Chapter III

Materials and Methods

Experimental animals

Anticonvulsant efficacy and neurotoxicity experiments were performed on male Swiss albino mice weighing 18-25 g. Wistar rats weighing 250-350 g were used to study the effect of AMP on cortical amino acid neurotransmitters. Both of them were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom.

The animals were acclimatized in laboratory for a week before the experiments, they were maintained under natural light/dark cycle at a control temperature (25°C) and were allowed free access to standard food and tab water. In anticonvulsant efficacy studies, the experiment with mice was completed within the week to minimize the effect of increasing age on seizure susceptibility (Loscher and Nolting, 1991). In all experiments, each animal was used for only one experiment and the experiments were carried out between 8.00 a.m. – 6.00 p.m.

Equipments and Chemicals

Equipments

1. Electroshock apparatus with corneal electrode (King Mongkut Institute of Technology, North Bangkok, Thailand)
2. Roto thread mill (King Mongkut Institute of Technology, North Bangkok, Thailand)
3. Stereotaxic apparatus (Narishige, Japan)
4. Automatic infusion pump (CMA 100, Carnegic, Sweden)
5. Horizontal microdialysis probe (Homofilter PNF-140, Asahi Medical Co., Tokyo, Japan)
6. pH meter (Suntex, Japan)
7. Centrifuge (International Equipment Company, U.S.A.)
8. Automatic mixer (Vertex, U.S.A.)
9. HPLC system
   - Pump with gradient system (LC-10 AD Shimadzu, Japan)
   - C^{18} Reverse- phase column 250x4.6 mm., particle size 5 μm, Spherisorb ODS(2) (Phenominex®, U.S.A.)
   - Column oven (model 2155, LKB, Sweden)
   - Fluorescence detector (Water 470, U.S.A.)
   - Analog digital instruments (Maclab TM/4, AD instruments, Australia)

- Macintosh® computer (Model LC 630, Apple computer, Inc., U.S.A.) with Chart TM V. 3.2.8 program for data recording system and Peak TM v. 1.3 for processing system.

Chemicals

1. N–(4-Amino-2-Methylphenyl)Phthalimide, (AMP). It was kindly supplied by Wanchai Pleumpanupat (Department of Chemistry, Faculty of Science, Chulalongkorn University, Thailand).
2. γ-Amino-n-butyric acid (SIGMA, U.S.A.)
3. l-Aspartic acid (SIGMA, U.S.A.)
4. Calcium chloride-2-hydrate (Reidel de Haen, Germany)
5. D(+)- Glucose monohydrate (Reidel de Haen, Germany)
6. L-Glutamic acid (SIGMA, U.S.A.)
7. Glycine (SIGMA, U.S.A.)
8. L-Homoserine (SIGMA, U.S.A.)
9. Magnesium sulfate-6-hydrate (Reidel de Haen, Germany)
10. 2-Mercaptoethanol (MERCK, U.S.A.)
11. Methanol HPLC grade (SIGMA, U.S.A.)
12. o-Phthaldialdehyde(OPA) (SIGMA,U.S.A.)
13. Polyetheleneglycol (PEG) (Witayasum, Thailand)
14. Potussium chloride (Reidel de Hean, Germany)

15. Sodium dihydrogen phosphate-2-hydrate (Reidel de Hean, Germany)
16. Sodium hydrogen carbonate (Reidel de Hean, Germany)
17. Di-Sodium hydrogen phosphate-2-hydrate (Reidel de Hean, Germany)
18. Sodium hydroxide (Reidel de Hean, Germany)
19. Valproic Acid (SIGMA, U.S.A.)

Experimental Methods

1. Preparation and administration of test substances

The test substances, VPA and AMP, which are insoluble in water were dissolved in PEG 400. They were administered intraperitoneally. In mice, the volumes of an intraperitoneal (i.p.) administration were 0.1 ml/25 g B.W., while the volume of intraperitoneally injection in rats was 0.05 ml/25 g B.W.

2. Anticonvulsant efficacy

2.1 Maximal Electroshock Seizure (MES) test

The test was modified from the method of Toman, Swinyard, and Goodman (1946). The MES was elicited by passage of the minimum current

from electroshock apparatus (current = 55 mA., frequency = 50 Hz., duration 0.2 sec.) through the brain via corneal electrodes after the pretreatment with test substances. The end point of the test was generalized seizure with tonic hindlimb extension exceed angle with plane of the body.

The test substance was considered to possess anticonvulsant activity if previously described convulsion did not occur within 0.2-0.5 sec after the MES was performed.

2.2 Determination of the median effective dose (ED_{50})

Anticonvulsant activity of test substance was performed on 10 groups of 8 mice each. Two groups (normal saline solution (NSS) and PEG 400) were used as control groups. The other 8 groups were used for the determination of the median effective dose (ED_{50}). The test substances, VPA and AMP, were intraperitoneally injected. When pretreated time (30 min) was due, MES was performed as previously described in 2.1

2.3 Duration of protection against MES

For determining the duration of action of AMP, mice were divided into 3 groups according to pretreated time (30 min, 1 and 3 hr). Each group was then divided into 5 subgroups of 8 animals each, for the determined of the ED_{50} of test substance VPA and AMP were given intraperitoneally to

respective groups of animals. When pretreated time was due, MES was performed as previously described in 2.1 and the ED_{50} of VPA and AMP at different times were calculated.

2.4 Chemically induced seizure tests

Chemoshock was induced by an injection of either pentylenetetrazole or strychnine into the experimental animals. Abolition of seizures by pretreatment of test substances indicated their convulsant activity.

2.4.1 Anticonvulsant activity against Pentylenetetrazole induced Seizure Test (PTZ)

The PTZ seizures were elicited by a subcutaneous injection of PTZ 70 mg/kg B.W. to the animals after the pretreated time of test substances. 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 and Nolting, 1991).

Male Swiss albino mice were divided into 10 groups of 8 animals each for determination of the ED_{50} against PTZ seizures. Two groups were used as a control (PEG 400 and NSS 0.1 ml/25 g B.W., i.p.). The other 8 groups were used for the test substances, VPA (200, 300, 400, 500 mg/kg B.W.)

and AMP (15, 20, 30, 70 mg/kg B.W.), which were given intraperitoneally at the optimal pretreated time from 2.1.

2.4.2 Anticonvulsant activity against Strychnine induced convulsion

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

Anticonvulsant activity of test substance was tested on male Swiss albino mice which were divided into 9 groups of 8 animals each for the determination of the ED_{50} against strychnine seizures. Two groups were used as a control (PEG 400 and NSS 0.1 ml/25 g B.W., i.p.). The other 8 groups were used for the test substance, VPA (200, 300, 400, 500 mg/kg B.W.) and AMP (15, 20, 30, 70 mg/kg B.W.), which were given intraperitoneally at the optimal pretreated time from 2.1.

3. Toxicity test

3.1 Acute toxicity

Mice were divided into 7 groups of 8 animals each for the determination of the median lethal dose (LD_{50}) and other effects, such as ataxia, sedation, hypnosis, respiratory tract secretion, etc., of VPA (500, 600, 800, 1000 mg/kg B.W.) and AMP (80, 100,120,140,160 mg/kg B.W.). The test compounds were intraperitoneally injected and the observation for lethality was made within 72 hrs.

3.2 Neurotoxicity

3.2.1 Rotorod test

The rotorod test was modified from the previously described by Dunham and Miya (1975); Swinyard and Woodhead (1982), carried out with a rod of 3.5 cm diameter, rotating at 16.5 rev/ min. Neurological deficit was indicated by inability of the animals to maintain their equilibrium for at least 1 min on the rotating rod in each of three successive trials. 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.

3.2.2 Determination of median neurotoxic dose (TD_{50})

Neurotoxicity of test substance was performed on 10 groups of 8 mice each. Two groups (NSS and PEG400) were used as control groups. The other groups were used for the determination of the median neurotoxic dose (TD_{50}) of the test substances. VPA and AMP were intraperitoneally injected. After the pretreated time was due, rotorod test was performed as previously described in 3.2.1.

3.2.3 Duration of neurotoxic effect

For determining the duration of neurotoxic effect, mice were divided into 3 groups according to pretreated time (30 min, 1, 3 hr). Each group of mice was then divided into 4 subgroups of 8 animals each. VPA and AMP were given intraperitoneally to respective groups of the animal. After the given pretreated time, rotarod test was performed as previously described in 3.2.1 and the percentage falling of mice at various times was calculated and plotted.

4. Potentiation of barbiturate sleeping time.

The effect on barbiturate sleeping time was used to evaluate the depressing effect of substance on the CNS. In this study, pentobarbital sodium (50 mg/kg B.W.) was intraperitoneally injected immediately to the animal after the administration of the test substance. The sleeping time was measured as the time between the loss and the recovery of righting reflex which

indicated by inability and ability of the animal to upright itself within 5 sec when placed on its back in three successive trials (Thomson, 1990).

Mice were divided into 6 groups of 8 animals each. Two groups (NSS and PEG 400, 0.1 ml/25 g B.W., i.p.) were used as control groups. The other 4 groups (VPA 100, 200 mg/kg B.W. and AMP 17 and 70 mg/kg B.W., i.p.) were used to test for the potentiation of barbiturate sleeping time.

5. Effect of test substances on some cortical amino acid neurotransmitter levels relating to convulsion in freely moving rats by microdialysis technique

Male Wistar rats weighing 250-350 g were divided into 6 groups of 5 animals each for the determination of effect of tested substance on the levels of aspartate, glutamate, glycine and GABA in rat cerebral cortex. Two groups were used as control (NSS and PEG 400, 0.4 ml i.p.). The other 4 groups were used for testing effect of test substance (VPA 200, 400 mg/kg B.W. i.p. and AMP 70, 100 mg/kg B.W. i.p.)

5.1 Microdialysis technique

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

5.1.1 Microdialysis probe implantation

Rats were 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 stereotaxis apparatus (Narishige, Japan). The surface of microdialysis probe (0.2 mm outer diameter, acrylic polymer with 50,000 molecular weight cut off) was totally covered with epoxy resin 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 experiment was started.

5.1.2 Collection of cerebrospinal fluid (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 mM $Mgso_4$ and 6 mM glucose, pH 7.4 (Benveniste and Huttemeier, 1990). The aCSF was continously perfused at the rate of 2 μl/min. Dialysate collected during the equillibration period of 60 min was discarded. After the equilibration period of 60 min the time first samples was 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 and removed to confirm the appropriate position of microdialysis probed by sectioning the specimen with a sharp blade and inspected visually. The data was valid only when the right position of microdialysis probe was confirmed.

5.2 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) was first published by Lindorth and Mopper (1979). The mobile phase used was 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 1 ml/min. At the end of the 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 derivatization procedure was performed by mixing 10 μl of homoserine solution (internal standard) and adding 50 μl of OPA solution at room temperature. Then 50μl injection to HPLC was made after a precise 2 min incubation period.

6. Calculation and statistical analysis

The ED_{50} was 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 % confidence interval was calculated by the method of Lichfield and Wilcoxon (1949).

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

(S.E.M.). Analysis of variance (Oneway ANOVA followed by Duncans Multiple range Test) was used to compare the data between various groups ($p<0.05$).