Chapter II

Materials and Methods

Animals

1. The experiments were performed on either male Swiss mice or male Wister albino rats weighing 20-25 g. (about 4 weeks of age) and 180-250 g. (about 11-12 weeks of age) respectively. Both of them were obtained from a commercial breeder (National laboratory animal centre. Mahidol University, Budamonton IV road, Salaya, Nakornpathom). Prior to the experiment the mice were acclimatized in the laboratory for 1 week. In anticonvulsive experiments with the respective group of mice were, then, carried out within a following week to minimize the effect of increasing age on seizure susceptibility.

They were kept in a group of 10 at a controlled temparature (25°C) and having free access to standard diet (F.E. Zeulig) and tap water . All experiments were carried out between 8.00-18.00 hr. at an ambient temperature of 25-30 °C.

Equipments

Electroshock apparatus with Corneal electrode (King Mongkut Insititue of Technology, North Bangkok, Thailand).

Stereotaxic apparatus (Narishige, Japan)

HPLC pump with gradient system (Thermo Separation Products, U.S.A.)

Fluorescence detector (Jasco FP-210, Japan)

Personal microcomputer for system controlling and data processing (Compaque prolinea 33, U.S.A.)

C_{18} reversed-phase column 200 x 4.6 m.m. particle size 5 μm. (Spherisorb ODS2, U.S.A.)

Horizontal microdialysis probe with a molecular weight cut off at 50,000 (Homofilter PNF-140, Asahi Medical Co., Tokyo, Japan)

Temparature-controlled water bath (Julabo model SW1, W.Germany)

Centrifuge (International Equipment Company, U.S.A.)

pH meter (Orion model 720A,U.S.A.)

Automatic mixer (Vertex, U.S.A.)

Chemicals

N(2'-propylpentanoyl)-2-pyrrolidinone (with supplement from Assis. prof. Chamnan Patarapanich, Ph.D. , et al.)

Polyethyleneglycol 400 (PEG 400 T. Chemical Ltd. Partnership, Thailand)

Valproic acid (Aldrich, U.S.A.)

Pentobarbitone sodium (Nembutal®, Sanofi (Thailand) Ltd.)

Pentylenetetrazol (Sigma, U.S.A.)

o-Phthaldialdehyde (Sigma, U.S.A.)

Mercaptoethanol (Merck, Germany)

Acetonitrile HPLC grade (Merck, Germany)

Methanol HPLC grade (Merck, Germany)

Na_2HPO_4 (Merck, Germany)

NaH_2PO_4 (Merck, Germany)

NaCl (Ridel de Haën, Germany)

$NaHCO_3$ (Ridel de Haën, Germany)

$CaCl_2$ (Ridel de Haën, Germany)

KCl (Ridel de Haën, Germany)

$MgSO_4$ (Ridel de Haën, Germany)

Glucose (Merck, Germany)

9-Anthryldiazomethane (Funakoshi Yakuhin.,Tokyo Japan)

Methods

1. Anticonvulsant activity studies.

1.1 Maximal electroshock seizures (MES).

1.1.1 The test was modified from the method of Toman, Swinyard, and Goodman (1946). The electric current for inducing the MES of mice was selected by varing the electric current from electroshock apparatus which stimulated the mice via a corneal electrode. The selected current was the minimum current that produced tonic-clonic sizures (current = 55 mA., frequency = 50Hz., duration = 0.2 seconds).

1.1.2 To determine the peak time of reponse, the mice were divided into 3 groups according to pretreated time (15, 30 and 60 minutes) . Various doses of drug were intraperitonealy given to each group. When pretreated time was due, maximal electroshock was performed by previously selected current (see 1.1.1). The pretreated drug was considered to possess anticonvulsant activity if no clonic-tonic seizure was observed. immediately after electroshock.

1.1.3 The percentage of non-seizure mice was calculated at each dose, then the bar charts were plotted between the percentage of non-seizure mice and doses. From these charts, the peak time of response of each drugs, selected by the minimum pretreated time that gave the maximum effect, was used to evaluate the potency of drug and to be further used as an optimal pretreated time in chemoshock model.

1.2 Pentylenetetrazole (PTZ) seizures.

1.2.1 The dose was determined by choosing the minimum dose which reached the end point. The end point of this chemoshock test was the 'threshold seizure' proposed by Swinyard, Brown, and Goodman (1952) (a clonic seizure with a seizure duration of at least 5 seconds, but without loss of righting reflexes).

1.2.2 The mice were divided into two groups and pretreated by

by a subcutaneous injection of PTZ in the predetermined dose (70 mg/kg). The pretreated time of each anticonvulsant drug was selected as previously described in 1.1.3. The animal was considered to be protected from convulsive effect of PTZ if a clonic seizure with a seizure duration of at least 5 seconds, without loss of righting reflexes did not occur.

1.3 Determination of the median effective dose (ED_{50}). The dose-response curve was plotted between log(dose) and probits which was tranformed from percentage protection in 1.2.2. The linear regression method was used to fit curve and ED_{50} was then calculated.

2. The degradation of VPP.

2.1 The stock solution of VPP was prepared in acetonitrile at a concentration of 0.1 M and kept at 0 °C in cold room.

2.2 Male Wista rats were used to prepare brain and liver homogenate. They were sacrificed by dislocation method. The 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 x g for 15 minutes, and supernatant was used for the experiments. Degradation experiments were performed at 37 °C and initiated by adding the stock solution of a test compound to give a final concentration of 1 mM. At appropiate time intervals, 15, 30, 60, 120, 240 min., aliquots of the solution were withdrawn and acetonitrile was added to precipitate the protien. After that the supernatant was analyzed for GABA and VPA contents by HPLC precolumn derivatization procedures as described in 4 and 5. The same procedure which is a modification of those described by Sasaki et al., (1991) was used in control experiments in which acetonitrile solution was used instead of VPP

2.3 The data were plotted between concentration vs. time and analyzed by student t-test

3. The effect of VPP on GABA levels in a rat cortex.

The male Wista rats were devided into two groups, namely, VPA and VPP groups. The animal was anesthezied by an intraperitoneal injection of 40 mg/kg of pentobarbital sodium and was placed in a stereotaxic apparatus. A dialysis tube (0.2 mm outer diameter, acrylic copolymer with a 50,000 molecular weight cutoff; Asahi Medical Co.) was implanted transversely into the cerebral cortex at the coordinates 2 mm. rostral to the bregma and 1 - 1.5 mm. in depth below the cerebral surface (Collins, 1978) according to the brain atlas of Pellegrino and Cushhman (1967). The outer surface of the dialysis tube has been covered by Epoxy resin, except for 5 mm wide. After the implantation of dialysis tube ,the inlet to the dialysis tube was connected to a constant-flow perfusion pump by polyethylene tubing. Artificial cerebrospinal fluid was pumped into the dialysis tube at a rate of 2 μl/min and the solution emerging from the other end of the tube was colllected every 10 minutes. The first sample was collected at 60 minutes after the infusion was started and was determined for the GABA concentration. At the equilibrium state of GABA concentration, 200 mg/kg of test compound, either VPP or VPA, was then injected by intraperitoneal injection and the sample was continueously collected for another three hours.

Each sample was then analyzed for GABA by using the HPLC procedure as described in 5. After the experiment, the brain was excised to confirm the position of microdialysis probe. The data was valid only from the right position of microdialysis probe. This method was modified from the method of Imperato and Di Chiara. (1984)

4. The determination of VPA.

VPA is composed of carboxylic acid and can be detected by a great number of procedures. The HPLC is a commonly used method with the aids of 9-anthryldiazomethane (ADAM), a fluorescent labelling reagent. ADAM has widely been applied in HPLC for precolumn derivatization of biologically significant

carboxylic acids such as fatty acid and prostaglandins (Ichinose et al.,1984). It can react with carboxylic acids at a room temperature without the presence of a catalyst and even in the presence of water. Therefore, it was chosen in this study for the determination of VPA.

For the HPLC analysis of the ADAM derivative of VPA, methanol-water (9:1) was used as the mobile phase with Sperisorb ODS2 C_{18} as the solid phase. ADAM solution was dissolved with agitation by adding a drop of acetone to 1 mg of ADAM in 1 ml of methanol and kept in a freezer (at-10°C) 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 cary 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 has been set as the follows: 1.0 ml/min of flow-rate with column temperature at 50° C, fluorescence excitation at 345 nm and emission at 416 nm.

5. The determination of GABA.

The precolumn fluorescence derivatization with o-Phthaldialdehyde was used to determine the amounts of GABA. The experimental method has been carried out in accord with that described by Lindroth and Mopper (1979). The 0.05 M phosphate buffers at pH 7.3 were prepared from analytical grade salts and triple distrilled water. Methanol (HPLC Grade) was employed as the organic modifier in the aqueous eluent. All mobile phase were degassed with continueous helium gas. The gradient run was started at 20 % methanol and increasing by 2% /min. for 20 minutes. The flow rate was 1 ml/min. At the end of the run, initial conditions were restored by running a reversed methanol gradient of 10% /min. A delay period of about 10 minutes is required for equilibration. The buffered reagent solution was prepared by dissolving 270 mg. of *o*-Phthaldialdehyde in 5 ml. of ethanol (99.5%) and added 200 µl. of 2-mercaptoethanol and then the borate buffer pH 9.5 was

added to adjust the volumn to 50 ml. The derivatization procedure was performed by mixing 20 µl. of sample with 100 µl. of buffered reagent solution at room temparature then 20 µl. were injected after 2 minutes.