Chapter III

Materials and Methods

Animals

The experiments were performed on either male Swiss mice or male Wistar albino rats weighing 18-25 g and 280-320 g, respectively. Both of them were obtained from the National Laboratory Animal Center, Mahidol University, Nakorn Pathom. The animals were acclimatized in the laboratory for a week before the experiments started. In behavioral studies, the experiments with mice were completed within the week to minimize the effect of increasing age on seizure susceptibility (Loscher and Nolting, 1991). The experimental animals were received standard pellet diet (F.E. Zeullig) and tap water ad libitum under natural light/dark condition. All experiments were carried out between 8.00 a.m.- 6.00 p.m. except for the sleeping time and locomotor experiments which were carried out between 8.00 - 12.00 a.m.

Equipments

1. Electroshock apparatus with corneal electrode (King Mongkut Institute of Technology , North Bangkok, Thailand)
2. Rotorod (King Mongkut Institute of Technology, North Bangkok, Thailand)
3. OPTO-VARIMEX(Columbus Instruments International Co., U.S.A.)
4. HPLC system .
   - Pump with gradient system (Thermo Separation Products,U.S.A.)

- C_{18} Reverse-phase column 250 x 4.6 mm., particle size 5 μm, Spherisorb ODS2 (Phenominex®, U.S.A.)
- Guard column with packing material., particle size 5 μm, Spherisorb ODS2 (Phenominex®, U.S.A.)
- Column oven (Model 2155, LKB)
- Fluorescence detector (Jasco FP-210, Japan)
- Analog Digital Instruments (Maclab™/4, AD instruments, Australia)
- Macintosh® computer (Model LC475, Apple computer, Inc., U.S.A.) with Chart™ v. 3.2.8 program for data recording system and Peak™ v. 1.3 for processing system .

5. Sterotaxic apparatus (Narishige, Japan)
6. Automatic infusion pump (CMA100, Carnegic, Sweden)
7. Horizontal microdialysis probe (Homofilter PNF-140, Asahi Medical Co., Tokyo, Japan)
8. pH meter (Suntex,Japan)

Chemicals

1. (N-Hydroxymethyl)-2-propylpentamide (HMV) was kindly supplied by Assist. Prof. Dr. Chamnan Patarapanich and co- worker (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University).
2. Valproic acid (VPA ; Aldrich, U.S.A.)
3. Polyethylene glycol 400 (PEG400 ; Witayasum, Thailand)
4. Pentylenetetrazole (PTZ ; Sigma Chem.Co.,USA)
5. Pentobarbital sodium (PB ; Nembutal®, Sanofi (France) Ltd.)
6. Methanol HPLC grade (E.Merck, Germany)

7. di-Sodium hydrogen phosphate-2-hydrate (Na_2HPO_4 $2H_2O$; Ridel de Haen, Germany)
8. Sodium dihydrogen phosphate-2-hydrate (NaH_2PO_4 . $2H_2O$; Ridel de Haen, Germany)
9. Sodium chloride (NaCl ; Ridel de Haen, Germany)
10. Sodium hydrogen carbonate ($NaHCO_3$; Ridel de Haen, Germany)
11. Calcium chloride-2-hydrate ($CaCl_2$ $2H_20$; Ridel de Haen, Germany)
12. Potassium chloride (KCl, Ridel de Haen ; Germany)
13. Magnesium sulfate-6-hydrate ($MgSO_4.6H_2O$;Ridel de Haen,Germany)
14. D(+)-Glucose monohydrate (Ridel de Haen ; Germany)
15. Boric acid (H_3BO_3 ; E.Merck, Germany)
16. o-Phthaldialdehyde (OPA ; Sigma Chem.Co.,U.S.A)
17. 2-Mercaptoethanol (Sigma Chem.Co.,U.S.A .)
18. l-Homoserine (Sigma Chem.Co.,U.S.A .)
19. γ-Amino-n-butylic acid (GABA ; Sigma Chem.Co.,U.S.A .)
20. Ethanol Absolute (E.Merck, Germany)

Preparation and administration of the test substances

The test substances (VPA and HMV) which are insoluble in water were dissolved in PEG400 and the ones soluble in water (PTZ, strychnine, bicuculline and PB) were dissolved in 0.9% Sodium Chloride (NSS). They were administered either intraperitoneally or orally (by means of gavage tube) except PTZ which was given by a subcutaneous (s.c.) injection. In mice, the volumes of an intraperitoneal (i.p.) and oral.(p.o.) administration were 0.1 ml/25 g B.W. and 0.3 ml/25 g B.W. respectively, while the volume of intraperitoneally injection in rats was 0.4-0.8 ml.

Experimental methods

1. Anticonvulsant Activity

1.1 Anticonvulsant activity against Maximal Electroshock Seizure Test (MES)

The MES was elicited by passage of an alternating electric current (current = 55 mA., frequency = 50 Hertz., duration = 0.2 sec.) from electroshock apparatus through the brain via corneal electrodes after the pretreatment with test substances. Convulsive seizure is characterized by an extensor thrust of the forelimbs and hindlimbs of the animal in rigid manner (tonic convulsion), tonic flexion changing into tonic extension of the hindlimbs within 0.2-0.5 sec. and then into generalized clonic convulsions (a continuous cycling motion of the extremities) followed by depression and recovery. The end point of test was generalized seizure with tonic hindlimb extension exceed a 90 ^{o} angle with the plane of the body (Swinyard and Woodhead, 1982).

The pretreated substance was considered to possess anticonvulsant activity if no clonic-tonic seizure was observed immediately after the electroshock.

1.2 Determination of the optimal pretreated time and anticonvulsant activity against MES

1.2.1 The peak time of maximal anticonvulsant activity which would be subsequently used as the optimal pretreated time was performed on mice. They were divided into 3 groups according to pretreated time (15, 30 and 60 min). Each

group of mice was then divided into 10 subgroups of 8 animals each. Two subgroups (NSS and PEG400, 0.1 ml/25 g B.W. i.p.) were used as control groups. The other 8 subgroups were used for determination of anticonvulsant activity, expressed as the median effective dose (ED_{50}) against MES, of test substances. VPA (100, 200, 300, 400 mg/kg B.W.) and HMV (50, 75, 100, 150 mg/kg B.W.) were intraperitoneally injected into the animals. When pretreated time was due, MES was performed by previously selected current (see 1.1). The minimum pretreated time that gives the maximal anticonvulsant activity of each test substance was selected and used as optimal pretreated time in other experiments.

1.2.2 Anticonvulsant activity of test substances, orally given(p.o.), against MES was performed on 11 groups of 8 mice each. Two groups (NSS and PEG400, 0.3 ml/25 g B.W. p.o.) were used as control groups. The other 8 subgroups were used for the determination of the ED_{50} of the test substances. VPA (200, 400, 500, 800 and 1000 mg/kg B.W.) and HMV (50, 75, 150 and 300 mg/kg B.W.) were given orally by gavage tube. After the optimal pretreated time selected according to 1.2.1 MES was performed as previously described in 1.1

1.3 Anticonvulsant activity against Pentylenetetrazole Seizure Test (PTZ)

1.3.1 The PTZ seizures were elicited by a subcutaneous injection of PTZ 70 mg/kg B.W. to the animals. The end point of this chemoshock test was a generalized clonic seizure with loss of righting reflex within 60 min after the injection of PTZ (Loscher et al.,1991).

The pretreated substance was considered to possess anticonvulsant activity if no generalized clonic seizure with loss of righting reflex occurred within a period of 60 min after the injection of PTZ.

1.3.2 Anticonvulsant activity of test substances against PTZ was performed on 2 sets of animals assigned for the oral (given by gavage tube) and intraperitoneal administration of the test substances. One set of animals was divided into various groups of 8 mice each, two groups (NSS and PEG400, 0.1 ml/25 g B.W. i.p. or 0.3 ml/25 g B.W p.o.) were used as control groups. The other 8 subgroups were used for the determination of the ED_{50} against PTZ of the test substances. VPA (50, 75, 100, 200, 250 mg/kg B.W., i.p and 125, 250, 375, 500 mg/kg B.W., p.o.) and HMV (25, 50, 75, 100 mg/kg B.W., i.p and 25, 50, 100, 200 mg/kg B.W, p.o.) were administered to different groups of animals. The PTZ test was performed after the optimal pretreated time (see 1.2.1) was due.

1.4 Anticonvulsant activity against Strychnine convulsion

Strychnine convulsion was induced by an intraperitoneal injection of strychnine sulfate 2 mg/kg B.W. after the pretreated time of test substances. The end point of strychnine convulsion was generalized seizure with tonic extension in 30 min after the injection of strychnine (Ticku and Rastogi, 1986).

1.5 Anticonvulsant activity against Bicuculline convulsion

Bicuculline convulsion was induced by an intraperitoneal injection of bicuculline 8 mg/kg B.W. after the pretreated time of test substances. The endpoint of bicuculline convulsion was generalized seizure with tonic extension of the hindlimbs in 30 min after the injection of bicuculline (Ticku and Rastogi, 1986).

2. Toxicity

2.1 Acute Toxicity test

Mice were divided into 6 groups containing 8 mice each for the determination of the median lethal dose (LD_{50}). Various doses of test substances (VPA 400, 800, 1600 mg/kg B.W. and HMV 400, 800, 1400 mg/kg B.W.) were intraperitoneally injected. General changes such as ataxia, sedation, hypnosis, respiratory secretion, etc. were observed and lethality within a period of 72 hours was noted.

2.2 Rotorod test

The rotorod test was modified from the one previously described by Dunham and Miya (1957), carried out with a rod of 3.5 cm diameter, rotating at 12 rev/min. Neurological deficit was indicated by inability of the animals to maintain their equilibrium for at least 1 min on the rotating rod. Untreated mice were able to maintain their balance on the rod for several minutes. Substance or vehicle-treated mice which were not able to maintain their equilibrium on the rod for 1 min were put back on the rod twice. Only animals which were not able to remain on the rod for 1 min in each of trials were considered to exhibit neurological deficit.

Neurotoxicity of test substances was performed on 10 groups of 8 mice each. Two groups (NSS and PEG400, 0.1 ml/25 g B.W. i.p.) were used as control groups. The other 8 groups were used for the determination of the TD_{50} of the test substances. VPA (100, 200, 400 600 mg/kg B.W.) and HMV (37.5, 75, 150, 200 mg/kg B.W.) were given intraperitoneally. After the optimal pretreated time selected according to 1.2.1, rotorod test was performed as previously described.

2.3 Locomotor Activity Test

Locomotor activity was assessed by OPTO-VARIMEX activity meter which is a plastic box (17 x 17 x 8 inches) equipped with two of couple detectors which are placed perpendicular to each other. Each movement of animal interrupts infra-red beams generated from these detectors and electric impulse signals were registered and processed by a personal computer (IBM compatible, 486 DX, Acermate, Acer peripherals Inc.,Taiwan).

Mice were divided into 6 groups of 8 animals each. Two groups (NSS and PEG400, 0.1 ml/25 g B.W. i.p.) were used as control groups. The others 4 groups (VPA 100, 200 mg/kg B.W. and HMV 35, 75 mg/kg B.W. i.p.) were used to test the effect on locomotor activity. After each mouse was placed singly in the OPTO-VARIMEX activity cage for familiarization with the environment for 45 min, the test substance was given and allowed the animal back into the cage immediately in order to record movement for another 90 min.

2.4 Barbiturate potentiation Test

The effect of test substances on barbiturate sleeping time was used to evaluate the depressing effect on CNS. In order to induce a loss of righting reflex, Pentobarbital sodium (PB) 50 mg/kg B.W. i.p. was given at the pretreated time to the animals after the administration of the test substances. The inability and ability of the animal to upright itself within 5 sec when placed on its back in three successive trials is taken as the criterion of loss and recovery, respectively, of the righting reflex (Thompson, 1990).

Mice were divided into 6 groups of 8 animals each. Two groups (NSS and PEG400, 0.1 ml/25 g B.W. i.p.) were used as control groups. The others 4 groups (VPA 100, 200 mg/kg B.W. and HMV 35, 75 mg/kg B.W. i.p.) were used to test for the potentiation of barbiturate sleeping time. The test substances were administered at the pretreated time described in 1.2.1. Then, the animals were observed continuously for the onset and duration of the loss of righting reflex after the administration of PB.

3. Effects on the rat cortical GABA

3.1. Microdialysis Experiment

The rat was initially anesthetized by PB 40 mg/kg B.W., i.p. and was maintained by supplementary doses of PB. The rat was fixed in the stereotaxic apparatus. An outer surface of microdialysis probe (outer diameter 0.2 mm., acrylic copolymer with a 50,000 molecular weight cut off) was totally covered with epoxy resin except the area 5 mm in length that contact the cortex of rat. The probe was implanted transversely into the cerebral cortex at the coordinates 2 mm. rostral to the bregma and 1-1.5 mm depth below the cerebral surface from dura of the cortex according to the brain atlas (Pellegrino, Pellegrino and Cushman ,1979). One side of probe was connected to a constant-flow perfusion pump by polyethylene tube, and the other side was placed into a collecting tube. The artificial cerebrospinal fluid (aCSF) pH 7.3 contained NaCl 120, $NaHCO_3$ 15, KCl 5 $CaCl_2$ 15, $MgSO_4$ 10 and glucose 60 mM (Benveniste and Huttemeier, 1990) was perfused into the probe by a perfusion pump at the constant flow rate of 2 μl/min. The dialysate emerging from the other end was collected every 20 min.

After surgery, the animal was left for an equilibration period of 60 min before the first dialysate was collected. The basal GABA level was determined from the first three successive dialysate samples collected (20 min for each collection). NSS, PEG400, VPA (100, 200 mg/kg B.W.) or HMV (75, 150 mg/kg B.W.) were intraperitoneally injected to the rats and the dialysate samples were continuously collected for another three hours. Determination of amino acid levels was done by means of high performance liquid chromatography (HPLC) technique.

After the experiment, the brain was excised to confirm the position of microdialysis probe by sectioning the specimen with a sharp blade and then inspected visually. The data were valid only when the right position of microdialysis probe was confirmed.

3.2 The HPLC Determination of GABA Level.

The experimental method used to determine the levels of rat cortical GABA was the precolumn fluorescence derivatization with o-Phthaldialdehyde (OPA) (Lindroth & Mopper ,1979). The mobile phase used was gradient run between 0.05 M phosphate buffer pH 7.3 in triple distilled water and methanol and degased with continueous helium gas. For the phosphate buffer, the pH was set by mixing equimolar solutions of Na_2HPO_4 and NaH_2PO_4 in different proportions. For gradient run, the mobile phase gradient was increased from 20% to 60% methanol in one linear step at the increment rate of 2% /min. and flow rate of 1 ml/min. Then, it was decreased from 60% to 20% methanol within 5 minutes and equilibrate time was about 10 minutes.

The buffer reagent solution of OPA was prepared by dissolving OPA (270 mg) in 5 ml absolute ethanol and adding 200 μl of 2-mercaptoethanol and then adjusting the volume to 50 ml with borate buffer pH 9.5 (H_3BO_3 0.4 M, pH adjusted with 1 M NaOH).

The GABA derivatization was accomplished by mixing a perfusate sample or GABA with 2 μl of Homoserine (internal standard) and OPA-buffer reagent solution in a volume ratio of 1:1:5 for exactly 2 min. prior to injection into the injector loop (50 μl) of HPLC system.

The Fluorescence detector was set by adjusting an emission wave length to 418 nm and an excitation wavelength to 330 nm. Analogue data from the detector were changed to digital data by MacLab, recorded by Chart program v. 3.2.8 and the area under curve was analyzed by Peak program v.1.3.

Calculation and Statistical Analysis

1. For the determination of ED_{50}, TD_{50} and LD_{50} , groups of 8 mice each were used to test the effect of test substances at various doses until at least 3 points were established between the limit of 0-100% response or non response and transform to probit unit by transformation table of Fisher and Yates (Diem and Lentner, 1972). The linear regression method was used to fit a curve between probit unit of response or non response and dose (log scale) by using Crikcet graph program (Macintosh® computer) The 95% confidence interval was calculated by the method of Litchfield and Wilcoxon (1949).

2. Statistical analysis was carried out using SPSS/PC+(1991) software. All numerical data are expressed as mean ± standard error of the mean (S.E.M.) Analysis

of variance (oneway ANOVA followed by Duncan's Multiple range Test) was used to compare the data between various groups ($p<0.05$). Student paired t-test was used to compare the data within one individual group ($p<0.05$).