Chapter II

Materials and Methods

Experimental animals

The experiments were performed on either male Swiss albino mice or male Wistar albino rats weighing 18-25 g and 250-350 g, respectively. Both of them were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. In behavioral studies, the animals were acclimatized in the laboratory for a week before the experiments. The experiments with mice were completed within a week to minimize with the effects of increasing age on susceptibility (Löscher and Nolting, 1991). The experimental animals were allowed free access to both food (F.E. Zeulig,Thailand) and water under natural light/dark condition. All experiments were carried out between 8 a.m.– 6.00 p.m. except for the barbiturate sleeping time which was carried out between 7.00 a.m.– 13.00 p.m. Each animal was used for only once.

Chemicals

1. Valproyl morpholine (VPM) was kindly suppied by Assist. Prof. Dr. Chamnan Patarapanich and coworker (Department of Phamaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand).
2. γ - Amino - n - butylic acid (GABA; Sigma, U.S.A.)
3. Aspartic acid (Sigma, U.S.A.)
4. Calcium chloride 2 hydrate ($CaCl_2.2H_2O$; Riedel de Haën, Germany)
5. Chloral hydrate (Witayasom, Thailand)
6. D – Glucose monohydrate (Riedel de Haën, Germany)
7. Glutamic acid (Sigma, U.S.A.)
8. Glycine (Sigma, U.S.A.)
9. Homoserine (Sigma, U.S.A.)
10. Magnesium sulfate 6 hydrate ($MgSO_4.6H_2O$; Riedel de Haën, Germany)
11. 2 – Mercaptoethanol (Merck, Germany)
12. Methanol, HPLC grade (Merck, Germany)
13. Pentobarbital sodium (PB; Nembutal^R, Sanofi (France) Ltd.)

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14. Pentylenetetrazole (PTZ; Sigma , U.S.A.)
15. *O* – Phthaldialdehyde (Sigma, U.S.A.)
16. Polyethyleneglycol 400 (PEG 400; Witayasom, Thailand)
17. Potassium chloride (KCl; Riedel de Haën Germany)
18. Sodium chloride (NaCl; Riedle de Haën, Germany)
19. Sodium dihydrogen phosphate 2 hydrate ($NaH_2PO_4.2H_2O$; Riedel de Haën, Germany)
20. Sodium hydrogen carbonate ($NaHCO_3$; Riedel de Haën, Germany)
21. di – Sodium hydrogen phosphate 2 hydrate ($Na_2HPO_4.2H_2O$; Riedel de Haën, Germany)
22. Sodium hydroxide (NaOH; Riedel de Haën, Germany)
23. Valproic acid (VPA; Sigma, U.S.A.)

Drug preparations and administrations

The tested substances (VPA and VPM) which are insoluble in water were dissolved in PEG400 (which was used as a vehicle) and the others, which soluble in water (Chloral hydrate, PTZ and pentobarbital sodium) were dissolved in 0.9% sodium chloride (NSS). The dose levels of tested substances were expressed as milligram of substance/kilogram of body weight (mg/kg B.W.) and they were administered intraperitoneally (i.p.) except PTZ which was given by a subcutaneous (s.c.). The volumes of injection were 0.1 ml/25 g B.W. in mice and 0.4 – 0.8 ml in rat.

Equipments

1. Electroshock apparatus with corneal electrode (King Mongkut Institute of Technology, North Bangkok, Thailand)
2. Rotorod (UGO Basile, Comerico, Italy)
3. Animal activity cage (UGO Basile7430, Comerico, Italy)
4. Automatic infusion pump (CMA/100, Carnegie, Sweden)
5. Stereotaxic Instruments (NARISHIGE, Japan)
6. Automatic mixer (Vortex, U.S.A.)

7. System for freely moving animal (CMA/120, Carnegie, Sweden)
8. HPLC system
   - C_1 Reverse - phase, 250X4.6 mm, particle size 5 μm, Spherisorb ODS2. (Attech[R] , U.S.A.)
   - Guard column with packing material, particle sizes 5 μm, Spherisorb ODS2. (Phenominex[R] , U.S.A.)
   - Column oven (Model 2155, LKB, Sweden)
   - Fluorescence detector (Water470, U.S.A.)
   - Pump with gradient system (LC – 10AD; Shimadzu, Japan)
   - Analog to Digital Instruments (Maclab [TM/4], AD Instruments, Australia)
   - Macintosh computer (Model LC 630, Apple computer, Inc., U.S.A) with software programs (Maclab [TM/4] ,AD Instruments, Australia); Chart[TM] V 3.2.8 for data recording system and Peak[TM] V1.3 for data processing system
   - Laser printer (Laser writer select 360, Apple computer, Inc., U.S.A.)
9. Microdialysis probe; horizontal type, molecular weight cut off 50,000 (Homofilter PNF- 140 , Asahi Medical Co., Tokyo Japan)
10. pH meter (Suntex, Japan)

Experiment methods

1. Anticonvulsant activity

1.1 Anticonvulsant activity against Maximal Electroshock Seizure (MES)

The MES was elicited by a passage of an alternating current (55 mA, 50 Hz, 0.2 sec) from electroshock apparatus through the brain via corneal electrodes after pretreatment with tested substances. The endpoint of MES test was generalized seizure with tonic hindlimb extention (Thompson, 1990; Löscher and Nolting, 1991).

1.2 Determination of the optimal pretreated time within the period of 60 min and anticonvulsant activity against MES.

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 divided into 12 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 10 subgroups were used for determination of anticonvulsant activity, expressed as the median effective (ED_{50}) against MES. The tested substances, VPA (50, 100, 200, 300, 400 mg/kg B.W.) and VPM (75, 100, 125, 150, 175 mg/kg B.W.) were intraperitoneally injected. When pretreated time was due, the pretreated time that given the maximal anticonvulsant activity of each tested substance was selected and used as optimal pretreated time in other experiments.

1.3 Anticonvulsant activity against pentylenetetrazole (PTZ)seizure.

PTZ seizure was induced by a subcutaneous injection of PTZ 70 mg/kg B.W. to the mice. The endpoint of PTZ seizure test was generalized clonic seizure with loss of righting reflex within 60 min after injection of PTZ (Löscher et al., 1991).

Male Swiss albino mice were divided into 12 groups of 10 mice each. Two groups (NSS and PEG400, 0.1 ml/25 g B.W. i.p.) were used as control groups. The other 10 groups were used for the determination of the ED_{50} of tested substances, VPA (50, 75, 100, 150, 200 mg/kg B.W..) and VPM (100, 150, 200, 250, 300, 400 mg/kg B.W.) were pretreated by intraperitoneally injected to the mice at the optimal pretreated time obtained from1.2.

2. Toxicity

2.1 Acute toxicity test

Male Swiss albino mice were divided into10 groups of 8 animals each for determination of the median lethal dose (LD_{50}) and other effects such as ataxia,

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sedation, hypnosis, respiratory secretion, etc., which were observed in 72 hours after the administration of VPA (500, 600, 700, 800,900 mg/kg B.W. i.p.) and VPM (500, 600, 700, 800, 900 mg/kg B.W. i.p.).

2.2 Rotorod test

The rotorod test, which was modified from the one previously described by Dunham and Miya (1957), was carried out with a rod of 3.5 cm diameter, rotating at 18 rev/min. Neurological deficit was indicated by inability of the animals to maintain their equilibrium for at least 2 minutes within 2 trials 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 2 minutes, were put back on the rod. Only animals which were not able to remain on the rod for 2 minutes in each of trials, were considered to exhibit neurological deficit.

Determination of median neurotoxic dose (TD_{50})

Neurotoxicity of tested substance was performed on 12 groups of 8 mice each. Two groups (NSS and PEG400 0.1 ml/25g B.W. i.p.) were used for control groups. The other 10 groups were used for determination of the tested substances, VPA (200, 300, 400, 500, 600 mg/kg B.W.) and VPM (75, 100, 125, 150, 175 mg/kg B.W.) were given intraperitoneally. After the optimal pretreated time obtained from 1.2 was due, rotorod test was performed.

2.3 Effect on locomotor activity

Activity cage (UGO Basile, Comerico, Italy) was used to access the locomotor activity of mice which were placed in a box (length 35 cm; width 23 cm; height 20 cm). The cage floor is made of evenly spaced stainless steel bars (3 mm diameter) that are spaced 11 mm apart. Each of the steel bar is insulated from each other. The odd bars are grounded and the even bars are active and wired out in four sets. The bridge that animal breaks with its paw disconnects one or more active bars with ground, thereby producing configurations which change as the animal moves. These changes in configuration are converted into pulses and subsequently count by a counter. The counters were detected at 15 min intervals. A baseline locomotor activity

of each mouse was established allowing a control period of 45 min before the administration of the tested substances. Motor activity was measured in an animal cage for a period of 180 min.

Mice were divided into 6 groups of 10 animals each. Two groups (NSS and PEG400 0.1 ml/25g B.W. i.p.) were used as the control groups. The other 4 groups (VPA 100, 250 mg/kg B.W. i.p.) and VPM (100, 200 mg/kg B.W. i.p.) were used to test the effects on locomotor activity. After each mouse was placed single in the UGO Basile activity cage for familiarization with the environment for 45 minutes, the tested substance was given and the animal was allowed back into the cage immediately in order to record its locomotor activity for another 180 min.

2.4 Effect on barbiturate sleeping time

The effect on barbiturate sleeping time of tested substances were used to evaluate the depressing effect on CNS. In this study, pentobarbital sodium 50 mg/kg B.W. was intraperitoneally injected immediately to the animal after the administration of the tested substances. The sleeping time was measured as the time between the loss and the recovery of righting reflex, the inability and ability, respectively of the animal to upright itself within 5 seconds when placed on its back in three successive trials (Thompson, 1990).

Mice were divided into 6 groups of 8 animals each. Two groups (NSS and PEG400 0.1 ml/25g B.W. i.p.) were used as control groups. The other 4 groups (VPA 100, 250 mg/kg B.W. i.p. and VPM 100, 200 mg/kg B.W. i.p.) were used to test for the potentiation of barbiturate sleeping time.

2.5 Hypnotic effect

Hypnotic effect defined as ability of the tested substance to induce sleep was determined in mice. Loss of righting reflexes as reflected by inability of mouse to upright itself within 5 seconds when placed on its back. As previously base on in 2.4, mice were divided into 5 groups of 8 animals, which was used for determination of the median hypnotic dose (HD_{50}) of VPM (200, 225, 250, 275, 300 mg/kg B.W. i.p.).

3. Effect on some cortical amino acid neurotransmitter levels relating to convulsion in freely moving rats by microdialysis technique

3.1 Experimental animals

Male Wistar rats weighing 250-350 g were divided into 6 groups of 5 animals each for determination the effect of tested substances on the levels of aspartate, glutamate, glycine and GABA in rat cerebral cortex. Two groups were used as control (NSS and PEG400, 0.4ml i.p.). The others 4 groups were used for testing the effect of tested substances (VPA 100, 250 mg/kg B.W. i.p. and VPM 100, 200 mg/kg B.W. i.p.).

3.2 Microdialysis technique

This technique was modified from Benveniste and Huttemeier (1990).

3.2.1 Microdialysis probe implantation

The rat was anesthetized with chloral hydrate (350 mg/kg B.W. i.p.) with supplementary doses as required to maintain surgical anesthesia. The anesthetized animals were then placed in a stereotaxic apparatus (Narishige, Japan). The surface of microdialysis probe (0.2 mm outer diameter, acrylic polymer with 50,000 molecular weight cut off) was covered with epoxy resin totally except the area of 5 mm in length that contacted the cerebral cortex of the rat. After the appropriate area of the skull was exposed, the probe was implanted transversely into the cerebral cortex at coordination of 2 mm rostral to the bregma and 1-1.5 mm inferior to the cerebral surface according to a stereotaxic atlas of rat brain (Pellegrino, Pelligrino and Cushman, 1979) and was fixed by polycarboxylate cement. After microdialysis probe implantation, the rats were allowed at least 24 hours for recovery before the experiments were started.

3.2.2 Collection of CSF samples

The rat was placed in the collecting sample instrument (CMA/120, Carnegie, Sweden) which allowed freely moving. One side of probe was connected to a constant flow infusion pump (CMA/100, Carnegie, Sweden) by polyethylene tube, and the other side was placed into a collecting tube. The perfusion fluid for this microdialysis

experiment was artificial cerebrospinal fluid (aCSF). The composition of aCSF was 120 mM NaCl, 15 mM $NaHCO_3$, 5 mM KCl, 15 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 6.0 mM glucose, pH 7.4 (Benveniste and Huttemeier, 1990). The aCSF was perfused at the rate of 2μl/min. The dialysate from the freely moving rat was left 60 minutes for equilibration before samples were collected.

Basal amino acid levels were determined from the first three successive dialysate samples collected (20 min for each collection). The dialysate samples were collected at 20, 40, 60, 80, 100, 120, 140, 160 and 180 min after administration of the tested substances. The dialysate samples were determined for amino acid levels by high performance liquid chromatography (HPLC) technique.

At the end of each experiment, the brain was exposed to confirm the appropriate position of microdialysis probe by sectioning the specimen with a sharp blade and then inspected visually. The data was valid only when the right position of microdialysis probe was confirmed.

3.3 Analysis of rat cortical amino acid levels

The experimental method used to determine the levels of rat cortical amino acid by precolumn fluorescence derivatization with O-Phthaldialdehyde (OPA) which was first published by Lindorth and Mopper (1979). The mobile phases were used gradient run between 0.05 M phosphate buffer, pH 7.3 in triple distilled water and methanol (HPLC grade). Both of the mobile phases were degassed with continuous helium gas. 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 for 20 minutes. The rate of mobile phase was flow 1 ml/min. At the end of run, initial condition was restored by the reversed methanol gradient run from 60% to 20% at the rate of 10%/min. A delay period of about 10 minutes was required for column equilibration.

The solution of OPA was maintained by an addition of 4 µl 2-mercaptoethanol every 4 days. The derivartization procedure was performed by mixing 10 µl of dialysate sample with 10 µl of homoserine solution (internal standard) and adding 50 µl of OPA solution at room temperature. Then 50 µl injection to HPLC was made after a precise 2 minute incubation period.

4. Calculation and statistical analysis

4.1 The ED_{50}, TD_{50}, LD_{50}, and HD_{50} were transformed from probit unit by transformation table of Fish and Yates (Diem and Lentner, 1972). The linear regression method was used to fit the curve between probit of response and dose (log scale) by using Crikcet graph program (AD Instruments, Australia). The 95 percent confidence interval was calculated by the method of Litchfield and Wilcoxon(1949).

4.2 Statistical analysis was carried out using SPSS/PC+(1991) software Data presented are expressed as mean ± standard error of the mean (S.E.M.). Analysis of variance (oneway ANOVA) followed by Duncan's Multiple rang Test) was used to compare the data between various groups($p<0.05$).