดยวิธีแก๊สโครมาโทกราฟฟีและแมสสเปกโทรเมตรี โดยใช้สารแยกอนุพันธ์คือ (*S*)- (-)- *N*- trifluoroacetyl-prolyl chloride (*l*-TPC) สำหรับแยกอนุพันธ์ของเมทแอมเฟตามีน และแอมเฟตามีน และใช้คอลัมน์ในการแยกคือ Rtx5 (5% diphenyl) ความยาว 30 เมตร เส้นผ่านศูนย์กลางภายใน 0.25 มิลลิเมตร และความหนา 0.25 ไมโครเมตร รวมทั้งยังได้ศึกษาวิธีการสกัดของเมทแอมเฟตามีน และแอมเฟตามีน โดยปราศจากการใช้สารแยกอนุพันธ์อีกด้วย

ผลการวิจัยของวิธีการใช้สารแยกอนุพันธ์พบว่า สามารถแยกโครมาโตรแกรมของโครงสร้าง *d* และ *l* ออกจากกันได้ดี กราฟมาตราฐานมีความเป็นเส้นตรง ความเข้มข้นต่ำสุดที่สามารถตรวจสอบได้ของ *l* แอมเฟตามีน, *d* แอมเฟตามีน, *l* เมทแอมเฟตามีน และ *d* เมทแอมเฟตามีน มีค่าตามลำดับดังนี้คือ 0.032, 0.110, 0.059, 0.101 µg/ml โดยใช้ค่าของ m/z 237 และ 251 สำหรับยืนยัน *d* และ *l* ของแอมเฟตามีน และ *d* และ *l* ของเมทแอมเฟตามีนตามลำดับ สำหรับผลการวิจัยของวิธีที่ไม่ได้ใช้สารแยกอนุพันธ์ สามารถแยกโครมาโตรแกรมของเมทแอมเฟตามีนและแอมเฟตามีน โดยใช้ค่าของ m/z 44 และ 58 สำหรับยืนยันแอมเฟตามีนและเมทแอมเฟตามีน

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LIST OF ABBREVIATIONS

AP	Amphetamine
AR	analytical regent
conc.	concentration
cpm	cycle per minute
° C	Degree Celsius
ca	Calculate
EA	Ethyl acetate
eV	electron volt.
Gas Chromatography (GC)	A chromatographic technique for the analytical separation of complex volatile mixture into their individual components.
HPLC	High Performance Liquid Chromatography
i.d.	inner diameter
IS	Internal standard
KOH	Potassium hydroxide
LOD	limit of detection
LLE	Liquid-Liquid Extraction
l-TPC	(*S*)- (-)- *N*- trifluoroacetyl-prolyl chloride
M	Molarity
MA	Methamphetamine
Mass spectrometer (MS)	A common detector for gas chromatography that gives structural information on the component entering into its ion source.
mg	miligram
Min	minute
ml	microlitter

LIST OF ABBREVIATIONS (CONTS.)

mm	millimeter
MTBE	Methyl tertiary-butyl ether
N	Mole
NaOH	Sodium hydroxide
rpm	round per minute
r^2	linear regression coefficient
SIM	Select Ion Monitoring
SPE	Solid Phase Extraction
STD	Standard
TIC	Total Ion Collection Chromatography
µl	microlitter
µg	microgram

CHAPTER I
INTRODUCTON

1.1 Introduction

In forensic toxicology there were difficulties for many years in the enantiomeric separation of amphetamine formed from drugs (referred to as “precursor drugs to amphetamines”) as well as the differentiation between illegal consumption of amphetamines and its analogues and the legitimate administration of prescribed amphetamines-generating drugs.

In forensic analysis in Thailand there have not been analysis of the enantiomers of amphetamine (AP) or methamphetamine (MA) in forensic sample as carried out in other countries (*e.g.*., USA [1], Australia [2], Japan [3], Taiwan [4]). Therefore there may be arrests of persons who are using legally prescribed amphetamine/methamphetamine-generating drugs. There is a group of compounds that are metabolized by the body to methamphetamine and/or amphetamine. This group of “precursor” drugs includes amphetaminil, benzphetamine, clobenzorex, deprenyl (selegiline), dimethylamphetamine, ethylamphetamine, famprofazone, fencamine, fenethylline, fenproporex, furfenorex, mefenorex, mesocarb, prenylamine. See Figure 1.1 [5] and Table 1.1 [4] show the molecular structures and a summary of some common of these compounds, respectively.

Another example is Vicks® Vapor Inhaler that contains *l*-methamphetamine [6,7]. This inhaler is legally sold in stores in the United States. A dose of 50 mg suffices for urine collected to test positive for unchanged methamphetamine and its major metabolite, amphetamine.

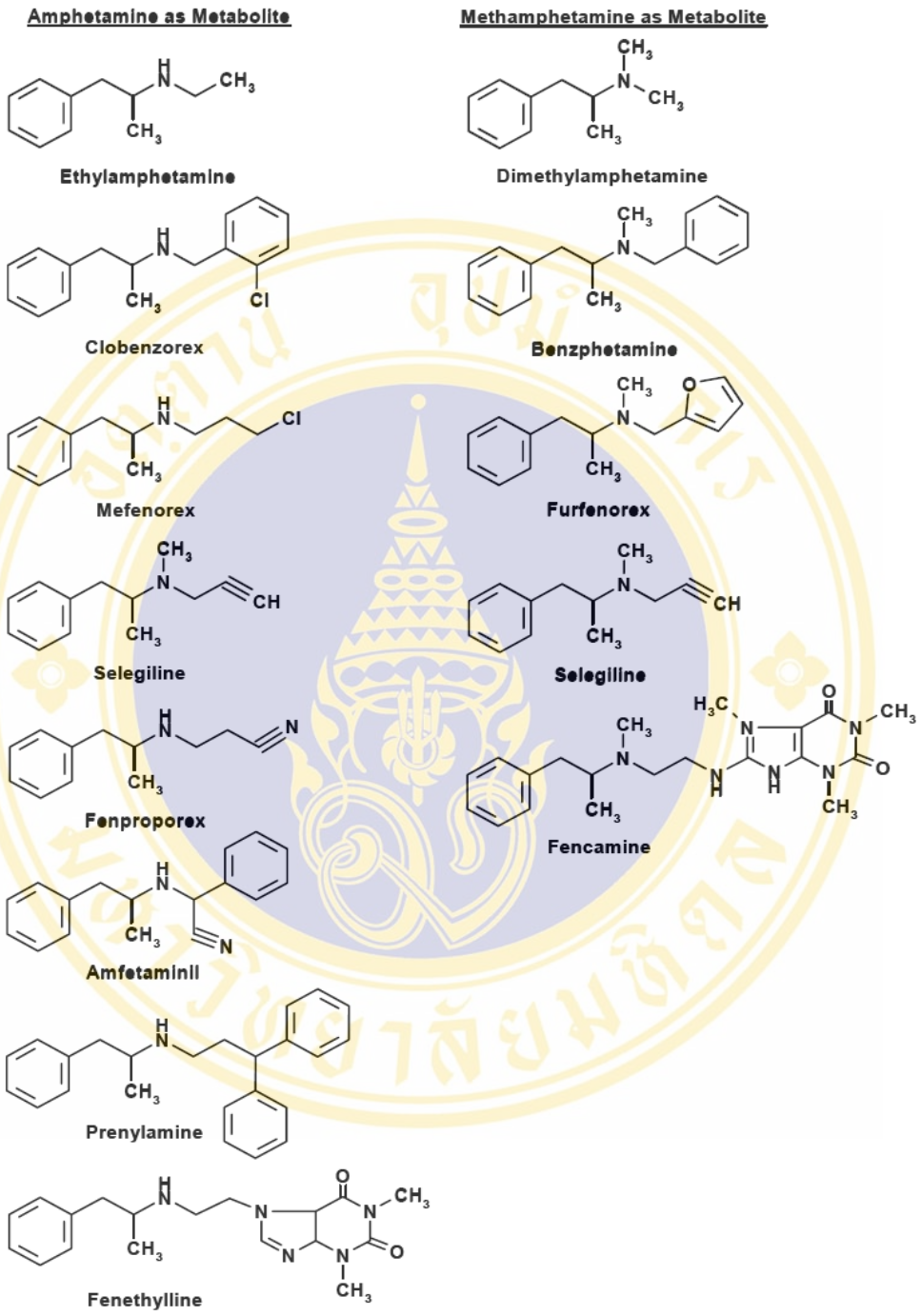


Figure 1.1 Structures of amphetamine and/ or methamphetamine-precursor drugs

Table 1.1 A summary of some common amphetamine/methamphetamine-generating drugs

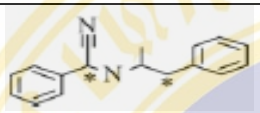
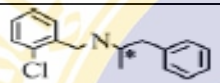
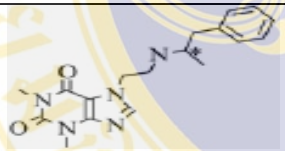
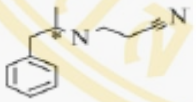
Brand name	**IUPAC name**	**Chemical structure**[a]	**Medical or illegal status**	**Important metabolites**
Amphetaminil	∝-[(1-Methyl-2-phenylethyl)amino] benzeneacetonitrile		Psychotropic drug	AP
Clobenzorex	*N*-[(2-Chlorophenyl)methyl]-∝-methylbenzeneethanamine		Treatment of obesity	AP; 4-Hydroxyamphetamine; 4-Hydroxyclobenzorex
Ethylamphetamine	*N*-Ethyl-∝−methylbenzeneethanamine		Schedule I drug in USA; no recognized medical use	AP;4-Hydroxyethylamphetamine
Fenethylline	3,7-Dihydro-1,3-dimethyl-7-[2-[(1-methyl-2-phenylethyl)amino]ethyl]-1H-purine-2,6-dione		Schedule I drug in USA;treatment of narcolepsy and children with attention deficit disorder	AP; Theophylline; Hippuric acid
Fenproporex	3-[(1-Methyl-2-phenylethyl)amino]-propanenitrile		Treatment of obesity	AP

Table 1.1 A summary of some common amphetamine/methamphetamine-generating drugs (Continued)

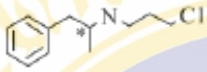
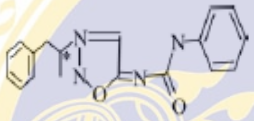
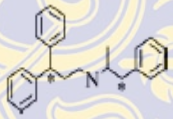
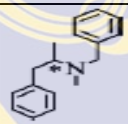
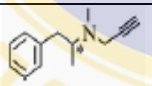
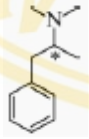
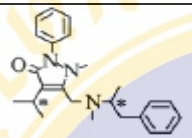
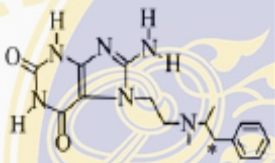
Brand name	**IUPAC name**	**Chemical structure**[a]	**Medical or illegal status**	**Important metabolites**
Mefenorex	*N*-(-3-Chloropropyl)-∝-methyl-benzeneethanamine		Treatment of obesity	AP; 4-Hydroxymefenorex
Mesocarb	3-(1-Methyl-2-phenylethyl)-*N*-(phenylaminocarbonyl)-sydnoneimine		A stimulant; treatment of phantom pain syndrome	AP; Hydroxymesocarb; Dihydroxymesocarb
Prenylamine	*N*-(1-Methyl-2-phenylethyl)-∝–phenylbenzenepropanamine		A coronary vasodilator; treatment of angina	AP; Norephedrine; Diphenylpropylamine
Benzphetamine	*N*,∝-Dimethyl-*N*-(phenylmethyl)-benzeneethanamine		Treatment of obesity	AP; MA; 1-(4-Hydroxyphenyl)-2-(*N*-methyl-*N*-benzylamino) propane
Deprenyl	*N*,∝-Dimethyl-*N*-2-propenylbenzeneethanamine		Treatment of Parkinson's Disease	MA; AP; Desmethyldeprenyl
Dimethylamphetamine	*N*,*N*-∝–Trimethylbenzeneethanamine		No recognized medical use; an illicit drug	MA; AP; Dimethylamphetamine-*N*-oxide

Table 1.1 A summary of some common amphetamine/methamphetamine-generating drugs (Continued)

Brand name	**IUPAC name**	**Chemical structure**[a]	**Medical or illegal status**	**Important metabolites**
Fampropazone	4-Isopropyl-2-methyl-3-[*N*-methyl-*N*-(∝⁻methyl-phenylethyl)-aminomethyl]-1-phenyl-3-pyrazolin-5-one		Antipyretic & analgesic	MA; AP; 3-Hydroxymethylpyrazolone
Fencamine	*N*-Methyl-*N*-(1-methyl-2-phenylethyl)-*N*-3,7-dihydro-1,3,7-trimethyl-8-[[2-[methyl(1-methyl-2-phenylethyl)amino]ethyl]amino]-1H-purine-2,6-dione		Treatment of depression	MA; AP
Furfenorex	*N*-Methyl-*N*-(1-methyl-2-phenylethyl)-2-furanmethanamine		Treatment of obesity	AP; MA; 1-Phenyl-2-(*N*-methyl-*N*-ɣvalerolactonylamino)propane

[a] The asterisks designate the asymmetric carbons and imply the possible stereoisomerism.

Development of a simple analysis of enantiomers of MA and AP is of interest to forensic toxicology. The simplest method for determination of the enantiomeric composition of chiral compounds involves measurement of the specific rotation of a solution of the enantiomeric mixture, but this is impractical for determination of trace amounts of the enantiomers. Various chromatographic procedures for HPLC or GC have been developed for enantiomeric resolution of amphetamine, after sample derivatization, have become available [2]**.** The derivatization affords greater selectivity and sensitivity in enantiomeric separation. Existing commonly used chromatographic methods for enantiomeric separation all employ derivatization.

This research will study suitable method of extraction, detection and quantification of *d* and *l* forms of MA and AP in urine of persons who have taken amphetamine/ methamphetamine directly or prescribed amphetamine/ methamphetamine-producing drugs(e.g. selegiline, Vicks® Vapor Inhaler) by GC-MS.

1.2 Aim of Study

- Enantiomeric analysis of amphetamine and methamphetamine present in urine as the indicator of the probable source of the drug for forensic purposes in order to distinguish people who take amphetamines/methamphetamine illegally from those taking prescribed drugs.
- To study suitable method of extraction, detection and quantification of *d* and *l* form of MA and AP.
- To provide fundamental data for forensic toxicology in Thailand.

CHAPTER II
LITERATURE REVIEW

This chapter gives a review of enantiomeric analysis of amphetamine and methamphetamine, definition of stereoisomers, chemical and pharmacological properties of amphetamine, methamphetamine, selegiline, Vicks® Vapor Inhaler, chiral derivatization, and basics of gas chromatography-mass spectrometry (GC-MS).

2.1 History of enantiomers analysis of amphetamine and methamphetamine

Immunoassays are frequently used for urine screening for amphetamines in order to differentiate between negative and presumptively positive samples. Positive results must be confirmed by a second independent method that is at least as sensitive as the screening test and that provides the highest level of confidence in the result. Without doubt, gas chromatography-mass spectrometry (GC-MS) is the most widely used method for confirmation of positive screening tests [8-12], since it provides high levels of specificity. The mandatory Guidelines for Federal Workplace Drug Testing in the US also demand GC-MS as confirmation method. Besides GC-MS, further methods like GC with other detectors, high-performance liquid chromatography (HPLC), high-performance thin-layer chromatography (HPTLC) or capillary electrophoresis (CE) have been used.

The great number of publications on amphetamine analysis published in the last five years indicates that it was necessary to improve the methods of extraction and derivatization, and the instrumental techniques. Reports on false positive MA results by GC-MS stimulated these efforts.

Sample preparation for the GC separation of the amphetamine enantiomers different chiral derivatization reagents were used: trifluoroacetyl-*S*-prolyl chloride (TPC) [13-16], heptafluorobutylryl-*S*-prolyl chloride (HFBP) [17] and 1*R*,2*S*,5*R*-(-)-menthyl-chloroformate (MCF) [18]. As demonstrated by Maurer et al. [19], chiral GC columns are also suitable for separation of enantiomers of AP and MA extracted from urine samples of patients treated with selegiline. The disadvantages of the chiral columns are the relative thermal lability, the insufficient separation power [17] and the laborious handling. The gas chromatograph must first be equipped with the chiral column, which can be used only for a specific analytical problem. Using GC-MS with direct interfaces, change of column needs a lot of time, since the MS must also be brought down and the vacuum must be completely restord. A drawback of the TPC reagent is that it is contaminated by the *R*-enantiomer by 0.7% [20] or even more. Furthermore, it is known, that the enantiomers react at different rates with the TPC reagent, but using stable isotopes as I.S. this should be compensated.

2.2 Stereoisomers

Isomers are two or more compounds with the same molecular composition, but different structures often results in different properties. There are two types of isomers- structural and stereoisomers .

Structural isomers are compounds with the same molecular formula, but different chemical structures shown in Figure 2.1 [21].

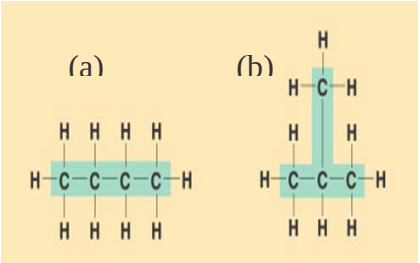


Figure 2.1 Example of structural isomer (a) butane and (b) isobutane

Stereoisomers describes compounds with the same molecular formula and chemical structure, but the atoms are orientated in different directions. There are two isomers, each a mirror image of the other, called enantiomers shown in Figure 2.2 [21].

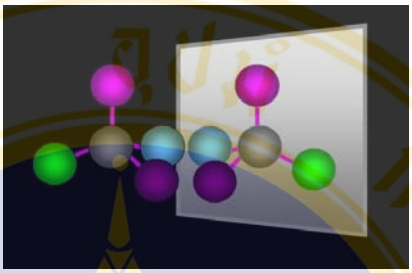


Figure 2.2 These two molecules are stereisomers or enantiomers

2.3 *d* and *l* form

2.3.1 The mean of *d*- and *l*- or (+)- and (-)-

d- and *l*- or (+)- and (-)-, they are systems of naming optical isomers . An optical isomer can be named by the direction in which it rotates the plane of polarized light (see Figure 2.3 [22]. If an isomer rotates the plane clockwise as seen by a viewer towards whom the light is traveling, that isomer is labeled (+).Its counterpart is labeled(-). The (+) and (-) isomers have also been termed *d*- and *l*-, respectively (for *dextrorotatory* and *levorotatory*) examples of *d* and *l* form show in Figure 2.4. [23]

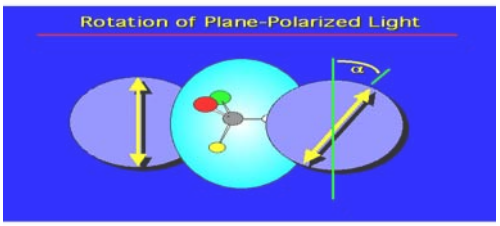


Figure 2.3 Rotation of Plane-Polarized Light

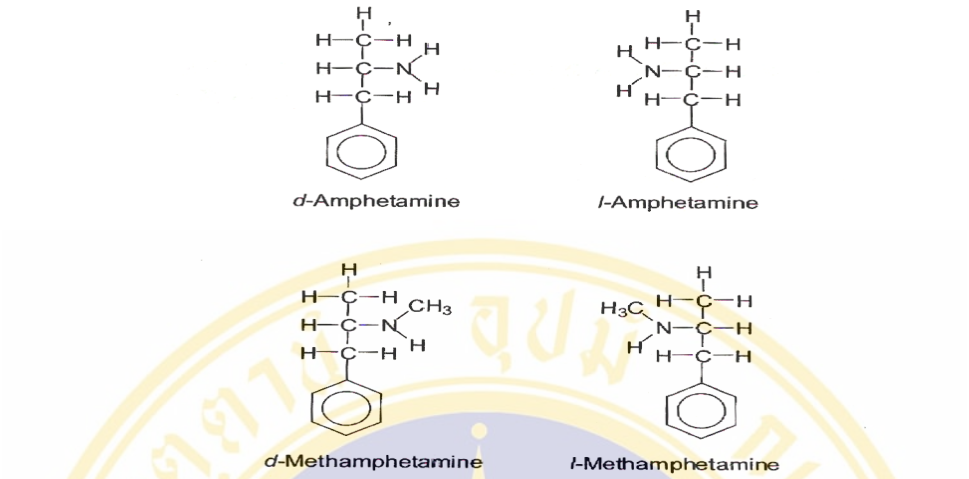


Figure 2.4 Structures of enantiomers of amphetamine and methamphetamine

2.3.2 Properties of *d* and *l* form

Amphetamine (AM, *R,S*-1-phenyl-2-propanamine) and methamphetamine (MA, *R,S*-*N*-methyl-1-phenyl-2-propanamine) are powerful stimulants of the central nervous system. They are drugs of abuse as well as doping agents in sports. The *S*-(+) -enantiomers of AM and MA have five times more psycho stimulant activity than the *R*-(-) –enantiomers [24].

Amphetamine and methamphetamine, the two most commonly encountered of these drugs, have an asymmetric center and thus exist as one of two possible enantiomers. The enantiomers have quite different pharmacological properties, and determination of the enantiomeric form of the drug is often an important consideration. The structures of amphetamine and methamphetamine enantiomers are shown in Figure 1.1. Substitutions have been made to amphetamine and methamphetamine for a variety of reasons. Many modifications were made to these drugs in attempts to maintain anorexic activity while limiting undesirable side effects. Others have been made to enhance the stimulatory activity or to avoid legal restrictions on the production and use of the drug.

Illicit amphetamine is produced as a racemate, with equal concentrations of both the *l*-isomer and the *d*-isomer giving an *l*/*d*-isomer ratio of approximately 100%, dependent on the manufacturing process. This has been demonstrated previously by the analysis of 17 specimens of illicit amphetamine powder, which was found to have a mean *l*/*d*-isomer ratio of 89.2% [25]. Dexamphetamine, the pharmaceutical preparation of the *d*-isomer of amphetamine, has an *l*/d-isomers ratio of approximately only 5% due to some residual impurity from its manufacture and purification [26]. The determination of the *l*/*d*-isomer ratio above 50% in using primarily illicit amphetamine [14].

After administration of racemic amphetamine the optical isomers were not excreted at the same rate. The ratio of the *d*-isomer to the *l*-isomer in the urine decreased from 0.99 during the first 12 hr to 0.46 at 48–60 hr [27]. Although the total urinary excretion of amphetamine is pH-dependent, the differential excretion of the *d*- and *l*-isomers occurred whether the urine was acid or alkaline. Racemic methylamphetamine was metabolized to amphetamine and the main isomer excreted in the urine was *d*-amphetamine, whereas
l-amphetaminewas formed to a lesser extent.

2.4 Physical properties of amphetamine, methamphetamine, selegiline, Vick® nasal inhalers.

2.4.1 Amphetamine

IUPAC name: 1-phenylpropan-2-amine

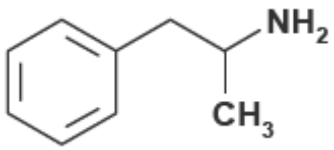


Figure 2.5 Structure of amphetamine

Chemical formula of amphetamine is $C_9H_{13}N$ (see Figure 2.5). Molecular weight is 135.2084. Melting point is 156.5-158.5^{0}C and boiling point is 203^{0}C. Bioavailability in human is 4L/kg; low binding to plasma proteins (20%) and metabolism hepatic & renal clearance. Elimination half life is 10-13 hours. Excretion significant portion unaltered. Legal status is in schedule I of abuse drug control in Thailand.

Physiological effects are short-term and long-term, physiological effects of short-term are physical energy, increased sexual drive/response, involuntary bodily movements, nausea, itchy, blotchy, increased heart rate, irregular heart rate, and headaches. Long-term abuse or overdose effects can include tremor, restlessness, changed sleep patterns, gastrointestinal narrowing, and weakened immune system, fatigue , depression and schizophrenia and dead .Routes of administration, amphetamine most frequently taken orally or intranasally (snorting) as the sulfate or phosphate salt in does ranging from 5-15 mg. in occasional users to 100 -2,000 mg per day in habitual users**.**

2.4.2 Methamphetamine

IUPAC name: (S)-N-methyl-1-phenyl-propan-2-amine

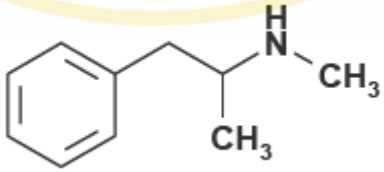


Figure 2.6 Structure of methamphetamine

Chemical formula is $C_{10}H_{15}N$ (see in Figure 2.6). Molecular weight is 149.24. Melting point is 175 ^{0}C and boiling point is 214 ^{0}C. Bioavailability, depends upon method of administration. Metabolism hepatic elimination half life is 4-12 hours, 8 hours on average. Legal status is in schedule I of abuse drug control in Thailand.

Shot-term effect is increased alertness, motivation, brain activity, etc. Long-term effect is cognitive impairment due to neurotoxicity, damage to immune system and dead.

Methamphetamine is an addictive stimulant drug that strongly activates central nervous system. It is closely related chemically to amphetamine, but the central nervous system effects of methamphetamine are greater [28]. Both drugs belong to a group of structurally related drugs called sympathomimetic amines (SMAs) that the brain stimulants [6]. They have some medical uses, primarily in the treatment of obesity , nasal congestion, narcolepsy, and depression but their therapeutic use is limited**.** Methamphetamine as the hydrochloride salt is most frequently prepared for injection or for smoking (“ice”) but is also available in tablet form .

2.4.3 Metabolism and excretion of amphetamine and methamphetamine

Once a drug is exposed to a biological system, it may be converted to different forms as a result of degradation and enzymatic reactions reactions. The biotransformation process is divide into two categories: metabolic reactions and conjugation and hydrolysis; conjugation reactions combine products of metabolism with endogenous constituents such as glucoronic acid, sulfuric acid and amino acids (*e.g.*, glycine)

Metabolism of drug is facilitated by enzymatically catalyzed reactions in the blood, kidney, intestinal mucosa and to the great extent, liver.

The overall purpose of these transformations is to produce derivatives of higher solubility in the aqueous phase so that they may be readily excreted through the kidney. These derivatives, in general, are less toxic than their parent compounds and more easily excreted. The drugs and the metabolites in biological samples are present in much lower concentrations. In some case, only metabolites are found in the urine without the parent compound. Basic knowledge of drug metabolism is essential to

effective handing of biological samples for the analysis of abused drugs and their metabolites [29].

Methamphetamine begins to be metabolized to amphetamine almost immediately when it enters the body. This metabolism to amphetamine occurs both in the liver and also throughout the entire body. The enzyme, monoamine oxidase, which breaks methamphetamine into amphetamine, is the same enzyme that body. Therefore, we begin to see measurable concentrations of amphetamine metabolite almost immediately after methamphetamine are administered to the body [30].

The major metabolic pathway for amphetamine involves deamination by cytochrome P_{450} to para-hydroxyamphetamine and phenylacetone; this latter compound is subsequently oxidized to benzoic acid and excreted as glucuronide or glycine (hippuric acid) conjugate. Smaller amounts of amphetamine are converted to norephedrine by oxidation. Hydroxylation produces an active metabolite, O-hyroxynorephedrine, which acts as a false neurotransmitter and may account for some drug effect, especially in chronic users [31].

Following oral doses of 2.5-15 mg of amphetamine, peak plasma levels of 30-170 µg/ml are reached in 2 hours and plasma elimination half-life range from 8-12 hours. Blood concentrations in fatalities are normally above 500µg/ml.

Amphetamine and methamphetamine begin to appear in the urine within 20 minutes of administration. Methamphetamine is excreted as the unchanged drug (44%) and as its major metabolites amphetamine (6-20%) and 4-hydroxymethamphetamine (10%). The metabolic pathway of methamphetamine is summarized in Figure 2.7 [5]. As with amphetamine, acidic urine increase both the rate of excretion and the percentage of unchanged drug excreted [32,33].

Amphetamine is excreted as the unchanged drug, typically 20-30% of the dose, and as deaminated (hippuric acid and benzoic acid) and hydroxylated metabolites, partly as conjugates, typically adding up to 25 % of the dose. The rate of excretion

and the fraction of the dose excreted as unchanged drug vary according to pH of the urine. In alkaline urine about 45% of the dose is excreted in 24 hours, 2% of the dose as the unchanged drug, while in acid urine, up to 78% of the dose may be excreted in 24 hours, 68% as the unchanged drug [32,34]. The recommended target analytes are, therefore, the unchanged drugs. The major and minor metabolic pathways of amphetamine are summarized in Figure 2.8 [5,32] metabolized of AP.

After chronic administration, abusers have shown amphetamine concentrations in urine of 1-90 microgram/milliliters and methamphetamine concentration of 25-300 microgram/milliliters [32,35].

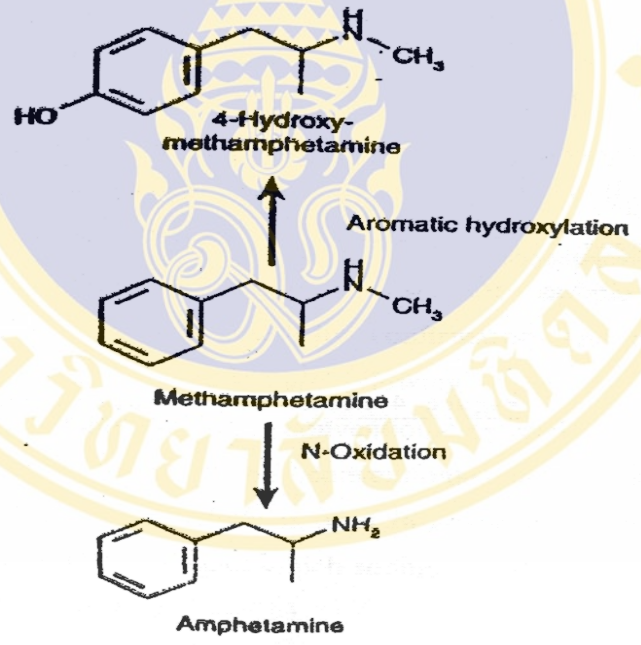


Figure 2.7 Metabolic pathway of methamphetamine

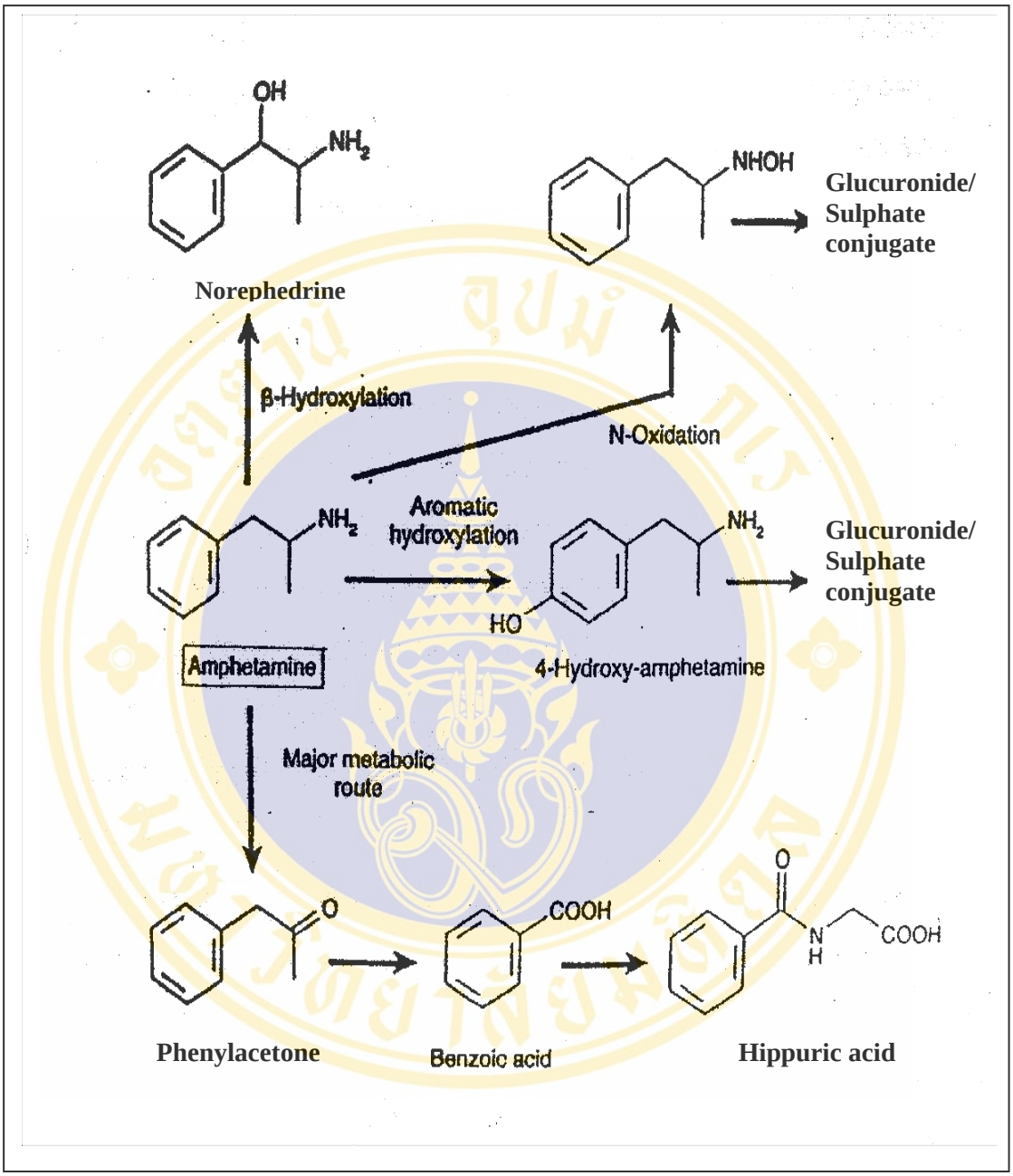


Figure 2.8 Metabolic pathway of amphetamine

2.4.4 Selegiline

ELDEPRYL (selegiline hydrochloride) is a levorotatory acetylenic derivative of phenethylamine. It is commonly referred to in the clinical and pharmacological literature as *l*-deprenyl.

The chemical name is (R)-(-)-N,2-dimethyl-N-2-propynylphenethylamine hydrochloride. It is a white to near white crystalline powder, freely soluble in water, chloroform, and methanol, and has a molecular weight of 223.75 and boiling point equal 80^0C. The structural formula is as follows in Figure 2.9.

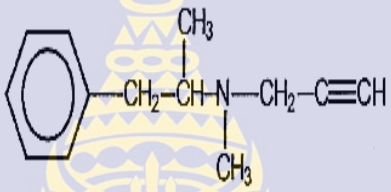


Figure 2.9 Struture of selegiline

Selegiline is used in at least 42 countries as an anti-Parkinsonian drug. Selegiline is metabolized to N-desmethylselegiline, R(-)-methamphetamine, R (-)-amphetamine and their conjugated p-hydroxy derivatives (see Figure 2.10). For forensic purposes, it is necessary to differentiate the intake of selegiline from abuse of amphetamine and methamphetamine by an enantioselective procedure.

Pharmacokinetics of selegiline presented by Stewart A. and William J. [36], selegiline is a relatively selective, irreversible MAO B inhibitor. It is considered a "suicide inhibitor" because it forms a covalent bond with MAO, and loss of MAO inhibition is dependent on generation of new enzyme. Selegiline is lipophilic and readily absorbed from the gastrointestinal tract. The absolute bioavailability of selegiline is roughly 10%. Ninety-four percent is bound to plasma proteins, with strong binding to macroglobulins. Maximal concentrations are achieved approximately 1/2 to 2 hours after oral administration. Studies of platelets in PD patients have shown that within two to four hours after a single 5 mg dose of

selegiline, MAO B activity is inhibited by 86%, whereas within 24 hours of a 10 mg dose MAO B activity is inhibited by almost 98%. Selegiline readily crosses the blood–brain barrier and accumulates in brain regions rich in MAO B, including the striatum, thalamus, cortex, and brain stem.

Selegiline is metabolized in the liver by the microsomal cytochrome P-450 system to (-)-desmethylselegiline (DES), and *l*-(-)-methamphetamine (*1*-MA); the latter compound is further metabolized to amphetamine and p-hydroxylated metabolites. These metabolites are conjugated with glucuronic acid to form inactive metabolites. Selegiline may also be metabolized outside the liver. Three metabolites have been identified in serum and urine: *l*-(-)-methamphetamine, *l*-(-)-amphetamine, and (-)-desmethylselegiline (DES). Desmethylselegiline has activity as an irreversible MAO B inhibitor, but it is much less potent than selegiline in vitro. Metabolic pathways of selegiline in humans shown in Figure 2.10 [37].

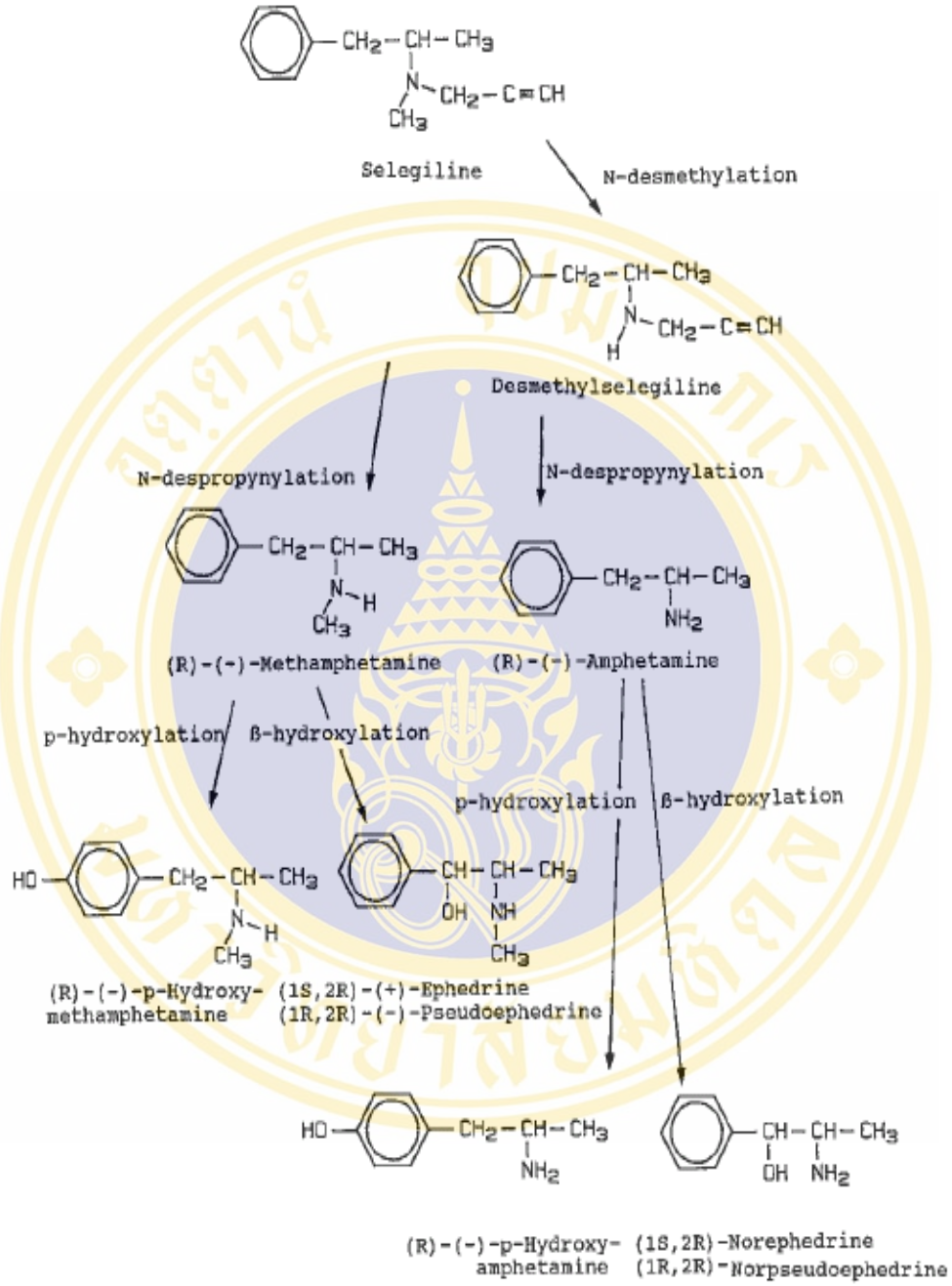


Figure 2.10 Metabolic pathways of selegiline in humans

2.4.5 Vicks® Vapor Inhaler

All the legal Vicks® Vapor Inhaler use merely *l*-MA and a dose of 50 mg suffices the urine collected thereupon to test positive for unchanged MA and its major metabolite.

Ingredients of Vicks® Vapor Inhaler are the active ingredient 50 mg Levmetamfetamine (nasal decongestant) and the inactive ingredients bornyl acetate, camphor, lavender oil, and menthol. Vicks® Vapor Inhaler temporarily relieves nasal congestion due to colds, hay fever, upper respiratory and allergies sinusitis

Figure 2.11 Vicks® Vapor Inhaler [7]

2.5 Derivatization

Derivatization serves several important functions in GC/MS analysis. It can dramatically affect the volatility of compound, improve its chromatographic behavior, and enhance the uniqueness of a compound ´s mass spectrum. Large derivatives yield molecular and fragment ions with higher masses. While they are often advantageous for analysis of smaller drug molecules, such as amphetamine [38].

GC separation of enantiomers can be performed either direct (use of a chiral stationary phase) or indirect (off-column conversion into diastereomeric derivatives and separation by non-chiral stationary phases) [39].

Chiral GC column are designed to separate enantiomers without the need for chiral derivatization. Although effective, these columns have several drawbacks. Typically, chiral columns are more expensive than the corresponding achiral columns. Additionally, chiral columns tend to degrade more readily than achiral columns at the

high temperatures typically encountered in GC procedures and thus must be replaced more frequently. In addition, since they have a specific purpose, they are typically dedicated and not used for general analysis.

Separation of the enantiomers of amphetamines is an important analytical technique that can be very useful in the interpretation of laboratory results. Assessment of enantiomer composition of the drug used, time since administration, origin of the drug, etc., are all parameters that can be elucidated by knowing the enantiomer composition of the drug in the biological sample. Enantiomer analysis has been described by a number of investigators. One method used the derivatizing reagent (-)-methyl chloroformate [21]. Methamphetamine enatiomers were not separated effectively at all. Athough the method provided good quantitative results, its utility was limited by the inability to separate the enantiomers effectively.

The most common chiral derivatizing reagent used with the amphetamines is *N*-trifluoroacetyl-*l*-prolyl chloride (*l*-TPC), pentafluoropropionyl-*l*-prolyl chloride (*l*-PPC) and heptafluorobutyryl-*l*-prolyl chloride (*l*-HPC). All three of these reagent have been used with success in a number of laboratories. Derivatization of amphetamines with *l*-TPC can be carries out at room temperature for 15 minutes [40], although some investigators carry out the reaction at high temperatures (85-90^{0}C) for 10 minutes [41]. For an example of enantiomeric separation of amphetamine and related compounds using this reagent *l*-TPC derivatives of amphetamine and methamphetamine can also be formed by coinjection of the drug extract and derivatizing reagent [42].

In this experiment used indirect method (diastereomeric derivatives) and derivatization is typically accomplish by reconstituting the dried extract with a solvent containing a derivatizing reagent (*S*)- (-)- *N*- trifluoroacetyl-prolyl chloride (*l*-TPC) molecular formula is $C_7H_7ClF_3NO_2$, boiling point = 80^{0}C and MW = 229.58 shown in Figure 2.12 [43] and then allowing the reaction to occur, usually at elevated temperatures, for a specific period of time. While effective, this method requires time for evaporation of the extract, reconstitution, and incubation to derivatize the analytes

of interest. Extractive derivatization of drug is another method that has been successfully employed in a number of cases [44]. As the name implies, this procedure combines extraction with derivatization, thus saving time. Use of the same derivatizing reagent for different drug assays has several advantages. From a practical standpoint, preparation and use of one derivatizing reagent saves significant time and effort in the laboratory with respect to personnel.

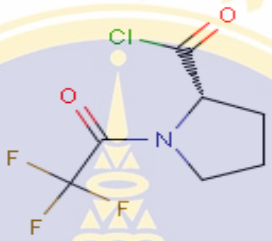


Figure 2.12 (*S*)- (-)- *N*- trifluoroacetyl-prolyl chloride (*l*-TPC)

Derivatization of the amphetamines and their metabolites, Isolation was performed by liquid-liquid extraction (LLE) usually at an alkaline pH. LLE and SPE are discussed in the review of Franke and Zeeuw [45]. Derivatization of the amphetamines is necessary to improve their GC properties, to form more characteristic mass spectral fragment ions, to introduce halogen atoms for sensitive negative chemical ionization (NCI) or electron capture detection (ECD).

Evaporation steps after extraction and/or derivatization may lead to loss of the amphetamines because of their high volatility. Therefore, addition of hydrochloric acid before evaporation is recommended to form less volatile hydrochlorides [46,47]. However, this can also cause problems. In our experience, traces of the acid in the GC system may impair detection of basic drugs. Evaporation of aqueous HCl requires high temperatures or longer evaporation times, so that evaporation losses of the amphetamines may increase. Use of alcoholic solutions of HCl (e.g., methanolic, isopropanolic) may help. However, it should be possible to renounce use of HCl reported from Dallakian [48] reported that evaporation at 48^0C without addition of HCl did not lead to any loss of amphetamine. This is in accordance with our results

concerning LLE of AM from urine. Careful evaporation allowed recoveries of over 80 % with coefficients of variation (C.V.s) of less than 5% [49].

2.6 Gas chromatography and mass spectrometry (GC-MS)

2.6.1 History and instrumentation of GC-MS [50]

Gas chromatography-mass spectrometry (GC-MS) is a method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample. Applications of GC-MS include drug detection, fire investigation, environmental analysis, and explosives investigation. GC-MS can also be used in airport security to detect substances in luggage or on human beings. Additionally, it can identify trace elements in materials that were previously thought to have disintegrated beyond identification.

The GC-MS has been widely heralded as a "gold standard" for forensic substance identification because it is used to perform a specific test. A specific test positively identifies the actual presence of a particular substance in a given sample. A non-specific test, however, merely indicates that a substance falls into a category of substances. Although a non-specific test could statistically suggest the identity of the substance, this could lead to false positive identification.

History, the use of a mass spectrometer as the detector in gas chromatography was developed during the 1960s. These sensitive devices were bulky, fragile, and originally limited to laboratory settings. The development of affordable and miniaturized computers has helped in the simplification of the use of this instrument, as well as allowed great improvements in the amount of time it takes to analyze a sample. In 1996 the top-of-the-line high-speed GC-MS units completed analysis of fire accelerants in less than 90 seconds, whereas first-generation GC-MS would have required at least 16 minutes. This has led to their widespread adoption in a number of fields.

Instrumentation, the GC-MS is composed of two major building blocks: the gas chromatograph and the mass spectrometer. The gas chromatograph uses the difference

in the chemical properties between different molecules in a compound to separate the molecules. The molecules take different amounts of time (called the retention time) to come out of the gas chromatograph, and this allows the mass spectrometer downstream to evaluate the molecules separately in order to identify them. The mass spectrometer does this by breaking each molecule into ionized fragments and detecting these fragments using their charge to mass ratio. Each molecule has a specific fragment spectrum which allows for its detection.

These two components, used together, allow a much finer degree of substance identification than either unit used separately. It is possible to make an accurate identification of a particular molecule by gas chromatography or mass spectrometry alone. The mass spectrometry process normally requires a very pure sample while gas chromatography can be confused by different molecular types that both happen to take about the same amount of time to travel through the unit (i.e. have the same retention time). Sometimes two different molecules can also have a similar pattern of ionized fragments in a mass spectrometer (mass spectrum). Combining the two processes makes it extremely unlikely that two different molecules will behave in the same way in both a gas chromatograph and a mass spectrometer. So when an identifying mass spectrum appears at a characteristic retention time in a GC-MS analysis, it is usually taken as proof of the presence of that particular molecule in the sample.

Analysis, the primary goal of chemical analysis is to identify a substance. This is done by comparing the relative concentrations among the atomic masses in the generated spectrum. Two kinds of analysis are possible, comparative and original. Comparative analysis essentially compares the given spectrum to a spectrum library to see if its characteristics are present for some sample in the library. This is best performed by a computer because there are a myriad of visual distortions that can take place due to variations in scale. Computers can also simultaneously correlate more data (such as the retention times identified by GC), to more accurately relate certain data.

Another analysis measures the peaks in relation to one another, with the tallest peak receiving 100% of the value, and the others receiving proportionate values, with all values above 3% being accounted for. The parent peak normally indicates the total mass of the unknown compound. This value can then be used to fit to a chemical formula containing the various elements assumed to be present in the compound. The isotope pattern in the spectrum, which is unique for elements having many isotopes, can also be used to identify the various elements present. Once a chemical formula has been matched to the spectrum, the molecular structure and bonding can be identified, and needs to be consistent with a substance with the characteristics recorded by GC-MS. The fitting is normally done automatically by programmes which come with the machine, given a list of the elements which could be present in the sample.

A “full spectrum” analysis considers all the “peaks” within a spectrum. However, selective ion monitoring (SIM) which looks only at a few characteristic peaks associated with a candidate substance, can also be done. This is done on the assumption that at a given retention time, a set of ions is characteristic of a certain compound. This is a fast and efficient analysis, especially if you have some prior information about a sample or are looking for a specific compound. When the amount of information collected about the ions in a given gas chromatographic peak is reduced, the sensitivity of the analysis goes up. So, SIM analysis allows a smaller quantity of a compound to be detected and measured, but the degree of certainty about the identity of that compound is reduced.

2.6.2 Applications and criminal forensics

GC-MS can analyze the particles from a human body in order to help link a criminal to a crime. The analysis of fire debris using GC-MS is well established, and there is even an established American Society for Testing Materials (ASTM) standard for fire debris analysis. Law enforcement, GC-MS is increasingly used for detection of illegal narcotic, and may eventually supplant drug-sniffing dogs.

CHAPTER III
MATERIALS AND PROCEDURES

3.1 Materials

3.1.1 Instrumentation

The GC-MS system used for the chromatographic experiments and other instruments used for the preparation of solution and sample clean-up are given as follows.

Table 3.1 Instruments

Instrument	Model	Company
1. GC-MS	GC-MS 2000	Thermo Finnigan (Germany)
1.1 Gas chromatograph (GC)	Trace2000	Germany
1.2 Mass spectrometer (MS)	PolarisQ; Ion Trap	Germany
1.3 Injector	AS2000	Germany
1.4 Column	Rtx-5MS (5% diphenyl-95% dimethylpolysiloxane (30 m x 0.25 mm x 0.25 µm)	Restex (USA)
1.5 Data system	Xcalibur 1.4	Germany
2. Centrifuge	Cetromic CE 95	J.P.Selecta (UK)

Table 3.1 Instruments (Continued)

Instrument	Model	Company
3. Heating box	Boekel	Boekel Scientific (USA)
4. Shaker	GFL 3006	Perch (Germany)
5. Vortex Mixer	G-560 E	Scientific industries (USA)
6. Autopipette	Eppendorf Research	Eppendorf (Germany)

3.1.2 Reagents

All chemicals and organic solvents were analytical or GC grade.

Table 3.2 List of reagents and suppliers

Chemical	Supplier	Grade
1. Potassium hydroxide	Ajax Finechem (AUS)	AR
2. Sodium sulfate anhydrous	Merck (Germany)	AR
3. Sodium hydroxide	Merck (Germany)	AR
4. Methyl tertiary-butyl ether (MTBE)	LABSCAN (Thailand)	GC
5. Ethyl acetate	LABSCAN (Thailand)	GC
6. (*S*)- (-)- *N*- trifluoroacetyl-prolyl chloride (*l*-TPC)	Aldrich (USA)	AR
7. Diphenylamine	Sigma-Aldrich (USA)	AR
8. Standard of 1mg/ml of racemic *d,l*-MA, *d,l*-AP and *d*-MA and *l*-AP	Sigma-Aldrich (USA)	AR

3.2 Urine samples

Urine samples (n = 26) were obtained from Toxicology Department of Thunyaruk Hospital, Toxicology Department of Ramathibodi Hospital and Toxicology Department of Maharaj Hospital, Chiangmai. One urine samples from subject who took selegiline was obtained from National Doping Control Centre (NDCC), Mahidol University. Urine sample were obtained from a volunteer who inhaled Vicks® Vapor Inhaler. The urine samples were stored in glass bottles and kept at -20 ^{0}C until analyzed [13].

A horse urine that had been tested positive for amphetamine/methamphetamine was also obtained from NDCC, Mahidol University. This sample was used in the development of the derivatizing procedure and GC-MS chromatography.

3.3 Preparation of standard solutions and other reagents

3.3.1 Standard solution

Stock standard solution of 1000 µg/ml (ppm) of racemic *d,l*-MA, *d,l*-AP and *d*-MA and *l*-AP were prepared in methanol.

3.3.1.1 Standard 100 µg/ml of racemic *d,l*-MA, *d,l*-AP, *d*-MA and *l*-AP

2 ml of 100 µg/ml of racemic *d,l*-MA, *d,l*-AP, *d*-MA and *l*-AP were prepared by transferring 200 µl from the stock standard solutions (1000 µg/ml) and adding 1.8 ml methanol for each of the standard. The bottles were sealed with Parafilm® and wrapped with foil and stored at 4 ^{0}C. This solution was used as the stock solution for preparation of working solutions used in the calibration standard and GC-MS studies.

3.3.2 Internal standard solution

The stock internal standard (IS) solution was 1000 µg/ml diphenylamine in methanol. The solution was sealed with Parafilm®, wrapped with foil and stored at 4 ^{0}C. The stock solution was diluted to give suitable working solution for the preparation of the calibration standard and for urine samples.

3.3.3 Potassium hydroxide, 5 M

Potassium hydroxide pellets (ca.7 g) was dissolved in deionized-distilled water and made up to 25 ml. This solution was used for adjustment of pH of reagent solutions.

3.3.4 Sodium hydroxide, 0.01 M

Sodium hydroxide pellets (0.16 g) was dissolved in deionized-distilled water and made up to 400 ml. This solution was used as the reducing agent for the derivatizing reagent (*l*-TPC).

3.4 Calibration curve of derivatization method and limit of detection

3.4.1 Preparation of calibration curve

Blank urine sample volume 2 ml were spiked with standard solutions of racemic *d,l*-MA, *d,l*-AP (1,10 and 20 µg/ml) by transferring 20, 200 and 400 µl from the stock standard solution (100 µg/ml), respectively and all samples contained 10 µl of 1mg/ml of diphenylamine as the internal standard**.** These samples were subjected to liquid-liquid extraction and derivatization procedure as described in Section 3.6.1.

3.4.2 Limit of detection

The limit of detection is the lowest concentration of an analyte that an analytical process can reliably detect [51]. Several methods for determining the detection limit have been proposed. In this work calculation of limit of detection are based on the recommendation of the International Conference on Harmonisation (ICH) Validation of Analytical Procedures Guideline [52] as described in Appendix.

3.5 Non derivatization method

3.5.1 Extraction procedure for non derivatized sample

The volume of urine is 2 ml. The extraction procedure is shown below.

- Add 10 μL of 1mg/ml methanolic solution of diphenylamine (IS) using a micropipette
- Vortex for 3 sec.
- Add 200 μL of 5 M KOH.
- Vortex for 3 sec.
- Add ~2 g of anhydrous sodium sulfate.
- Add 1 ml of *tertiary*-butyl methyl ether.
- Cap tubes with teflon-lined screw cap and examine for leakage.
- Shake horizontally for 15 min at 300 cpm.
- Centrifuge for 15 min at 2,500 rpm.
- Transfer ~ 200 μL of the upper organic phase into the insert of a GC vial.
- Inject 1 μL onto GC-MS.

3.6 Derivatization method

3.6.1 Extraction procedure with derivatization of sample

The volume of urine is 2 ml. The extraction and derivatizing procedures are shown below.

- Add 10 μL of 1mg/ml methanol solution of diphenylamine (IS) using a micropipette
- Vortex for 3 sec.
- Add 200 μL of 5 M KOH.
- Vortex for 3 sec.
- Add ~2 g of anhydrous sodium sulfate.
- Add 2 ml of *tertiary*-butyl methyl ether.
- Cap tubes with teflon-lined screw cap and examine for leakage.
- Shake horizontally for 15 min at 300 cpm.
- Centrifuge for 15 min at 2500 rpm.
- Transfer upper organic phase to new test-tube.
- Add 50 μL of 0.1 M (*S*)-(-)-*N*-(trifluoroacetyl)-prolyl chloride (*l*-TPC) in dichloromethane.
- Vortex for 3 sec.
- Stand at room temperature for 15 min**.**
- Add 3 ml 0.01 M NaOH and shake horizontally for 15 min on the shaker.
- Centrifuge sample at 2500 rpm for 15 min.
- Transfer upper organic phase into new test-tube.
- Evaporate the sample in the tube to dryness under N_2 stream at 45 ^{0}C in a fume hood.
- Dissolve the residue in 100 μL of ethyl acetate.
- Transfer sample into the insert of a GC vial and inject 1 μL onto GC-MS.

3.6.2 Development of GC temperature program for separation of derivatized AP and MA

The following temperature programs were tested.

Program 1: (as described in Reference [4])
Initial temperature 60 ^{0}C; hold 5 min; ramp 25 ^{0}C/min to 250 ^{0}C; hold 5.4 min.
Total runtime = 18 min

Program 2: (as used by NDCC, Mahidol University)
Initial temperature 70 ^{0}C; hold 1 min; ramp 25 ^{0}C/min to 300 ^{0}C; hold 1 min.
Total runtime = 11.2 min

Program 3:
Initial temperature 70 ^{0}C; hold 1 min; ramp 25 ^{0}C/min to 250 ^{0}C; hold 3.5 min; ramp 25 ^{0}C/min to 300 ^{0}C; hold 1.5 min.
Total runtime = 15.2 min

Samples used in this experiment were:

(a) Solution 1 ppm of *l*-AM and *d*-MA in negative control urine.
(b) Negative control urine.
(c) Horse urine, positive for MA.

All the above samples were extracted and derivatized as described in Section 3.6.1

3.7 Optimal parameters for the gas-chromatograph and mass spectrometer

Table 3.3 Parameters of gas-chromatograph and mass spectrometer for derivatized and non-derivatized AP and MA

Parameters	Condition
Instrument identification	
GC	TRACE GC 2000
MSD	PolarisQ (Ion trap)
Injector	AS2000, Autosampler
Carrier gas	
Type	Helium, head pressure 48 kPa at 70 ^{0}C
Mode	Constant flow
Flow rate	1.0 ml/min
Column	
Type	Rtx-5MS (Restex, USA) 5% diphenyl-95% dimethylpolysiloxane
Length	30 m
Internal diameter	0.25 mm
Film thickness	0.25 μm
Split	
Mode	Splitless
Split flow	50 ml/min
Splitless time	1 min
Gas saver	
Mode	On
Gas saver flow	30 ml/min
Gas saver time	3 min
Temperature	
Injector port	280^{0}C
Non-derivatized	70^{0}C, hold for 1 min, rising by 25^{0}C/min to 300^{0}C, hold 1 min

Table 3.3 Parameters of gas-chromatograph and mass spectrometer for derivatized and non-derivatized AP and MA (Continued)

Derivatized	70^0C, hold for 1 min, rising by 25^0C/min to 250^0C, hold for 3.5 min, rising by 25^0C/min to 300^0C, hold for 1.5 min
Interface	275^0C
Detector	Ion trap Mass Spectrometer, PolarisQ
Ionisation mode	Electron impact
Electron Energy	70 eV
Solvent delay	3 min
Source temperature	200 ^{0}C
Acquisition mode	Full scan
Low mass	40
High mass	450
Micro scan	3
Max Ion Time	25 msec
Total run time	
Non-derivatized	11.2 min
Derivatized	15.2 min
Computer Software	Xcalibur 1.4

3.8 Study of some factors in the extraction and derivatizing steps

3.8.1 Study effect of drying process in the non derivatized method

Samples used in this experiment are negative control urine, positive urine and standard solution (5 ppm) of *d,l*-AP, *d,l*-MA in negative control urine. The samples were extracted as described in Section 3.4.1 but after centrifugation the organic layer was transferred to another test-tube. The sample was then dried under nitrogen gas and heated at 45 ^{0}C and then the residue dissolved in 100 µL ethyl acetate.

3.8.2 Study of the effect of NaOH in the derivatization method

3.8.2.1 Effect of NaOH

Samples were extracted and derivatized as described in Section 3.5.1

3.8.2.2 Effect of not adding NaOH

The extraction and derivatizing procedures were as described in Section 3.5.1 but the NaOH solution was not added after the addition of the derivatizing agent (*l*-TPC). The organic phase was then dried under nitrogen gas and the residue dissolved in 100 µL ethyl acetate. It was then transferred to the GC-insert.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Introduction

In this work two methods for extraction, separation and detection of amphetamine and methamphetamine in human urine were studied. The first method is a simple liquid-liquid extraction followed by gas chromatography with mass spectrometry detection. In the second method, the amphetamines are first extracted and then derivatized with a chiral reagent, *l*-TPC. The derivatized products are then separated by gas chromatography with mass spectrometry detection. It is now possible to separate the *d*- and *l*-enantiomers.

4.2 Identification of amphetamine and methamphetamine

Compounds are identified from comparison of their mass spectra with the mass spectra of known standards. However due to co-eluting compounds exact match of the spectra may not be possible. Thus a small number of mass fragments, unique for that particular compound, are selected for comparison between the suspected compound and the pure standard. The ion fragment with the greatest abundance is selected as the quantifying ion which is used for calculating the concentration. Two or more ions, the qualifier ions, are selected. In this work the quantifying ions are m/z 237 and 251 [4] for the diastereomeric pairs of AP-*l*-TPC and MA-*l*-TPC, respectively. The quantifier ions for the underivatized AP and MA are m/z 44 and 58, repectively. [24,38]

Qualifier and quantifier ions for AP and MA are shown in the table below.

Table 4.1 Qualifier and quantifier ions selected for AP and MA

Compound	Qualifier ions (m/z)	Quantifier ions (m/z)
AP	91,42	44
MA	91,56	58
l-AP-*l*-TPC	166[a],194	237
d-AP-*l*-TPC	166[a],194	237
l-MA-*l*-TPC	166[a],194	251
d-MA-*l*-TPC	166[a],194	251

[a] Base peak

4.3 Optimization for non-derivatized AP and MA

4.3.1 GC condition

The choice of split or splitless injection of samples onto the gas chromatographic column is an important consideration. Splitless injection is undoubtedly the method most often used in the analysis of biological extracts as in this work. This is because of the sensitivity achievable by splitless injection over split injections [38].

4.3.2 GC temperature program

To find the optimum GC temperature program for non-derivatized AP and MA, two temperature programs were compared as shown in Figure 4.1.

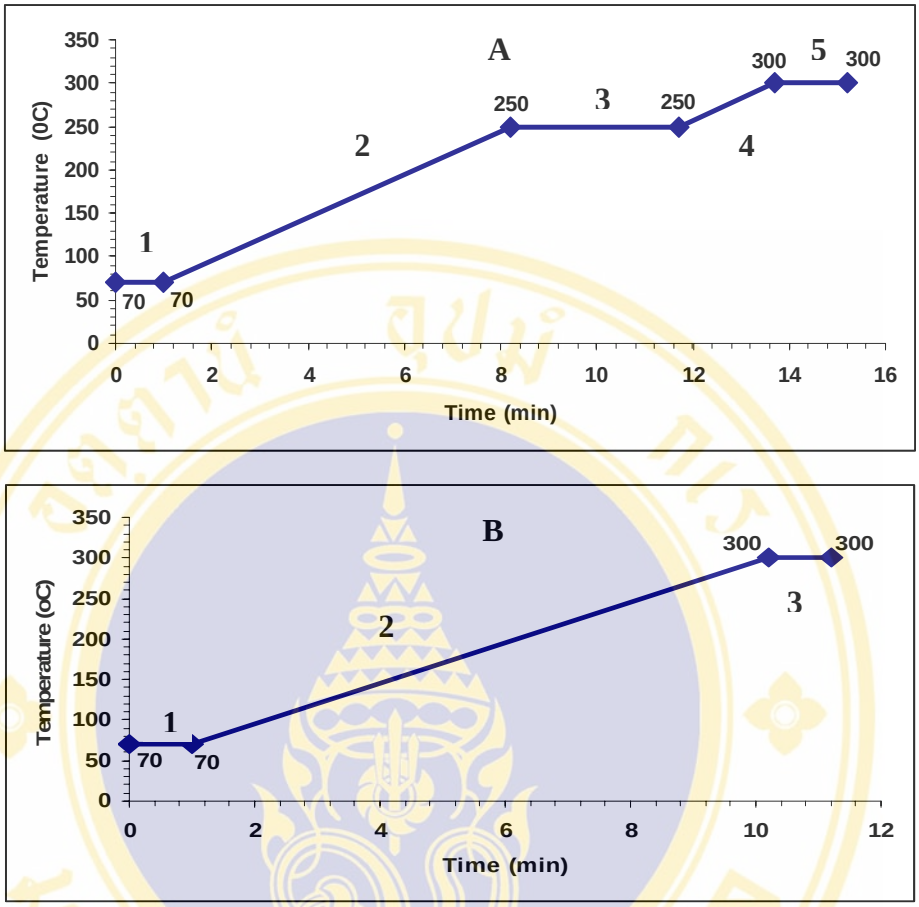


Figure 4.1 Comparison of GC temperature Programs A and B, for AP and MA.

Temperature Program A is as follow:

1: initial temperature 70^{0}C, hold 1 min.

2: ramping at 25^{0}C/min to 250^{0}C.

3 hold 3.5 min at 250^{0}C.

4: ramping 25^{0}C/min to 300^{0}C.

5: hold 1.5 min at 300^{0}C (to clean column)

The temperature program for Program B is as follow:

1: initial temperature 70^{0}C, hold 1 min.

2: ramping at 25^{0}C/min to 300^{0}C.

3: hold 1 min at 300^{0}C (to clean column)

The EIC (Extract Ion Chromatogram) for the two GC temperature programs are shown below.

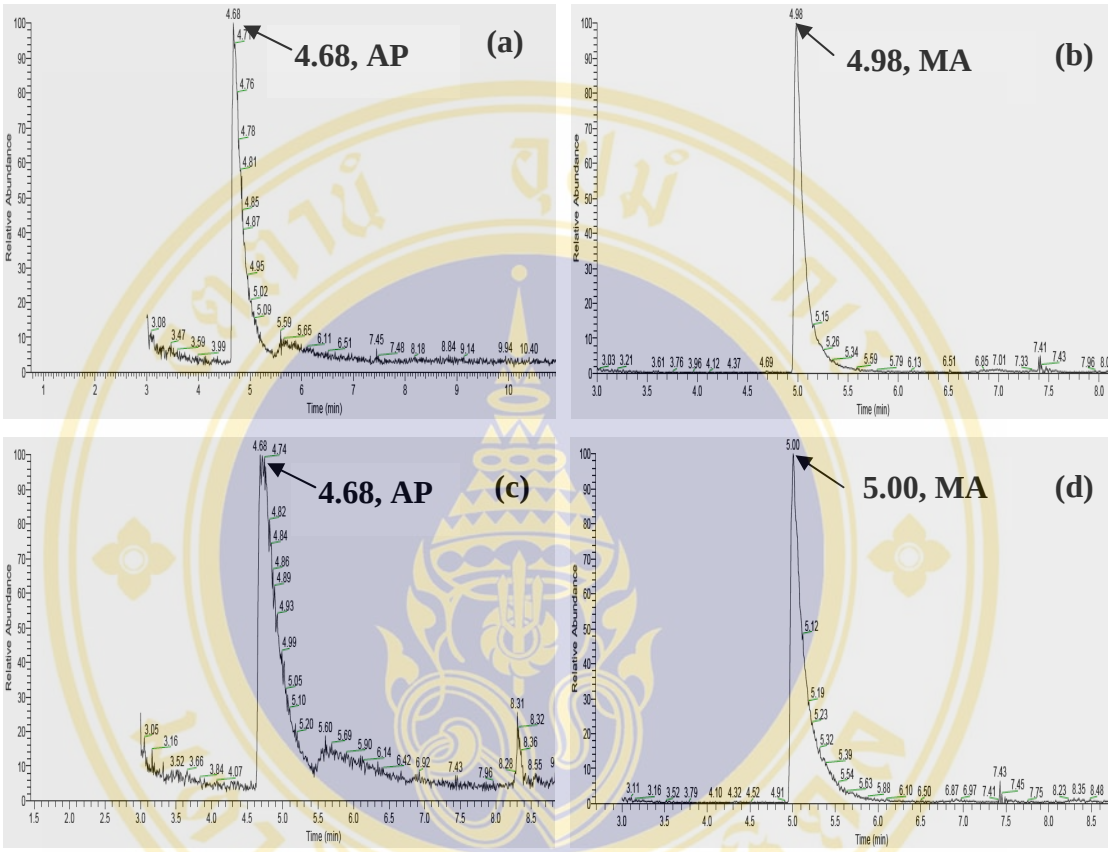


Figure 4.2 Examples of EIC (Extract Ion Chromatogram) of non-derivatized standard mixture (5 ppm) using GC temperature Program A (Figure (a) and (b)) and using GC temperature Program B (Figure (c) and (d)). The mass selected is m/z 44 and 58 for AP and MA, respectively.

GC temperature Program B can separate AP and MA with total runtime of 11.2 minutes. Temperature Program A has a total runtime of 15.2 minutes. This longer runtime was tested to observe whether an extended temperature program can separate the *d* and *l* form of AP and MA.

Optimum temperature program for non derivatized samples is temperature Program B because of total runtime is shorter than type A with good separated of AP and MA.

4.3.3 Non derivatizing procedure

4.3.3.1 Study of the effect of drying samples

This study compared the amount of sample detected when a drying step was included as described in Section 3.5.1. The same GC temperature Program A was used as described in Section 4.3.2. Results for dry and not drying samples are shown in Figure 4.3.

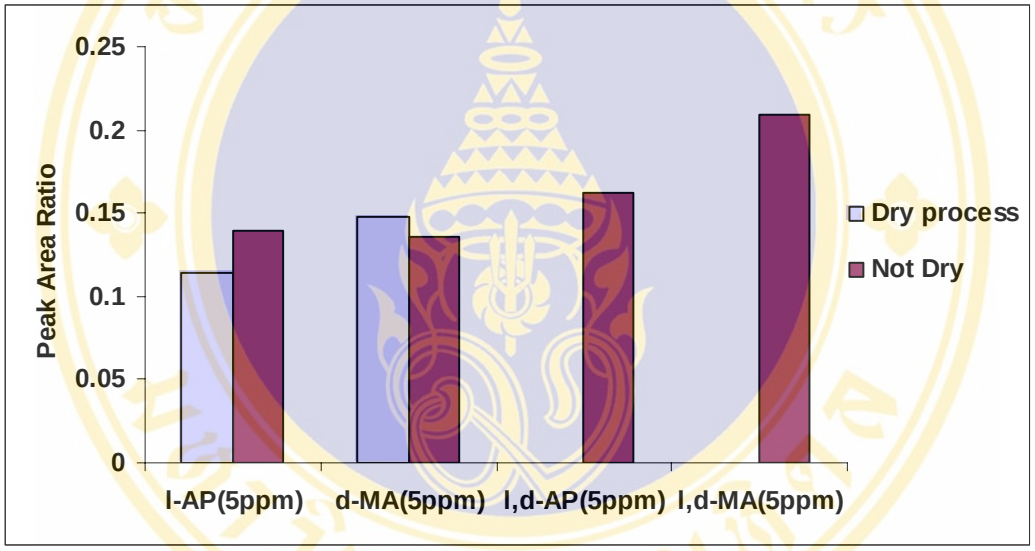


Figure 4.3 Peak area ratio of samples. The mass selected is m/z 44 for AP, m/z 58 for MA and m/z 169 for IS (diphenylamine).

Blank urine spiked with *l*-AP, *d*-MA, *d,l*-AP and *d,l*-MA at the level of 5 ppm were extracted with 2 ml MTBE and analyzed using GC temperature Program A. The results show that no amphetamines could be detected, except for the 5 ppm d-AP sample, when the samples were dried using nitrogen gas and heated at 45 ^{0}C. The amphetamines are volatile and are lost when the extract is dried. Therefore in this work the extracts for the non-derivatized amphetamines were directly injected into the GC.

4.4 The study of optimization for the derivatization step

4.4.1 GC temperature program

In this study three temperature programs were tested to find optimum GC temperature program for derivatization method.

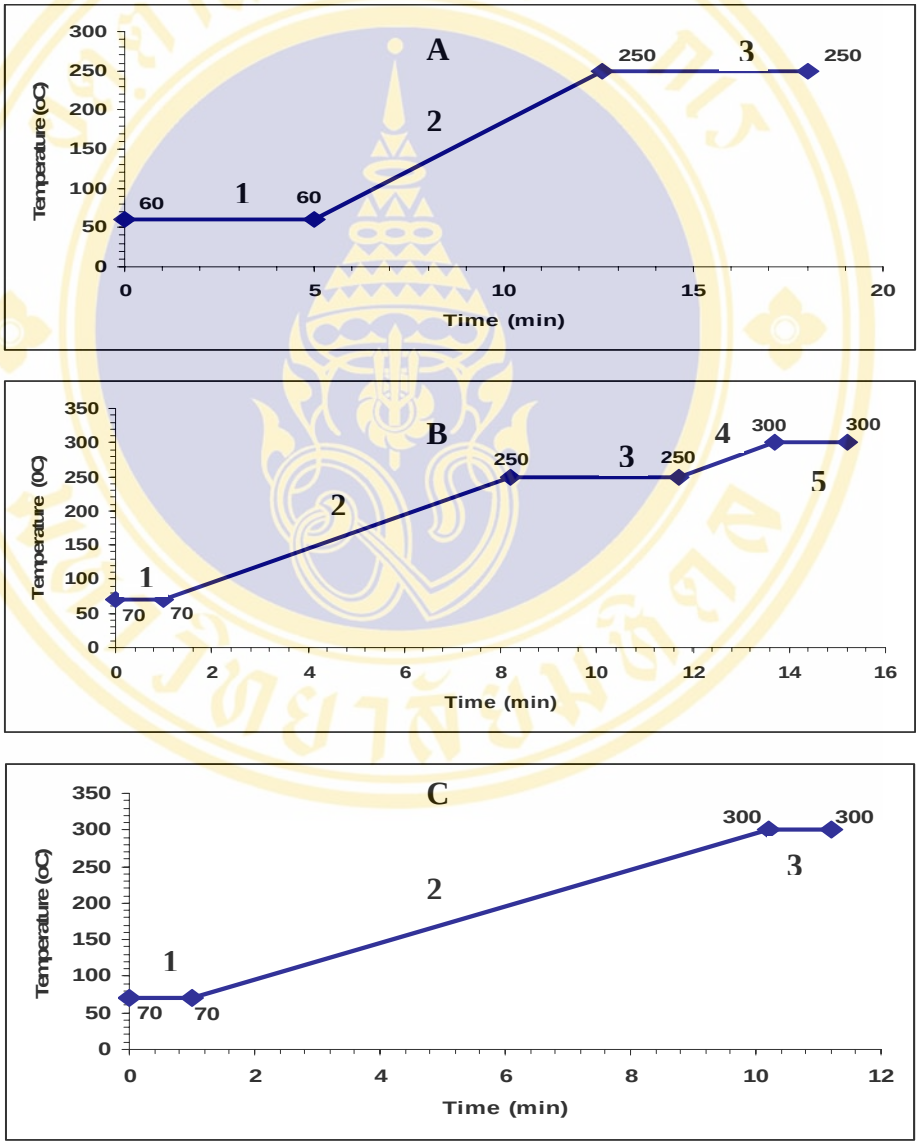


Figure 4.4 Comparison of GC temperature Programs A, B and C for separation of the *l*-TPC derivatives of AP and MA.

Temperature Program A is as follow:

1: initial temperature 60^0C, hold 5 min.

2: ramping at 25^0C/min to 250^0C.

3: hold 5.4 min at 250^0C (to clean column).

Temperature Program B is as follow:

1: initial temperature 70^0C, hold 1 min.

2: ramping at 25^0C/min to 250^0C.

3: hold 3.5 min at 250^0C.

4: ramping 25^0C/min to 300^0C.

5: hold 1.5 min at 300^0C (to clean column).

The temperature program for Program C is as follow:

1: initial temperature 70^0C, hold 1 min.

2: ramping at 25^0C/min to 300^0C.

3: hold 1 min at 300^0C (to clean column)

The EIC (Extract Ion Chromatogram) for three GC temperature programs are shown below.

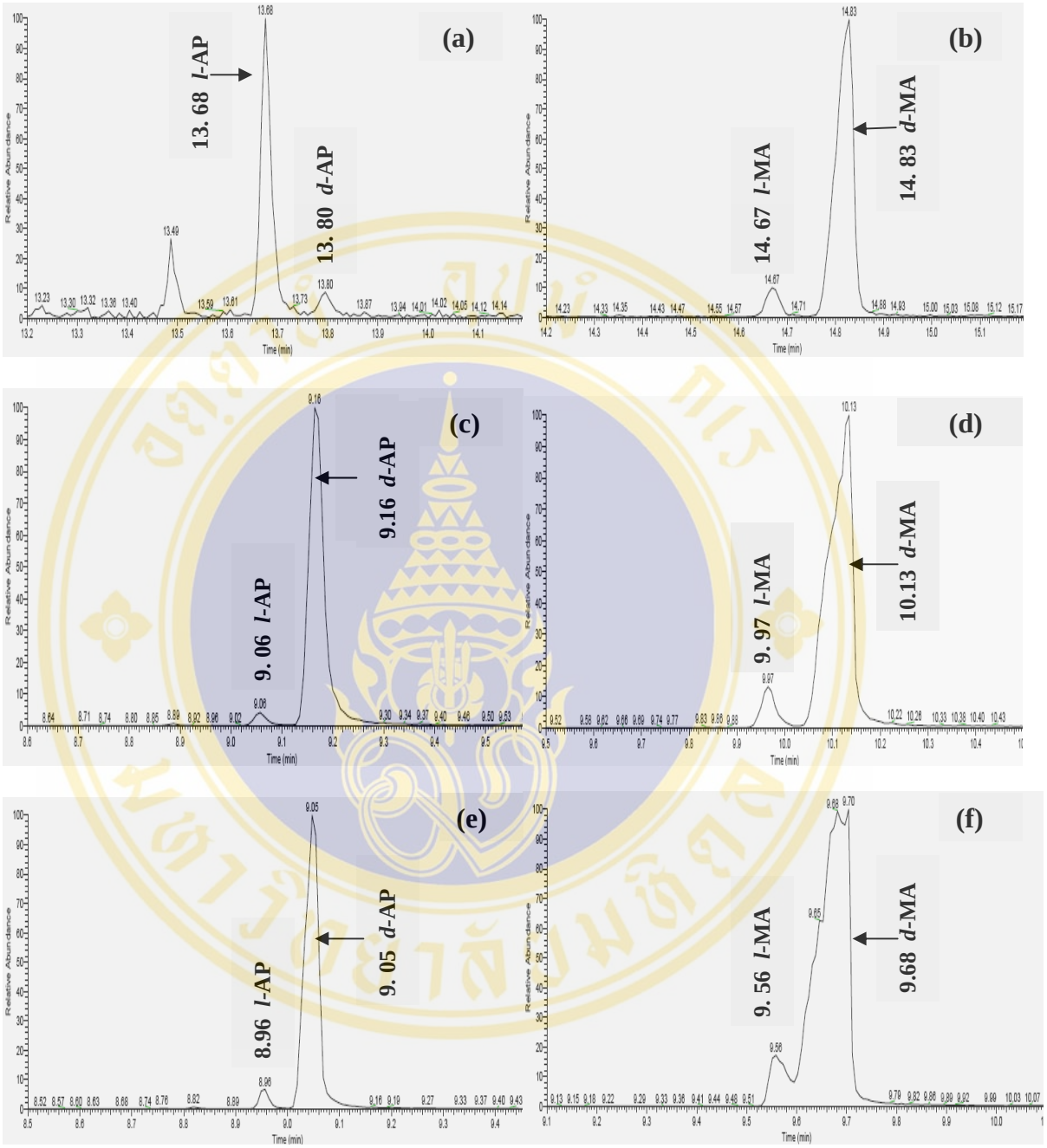


Figure 4.5 Example of EIC (Extract Ion Chromatogram) for derivatization method of GC temperature Program A shown in Figure (a): STD of *l*-AP (1 ppm) and Figure (b): Horse urine and using GC temperature Program B shown in Figure (c) and (d) are Human urine and using GC temperature C shown in Figure (e) and (f) are Human urine. The mass selected is m/z 237 and m/z 251 for detected AP-*l*-TPC and MA-*l*-TPC, respectively.

GC temperature Program A has a total runtime of 18 min with good separation of *d* and *l* form. Program C has a total runtime of 11.2 min but without baseline separation of *d* and *l* form. Program B has a total runtime of 15.2 min but with good baseline separation of *d* and *l* isomers.

The GC temperature Program B was selected for separation of the enantiomers because it had a shorter runtime than Program A.

4.4.2 Derivatizing procedure

4.4.2.1 Effect of NaOH

The effect of adding or not adding solution of NaOH after the reaction with TPC was studied. The results are shown in Table 4.2.

Table 4.2 Peak area of samples with and without addition of NaOH solution.

Sample	Peak area	
	With NaOH	**Without NaOH**
STD of *d*-MA (1 ppm)	38,944	ND*
Positive horse urine *l*-AP	10,702	ND
Positive horse urine *d*-AP	156,261	ND
Positive horse urine *l*-MA	201,172	ND
Positive horse urine *d*-MA	2,332,014	ND

* ND = Not Detected

The results of using NaOH can detected in all samples of this experiment (*l*-AP, *d*-MA and horse urine) but not using NaOH can't detected samples except *l*-AP although found only *l*-AP may be causes its process has the excess of TPC after derivatizing reaction, TPC can be reacted with NaOH so that TPC excess can be interfered to the result of chromatogram. TPC excess can be effected to function group of R-COOH, R-OH, R-SH and R-NH_2 [53].

4.5 Conclusion of optimization for using in non derivatization and derivatization method.

The optimum condition for non derivatization and derivatization method shown in the Table 4.3

Table 4.3 The optimum condition for non derivatization and derivatization method

Parameters	**Condition**	
	Non derivatization method	**Derivatization method**
Injection mode	Spiltless	Spiltless
GC temperature program	Temp (^{0}C) / Ramp (^{0}C/min) / Hold (min) 70 / 0 / 1 300 / 25 / 1	Temp (^{0}C) / Ramp (^{0}C/min) / Hold (min) 70 / 0 / 1 250 / 25 / 3.5 300 / 25 / 1.5
Column of GC	Rtx-5MS (5%diphenyl-95%dimethylpolysiloxane) Length : 30 m Internal diameter : 0.25 mm Film thickness : 0.25 µm	Rtx-5MS (5%diphenyl-95%dimethylpolysiloxane) Length : 30 m Internal diameter : 0.25 mm Film thickness : 0.25 µm
Scan mode	Full scan (40-450)	Full scan (40-450)
Total run time	11.2 min	15.2 min
Shaking time	15 min ; 1 time*	30 min ; 2 times*
Centrifuge time	25 min	50 ; 2 times*
Solvent	Not used	Ethyl acetate
Extractant	MTBE	MTBE
Derivatizing agent	Not used	*l*-TPC

***** time = Number of shaking or centrifuge of procedure as followed non derivative and derivative method

4.6 Internal standardization and limit of detection (LOD)

4.6.1 Calibration graph

A three-point calibration curve was constructed for each standard by analyzing 2 ml blank urine samples, which were spiked with various volume of mixed standard solution (*d*,*l*-AP and *d*,*l*-MA). The spiked sample were extracted and derivatized as described in Section 3.4.1. The calibration was linear in the range tested, 1-20 µg/ml urine. The calibration data for each analyte are shown in Table 4.4 and Figure 4.6.

Table 4.4 Calibration data, linear regression equation and detection limit of *d*,*l*-amphetamine and *d*,*l*-methamphetamine

Standard	Spiked amount (µg/ml) in urine	Linear regression equation (y = mx+c)	r^2	LOD (µg/ml) or ppm
***l*–AP**	1-20	Y=0.021X +0.0293	0.9959	0.032
***d*-AP**	1-20	Y=0.0233X+0.0433	0.9924	0.110
***l*-MA**	1-20	Y=0.0098X+0.0239	0.9859	0.059
***d*-MA**	1-20	Y=0.0069X+0.0250	0.9758	0.101

4.6.2 Limit of detection (LOD)

Limits of detection for *l*-AP, *d*-AP, *l*-AP and *d*-MA were 0.032, 0.110, 0.059, 0.101 µg/ml, respectively.

4.7 Analysis in human urine of the *d,l*-enantiomers of amphetamine and methamphetamine

In this study 28 samples of human urines, 26 samples from persons who have been tested positive for amphetamines, one from a person who inhaled Vicks® Vapor Inhaler and one sample from an excretion urine from a subject who had taken selegiline drug, were analyzed for the *d,l*-enantiomers of amphetamine and/or methamphetamine. The concentration of *d,l*-enntiomers of AP and MA are shown in Table 4.5.

Table 4.5 The concentration and % concentration of *d,l*-amphetamine and *d,l*-methamphetamine in urine samples

Human urine	Amphetamine				Methamphetamine			
	Concentration (µg/ml) in urine				Concentration (µg/ml) in urine			
	Conc. *l*-AP	%Conc. *l*-AP	Conc. *d*-AP	%Conc. *d*-AP	Conc. *l*-MA	%Conc. *l*-MA	Conc. *d*-MA	%Conc. *d*-MA
TH 1	0.019	0.410	4.635	99.590	0.857	1.440	58.575	98.560
TH 2	0.015	0.420	3.636	99.580	0.361	1.320	26.972	98.680
TH 3	ND	ND	0.367	100.000	0.671	2.840	22.982	97.160
TH 4	ND	ND	1.623	100.000	0.350	2.170	15.783	97.830
TH 5	0.140	0.520	27.009	99.480	2.455	1.610	150.154	98.390
TH 6	0.003	0.301	1.103	99.699	0.080	1.750	4.463	98.250
TH 7	0.009	0.577	1.541	99.423	0.220	1.320	16.519	98.680
TH 8	0.013	0.530	2.399	99.470	0.499	1.460	33.729	98.540
TH 9	0.019	0.410	4.648	99.590	0.878	1.480	58.332	98.520
CMU 1	ND	ND	0.896	100.000	0.165	1.240	13.123	98.760
CMU 2	0.042	0.430	9.770	99.570	2.441	2.630	90.380	97.370
CMU 3	0.063	0.500	12.428	99.500	1.863	2.660	68.276	97.340
CMU 4	0.043	0.430	9.915	99.570	1.419	2.080	66.707	97.920
CMU 5	0.084	0.420	19.877	99.580	2.827	3.040	90.191	96.960

Table 4.5 The amount of concentration and % concentration of *d,l* of amphetamine and *d,l* of methamphetamine in urine samples (Continued)

Human urine	Amphetamine				Methamphetamine			
	Concentration (µg/ml) in urine				Concentration (µg/ml) in urine			
	Conc. *l*-AP	%Conc. *l*-AP	Conc. *d*-AP	%Conc. *d*-AP	Conc. *l*-MA	%Conc. *l*-MA	Conc. *d*-MA	%Conc. *d*-MA
CMU 6	0.034	0.420	8.134	99.580	3.383	3.290	99.502	96.710
CMU 7	ND	ND	0.182	100.000	0.252	1.110	22.494	98.890
CMU 8	0.006	1.301	0.433	98.699	0.074	3.140	2.294	96.860
CMU 9	0.006	0.949	0.623	99.051	0.390	1.260	30.452	99.740
CMU 10	0.039	0.460	8.432	99.540	5.189	3.700	135.171	96.300
CMU 11	0.013	0.470	2.748	99.530	0.040	0.090	46.980	99.910
RA 1	ND	ND	1.245	100.000	0.149	1.010	14.639	98.990
RA 2	ND	ND	3.011	100.000	0.565	1.150	48.738	98.850
RA 3	ND	ND	1.852	100.000	0.109	1.160	9.309	98.840
RA 4	0.069	0.390	17.700	99.610	3.246	3.320	94.503	96.680
RA 5	ND	ND	1.353	100.000	0.143	1.300	10.878	98.700
RA 6	0.021	0.290	7.439	99.710	3.500	3.270	104.837	96.730
Vicks®	ND	ND	ND	ND	0.153	100.000	ND	ND
Selegiline	0.125	55.670	0.100	44.330	3.012	86.480	0.471	13.520

ND = Not Detected

TH are samples from Thunyaruk Hospital, Phatumthani

CMU are samples from Maharaj Hospital, Chiangmai

RA are samples from Ramathibodi Hospital, Bangkok

The results shown the concentration of MA is greater than AP in all 28 samples. The concentration of *d*-AP is greater then *l*-AP in all 26 samples from persons who had tested positive for amphetamine/methamphetamine. The selegiline-positive sample had *l*-AP > *d*-AP and the sample from the person using Vick's Inhalor had no amphetamine detected. The concentration of *d*-MA is greater than *l*-MA in the same

26 positive samples as for *d*-AP. The selegiline and Vick samples had, as expected *l*-MA > *d*-MA.

The results show concentration of MA is greater than AP because AP is a metabolite of MA [5]. Most illicit amphetamine/methphetamine-drugs found in Thailand are methamphetamines [54-56]. Vicks® Vapor Inhaler contains only *l*-MA and l-MA was found. Selegiline can be metabolized to AP and MA [37]. Both *d*- and *l*-forms of AP and MA were detected in the urine but the amount of the *l*-isomers are greater than the *d*-isomers. This ratio is an indicator than the AP and MA may be metabolites of prescribed drugs. Summary of concentration *d,l* of amphetamine and *d,l* of methamphetamine in urine samples shown in the below table.

Table 4.6 Summary of concentration *d,l* of amphetamine and *d,l* of methamphetamine in urine samples

Urine samples	**Number of samples**	**Concentration**
All samples	28	Conc. MA > AP ~ (17:1)
1. Positive urine for AP/MA (illicit drug)	26	Conc. *d* > *l* (9:1) of AP and MA
2. Selegiline sample (prescribed drug)	1	% Conc. *l* > *d* (5:1) of AP and MA
3. Vicks® Vapor Inhaler	1	% Conc. *l* > *d* (10:1) of MA

The TIC (Total Ions Chromatogram) of standard samples and TIC of urine samples and the mass spectra of pure standards are shown in Figures 4.7, 4.8 and 4.9, respectively.

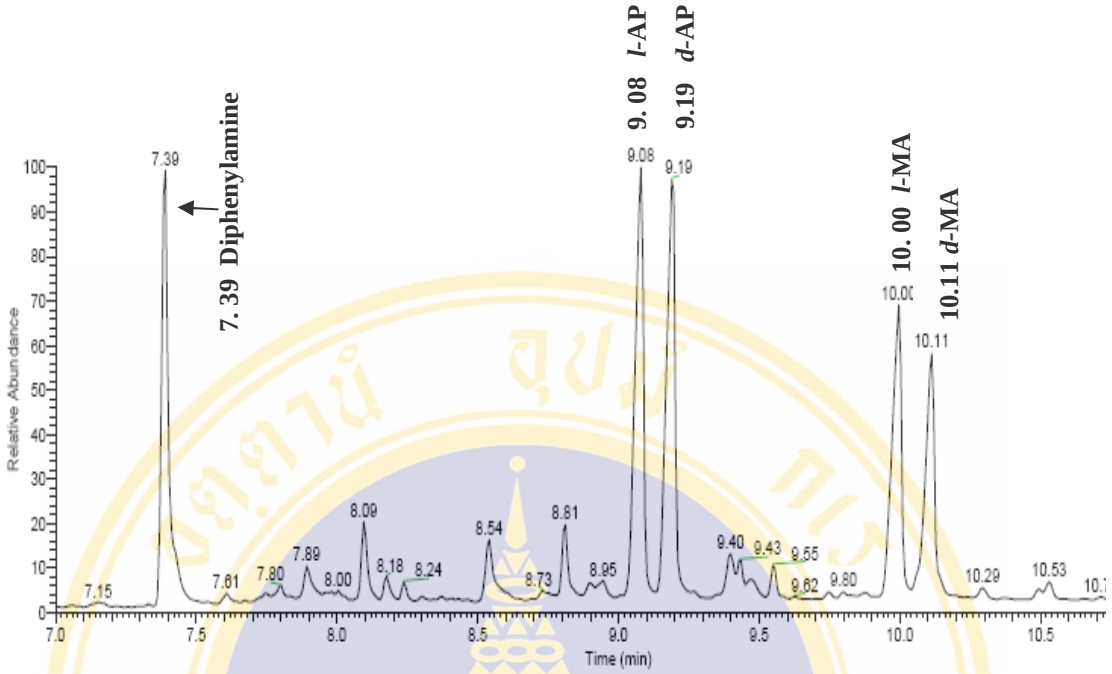


Figure 4.6 TIC of standard mixture (10 ppm) of *d*,*l*-AP and *d*,*l*-MA by using GC temperature Program B as followed Section 3.6.2, IS (RT=7.39min), *l*-AP (RT=9.08), *d*-AP (RT=9.19), *l*-MA (10.00) and *d*-MA (10.11)

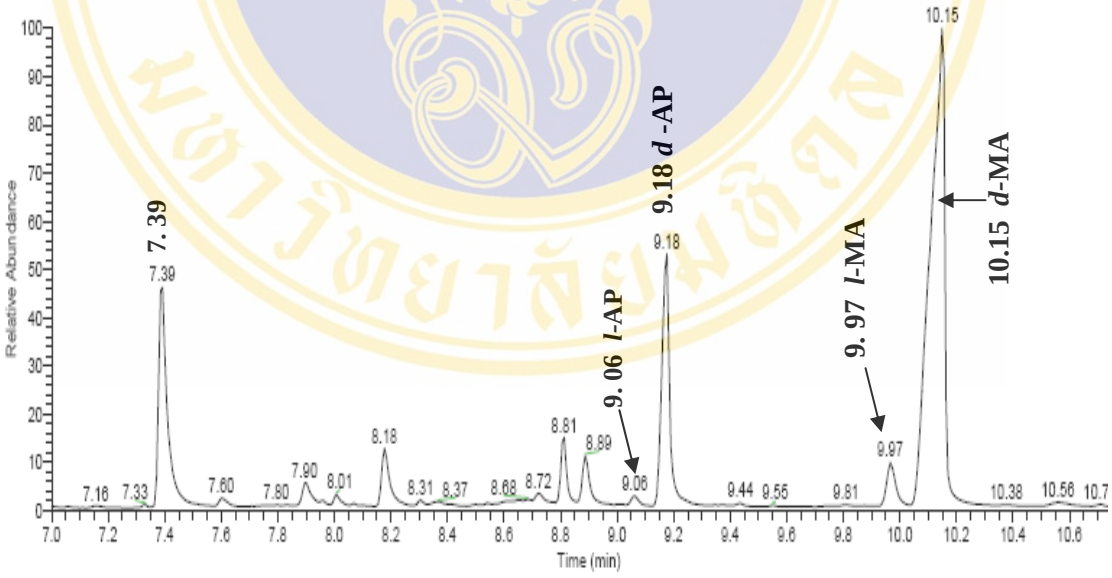
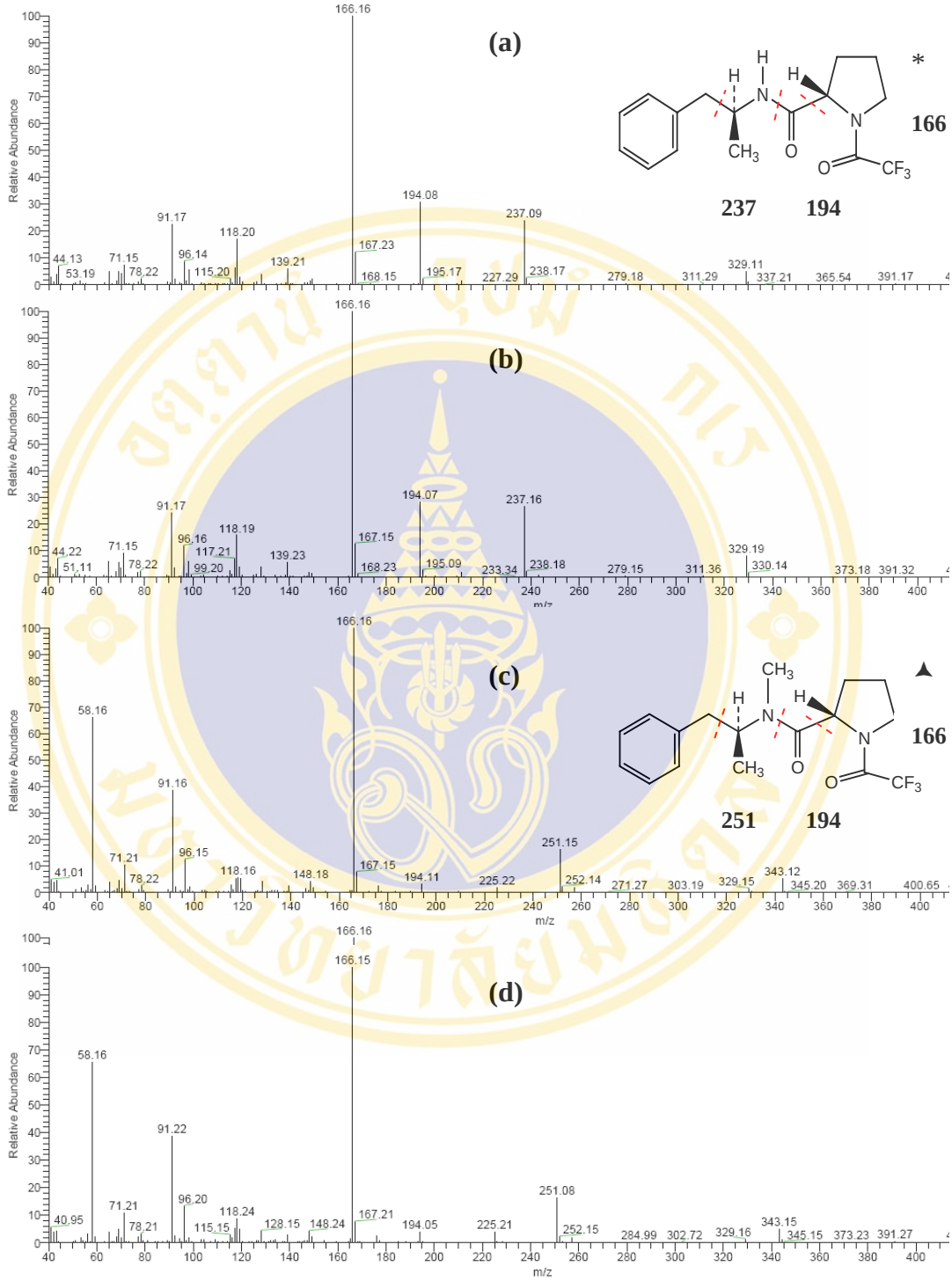


Figure 4.7 TIC of urine sample of who took amphetamine themselves, IS (RT=7.39min), *l*-AP (9.06), *d*-AP (9.18), *l*- MA (9.97) and *d*-MA (10.15)



* is *l*-AP-TPC or *d*-AP-TPC , ▲ is *l*-MA-TPC or *d*- MA-TPC

Figure 4.8 Mass spectrum of (a) *l*-AP-TPC, (b) *d*-AP-TPC, (c) *l*-MA-TPC and (d) *d*- MA-TPC of standard mixture (10 ppm) of (*d*,*l*-AP and *d*,*l*-MA)

This derivatization method and GC condition was used in this experiment can good separated *d* and *l* form and found retention time of IS (7.39min), *l*-AP (9.08), *d*-AP (9.19), *l*-MA(10) and *d*-MA (10.11) in standard mixture (10 ppm) of *d,l*-AP and *d,l*-MA and using quantifier ion of m/z 237 for detected the diastereomeric pairs of AP-*l*-TPC and m/z 251 for MA-*l*-TPC. The results of derivatization method examined in 28 samples can good baseline separated *d* and *l* isomers of AP and MA in all samples, the concentration and % concentration shown in Table 4.5.

The EIC (Extract Ion Chromatogram) and mass spectrum of Selegiline sample and Vicks® Vapor Inhaler sample shown in Figure 4.10, 4.11 and 4.12, respectively. The mass selected is m/z 237 and m/z 251 for detected AP-*l*-TPC and MA-*l*-TPC, respectively.

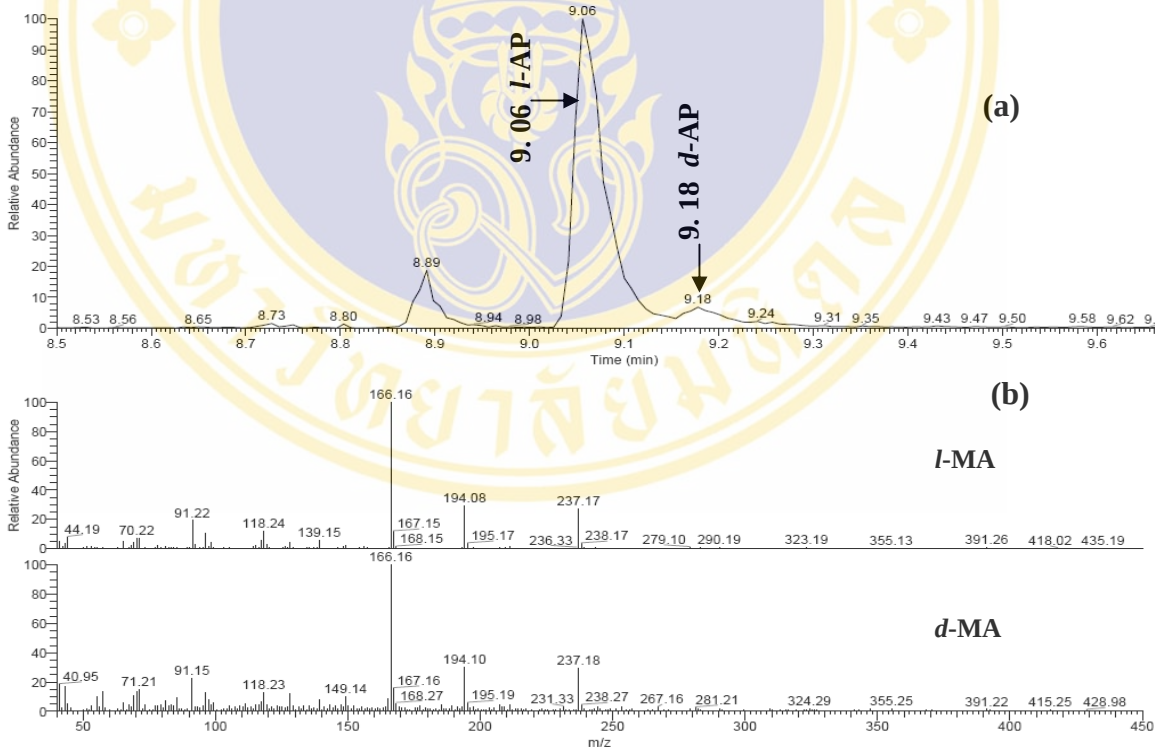


Figure 4.9 EIC chromatogram (a) and mass spectrum (b) of *d,l* of AP in Selegiline sample

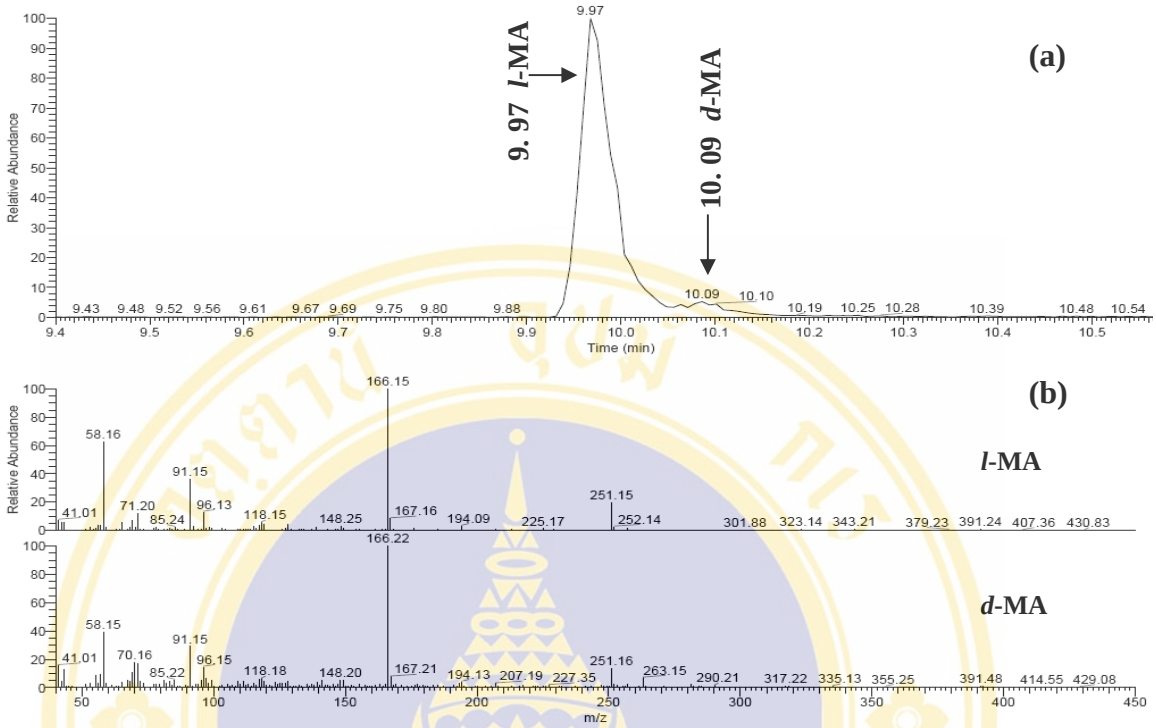


Figure 4.10 EIC chromatogram (a) and mass spectrum (b) of *d*,*l* of MA in Selegiline sample

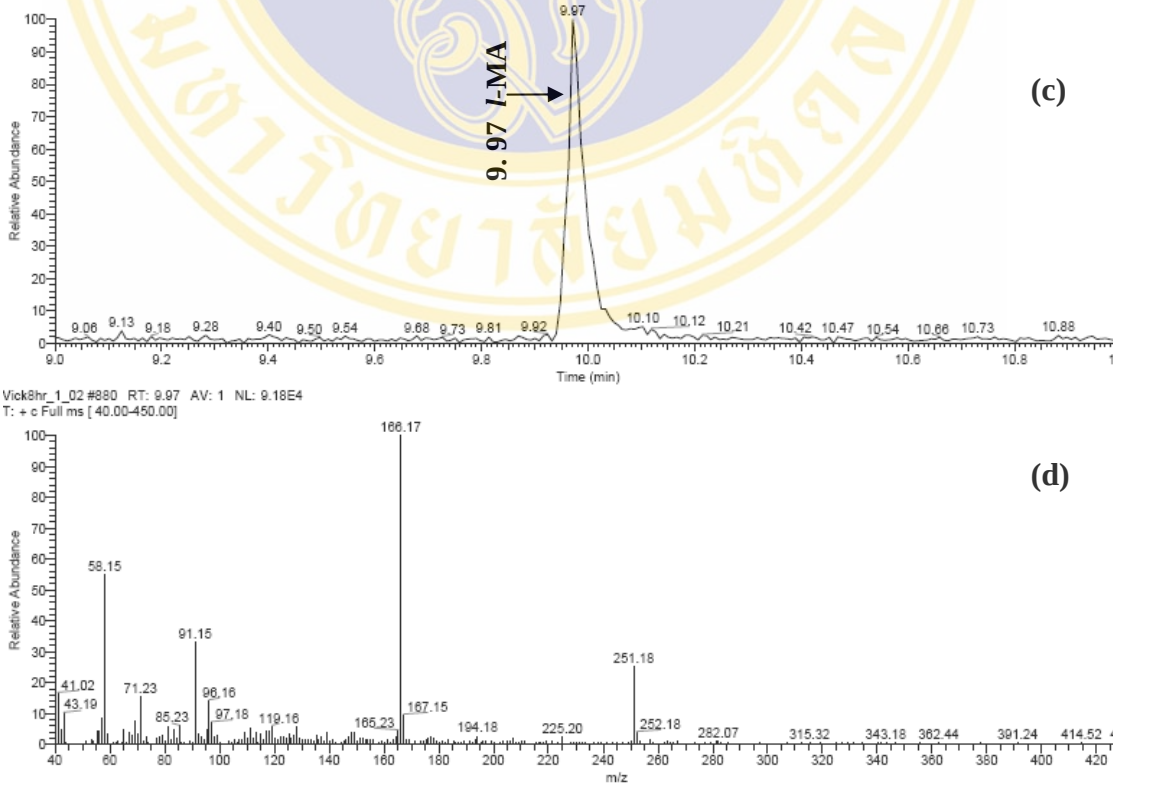


Figure 4.11 EIC chromatogram (c) and mass spectrum (d) of *l*-MA in Vicks® Vapor Inhaler sample

CHAPTER IV
CONCLUSION

In this study optimal conditions for liquid-liquid extraction and GC-MS separation and quantification of amphetamines and methamphetamine in human urine samples were investigated. In order to separate the enantiomers of amphetamines and methamphetamines derivatizing with the chiral reagent, *l*-TPC, was carried out.

It was also found that after the derivatizing step washing with a solution of NaOH was necessary. This destroyed the excess *l*-TPC in the solution and also HCl, a product from the derivatizing process. The HCl can hydrolyze the amphetamine-TPC compound and so reduce the amount of derivatized product so formed.

For the liquid-liquid extraction, it was found that there was substantial loss of the amphetamine/methamphetamine on drying the organic layer. However when derivatized with *l*-TPC there was no loss on drying. It is recommended that for simple detection of amphetamine/methamphetamine no drying step should be carried out to prevent possible loss of the compounds.

The GC column used was Rtx-5MS (5% diphenyl) capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness). Two GC temperature programs were employed; one for non-derivatized amphetamine and methamphetamine and a second oven temperature program for separation of the *d,l*-enatiomers. The mass spectrometer detector used in this work was an ion-trap instrument operating in the full scan mode, with mass range m/z 40 – 450.

The proposed analytical method is simple and can separate the enantiomers of amphetamine and methamphetamine.

The detection limit for *l*-AP, *d*-AP *l*-MA, *d*-MA are 0.032, 0.110, 0.059 and 0.101 µg/ml, respectively.

The results show the concentration methamphetamine is greater than amphetamine in the 26 samples of urine from persons found to be positive for use of amphetamine or methamphetamine. Also the main isomer found in these samples is the *d*-isomer indicating the origin of the drug. The urine from subject inhaling the Vicks® Vapor Inhaler contained mainly *l*-methamphetamine, the ingredient of the inhaler. Also the excretion urine from the subject who had taken selegiline showed amphetamine and methamphetamine, the metabolites of the drug. However the ratio of *l*- to *d*-isomer is much greater than for the samples from users of amphetamine/methamphetamine. Hence the *l*- to *d*- ratio can be used to differentiate between amphetamine/methamphetamine from legal prescribe drugs and those from illegal drugs.

Suggestion for future work

The following are suggestion for further work.

1. Study the *l*- to *d*- ratio of amphetamine/methamphetamine from other prescribed drugs.
2. Study in other forensic samples (*e.g.* blood, saliva, etc.)

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APPENDIX

APPENDIX

DETERMINATION OF DETECTION LIMIT

The limit of detection is the lowest concentration of an analyte that an analytical process can reliably detect [51]. Several methods for determining the detection limit have been proposed. In this work calculation of limit of detection are based on the recommendation of the International Conference on Harmonisation (ICH) Validation of Analytical Procedures Guideline [52] .The detection limit (LOD) of this experiment is based on ICH guideline, *i.e.*, detection limit is expressed as:

$$LOD = \frac{3 \times (\text{Mean of Peak area ratio of Blank}) \times (\text{Conc. of STD})}{(\text{Mean of Peak area ratio of STD} - \text{Mean of Peak area ratio of Blank})}$$

Table 5 The data for calculation limit of detection

Sample	Peak area ratio			
	***l*-AP**	***d*-AP**	***l*-MA**	***d*-MA**
Blank1	0.0002	0.0016	0.0004	0.0007
Blank2	0.0006	0.0020	0.0004	0.0009
Blank3	0.0005	0.0021	0.0008	0.0008
Mean of Blank	0.0004	0.0019	0.0005	0.0008
STD1(1ppm)	0.0437	0.0528	0.0280	0.0252
STD2(1ppm)	0.0425	0.0548	0.0269	0.0256
STD3(1ppm)	0.0417	0.0527	0.0265	0.0239
Mean of STD	0.0426	0.0534	0.0271	0.0249
LOD (µg/ml)	0.0315	0.1101	0.0588	0.1011

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