Chapter II

Materials and Methods

Experimental animals

The experiments were preformed on Male Wistar albino rats. The animals weighing 100-150 g. were used to determine the median effective dose of VHA by MES while those weighing 250-350 g. were used to study the effect of test compounds on cortical amino acid neurotransmitters. All rats were obtained from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakornpathom. The animals were acclimatized in the laboratory for a week before the experiments were started. All animals were maintained under natural light/dark cycle at control temperature (25^{o}C) and were allowed free access to both food (C.P. Mice Food, Thailand) and water. All experiments were carried out between 8 a.m. – 6.00 p.m. Each animal was used for only once. All animal care and handling was conducted in compliance with the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

Chemicals

1. Valproyl hydroxamic acid (VHA) was kindly supplied by Assist. Prof. Dr. Chamnan Patarapanich and coworker (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand).

2. γ-Amino-n-butylic acid (GABA; Sigma, U.S.A.)

3. Calcium chloride-2-hydrate ($CaCl_2.2H_2O$; Riedel de Haën, Germany)

4. Chloral hydrate (Witayasom, Thailand)

5. D-Glucose monohydrate (Riedel de Haën, Germany)

6. Glycine (Sigma, U.S.A.)

7. L-Aspartic acid (Sigma, U.S.A.)
8. L-Glutamic acid (Sigma, U.S.A.)
9. L-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. *O*-Phthaldialdehyde (OPA; Sigma, U.S.A.)
14. Polyethyleneglycol 400 (PEG 400;Witayasom, Thailand)
15. Potassium chloride (KCl; Riedel de Haën, Germany)
16. Sodium chloride (NaCl; Riedel de Haën, Germany)
17. Sodium dihydrogen phosphate 2 hydrate ($NaH_2PO_4.2H_2O$; Riedel de Haën, Germany)
18. Sodium hydrogen carbonate ($NaHCO_3$; Riedel de Haën, Germany)
19. di-Sodium hydrogen phosphate 2 hydrate ($Na_2HPO_4.2H_2O$; Riedel de Haën, Germany)
20. Sodium hydroxide (NaOH; Riedel de Haën, Germany)
21. Valproic acid (VPA; Sigma, U.S.A.)

Drug preparations and administrations

The tested substances (VPA and VHA) which are insoluble in water were dissolved in PEG400 and the others which are soluble in water (Chloral hydrate) was

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.). The volumes of injection was kept at 0.4-0.8 ml.

Equipments

1. Electroshock apparatus with corneal electrodes (King Mongkut Institute of Technology, North Bangkok, Thailand)
2. Automatic infusion pump (CMA/100, Carnegie, Sweden)
3. Stereotaxic Instruments (NARISHIGE, Japan)
4. Automatic mixer (Vortex, U.S.A.)
5. System for freely moving animal (CMA/120, Carnegie, Sweden)
6. HPLC system
   - C_1 Reverse – phase, 250 x 4.6 mm, particle size 5 μm, Spherisorb ODS2. (Attech^{R}, U.S.A.)
   - Guard column with packing material, particle sized 5 μm, Spherisorb ODS2.(Phenominex^{R}, U.S.A.)
   - Column oven (Model 2155, LKB, Sweden)
   - Fluorescence detector (Water 470, 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} V3.2.8 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.)

7. Microdialysis probe; horizontal type, molecular weight cut off 50,000 (Homofilter PNF – 140, Asahi Medical Co., Tokyo Japan)

8. pH meter (Suntex, Japan)

Experimental methods

1. Median effective dose of VHA with anticonvulsant activity against Maximal Electroshock Seizure (MES) in rats.

The MES was elicited by the passage of an alternating electric current (60 Hz.) from electroshock apparatus through the brain via corneal electrodes after the pretreated time of tested substances. The current intensities used to produce tonic-clonic seizures were 160 mA., 0.2 second duration in rats. The endpoint of MES test was generalized seizure with tonic hindlimb extention in both species (Loscher and Nolting, 1991; Cereghino and Kupferberg, 1993). In mice, the optimal pretreated time for protection against electroshock of VPA was 30 min and anticonvulsant activity of VPA was also demonstrated in rats in which the ED_{50} was 233 mg/kg B.W. (Sooksawate, 1995). The optimal pretreated time of the VHA preformed in mice using the maximal electroshock seizure (MES) was 15 min (Thongsathean, 1999). Male Wistar rats were divided into 3 groups of 8 animals each for the determination of the ED_{50} against MES. All of groups were used for the test of anticonvulsant activity of VHA in 3 dose levels of 80, 100 and 120 mg/kg B.W. i.p.

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

2.1 Experimental animals

Male Wistar rats weighing 250-350 g. were divided into 6 groups of 5 animals each for determination of 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, 0.4 ml i.p.). The others 4 groups were used for testing the effect of tested substances (VPA 200, 400 mg/kg B.W. i.p. and VHA 100, 200 mg/kg B.W. i.p.).

2.2 Microdialysis technique

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

2.2.1 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 stereotaxic 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.

2.2.2 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 continuously perfused at the rate of 2 μl/min. Dialysate collected during the equilibration 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 probe by sectioning the speciment with a sharp blade and the inspected visually. The data was valid only when the right position of microdialysis probe was confirmed.

2.3 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) 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 50 μl injection to HPLC was made after a precise 2 min incubation period.

4. 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 percent confidance interval was calculated by the method of Litchfield 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 Duncan's Multiple range Test) was used to compare the data between various groups ($p<0.05$).