Chapter II

Materials and methods

Experimental animals

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

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 (F.E. Zeulig) 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 experiment were carried out between 8.00 a.m. - 6.00 p.m.

Equipments

1. Electroshock apparatus with corneal electrode (King Mongkut Institute of Technology, North Bangkok, Thailand)
2. Roto treated 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, spherisorp ODS(2) (Phenominex®, U.S.A.)
   - Guard column with packing material, particle size 5 μm, Spherisorb ODS(2) (Phenominex®, U.S.A.)
   - Column oven (model 2155, LKB)
   - Fluorescence detector (RF-10A Shimadzu, Japan)
   - Analog digital instruments (Maclab TM/4, AD instruments, Australia)
   - Macintosh® computer (Model LC 630, 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.

Chemicals

1. N- (2-Propylpentanoyl) Urea. It was supplied by Assist. Prof. Dr. Chamman Patarapanich (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Science,Chulalongkorn University, Thailand).
2. Acetonitrile (SIGMA, U.S.A.)
3. 9-anthryldiazomethane (Funakoshi, Japan)
4. γ-Amino-n-butyric acid (SIGMA, U.S.A.)
5. l-Aspartic acid (SIGMA, U.S.A.)
6. Boric acid (MERCK, Germany)
7. Calcium chloride-2-hydrate (Reidel de Haen, Germany)
8. Ethanol absolute (MERCK, Germany)
9. D (+) -Glucose monohydrate (Reidel de Haen, Germany)
10. l-Glutamic acid (SIGMA, U.S.A.)
11. Glycine (SIGMA, U.S.A.)
12. L-Homoserine (SIGMA, U.S.A.)
13. Magnesium sulfate-6-hydrate (Reidel de Haen, Germany)
14. 2-Mercaptoethanol (MERCK, U.S.A.)
15. Methanol HPLC grade (MERCK, U.S.A.)
16. O-Phthaldialdehyde (OPA) (SIGMA, U.S.A.)
17. Polyethyleneglycol (PEG) (Witayasum, Thailand)
18. Potassium chloride (Reidel de Hean, Germany)
19. Sodium dihydrogen phosphate-2-hydrate (Reidel de Haen, Germany)
20. Sodium hydrogen carbonate (Reidel de Haen, Germany)
21. di-Sodium hydrogen phosphate-2-hydrate (Reidel de Haen, Germany)
22. Sodium hydroxide (Reidel de Haen, Germany)
23. Valproic Acid (SIGMA, U.S.A.)

Experimental Methods

1. Preparation and administration of test substances

The test substances (VPA and VPU) which are insoluble in water were dissolved in PEG400. They were administered either intraperitoneally or orally (by means of gavage tube). 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 0.05 ml/25 g B.W.

In degradation studies, the stock solution of test substance was prepared in methanol at concentration of 0.1 M and kept at $0^{\circ}C$ in cold room.

2. Anticonvulsant efficacy

2.1 Maximal Electroshock Seizure (MES)

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 Hertz., 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 median effective dose (ED_{50})

2.2.1 Intraperitoneal route

Anticonvulsant activity of test substance was performed on 10 groups of 8 mice each. Two groups (normal saline solution (NSS) and PEG400) were used as control groups. The other 8 groups were used for the determination of the medain effective dose (ED_{50}). The test substances, VPA(100, 200, 300, 400 mg/kg B.W.) and VPU (50, 100, 200, 300 mg/kg B.W.) were intraperitoneally injected. When pretreated time (30 min) was due , MES was performed as previously described in 2.1.

2.2.2 Oral route

The test substances were orally given (p.o.) to 220 mice which were divided into 3 groups according to pretreated time (30, 60 and 120 min). Each group was then divided into 9 subgroups of 8 animals each. One subgroup was used as a control group for the administration of PEG400. The other 8 subgroups were used for the determination of anticonvulsant activity of test substances, expressed as the ED_{50} against MES. VPA (200, 400, 600, 800 mg/Kg B.W.) and VPU (200, 400, 600, 800 mg/Kg B.W.) were given orally by a gavage

tube to the animals. When pretreated time 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 VPU, 300 mice were divided into 5 groups according to pretreated time (1, 3, 6, 9 and 12 hr). Each group was then divided into 6 subgroups of 10 animals each, for the determination of the ED50 of test solution. VPA (300, 350, 400 mg/kg B.W.) and VPU (200, 300 and 400 mg/kg B.W.) 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 VPU at different times were calculated.

3. Neurotoxiccity

3.1 Rotorod test

The rotorod test was modified from the one previously described by 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 Determination of median neurotoxic dose (TD_{50})

Neurotoxicity of test substances was performed on 10 groups of 8 mice each. Two groups (NSS and PEG400) were used as control groups. The other 8 groups were used for the determination of the median neurotoxic dose (TD_{50}) of the test substances. VPA (200, 300, 400, 600 mg/kg B.W.) and VPU (200, 400, 600, 800 mg/kg B.W.) were intraperitoneally injected. After the pretreated time was due, rotorod test was performed as previously described in 3.1.

3.3 Duration of neurotoxic effect

For determining the duration of neurotoxic effect 360 mice were divided into 8 groups according to pretreated time (1/2, 1, 2, 3, 4, 5, 9, and 12 hr). Each group of mice was then divided into 4 subgroups of 10 animals each. VPA (300, 400 mg/kg B.W. and VPU (300, 400 mg/kg B.W.) were given intraperitoneally in to respective groups of the animal. After the given pretreated time, rotorod test was performed as previously described in 3.1. and the percentage falling of mice at various times was calculated and plotted.

4. The degradation of VPU

4.1 Degradation experiment using brain and liver homogenates

Rats were used to prepare brain and liver homogenates. They were sacrificed by dislocation method. Brain and liver were immediately removed and homogenized at 0-5°C in glass-Teflon homogenizer to prepare 40%

homogenate in a pH 7.4 isotonic phosphate buffer, then centrifuged at 2500 g for 15 min, and the supernatant was used for the experiments. Degradation experiment were performed at 37° ± 0.2°C and initiated by adding the stock solution of a test compound to give a final concentration of 1 mM. At appropriate time intervals of 15, 30, 60, 120, 240 min, aliquots of the solution were withdrawn and acetonitrile was added to precipitate the protein. After that, the supernatant was analyzed for VPA contents by high performance liquid chromatography (HPLC) technique.

4.2 VPA analysis

A fluorescent labeling reagent, 9-anthyldiazomethane(ADAM) has widely been applied in HPLC for precolumn derivertization of biologically significant carboxylic acids such as fatty acid and prostaglandins. This accounts for its application in the determination of VPA (Ichinose et. al., 1984).

The reagent of ADAM solution was dissolved with agitation by adding a drop of acetone to 1 mg of ADAM in 1 ml of methanol and kept at-10°C for not longer than 10 hours.

The derivatization procedure was performed by adding 40 µl of ADAM solution to 40 µl of sample. In order to carry out the esterification, the mixed solution was vibrated by means of the automatic mixer for 1 min and stood for 1 hour. Then a portion of solution was injected to 20 µl loop for HPLC analysis.

The condition for the analysis was methanol-water (85:15), as a mobile phase, with 1.0 ml/min of flow-rate, and the solid phase of Sperisorb ODS (2) C18. The fluorescence detector was set excitation to 345 nm and emission to 416 nm. Output data from detector analog were converted to digital by Maclab, recorded by chart program V.3.2.8 and being analyzed for the area under curve by Peak program V.1.3.

5. Effects on some cortical amino acid neurotransmitter levels in awake rats by microdialysis technique.

Male albino rats (Wistar strain, weighing 280-350 g) were divided into 6 groups of 5 animals each for determining the effect of test substances on the levels of glutamate, aspartate, glycine and GABA in rat cerebral cortex. Two groups were used as control groups (PEG400 and NSS). The others 4 groups (VPA 200, 400 mg/kg B.W. and VPU 200, 400 mg/kg B.W.) were used for testing the effect of test substance.

5.1 Microdialysis experiment

Rats were anesthetized with chloral hydrate (350 mg/kg B.W., i.p.) and was fixed in a stereotaxic head holder. Cover the horizontal microdialysis probe (outer diameter 0.2 mm., acrylic copolymer with a 50,000 molecular weight cut off) with epoxy resin totally except the area 5 mm in length that contact the cortex of rats. 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 the rat brain (Pellegrino, Pellegrino and Crushman, 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.

5.2 Collection of CSF samples

Rats were placed in the collecting sample instrument, which allowed freely moving. One side of probe was connected to a constant flow perfusion pump by polyethylene tube, and the other was placed into a collecting tube. The artificial cerebrospinal fluid (aCSF) pH 7.3 containing 120 mM NaCl, 15 mM $NaHCO_3$, 5 mMKCl, 1.5 mM $CaCl_2$, 1 mM $MgSO_4$, 6 mM Glucose (Benveniste and Huttemeier, 1990) was perfused into the probe by perfusion pump at the constant flow rate of 2 μl/min. The dialysate from the freely moving rats were left 60 minutes for equilibration before samples were collected.

Basal amino acid levels (glutamate, aspartate, glycine and GABA) were determined from the first three successive dialysate samples collected (20 min for each collection). Then each of test substances VPA (200,400 mg/kg B.W.) and VPU (200, 400 mg/kg B.W.) were individually injected into the rats by intraperitoneal route. The aCSF samples were collected at 20, 40, 60, 80, 100, 120, 140, 160 and 180 min after injection. Determination of amino acid levels was done by means of HPLC technique.

At the end of each 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 was valid only from the right position of microdialysis probe.

5.3 Amino acid analysis

The experimental method used to determine the levels of rat cortical amino acid was the precolumn fluorescence derivatization with O-Phthaldialdehyde (OPA) which was firstly published by Lindroth and Mopper (1979). The mobile phases used were 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 the phosphate buffer, the pH was set by mixing equimolar solution of Na_2HPO_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 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 10 ml with borate buffer pH 9.5 (H_3BO_3 0.4 M adjusted pH with 1 M NaOH). The reagent strength was maintained by an addition of 4 µl. 2-mercaptoethanol every 4 days.

The derivertization procedure was performed by mixing 10 µl of dialysate sample with 10 µl of 2 µM homoserine solution (internal standard) and

adding 50 μl of buffer reagent solution at room temperature. Then 50 μl injection to HPLC was made after a precise 2 min. incubation period.

The fluorescence detector was set to the condition of emission wave length of 418 mM and excitation wave length of 330 nm. Output data from detector analog were converted to digital by Maclab, recorded by Chart program V. 3.2.8 and being determined for the area under curve by Peak program V.1.3.

Calculation and Statistical Analysis

1. For determination of ED_{50} and TD_{50} groups of 8-10 mice each were used to test the effect of test substances at various dose until at least 3 points were established between the limit of 0-100 percent 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 Crickcet graph program (Macintosh® computer). The 95 percent confidence interval was calculated by the method of Litfield and Wilcoxon (1949).

2. Statistical analysis was carried out using SPSS/PC+(1991) softwere. All numerical data are expressed as mean ± standard error of 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 ($p<0.05$).