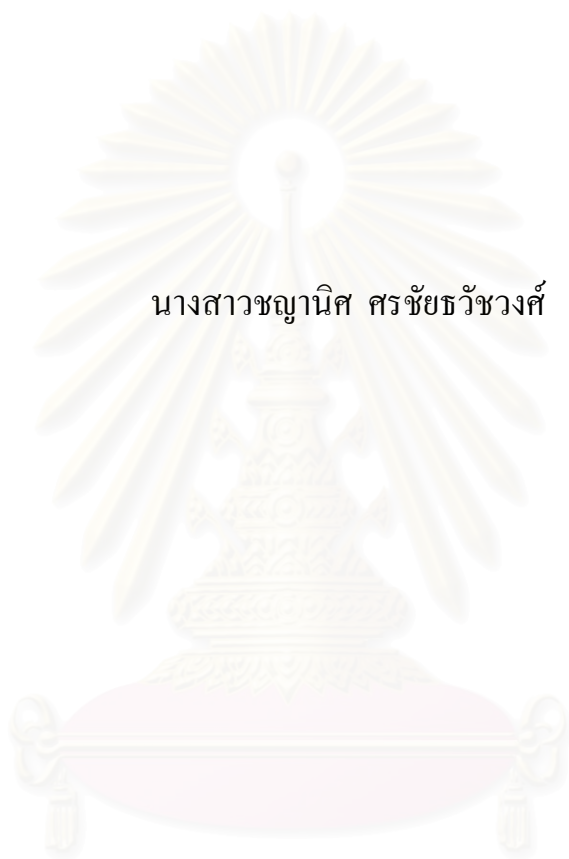การวิเคราะห์แอมเฟตามีน เมทแอมเฟตามีนและอีเฟดรีนในปัสสาวะ
ด้วยวิธีแก๊สโครมาโทกราฟี/แมสสเปกโทรเมตรี ที่ใช้การสกัดแบบไอออนแพร์

นางสาวชญานิศ ศรชัยธวัชวงศ์

DETERMINATION OF AMPHETAMINE, METHAMPHETAMINE AND

EPHEDRINE IN URINE BY GAS CHROMATOGRAPHY/

MASS SPECTROMETRY USING ION-PAIR EXTRACTION

Miss Chayanid Sornchaithawatwong

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Pharmacy

Department of Pharmaceutical Chemistry

Faculty of Pharmaceutical Sciences

Chulalongkorn University

Academic Year 2001

ISBN 974-03-1620-4

Thesis Title   DETERMINATION OF AMPHETAMINE,

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By   Miss Chayanid Sornchaithawatwong

Field of Study   Pharmaceutical Chemistry

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ชญานิศ ศรชัยธวัชวงศ์ : การวิเคราะห์แอมเฟตามีน เมทแอมเฟตามีน และอีเฟดรีนใน
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(DETERMINATION OF AMPHETAMINE, METHAMPHETAMINE AND
EPHEDRINE IN URINE BY GAS CHROMATOGRAPHY/MASS SPECTROMETRY
USING ION-PAIR EXTRACTION) อ. ที่ปรึกษา : อ. ดร. วัลลภา ทาทอง, 91 หน้า.
ISBN 974-03-1620-4.

ได้พัฒนาวิธีวิเคราะห์แอมเฟตามีน เมทแอมเฟตามีน และอีเฟดรีนในปัสสาวะซึ่งเป็นวิธีที่
ง่ายโดยการเตรียมตัวอย่างด้วยการสกัดแบบไอออนแพร์ และวิเคราะห์ด้วยวิธีแก๊สโครมาโทกราฟี/
แมสสเปกโทรเมตรี แบบอิเล็กตรอนอิมแพคท์ไอออไนเซชั่น และซีเล็กเต็ด ไอออน มอนิเตอริ่ง
3', 3", 5', 5"– เตตระโบรโมฟีนอล์ฟทาลีน เอทิล เอสเทอร์ เป็นสารมีสีที่มีโปรตอนเดี่ยว เมื่อทำ
ปฏิกิริยากับยากลุ่มแอมเฟตามีนในตัวกลางของเหลวที่มีค่าพีเอช 9.2-9.5 จะเกิดสารประกอบเชิง
ซ้อนที่มีสีม่วงแดง ซึ่งละลายในไดคลอโรมีเทน นำสารสกัดไดคลอโรมีเทนที่ได้ไปสกัดกลับแล้วจึง
นำไประเหยแห้ง และเตรียมเป็นอนุพันธ์โดยใช้เพนตะฟลูออโรโพรพิโอนิกแอนไฮไดรด์ นำตัว
อย่างที่เตรียมเป็นอนุพันธ์ไประเหยแห้ง แล้วละลายในเอทิล อะซีเทต และฉีดเข้าเครื่องแก๊สโครมา-
โทกราฟ

กราฟมาตรฐานเป็นเส้นตรงในช่วง 50-2500 นาโนกรัมต่อมิลลิลิตร (แอมเฟตามีน และ
เมทแอมเฟตามีน) และ 100-2500 นาโนกรัมต่อมิลลิลิตร (อีเฟดรีน) ความเข้มข้นต่ำสุดที่ตรวจพบ
ได้ คือ 16.45 นาโนกรัมต่อมิลลิลิตร (แอมเฟตามีน) 15.46 นาโนกรัมต่อมิลลิลิตร (เมทแอมเฟตา-
มีน) และ 22.21 นาโนกรัมต่อมิลลิลิตร (อีเฟดรีน) และความแม่นยำของวิธีวิเคราะห์มีค่าเบี่ยงเบน
มาตรฐานสัมพัทธ์ไม่มากกว่า 10 เปอร์เซ็นต์สำหรับแต่ละตัวยา ได้นำวิธีวิเคราะห์นี้มาวิเคราะห์ตัว
อย่างปัสสาวะจำนวน 10 ตัวอย่าง พบว่าปัสสาวะ 4 ตัวอย่างให้ผลบวกจากการตรวจยืนยันผล คือ มี
ปริมาณเมทแอมเฟตามีนมากกว่า 500 นาโนกรัมต่อมิลลิลิตร และแอมเฟตามีนมากกว่า 200 นาโน-
กรัมต่อมิลลิลิตร

ภาควิชา.......เภสัชเคมี.......	ลายมือชื่อนิสิต………….……………………………
สาขาวิชา......เภสัชเคมี......	ลายมือชื่ออาจารย์ที่ปรึกษา………………………..
ปีการศึกษา......2544……..	ลายมืออาจารย์ที่ปรึกษาร่วม………………………..

A simple method for analysis of amphetamine, methamphetamine and ephedrine in urine was developed using ion-pair extraction and gas chromatography/mass spectrometry with electron impact ionization and selected ion monitoring (GC/MS-SIM). The 3', 3'', 5', 5''- tetrabromophenolphthalein ethyl ester, a monoprotic dye, reacted with amphetamines in the liquid medium of pH 9.2-9.5 to form the red-violet complexes, which were soluble in dichloromethane. The dichloromethane extract was back-extracted and the extract was evaporated to dryness and derivatised with pentafluoropropionic anhydride (PFPA). The derivatised sample was evaporated to dryness, reconstituted in ethyl acetate, and injected into the GC.

The calibration curves were linear between 50-2500 ng/ml (amphetamine and methamphetamine) and 100-2500 ng/ml (ephedrine). The detection limits were 16.45 ng/ml (amphetamine), 15.46 ng/ml (methamphetamine) and 22.21 ng/ml (ephedrine) and the reproducibility was not more than 10 % RSD for each drug. The method was applied to 10 urine samples. Four samples were positively confirmed with greater than 500 ng/ml of methamphetamine and 200 ng/ml of amphetamine.

Department…Pharmaceutical Chemistry….　　　Student's signature………………

Field of study..Pharmaceutical Chemistry….　　　Advisor's signature……………...

Academic year…2001….　　　Co-advisor's signature…………..

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my advisor, Dr. Walapa Tatong, for her support, advise, understanding and help during my study.

I am so grateful to Mrs. Duangporn Abhighantaphan, Director of Division of Narcotics, Department of Medical Sciences, Ministry of Public Health for her kindness support and supplying the standards and urine samples. I would like to thank the staffs of the Division of Narcotics for their hospitality.

I also would like to thank Assistant Professor Dr. Mitr Pathipvanich, Associate Professor Nuansri Niwattisaiwong and Miss Somsiri Prateepachitti for serving as my research committee.

I also would like to acknowledge the Graduate School of Chulalongkorn University for granting partial financial support. I am thankful to the staffs of the Department of Pharmaceutical Chemistry, Chulalongkorn University for their helpfulness. I am deeply grateful to all of my friends for their encouragement and helpfulness.

With deepest love and appreciation, I would like to thank my parents and my sister for their constantly support and encouragement.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	percent
σ	standard deviation of regression line
μg	microgram
μl	microliter
°C	degree Celsius
A	absorbance
AP	amphetamine
EI	electron impact ionization
EP	ephedrine
g	gram
GC/MS	gas chromatography/mass spectrometry
hr	hour
IS	internal standard
LOD	limit of detection
LOQ	limit of quantitation
M	molar
m/z	mass to charge ratio
MA	methamphetamine
mg	milligram
min	minute
ml	milliliter
mM	millimolar
nm	nanometer
PAR	peak area ratio
PFPA	pentafluoropropionic anhydride
PT	phentermine
r	correlation coefficient

RSD	relative standard deviation
RT	retention time
S	slope of regression line
SIM	selected ion monitoring
TBPE	3', 3'', 5', 5'' tetrabromophenolphthalein ethyl ester
TFAA	trifluoroacetic anhydride
UV	ultraviolet

CHAPTER I

INTRODUCTION

Amphetamines are central nervous system stimulants that produce wakefulness, alertness, increased energy, reduced hunger, and an overall feeling of well being. The term " amphetamines " includes many drugs, but amphetamine and methamphetamine are the most common. Nowadays, the only major clinical uses of amphetamine and methamphetamine are for the treatment of narcolepsy and for treating children with attention deficit hyperactivity disorder (ADHD) [1].

Over the past few years, the growing problem of stimulant abuse in Thailand has been dramatic. The illicit stimulant tablets in Thailand contain a combination of few common active ingredients, methamphetamine, ephedrine, and caffeine [2]. (Figure 1).
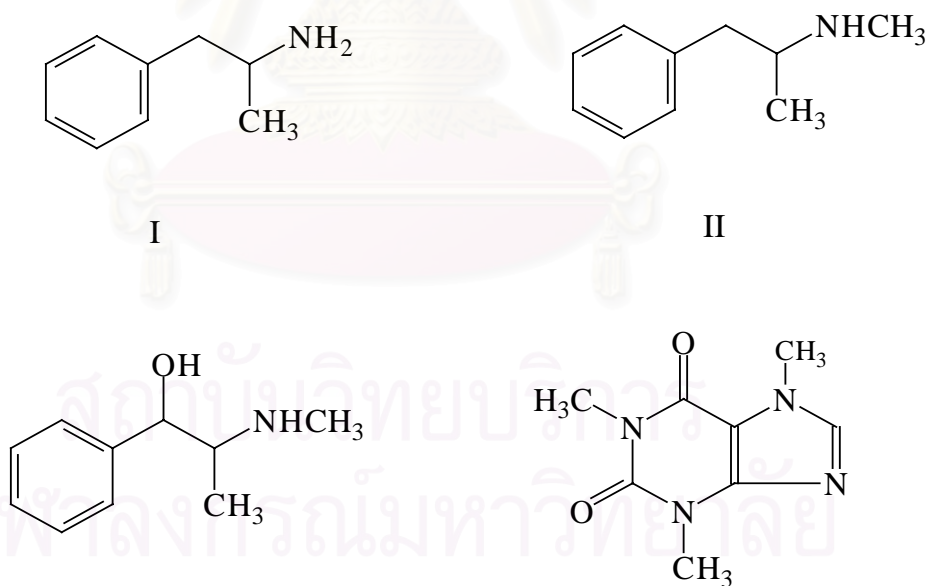
Figure 1 Structure of amphetamine (I), methamphetamine (II), ephedrine (III), and caffeine (IV).

Methamphetamine

Methamphetamine hydrochloride found in illicit stimulant tablets is produced by clandestine laboratories using ephedrine as a precursor [3]. The free base of methamphetamine is colorless or straw-colored oil with a mild " fishy " ammoniacal odor, which is not very stable. Therefore it is now often found as powder in the form of methamphetamine hydrochloride. It is white, odorless crystalline powders with a bitter taste and freely soluble in water and alcohol [4].

In Thailand, oral consumption of methamphetamine is the most common route of administration among labor groups, while smoking is the favorite route of administration among youth groups.

Methamphetamine is moderately weak base with a pKa of 10.1 and is negligibly absorbed from the acidic gastric contents, but rapidly absorbed from the intestinal fluids [5]. Methamphetamine is either deactivated by the liver or excreted unchanged in the urine. Under normal conditions, methamphetamine is excreted as the unchanged drug (44%) and as its major metabolite amphetamine (6-20%) and p-hydroxymethamphetamine (10%). The rate of excretion and the fraction of the dose excreted as unchanged drug vary according to the pH of the urine and are both increased in acidic urine [6].

Amphetamine is colorless and moderately weak base with a pKa of 9.9. It is excreted as the unchanged drugs, 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. In alkaline urine about 45 % of the dose is excreted in 24 hours and 2 % of the dose as the unchanged drug, while in acidic urine, up to 78 % of dose may be excreted in 24 hours and 68 % as the unchanged drug [6].

Amphetamine and methamphetamine begins to appear in the urine within 20 minutes of administration and can be detected for as long as 2-4 days after the last dose. The metabolic pathway of amphetamine and methamphetamine is summarized in Figure 2. In Thailand, amphetamine and methamphetamine are classified as schedule-I narcotic substance according to the Ministry of Public Health Notification No. 135 B.E. 2539 [7].
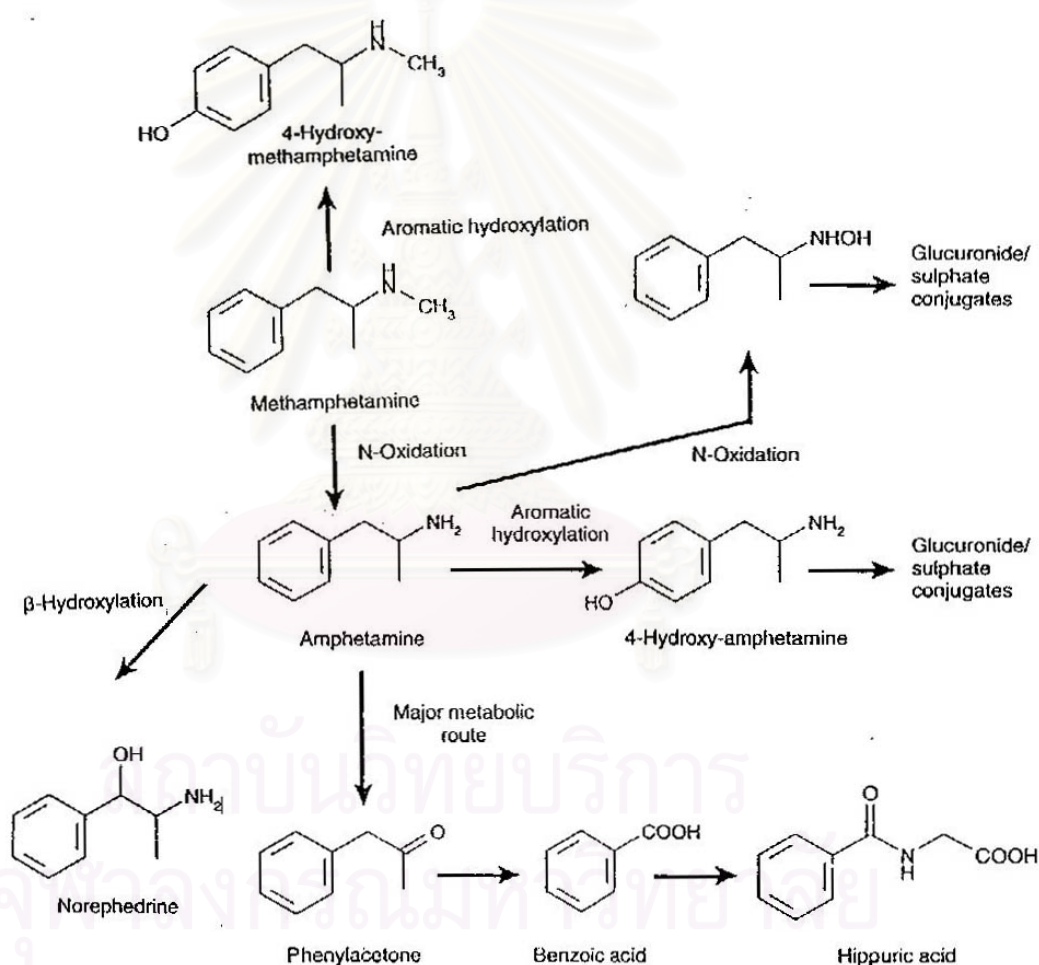
Figure 2 Metabolic pathway of amphetamine and methamphetamine

Ephedrine

Ephedrine is a naturally occurring alkaloid found in plants of the Ephedra species, although it is now produced synthetically. Ephedrine is a mild CNS stimulant and also stimulates the α- and β–receptors of the peripheral autonomic nervous system. Increased blood pressure, cardiac stimulation and increased pulse pressure have been noted with ephedrine use, although the heart rate may not increase. Ephedrine also causes relaxation of the bronchial muscles. Although the effect is milder and lasts longer than epinephrine, it is used occasionally to treat mild forms of asthma. Ephedrine is an effective decongestant in oral formulations as well as in nose drops. However, the use of ephedrine as a decongestant has been replaced by analogs with less potent CNS and cardiac stimulant effects, such as phenylephrine and pseudoephedrine [8].

In Thailand, ephedrine is a popular starting material for the illicit synthesis of methamphetamine tablets. The government of Thailand has placed controls on ephedrine by removing all pharmaceutical ephedrine from the market and classifying ephedrine as schedule-II psychotropic substance according to the Psychotropic Substances Act B.E. 2518 [7].

Ephedrine is rapidly and completely absorbed after oral administration. Up to 95 % of an oral dose of ephedrine may be excreted in the urine in 24 hours; 55-75 % as unchanged drug and the rest as metabolites, such as phenylpropanolamine (norephedrine), benzoic acid and hippuric acid. The urinary excretion of ephedrine is pH dependent and is increased in acidic urine. In alkaline urine, excretion is reduced to 20-35 % of the dose [9].

Urine testing

There are generally two purposes for analysis of methamphetamine in urine samples. Firstly, for forensic purposes, a positive analytical result for a sample taken would usually result in legal proceedings and a punitive outcome for the defendant whose sample was analysed. Secondly, for treatment and rehabilitative purposes, a positive analytical result in this context would not necessarily involve subsequent legal proceedings but might serve as a reliable indicator on which to base future medical treatment of the sample donor.

Urinary determination of methamphetamine and its metabolite amphetamine comprises two steps, screening test and confirmatory test. The initial screening test is performed to establish potential positive samples and would provide a rapid means of eliminating negative samples. For screening test, laboratories are advised to use immunoassay techniques such as radioimmunoassay (RIA), enzyme-multiplied immunoassay (EMIT), fluorescence polarization immunoassay (FPIA) and latex agglutination inhibition (LAI), at a drug cutoff level of 1,000 ng/ml [5,10]. Drug cutoff level is minimum concentration of methamphetamine that must be present in urine, before laboratories will report the drug testing results as positive. A positive result with immunoassay should then be followed by confirmation analysis using a method based on different chemical or physical principle. This is particularly important in the analysis of amphetamine and methamphetamine because there is a large number of amphetamine-like drugs, some of which may cross-react with antibodies targeted towards amphetamine and methamphetamine. The confirmatory test can be done by a variety of analytical techniques. Spectrophotometric, chromatographic and capillary electrophoretic methods have all been reviewed in the literature [11-14]. Among these analytical methods, chromatographic method is currently commonplace. Various chromatographic method have been employed for urinary confirmation of methamphetamine, including thin-layer chromatography (TLC) [15-17], high-performance liquid chromatography (HPLC) [17-20], gas chromatography (GC) [21-26] and gas chromatography/mass spectrometry (GC/MS) [16, 26-41].

Thin layer chromatography (TLC) is a very informative and comprehensive technique for initial screening purpose. The parent drugs and their metabolites can be detected simultaneously, giving characteristic colors and R_f values. Inexpensive procedure and simplicity are the advantage of TLC. A great disadvantage of TLC is insensitivity, non-specificity due to the close migration of the other phenethylamines, the lack of quantitative results without significant and coupling to other techniques [13].

Lillsunde and Korte [16] described TLC procedure using plates without fluorescence indicator and mobile phase of methanol-ammonia (50:0.5) for screening test and the analytes were confirmed by GC/MS. Fast black K salt was used as a spray reagent. Although it produces colored spots for a wide range of substance, it was especially suited for amphetamine-related compounds. Primary amines produced violet spots, and secondary amines produced orange ones. Talwar et al. [17] described the comparison of the commercial TLC system (Toxi-Lab) with the HPLC method for detecting amphetamines in immunoassay positive urine. The Toxi Lab system was far less sensitive and specific than the HPLC method. They concluded that the Toxi-Tube of the Toxi-Lab system was suitable for the extraction of urine procedure while the HPLC method was suitable for confirmation analysis of urine extract. The mean recovery of amphetamines was 78% (n=5).

HPLC has not been widely used for analysis of amphetamines in urine because of their low and non-specific UV absorptivities and low natural fluorescence. Additionally, primary and secondary amines often show poor chromatographic performance, which can be improved by derivatisation. To improve both chromatographic behavior and detectability of the amphetamines, a great number of procedures involving precolumn or postcolumn derivatisation using different reagents have been developed. Since the most specific MS detection for liquid chromatography (LC) has not been widely available, some authors use others less specific detectors for their procedures. Napthaquinone–4–sulphonate (NQS) was used as a reagent for precolumn derivatisation of amphetamine and methamphetamine [17-18]. Primary and secondary amine groups of amphetamine react with NQS in alkaline solution to form intense coloured compounds, that can be determined colourimetrically and can be separated using normal – phase HPLC. Talwar et al. [17] reported the limit of detection at 450 nm and 260 nm of 105 and 60 μg/l amphetamine and 90 and 65 μg/l for methamphetamine. Bougusz et al. [20] analysed by atmospheric-pressure chemical ionization (APCI) HPLC-MS and HPLC with diode array detection (DAD) for the determination of phenylisothiocyanate derivatives of amphetamine and its analogues, and other sympathomimetic amines in serum, blood and urine. The HPLC-ACPI-MS was far more specific and sensitive for all the drugs tested and the authors concluded that MS detection was preferable over simple UV absorbance detection.

Nevertheless, some papers were published concerning drug testing using GC with various detectors (FID, NPD, and ECD). Jonsson et al. [22] reported GC detection using NPD of amphetamines after derivatisation with methyl chloroformate as a derivatising agent. The limit of detection was 0.02 and 0.01 μg/ml urine for amphetamine and methamphetamine, respectively. There was a good correlation ($r^2 =$ 0.995).

Fourier transformation infrared spectroscopy (FTIR) is a further detection mode for GC, which can be coupled in line with MS. Platoff Jr. et al. [25] described such a technique for quantitative/qualitative GC/FTIR and quantitative GC/MS determination of amphetamine, methamphetamine and related analogues in human urine. The simultaneous use of both techniques should provide more specificity than when each single technique is used. The detection limit was 25 ng/ml for both amphetamine and methamphetamine and correlations were greater than 0.98. Kalasinsky et al. [24] used GC with FTIR spectroscopy for detection of amphetamines. The authors stated that the main drawback of the GC-FTIR technique was that the detector responded to everything that was eluting from the column and that the extracts needed to be very clean.

Most of the GC/MS procedures for the determination of amphetamine and methamphetamine in urine followed the same principle: after sample preparation followed by derivatisation, the analytes were separated on fused- silica capillary (FSC) columns and detected in the selected ion monitoring (SIM) mode, most often using deuterated internal standard.

GC/MS utilizes three separate characteristics of a drug to make a specific identification: retention time, molecular weight and fragmentation spectrum.

Accordingly, GC/MS is presently the only acceptable method of analysis for performing the confirmatory test. GC/MS analysis is regarded as the most reliable and legally defensible method of testing for drugs of abuse in urine [42]. For a urine sample to be judged positive, the concentration of methamphetamine must exceed a cut off of 500 ng/ml and at least 200 ng/ml of amphetamine, as metabolite, must also be present [43].

Derivatisation

Volatility and thermal stability of the compounds are required in GC and GC/MS analysis. Derivatisation is mandatory for polar and thermolabile compounds to make them amenable to chromatographic analysis. The reduction in polarity can also improve the chromatographic properties of the compounds by minimizing the undesirable and non-specific column adsorption and, therefore, allowing the detection of better peak shapes and a reduction in appearance of ghost peaks. The resolution of closely related compounds not separated in the underivatised form can also be increased by using the appropriate derivative.

The preparation of a derivative may also be performed when the mass spectrum of the underivatised molecule shows poor diagnostic ions. The chemical structure of the substance is changed after derivatisation and, in consequence, the fragmentation pattern can be radically altered. Mass spectra with ions of higher m/z ratios and higher abundance can be obtained. High-mass ions have greater diagnostic value, since they are more specific than low-mass ions, which can be easily affected by interference from the fragment ions of contaminants such as those due to column bleeding. For

identification purpose, the monitoring of high abundant mass ions is preferred for it is less subjected to background interference. An increase in the abundance of the molecular ion or a related ion can also be used for determination of the molecular mass. The preparation of more than one derivative can give helpful additional information to determine the molecular mass.

In GC/MS, derivatisation can also be used to enhance the detectability of a compound by introducing groups with high electron affinity, such as halogen atoms, that can produce an increase in the ionization efficiency and make possible highly sensitive analyses. GC/MS can be used for screening analyses of a structurally related group of compounds by monitoring a common and characteristic fragment ion. Derivatisation can be used to favor the formation of high stability fragments that can be used for this purpose.

The requirements for a successful derivatisation reaction are a single derivative should be formed for each compound, the derivatisation reaction should be simple and rapid, and should occur under mild conditions, the derivative should be formed with a high and reproducible yield and should be stable in the reaction medium and in quantitative analyses, and the calibration curve should be linear.

Acylation is the commonly use derivatisation methods for the determination amphetamines. It consists of the introduction of an acyl group in a molecular holding the reactive hydrogen. Acylated derivatives can be obtained from a great variety of functional groups: alcohols, amines, amides, thiols, phenols, enols, sulfonamides, unsaturated compounds and aromatic rings [44].

The commomly used derivatising agents of acylation of amphetamines are heptafluorobutyric anhydride (HFBA) [16, 24, 26, 28-29, 31, 38], pentafluoro-propionic anhydride (PFPA) [28], trifluoroacetic anhydride (TFAA) [22-23, 28, 34], 4-carbethoxyhexafluorobutyryl chloride (4-CB) [36-37], and N-methyl-bis-trifluoro-acetamide (MBTFA) [27]. The advantage of these derivatising agents is that the mass spectra frequently have abundant ions of high m/z values. Thurman [38] studied the derivatisation of sympathomimetic amines using 4-CB and HFBA and found that the 4-CB reagent partially derivatised the hydroxy-containing synpathomimetic amines, while the HFBA completely derivatised all the sympathomimetic amines.

Other derivatising agents of amphetamines are (-)-menthyl chloroformate [39], propyl chloroformate [32, 41], ethyl chloroformate [35], and N-methyl-N-tert-butyldimethylsilyl trifluoroacetamide (MTBSTFA) [40].

Melgar and Kelly [40] used MTBSTFA, a sylilating reagent, to derivatise active hydrogens of hydroxy, carboxyl and thiol groups as well as primary and secondary amines and found that the reaction by-products formed were neutral and volatile. Besides the resulting derivatives were stable and possessed suitable high molecular weight ions. Meatherall [41] described that the reaction of propyl chloroformate and amphetamines was completed in less than 1 minute at room temperature. The resulting carbamate was water-stable, thus allowing the removal of reaction by-products with aqueous washing. The advantages of using chloroformates to derivatise amphetamines have not been exploited, thus these reagents have not been widely used.

Sample preparation

In general, very little sample preparation is required for initial immunoassay tests. It is unnecessary to hydrolyse the urine samples because immunoassays can detect both the free and conjugated forms of the drug and metabolites. It may be necessary to centrifuge the urine to remove turbidity.

For chromatographic procedures, good sample preparation is extremely important. This is necessary because urine is a complex matrix containing a mixture of large amounts of numerous organic and inorganic compounds in which methamphetamine and amphetamine are found in minute amounts. Sample preparation usually involves extraction and purification of the analytes. The procedure should be efficient, since a good recovery is necessary to extract the small amounts present, and selective, to ensure that interfering substances in the urine are removed.

Various sample preparation procedures have been employed for the analysis of methamphetamine and amphetamine in urine. These are liquid-liquid extraction (LLE) [18,20-23, 25, 28, 32, 37-41], solid-phase extraction (SPE) [18-19, 26-27, 29-31] and solid-phase microextraction (SPME) [33-35].

Extraction performed by LLE usually at an alkaline pH, at which the amphetamines are unionized. Various organic solvents are used for the extraction such as isooctane [22], n-hexane [18], ethyl acetate [23], cyclohexane [40, 28], hexane/chloroform [32] and n-butylchloride [21]. The advantages of LLE are simplicility and low cost. Being a time consuming procedure, required large volume of solvents and often faced the problems of emulsion formation are the disadvantages.

SPE has become popular due to its efficiency and effectiveness. This method offers advantages in the reduction of the volume of solvents required, saving of time and avoidance of the problems caused by emulsion formation which sometimes arise during liquid-liquid extraction. These advantages are offset by the cost of the cartridges used [4].

Lee et al. [30] studied the recoveries of methamphetamine and amphetamine from spiked urine using various types of SPE columns, including C8, C18, Strong cation exchanger (SCX), and C8-SCX mixed mode. The highest recoveries of more than 86 % and 88 % for methamphetamine and amphetamine, respectively, were achieved from a mixed adsorbent (C8-SCX) using a mixed elution solvent of CH_2Cl_2-i-propanol-NH_4OH (78:20:2) at pH 6.

Solid phase microextraction (SPME) is a solvent-free technique that preserves all the advantages of SPE such as simplicity, easy automation, and at the same time, eliminates the disadvantages of SPE such as plugging and the use of solvent [45]. No special thermal desorption module is used, and no modification of the gas chromatography is needed. SPME with thermal desorption completely eliminates organic solvents from extraction and injection, and it investigates both processes into a single step. The geometry of SPME enables the placement of the sorbent (fiber coating) into a sample (such as gaseous or aqueous matrices) or the headspace above the sample to extract analytes.

Centini et al. [33] extracted amphetamines from urine samples by SPME with polydimethylsiloxane fibers 100 micron film thickness using headspace prior to GC/MS analysis without derivatisation. The correlation coefficent (r) was greater than 0.946 for both amphetamine and methamphetamine. Jurado et al. [34] developed a direct on fiber TFAA derivatisation of amphetamine and methamphetamine using SPME with polydimethylsiloxane fiber, and headspace-GC/MS. Recoveries data were 71.89-103.24% with correlation coefficients of 0.9946-0.9999.

Another interesting method of extraction is ion-pair (ion association) extracion which is based on the formation of associates composed of the colorless (or colored) analyte ion and the colored (or colorless) reagent ion of opposite charge (counter ion). The light absorption of these associates can be measured directly in the reaction solution, provided that the absorption maximum is different enough from the absorption maximum of the reagent. These associates can also be extracted from the aqueous solutions into an organic solvent immiscible with water (e.g. chloroform, dichloromethane, and toluene). The concentration of the original analyte is then determined by spectrophotometry of the organic layer, since only the amount of the counter ion equivalent to the amount of the analyte is tranferred to the organic layer (by ion-pair extraction spectrophotometry) [46].

Amphetamines are basic drugs (positive charge), reacting with acid dye (negative charge) to form colored ion-pair complexes in a suitable condition. Various acid dyes have been reported for the analysis of basic drugs and trace amounts of cation. These are sudan III [47], 3', 3'', 5', 5''-tetrabromophenolphthalein ethyl ester (TBPE) [48-53], bromthymol blue [53], and sodium 1,2-napthoquinone-4-sulphonate [54]. The structures of these dyes were shown in Figure 3.

TBPE was used for quantitation of basic drugs in pharmaceutical preparations [51-52] and biological samples [48]. Furthermore, it was applied for screening test of basic drugs in biological [49-50]. Sodium 1,2-napthoquinone-4-sulphonate was also used for determination of methamphetamine in urine [54].

The advantages of ion-pair extraction are ease of performance, economy, less time consumption and less organic solvent required than LLE.

The focus of this study was therefore to develop methods for sample preparation and analysis of amphetamine, methamphetamine and ephedrine in urine using the techniques of ion-pair extraction and GC/MS, respectively. The goal was to develop a sample preparation method that was both efficient and required little additional sample cleanup, while maintaining the advantage of short preparation time. The development and optimization of a GC/MS method would provide accurate quantitation of those drugs of interest.
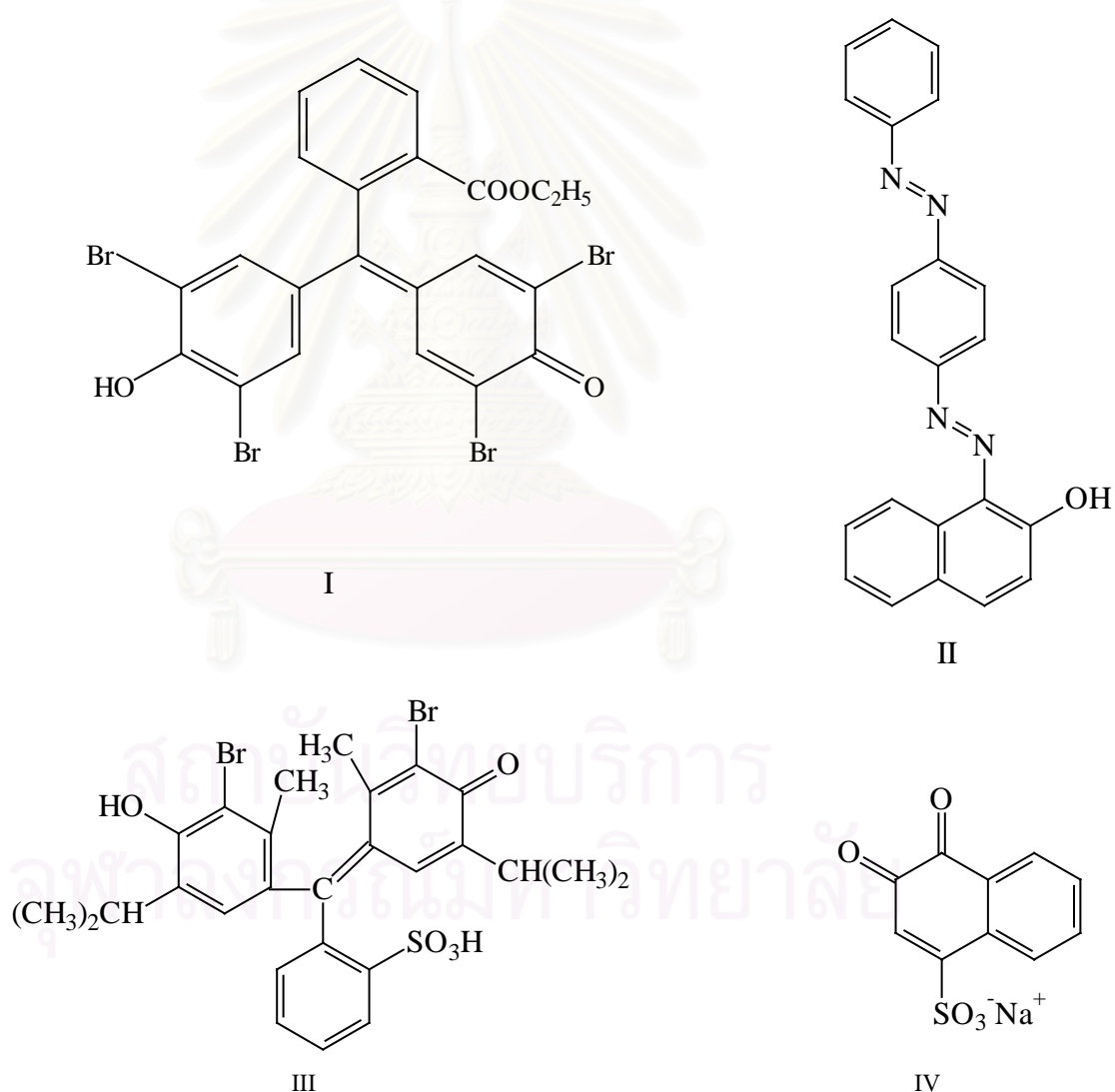
Figure 3 Structures of 3', 3'', 5', 5''-Tetrabromophenolphthalein ethyl ester (I),

SudanIII (II), Bromthymol blue (III) and Sodium 1,2-napthoquinone-4-
sulphonate (IV).

The focus of this study was therefore to develop methods for sample preparation and analysis of amphetamine, methamphetamine and ephedrine in urine using the techniques of ion-pair extraction and GC/MS, respectively. The goal was to develop a sample preparation method that was both efficient and required little to no additional sample cleanup, while maintaining the advantage of short preparation time. The development and optimization of a GC/MS method would provide accurate quantitation of those drugs of interest.

CHAPTER II

EXPERIMENTS

Materials and Methods

Chemical compounds and reagents

All chemical compounds and reagents were of analytical grade and used as received.

- Acetic anhydride (Analar, England)
- Amphetamine sulfate (obtained from the Narcotics Division, Medical Sciences Department, Thailand)
- Boric acid (Merck, Germany)
- Bromthymol blue (Merck, Germany)
- Chloroform (J.T. Baker, USA)
- Chlorpseudoephedrine hydrochloride (obtained from the Narcotics Division, Medical Sciences Department, Thailand)
- Dichloromethane (Mallinckrodt, France)
- Ephedrine hydrochloride (obtained from the Narcotics Division, Medical Sciences Department, Thailand)
- Ethyl acetate (J.T. Baker, USA)
- Helium gas, ultra high purity 99.999 % (TIG, Thailand)
- Hydrochloric acid (Merck, Germany)
- Methamphetamine hydrochloride (obtained from the Narcotics Division, Medical Sciences Department, Thailand)
- Methanol (J.T. Baker, USA)
- Pentafluoropropionic anhydride (Fluka Chemie AG, Switzerland)
- Phentermine hydrochloride (obtained from the Narcotics Division, Medical Sciences Department, Thailand)
- Potassium carbonate (Merck, Germany)
- Potassium chloride (Merck, Germany)
- Potassium dihydrogen phosphate (Merck, Germany)
- Sodium hydroxide (Merck, Germany)

- Sodium sulfate (Merck, Germany)

- Sudan III (Fluka Chemie AG, Switzerland)

- Sulfuric acid (Merck, Germany)

- 3', 3'', 5', 5''- Tetrabromophenolphthalein ethyl ester (Fluka Chemie AG, Switzerland)

- Trifluoroacetic anhydride (Fluka Chemie AG, Switzerland)

Apparatus

- Analytical balance (Mettler Toledo, AT 200, Switzerland)

- Centrifuge (Heltich, EBA 12, Germany)

- Gas Chromatograph/Mass Spectrometer (Hewlett Packard Co., Palo Alto, CA) consisting of

    a) Autosampler, Hewlett Packcard model 7376

    b) Gas Chromatograph, Hewlett Packcard model 5890 Seris II

    c) Mass Spectrometer, Hewlett Packcard model MSD 5971

    d) GC- column, DB-1 (25 m x 0.25 mm x 0.50 μm) , J&W Scientific

    e) Vacuum pump, Edwards

    f) MS Chemstation software, version G1034C

    g) Hewlett Packard Laser 4L printer

- Heating Block (Yamato model HF-21, Japan)

- Nitrogen evaporator (Jet Air Vaporizer, Japan)

- Micropipet (Eppendorf, Germany)

- pH-meter (Mettler Toledo, Delta 350, Switzerland)

- Ultraviolet Spectrophotometer (Shimadzu 2401 PC, Japan)

- Vortex (Genei Scientific Industries Inc., USA)

Methods

This study composed of four experimental steps as follows:

1. Determination of the conditions for the ion-pair formation
2. Optimization of GC/MS analysis method
3. Method validation
4. Assay application

1. Determination of the conditions for the ion-pair formation

The following solutions were prepared:

<u>0.2 M Phosphate buffer solution</u> (pH 7-8)

Dissolved approximately 2.72 g of monobasic potassium phosphate (KH_2PO_4), accurately weighed, in a 100-ml volumetric flask with water and adjusted to volume. Placed 25 ml of the monobasic potassium phosphate solution in a 100-ml volumetric flask and adjusted to the desired pH with hydrochloric acid (HCl) or 1 M sodium hydroxide (NaOH), and then added water to volume.

<u>0.2 M Borate buffer solution</u> (pH 8.5 - 11)

Dissolved approximately 1.24 g and 1.49 g of boric acid and potassium chloride (KCl), respectively, in a 100-ml volumetric flask with water and adjusted to volume. Placed 25 ml of the prepared solution in a 100-ml volumetric flask and adjusted to the desired pH with hydrochloric acid (HCl) or 1 M sodium hydroxide (NaOH), and then added water to volume.

Stock solution of standard amphetamine (AP)

Dissolved approximately 35 mg standard amphetamine sulfate (equivalent to 25 mg amphetamine), accurately weighed, in a 25–ml volumetric flask with methanol and adjusted to volume to obtain amphetamine concentration of 1 mg/ml.

Stock solution of standard methamphetamine (MA)

Dissolved approximately 31 mg standard methamphetamine hydrochloride (equivalent to 25 mg methamphetamine), accurately weighed, in a 25–ml volumetric flask with methanol and adjusted to volume to obtain methamphetamine concentration of 1 mg/ml.

Stock solution of standard ephedrine (EP)

Dissolved approximately 31 mg standard ephedrine hydrochloride (equivalent to 25 mg ephedrine), accurately weighed, in a 25–ml volumetric flask with methanol and adjusted to volume to obtain ephedrine concentration of 1 mg/ml.

Standard solution of amphetamine

Transferred 15 milliliters of stock solution of standard amphetamine to a 100–ml volumetric flask and adjusted to volume with methanol to obtain amphetamine concentration of 150 µg/ml.

Standard solution of methamphetamine

Transferred 2 milliliters of stock solution of standard methamphetamine to a 100–ml volumetric flask and adjusted to volume with methanol to obtain methamphetamine concentration of 20 μg/ml.

Standard solution of ephedrine

Transferred 4 milliliters of stock solution of standard ephedrine to a 100–ml volumetric flask and adjusted to volume with methanol to obtain ephedrine concentration of 40 μg/ml.

Standard solution of the mixture of amphetamine, methamphetamine and ephedrine

Transferred 2 milliliters of each of stock solutions of standard amphetamine, methamphetamine and ephedrine to a 100-ml volumetric flask and adjusted to volume with methanol to obtain 20 μg/ml of amphetamine, methamphetamine and ephedrine, each.

1.1 Determination of the suitable acid dye

1.1.1 Solubility of the acid dye in organic solvent

The following organic solvents were used: dichloromethane, chloroform and ethyl acetate.

Procedure

Dissolved separately10 mg each of TBPE, sudan III and bromthymol blue in 100 ml each of the organic solvents listed above. Mixed and observed solubility of the dye.

1.1.2 Characteristics of ion-pair complex

Procedure

Separately transferred the following solutions: 5 ml of sudan III and TBPE solutions in chloroform, dichloromethane, or ethyl acetate, and bromthymol blue solution in ethyl acetate to each of the tubes containing 3 ml water, 1 ml of buffer pH range 7-11, and 1 ml of MA standard solution. All tubes were shaken for one minute. After centrifugation for 10 minutes, the organic layer was separated. The color and stability of the complexes were observed.

1.2 Study of factors affecting the ion- pair complex formation

TBPE stock solution A (1.0×10^{-4} M)

Dissolved 16.5 mg of TBPE, in dichloromethane to make 250 ml

TBPE stock solution B (4.0×10^{-4} M)

Dissolved 26.4 mg of TBPE in dichloromethane to make 100 ml

1.2.1 Effect of pH

Procedure

AP, MA, and EP were extracted from the AP standard solution, MA standard solution, EP standard solution and standard solution of the mixture of three drugs, respectively, with the TBPE stock solution A in dichloromethane. These standard solutions were adjusted to have pH range 7-11. The dichloromethane extracts were separated and scanned for maximum absorbances, using reagent blank as a reference.

1.2.2 Effect of the TBPE concentration

TBPE solution

From TBPE stock solution A, diluted quantitatively with dichloromethane to obtain solutions having known concentrations of about 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0 and 8.0 x 10^{-5} M.

From TBPE stock solution B, diluted quantitatively with dichloromethane to obtain solutions having known concentrations of about 12.0, 16.0, 20.0, 24.0, 28.0 and 32.0 x 10^{-5} M.

Procedure

Four sets of 8 test tubes, containing a mixture of 3 ml water and 1 ml borate buffer (pH 9.5), were prepared. Transferred separately 1 ml of AP standard solution, 1.5 ml of MA standard solution, 1 ml of EP standard solution and 1 ml mixture of the three drugs to each of the tubes in the first set, the second set, the third set and the

fourth set, respectively. For the first set, 5 ml of various concentrations of TBPE solution (4.0×10^{-5}–3.2×10^{-4} M) were added separately to each tube. Did the same for the other sets, but using the TBPE solution in the concentration range 1.0×10^{-5}–6.0×10^{-5} M for the second and the third sets and 3.0×10^{-5}–8.0×10^{-5} M for the fourth set. All tubes were shaken for one minutes. After centrifugation for 10 minutes, the organic layer (dichloromethane) was separated and its absorbances was measured at 565 nm for AP-TBPE and MA-TBPE, at 554 nm for EP-TBPE, and at 562 nm for the mixture-TBPE of AP, MA and EP, using a reagent blank as a reference.

1.2.3 Effect of shaking time

Procedure

Transferred 5 ml of the TBPE stock solution A to each of the 5 test tubes, containing 3 ml water, 1 ml borate buffer (pH 9.5) and 1 ml of MA standard solution. The tubes were shaken for 0.5, 1, 1.5, 2, and 3 minutes, respectively. After centrifugation for 5 minutes, the organic layer (dichloromethane) was separated and its absorbances was measured at 565 nm for MA-TBPE, using a reagent blank as a reference.

1.2.4 Effect of sodium salt

Procedure

Transferred 1 ml of MA standard solution to each of the tubes containing 100, 200, 300, 400 and 500 mg of sodium tetraborate decahydrate powder, respectively. The tubes were shaken for about 1 minute. Then 5 ml of the TBPE stock solution A was added to each tube. The content in each tube was shaken for 1 minute. After

centrifugation for 10 minutes, the organic layer (dichloromethane) was separated and its absorbance was measured at 565 nm for MA-TBPE, using a reagent blank as a reference.

2. Optimization of the GC/MS analysis method

Preparation of standard stock solutions

Stock solutions of standard AP, MA and EP were separately prepared in methanol to have concentration of 1000 μg/ml of free base and were stored at 4 °C until use.

Preparation of standard solutions

Independenttly prepared from the standard stock solution of AP, MA and EP, diluted quantitatively with methanol to obtain solutions having known concentration of 1, 10, 100 μg/ml.

2.1 Selection of suitable derivatising reagent

Three derivatising reagents were investigated including TFAA, PFPA, and AA

Procedure

Step 1: Transferred separately to a test tube 30 μl of the standard solution (100 μg/ml) of AP, MA and EP. Evaporated the solution carefully under a gentle stream of nitrogen. Then added 100 μl of a mixture of TFAA and ethyl acetate (1:1) to the residue, and mixed. Sealed the tube and incubated in a thermostatic block heater at

60 °C for 20 minutes. Finally, the reaction mixture was evaporated to dryness under a stream of nitrogen at 40 °C. Reconstituted in 100 µl ethyl acetate. The derivative (1 µl) was then injected into the GC column by splitless injection using the autosampler. Data acquisition was performed in SCAN mode. All evaporation steps were stopped immediately after evaporation of the solvent to avoid the loss of amphetamines.

Step 2: Repeated the procedure in step 1, but replaced a mixture of TFAA and ethyl acetate (1:1) with a mixture of PFPA and ethyl acetate (1:1), and incubated at 70 °C for 30 minutes.

Step 3: Repeated the procedure in step 1, but replaced a mixture of TFAA and ethyl acetate (1:1) with 50 µl of AA and 20 µl of triethylamine, and incubated at 70 °C for 2 hrs.

Several different GC column temperature programs were studied in order to achieve optimal separation. Finally the optimum GC/MS conditions were as follows: The GC was equipped with 70 eV electron impact ionization and a DB-1 crosslinked 100 % polymethylsiloxane fused silica capillary column (25 m x 0.25 mm x 0.5 µm film thickness). Helium was used as the carrier gas with head pressure of 60 p.s.i. The injection port and the transfer line were maintained at 200 and 280 °C, respectively. The column temperature was initially set at 70 °C, increased to 140 °C at 35 °C /min and held for 5 min, increased to 150 °C at 3 °C /min, then increased to 250 °C at 30 °C /min and finally held at 250 °C for 3 min. Scan mass range was 40-500 amu.

2.2 Selection of suitable internal standard

Preparation of phentermine standard solution

Phentermine (PT) standard solution was prepared in methanol to have concentration of 100 μg/ml of free base.

Preparation of chlorpseudoephedrine standard solution

Chlorpseudoephedrine (CP) standard solution was prepared in methanol to have concentration of 100 μg/ml of free base.

Procedure

In a test tube, a 3 ml of blank urine was spiked with standard solution of AP, MA, EP and PT or CP of concentration of 1000 ng/ml, each. The sample was adjusted to pH 9.2-9.5 with 100 mg sodium tetraborate decahydrate (borax). Then, 1 ml of 1.0×10^{-4} M TBPE solution was added and mixed for 1 min using a vortex. After centrifugation for 2 min, the organic layer was transferred to a test tube. Then an additional clean-up step was performed which was based on back-extraction into acid. To the extract sulfuric acid (0.15 M; 1 ml) was added and the tube was capped, shaken and centrifuged as before. The upper aqueous phase was transferred to a test tube into which sodium hydroxide (1 M; 1 ml) and 2 ml of dichloromethane were added. The tube was capped, vigorously vortexed and centrifuged. The organic solvent was transferred to a test tube. Methanolic hydrochloric acid (9:1, v/v; 50 μl) was added and the extract was evaporated to dryness under a gentle stream of nitrogen at 37 °C. The dried residue was derivatised with 100 μl of a mixture of PFPA and ethyl acetate (1:1) at 70 °C for 30 min. The mixture was then evaporated to dryness under a gentle stream of nitrogen at 37 °C. The residue was reconstituted in 100 μl ethyl acetate.

Then, 1 µl of derivative was injected into the GC column by splitless injection using autosampler with the GC/MS conditions as described in section 2.1. Data acquisition was performed in SCAN mode.

2.2 System suitability

Procedure

Six replicate injections of urine sample spiked with 1000 ng/ml each of AP, MA, EP and PT. Data acquisition was performed in SCAN mode and calculated the percent of relative standard deviation (% RSD). The tailing factor (T), number of theoretical plates (N) and the resolution (R) were calculated as follows:

$$T = \frac{W_{0.05}}{2f}$$

Where $W_{0.05}$ is the width of peak at 5 % height and f is the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5 % of the peak height from the baseline.

$$N = 5.54 \ (t/W_{h/2})^2$$

Where t is the retention time of the substance and $W_{h/2}$ is the peak width at half-height.

$$R = \frac{2(t_2 - t_1)}{W_1 + W_2}$$

Where t_1 and t_2 are the retention times of the first and second adjacent peaks and W_1 and W_2 are their baseline peak widths.

2.4 Quantification

Injected 1 µl of the derivatised sample with PT as the internal standard, into the GC column by spiltless mode using autosampler. Data acquisition was performed in SIM mode. The GC/MS conditions were those described in section 2.1.

3. Method Validation

For the rest of the study, the procedures were as follows:

Sample preparation

A 3 ml of blank urine and spiked urine samples were adjusted to have pH 9.2-9.5 with 100 mg sodium tetraborate decahydrate. Then, 1 ml of 1.0×10^{-4} M TBPE solution was added, and mixed for 1 min using a vortex. After centrifugation for 2 min, the organic layer was transferred to a test tube. Then an additional clean-up step was performed which was based on back-extraction into acid. To the extract sulfuric acid (0.15 M; 1 ml) was added and the tube was capped, shaken and centrifuged as before. The upper aqueous phase was transferred to a test tube into which sodium hydroxide (1 M; 1 ml) and 2 ml of dichloromethane were added. The tube was capped, vigorously vortexed and centrifuged. The organic solvent was transferred to a test tube. Methanolic hydrochloric acid (9:1, v/v; 50 µl) was added and the extract was evaporated to dryness under a gentle stream of nitrogen at 37 °C. Added 100 µl of a mixture of PFPA and ethyl acetate (1:1) to the dried residue. Sealed the tube and

incubated in a thermostatic block heater at 70 $^\circ$C for 30 min. Evaporated the mixture to dryness under a gentle stream of nitrogen at 37 $^\circ$C. Redissolved the residue in 100 µl of ethyl acetate. The derivatised sample was transferred to a conical micro vial and placed in the autosampler for GC/MS analysis.

GC/MS conditions

Column: DB-1 (25 m x 0.25 mm x 0.5 µm film thickness)

Injection port temperature: 200 $^\circ$C

Transfer line temperature: 280 $^\circ$C

Column temperature: initially set at 70 $^\circ$C, increased to 140 $^\circ$C at 35 $^\circ$C/ min, and held for 5 min, 150 $^\circ$C at 3 $^\circ$C/min, and to 250 $^\circ$C at 30 $^\circ$C/min and finally held at 250 $^\circ$C for 3 min.

Injection volume: 1 µl, splitless mode

Carrier gas: Helium, head pressure 60 p.s.i.

Ionization mode: electron impact, 70 eV

Data acquisition: selected ion monitoring (SIM)

Quantification

From the TIC of SIM scan, peak area of quantitation ion of each PFPA derivative was automatically integrated and used to calculate peak area ratio (PAR).

$$PAR = \frac{\text{peak area of quantitation ion of analyte}}{\text{peak area of quantitation ion of internal standard}}$$

3.1 Selectivity

Procedure

Blank human urine samples from six different human sources were evaluated to determine the presence of any interferences across the retention windows of PFPA derivatives of AP, MA, EP and PT (IS).

3.2 Linearity

Procedure

Three stock solutions of AP, MA and EP and a stock solution of PT in methanol were independently prepared and appropriate volumes added to a 3 ml aliquots of blank urine to give three replicate spiked urine samples, containing standard mixture of concentration ranges of 50-2500 ng/ml for AP and MA, and 100-2500 ng/ml for EP. Each urine standard was additionally spiked with phentermine, the internal standard, at a concentration of 1000 ng/ml. Urine standards were then subjected to sample preparation and analyzed as described in section 3 under sample preparation. Peak area ratios and concentrations of each analyte was plotted and the relationship between these variables was explained by regression analysis.

3.3 Limits of detection and quantitation

Procedure

Urine standards containing 10, 25, 50,100, and 200 ng/ml of AP, MA and EP were independently prepared by serially spiking these three drugs into 3 ml blank urine to give three replicate urine standards. Each urine standard was additionally spiked with the internal standard, PT, at a concentration of 1000 ng/ml. Urine standards were subjected to sample preparation and analyzed as described in section 3.

LOD and LOQ were calculated as follows:

$$LOD \quad = \quad 3.3 \ \sigma/S$$
$$LOQ \quad = \quad 10 \ \sigma/S$$

Where σ and S are standard deviation and slope of the regression line, respectively.

3.4 Precision

Precision of the method was determined in term of percentage of relative standard deviation (%RSD)

3.4.1 Within-run

Procedure

Five replicates of urine samples spiked with AP at 50, 100, 1000 and 2000 ng/ml; MA at 50, 100, 1000, 2000 ng/ml; EP at 100, 250, 1000 and 2000 ng/ml, and internal standard, PT, at 1000 ng/ml were prepared and carried out the entire procedure as described in section 3.

3.4.2 Between-run

Procedure

The between-run was evaluated over three days with five replicates of urine samples being prepared in the same manner as those described in within-run.

3.5 Accuracy

The accuracy of the proposed method was evaluated as the percentage of deviation of the mean from the true value.

Procedure

The amount of analyte added and found in spiked urine sample obtained from section 3.4 were used to calculate the accuracy of the developed method.

3.6 Extraction efficiency (Recovery)

<u>Procedure</u>

Set A: five replicates of urine samples spiked with AP at 100, 1000 and 2000 ng/ml; MA at 100, 1000, 2000 ng/ml; and EP at 250, 1000, 2000 ng/ml and PT, the internal standard at 1000 ng/ml, were prepared and carried out the entire procedure as describe in section 3.

Set B: five replicates of urine samples were prepared and extracted with the TBPE solution. The organic layer was separated and evaporated to dryness. The residue was spiked with AP, MA, EP and PT in the same manner as the replicates in Set A. Organic solvent was evaporated to dryness prior to derivatization with PFPA. Then followed the rest of procedures described in section 3.

Extraction efficiency was calculated by comparing peak area ratio obtained from spiked urine standard, set A with those obtained from set B.

3.7 Stability of the analyte

3.7.1 Freeze and Thaw Stability

<u>Procedure</u>

Three aliqouts of spiked urine samples at each of the low and high concentrations (100 and 2000 ng/ml for AP and MA; 250 and 2000 ng/ml for EP) were stored at $-20\ ^{\circ}$C for 24 hrs and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 12 to 24 hrs at $-20\ ^{\circ}$C. The freeze

thaw cycle was repeated two more times, then analyzed on the third cycle. Each aliquot was analyzed in triplicate. Comparison of the results obtained with those obtained from the zero cycle.

3.7.2 Short-term stability

<u>Procedure</u>

Three aliqouts of spiked urine samples at each of the low and high concentrations (AP and MA at 100 and 2000 ng/ml, and EP at 250 and 2000 ng/ml) were stored at -20 °C for 24 hrs, thawed at room temperature and kept at this temperature from 4 to 7 hrs and analyzed. Each aliquot was analyzed in triplicate. Comparison of the results obtained with those obtained at the time of freshly prepared.

3.7.3 Long-term stability

<u>Procedure</u>

Four aliqouts of spiked urine samples at each of the low and high concentrations (AP and MA at 100 and 2000 ng/ml, and EP at 250 and 2000 ng/ml) were stored at -20 °C for three months, thawed and analyzed. Each aliquot was analyzed in triplicate. Comparison of the results obtained with those obtained from the freshly prepared of long-term stability testing.

3.7.4 Stock solution stability

Procedure

The methanolic stock standard solutions of AP, MA, EP and PT were independently prepared to have a concentration of 1000 µg/ml. Each solution was analyzed immediately in triplicate by the developed method and was stored at 4 °C. The solutions were reanalyzed after 3 months of storage at 4 °C. The results obtained from these two occasions were compared.

3.7.5   Autosampler stability

Procedure

The stability of processed samples in the autosampler was determined at the operating temperature of the GC/MS system. Five replicates of spiked urine samples at each of the low and high concentrations (AP and MA at 100 and 2000 ng/ml, and EP at 250 and 2000 ng/ml) were prepared and analyzed at the time zero, 6 and 12 hrs. Comparison of the results obtained at the time 6 and 12 hrs with those obtained at time zero hour.

4. Assay application

The developed method was used to analyte AP, MA and EP in 10 human urine samples. These samples were screened by immunoassay technique; such as EMIT and PFIA. In this study, the samples that gave positive and negative screening test were gone through the confirmatory test for quantitative analysis by GC/MS.

Procedure

A batch of urine samples, consisting of 10 urine samples and 3 quality control spiked urine samples was formed. The quality control spiked urine samples contained AP, MA and EP at each of the concentrations 100, 1000 and 2000 ng/ml for AP and MA and 250, 1000 and 2000 ng/ml for EP. A 1000 ng/ml PT was added to all samples. The samples were prepared and analyzed according to the procedure described in section 3.

RESULTS AND DISCUSSION

1. Determination of the conditions for the ion-pair formation.

1.1 Determination of the suitable acid dye

A thorough investigation was conducted in order to select the suitable acid dye for using as an ion-pair reagent for sample preparation of urine samples prior to the GC-MS analysis.

Criteria for selecting the suitable acid dye are:

1) The dye is soluble in organic solvent.
2) The dye reacts with amphetamine, methamphetamine and ephedrine at room temperature to form colored ion-pair complexes in the organic solvent.
3) The ion-pair complex formed has a clear intense color, different from the color of the original dye solution and the color of urine.
4) The reaction time should be short. Full color development upon shaking is within 1 minute.
5) The complex is stable at room temperature for at least 10 minutes.

1.1.1 Solubility of the acid dye in organic solvents

The solubility of TBPE, sudan III and bromthymol blue in each of the organic solvents was presented in Table 1. TBPE and sudan III dissolved in dichloromethane

(CH$_2$Cl$_2$), chloroform (CHCl$_3$) and ethyl acetate (EtOAc) but bromthymol blue only dissolved in EtOAc.

Table 1 Solubility of TBPE, sudan III and bromthymol blue in organic solvents

Dye	Organic solvent		
	Dichloromethane	Chloroform	Ethyl acetate
TBPE	soluble (yellow solution)	soluble (yellow solution)	soluble (blue-green solution)
Sudan III	soluble (red solution)	soluble (red solution)	soluble (red solution)
Bromthylmol blue	insoluble	insoluble	soluble (yellow solution)

1.1.2 Characteristics of ion-pair complex

At room temperature and at any pH, methamphetamine did not react with sudan III and bromthymol blue in all organic solvents.

The MA-TBPE was red-violet complexes. Using TBPE solution in ethyl acetate as an ion-pair reagent, it was difficult to notice the formation of red-violet complexes of the MA in blue-green organic phase. TBPE solution in dichloromethane or chloroform was a clearly intense yellow solution. Therefore, it was very easy to see the red-violet complexes of TBPE and the drugs in the yellow organic layer. Consequently, it was very easy to separate the complexes in organic layer from the aqueous layer.

In the optimum condition, TBPE and MA were in the deprotonated and protonated forms, respectively (TBPE anion and MA cation). These ions reacted to form colored ion-pair complexes which dissolved into an organic solvent.

TBPE in chloroform had lower extractability than TBPE in dichloromethane with respect to the same amount of MA as shown in Figure 4.

Therefore, TBPE in dichloromethane was selected as the acid dye for sample preparation of amphetamine, methamphetamine and ephedrine in human urine samples prior to GC-MS analysis.
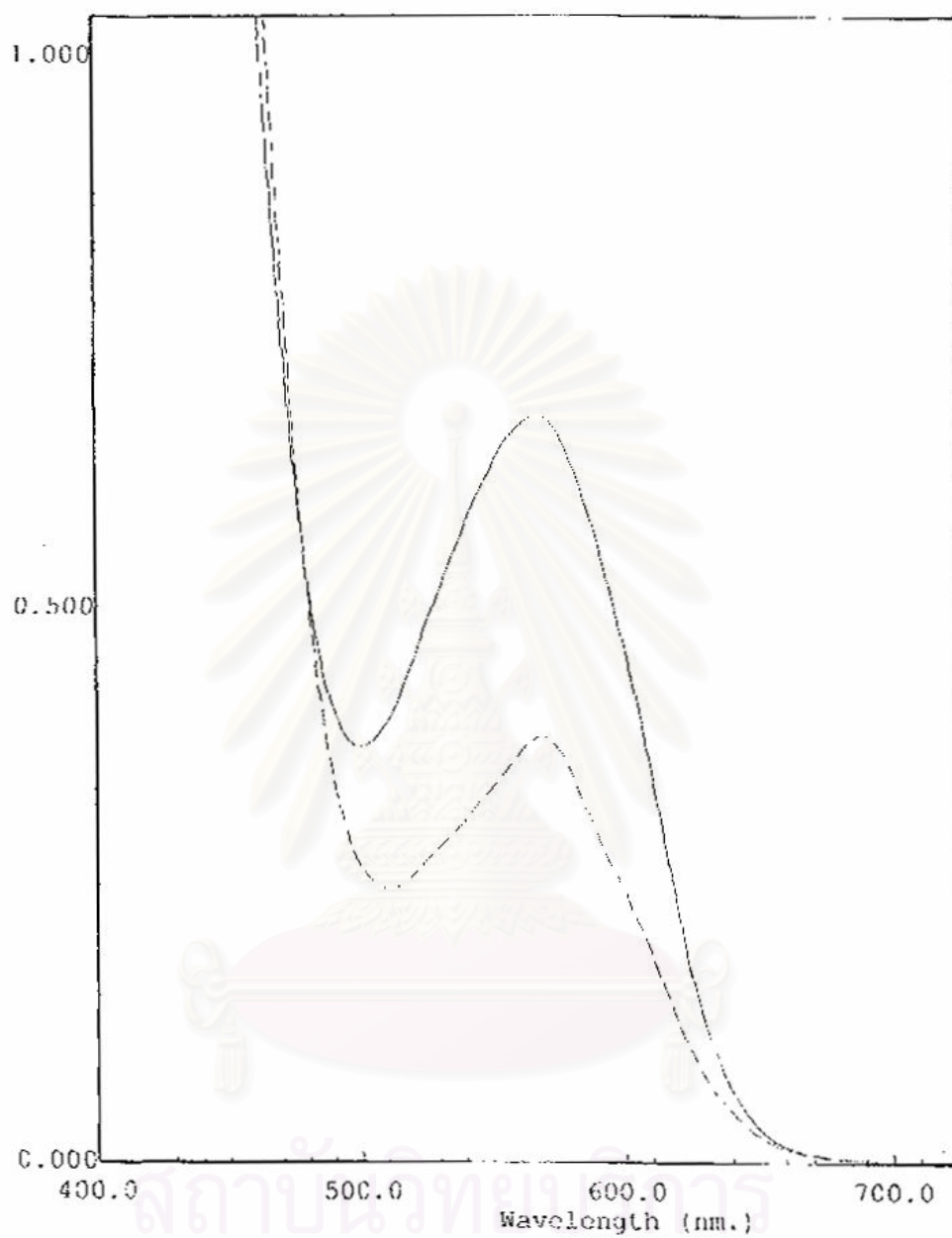
Figure 4 Effect of an organic solvent on the absorbance of methamphetamine-TBPE

(4 μg/ml). (-----) dichloromethane; (- - - -) chloroform.

1.2 Study of factors affecting the ion-pair complex formation.

1.2.1   Effect of pH

The AP-TBPE, MA-TBPE, EP-TBPE and mixture of AP, MA and EP-TBPE complexes in dichloromethane absorbed strongly in the visible region and exhibited absorption maxima at 565 nm for AP-TBPE and MA-TBPE, 554 nm for EP-TBPE and 562 nm for the mixture-TBPE as displayed in Figure 5. The TBPE in dichloromethane was yellow and slightly absorbed in the visible region of 540-570 nm. The extracted ion-pair complexes were stable upon standing at room temperature for up to 1 hour.

TBPE is a monoprotic dye with an acid dissociation constant (K_a) of about 10^{-4} [48].   AP, MA and EP are basic compounds with pK_a of 9.9, 10.1, and 9.6, respectively. In order to establish the optimum pH range for the extraction of AP, MA and EP with TBPE, the AP-TBPE, MA-TBPE and EP-TBPE complexes were extracted into dichloromethane from the series of aqueous solutions buffered to have a pH range of 7-11. As shown in Figure 6, the maximum and constant absorbance of these complexes was obtained in the pH range of 9.0-10.0.

At lower pH, the concentration of the TBPE anion was reduced while at higher pH, the concentration of AP, MA, and EP cations were reduced as well as the instability of TBPE. Therefore, the reduction in absorbances of the extracts was observed when pH of aqueous solutions was outside the optimum pH range. Consequently, the subsequent extraction of AP, MA and EP was performed at pH 9.2-9.5. Variation of pH in this range did not significantly shift the wavelength of maximum absorbance (λmax).
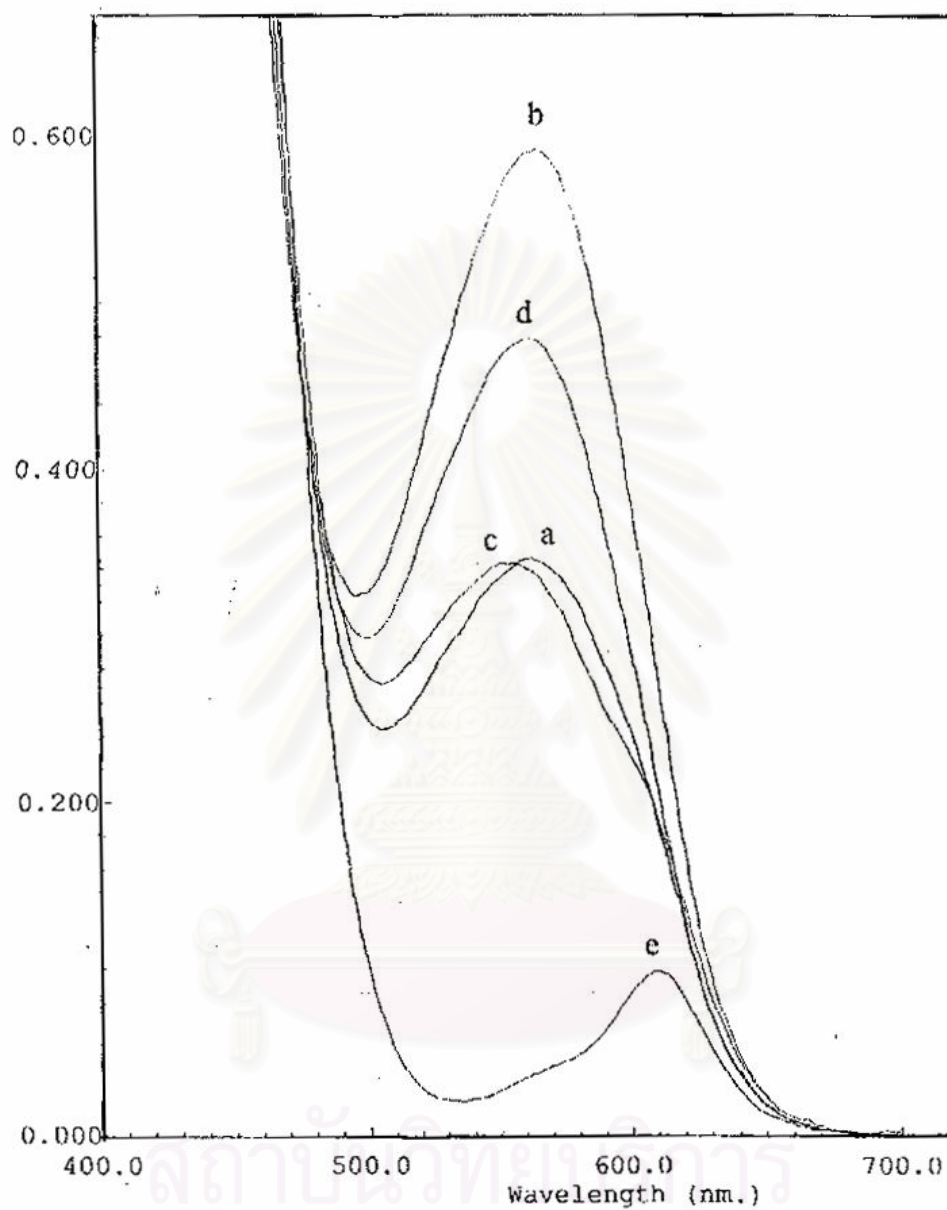
Figure 5 Absorption spectra of amphetamine-TBPE, methamphetamine-TBPE,

ephedrine-TBPE, and reagent blank in dichloromethane.

(a) AP-TBPE (30 μg/ml); (b) MA-TBPE (4 μg/ml); (c) EP-TBPE

(8 μg/ml); (d) Mixture-TBPE (4 μg/ml of AP, MA and EP,each) and

(e) reagent blank.
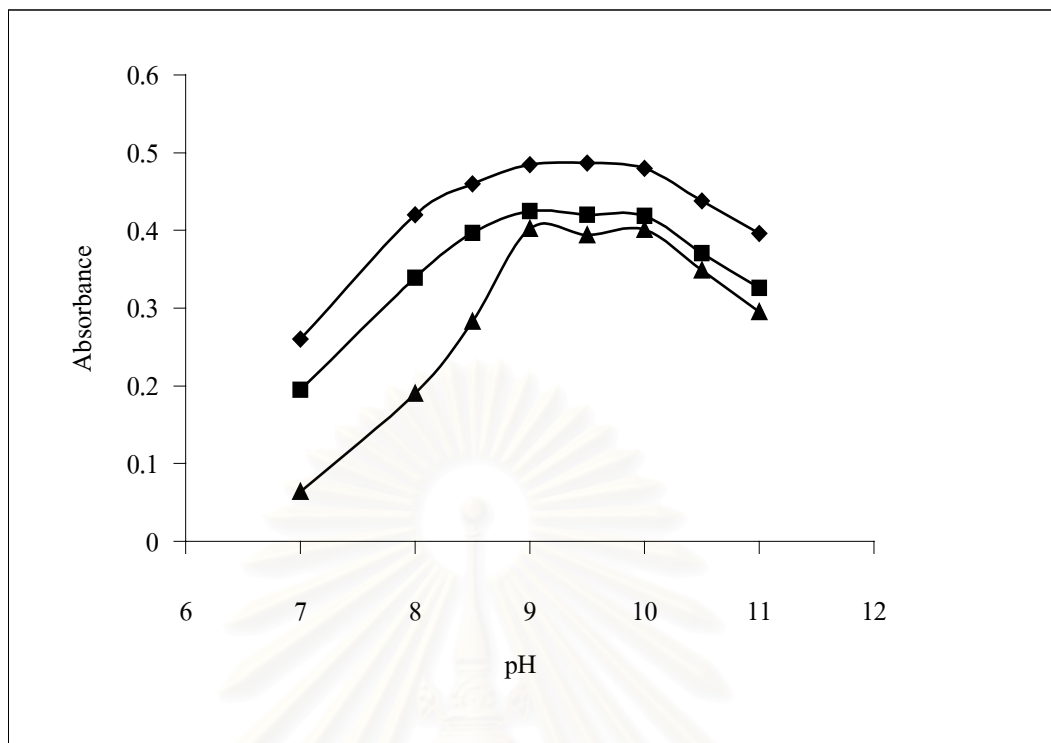
Figure 6 Effect of pH on the extraction of amphetamine (30 μg/ml),

methamphetamine (4 μg/ml), and ephedrine (8 μg/ml) with TBPE

in dichloromethane (AP;   MA;   EP).

1.2.2   Effect of the TBPE concentration

The influence of TBPE concentration on the extractions of AP (30 μg/ml), MA (6 μg/ml), EP (8 μg/ml) and mixture of AP, MA and EP of 4 μg/ml, each   was examined. For AP, MA and EP, and the mixture, the concentration of TBPE solution used ranged from 4.0×10^{-5} to 3.2×10^{-4} M, 1.0×10^{-5} to 6.0×10^{-5} M, and 3.0×10^{-5} to 8.0×10^{-5} M, respectively. The results obtained for AP, MA, EP and the mixture of three drugs were shown in Figure 7, 8 and 9, respectively.

For all three drugs, the absorbances at the λmax increased along with an increase in the TBPE concentration up to about 2.27×10^{-4} M for AP (30 μg/ml),

3.62x 10^{-5} M for MA (6 μg/ml), 4.24x10^{-5} M for EP (8 μg/ml)and 5.44x10^{-5} M for mixture of 4 μg/ml each of AP, MA and EP.

From these results, the excess amount of TBPE needed for extraction of all three drugs of 1000 ng/ml each in urine sample was found to be about 7x10^{-5} mmole.
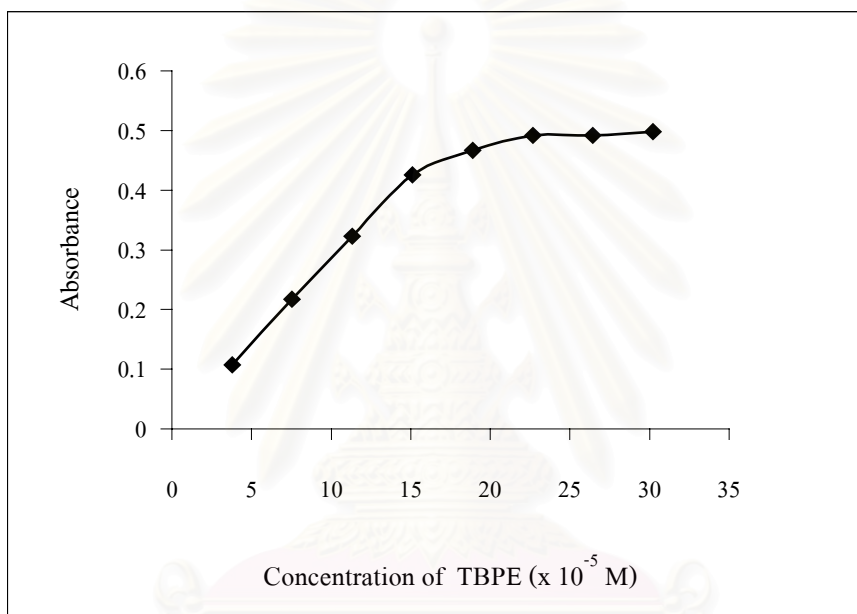
Figure 7 Effect of TBPE concentration on the extraction of amphetamine

(30 μg/ml) with dichloromethane.
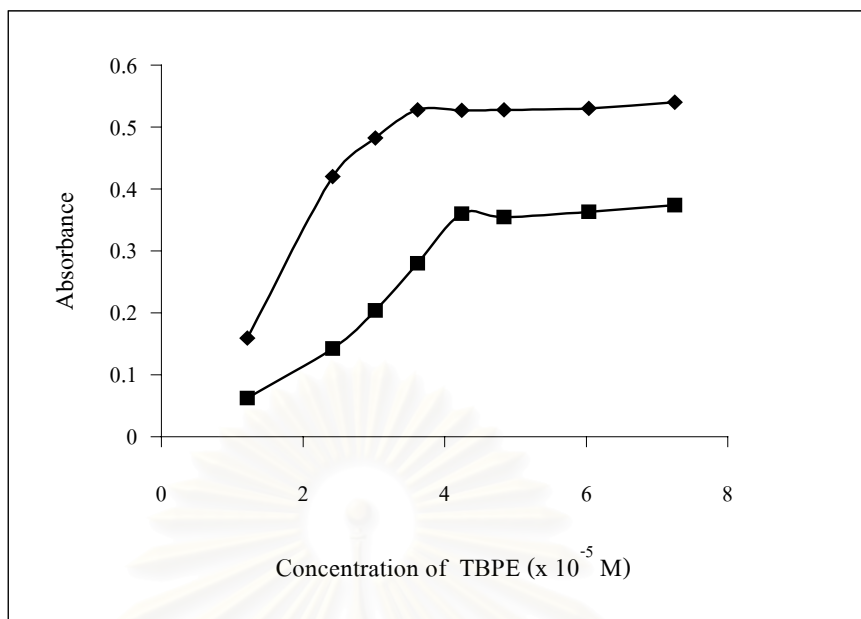
Figure 8 Effect of TBPE concentration on the extraction of methamphetamine

(6 μg/ml), and ephedrine (8 μg/ml) with dichloromethane. (   ) MA;

(   ) EP.
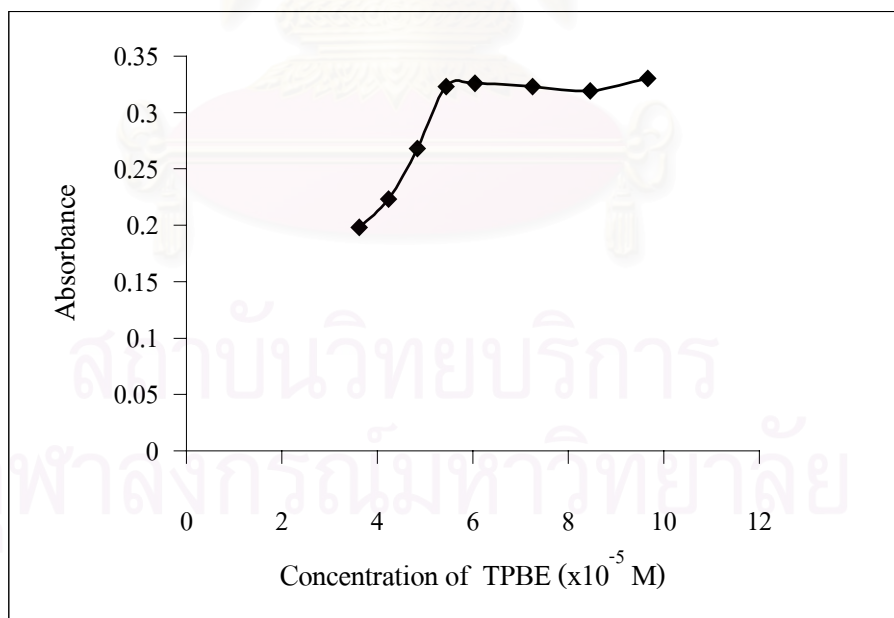
Figure 9 Effect of TBPE concentration on the extraction of a mixture of 4 μg/ml

each of amphetamine, methamphetamine and ephedrine

with dichloromethane.

1.2.3 Effect of shaking time

Complete extraction of each of the complexes of AP-TBPE, MA-TBPE, and EP-TBPE into dichloromethane was found to be about 30 seconds of shaking. Continued shaking up to 3 minutes produced no further change in the color intensity, neither did the absorbance, as shown in Figure 9. The color intensity of dichloromethane extracts remained constant for up to 1 hour. Therefore, the shaking time of 1 minute was applied for this study.
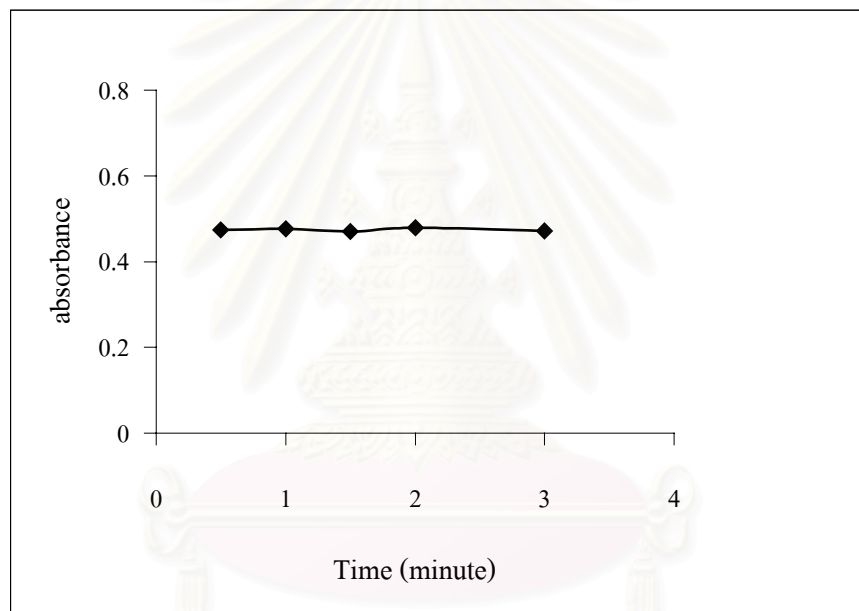
Figure 10 Effect of shaking time on the extraction of methamphetamine (4 μg/ml)
with TBPE in dichloromethane.

1.2.4 Effect of sodium salt

In order to save time in preparing the buffer solution pH 9.5 and solve a problem of dilution effect on urine sample, sodium tetraborate decahydrate in powder form was used for adjusting the pH of urine sample to 9.2-9.5. As shown in Figure 10

and 11, there was no difference in the absorbance of MA-TBPE in dichloromethane whether adjusting urine pH by using sodium tetraborate decahydrate in powder form (100 mg) or using borate buffer pH 9.5, given that MA was the same concentration. The effect of the amount of sodium tetraborate decahydrate (100-500 mg) on the formation of the ion-pair complex was also studied and was found to be insignificant as shown in Figure 12. Consequently, 100 mg of sodium tetraborate decahydrate powder was applied for this study.
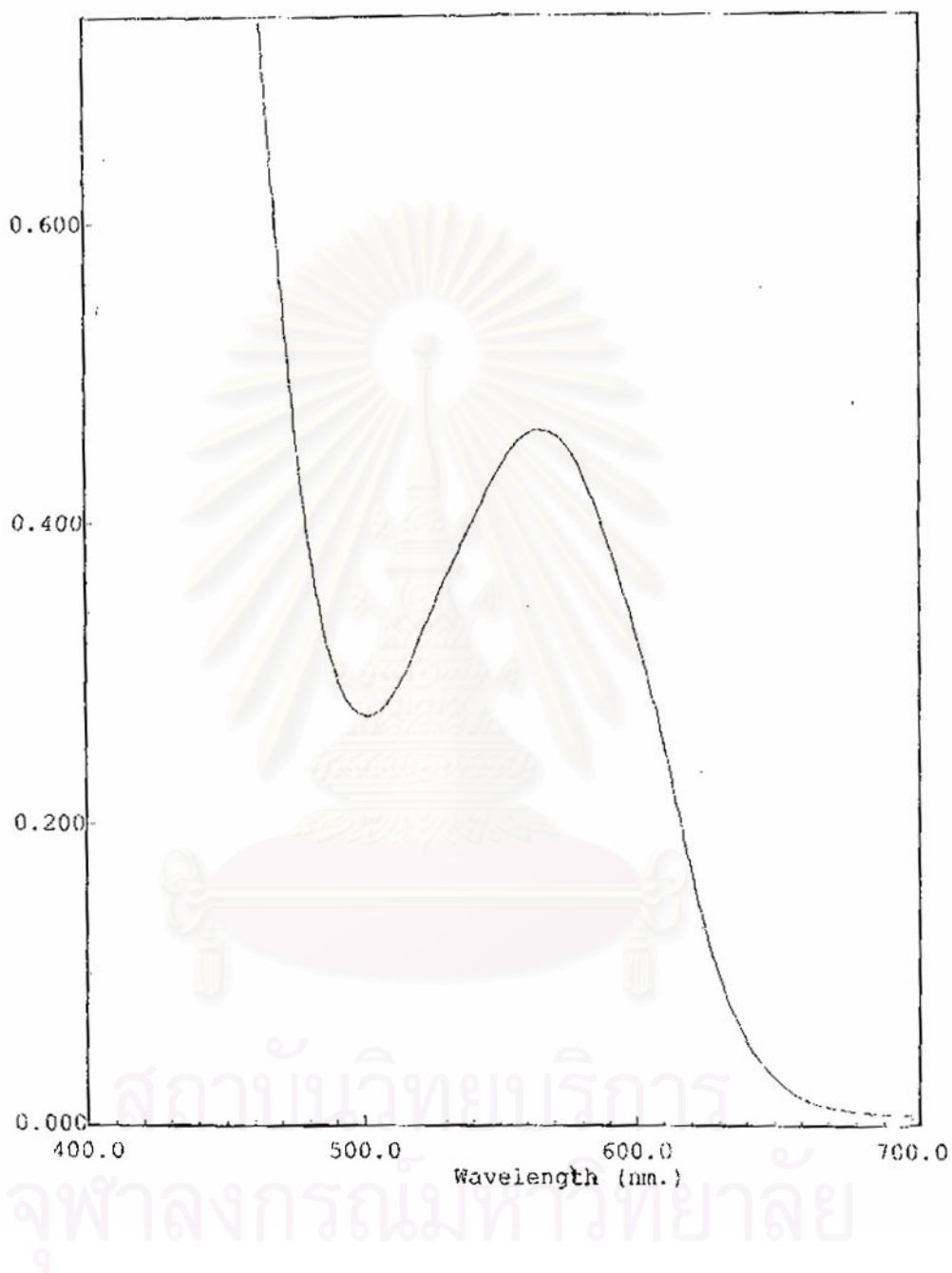
Figure 11 Absorption spectrum of methamphetamine-TBPE (4 μg/ml) in
dichloromethane adjusting pH with sodium tetraborate decahydrate
powder (100 mg).
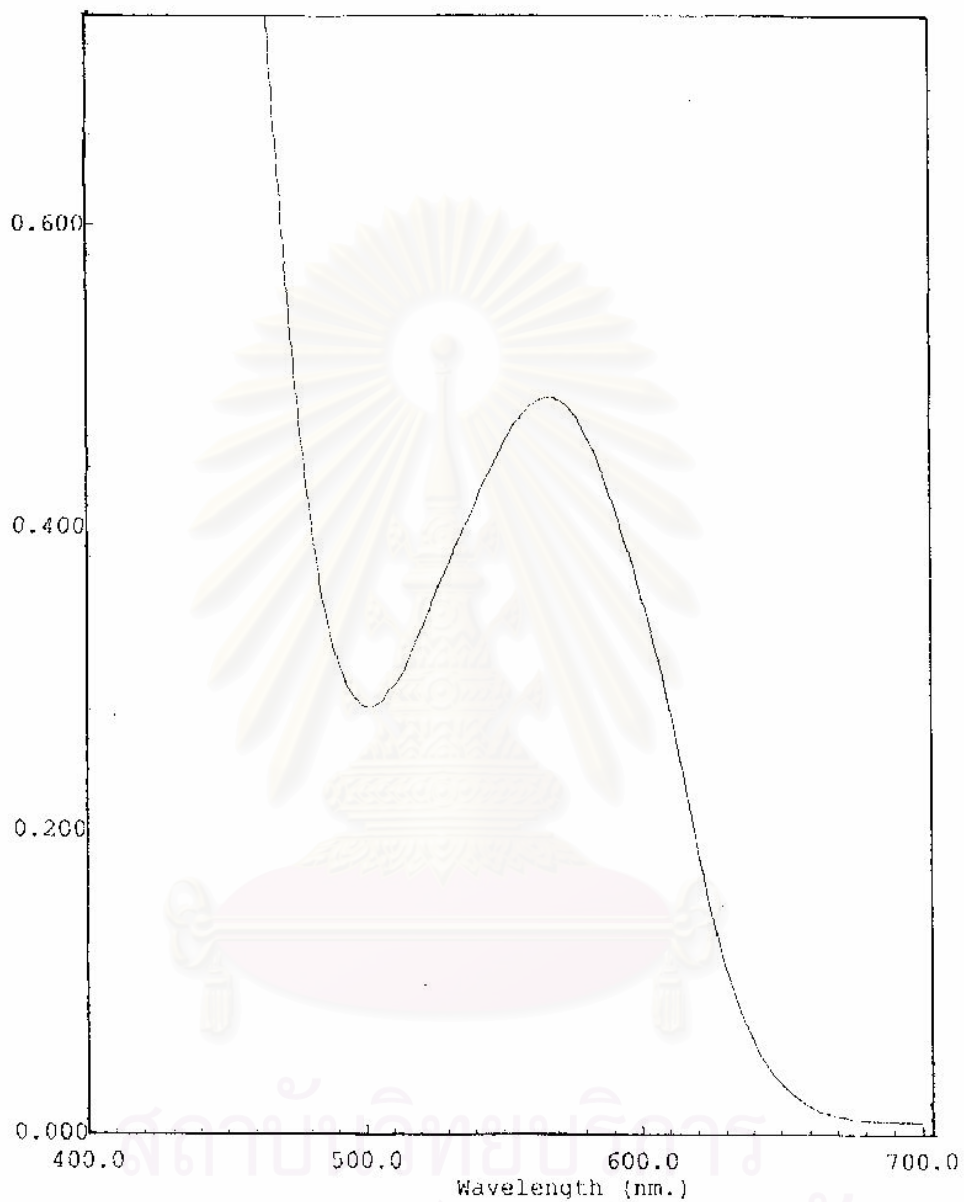
Figure 12 Absorption spectra of methamphetamine-TBPE (4 μg/ml) in

dichloromethane adjusting pH with borate buffer solution (pH 9.5).
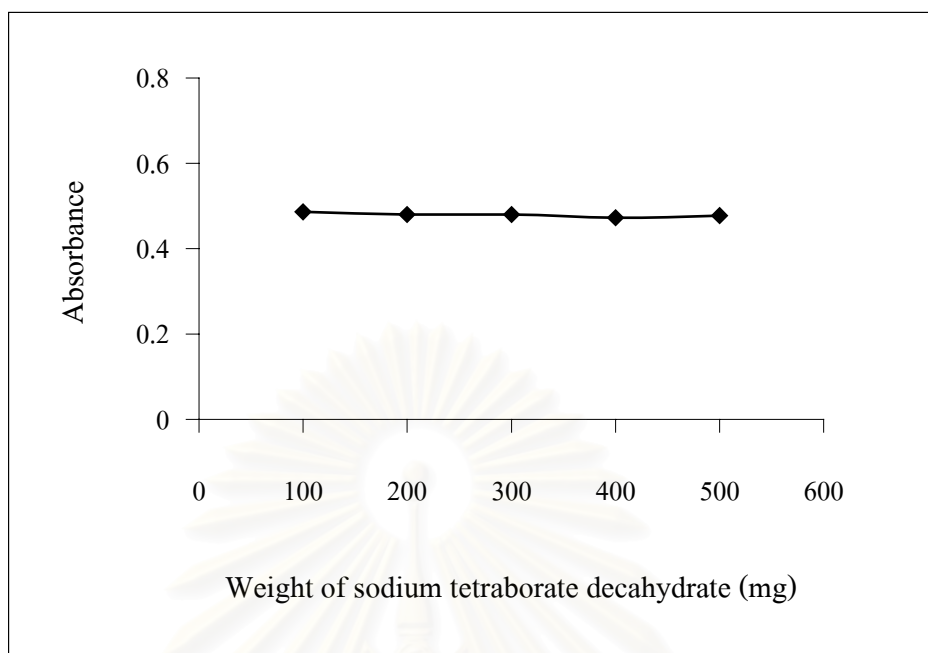
Figure 13 Effect of sodium salt on the extraction of methamphetamine (4 µg/ml)
with TBPE.

2. Optimization of the GC/MS analysis method

2.1 Selection of suitable derivatising reagent

Acylation is a widely used derivatisation procedure for sample analysis by GC
and GC/MS. The popularity of acylating reagents is enhanced by their ease of use and
formation of derivatives. In acylation, an active hydrogen of a parent compound is
replaced by an acyl group such as acetyl, trifluoroacetyl, or pentafluoropropionyl.
Compared to their parent compounds, acyl derivatives are more volatile, less polar, and
more thermally stable. As a result, separation is improved and detection is enhanced in
both GC and GC/MS analyses.   Besides, acyl derivatives tend to direct the
fragmentation patterns of compounds in MS applications, and so provide helpful
information on the structure of these compounds.

In this study, most commonly used acylating reagents: TFAA, PFPA, and AA, were compared with respect to reaction time and stability of acyl derivatives.

AP, MA and EP react with TFAA, PFPA and AA according to reactions shown in Figure 14.
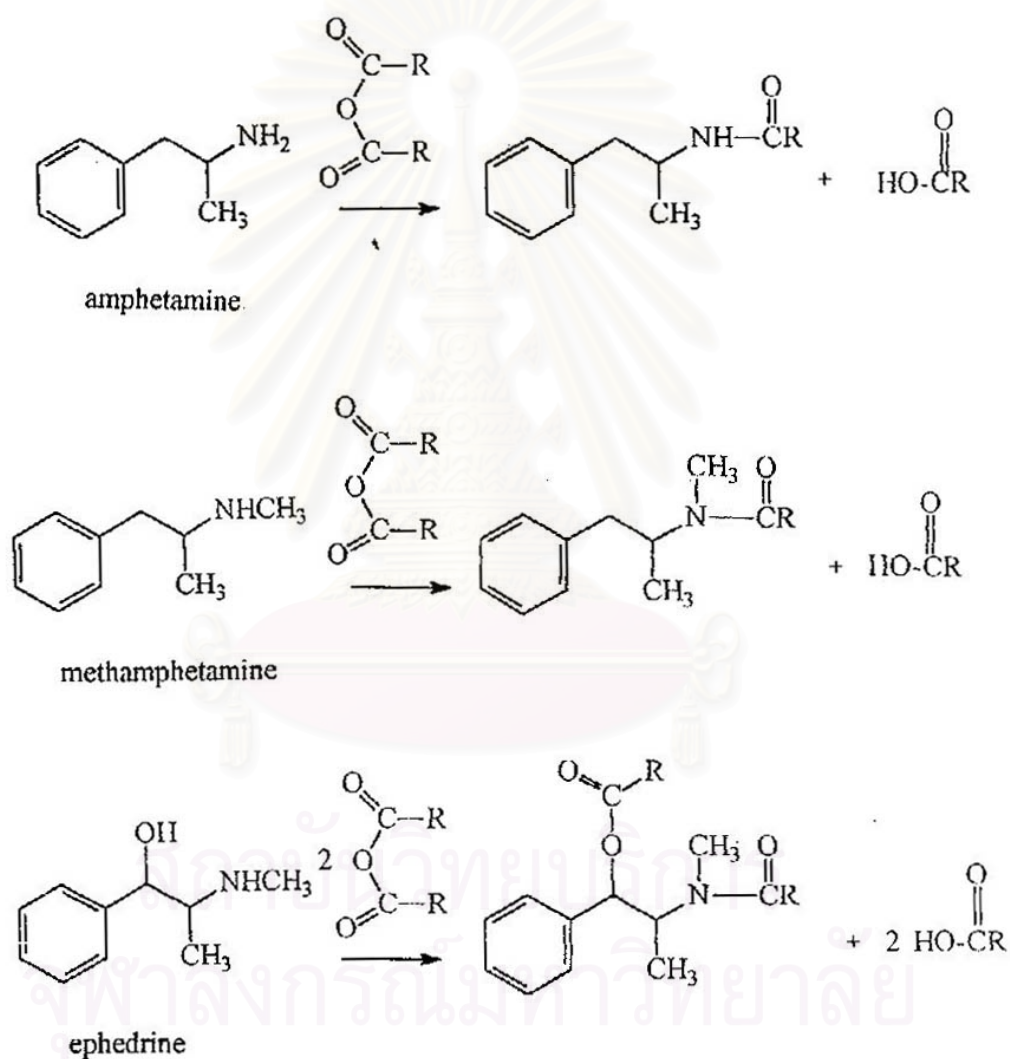
Figure 14 Acylation of amphetamine, methamphetamine and ephedrine.

TFAA: R = CF_3; PFPA: R = C_2F_5; AA: R = CH_3.

Ethyl acetate was used as a solvent for the acid-by product which must be removed, via a stream of nitrogen, prior to GC/MS analysis to prevent deterioration of the column.

Although TFAA rapidly reacted with AP, MA and EP at 60 $^\circ$C, derivatives formed were not quite stable and needed to be analysed by GC/MS as soon as possible after reconstituting in ethyl acetate. When PFPA or AA was used as derivatizing reagent for the drugs under investigated, derivatives obtained were stable for up to 6 hrs. However, it took almost 2 hrs, even when triethylamine was added to promote reactivity to completely derivatise the drugs with AA. Therefore PFPA was chosen as a suitable acylating agent for further experiment. Figure 15 shows the total ion chromatogram (TIC) of full-scan analysis of a mixture of PFPA derivatised AP, MA and EP. Mass spectra of AP-PFPA, MA-PFPA and EP-PFPA are respectively shown in Figure 16, 17 and 18. Mass-to-charge ratios (m/z) and retention times of derivatives are presented in Table 2.

Table 2 Mass-to-charge ratios (m/z) and retention times of amphetamine-PFPA,
         methamphetamine-PFPA and ephedrine-PFPA

Derivatised drug	m/z*	Gas chromatographic Retention time, min
Amphetamine-PFPA	91, 118, <u>190</u>	6.67
Methamphetamine-PFPA	91, 118, 160, <u>204</u>	9.37
Ephedrine-PFPA	119, 160, <u>204</u>	9.63

* underlined value indicates base peak
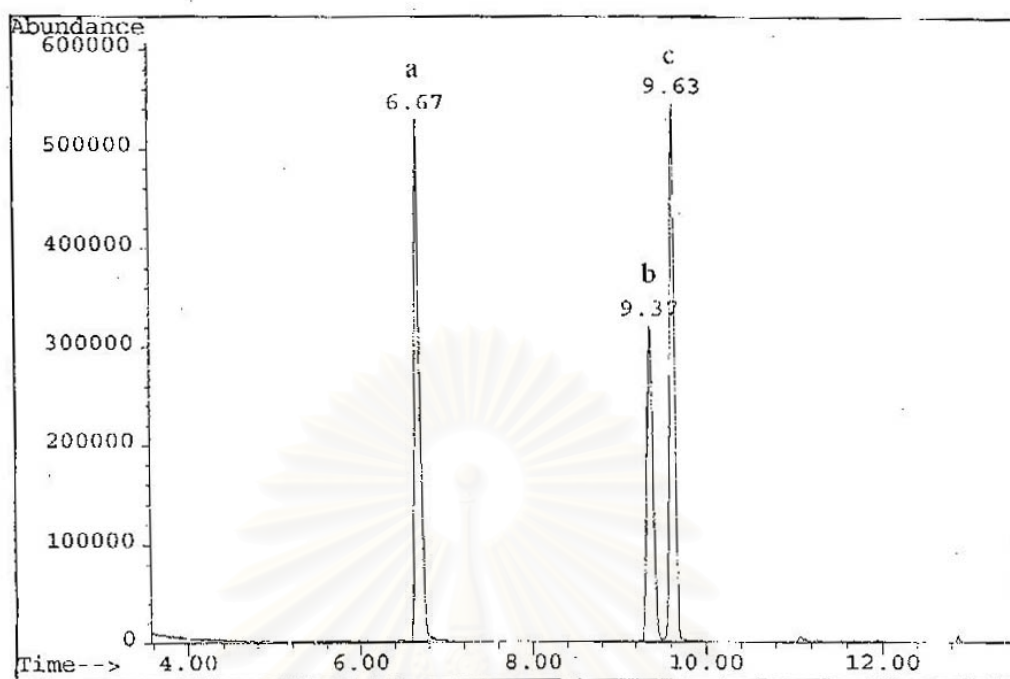
Figure 15 TIC of full-scan analysis of derivatives; a: amphetamine-PFPA,

b: methamphetamine-PFPA, and c: ephedrine-PFPA.
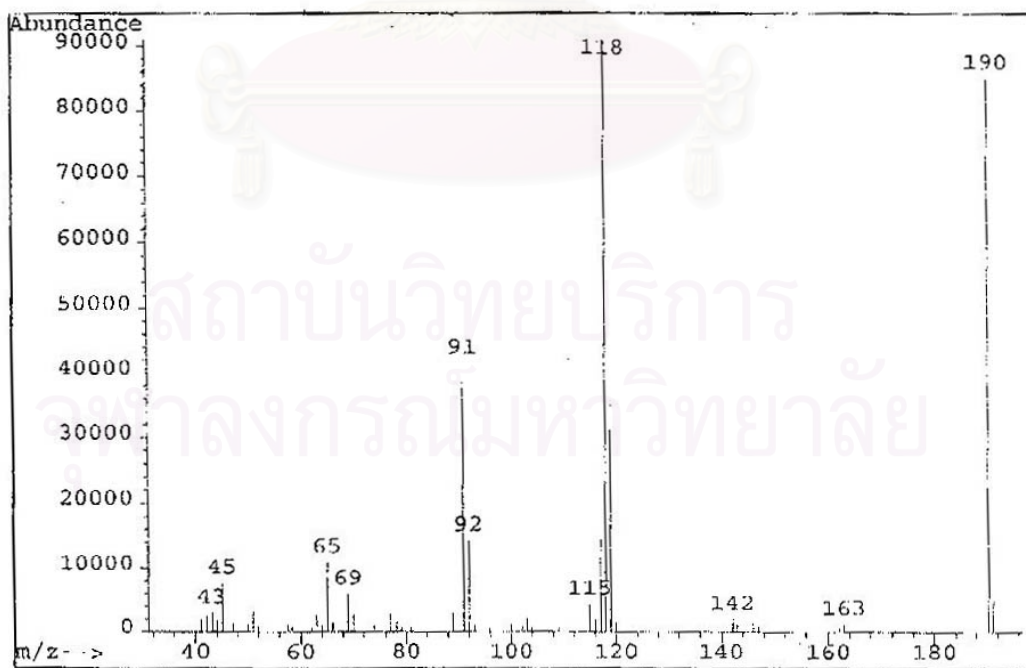
Figure 16 Mass spectrum of amphetamine-PFPA.
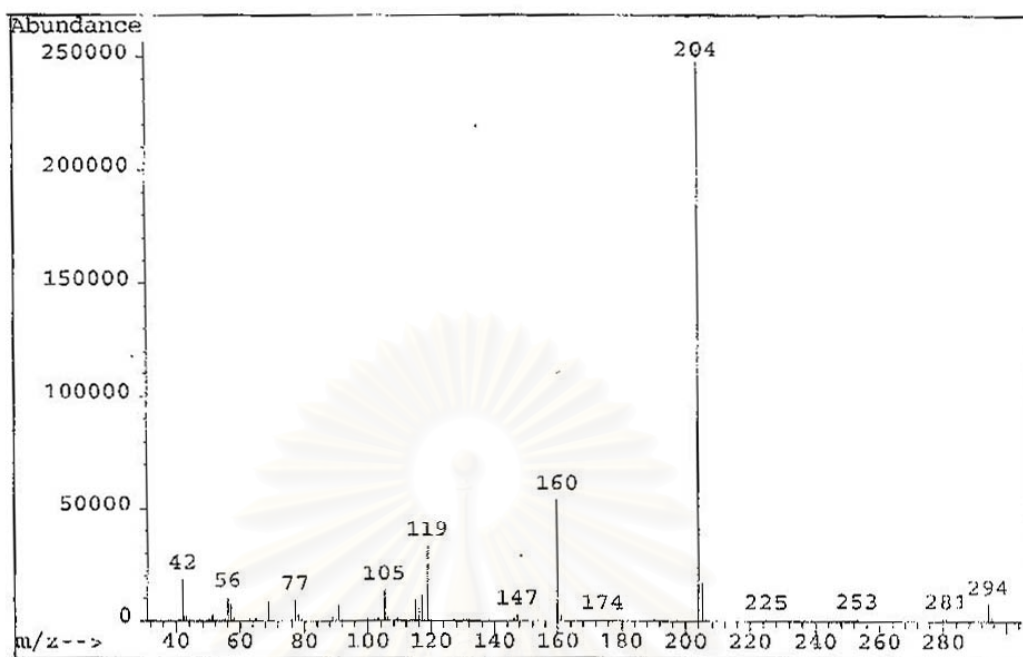
Figure 17 Mass spectrum of methamphetamine-PFPA.
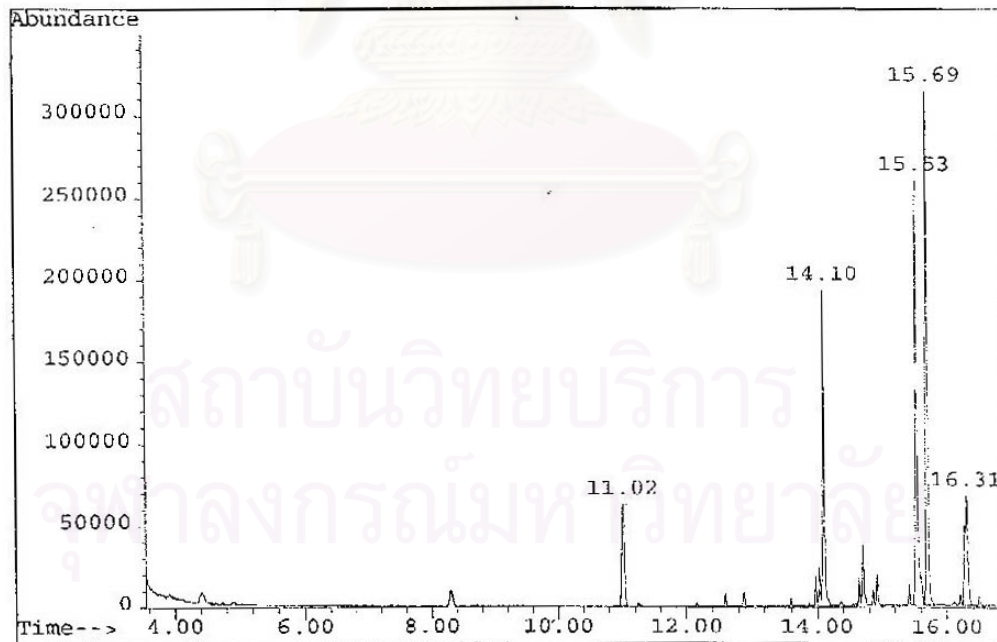
Figure 18 Mass spectrum of ephedrine-PFPA.

2.2 Selection of suitable internal standard

Quantitative analysis by GC/MS requires an internal standard to be added to the sample prior to extraction. An internal standard also permits the measurement of relative retention times. Internal standard should resemble the target analytes such that they can be extracted, derivatised and analysed under the same conditions as the target analytes, but be readily distinguished from them during the chromatographic procedure.

With an internal standard, GC/MS assay reliability benefits through correction of losses which may occur during extraction, purification and derivatisation.

For quantitative analysis of amphetamines in urine performed by GC/MS, the preferred internal standards are deuterium-labeled analogue of amphetamine and methamphetamine because of closeness in properties to the analyte.

However, these deuterium-labeled analogues are expensive and may not readily available. In this study, two amphetamine analogues: phentermine and chlorpseudoephedrine were investigated.

Figure 19, 20 and 21 show the TIC of full-scan of derivatised samples of blank urine, spiked urine with AP, MA, EP and chlorpseudoephedrine, and spiked urine with those three drugs and phentermine, respectively. Both chlorpseudoephedrine-PFPA and phentermine-PFPA were completely resolved from AP, MA, EP and other endogenous substances in urine. However, the retention time of chlorpseudoephedrine-PFPA, 12.79 min, was quite long comparing to the retention time of 7.10 min of phentermine-PFPA. Therefore, in order to save analysis time, phentermine-PFPA was chosen as the internal standard for subsequent experiment.

The mass spectrum of phentermine-PFPA is shown in Figure 22 with m/z values of 59, 91, 132, 164 and the base peak of 204 amu.
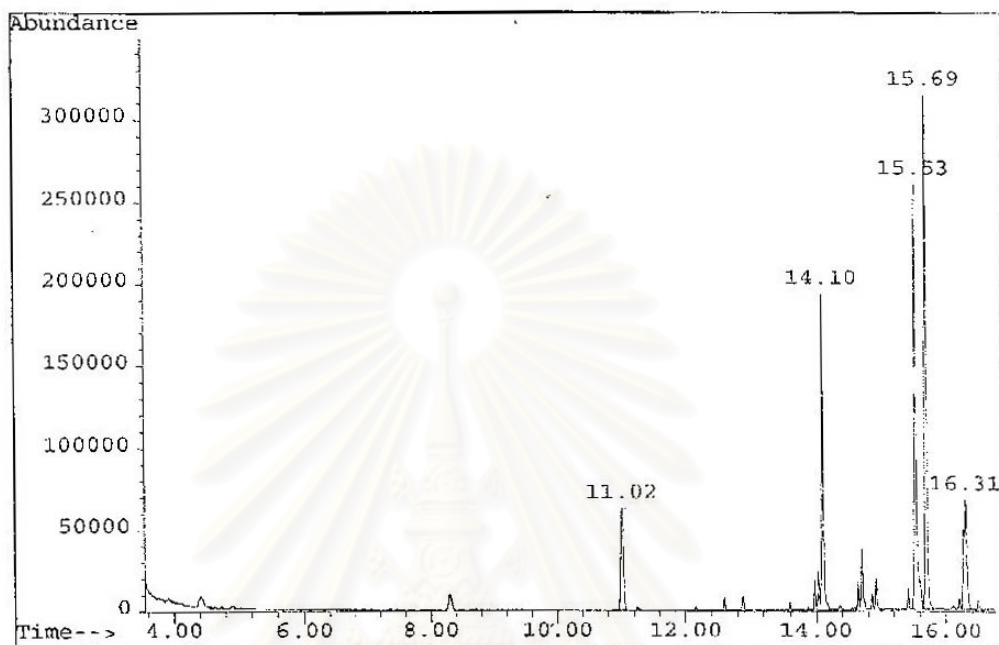
Figure 19 TIC of full-scan of blank urine sample after derivatising with PFPA
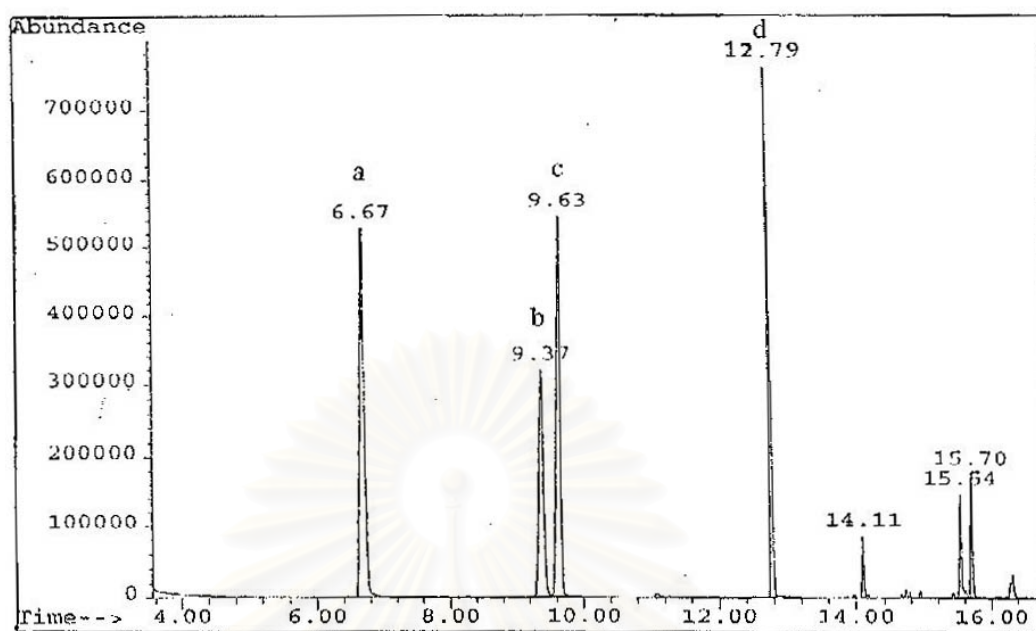
Figure 20 TIC of full-scan of blank urine spiked with amphetamine,

methamphetamine, ephedrine and chlorpseudoephedrine 1000 ng/ml each,

after derivatising with PFPA. a: AP-PFPA, b: MA-PFPA, c: EP-PFPA, and

d: CP-PFPA.

Figure 21 TIC of full-scan of blank urine spiked with 1000 ng/ml each of
amphetamine, methamphetamine, ephedrine and phentermine, after
derivatising with PFPA. a: AP-PFPA, b: PT-PFPA, c: MA-PFPA, and
d: EP-PFPA.
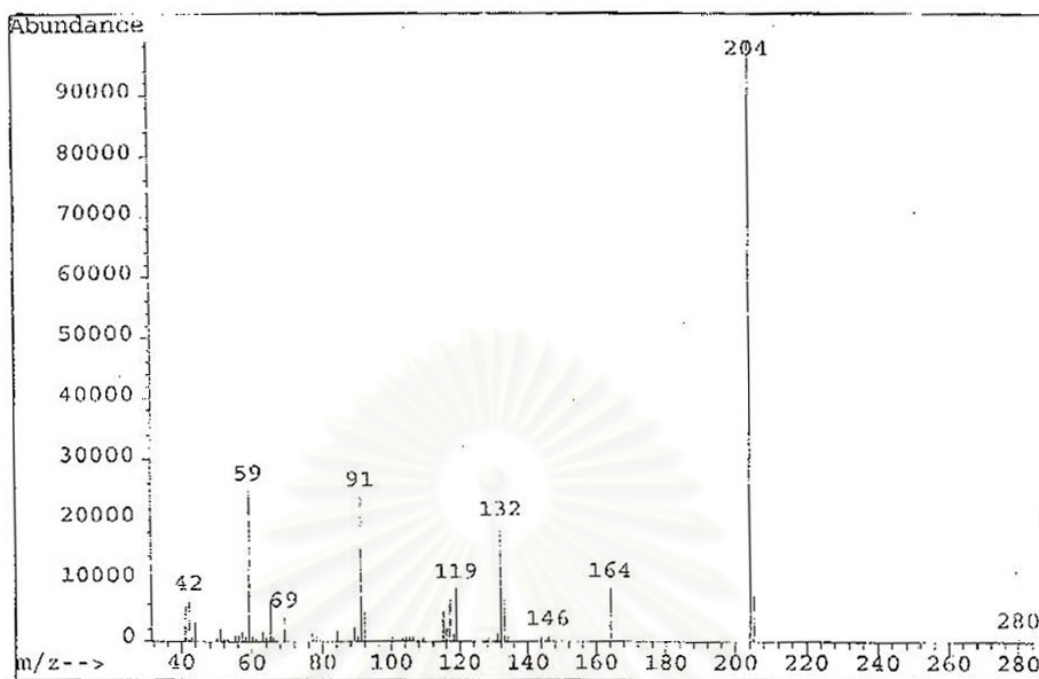
Figure 22 Mass spectrum of phentermine-PFPA.

2.3 System suitability

System suitability tests are an integral part of gas-liquid chromtographic methods. They are used to verify that the resolution and reproducibity of chromatographic system are adequate for the analysis to be done. The tests are based on concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such [55].

1. % RSD of the peak area ratio of six replicate injections of the control solution must not exceed 2.0 % for MA-PFPA.

2. The tailing factor for MA-PFPA must not exceed 2.0.

3. There must be not less than 80,000 theoretical plates for MA-PFPA.

4. The resolution between MA-PFPA and EP-PFPA must not less than 2.0.

2.4 Quantification

In order to increase sensitivity of the MS detector, the selected ion monitoring (SIM) mode was selected for quantitative analysis of AP, MA, and EP in urine samples, using PT as the internal standard.

SIM allows the MS to detect specific compounds with very high sensitivity. In SIM mode, the instrument is set to acquire data at masses of interest instead of stepping the mass filter over a wide range of masses, Because the MS collects data at only the masses of interest, it responds only to those compounds possessing those masses in the mass spectrum. In essence, the instrument is focused only the compounds of interest. Also, because only few masses are monitored, much more time may be spent looking at these masses, with the attendant increase in sensitivity, accuracy, and precision.

In SIM mode for this study, quantitation ions (target ions) along with qualifier ions (confirmimg ions) for PFPA derivatives of AP, MA, EP and PT are listed in Table 3.

Table 3 m/z values of quantitation and qualifier ions of amphetamines-PFPA in the SIM mode

	AP-PFPA	MA-PFPA	EP-PFPA	PT-PFPA
Quantitation ion	190	204	204	204
Ion qualifier # 1	91	91	119	91
Ion qualifier # 2	118	118	160	132

Quantitation ion (typically the base peak) is the mass whose response is used in calculations to determine amount detected while qualifier ion is the mass whose response is compared to that of quantitation ion for verification of compound identity. Figure 23 shows the TIC of PFPA derivatives of AP, MA, EP and PT, running in SIM mode. Retention times of AP-TBPE, MA-TBPE and EP-TBPE were 6.66, 7.09, 9.36 and 9.61 min, respectively. As expected, the background signal obtained from SIM mode was quite low with less interference from endogenous substance in urine. Therefore, data acquisition in SIM mode was performed for quantitative analysis of amphetamines in urine in this study.
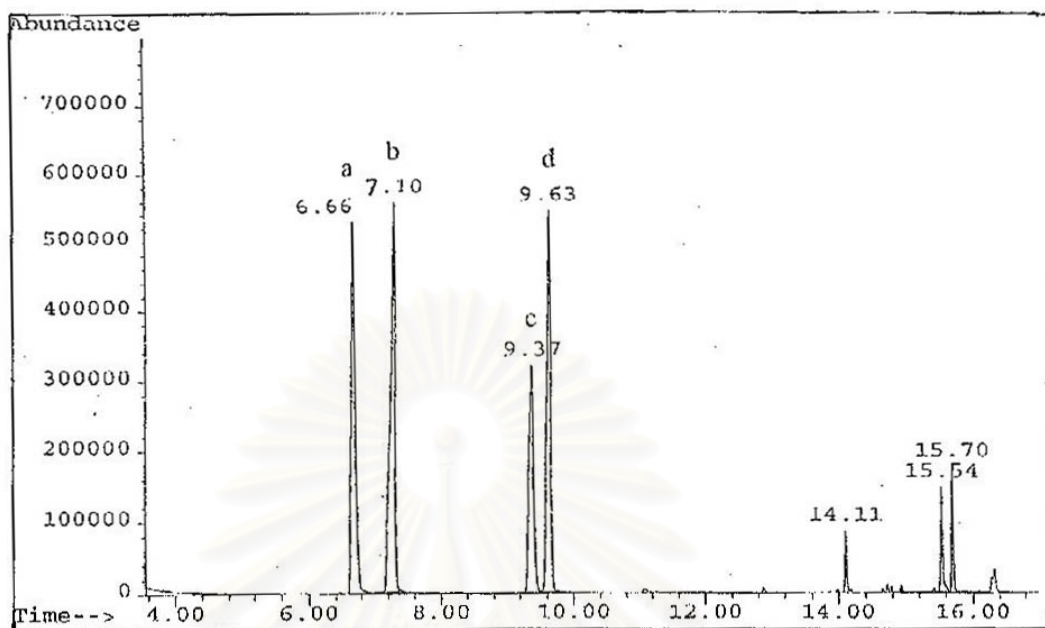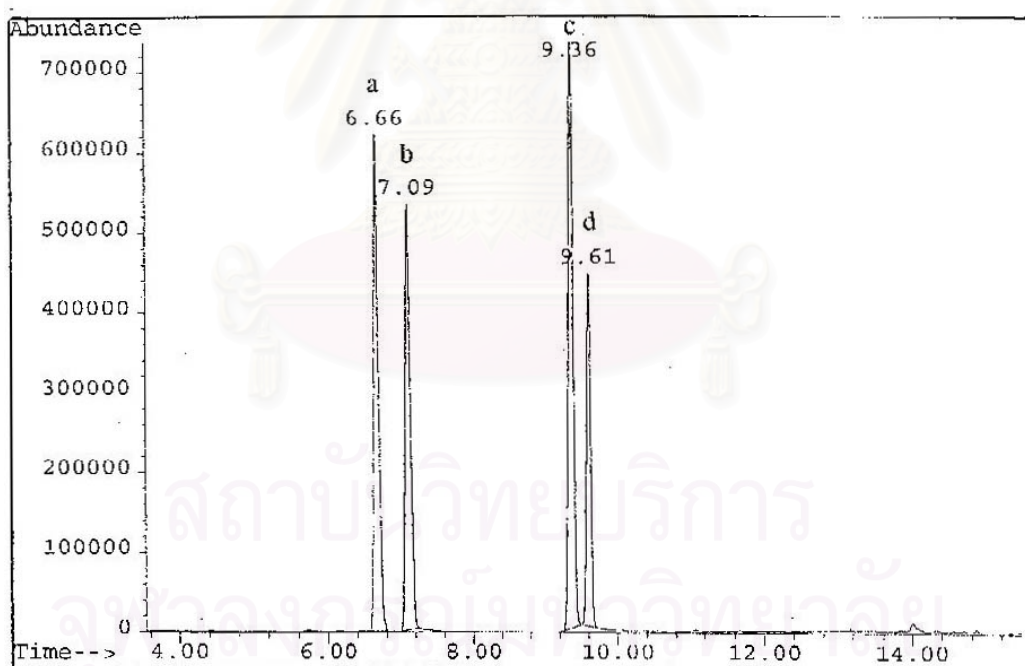

Figure 23 TIC of SIM scan of blank urine sample spiked with 1000 ng/ml each of amphetamine, methamphetamine, ephedrine and phentermine, after derivatising with PFPA. a: AP-PFPA, b: PT-PFPA, c: MA-PFPA, and d: EP-PFPA.

3. Method Validation

Analytical method validation includes all of procedures recommended to demonstrate that a particular method for the quantitative measurement of an analyte in urine is reliable and reproducible. The parameters essential to the validation include selectivity, linearlity, within-run and between-run precision and accuracy, limit of detection (LOD), limit of quantitation (LOQ) and the stability of the analyte in urine, as follows:

3.1 Selectivity

Selectivity is the ability of an analytical method to differentiate and quantitate the analyte in the presence of other constituents in the sample. Blank urine samples from six different human sources were evaluated to determine the presence of any interferences across the retention windows of PFPA derivatives of AP, MA, EP and PT. A representative TIC of PFPA derivatised blank urine sample, running in SIM mode, is shown in Figure 24. Comparison of Figure 23 with Figure 24, it was clear that responses of interferences in urine sample were outside the retention windows of AP-PFPA (6.66 min), PT-PFPA (7.09 min), MA-PFPA (9.36 min) and EP-PFPA (9.61 min). The results obtained were strongly supported that the method was selective for PFPA derivatives of AP, MA, EP and PT.
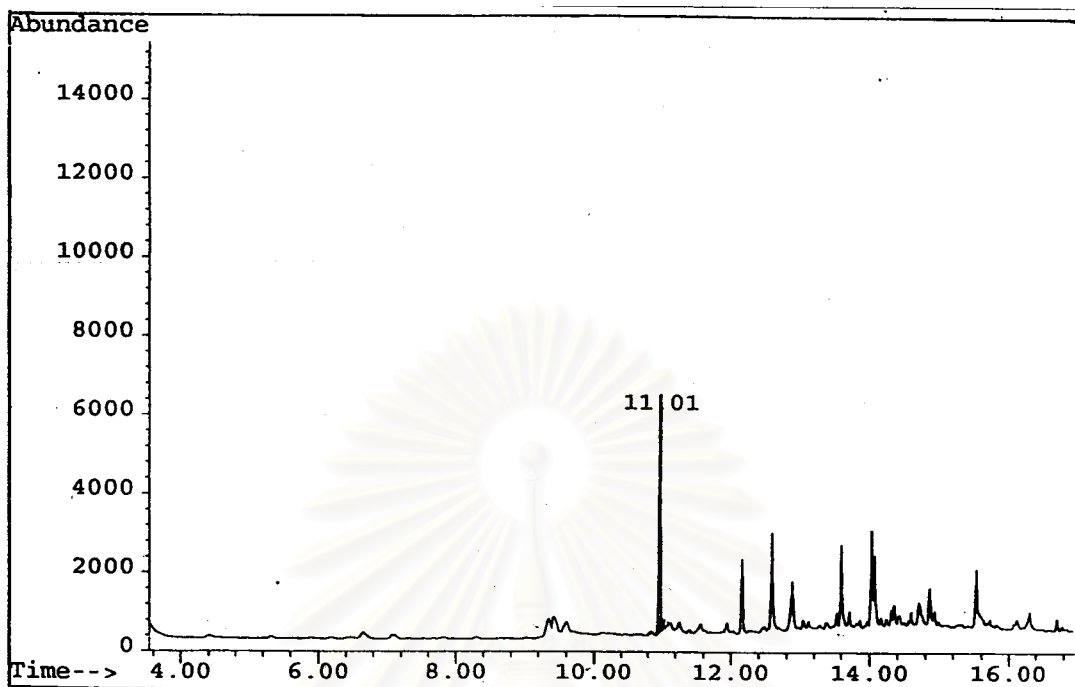
Figure 24 TIC of SIM scan of a blank urine sample after derivatising with PFPA.

3.2 Linearity

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Linearity can be expressed as a calibration curve which is the relationship between instrument response and known concentration of the analyte. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking with known concentrations of the analyte.

Figure 25 and 26 illustrate repectively the calibration curves of the PFPA derivatives of AP and MA over a linear dynamic range of 50-2500 ng/ml. The

calibration curve of EP-PFPA over a linear dynamic range of 100-2500 ng/ml is illustrated in Figure 27. The calibration curves have correlation coefficients of 0.9989, 0.9995 and 0.9980 for AP, MA and EP, repectively.
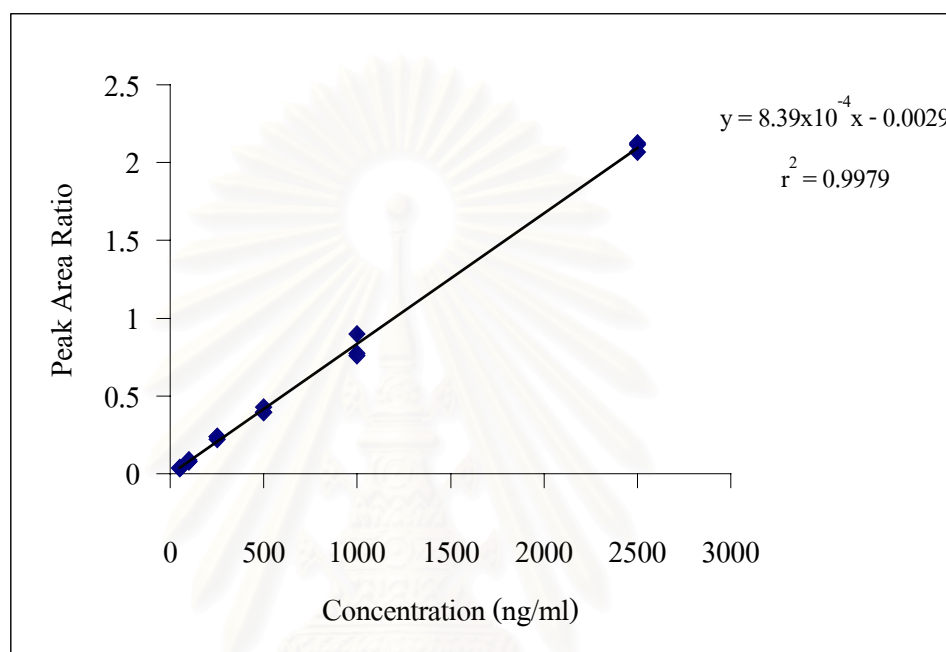
Figure 25   Calibration curve of amphetamine-PFPA.
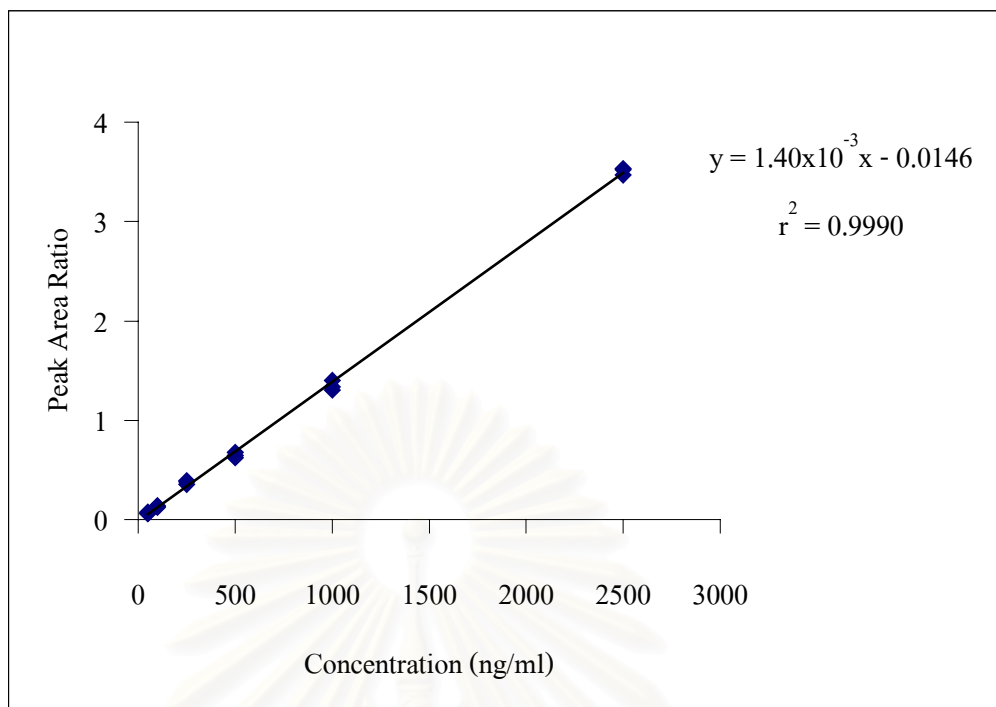
Figure 26 Calibration curve of methamphetamine-PFPA.
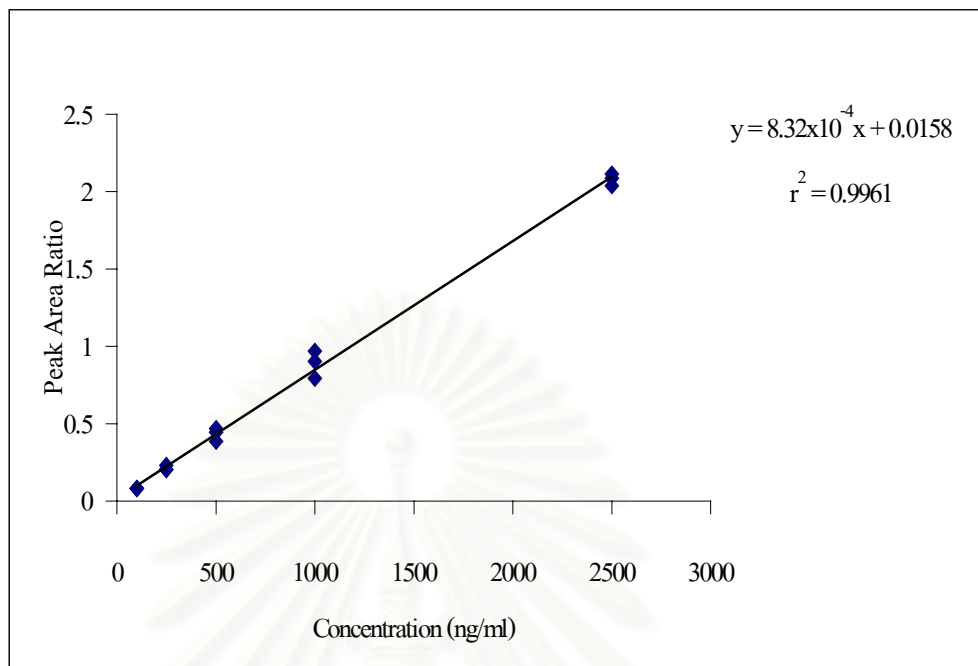
Figure 27 Calibration curve of ephedrine-PFPA.

3.3 Limit of Detection (LOD) and Limit of quantitation (LOQ)

Limit of detection (LOD) is the lowest concentration of an analyte in a sample that can be detected, but not necessary quantitated under the stated conditions of the test.

Limit of quantitation (LOQ) is the lowest concentration of an analyte that can be determined with acceptable precision and accuracy under the stated conditions of the test. For this study, the acceptable precision was 20% and % deviation of mean (accuracy) was 20% [56]. Figure 28, 29 and 30 illustrate the linear relationship between peak area ratio and concentration for PFPA derivative of AP, MA and EP, respectively. Slopes (S) and residual standard deviations of regression lines (σ) of

these PFPA derivatives were shown in Table 4. LOD and LOQ of each analyte were calculated and shown in Table 4 as well.

Table 4 LOD and LOQ of PFPA derivatives of amphetamine, methamphetamine and
ephedrine in spiked urine

	Amphetamine	Methamphetamine	Ephedrine
σ	0.004302	0.006137	0.006225
S	8.63×10^{-4}	1.31×10^{-3}	9.25×10^{-4}
LOD (ng/ml)	16.45	15.46	22.21
LOQ (ng/ml)	49.85	46.85	67.30

The response of each analyte at LOQ should be identifiable, discrete, and reproducible with a precision of 20 %. In order to comply with the criteria, the LOQ for AP, MA and EP were set at 50, 50 and 100 ng/ml, respectively.
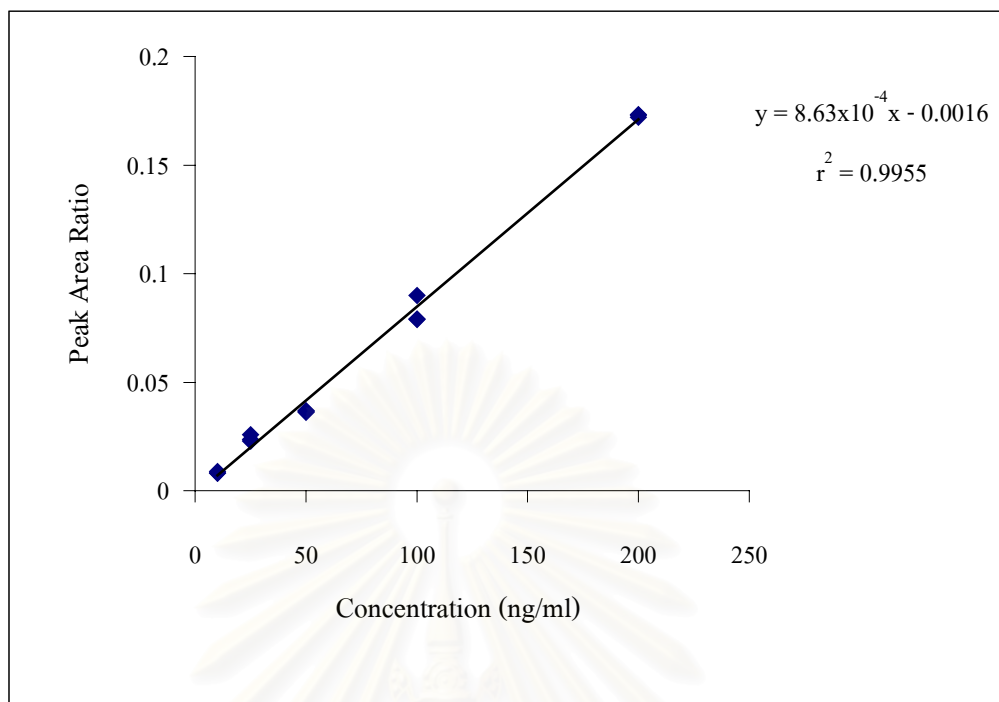
Figure 28 Peak area ratio VS concentration of amphetamine-PFPA.
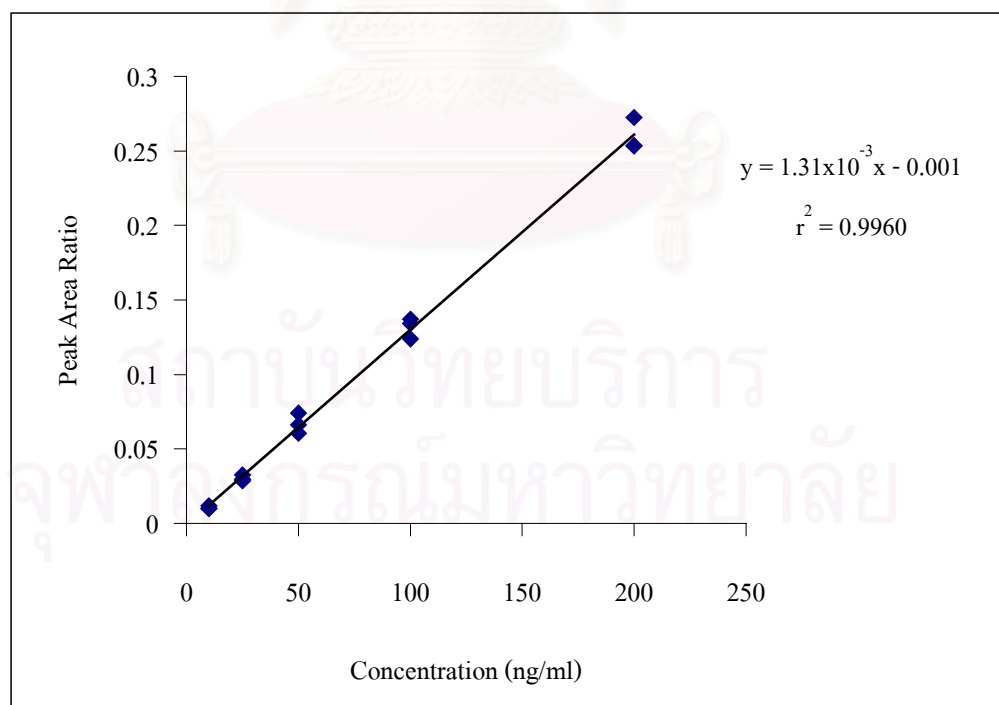
Figure 29 Peak area ratio VS concentration of methamphetamine-PFPA.

$$y = 9.25 \times 10^{-4} x - 0.0021$$

$$r^2 = 0.9917$$
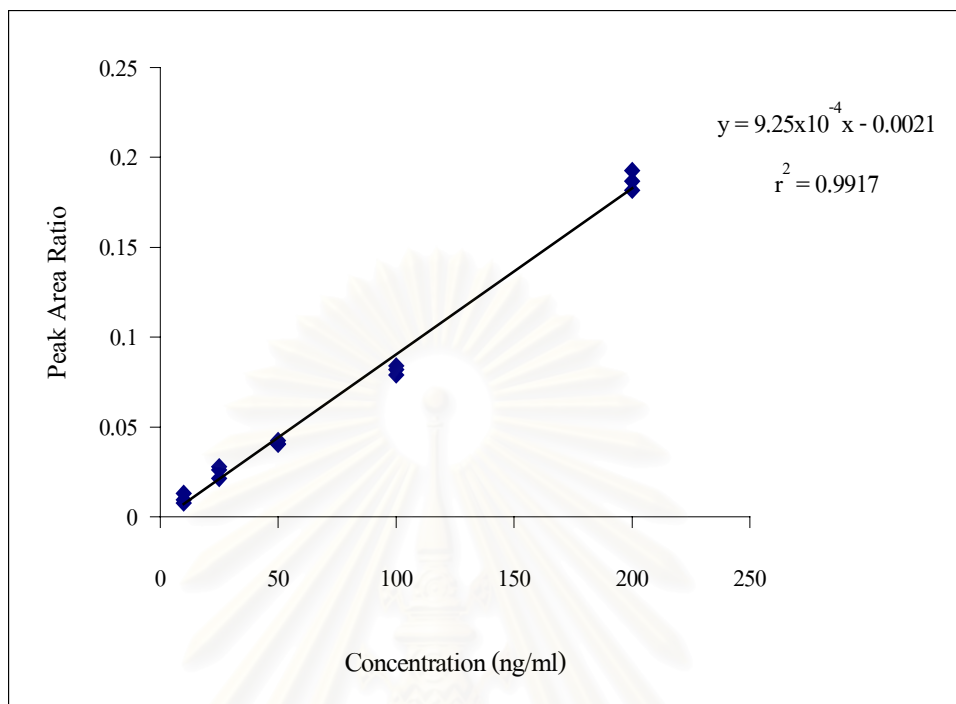
Figure 30 Peak area ratio VS concentration of ephedrine-PFPA.

3.4 Precision

The precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of urine. The precision of an analytical method is usually expressed as the relative standard deviation (RSD) or coefficient of variation (CV). Precision is further subdivided into within-run or repeatability and between-run or intermediate precision. The within-run assesses precision during a single analytical run using the same analyst with the same equipment.

The between-run measures precision with time, as on different days, and may involve different analysts, equipments, reagents and laboratories.

The within-run and between-run precision were determined by analysing five replicates of urine samples spiked with AP and MA at 50, 100, 1000 and 2000 ng/ml; EP at 100, 250, 1000 and 2000 ng/ml; and PT, the internal standard, at 1000 ng/ml. The LOQ at 50 ng/ml for AP and MA and 100 ng/ml for EP were included in the study. As shown in Table 5-7, %RSD of within-run and between-run for AP, MA and EP were less than 15 % for all four concentrations.

Therefore, the precision of the proposed method was acceptable according to the criteria that the %RSD obtained at each concentration level should not exceed 15 % except for the LOQ, where it should not exceed 20 % [56].

Table 5 Within-run and between-run precision and accuracy of amphetamine in
        spiked urine samples.

Nominal concentration (ng/ml)	Amphetamine			
	% RSD		% Deviation of mean (accuracy)	
	Within-run	Between-run	Within-run	Between-run
50	4.73	6.67	1.96	4.20
100	5.70	8.14	5.03	4.69
1000	3.70	8.67	1.76	2.62
2000	3.21	5.17	4.35	2.60

Table 6 Within-run and between-run precision and accuracy of methamphetamine
in spiked urine samples.

Nominal concentration (ng/ml)	Methamphetamine			
	% RSD		% Deviation of mean (accuracy)	
	Within-run	Between-run	Within-run	Between-run
50	7.16	6.32	1.98	3.74
100	3.45	7.57	1.29	4.24
1000	3.68	6.63	1.94	3.94
2000	5.28	7.48	1.78	3.11

Table 7 Within-run and between-run precision and accuracy of ephedrine in spiked
urine samples.

Nominal concentration (ng/ml)	Ephedrine			
	% RSD		% Deviation of mean (accuracy)	
	Within-run	Between-run	Within-run	Between-run
100	6.69	5.73	4.37	1.49
250	8.64	9.99	1.05	1.01
1000	2.67	9.68	1.73	1.66
2000	5.55	4.71	1.24	1.75

3.5 Accuracy

The accuracy of an analytical method is the closeness of mean test results obtained by the method to the true value of the analyte. The mean value should be within 15 % of the actual value except at LOQ, where it should not deviate by more than 20 % [56].

The accuracy of AP, MA and EP were listed in Table 5, 6 and 7, respectively. For within-run and between-run, The accuracy or % deviation of the mean from the true value at LOQ for AP (50 ng/ml), MA (50 ng/ml) and EP (100 ng/ml) were all less than 20 %. At other concentration levels of the three drugs investigated, the accuracy were less than 15% for both within-run and between-run. Therefore, the accuracy of the proposed method was within the acceptance criteria.

3.6 Extraction effienciency (Recovery)

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from urine, compared to the detector response obtained for the true concentration of the pure standard. Recovery relates to the extraction efficiency of an analytical method within the limits of variability. The extraction efficiencies or recoveries of AP, MA and EP were determined by analyzing replicates of urine samples (n = 5) spiked with AP, MA and EP at 100, 1000 and 2000 ng/ml and the internal standard at 1000 ng/ml. As shown in Table 8, the ranges of recoveries of AP, MA and EP were 95.29-97.31 %, 96.08-98.33 % and 68.26-71.50 %, respectively. Extraction efficiencies of ephedrine were much lower than those of AP and MA at all concentrations studied while those of AP and MA were comparable. This might be due to the ionization of EP (pKa 9.6) was lower than those of AP (pKa 9.9) and MA (pKa 10.1) at optimum pH range 9.2-9.5 and resulted in lower amount of

ephedrine cation available for reacting with TBPE. Furthermore, the hydroxyl group on beta-carbon of EP might influence the reaction with TBPE by steric effect.

Table 8 Extraction efficiency of amphetamine, methamphetamine and ephedrine in spiked urine (n=5).

Amphetamine		Methamphetamine		Ephedrine	
Nominal concentration	% recovery	Nominal concentration	% recovery	Nominal concentration	% recovery
100	96.24	100	96.60	250	68.34
1000	97.31	1000	98.33	1000	71.50
2000	95.29	2000	96.08	2000	68.26

3.7 Stability of the analyte

Drug stability in urine is a function of the storage conditions, the chemical properties of the drug, the matrix and the container system. Stability procedures should evaluate the stability of the analytes in urine after long-term and short-term storage, and after going through the freeze and thaw cycles. The procedure should also include an evaluation of analyte stability in stock solution.

3.7.1 Freeze and Thaw Stability

The stability of AP, MA and EP in urine after three freeze-thaw cycles was evaluated. As shown in Table 9, the concentrations of AP, MA and EP were obtained within 92.43 % -103.86 % of the concentration at the zero cycle. These three drugs

were therefore considered to be stable in urine during three freeze-thaw cycles ($p > 0.05$).

Table 9 Concentration of amphetamine, methamphetamine and ephedrine in urine

after going through freeze and thaw cycles. (n=3)

Freeze-Thaw cycle	Target concentration (ng/ml)[a]					
	Amphetamine		Methamphetamine		Ephedrine	
	100	2000	100	2000	250	2000
0	102.29	2023.83	97.26	1944.31	249.96	2074.96
1	95.08 (92.95)	2075.81 (102.57)	93.18 (95.81)	1883.56 (96.88)	231.05 (92.43)	1948.43 (93.90)
2	97.09 (94.92)	2086.92 (103.12)	94.81 (97.84)	1851.43 (95.22)	233.25 (93.31)	1970.99 (94.99)
3	97.80 (95.61)	2102.02 (103.86)	98.99 (101.78)	1880.71 (96.73)	245.96 (98.40)	2049.70 (98.78)

[a] Value in the parenthesis represented the percentage of the analyte at each cycle comparing to that at zero cycle.

3.7.2 Short-term stability

The stability of AP, MA and EP in urine after three aliquots were thawed at room temperature and kept at room temperature for 4 and 7 hours. The concentration of AP, MA and EP in urine (90.00 % - 103.87 % of the zero hour) did not change significantly ($p > 0.05$), as presented in Table 10.

Table 10 Stability of amphetamine, methamphetamine and ephedrine in urine stored at
room temperature

Hours at room temperature	Target concentration (ng/ml)[a]					
	Amphetamine		Methamphetamine		Ephedrine	
	100	2000	100	2000	250	2000
0	97.80	2102.02	98.99	1880.71	245.96	2049.70
4	91.89 (93.96)	2126.92 (101.85)	89.10 (90.00)	1932.55 (102.76)	236.44 (96.13)	1941.75 (94.73)
7	100.57 (102.83)	2001.20 (95.20)	102.82 (103.87)	1944.16 (103.37)	232.60 (94.57)	2005.57 (97.85)

[a] Value in the parenthesis represented the percentage of the analyte at the specified
time comparing to that of freshly prepared

3.7.3 Long-term stability

Four aliquots of spiked urine samples at each of the low and high
concentrations of AP, MA, and EP were used for this study. The long-term stability of
AP, MA, and EP was evaluated by analyzing a freshly prepared aliquot and aliquots
stored at –20 $^\circ$C for every 1 month cycle, up to 3 months. Concentrations of AP, MA,
and EP were in the range of 91.91 – 104.98 % of the concentration of the freshly
prepared. The concentration did not change significantly ($p > 0.05$), as shown in Table
11.

Table11 Long-term stability of amphetamine, methamphetamine and ephedrine in
        urine stored at –20 °C

Months at storage	Target concentration (ng/ml)[a]					
	Amphetamine		Methamphetamine		Ephedrine	
	100	2000	100	2000	250	2000
0	102.29	2023.83	97.26	1944.31	249.96	2074.96
1	103.17 (100.86)	1860.14 (91.91)	94.65 (97.32)	1843.74 (94.83)	240.46 (96.20)	2176.99 (104.92)
2	106.86 (104.47)	1976.68 (97.67)	92.25 (94.85)	1933.81 (99.46)	261.75 (104.72)	2178.39 (104.98)
3	103.47 (101.15)	2058.96 (101.74)	94.68 (97.35)	1875.09 (96.44)	258.12 (103.26)	2138.71 (103.07)

[a] Value in the parenthesis represented the persentage of the analyte at the specified time
comparing to that of freshly prepared

3.7.4 Stock solution stability

The stability of AP, MA, and EP in methanolic stock solutions was evaluated.
Means peak area ratio (n=3) of AP, MA, and EP obtained from freshly prepared
solutions were compared with those obtained from solutions that had been stored at
4 °C for 3 months. As shown in Table 12, after 3 months storage at 4 °C, means peak
area ratio were 98.67 for AP, 102.46 for MA and 103.66 for EP, with respect to those
of freshly prepared solutions. The results indicated that methanolic stock solutions of
these drugs were stable for up to 3 months of storage at 4 °C.

Table 12 Stock solution stability of amphetamine, methamphetamine and ephedrine in
methanol stored at 4 $^\circ$C

Sample No.	Peak Area Ratio [a]					
	Amphetamine		Methamphetamine		Ephedrine	
	Freshly prepared	3 months	Freshly prepared	3 months	Freshly prepared	3 months
1	0.8112	0.7742	1.2709	1.311	1.4165	1.5587
2	0.8937	0.9603	1.2004	1.1059	1.4373	1.2280
3	0.8584	0.7945	1.1920	1.3364	1.2020	1.4175
Mean	0.8544	0.8430	1.2211	1.2511	1.3519	1.4014
% relative to freshly prepared	-	98.67	-	102.46	-	103.66

[a] Calculated from the ratio of peak area of the analyte to peak area of phentermine

3.7.5 Autosampler Stability

The stability of processed samples in the autosampler was studied at room temperature for 6 and 12 hours. The mean concentrations of the low and high spiked urine samples of each drug were within 15 % of their target concentrations and within 97.79 % - 100.99 % of the zero hour (Table13, 14). These results indicated that AP, MA and EP were stable in the processed urine for at least 12 hours at room temperature ($p > 0.05$).

Table 13 Autosampler stability of the low spiked urine sample of amphetamine,

methamphetamine and ephedrine in urine at room temperature

Hours in autosampler	Target concentration (ng/ml)[a]					
	Amphetamine		Methamphetamine		Ephedrine	
	100	% recovery	100	% recovery	250	% recovery
0	92.96	92.96	92.38	92.38	235.88	94.35
6	90.96 (97.85)	90.96	92.57 (100.21)	92.57	230.67 (97.79)	92.27
12	92.42 (99.42)	92.42	92.62 (100.26)	92.62	229.98 (97.50)	91.99

[a] Value in the parenthesis represented the percentage of the analyte at the specified time comparing to that of freshly prepared sample.

Table 14 Autosampler stability of the high spiked urine sample of amphetamine,

methamphetamine and ephedrine in urine at room temperature

Hours in autosampler	Target concentration (ng/ml)[a]					
	Amphetamine		Methamphetamine		Ephedrine	
	2000	% recovery	2000	% recovery	2000	% recovery
0	2096.17	104.81	2040.86	102.04	1968.79	98.44
6	2117 (100.99)	105.85	2007.14 (98.35)	100.36	1930.25 (98.04)	96.51
12	2116.63 (100.98)	105.83	2031.50 (99.54)	101.58	1941.04 (98.59)	97.05

[a] Value in the parenthesis represented the percentage of the analyte at the specified time comparing to that of freshly prepared sample.

4. Assay application

The developed assay method was successfully applied to ten urine samples of truck drivers who were suspected of taking illicit methamphetamine tablets. These urine samples were kindly supplied by the Division of Narcotics, Department of Medical Sciences. The results of confirmatory test were summarized in Table 15. A representative TIC of a urine sample was shown in Figure 31. Seven samples were found to have MA greater than 500 ng/ml but only 4 samples out of these 7 samples were found to have AP higher than 200 ng/ml. Therefore only these 4 urine samples (#1, 3, 7 and 9) were positive on MA confirmatory test, according to the guidelines [9].

For sample # 10, only EP and the unknown peak at 11.10 minute were found as shown in Figure 32. EP found was very concentrate (5582.4 ng/ml). Not surprisingly,

sample # 10 was positive on screening test. After comparison of the unknown peak with the peak of pseudoephedrine-PFPA (Figure 33) with respect to the retention time and mass spectrum, the unknown peak was finally confirmed to be pseudoephedrine-PFPA.

Table 15 Analysis of amphetamine, methamphetamine and ephedrine in urine samples

Urine sample #	Concentration found (ng/ml)[a]		
	Amphetamine	Methamphetamine	Ephedrine
1	508.9	1455.5	ND
2	ND	423.8	ND
3	1455.4	2373.7	ND
4	67.9	394.7	ND
5	121.2	1159.1	ND
6	100.8	850.1	ND
7	608.2	2342.9	ND
8	170.4	1391.0	ND
9	432.5	1738.7	ND
10	ND	ND	5582.4

[a] ND was not detected
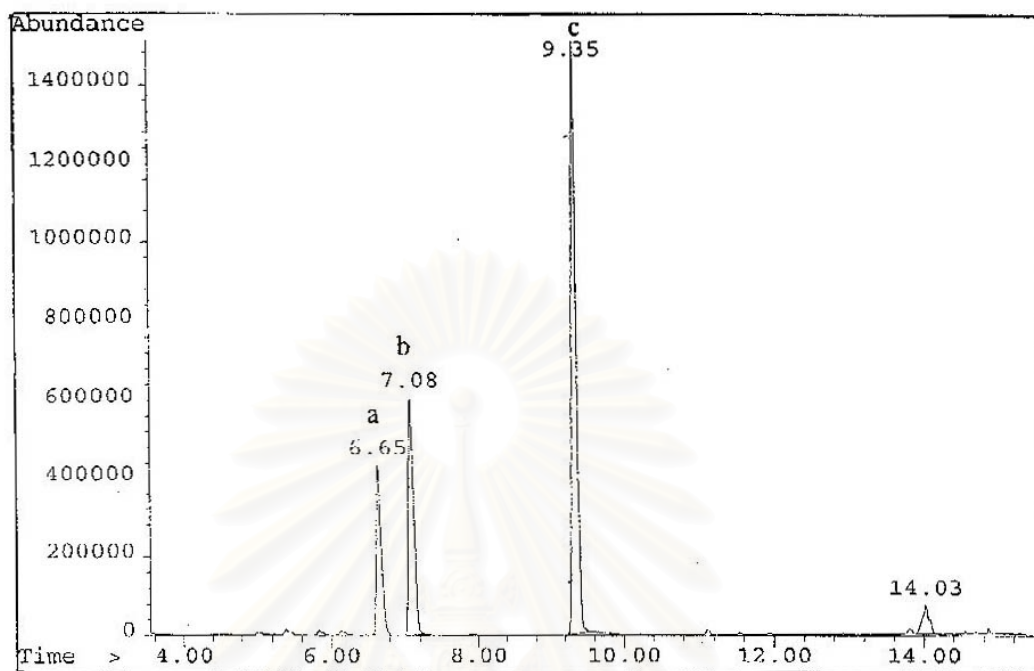
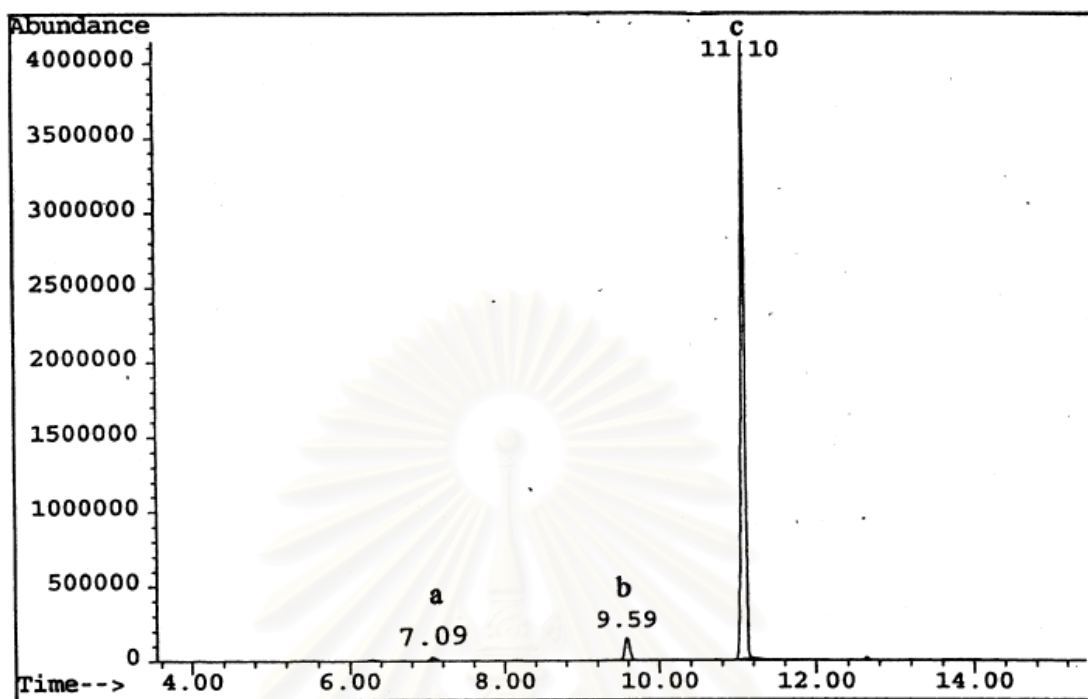Figure 31 TIC of the comfirmatory test positive urine sample. a: amphetamine-PFPA,

b: phentermine-PFPA and c: methamphetamine-PFPA.

Figure 32 TIC of the urine sample #10. a: phentermine-PFPA, b: ephedrine-PFPA,
and c: pseudoephedrine-PFPA.
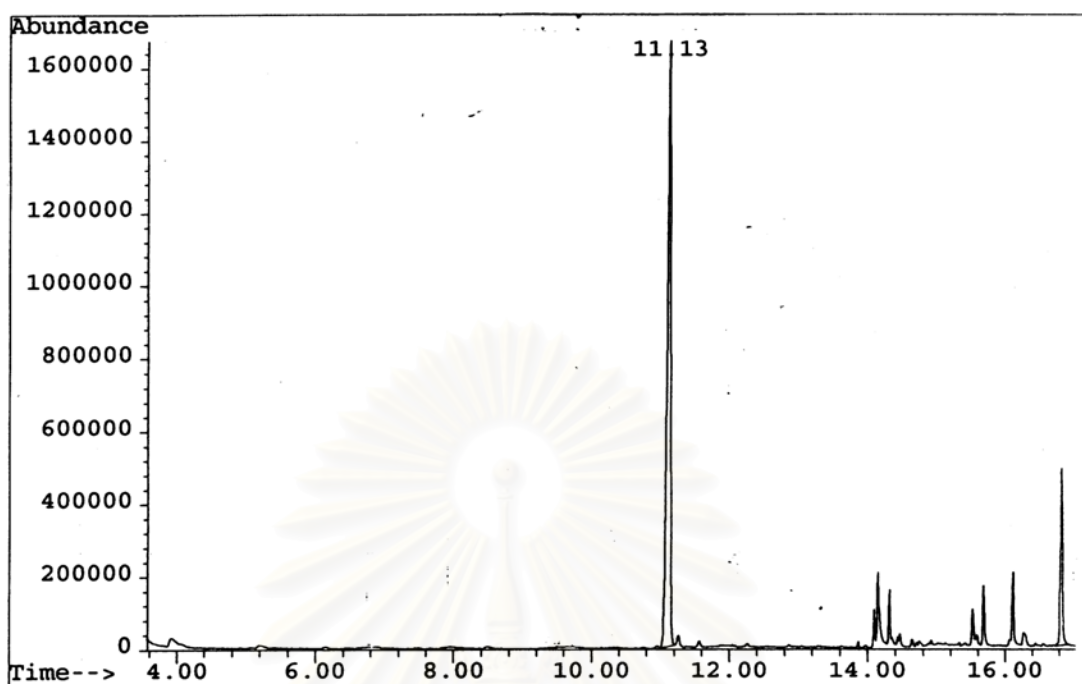
Figure 33 TIC of full scan of blank urine spiked with pseudoephedrine after

derivatising with PFPA.

CHAPTER IV

CONCLUSION

An analysis method which is rapid, sensitive and reproducible to determine urine levels at 50 ng/ml of AP and MA and 100 ng/ml of EP has been developed. This method involved ion-pair extraction using TBPE to obtain recoveries (n=5) of 95 %, 96 % and 68 % for AP, MA and EP, respectively and followed by GC/MS. The method utilities phentermine as the internal standard. In this study, AP, MA, EP, and the internal standard were recovered from 3 ml of human urine. The samples were then derivatised using pentafluoropropionic anhydride (PFPA) followed by analysis by GC/MS with selected ion monitoring (SIM) for the ion m/z 190 of PFPA-amphetamine, m/z 204 of PFPA-methamphetamine and m/z 204 of PFPA-ephedrine. The correlation coefficients were 0.9989, 0.9995, and 0.9980 with concentration ranges of 50-2500, 50-2500, and 100-2500 ng/ml in urine for AP, MA and EP, respectively. The method has also been validated at 50, 100, 1000 and 2000 ng/ml of AP and MA, and 100, 250, 1000 and 2000 ng/ml of EP in urine. The within-run and between-run relative standard deviation (%RSD) were not higher than 10 % for all concentrations of each drug. Eventually, it was successfully applied to urine samples taken from the suspects.

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