CHAPTER 3
MATERIALS AND METHODS

1. Preparation of anhydrobarakol hydrochloride

The method used for preparation of anhydrobarakol hydrochloride was modified from Kaokeaw, 1993.

1.1 Plant materials

Fresh young leaves and flowers of Khi-lek (*Cassia siamea*, Lamk.) were obtained from Klongtoey market locates in Bangkok, Thailand. The plant was identified and confirmed by comparison with the herbarium specimens in the Botany Section, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand and with the Standard of Asean Herbal Medicine, Jakarta, Indonesia as reference.

1.2 Extraction and purification of anhydrobarakol hydrochloride

Three kilograms of fresh young leaves and flowers of *Cassia siamea*, Lamk. were blended into small pieces in a blender and mixed with sulfuric acid (0.5%, AR Grade, BDH Chemical, UK). The mixture was then heated until boiling and left to room temperature for 30-60 minutes prior to filtration. The supernatant was alkalinized with sodium bicarbonate (1N, AR Grade, Merck, Germany), and adjusted to pH 7-8 prior to extraction with chloroform (1 volume of supernatant : 2 volume of chloroform, AR Grade, Merck, Germany). The chloroform extract was evaporated under reduced pressure down to the volume of 200-300 milliliters, then, mixed witn equal volume of cold distilled water (4°C) and shaked vigorously until precipitation of the yellow lemon needled-shape crystals. The solution was stored in the refrigerator for 20-30 minutes for complete precipitation. The crystal was separated by filtration and purified by recrystallization in acetic acid (5%, AR grade, Merck, Germany) then, neutralized with strong ammonium hydroxide solution (AR Grade, Merck, Germany). The crystal was filtered and dissolved

in a few drops of ethanol (AR Grade, Merck, Germany). The concentrate hydrochloric acid (AR Grade, Merck, Germany) was added later. The presumed lemon yellow needle-shaped crystals of anhydrobarakol hydrochloride was slowly precipitated after administration of concentrate hydrochloric acid. Purification of the crystal was accomplished by recrystallized it in absolute ethanol (Figure 4).

1.3 The chemical structure identification of anhydrobarakol hydrochloride

The chemical structure of the presumed anhydrobarakol hydrochloride obtained from 1.2 was verified by the following procedures.

1.3.1   Infrared spectrum

The infrared spectrum of the purified sample was registerd in KBr pellets with Jasco IR-700 spectrophotometer.

1.3.2   Ultraviolet spectrum

The ultraviolet spectrum of the purified sample was determined in ethanol on a Shimudzu UV-160 Spectrophotometer.

1.3.3   NMR spectrum

^{1}H-NMR spectrum was recorded on a Cryomagnetic for spectroscopy BZH 200/52 (200 MHz) spectrometer, using tetramethylsilane (TMS) as the internal standard.

1.3.4   Mass spectrum

Mass spectrum was measured with JEOL JMS-DX 300 mass spectrometer.

The results obtained from each procedured were compared to those of previous works (Bycroft, 1970 and Kaokeaw, 1993).
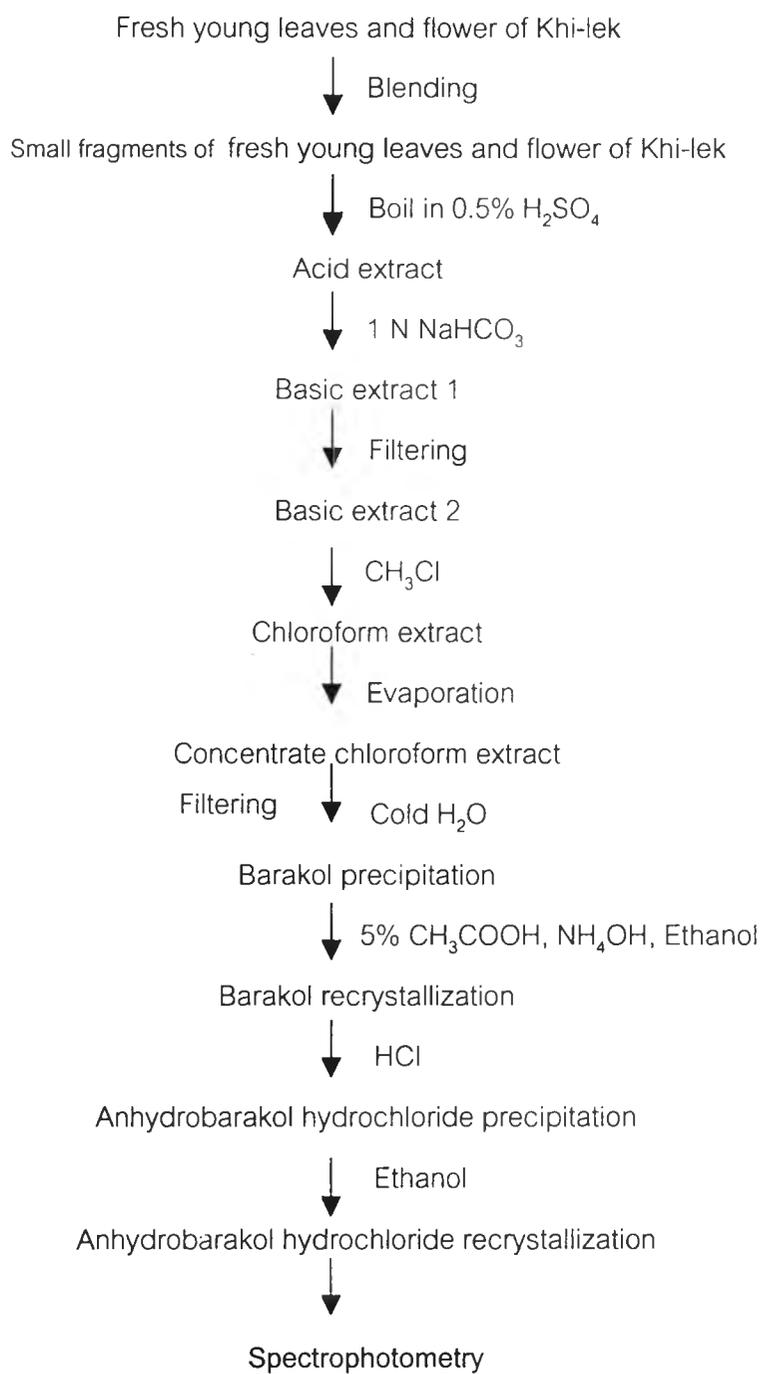
Fresh young leaves and flower of Khi-lek

↓ Blending

Small fragments of fresh young leaves and flower of Khi-lek

↓ Boil in 0.5% H_2SO_4

Acid extract

↓ 1 N $NaHCO_3$

Basic extract 1

↓ Filtering

Basic extract 2

↓ CH_3Cl

Chloroform extract

↓ Evaporation

Concentrate chloroform extract

Filtering ↓ Cold H_2O

Barakol precipitation

↓ 5% CH_3COOH, NH_4OH, Ethanol

Barakol recrystallization

↓ HCl

Anhydrobarakol hydrochloride precipitation

↓ Ethanol

Anhydrobarakol hydrochloride recrystallization

↓

Spectrophotometry

Figure 4 Flow chart diagram demonstrating a step-wise preparations of
anhydrobarakol hydrochloride

2. Effect of anhydrobarakol hydrochloride on rat locomotor activity

The test method was modified from that of Kaokeaw, 1993 and Lu 1998.

2.1 Animals

Male Sprague Dawley Rats weighing 250-300 gm. were kept on 12 hours light / 12 hours dark cycle and had free access to food and water until the start of experiments. Each experiment employed 6 rats.

2.2 Apparatus

The Opto-Varimex animal activity meter was used (Columbus Instruments, USA) for measure of locomotor activity (Figure 5). The apparatus consisted of an acrylic monitor cage (43.2 x 44.4 x 30.5 cm) which was surrounded by horizontal and vertical sensors and were detectable by rats. The apparatus also consisted of fifteen infared beams, spaced 2.65 cm apart on each axis. Two infrared beams were set as sensitivity of monitors. The cage was enclosed in a ventilated cabinet. Measurement was performed during 08.00-10.00 h.

2.3 Procedure

The experiment was carried out as the followings;

Day 1 : The rat was placed into the test chamber for 30 minutes as to allow it to get acquainted with new environmental condition. Then the rat was injected intraperitoneally (i.p.) with 1.0 ml normal saline and left for 60 minutes for locomotion activity recording. The number of activity counts were plotted against time at 10 minutes intervals.

Day 2 : The rat was injected intraperitoneally (i.p.) with 1.0 ml anhydrobarakol hydrochloride at doses of 10, 20, 40, 60, 80 and 100 mg/kg bodyweight and left for 60
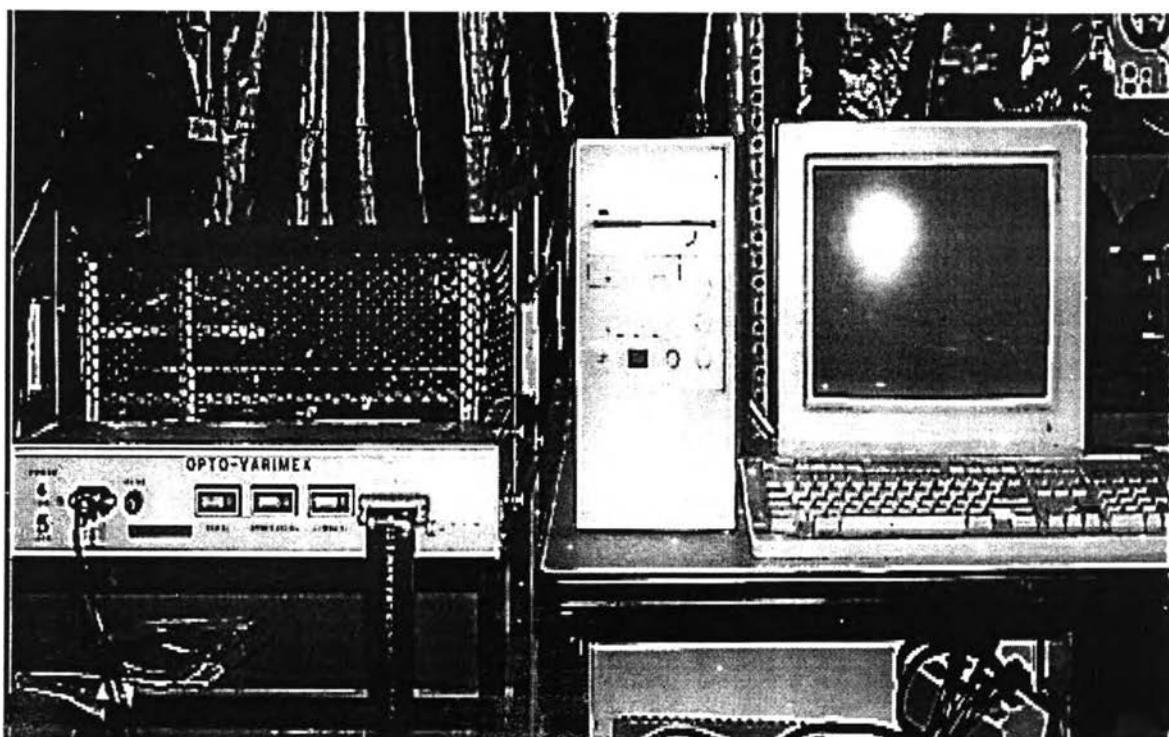
Figure 5 Opto-varimex system using for analysis of locomotor activity.

minutes for locomotion activity recording. The number of activity counts were plotted against time at 10 minutes intervals.

2.4 Statistical Analysis

Comparisons of mean value between the control and the treated rat were made calculated using paired t-test (two tailed) and were considered as significant difference when P values are less than 0.05. Data present as mean±standard error of mean (S.E.M).

3. Determination of monoamines in the brain nuclei samples by high performance liquid chromatography with electrochemical detection

The method used in this section was modified according to Palkovitz (1973) and Renner and Luine (1986).

3.1 Treatment of the rat

Male Sprague Dawley Rats weight 250-300 gm. were kept on 12 hours light / 12 hours dark cycle and had free access to food and water until the start of the experiments. The animals were separated into two main groups.

- Control group : On the day of experiment the animals were injected intraperitoneally (i.p.) with 1.0 ml normal saline.
- Treatment group : On the day of experiment the animals were injected intraperitoneally (i.p.) with 1.0 ml anhydrobarakol hydrochloride to give 10 mg/kg body weight or 60 mg/kg body weight dosages.

3.2 Instrumentation and chromatography

The chromatographic system was from GBC Scientific Equipment, Pty., Ltd., Australia. It consisted of a Model 1150 HPLC pump, LC 1260 EC detector, LC 1460

online solvent degasses, LC 1445 system organizer and Rheodyne syringe loading injector Model 7725i (Rheodyne L.P., C.A., U.S.A.). Column was GBC ODS2 C18, 5 μm, 46x150 mm with GBC ODS2 C18 guard column (Figure 6). Electrochemical detection was provided by glassy carbon and a Ag/AgCl electrode. The detector was set at +0.65 V and sensitivity at 1 nA.

Mobile phase was prepared from 6.8 g sodium acetate (HPLC grade, Merck, Germany), 100 mg disodium EDTA (HPLC grade, Merck, Germany), and 0.85 g heptane sulfonate (HPLC grade, Fluka, Switzerland). They were dissolved in 970 ml of double distilled deionized water. The pH was decreased to 3.5 with glacial acetic acid (AR grade, Merck, Germany), after which 40 ml of acetonitrile (HPLC grade, Merck, Germany) was added. The mobile phase was filtered under vacuum using a 0.2 μm nylon filter (Sartorius, Germany) and degassed. The mobile phase was recirculated and was only changed after 3-4 months of continuous use. The pump flow rate was only 1.0 ml per minute, and back pressure was approximately 1200 psi.

3.3 Standard monoamines

All standards were prepared in double distilled deionized water. Dopamine HCl (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), dihydroxybenzylamine HBr (DHBA), serotonin creatine sulfate (5-HT) and 5-hydroxyindole acetic acid (5-HIAA) were obtained from RBI (Research Biochemicals International, U.S.A.). Stock concentrations DA, DOPAC and DHBA were prepared in 0.1 M $HClO_4$ (Percholic acid, AR Grade, Merck, Germany). 5-HT and 5-HIAA were prepared in 0.9% saline solutions. Stock solutions of all standards were prepared at 1×10^{-3} M.

3.4 Method

After injection with normal saline or anhydrobarakol hydrochrolide for 30 minutes the rats were sacrificed by rapid decapitation at 09.00 hr. The brain was removed and quickly frozen on dry ice or liquid nitrogen. Then, the brain was cut as serial section at
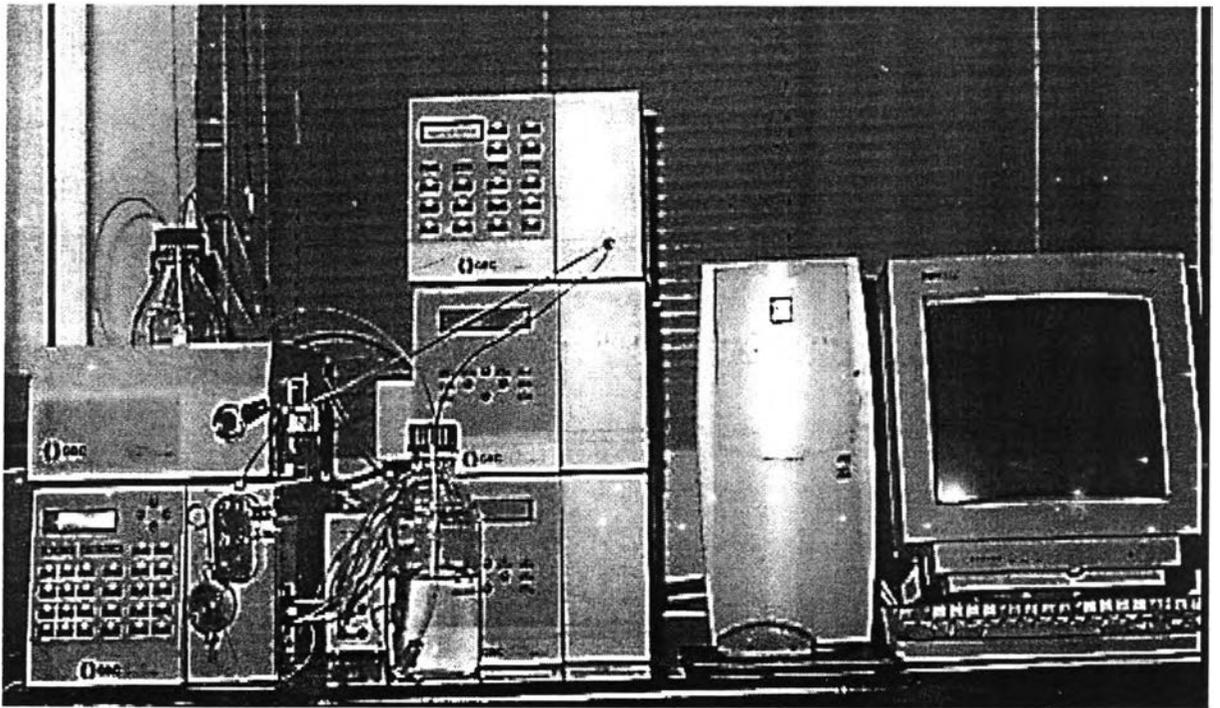
Figure 6 Chromatographic instrument used for quantitative analysis of

catecholamines.

300 μm in an Bright Cryostat at −10 °C beginning with bregma 3.2 mm according to the Paxinos and Watson rat brain atlas (Paxinos and Watson, 1982). The sections were thawed and mounted on glass slides and either punched or stored at −40°C until assayed. The brain nucleis were selected from previous work (Bhengsri, 1996) and removed by punching needle 1000 and 500 μm diameter at Caudate putamen (bregma 1.7 mm; head 1, 1.4 mm; head 2, -0.3 mm; body (middle) 1, -0.6 mm; body (middle 2); −1.3; tail 1 and −1.6; tail 2) (Figure 7), Hippocampus and Substantia nigra (bregma −4.8, -5.1 and −5.4 mm) (Figure 8), Dorsal raphe nuclei and Median raphe nuclei (bregma −7.3 and −7.6 mm) (Figure 8), raphe nucleus 1 (bregma −10.3) (Figure 8), and raphe nucleus 2 (bregma −12.8) (Figure 8). Tissue was expelled with 1.5 ml Ependorf tube containing $5x10^{-10}$ M DHBA in 60 μl of 0.1 M $HClO_4$ (AR Grade, Merck, Germany). The tube was frozen on dry ice, thawed and centrifuged at 17,000 rpm for 10 minutes at 2°C. Following centrifugation, the supernatant was removed and 40 μl was injected into HPLC-ECD system (Figure 9). The pellet was dissolved in 100 μl of 0.2 M NaOH (AR Grade, BDH, UK). Protein measurement was done according to the method of Bradford (1976).

3.5 Calculation

Amine concentrations were calculated from the standard values and corrected for percent recovery and injection volume using WinChrom Chromatography System Software version 1.3 (GBC Scientific Equipment, Pty., Ltd., Australia). The amine concentrations were divided by μg protein to yield pg amine/μg protein.

3.6 Statistical Analysis

Results as mean values were compared by using One-way Analysis of Variance (ANOVA). Significant different results in the ANOVA were further analyzed by post-hoc testing using Duncan-multiple range test at p-value $\leq$ 0.05. Data present as mean±standard error of mean (S.E.M).
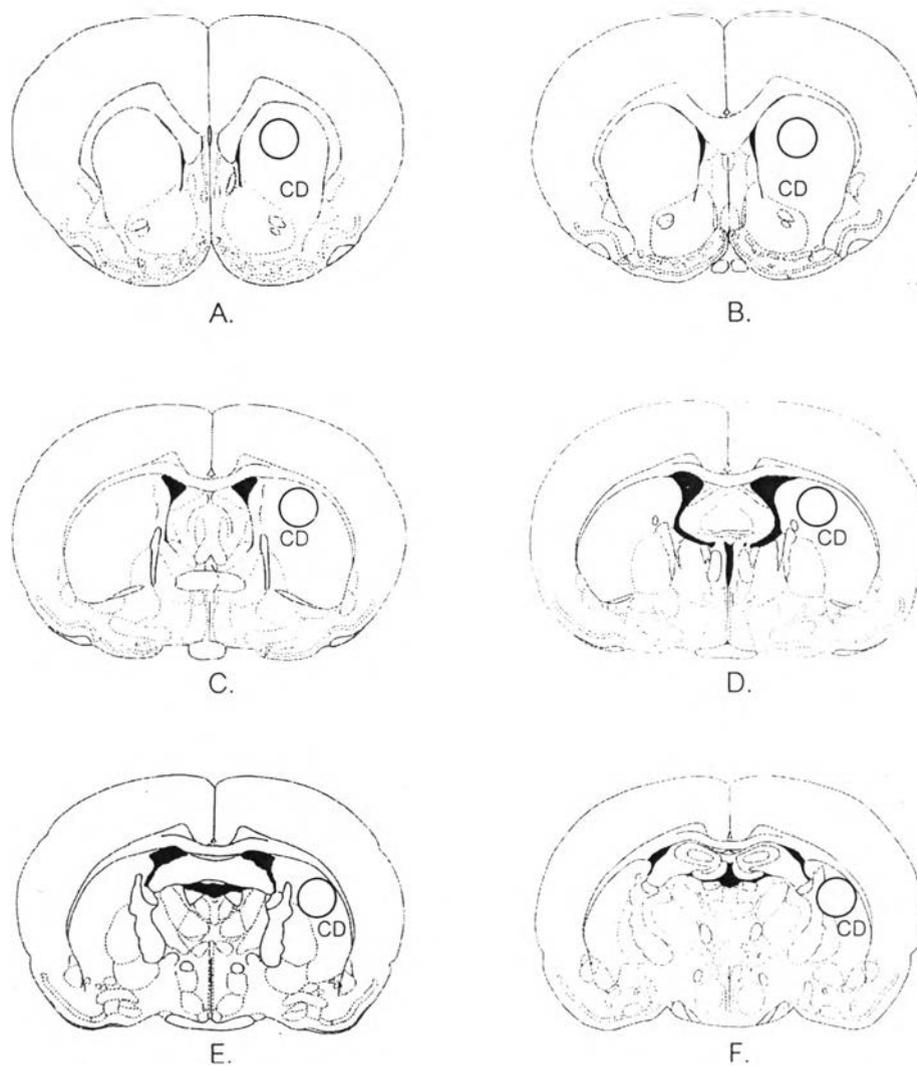
Figure 7 The illustration of selective brain section in any area, A. caudate (CD) head 1 (bregma; 1.7 mm), B. caudate head 2 (bregma; 1.4 mm), C. caudate middle 1 (bregma; -0.3 mm), D. caudate middle 2 (bregma; -0.6 mm), E. caudate tail 1 (bregma; -1.3 mm), F. caudate tail 2 (bregma; -1.6 mm). All punching areas (circle) diameter is 1,000 μm.
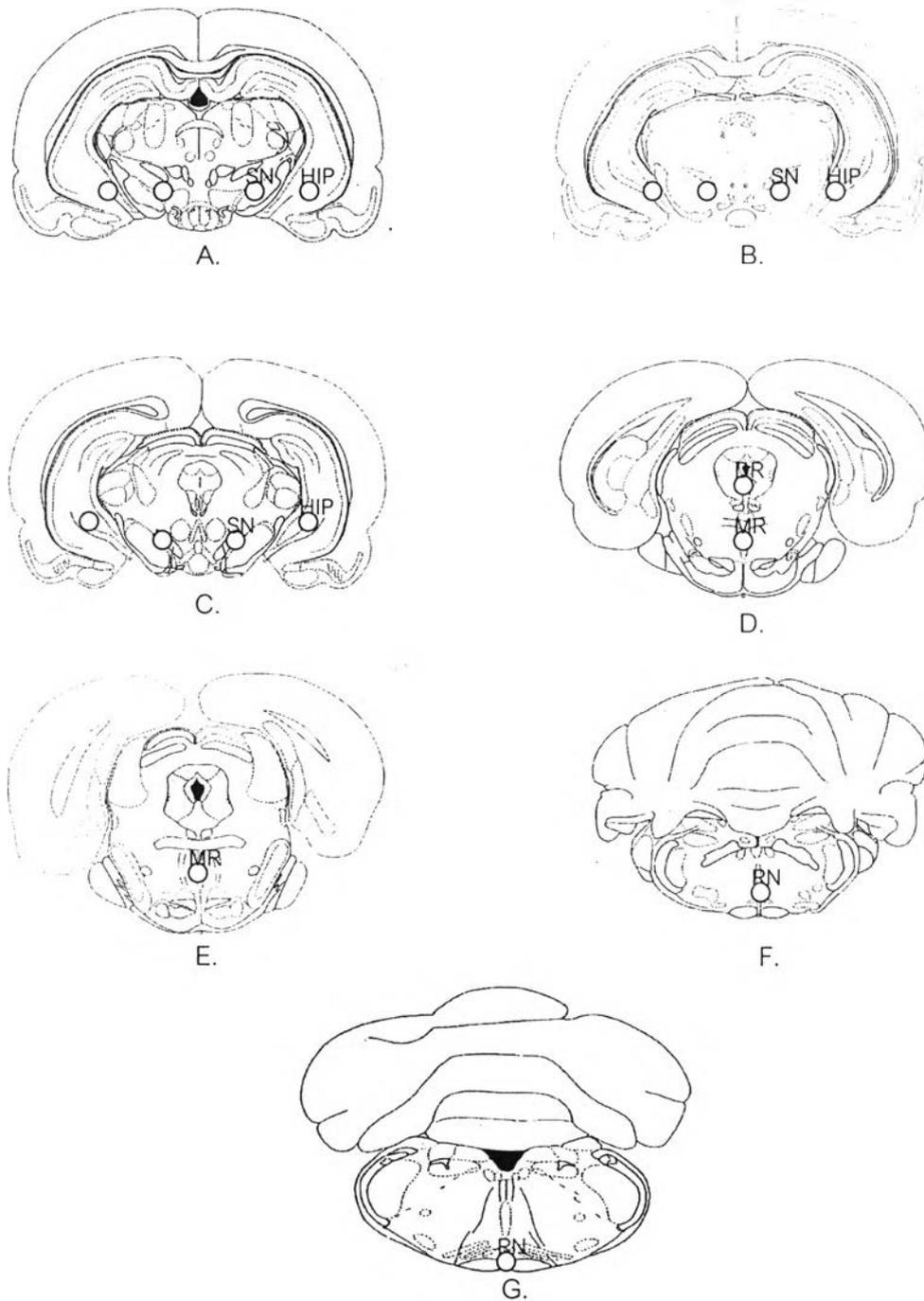
Figure 8 The illustration of selective brain section in any area, A. Substantia nigra (SN) 1 and Hippocampus (HIP) 1 (bregma; -4.8 mm), B. Substantia nigra 2 and Hippocampus 2 (bregma; -5.1 mm), C. Substantia nigra 3 and Hippocampus 3 (bregma; -5.4 mm), D. Dorsal raphe nuclei (DR) and Median raphe nuclei (MR) 1 (bregma; -7.3 mm), E. Median raphe nuclei 2 (bregma; -7.6 mm), F. Raphe nucleus 1 (bregma; -10.3 mm), F. Raphe nucleus 2 (bregma; -12.8 mm). All punching area (circle) diameter is 500 μm.
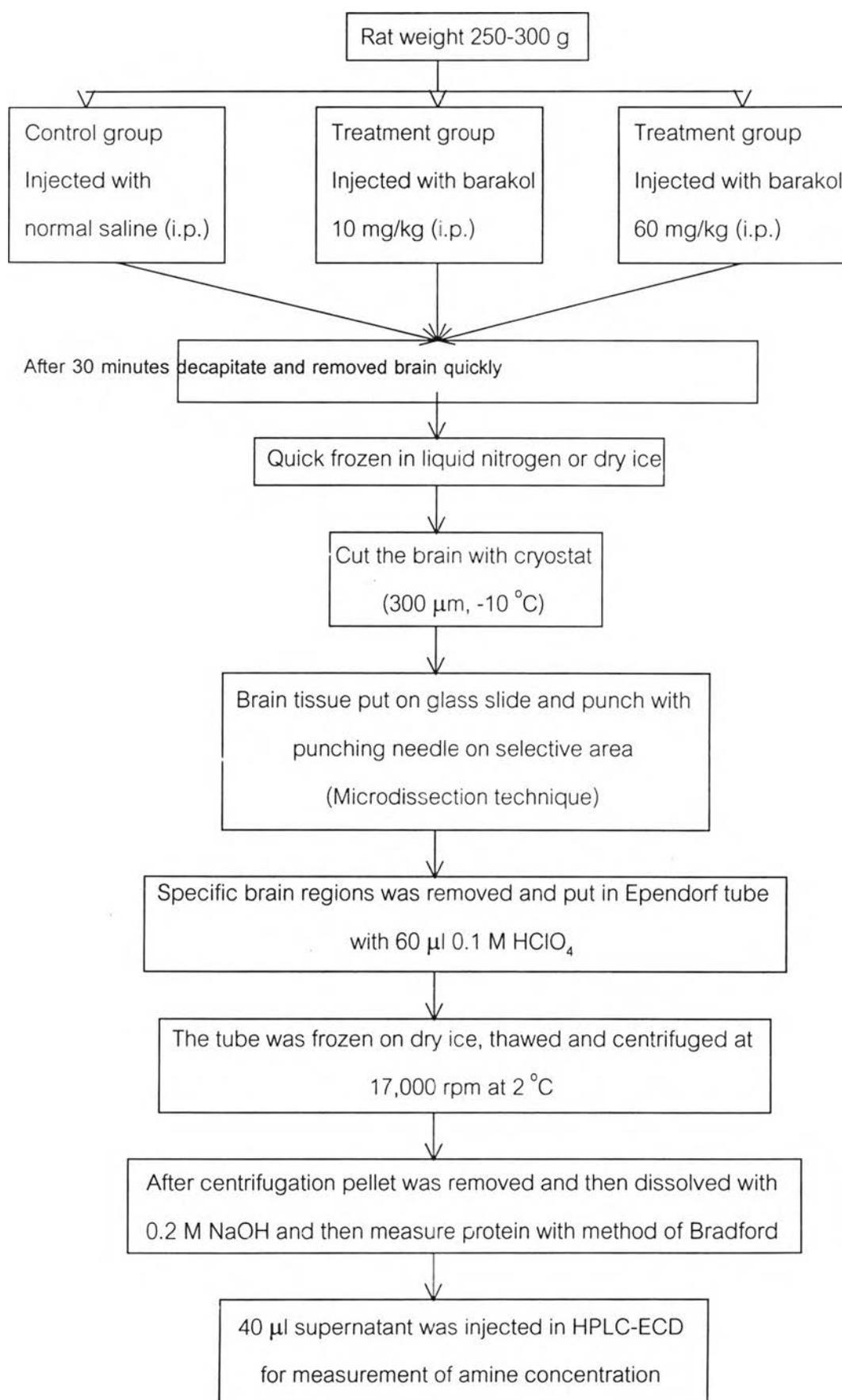
Figure 9 Flowchart diagram show step-wise of determination of monoamines in brain

nuclei by high performance liquid chromatography with electrochemical

detection.