CHAPTER 2

MATERIALS AND METHODS

2.1 Apparatus and chemicals

2.1.1 Apparatus

1) Gas Chromatograph (GC), 6890N Series GC system, Agilent Technology, U.S.A.

a) GC capillary-column, HP-5MS (30m× 0.25mm× 0.25μm), J&W Scientific

2) Mass Spectrometer (MS), 5973*inert* Mass Selective Detector Series MS system, Agilent Technology, U.S.A.

a) Vacuum Turbo-V70 pumps

b) Electron impact ionization (EI)

c) Quadrupole mass filter

3) Multi Purpose Sampler MPS 2, Gertsel, Germany

a) Solid phase microextraction fiber (SPME), Supelco, U.S.A.

- 100 μm Polydimethylsiloxane (PDMS)

- 65 μm Polydimethylsiloxane/Divinylbenzene (PDMS/DVB)

4) Hot air oven, Memmert, Germany

5) Analytical balance

a) Adventurer, Ohaus, U.S.A.

b) Mettler Toledo, Switzerland

6) Autopipette, Socorex, Switzerland

7) Crimper, 20 mm cap, Agilent, U.S.A

8) Crimper, 11 mm cap, Agilent, U.S.A

9) Decrimper, 20 mm cap, Agilent, U.S.A

10) Decrimper, 11 mm cap, Agilent, U.S.A

11) Vial 10 ml, 18 mm screw top, Gertsel, Germany

12) Screw caps with septa for 18 mm vials, magnetic, septum silicone blue transparent / PTFE white, Gertsel, Germany

2.1.2 Chemicals

1) Amphetamine (AM) sulfate, purity 99%, Alltech, U.S.A.

2) Methamphetamine (MA) hydrochloride, purity 99%, Alltech, U.S.A.

3) Methylenedioxyamphetamine (MDA), purity 99.74%, Lipomed, Switzerland

4) Methylenedioxymethamphetamine (MDMA), purity 99.72%, Lipomed, Switzerland

5) Methylenedioxyethylamphetamine (MDE), purity 99.58 %, Lipomed, Switzerland

6) Ketamine, purity 99.248%, Lipomed, Switzerland

7) Hydrochloric acid (HCl) 37%, AR grade, Merck, Germany

8) Potassium carbonate (K_2CO_3), AR grade, Fisher Scientific, UK.

9) Methanol (MeOH), HPLC grade, Fisher Scientific, UK.

10) Benzaldehyde, AR grade, B.D.H., UK.

11) Distilled water (DW)

12) Acetone, AR grade, B.D.H., UK.

13) Helium (He) gas, 99.999% (UHP grade), TIG, Thailand

14) Nitrogen gas, 99.99% (HP grade), TIG, Thailand

2.2 Preparation of solutions

2.2.1 Preparation of standard solutions

2.2.1.1 Stock standard solutions

A stock standard solution of AM was prepared by dissolving 6.41 mg of AM sulfate (Alltech, purity 99%) in MeOH in a 5 ml volumetric flask. The final concentration of AM was 1.00 mg/ml.

MA was prepared by dissolving 6.36 mg of MA hydrochloride (Alltech, purity 99%) in MeOH in a 5 ml volumetric flask. The final concentration of MA was 1.012 mg/ml.

2.2.1.2 Working standard solutions

Working standard solutions were prepared by diluting a stock standard solution to 20,000 ng/ml in MeOH before conducting serial dilutions with 1 M HCl. The working standard solution concentrations are shown in Table 2.1.

Table 2.1 Working standard solution preparation

Standards	Working standard solution concentrations (ng/ml)		
	First dilution in MeOH	Second dilution In 1 M HCl	Serial dilutions in 1M HCl
AM	20,000	1000	850, 700, 550, 400, 250
MA	20,000	1000	500 , 250, 100, 50

2.2.1.3 Internal standard solution

Benzaldehyde was selected for use as an internal standard (INS) for AM and MA analysis in this study. It was sensitive and stable under this analysis condition and its retention time (RT) did not overlap with the retention time of AM, MA and other amphetamine derivatives.

Benzaldehyde at 0.5 ml (99.99% v/v) was pipetted into a 500 ml volumetric flask before MeOH was added. After mixing, 1 ml of this solution was pipetted into a 1,000 ml volumetric flask and the solution was diluted with distilled water, yielding a working solution of 0.0001% v/v in distilled water.

2.2.2 Potassium carbonate

The 5 M K_2CO_3 was prepared by dissolving 691.05 g in 1,000 ml of distilled water in a 1,000 ml volumetric flask, then, serial dilutions to 3, 1, 0.5 and 0.25 M were conducted.

2.2.3 Hydrochloric acid

The 1 M HCl was prepared by pipetting 48.25 µl of concentrated HCl into 1,000 ml of distilled water in a 1,000 ml volumetric flask.

2.3 GC-MS conditions

The conditions of GC-MS followed the study of Gentili et al. [21], with some modifications. GC parameters consisted of an HP-5MS column (30m× 0.25mm× 0.25µm), and the temperature was held initially at 60 °C for 2 min, then gradually increased by 20 °C/min to 250 °C, and finally held at 250 °C for 1 min. The temperature of the injection port was set at 250 °C. The splitless injection mode was used (purge flow 60 ml/min, purge time 0.5 min). Helium was used as carrier gas at a flow rate of 1.0 ml/min. MS parameters consisted of electron impact ionization, quadrupole and ion source, and transfer line temperatures were set at 150, 230 and 280 °C, respectively. The mass spectra of amphetamine derivatives were collected by the total ion chromatogram (TIC) using scan mode (mass range 35-550).

2.4 Optimization of SPME conditions

The HS-SPME was used for specimen preparation in this study. The condition initially followed the study of Gentili et al. [21]. The optimization of HS-SPME conditions was then conducted. The following parameters were studied; total volume of solution in a 10 ml vial, K_2CO_3 concentration, type of SPME polymer, and incubation, extraction and desorption time. The experiment started with adding 150 µl of K_2CO_3, 200 µl of AM or MA and 150 µl of benzaldehyde into a 10 ml vial. The vial was heated by a heat block at 90 °C for 5 min of incubation. The SPME fiber was introduced into the vial, allowing extraction for 5 min. The SPME fiber was removed from the vial and inserted into the GC injection port for 5 min desorption time. After analysis, the SPME fiber was cleaned up with 270 °C bake out for 5 min. Each parameter was optimized and the proper conditions were chosen for future experiments.

2.4.1 Optimization of total volume of preparation

The total volume of solution preparation was determined in a 10 ml vial, which affected headspace above the sample. A working standard solution of MA (250 ng/ml) was analysed by making a total volume of 0.5, 1, 2, 3 and 4 ml with distilled water. The samples were analysed in five replicates at each volume. The results are shown in Table 3.3 and Figure 3. 7.

2.4.2 Optimization of potassium carbonate concentration

To increase the sensitivity of the analytical method, the analysate had to be volatile. Since the pH of amphetamine derivatives was alkaline, adjusting matrix pH to alkaline was efficient for extraction [49]. An addition of salt into the solution increased pH and the ionic strength of the solution, making the target compounds less

soluble and more responsive in SPME [21]. The concentration of K_2CO_3 was varied in a fix volume of 1,650 µl, in order to make a total volume of 2 ml. A working standard solution of MA (250 ng/ml) was analysed by adding 0.25, 0.5, 1, 3 and 5 M K_2CO_3. The samples were analysed in five replicates at each concentration. The results are shown in Table 3.4 and Figure 3.8.

2.4.3 Comparison of SPME fibers

The affinity of the SPME fiber for analysis was an important factor. By following the principle of ‘‘like dissolves like’’, fibers of different coating polymers were selected in accordance with different compounds [50]. Although six kinds of fibers were commercially available, two of them [100 µm polydimethylsiloxane (PDMS) and 65 mm polydimethylsiloxane/divinylbenzene (PDMS/DVB)] were commonly used for analyzing drugs and poisons. In this study, extractions of AM derivatives were determined by using two kinds of SPME fiber. The results are shown in Table 3.5 and Figure 3.9.

2.4.4 Optimization of incubation time

The incubation process in the SPME technique allowed the sample to be volatile. The sample was equilibrated in the incubation time and a maximum response was determined. By following the published data [21], the incubation temperature was set at 90 °C [21]. MA (250 ng/ml) was analysed with various incubation times (3, 5, 10, 20 and 30 min). The results are shown in Table 3.6 and Figure 3.10.

2.4.5 Optimization of extraction time

After incubation, the samples were volatile in the headspace of the vial. Insertion of SPME fiber into the headspace allowed the volatile compounds to be absorbed into the fiber. Extension of this extraction time increased the chances of

detection. In this experiment, the PDMS/DVB was used to determine 250 ng/ml of MA at various extraction times (1, 3, 5, 10, 20 and 30 min). The extraction temperature was 90 °C [21]. The result is shown in Table 3.7 and Figure 3.11.

2.4.6 Optimization of desorption time

Desorption is a step when analytes are released from the SPME fiber and reach the GC. To determine a suitable desorption time, MA (250 ng/ml) was analysed with PDMS/DVS fiber. After the extraction period, the SPME needle was inserted into the GC injection port for 1, 3, 5 or 10 min. The result is shown in Table 3.8 and Figure 3.12.

2.4.7 Determination of carry over effect

Carry over would cause a false positive result. To avoid the carry over effect, the SPME fiber was kept in a bake out station at 270 °C for a further 5 min before the next cycle of analysis. A bake out station was used to elute remaining compounds from SPME fiber after desorption. The bake out temperature had to be higher than the desorption temperature, and nitrogen flow was used to chase the remaining analysates. Three high concentrations of MA (10,000, 20,000 and 30,000 ng/ml) were determined, followed by blank vials to observe the carry over effect. The result is shown in Table 3.9.

2.5 Validation of the analytical method by HS-SPME-GC-MS

According to US FDA guidelines, the analytical method has to be validated [48]. Validation experiments include assessments of linearity, accuracy and precision, limit of detection (LOD) and limit of quantitation (LOQ).

The 200 µl of working standard solution was spiked into 20 mg of negative hair sample. Then, the spiked hair sample went through the process of sample extraction before analysis by HS-SPME-GC-MS. The sample was incubated at 60 °C for 1 hr. After cooling to room temperature, 150 µl of internal standard was added to the sample. The 350 µl of supernatant were then pipetted into 1,650 ml of 1 M K_2CO_3 and rapidly sealed.

2.5.1 Linearity

To determine linearity for the standard curve, various concentrations of AM and MA were analysed. The concentrations of AM used in this study were 2.5, 4, 5.5, 7, 8.5 and 10 ng/mg of hair. The concentrations of MA used to create a standard curve in this study were 0.5, 1, 2.5, 5 and 10 ng/mg of hair. The experiment for intra-day linearity was conducted in triplicate, while inter-day linearity was conducted on three consecutive days. The ratios of AM and MA peak area to internal standard peak area were plotted against the sample concentrations (Figures 3.13-14). The correlation coefficient (r^2) was calculated from this result and is shown in Tables 3.11-12.

2.5.2 Accuracy and Precision

The accuracy determines how close the measured concentration is to a known amount of drug. The precision is the variability of the measured concentration. The minimum requirements for the evaluation of accuracy and precision consist of low, medium and high concentrations of the target compound.

Intra-day accuracy and precision were analyzed in seven replicates for each concentration. Inter-day accuracy was analyzed in triplicate for each concentration on four separate days [48]. The concentrations of the target compound were determined using the standard curves prepared and analysed on each day.

In this study, three concentrations of AM (2.5, 5 and 10 ng/mg of hair) and MA (1, 2.5 and 5 ng/mg of hair) were prepared by another researcher. These target compounds were spiked into negative hair samples, then submitted for analysis as described earlier. The analysis was conducted in triplicate for each evaluated concentration and performed for 4 consecutive days. The results for AM and MA were calculated from the relevant standard curves. The percentage of relative recovery (%RR) was calculated from measured concentration divided by known concentration multiplied by 100. The %RR indicated the accuracy of the analysis method [48]. An acceptable accuracy was within ±15% RR [50].

Precision is determined by the percentage coefficient of variation (%CV), which is calculated from standard deviation (SD) divided by the mean of that dataset and multiplied by 100. An acceptable precision criterion was a %CV of lower than 15. For inter-day precision, the %CV was calculated from a pooled SD and mean. A % CV of lower than 15 was also acceptable for inter-day precision [48].

2.5.3 Limit of detection and limit of quantitation

The limit of detection (LOD) is the lowest concentration of substance that can be detected, but the concentration cannot be measured. Usually, the relative recovery and coefficient of variation at that concentration are more than 20% [48]. The limit of quantitation (LOQ) is the lowest concentration that can be measured with a definite level of certainty.

In this experiment, AM and MA were analysed in ten replicates for each evaluated concentration of 2.5, 2.0, 1.5 ng/mg of hair and 0.5, 0.3, 0.25 ng/mg of hair, respectively. The fortified concentration, which showed a relative recovery and coefficient of variation of less than 20% [50], was considered an acceptable LOQ.

2.6 Analysis of hair samples from drug abusers

After the analytical method had been validated, it was applied to real hair samples from drug abusers and drug-free subjects. This human experiment was approved by the Ethic Committee of the Faculty of Medicine, Chiang Mai University.

2.6.1 Subjects

For the study group, thirty hair samples were obtained from the "Reducing Youth Drug-Related HIV/STD Risk Behaviors in Thailand: Phase II Intervention project". The inclusion criteria were age 18-45 years old and experience of using YABA at least 3 times in the past 3 months. The history of previous use, frequency of use and amount used was collected from the subjects' self-report. The data of each individual were recorded in a prepared form. The hair samples were analyzed by the validated method described earlier. An estimated 50 hairs were cut with clean scissors from the vertex posterior of the scalp. All samples collected were over 3 centimeters (cm) in length, for analyzing the past 3 months.

For the control group, thirty hair samples from drug-free subjects were used. After gaining their permission, hair samples were obtained from the laboratory staff, who had no drug abuse history.

2.6.2 Sample preparation

Each hair sample was cut into 3 sections of 1 cm in length and washed separately with 5 ml of distilled water, then, vortexed for 1 min before decanting the washed solution. This process was repeated three times before the hair was washed with 5 ml of acetone for the last time [27]. The washed samples were dried in an incubator at 50 °C. Each hair sample was cut into small pieces of an estimated 1 mm in length and left for further analysis.

2.6.3 Analytical procedure

Twenty milligrams of cut and dried hair sample were extracted with 200 µl of 1 M HCl in a closed headspace vial (20 ml) at 60 °C for 1 h [21]. After cooling to room temperature, 150 µl of benzaldehyde (0.0001% v/v) was added into the vial for INS. The extract was separated and placed into a 10 ml GC vial containing 1,650 µl of 1 M K_2CO_3, which was rapidly sealed. The extracted hair sample was analyzed by HS-SPME-GC-MS and incubated at 90 °C for 5 min. The PDMS/DVB fiber was inserted into the vial headspace for 10 min extraction. After that, the SPME needle was inserted into the GC injection port for 5 min desorption. The condition of GC is shown in Table 3.1.

2.7 Statistical analysis

Descriptive analysis including; age, concentration range, min, max, median, and SD was described. Linear regression was used in order to determine correlation of the amount of use from self-reports and hair concentration in method analysis.

Total volume determination of preparation, K_2CO_3 concentration, and incubation, extraction and desorption time was compared using ANOVA for statistical analysis. The type of SPME polymer used the t-test. The Chi-square test was used for determining the amount of drug use and positive results. Correlation between the amount of hair concentration used linear regression (95% CI, α =0.05).