CHAPTER 1

INTRODUCTION

Drug abuse is an important problem in Thailand and worldwide. At present, the most serious drug abuse in Thailand is of amphetamine and methamphetamine (YABA). Information from drug cases throughout the country during 2000-2006 showed that YABA addiction had the highest prevalence [1]. The Office of Narcotic Control Board (ONCB) estimated that there are about 7,312,200 Thais ubusing YABA [2]. A study by Adam indicated that YABA in Thailand appeared as an orange tablet bearing a WY symbol, and it consisted of methamphetamine (MA) 21.76-22.79 mg%, and caffeine 61.8-62.42 mg%. Some tablets also contained amphetamine (AM), ephedrine, theophylline and phenacetin [3]. YABA can cause personality changes and lead to criminal offences. Its use is widely spread both in rural and urban areas and has become a fashion for some young people.

1.1 Methamphetamine/Amphetamine

MA, which is an important substance in YABA, is a psychostimulant compound. It has been used under the brand name of Desoxyn for attention-deficit hyperactivity disorder or narcolepsy [4]. However, it also is used illegally for recreational and other non medication purposes.

AM was first synthesized in Germany in 1887 by Lazăr Edeleanu. During that time, MA was first synthesized from ephedrine in 1893 by Nakayoshi Nagai. In 1919,

crystallized MA was synthesized by Akira Ogata via the reduction of ephedrine using red phosphorus and iodine [5]. AM and MA structure are shown in Figure 1.1

Amphetamine Methamphetamine

Figure 1.1 AM and MA structure; from http://leda.lycaeum.org.

The physical properties of MA are shown in Table 1.1

Table 1.1 Physical properties of MA [6]

Properties	MA
Synonyms	α-Methylbenzeneethanamine
Molecular formula	$C_{10}H_{15}N$
Molecular weight	149.2
Boiling point	214 ° C
Appearance	Colorless
Soluble	1:50 of water; soluble in ethanol, chloroform and ether; readily soluble in acids

Route of administration

Oral administration is the usual route for medical purposes. In recreational abuse, it can be inhaled (smoked), swallowed, snorted, dissolved in water and injected, or

inserted into the anal passage or vagina [7]. The potential for addiction is greater when delivered by a means that increases blood concentration quickly. In general, inhalation is the fastest route to increasing blood concentration followed by intravenous injection, insertion and swallowing. Inhalation is commonly carried out by smoking from a glass pipe or aluminum foil heated by a flame underneath.

Metabolic pathway

MA is changed to AM by demethylation and to p-hydroxymethamphetamine by hydroxylation (Figure 1.2). About 70% of a dose is excreted in urine within 24 h after oral administration. Under normal conditions, up to 43% of a dose is excreted as unchanged drug, and up to 15% and 5 % is excreted via p-hydroxymethamphetamine and amphetamine pathways, respectively [6]. Excretion of unchanged drug depends on the pH of the urine, which increases in acid and reduces in alkaline. The plasma half-life of MA is about 9-10 h [8].

Pharmacological effects and toxicity

MA is a central nervous system stimulant, which acts as a dopaminergic and adrenergic reuptake inhibitor. It rapidly enters the brain and triggers a cascading release of norepinephrine, dopamine and serotonin [9]. This inversion leads to a release of these transmitters from the vesicles to the cytoplasm and from the cytoplasm to the synapse. It also indirectly prevents the reuptake of these neurotransmitters, causing them to remain in the synaptic cleft for a longer period [9]. The methyl group is responsible for the potentiation of effects, as compared to the related compound, AM, rendering the substance more lipid soluble and easily transported across the blood brain barrier, and it is also more stable against enzymatic degradation by monoamine oxidase (MAO).

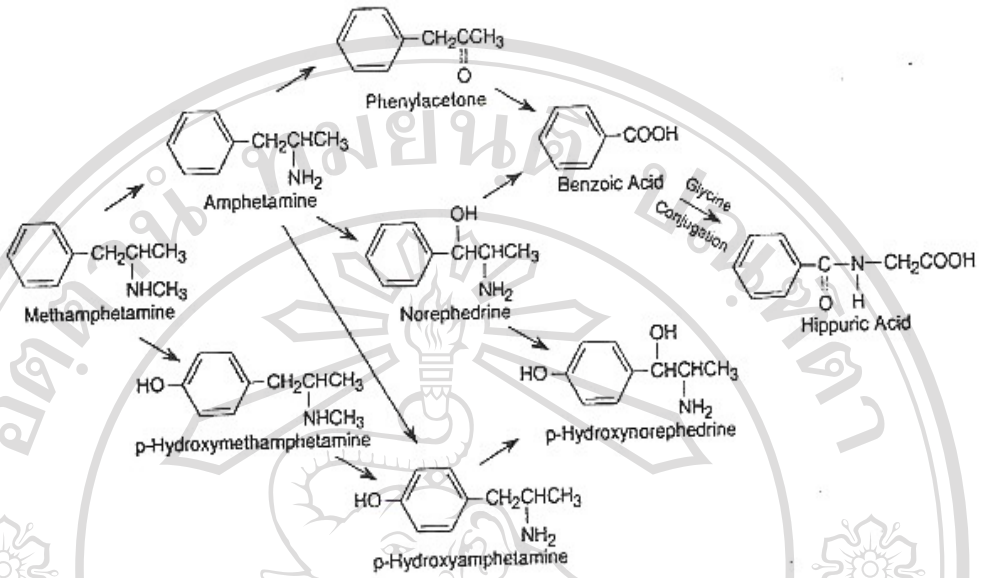

Figure 1.2 Metabolic pathway of MA [10].

Common acute side effects include euphoria, increased energy, diarrhea, nausea, loss of appetite, insomnia, tremor, agitation, compulsive fascination with repetitive tasks, talkativeness, irritability, panic attacks, increased libido and dilated pupils [11]. Side effects associated with chronic use include drug craving, weight loss, withdrawal-related depression and anhedonia or amphetamines psychosis. High dose MA consumption leads to sudden fatality [12].

Tolerance

Tolerance to MA is not completely understood, but known to be sufficiently complex that it cannot be explained by any single mechanism. The extent of tolerance and the rate at which it develops varies widely between individuals, and even within one person it is highly dependent on dosage, duration of use and frequency of administration [13]. Many cases of narcolepsy are treated with MA for years without

escalating doses or any apparent loss of effect. Prolonged over stimulation of dopamine receptors caused by MA may eventually cause the receptors to down regulate in order to compensate for increased levels of dopamine within the synaptic cleft [13].

1.2 Diagnosis of MA abuse

Any non medical use of MA is considered as abuse, especially when used for recreation or illegal purposes. Diagnosis of MA abuse relies on the history of abuse, physical examination and detection of MA in the body. The history of abuse is always under reported, particularly when involving a legal issue. Therefore, reliability is at a very low level. Clinical manifestation from MA can be observed by physicians, but it is non specific for MA abuse. The detection of MA or its metabolites in the body is crucial for diagnosing MA abuse, and biological specimens commonly used in the laboratory are blood and urine. By law, laboratory results must be confirmed by a specific method. Generally, a biological specimen from a subject has to be screened by a sensitive laboratory technique, such as color test immunoassay or thin layer chromatography. If a screening test shows a positive result, that specimen will be subjected to a more specific confirmation test such as high performance-liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS) [14].

Blood sampling is the first choice in forensic work, since it represents acute use, and its concentration offers good correlation with clinical manifestation and dosing [15]. However, its window of detection is narrow and blood specimen collection is not convenient for other drug monitoring purposes. Urine is most widely used for drug abuse monitoring, despite the detection time of substance in urine taking

longer than in blood. Urine collection is less invasive than taking a blood specimen. However, MA and amphetamine derivatives can be detected in urine for approximately 2-3 days [16] depending on dosage, urine pH and analytical techniques. This limitation leads to exploration of other biological matrixes to monitor drug abuse. Hair is a tissue in which many substances are deposited and it can be used to detect many compounds [17, 18]. Considering drug abuse, hair analysis has been applied to forensic and drug monitoring work for a period of time. Yet, the introduction of hair analysis into Thailand has not been popular and it still needs more laboratory verification to support its use.

1.3 Hair analysis

In recent years, the analysis of drugs in hair samples has become more popular, due to its potential applications in forensic and clinical toxicology [19]. Hair analysis has become both an alternative and a complementary approach to drug abuse detection and offers some advantages over urine assays. The collection of hair specimens is less embarrassing and intrusive for studied subjects. Hair analysis can monitor long-term exposure, and the detection window of drug abuse in hair tests is considerably wider than that of urine assays. The detection window is limited only by the length of the hair and typically ranges from weeks to several months or years [19].

In the 1960s and 1970s, hair analysis was used to evaluate exposure to toxic heavy metals, such as arsenic, lead or mercury. This was achieved using atomic absorption spectroscopy, which allowed detection in the nanogram range. At that time, the examination of hair for organic substances, especially drugs, was not possible because the analytical methods were not sensitive enough. Examination by

means of drugs marked with radioactive isotopes, however, established that these substances can move from blood to hair and are deposited there. In 1979, Baumgartner et al. published the first report on the detection of morphine in the hair of heroin abusers by using radioimmunoassay (RIA) [19]. They found that differences in the concentration of morphine along the hair shaft correlated with the time of drug use. Furthermore, gas chromatography coupled with mass spectrometry (GC–MS) is the method of choice for hair analysis [20]. Gentili et al. 2004 performed a headspace solid-phase microextraction, which was a soventless extraction technique. This SPME method was reliable and feasible with gas chromatography-mass spectrometry [21].

Hair growth

Hair grows in cycles, alternating between periods of growth and quiescence. A follicle that is actively producing hair is called the anagen phase. Hair is produced during 4 to 8 years for head hair (<12 months for non–head hair) at a rate of approximately 0.22 to 0.52 mm/day or 0.6 to 1.42 cm/month [22]. After this period, the follicle enters a relatively short transition period of about 2 weeks, known as the catagen phase, during which cell division stops and the follicle begins to degenerate. Following the transition phase, the hair follicle enters a resting or quiescent period, known as the telogen phase, during which the hair shaft stops growing completely and hair growth begins to shut down, as shown in Figure 1.3. On the scalp of an adult, approximately 85% of the hair is in the growing phase and the remaining 15% is in a resting stage.

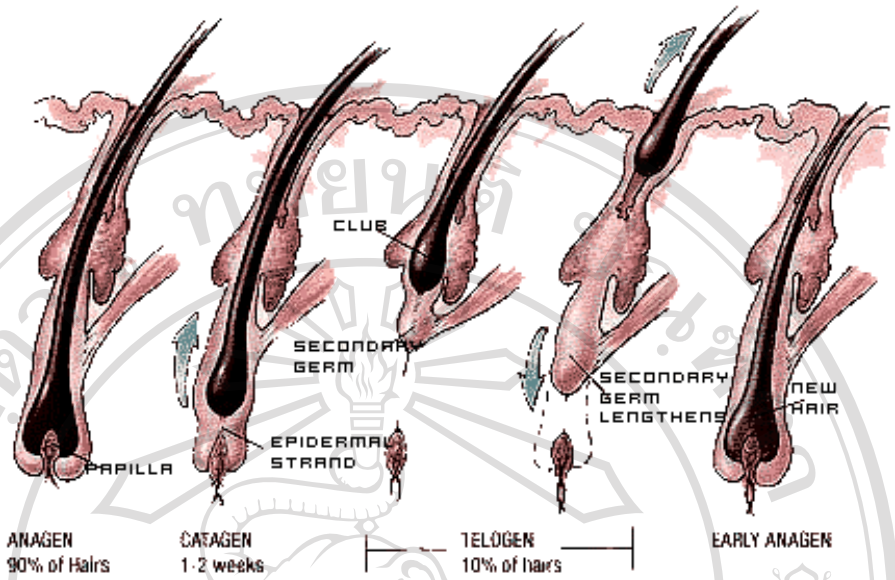

Figure 1.3 Diagram illustrating hair growth sequences [23]

from; http://hairtransplantsurgery.ie.

Mechanisms of drug incorporation into hair

Drugs enter into hair by two processes [24]: contamination from the external environment and incorporation into the growing hair shaft from blood that supplies the hair follicle (Figure 1.4). Drugs can enter the hair from exposure to chemicals in aerosols, smoke or secretions from sweat and sebaceous glands. Sweat is known to contain drugs that are present in blood. The exact mechanism by which chemicals are bound into hair is not known. It has been suggested that passive diffusion may be augmented by drug binding to intracellular components of the hair cells, such as the hair pigment melanin. Another proposed mechanism is the binding of drugs with sulfydryl-containing amino acids that are present in hair. There is an abundance of amino acids, such as cystine, in hair; these form cross-linking SS bonds to stabilize the protein fiber network.

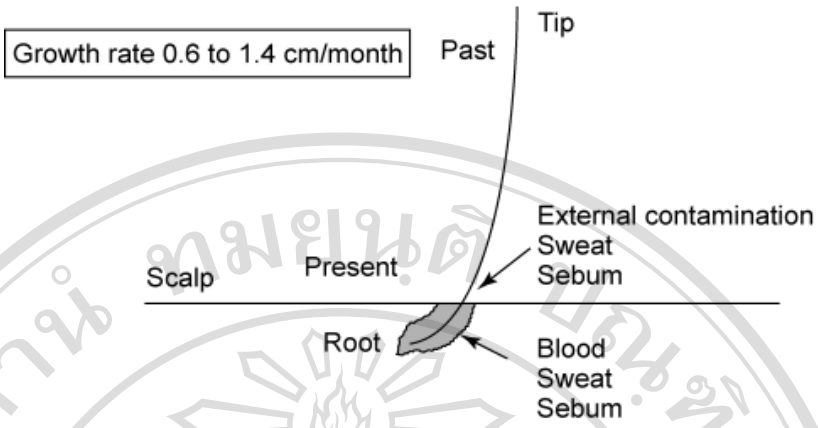

Figure 1.4 Possible model for drug incorporation into hair [24].

Steps in hair analysis

Decontamination

As normal hygienic practices do not sufficiently remove externally deposited drugs, there is a consensus that hair specimens have to be treated by decontamination procedures in the laboratory before extraction and analysis of drugs [25, 26]. Among the agents used for cleaning hair, there are detergents and organic solvents such as water followed by acetone [27, 28], dichloromethane [29], methanol [30, 31], and dichloromethane followed by water and methanol [32], and hexane followed by methanol, and acetone [33]. As decontamination with organic solvent, such as water and MeOH, does not produce hair swelling, it is possible that not all external incorporated drugs will be removed.

Extraction

To quantify how much of a drug remains in hair after washing, it is necessary to release the incorporated drugs from the hair matrix. The hair sample can be pulverized in a ball-mill or cut into segments of about 1 mm in length. A variety of hair-extraction procedures have been described, including 0.01 M HCl [32], 1 M HCl [21],

NaOH incubation [34, 35], enzyme; pronase E in Tris buffer [28], methanol incubation [30, 33], phosphate buffer [36], and β-glucuronidase/arylsulfatase in pH 7.6 phosphate buffer [17].

Methanol incubation is a good extraction method because it produces hair swelling, but the extraction process takes several hours. NaOH is also a good extraction method because the protein matrix is completely destroyed; however, the chromatogram makes too much noise. The method using 1 M NaOH and 0.1 M HCl yields higher recoveries of morphine and codeine [37].

Cleanup

Solutions containing the analytes of interest, obtained after extraction of the drugs from the hair matrix, need to be clean to eliminate possible substance interference. A variety of extraction procedures have been described, including solid-phase extraction (SPE) [17, 38], liquid-liquid extraction (LLE) [30], solid-phase microextraction (SPME) [21, 34], and solid-phase dynamic extraction (SPDE) [35, 39].

The SPE for organic compounds has demonstrated several advantages over the LLE. The SPE provides cleaner extracts, higher selectivity, greater reproducibility, and avoidance of emulsion formation. Recently, SPME, a relatively new technique, gained considerable interest in the field of toxicology analysis. It consists of direct absorption of the analytes from the sample onto fused-silica fiber that is coated with an appropriate stationary phase. The advantage of this technique over SPE is that fewer solvents are used; consequently, fewer residues are generated, and the time of analysis is shorter [21, 34].

Analysis

The RIA technique was first used to detect opiates in hair samples of heroin abusers by Baumgartner et al. [19]. Since then, various methods using immunoassay or the chromatographic technique have been published. Immunoassays are used as a screening test because of their relative sensitivity, speed, and convenience. However, quantification by immunoassay is difficult to achieve, as the specificity of most kits is directed to a group of drugs and drug metabolites rather than a single substance. Therefore, presumptive analytes detected by immunoassay have to be identified by a chromatographic method. Chromatographic procedures have proved to be powerful tools for identifying and quantifying drugs in hair, due to their separation ability and sensitivity and specificity for detection, particularly when coupled with MS. At present, GC-MS is commonly used [21, 30, 32]. Moreover, the tandem mass spectrometry (MS/MS) represents a very powerful technique, due to its excellent selectivity and sensitivity [33, 35].

1.4 Solid-Phase Microextraction (SPME)

SPME was invented by Pawliszyn and co-workers in 1989. It has been widely used in different fields of analytical chemistry since its first applications to environmental and food analysis and is ideally suited for coupling with gas chromatography -mass spectrometry [40, 41].

SPME is a sample preparation technique, which is an essential step in analysis, greatly influencing the reliability and accuracy of results. The SPME technique is a quite simple and efficient; soventless sample preparation method [21]. It uses a fused-silica fiber that is coated on the outside with an appropriate stationary phase. Analytes

in the sample are directly extracted to the fiber coating. The fiber is coated with a thin polymeric film, which concentrates the organic analytes (or inorganic analytes such as volatile mercury and arsenic compounds) during absorption, or adsorption from the sample matrix.

Extraction procedure

The coated fiber is immersed directly into the sample or the headspace (HS) of the sample, where analysates are concentrated [42]. After equilibrium has been reached (from a few minutes to several hours depending on the properties of the analytes measured) or after a defined time, the fiber is withdrawn and transferred to a GC injection port. The fiber is exposed and the analyte is either desorbed thermally in the hot GC injector port or eluted by the mobile phase or carrier gas (He). With very complex matrices such as sledges, biological fluids and food products, or solid sample use, the SPME technique is mainly applied for the extraction of analysate from the sample HS. Schematic view of the HS- SPME apparatus is shown in Figure 1.5.

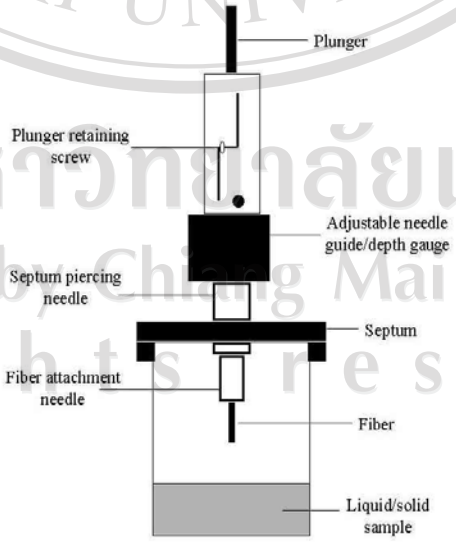

Figure 1.5 Schematic view of the headspace SPME apparatus [43].

1.5 Gas chromatography–mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) is an instrumental technique, comprising a gas chromatograph (GC) coupled to a mass spectrometer (MS), by which complex mixtures of chemicals may be separated, identified and quantified as shown in Figure 1.6 [44].

Gas chromatography (GC)

GC is commonly used for separation of thermally stable, volatile compounds [44]. Analysates are placed in the inlet/injector port of the GC, where they are vaporized and injected onto the head of the chromatographic column. The injector port is a self-sealing, heated body that allows vaporization of the sample and is then transferred to the capillary column. A glass liner is used in the port to allow for cleaning and to give a surface suitable for vaporization of the analyte of interest. The system in the injection port, which allows all or some of the analytes to go through the column, is called the split/splitless injector. The split injector mode allows for the expansion of the volatilized sample in the case of liquid injections, and prevents overloading of the column. The splitless mode is used for trace analysis and SPME fiber, where analytes are pre-concentrated onto a fiber and overloading of the column is unusual.

The sample is transported through the column by a gaseous mobile phase. The carrier gas used must be chemically inert to prevent interaction with analytes; helium is commonly used. The column itself contains a liquid stationary phase, which is adsorbed onto the surface of a thin fused-silica capillary tube. There have been many ways of adhering the liquid stationary phase to the walls of the capillary columns. Most columns are fused-silica open tubular (FSOT) columns; these have much thinner

walls than the glass capillary columns, and are given strength by the external polyimide coating. The columns are flexible and can be wound into coils; this gives them the advantages of physical strength, flexibility and low reactivity. The stationary phase is selected to separate the compounds of interest. There are many types of stationary phases to complement analytes of interest, with the choice for analytes based on the knowledge that 'like dissolves like' and, therefore, a polar column will retain polar molecules longer so it is better for separating polar compounds. Likewise, a non-polar column is used for non-polar analytes. Other stationary phases are designed to interact with different types of functional groups.

Mass spectrometry (MS)

After passing through the GC, the chemical continues to the MS. The molecules are blasted with electrons, which cause them to break into pieces and turn into positively charged particles called ions [44]. This is important because the particles must be charged to pass through the filter. In an MS filter such as quadrupole, the ions continue through the MS and travel through an electromagnetic field that filters the ions based on mass, as shown in Figure 1.7. Scientists using this instrument choose what range of mass should be allowed through the filter. The filter continuously scans through the range of mass as the stream of ions come from the ion source. An electron multiplier detector counts the number of ions with a specific mass. This information is sent to a computer and a mass spectrum is created. The mass spectrum is a graph of the number of ions with a different mass that travels through the filter.

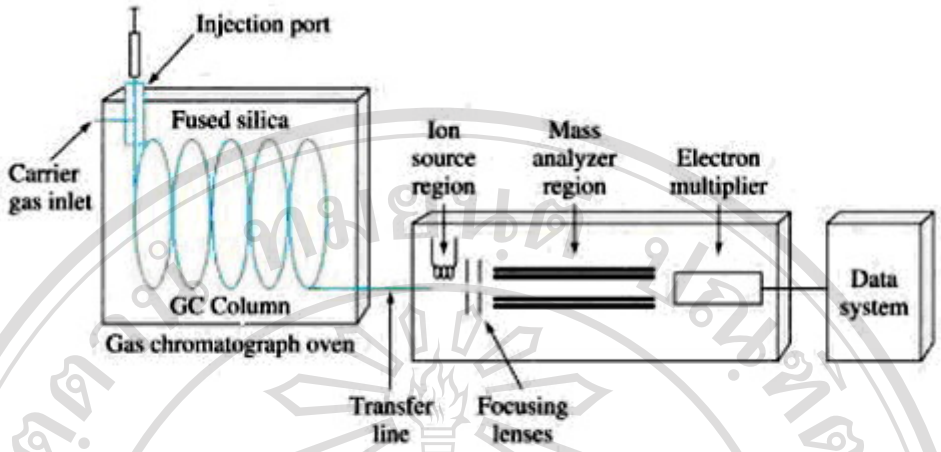

Figure 1.6 The GC-MS system [45].

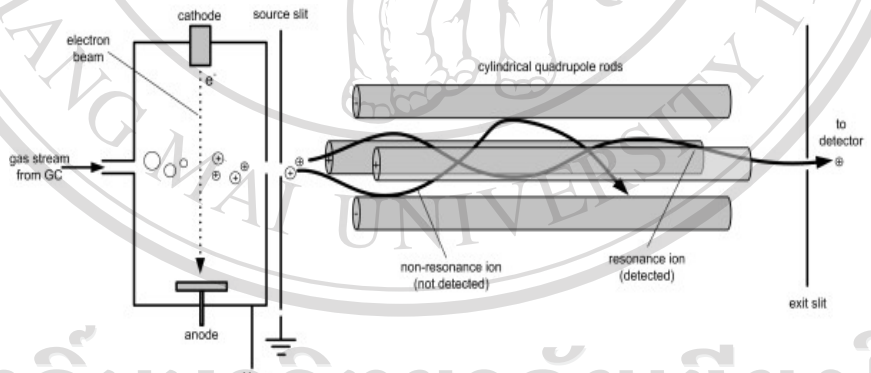

Figure 1.7 Schematic view of the ion source based on electron impact ionization and the quadrupole mass filter typically found in a GC-MS instrument [46].

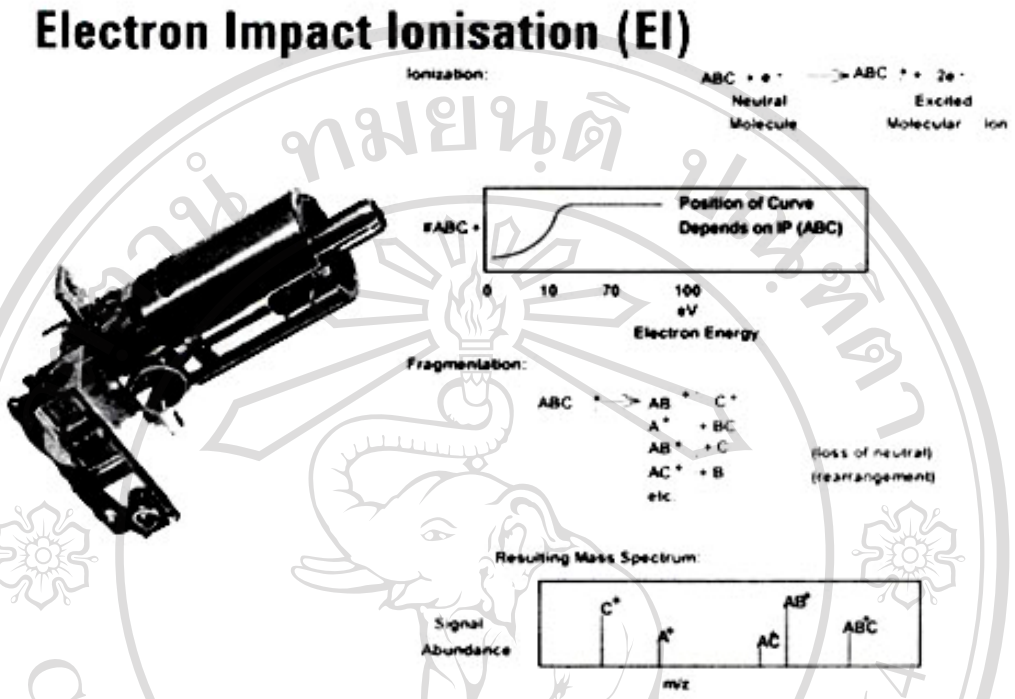

Figure 1.8 Electron Impact Ionization [47].

1.6 Method validation

Method validation is the process of proving that an analytical method is acceptable for its intended purpose. The Food and Drug Administration (FDA) [48] provides a framework for performing such validations. In general, methods for regulatory submission must include studies on linearity, accuracy, precision, and detection and quantitation limit.

A linearity study verifies that the sample solutions are in a concentration range where analyte response is linearly proportional to concentration. For assay methods, this study is generally performed by preparing standard solutions at five concentration

levels, from 50 to 150% of the target analyte concentration. Five levels are required to allow detection of curvature in the plotted data.

The accuracy of a method is the closeness of the measured value to the true value for the sample. Accuracy can be assessed by analyzing a sample of known concentration and comparing the measured value to the true one.

The precision of an analytical method is the amount of scatter in the results obtained from multiple analyses of a homogeneous sample. The precision study is on instrument precision or injection repeatability [48].

The detection limit of a method is the lowest analyte concentration that produces a response detectable above the noise level of the system. The detection limit needs to be determined only for impurity methods in which chromatographic peaks near the detection limit are observed. The quantitation limit is the lowest level of analyte concentration that can be accurately and precisely measured. This limit is required only for impurity methods and is determined by reducing the analyte concentration to a level where the precision is unacceptable.

1.7 The scope and aims of this research

The abuse of YABA is one of the most important problems among Thai people. Analysis by using urine could not detect cases where abuse had occurred daily or for more than one week. The analysis of drugs in hair samples has become an alternative and a complementary approach to drug abuse monitoring for long-term exposure. The concentration in hair is quite low; therefore, the analysis must be sensitive. HS-SPME-GC-MS is a method that is sensitive enough to detect drugs in hair. However, it had not been introduced or validated in Thailand. Therefore, this analytical technique needed to be verified and validated.

The scope and aim of this thesis was to verify and validate a technique to determine AM and MA in the hair of YABA abusers by using automated headspace solid-phase microextraction and gas chromatography-mass spectrometry.

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

1. To develop and validate a method for the detection of amphetamine and methamphetamine in human hair by using HS-SPME-GC-MS.
2. To determine the relationship between amphetamine and methamphetamine in human hair by using this verified technique and study the history of drug abuse.