1. INTRODUCTION

Drug abuse is an increasing problem worldwide. It has been recognized in prisons, hospitals, schools, and among the working population[1]. Amphetamine(AMP) and methamphetamine (MAMP), commonly known as "Yaba" in Thai, are significant drugs of abuse in Thailand. Illicit methamphetamine tablets from various areas over Thailand have been studied for their constituents. The varieties of illicit samples have been found to contain the following: only methamphetamine; methamphetamine mixed with caffeine; methamphetamine mixed with ephedrine and caffeine; ephedrine and caffeine; only caffeine; and only amphetamine [2]. Illicit use of these drugs is typically detected by identification of the nonmetabolized compounds in urine. Recent survey of "Yaba" abuse in schools from 69 provinces based on analysis of urine samples revealed that 1.16% students over Thailand consumed Yaba. Methamphetamine and ephedrine are two main groups of "Yaba" in Thailand [3]. The abuse of Yaba is thus a serious drug problem.

1.1 Amphetamine

Amphetamine was initially synthesized in 1887, although its impact on human biology was not investigated for nearly half a century. It was introduced into clinical medicine in the 1930s, to be followed by related compounds. The first clinical use of amphetamine was its incorporation into a benzedrine inhaler marketed for nasal congestion in 1932. Amphetamine tablets, promoted for the treatment of narcolepsy, followed by 1935, and by 1936 reports of self-administered inappropriate stimulant use occurred. The first marketing as an anorexiant followed in 1938 [4]. Amphetamine abuse case was first reported in Thailand in 1957 [5]. In 1979 the FDA proposed to withdraw its approval for use of amphetamines for weight reduction. The State of New York banned amphetamine for weight control in 1981 [6].

Amphetamine, racemic β-phenylisopropylamine, has powerful central nervous system(CNS) stimulant action in addition to the peripheral α and β actions common to

indirectly acting sympathomimetic drugs. It is effective after oral administration and its effects last for several hours [7]. The structural formula of amphetamine is figure1.1. Physical properties of amphetamine are listed in Table 1.1 [8].

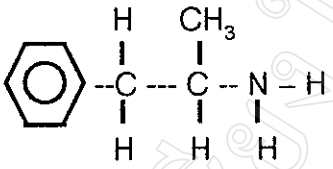

Figure 1.1 Amphetamine structure

Table 1.1 Physical properties of amphetamine [8]

Amphetamine	
Chemical name	(±)-α-Methylphenethylamine
Molecular formula	$C_9H_{13}N$
Molecular weight	135.2
Appearance	Colorless
Boiling point	200° to 203° C
Soluble	1 in 50 of water; very soluble in ethanol, chloroform, and ether; freely soluble in acids
Amphetamine Sulphate	
Molecular formula	$(C_9H_{13}N)_2H_2SO_4$
Molecular weight	368.5
Appearance	White crystalline powder
Melting point	About 300° C
Soluble	1 in 9 of water and 1 in 515 of ethanol; practically insoluble in chloroform and ether

Cardiovascular Responses

Amphetamine given orally raises both systolic and diastolic blood pressures. Heart rate is often reflexly slowed; with large doses, cardiac arrhythmias may occur. Cardiac output is not enhanced by therapeutic doses, and cerebral blood flow is little changed. The *l* isomer is slightly more potent than the *d* isomer in its cardiovascular actions.

Central Nervous System (CNS)

Amphetamine is one of the most potent sympathomimetic amine in stimulating the CNS. It stimulates the medullary respiratory center, lessens the degree of central depression caused by various drugs, and produces other signs of stimulation of the CNS. These effects are thought to be due to cortical stimulation and possibly to stimulation of the reticular activating system. In contrast, the drug can obtain the maximal electroshock seizure discharge and prolong the ensuing period of depression. In elicitation of CNS excitatory effects, the *d* isomer (dextroamphetamine) is three to four times as potent as the *l* isomer.

In man, the psychic effects depend on the dose and the mental state and personality of the individual. The main results of an oral dose of 10 to 30 mg are as follows: wakefulness, and a decreased sense of fatigue; elevation of mood, with increased initiative, self-confidence, and ability to concentrate; often elation and euphoria; increase in motor and speech activity. Performance of only simple mental tasks is improved; and, although more work may be accomplished, the number of errors may increase. Physical performance in athletes, for example, is improved, and the drug is often abused for this purpose. These effects are not invariable, and may be reversed by overdosage or repeated usage. Prolonged use or large doses are nearly always followed by mental depression and fatigue. Many individuals given amphetamine experience headache, palpitation, agitation, confusion, dysphoria, apprehension, delirium, or fatigue.

Depression of Appetite

Amphetamine and similar drugs have been widely used in treatment of obesity, although the wisdom of this use is best questionable. Weight loss in obese humans treated with amphetamine is almost entirely due to reduced food intake and only in small measure to increased metabolism. The site of action is probably in the lateral hypothalamic feeding center: injection of amphetamine into this area, but not into the ventromedial satiety center, suppresses food intake . In man, tolerance to the appetite suppression develops rapidly. Hence, continuous weight reduction is not usually observed in obese individuals without dietary restriction [7].

Behavioral effects: use, misuse, and abuse

The psychologic effects of amphetamine usually include a general increase in alertness, wakefulness and sense of well-being; however, increased irritability, tension and tremors may also occur. Amphetamines can counteract the impairing effect of fatigue, boredom and depressant drugs on the performance of many mental and physical tasks. However, contrary to widespread belief, complex intellectual functioning (involving comprehension, problem solving and judgment) is not improved by amphetamine in normal rested people. The somatic effects of amphetamines include improvement in motor coordination and performance in activities requiring physical exertion[6].

Pharmacokinetics

Absorption: Oral, rapid.

Onset of action: Oral, within 30 minuts.

Plasma half-life: Elimination half-life of amphetamine is dependent on urinary pH and urine volume, with more unchanged amphetamine excreted in acidic urine.

- Half-life with urine pH of < 5.6 is 7-8 hours.
- Up to 30 hours or longer with alkaline urine.

Peak effect: Within 2-4 hours of oral ingestion.

Duration of action: Dependent on half-life.

Elimination: metabolism is hepatic by deamination and hydroxylation. Amphetamine and closely related compounds are weak bases; urinary excretion of nonmetabolised compound is pH influenced.

Active metabolites:

- Hydroxylated compounds may continue to be significantly active by functioning as "false neurotrasmitters".
- Amphetamine is a pharmcologically active metabolite of methamphetamine [9].

The metabolic pathway of amphetamine is shown in Figure 1.2 [10].

Therapeutic uses

Therapeutic use of amphetamine has been attended by growing controversy because of widely publicized, abusive, self-medication. In general, the use of amphetamine is accepted in the treatment of obesity, narcolepsy, and hyperkinetic behavior in children with organic brain damage. Clinicians have found it useful in treating certain psychotic patients and in withdrawing amphetamine users from high doses. Amphetamine use in narcotic mixtures to treat severe pain, and occasional use to treat disabling menstrual cramps is defended by some clinicians.

Clinical toxicity

Toxic effects are often extensions of known pharmacodynamic actions. Central toxic effects include nervousness, agitation, tremor, insomnia, fever confusion, and in some patients, delirium, and panic state. Stimulation of the cardiovascular system may provoke headache, tachycardia, palpitations, and even cardiac arrythmias. Both hypertension and hypotension have been reported. Excessive sweating may occur. Abdominal cramps, nausea and vomiting, and diarrhea have been reported.

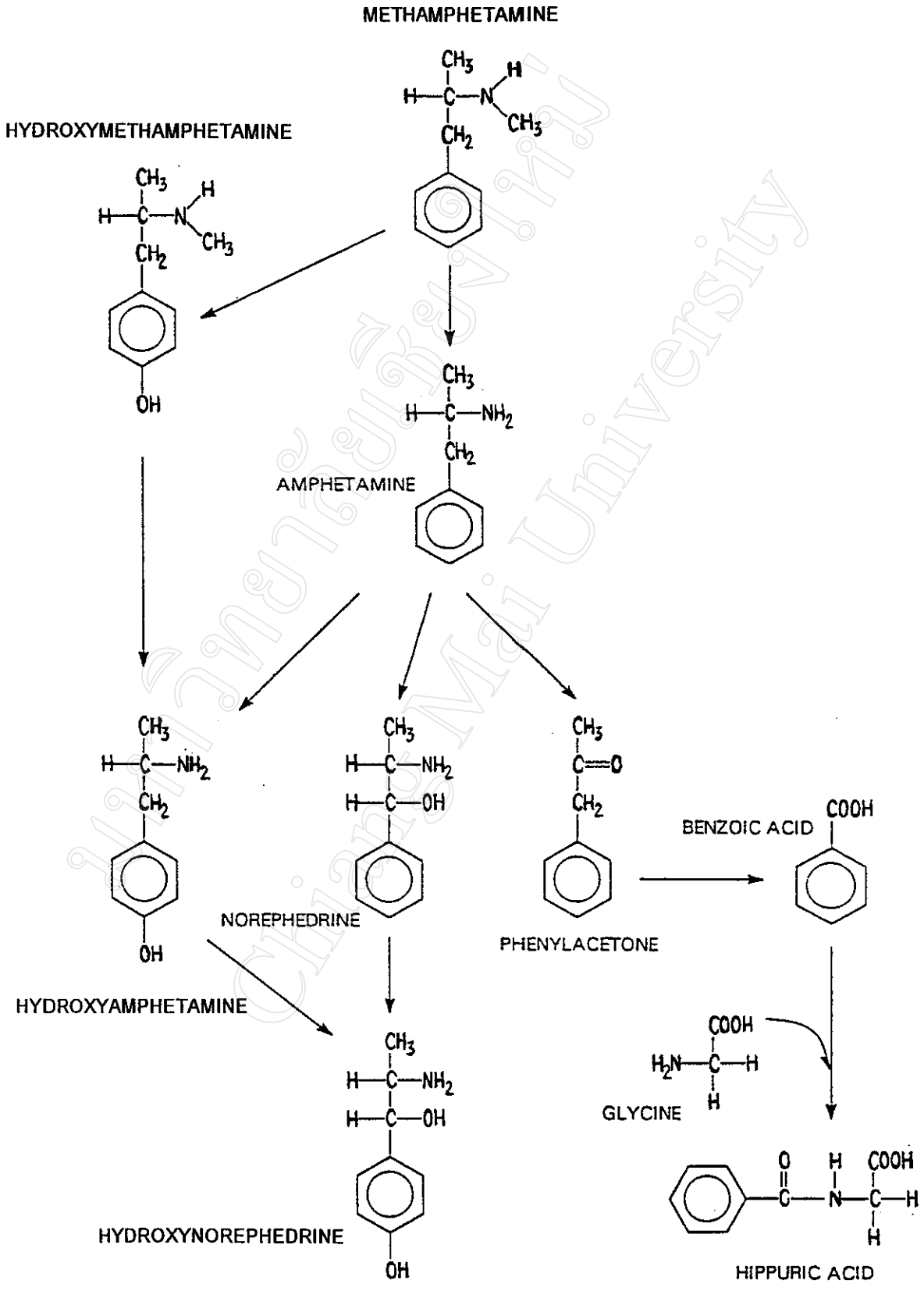

Figure 1.2 Metabolic pathway for amphetamine and methamphetamine

The toxic dose varies widely, but excessive symptoms are rare in doses of less than 15 to 30 mg. Nontolerant users have survived doses of 400 to 500mg, but death has resulted from as little as 120 mg given intravenously [4].

1.2 Methamphetamine

Methamphetamine is closely related chemically to amphetamine and ephedrine. The structural formula of methamphetamine is as Figure 1.3. Physical properties of methamphetamine are listed in Table 1.2 [8]

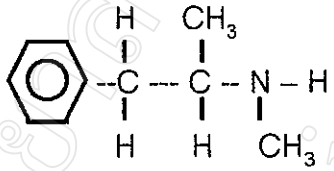

Figure 1.3 Methamphetamine structure

Table1.2 Physical properties of methamphetamine [8]

Methamphetamine	
Chemical name	(+)-*N*, α Dimethylphenethylamine
Molecular formula	$C_{10}H_{15}N$
Molecular weight	149.2
Appearance	Colorless
Boiling point	about 214 °C
Soluble	slightly soluble in water; miscible with ethanol, chloroform, and ether

Methamphetamine Hydrochloride

Molecular formula	$C_{10}H_{15}N.HCl$
Molecular weight	185.7
Melting point	172 to 174 °C
Appearance	White crystals or crystalline power
Soluble	1 in 2 of water, 1 in 4 of ethanol, and 1 in 5 of chloroform; practically insoluble in ether.

Small doses have prominent central stimulant effects without significant peripheral actions; somewhat larger doses produce a sustained rise in systolic and diastolic blood pressures, due mainly to cardiac stimulation. Cardiac output is increased, although the heart rate may be reflexly slowed. Venous constriction causes peripheral venous pressure to increase. These factors tend to increase the venous return and, therefore, the cardiac output. Pulmonary arterial pressure is raised, probably secondary to increased output. Renal blood flow is also enhanced. Although moderate doses stimulate cardiac contraction, excessive doses depress the myocardium [7]. The metabolic pathway of methamphetamine is shown in Figure 1.2 [8].

1.3 Ephedrine

Ephedrine is a sympathomimetic agent. It is metabolized by *N*-demethylation to norephedrine, and by oxidative deamination and conjugation. Ephedrine is itself a metabolite of methylephedrine [11]. The structural formula of ephedrine is as Figure 1.4. Physical properties of ephedrine are listed in Table 1.3 [8].

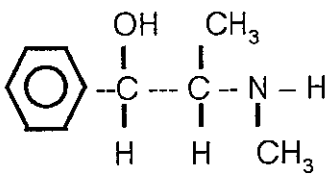

Figure 1.4 Ephedrine structure

Table 1.3 Physical properties of ephedrine [8]

Anhydrous Ephedrine	
Chemical name	(1*R*,2*S*)-2-Methylamino-l-phenylpropan
Molecular formula	$C_{10}H_{15}NO$
Molecular weight	165.2
Appearance	Colorless crystals, or white crystalline powder
Melting point	About 38 °C
Soluble	1 in 20 of water; very soluble in ethanol; soluble in chloroform; freely soluble in ether.
Ephedrine Hydrochloride	
Molecular formula	$C_{10}H_{15}NO.HCl$
Molecular weight	201.7
Appearance	Colorless crystals or white crystalline powder
Melting point	217 to 220 °C
Soluble	1 in 3 to 1 in 4 of water and 1 in 14 to 1 in 17 of ethanol; very slightly soluble in chloroform; practically insoluble in ether.

Pharmacological action

Ephedrine does not contain a catechol moiety, and it is effective after oral administration. The drug stimulates heart rate and cardiac output and variable increases peripheral resistance; as a result, ephedrine usually increases blood pressure. Stimulation of the α-adrenergic receptors of smooth muscle cells in the bladder base may increase the resistance to the outflow of urine. Activation of β-adrenergic receptors in the lungs promotes bronchodilatation. Ephedrine is potent stimulator of the CNS , but less so than amphetamine. After oral administration, effects of the drug

may persist for several hours. Ephedrine is eliminated in the urine largely as unchanged drug with a half-time of about 3 to 6 hours [7].

Pharmacokinetics

Onset of action: within 1 hour.

Plasma half-life: elimination increased in acid urine, with elimination half-life roughly 2 hours; alkaline urine will lengthen elimination half-life up to 10-flod.

Peak effect: for most compounds peak effects are seen between 1 and 3 hours.

Duration of action: clinical effects generally last less than 8 hours.

Elimination: major portion of compound excreted unchanged in urine.

Active metabolites: depends on agent [9].

Therapeutic use and toxicity

In the past, ephedrine was used to treat Stokes-Adams attacks with complete heart block and as a CNS stimulant in narcolepsy and depressive states. It has been replaced by alternative modes of treatment in each of these disorders. Its use as a bronchodilator in patients with asthma has become much less extensive with the development of selective β_2 agonists. Ephedrine has been used to promote urinary continence, although its efficacy is not clear. Indeed, the drug may cause urinary retention, particularly in men with prostatic hypertrophy. Ephedrine has also been used to treat the hypotension that may occur with spinal anesthesia [7].

1.4 Determination of Amphetamine, Methamphetamine, and Ephedrine

Since amphetamine and methamphetamine are classified as type 1 narcotic substances according to Ministry of Public Health Notification No135 B.E. 2539 [12] and ephedrine is classified as type 2 psychotropic substance according to Psychotropic Substance Act B.E. 2518 [13], highly precision and accuracy are required for analysis of these substance. The detection of amphetamine, methamphetamine and ephedrine in urine is important because the results can be used as evidence in court.

As a first step of the investigation in any toxicological laboratory the immunological screening methods [14,15] are used. Immunoassay, which can screen a large number of samples for amphetamine, methamphetamine, and ephedrine, are not entirely specific, cross-reacting with other amphetamine-like substances. The confirmation is obtained by thin-layer chromatography (TLC) [16], high-performance liquid chromatography (HPLC) [17], gas chromatography(GC) [18], or gas chromatography - mass spectrometry (GC-MS) [19-41]. GC-MS with electron impact(EI) ionization is the most common system used for confirmation. The National Institute on Drug Abuse (NIDA) has set stringent guidelines for laboratories to follow for accurate and precise analysis of the five classes of drugs of abuse (amphetamine, cannabinoids, cocaine, opiates, and phencyclidine). These guidelines require GC-MS identification of the drugs of abuse, as well as quantitation by GC-MS [19].

1.5 Sample Preparation

Preparation of a sample to determine the presence of amphetamine can be a relatively simple process. The amine group has a relatively high p*K* value and simply making the solution alkaline readily converts the amine group from the very polar - NH_3^+ to the far less polar -NH_2 form. This loss of hydrophilic character coupled with the already hydrophobic nature of the remainder of the molecule makes it easily extracted into a non-polar organic solvent. This process can be accomplished in a few minutes and the sample can be ready for analysis by a variety of analytical procedures, including sophisticated techniques such as GC-MS. Unfortunately, there are a number of other biological molecules that are chemically related to the amphetamines which are also extracted during a process as simple as that just described. This is true of all biological tissues and fluids, including the aqueous environment of a urine sample. In order to overcome some of the problems of the analysis of complex mixtures within a sample, extraction schemes have been developed using liquid-liquid [19-27] and solid

phase extraction techniques [28-34]. Solid phase extraction (SPE) has become popular owing to the relative simplicity of the procedure. Another reason is that with multiple-step extraction procedures, the time and solvent used are often less than for comparable liquid-liquid extraction methods. Manual manipulation of the sample is also generally less with solid phase extraction and it is therefore easier to develop and conduct the extraction in a consistent manner [9]. In this work, samples preparation by solid phase extraction method is described.

1.5.1 Solid phase extraction

Solid phase extraction, similar to low pressure liquid chromatography, is the basis for the design of a practical sample preparation technique consisting of small, disposable extraction columns filled with a variety of sorbents. To prepare a column for use, the sorbent is conditioned with an appropriate solvent. After conditioning, same solutions are then aspirated through the columns at a flow rate of 5 ml/min or less, using vacuum maintained by a water aspirator or a vacuum pump. Column eluates and washes are collected in a trap inserted between the manifold and the water aspirator. After the washes are completed, collection tubes are placed under each column using a specially designed rack. Appropriate elution solvents are added and forced or aspirated through the columns. The analyte eluates are collected and analyzed directly, or evaporated and then reconstituted in an appropriate solvent for further analysis. Figure 1.5 illustrates the process of solid phase extraction. Multiple extraction columns can be processed simultaneously with specially designed manifolds, as illustrated in Figure 1.6 [42].

1.6 Derivatization

Derivatization is usually carried out in order to increase the volatility of substance with boiling points that are too high, to reduce the adsorption of solutes on the support and column surface and to improve the separation. Special derivatives

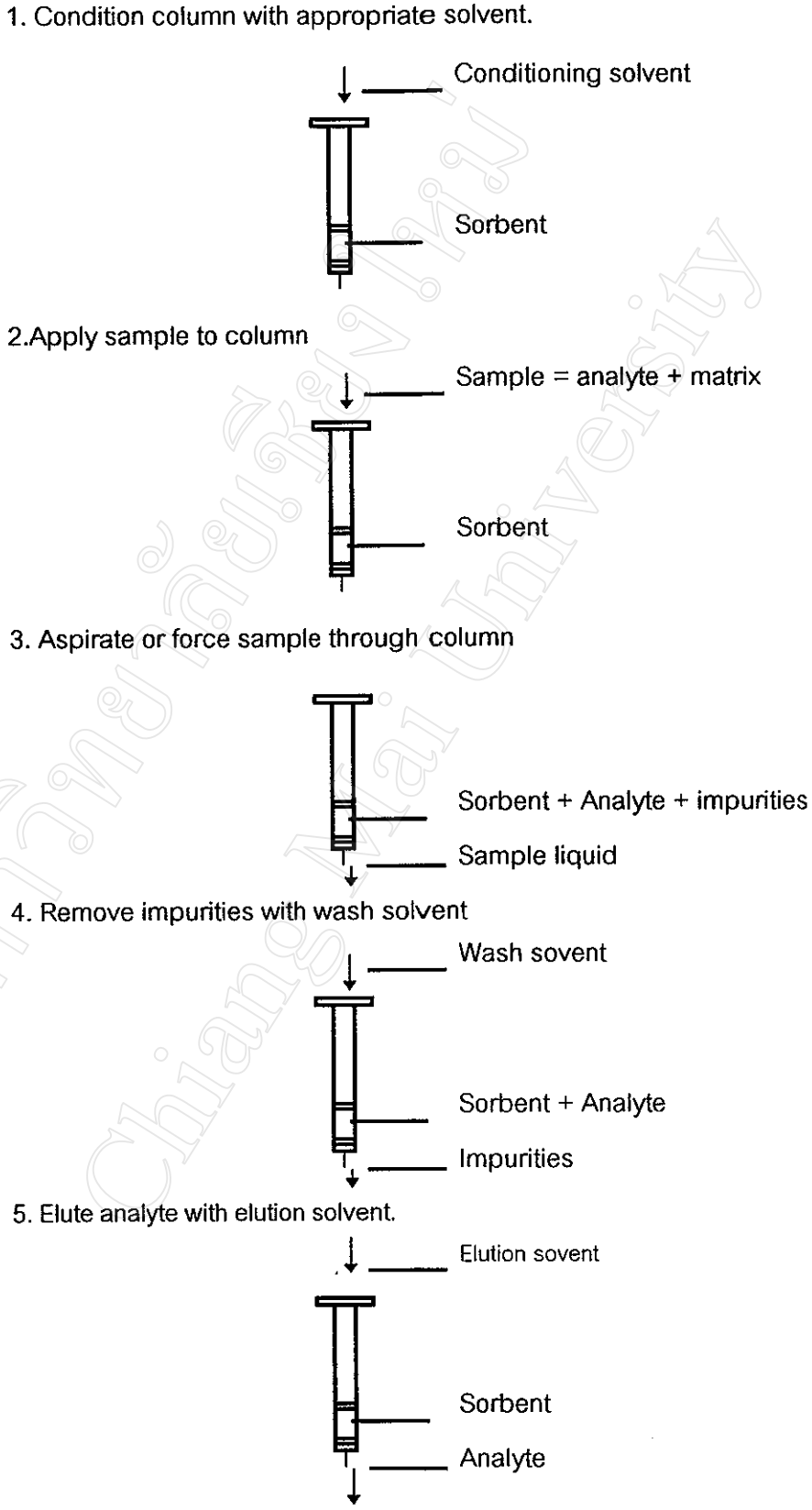

Figure 1.5 Solid-phase extraction process

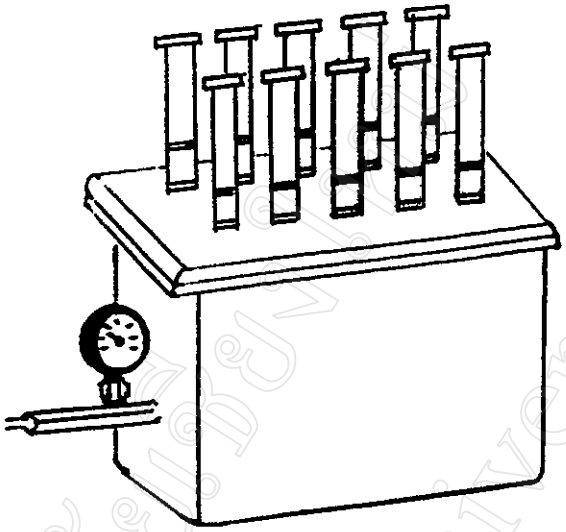

Figure 1.6 Vacuum manifold column processor

often provide for the selective detection of certain species of compounds or compounds or the separation of chemically very similar compounds, such as optical isomers.

Substances with high molecular weights and several functional groups in the molecule are usually not amenable to GC. Polar functional groups reduce the volatility of compounds, which results in excessively long retention times or non-elution of the compounds. The volatility can be enhanced by decreasing the polarity by blocking the polar groups, so that the derivatives can be chromatographed with reasonable retention times.

Many substances cannot be analyzed by GC owing to their thermal instability. Such substances decompose in the sample inlet port and produce several peaks in the chromatogram. These difficulties can also be overcome by using suitable derivatives.

Tailing of peaks can also result when the concentration of the solute in the chromatographic system is too high. At high solute concentrations, the sorption

isotherm is not linear and the peak is skewed. Even in these instances, the effect can be suppressed by using suitable derivatives.

The adsorption of the solute on the support surface or column wall usually results in non-linearity of the calibration graph, especially when working at low solute concentrations in the sorbent and employing peak heights as quantitation parameters. Adsorption on the support and the tailing effect are often caused by carboxylic and hydroxy groups, especially with polyfunctional compounds of higher molecular weight. Amino and imino groups also interact with the support. It is therefore desirable to convert these groups in to groups of lower polarity before the proper analysis.

Derivatization is of great importance in improving the separation of closely related compounds, and it frequently makes it possible to resolve compounds that cannot otherwise be separated.

The preparation of derivatives before GC analysis is a potential source of errors that may affect the entire analytical procedure. For this reason, a thorough knowledge of the reactions used and the factors that influence their results is necessary. Special attention must be paid to the purity of solvents and reagents, the stability of the derivatives and reaction rates[43].

Most gas chromatographic-mass spectrometric confirmation methods for amphetamine include a derivatization step. Reaction of the primary amine of amphetamine and the secondary amine of methamphetamine is beneficial in reducing the volatility of these two drugs in order to provide symmetrical peaks and thereby produce acceptable quantitation. Derivatization is also used to improve the mass spectral characterization of the amphetamine. The base mass spectral ion that is usually used for quantitation is shifted to a higher mass-to-charge (*m/z*) ratio, thereby removing it from potential low mass interference [26].

Amphetamine is commonly react with heptafluorobutylric anhydride [24, 28-30,32,35], 4-carbethoxyhexafluorobutyryl chloride [19,21,23] trifluoroacetic

anhydride, pentafluoroproprionic anhydride [15,27], *N*-methyl-*N*-t-butyldimethylsilyl trifluoroacetamidem [25], (-)-menthyl chlorformate [22], propylchloroformate [26], and *N*-methyl-bis-trifluoroacetamine [34]. For each of these methods, the urine extract is dried to a residue, dissolved in the derivatizing reagent, then heated for approximately 30 min. The excess reagent is evaporated, and the derivatives are redissolved in organic solvent prior to analysis. In this study employing derivatization with heptafluorobutylric anhydride has been employed.

1.7 Gas Chromatography-Mass Spectrometry (GC-MS)

The power of mass spectrometry (MS) as a detection method lies in enormous amount of useful information it provides. In routine use, searching a library of "known" spectra allows for rapid identification of unknowns in a capillary gas chromatogram. For trace analysis, this technique is limited only by the ability of the instrument to obtain a characteristic spectrum form a small amount of sample. This ability is affected by instrument design limitation and by variation in ion chemistry [44].

MS is an analytical technique for determining chemical composition and molecular structure. The sample is converted into ions and neutral particles by an ionization process; the resulting ions are separated according to their mass-to -charge ratios (m/z) (Figure1.7). The fragmentation pattern of each chemical compound is a "fingerprint" of that compound and can be used to identify it by comparison with previously acquired libraries of fingerprints. MS was used to analyze components separated by chromatography as early as in 1960s. As this became more common, it became a priority to couple the two techniques together. By the late '60s, this was happening. Microcomputers were developed and finally provided a means to store and manipulate the data the instruments were capable of producing.

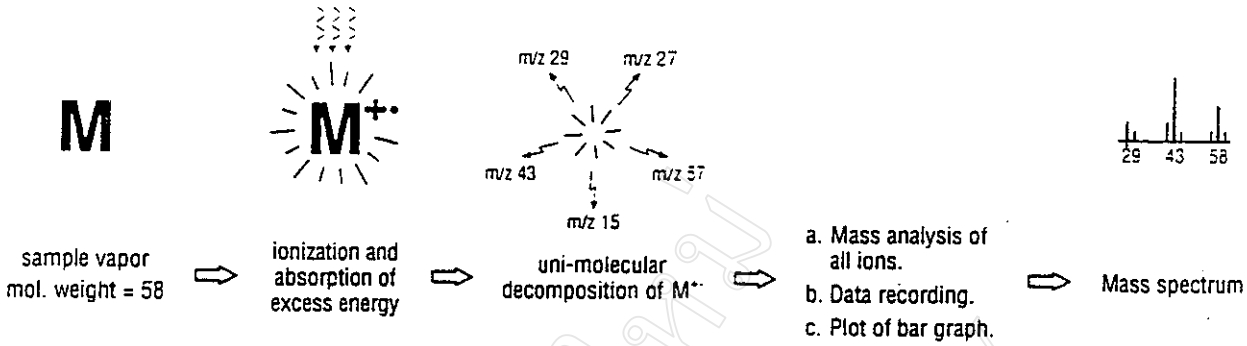

Figure 1.7 Process of mass spectrometry

GC-MS is the combined technique of GC and MS. The GC separates mixtures into discrete bands of compounds and then introduces those bands into the MS. The analyte bands are ionized and the MS analyzes the ions generated in the selected mass range. The GC-MS can provide data into domains, a reconstructed total ion current chromatogram is generated which provides quantitation information and a mass spectrum is available at any point in the chromatogram which provides the qualitative information.(Figure 1.8)

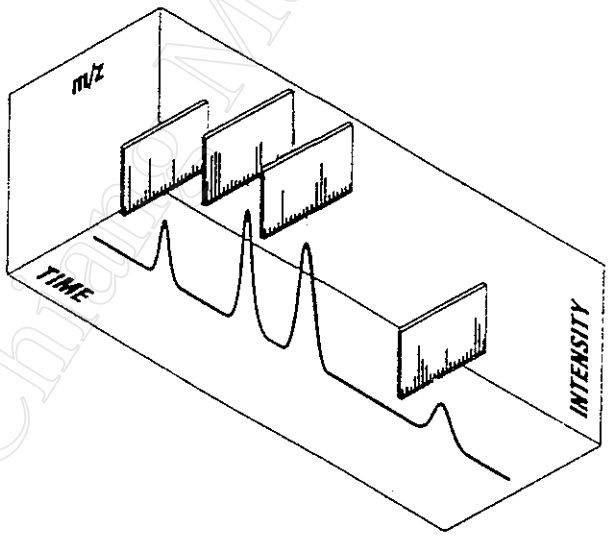

Figure 1.8 Data generated by a GC-MS system

1.7.1 System configuration

A GC-MS system is composed of an injector, a column, GC oven, transfer line, mass analyzer, pump, and data system, as shown in Figure 1.9. An inlet device

introduces the sample. The advantage to using a GC as the inlet is that it separates complex mixtures into their individual components before the sample is introduced into the mass spectrometer. There are many ionization techniques available for use in MS, commonly electron ionization (EI) and chemical ionization (CI). The principal types of mass analyzer are magnetic sector, quadrupole, and ion trap. The detector used in GC-MS is an electron multiplier, the detector collects the ions which have been produced and amplifies them into a measurable signal. A data system controls instrument parameters and allows acquisition, manipulation, interpretation, and display of the data.

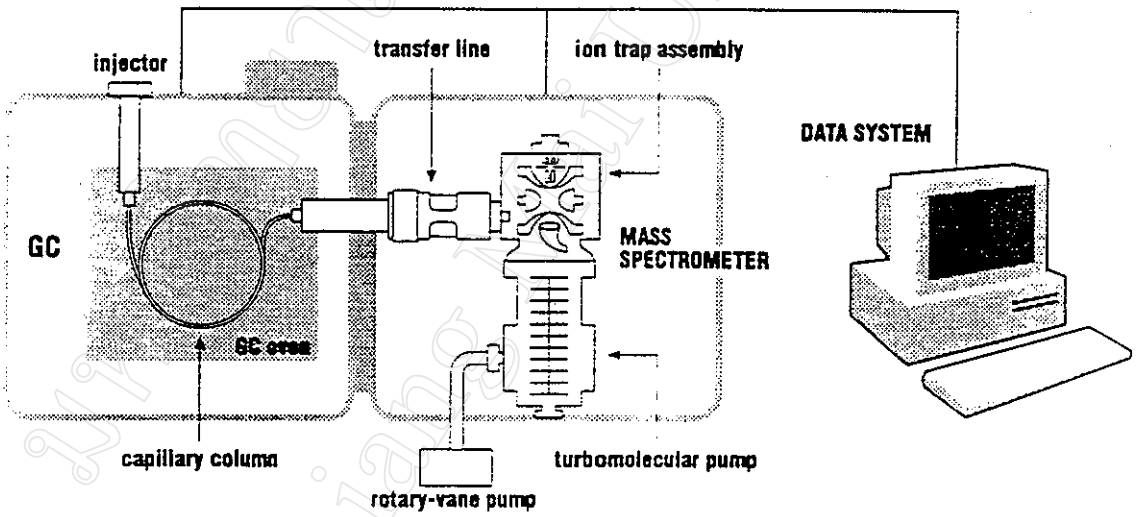

Figure 1.9 The GC-MS system

Both the GC and MS have been optimized for a smooth interface. The GC uses narrow bore capillary columns because of their separating power, but especially because of the low carrier gas flows which they require. Because the MS operates under vacuum, it can only accept very low gas pressure due to column flow.

1.7.2 Electron ionization

Electron ionization is the most common ionization technique. Electrons from a hot wire filament such as rhenium are focused across the ionization chamber where they collide with sample molecules. The electrons have on average an energy of 70 eV. This is sufficient to break the bonds in most organic molecules. Ionization consists of the sample molecules coming in close proximity to an energetic electron, losing an electron from its outer shell (valence electron) to form a molecular ion and then undergoing a possible fragmentation. Electron ionization produces a high degree of fragmentation. This yields structural information, where identification can be based on pattern recognition. The molecular ion is usually weak or perhaps not even present.

Chemical ionization is used when it becomes necessary to confirm the molecular weight. This is possible with chemical ionization because it is a soft ionization technique relying on about 10 eV. This energy is produced by first bombarding a reagent gas with the electron beam which produces low energy reagent gas molecules. Secondly, the reagent gas ions react with the sample molecules to create sample ions, indicative of the molecular weight.(Figure 1.10)

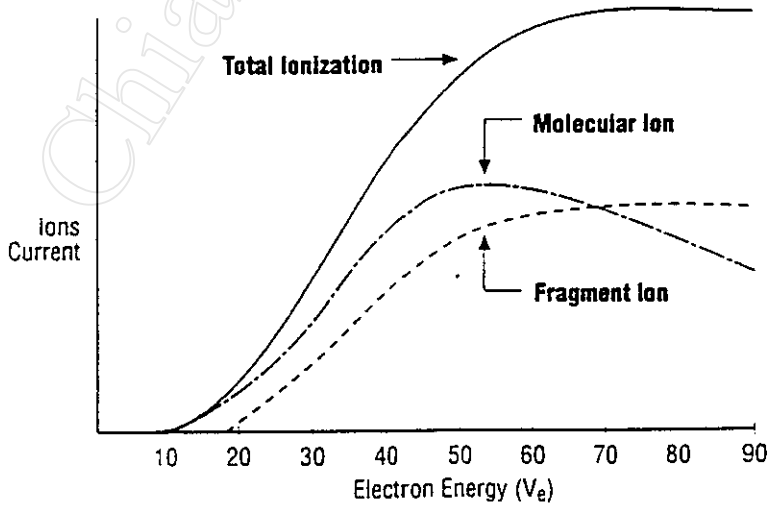

Figure 1.10 Energy versus ionization plot

1.7.3 Mass analysis

A mass spectrometer is classified according to the technique used to separate the ions, as shown in Figure 1.11 [45].

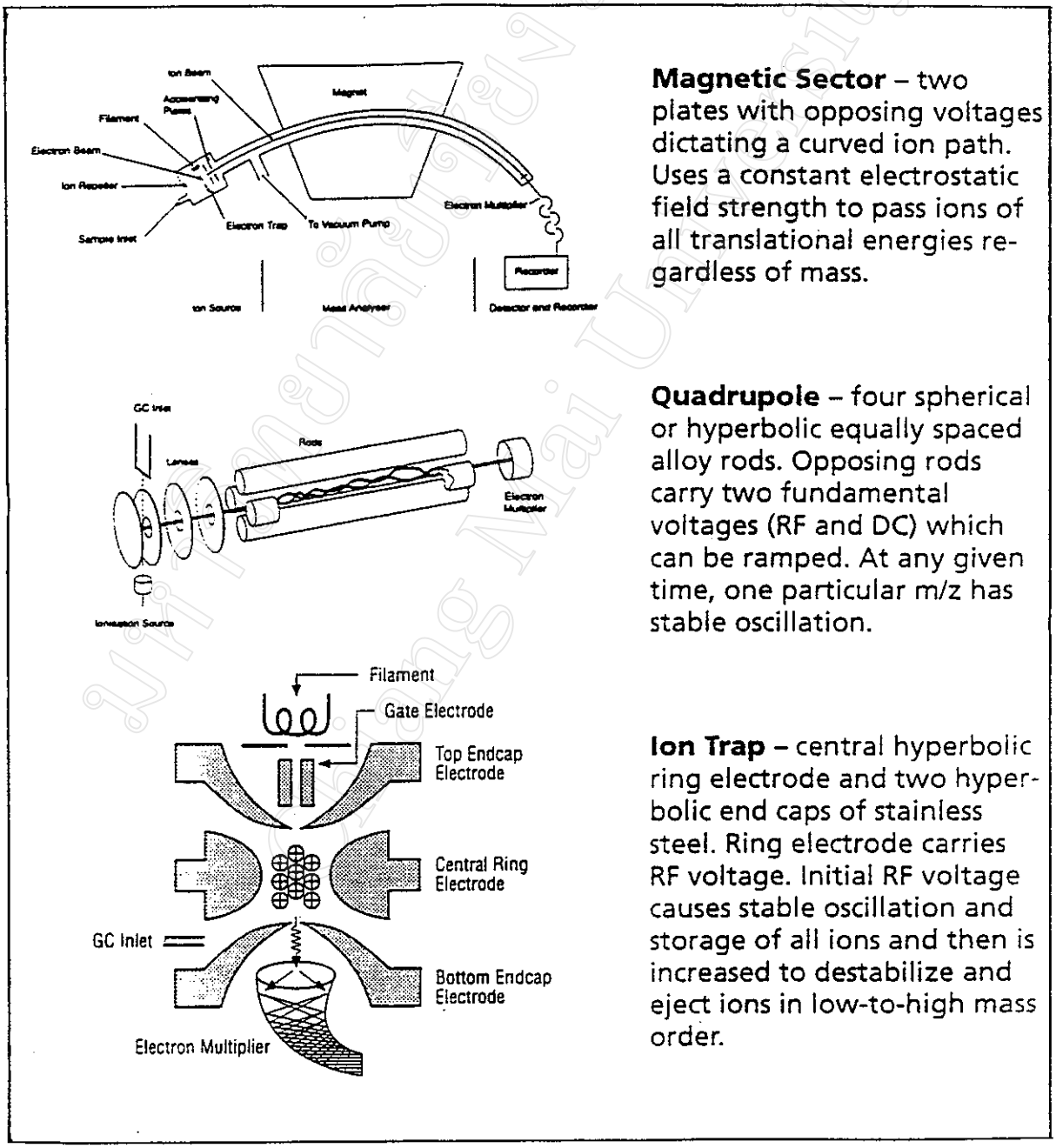

Figure 1.11 Three common types of mass analyzers

1.8 The Scope and Aims of This Research

In this work the development of gas chromatography-mass spectrometry with electron impact ionization as a method for the simultaneous determination of amphetamine, methamphetamine, and ephedrine was carried out after the analytes had been extracted by suitable solid phase extraction in positive urines, and derivatized to heptafluorobutyric anhydride derivatives.

The aims of this research work can be summarized as follows:

1. To optimize conditions for solid phase extraction of amphetamine, methamphetamine, and ephedrine in positive urines and derivatize them to heptafluorobutyric anhydride derivatives prior to gas chromatography-mass spectrometry analysis.

2. To study and obtain the optimum gas chromatography-mass spectrometry conditions for analysis of amphetamine, methamphetamine, and ephedrine, in selected urine samples.