# CHAPTER II

# MATERIALS AND METHODS

## Drugs and Reagents for Treatment

4-Acetaminophenol (Acetaminophen; N-acetyl-para-aminophenol) was purchased from Sigma (St. Louis, MO,USA) and propylene glycol (1,2-propanediol) from Carlo Erba.

## Animals and Treatment

### *Animals*

Adult male Wistar rats, supplied by Animal center at Salaya Campus, Mahidol University and weighing 180-200 g at the beginning of the experiments were used. Rats were housed and maintained in a room with a 12 hours light/dark cycle and provided with commercial rat chow and tap water *ad libitum* during the study.

### *Drug Treatment*

The rats, divided into several experimental groups of 5-6 animals each, received paracetamol or vehicle intraperitoneally (i.p.). Paracetamol, dissolved in vehicle, which consisted of 12.5% of 1,2-propanediol in 0.9% sterile saline was injected i.p. in a volume of 10 ml/kg. In all experiments, an equal volume of vehicle was used as control for injections and the same procedure was followed as for treated groups. To study the acute effect of drug administration, the rats were sacrificed after injection i.p. with

paracetamol 300 and 400 mg/kg for 90 min. In chronic experiment, the rats were sacrificed after injection i.p. with paracetamol 200, 300, and 400 mg/kg/day for 15 and 30 days. To minimize the influence of circadian rhythm, all groups of rats were treated at the same time of the day.

After the last injection, 90 min in acute treatment and 24 hr in chronic treatment, the rats were conducted to evaluate the antinociceptive activity by using the tail flick test. For studying 5-$HT_{2A}$ receptor sites and 5-HT uptake sites, the rats were sacrificed by decapitation. The brains were rapidly removed, dissected on a glass over an ice bath into two regions ; frontal cortex and brain stem by a modification of the method of Glowinsky and Iversen (1966). The brains were kept frozen at -80°C until required for analysis.

**The Tail-flick Test**

The tail flick test was performed using the Tail Flick Analgesia Meter of Harvard (England). It could be elicited under light anesthesia by injection with sodium pentobarbital (25 mg/kg) i.p. during the test. The tail flick test is a heat nociception test initially employed by D'Amour and Smith (1941) and further described in detail by Grossman et al. (1973) and Carlsson et al. (1986). The test is based on a segmentally mediated reflex movement of the tail.

The distal 4 cm portion of the rat tail was stained with a black ink marker pen in order to absorb a maximum amount of heat . The tail was then placed over the Tail Flick Analgesia Meter which the light of beam 2.5 ampere was focused to the rat tail. Latency to reflex response, i.e. the rat flicking away the tail, was the measured end point

in each exposure. Ten measurements were made in each animal with 3-minute intervals and the mean value of these measurements was used for calculation.

**Data Analysis of Tail-flick Test**

The tail flick latency in each group of treatment was measured as means ± S.E.M. Statistical significance was examined by using non-paired Student's *t*-test.

**Chemicals and Reagents for Studying 5-$HT_{2A}$ Receptor Sites and 5-HT Uptake Sites**

[phenyl-4-$^3$H]Spiperone (specific activity 23 Ci/mmol : used for studying 5-$HT_{2A}$ receptor sites) and [$^3$H]Imipramine (specific activity 20 Ci/mmol : used for studying 5-HT uptake sites) were purchased from Amersham International (Amersham U.K.) Ketanserin was gifted from Janssen Research Foundation (Bierse, Belgium). Fluoxetine HCl was purchased from RBI® . All other chemicals and reagents were of the purest commercially available grade, purchased mainly from E.Merck (Darmstadt, Germany) and May & Baker Ltd. (Dagenham, U.K.).

**Membrane Preparation for 5-$HT_{2A}$ Receptors**

Preparation of the membrane and radioligand binding assays of frontal cortex and brain stem was determined according to the method of Leysen et al.(1981) with minor modifications. The brains were weighed and homogenized in 1: 10 volume (wet g/volume) of ice cold 50 mM Tris HCl buffer pH 7.4 by a polytron homogenizer (Ultra Turrax T25) setting at 13,500 rpm for 10 second periods. The homogenate was

centrifuged at 900 x g at 4°C in a refrigerated centrifuge (Dupont, Sorvall RC 26 plus), the pellet was discarded, and the supernatant was further centrifuged at 40,000 x g for 15 minutes. The pellet was resuspended in 50 volumes of ice cold 50 mM Tris HCl buffer pH 7.4 and homogenized in the same way as above and centrifuged again at 40,000 x g for 15 minutes. The membrane pellet was kept at -80°C and used within two weeks for binding assays.

**Radioligand Binding Assays for 5-$HT_{1A}$ Receptors**

On the day of assay, the frozen membranes were allowed to thaw and then resuspended in ice cold 50 mM Tris HCl salt buffer pH 7.4 containing 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (Greese et al., 1977) and homogenized for 10 seconds with a polytron homogenizer. Seven concentrations of 50 µl of [$^3$H]spiperone was added to the suspension of membrane buffer with or without 100 µM of ketanserin to a final volume of 0.5 ml. [$^3$H]spiperone was added in concentrations ranging from 0.2 to 10 nM in frontal cortex and 0.2 to 8 nM in brain stem. Ketanserin was used to define nonspecific binding. Duplicate samples were incubated for 30 minutes at 37°C in a shaking water bath by which time of binding equilibrium with the ligand was reached. The bound ligand was separated by rapid filtration method. The aliquots were filtered through Whatman GF/C or GF/B glass fiber filters by vacuum pump. The filters were washed twice by two rinses with 3-5 ml of ice cold 50 mM Tris HCl salt buffer pH 7.4. The radioactivity trapped on the filters was counted by a liquid scintillation counter (Beckman LS 625) in 5 ml of scintillation fluid containing of 2,5-diphenloxazole (5g/l) and 1,4-bis(5-phenoxyoxazole-2-yl)benzene (0.1g/l) in toluene containing 35% Triton-X 100. The total binding and non-specific binding were performed in duplicate in the absence and presence of 100 µM

ketanserin respectively. Specific binding was defined as the binding obtained by subtracting the non-specific binding from the total binding. The protein concentrations of the membranes were determined by the method of Bradford et al., (1976) with bovine serum albumin as standard.

**Data analysis of 5-$HT_{2A}$ receptor sites**

The saturation curve was analyzed by the method of Scatchard and then using the non-linear least square regression analysis computer program, LIGAND (Munson and Rodbard, 1980) for analyzing the relationship between bound/free versus bound fraction. The dissociation equilibrium constant ($K_d$) and the maximum number of binding sites ($B_{max}$) were obtained. Statistical evaluation of the results was performed and compared by using the non-paired Student's *t*-test.

**Membrane Preparation for 5-HT Uptake Sites**

Preparation of the membrane and radioligand binding assays of frontal cortex and brain stem was determined according to the method of Raisman et al.(1980) with minor modifications. The brains were weighed and homogenized in 1: 10 volume (wet g/volume) of ice cold 50 mM Tris HCl buffer pH 7.4 containing 120 mM NaCl and 5 mM KCl by a polytron homogenizer (Ultra Turrax T25) setting at 13,500 rpm for 10 second periods. The homogenate was centrifuged at 900 x g at 4°C in a refrigerated centrifuge (Dupont, Sorvall RC 26 plus), the pellet was discarded, and the supernatant was further centrifuged at 40,000 x g for 15 minutes. The pellet was resuspended in 50 volumes of ice cold 50 mM Tris HCl buffer pH 7.4 and homogenized in the same way

as above and centrifuged again at 40,000 x g for 15 minutes. The membrane pellet was kept at -80°C and used within two weeks for binding assays.

**Radioligand Binding Assays for 5-HT Uptake Sites**

On the day of assay, the frozen membranes were allowed to thaw and then resuspended in ice cold 50 mM Tris HCl buffer pH 7.4 containing 120 mM NaCl and 5 mM KCl (Raisman et al., 1980) and homogenized for 10 seconds with a polytron homogenizer. Seven or eight concentrations of 35 µl of [$^3$H]imipramine was added to the suspension of membrane buffer with or without 100 µM of fluoxetine to a final volume of 0.25 ml. [$^3$H]imipramine was added in concentrations ranging from 0.2 to 6 nM in frontal cortex and 0.1 to 4 nM in brain stem. Fluoxetine was used to define nonspecific binding. Duplicate samples were incubated for 60 minutes at 0°C in ice box by which time of binding equilibrium with the ligand was reached. The bound ligand was separated by rapid filtration method. The aliquots were filtered through Whatman GF/C or GF/B glass fiber filters by vacuum pump. The filters were washed twice by two rinses with 3-5 ml of ice cold 50 mM Tris HCl buffer pH 7.4 containing 120 mM NaCl and 5 mM KCl. The radioactivity trapped on the filters was counted by a liquid scintillation counter (Beckman LS 625) in 5 ml of scintillation fluid containing of 2,5-diphenloxazole (5g/l) and 1,4-bis(5-phenoxyoxazole-2-yl)benzene (0.1g/l) in toluene containing 35% Triton-X 100. The total binding and non-specific binding were performed in duplicate in the absence and presence of 100 µM fluoxetine respectively. Specific binding was defined as the binding obtained by subtracting the non-specific binding from the total binding. The protein concentrations of the membranes were

determined by the method of Bradford et al., (1976) with bovine serum albumin as standard.

**Data Analysis of 5-HT Uptake Sites**

The saturation curve was analyzed by the method of Scatchard and then using the non-linear least square regression analysis computer program, LIGAND (Munson and Rodbard, 1980) for analyzing the relationship between bound/free versus bound fraction. The dissociation equilibrium constant ($K_d$) and the maximum number of binding sites ($B_{max}$) were obtained. Statistical evaluation of the results was performed and compared by using the non-paired Student's *t*-test.

**Chemicals and Reagents for HPLC Experiment**

All compounds used in the HPLC experiment were the HPLC or analytical grades. They were obtained from the commercial sources. Serotonin(5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA), octane sulfonic acid, sodium salt were obtained from Sigma (St. Luis, MO, USA); methanol, HPLC grade from Fisher Scientific (Fair Lawn, NJ, USA); sodium metabisulfite, sodium dihydrogen phosphate and sodium citrate from J.T. Baker (Philipsburg, NJ, USA); disodium ethylenediamine-tetraacetate from Mallinckrodt(St. Luis, MO, USA); perchloric acid from Farmitalia Carla Erba; sodium hydroxide and acetonitrile, HPLC grade from Merck (Germany). Water was distilled and filtered through Millipore all-glass filter apparatus (filter type, GV; pore size 0.22 μm).

**Standard Preparations**

**Stock standard solutions**

The stock solutions of the standard (5-HT, 5-HIAA and DHBA), 1 mg each, were prepared in 1 ml of 0.1 mM perchloric acid containing 0.1% sodium metabisulfite and 0.01% disodium EDTA. The amount of each compound was calculated as free base compound. All stock solutions were stored at -20°C.

**Working standard solutions**

The working standard solutions was diluted from 10 µl of 1 mg/ml stock standard solutions with 0.1 mM perchloric acid containing 0.1%sodium metabisulfite and 0.01% disodium EDTA to make final concentrations for 5-HT, 5-HIAA and DHBA. The working standard solutions was prepared freshly for each experiment.

**Chromatographic System**

The HPLC-ECD was a model M6000A solvent delivery system and a model 712 WISP Autosampler injection system (Water Assoc., Milford, MA, USA). The column was 12 cm x 4.6 mm I.D. stainless-steel packed with octadecylsilane (C18) on micropar-ticulate (3 µm particle size) silica gel (Li Chrosper® 100 RP 18, Merk Germany) and guarded with a 4 x 4 mm guard column. The electrochemical detector was a glassy carbon working electrode BAS LC-4B amperometric detector. The applied potential versus an Ag/AgCl reference electrode was 0.8 V. The peak height or peak area would be analyzed by computer.

The mobile phase was composed of 0.1 M monobasic sodium phosphate, 1 mM disodium EDTA, 1 mM sodium octanesulfonate and 4 % acetonitrile (adjusted pH 4 with saturated citric acid). Mobile phase was filtered through a 0.22 μM GV-filter type and degassed by ultrasonic agitation. All separations were performed isocratically at a flow rate 0.6 ml/min.

**Sample Collection and Platelet Preparation**

After 24 hr from the complete dose of treatment, the rats were anaesthetized by inhalation of ethyl-ether and approximately 10 ml of blood per animal was colleted from the heart into a plastic syringe containing 2 ml of anticoagulant (1% EDTA and 3.2% citric acid trisodium salt). Blood samples were centrifuged at 200 x g 5 min at room temperature, platelet rich plasma (PRP) was removed by a plastic pipette. This process was repeated three times. An aliquot, 1 ml of PRP was taken in eppendorf tube with the addition of 100 μl preserving solution containing 0.1 mM perchloric acid, 0.1% sodium metabisulfite and 0.01% EDTA and stored at -80°C for analysis of 5-HT and 5-HIAA by HPLC-ECD. The remainder of PRP was counted under the light microscopy using a x 40 objective and x 10 eye-pieces.

**Sample Preparation for Detection of 5-HT and 5-HIAA**

At the time of the experiment, frozen PRP samples were thawed at room temperature and 40 μl of 65 g/L perchloric acid solution was added to each tube to deproteinize the samples, plus 50 μl of 5 mM sodium metabisulfite and 50 μl of 10 μg/ml DHBA. The solution was vortexed for 10 seconds and precipitated for at least 2

hrs, centrifuged at 15,000 x rpm for 20 min at 4°C. The clear supernatant was then passed through filter before being injected 20 µl into HPLC system.

The retention time for 5-HIAA and 5-HT was 4.2 and 12 min, respectively. The 5-HIAA and 5-HT concentrations were expressed as ng/$10^8$platelets. All analyses were performed twice for each aliquots used.

**Calculation of the Levels of 5-HT and 5-HIAA**

All quantitations were based on peak heights of the chromatogram. By using external standard, concentrations were calculated by using the slope of the standard curve and intercept to the standard curve of each compound. Standard curves were prepared by analyzing a varying amounts of standard compounds.

**Statistical Analysis for HPLC Data**

Linear regression was applied for the standard curves of all compounds. All values were presented in means ± S.E.M. Statistical significance was examined by using non-paired Student's *t*-test.