2. EXPERIMENTAL

2.1 Apparatus and Chemicals

2.1.1 Apparatus

1) Gas Chromatography-Mass Spectrometry system, Saturn 2000, Varian, USA, consisting of

   a) Autosampler, Varian 8200CX

   b) Programmable Temperature Split/Splitless Injector, Model 1078

   c) Gas Chromatograph, Varian 3400CX

   d) Mass Spectrometer, Varian Saturn 2000 GC/MS/MS

   e) GC-column, DB-5MS (30m×0.25mm×0.25um), J&W Scientific

   f) Vacuum pump, Turbo-V70 pumps

   g) Saturn GC-MS Workstation, System Control Version 5.2, Hewlett Packard Laser Jet 5L printer.

2) Nitrogen evaporator, Turbo Vap® LV Evaporator, Zymark, USA

3) Vortex mixer, Vortex Genie2, Scientific Industries, USA

4) Hot air oven, Memmert, Germany

5) SPE vacuum manifold, J.T. Baker Inc., USA

6) Disposable extraction columns, Bakerbond spe, Octadecyl (C18), J.T.Baker Inc., USA

7) Analytical balance, AG204, Mettler-Toledo, USA

8) pH meter, Mettler Delta 345, Mettler-Toledo, USA

2.1.2 Chemicals

1) Methanol, HPLC grade, J.T.Baker Inc., USA

2) Dipotassium hydrogen phosphate, AR grade, Fluka , Switzerland

3) Potassium dihydrogen phosphate, AR grade, Carlo Erba, Italy

4) Amphetamine sulphate, obtained from the Narcotics Division, Medical Science Department

5) Methamphetamine hydrochloride, obtained from the Narcotics Division, Medical Science Department

6) Ephedrine hydrochloride, obtained from the Narcotics Division, Medical Science Department

7) Phenylpropanolamine hydrochloride, obtained from the Drugs Division, Medical Science Department

8) Phenylephrine hydrochloride, obtained from the Drugs Division, Medical Science Department

9) Chlorpheniramine maleate, obtained from the Drugs Division, Medical Science Department

10) Bromhexine hydrochloride, obtained from the Drugs Division, Medical Science Department

11) Heptafluorobutyric anhydride, puriss, Fluka, Switzerland

12) Ethylacetate, AR grade, Merck, Germany

13) Nitrogen gas, high purity 99.99%, TIG, Thailand

14) Helium gas, ultra high purity 99.999%, TIG,Thailand

15) Sodium hydroxide, AR grade, Merck, Germany

16) Hydrochloric acid 37%, AR grade, Merck, Germany

17) Ammonium hydroxide, AR grade, Merck, Germany

18) Methylene chloride, HPLC grade, J.T. Baker Inc., USA

19) Ethanol, AR grade, Merck, Germany

2.2 Preparation of Solutions

2.2.1 Preparation of Standard Solutions

2.2.1.1 Stock Standard Solution (1mg/ml)

25 mg of amphetamine, methamphetamine and ephedrine were each accurately weighed into a 25 ml volumetric flask and 10 ml of methanol was added. The solution was mixed well and filled up to the mark with additional methanol.

2.2.1.2 Working Standard Solutions without Internal Standard

0.1-1.0 ml of the stock standard solution was pipetted into a series of 10 ml volumetric flasks and the solution was diluted to the mark with methanol, yielding working standard solutions of 10-100 ug/ml concentration.

2.2.1.3 Working Standard Solution with the Presence of Internal Standard

0.1-1.0 ml of the stock standard solution was pipetted into a series of 10 ml volumetric flasks with internal standard solution 0.4 ml (1mg/ml) and the solution was diluted to the mark with methanol, yielding working standard solutions of 10-100 ug/ml with 40 ug/ml internal standard solution.

For HFBA derivatives, 0.1-2.0 ml of 10 ug/ml standard solution was pipetted into a series of 10 ml volumetric flasks with 1ml internal standard solution (10ug/ml) and the solution was diluted to the mark with methanol, yielding working standard solutions of 100-2000 ng/ml with 1000 ng/ml internal standard.

2.2.1.4 Internal Standard Solution (1mg/ml)

100 mg of phenylpropanolamine was accurately weighed into a 100 ml volume of methanol.

In order to select a suitable internal standard, four drugs, namely, phenylpropanolamine, phenylephrine, Chlorpheniramine maleate, and Bromhexine hydrochloride were employed. From Figure 2.1 , phenylpropanolamine was found to be the appropriate internal standard for this work because its retention time was reasonably within the region of methamphetamine hydrochloride and ephedrine hydrochloride.

2.2.2 Preparation of SPE Solutions

2.2.2.1 Phosphate Buffer Solutions

Phosphate buffer solutions from pH 5.0 to pH 9.0 were prepared by mixing 50 ml of 0.2 M potassium dihydrogen phosphate with quantities of 0.2 M sodium hydroxide specified in the following table and diluting to 200 ml with deionized water.

pH	5.0	6.0	7.0	8.0	9.0
0.2 M NaOH, ml	1.3	5.7	29.6	46.8	52.5

2.2.2.2 Rinse Solutions

Solution prepared by mixing ethanol, ammonium hydroxide and water with ratio 50:10:40, 40:10:50 and 20:10:70 (v/v), respectively, were used as rinsing solutions.

2.2.2.3 Elution Solvents

A series of mixed elution solvents, namely, CH_2Cl_2-*i*-propanol-NH_4OH (78:20:2), NH_4OH:ethyl acetate (2:98), diethyl ether and methanol, were used in this study.

2.2.2.4 Sample Preparation

0.5 ml of 100 ug/ml standard solution and 1.0 ml of 100 ug/ml internal standard were spiked into a 100 ml volume of a given urine sample.

2.3 Optimization of GC-MS Conditions

The procedure adopted was modified from literature [24,28-30,32,35,44]. Optimization was carried out to obtain an adequate separation, the shortest analysis time and mass matching possible for amphetamine, methamphetamine, ephedrine, and internal standard.

2.3.1 GC Parameters Optimization

GC parameters consist of injector temperature, initial temperature, final temperature, rate of ramp up, and flow rate of carrier gas. Optimal GC parameters were determined by injecting 1ul of standard amphetamine, methamphetamine, ephedrine, and phenylpropanolamine of 10 ug/ml. The results of the underivatized compounds are shown in Tables 3.1-3.6 and the results of the HFBA derivatives are shown in Tables 3.7-3.11

2.3.2 MS Parameters Optimization

The ion trap was first tuned using default software setting to obtain suitable mass calibrations and electron multiplier responses. The transfer line was held at 250°C. The ion trap temperature settings were subsequently manipulated as in Tables 3.12-3.13

2.4 Calibration Curves

The internal standard method was employed for the purpose of quantifying amphetamine, methamphetamine and ephedrine. Calibration curves for each amphetamine, methamphtamine and ephedrine at concentrations of an appropriate range were constructed by plotting the peak areas obtained from standard mixture chromatograms against corresponding concentrations.

2.5 Limits of detection

For full scan limits, LOD was selected based on a combination of the signal-to-noise (S/N) ratios and the selected limits were determined if results at the LOD had a S/N greater than 3:1. Each drug was analyzed in replicates of five [30].

2.6 Linearity, Repeatability and Reproducibility

The linearity plot was constructed for amphetamine, methamphetamine and ephedrine in the concentration 2-200 ug/ml for the underivatized drugs and 0.2-40 ug/ml for the HFBA derivatives with optimum GC-MS condition. The linear detector response (correlation coefficient, r) values obtained are shown in Tables 3.19-3.20

By injecting the standard mixture, 50 ug/ml each for the underivatized and 500 ng/ml each for the HFBA derivatives, six times in the same day and twice a day for three days, repeatability and reproducibility, respectively, were calculated. The data are presented in Tables 3.21-3.24

2.7 Optimization of SPE condition

The adsorbent C_{18} was used to extract amphetamine, methamphetamine and ephedrine from urine samples. The adsorbent was conditioned before use by sequentially passing 2 ml of methanol, deionized water and a buffer with the same pH value and volume as the sample through the cartridges. The cartridges were loaded by drawing 3 ml urine sample with 2 ml phosphate buffer. The cartridges were rinsed with a series of different rinsing solutions and the vacuum was held at 10 mm Hg. The cartridges were eluted with a series of elution solvents. The eluant was evaporated to dryness under a stream of nitrogen at 45° C. The residues were redissolved in 1 methanol for analysis. The results are shown in Tables 3.25-3.24

2.8 HFBA Derivatization

After evaporating the specimen from SPE and standard solution to dryness at 45° C under nitrogen, 50 ul of HFBA and 950 ul of ethyl acetate were added. The tube was tightly capped and heated at 70° C for 20 min.

2.9 Recovery Assay

The recoveries of the amphetamine, methamphetamine and ephedrine were examined at concentration 500 ng/ml. Three replicates were performed for the extraction of amphetamine, methamphetamine and ephedrine with SPE and HFBA derivatization. The recoveries data are shown in Table 3.28

2.10 Analysis of Urine Samples

After selecting the suitable condition for sample preparation based on the SPE method and HFBA derivatization, the amounts of amphetamine, methamphetamine and ephedrine in positive urine samples were determined. For the purpose of convenience, the steps for determination of amphetamine, methamphetamine and ephedrine in this work are summarized in the scheme shown in Figure 2.2

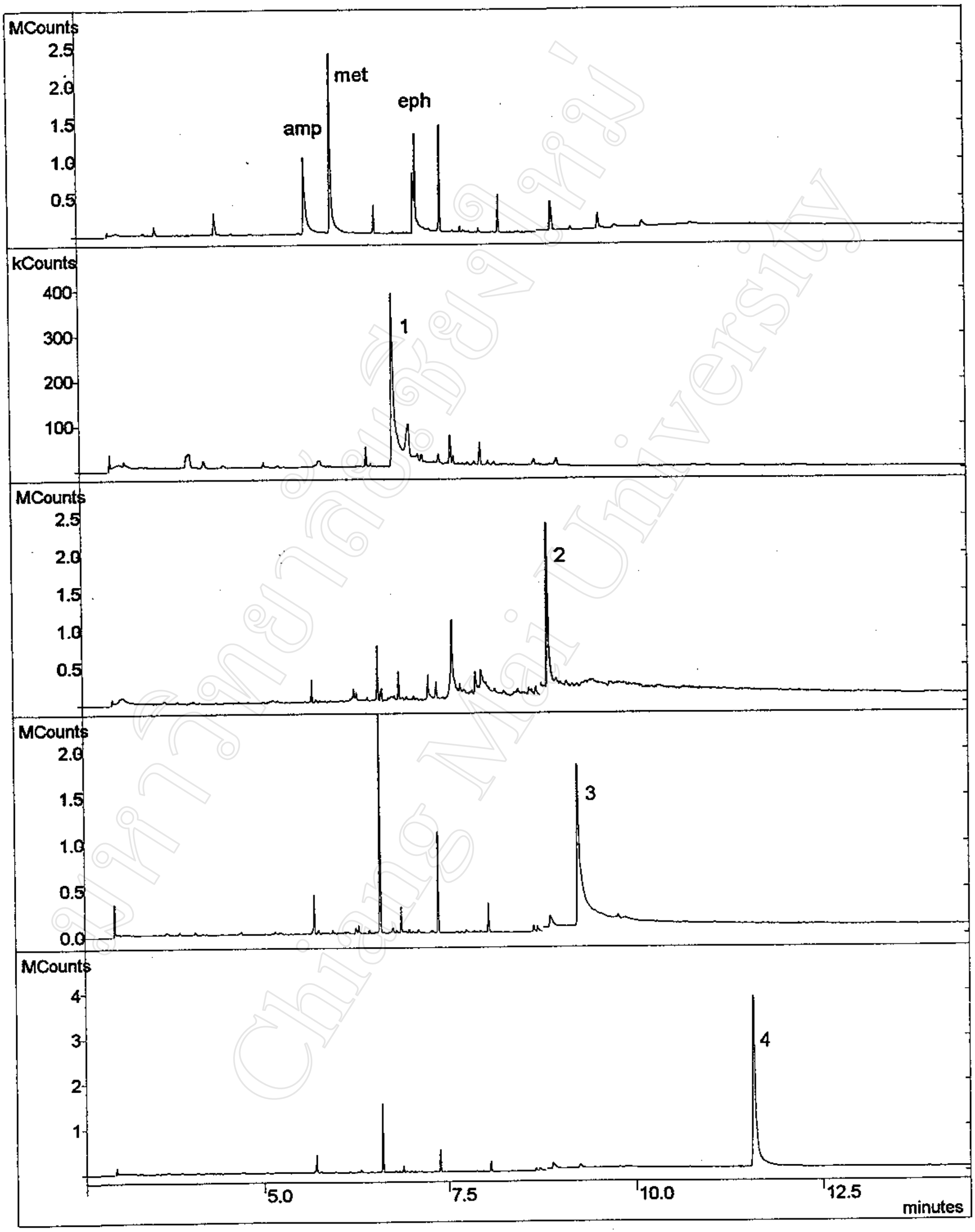

Figure 2.1 Chromatogram of selected drugs for internal standard selection

Peak identification: 1) phenylpropanolamine 2) phenylephrine

3) chlorphenniramine 4) bromhexine

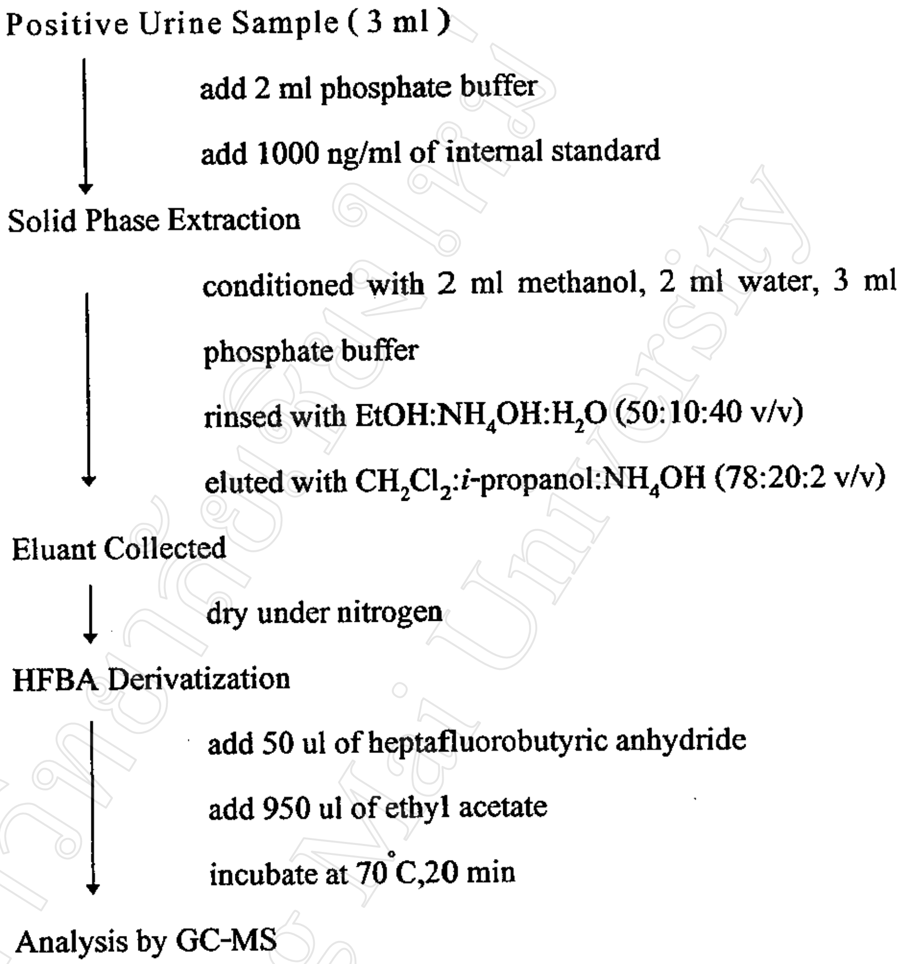

Figure2.2 Scheme of procedure for amphetamine, methamphetamine and ephedrine determination