Chapter II

Materials and Methods

Experimental animals.

Anticonvulsant efficacy and neurotoxicity experiments were performed using male Swiss albino mice weighing 18-25 g. Wistar albino rats weighing 330-360 g. were used to study the effect of test compounds on cortical amino acid neurotransmitters. Both of them were obtained from National Laboratory Animal Center, 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°C) and were allowed free access to both food (C.P. mice feed) and tab water except when they were removed from their cages for the experimental procedures. In anticonvulsant efficacy studies, the experiment with the respective groups of mice were then completed within a week to minimize the effect of increasing age on seizure susceptibility (Loscher and Nolting,1991.). All experiments were carried out between 8.00 -11.00 a.m. Each animal was used for only once.

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, Carnegie, Sweden)
5. pH meter (Suntex, Japan)
6. HPLC system (Shimatzu, Japan)
7. 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. 3 for processing system.

Chemicals

1. Amide 1C. It was supplied by Assist. Prof. Dr. Chamnan Patarapanich (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand)
2. γ-Amino butyric acid (Sigma, U.S.A.)
3. Boric acid (Merck, Germany)
4. Calcium chloride-2-hydrate (Reidel de Haen, Germany)
5. D(+) - Glucose monohydrate (Reidel de Haen, Germany)
6. di-Sodium hydrogen phosphate-2-hydrate (Reidel de Haen, Germany)
7. Ethanol absolute (Merck, Germany)
8. Glycine (Sigma, U.S.A.)
9. L-Aspartic acid (Sigma, U.S.A.)
10. L-Glutamic acid (Sigma, U.S.A.)
11. L-Homoserine (Sigma, U.S.A.)
12. Magnesium sulfate-6-hydrate (Reidel de Haen, Germany)
13. Methanol HPLC grade (Merck, Germany)
14. 2-Mercaptoethanol (Merck, Germany)
15. O-Phthaldialdehyde (OPA) (Sigma, U.S.A.)
16. Polyethyleneglycol 400 (PEG 400) (Witayasom,Thailand)
17. Potassium chloride (Reidel de Haen, Germany)
18. Sodium dihydrogen phosphate-2-hydrate (Reidel de Haen, Germany)
19. Sodium hydrogen carbonate (Reidel de Haen, Germany)
20. Valproic acid (Sigma, U.S.A.)

Experimental methods

Preparation and administration of the test substances.

The test substances solubilized in water were PTZ, strychnine, bicuculline and pentobarbital. VPA and Amide 1C, which are insoluble in water were dissolved in PEG 400. They were administered intraperitoneally in a volume of 0.1 ml /25 g. BW.and 2 ml/kg BW. in mice and rat respectively.

1. Anticonvulsant activity.

1.1 Maximal electroshock seizure test (MES)

The MES, convulsion induced by electric current, was elicited by passage of an alternating current (50 mA, 50 Hz. delivered for 0.2 sec.) from electroshock apparatus through the brain via corneal electrodes after pretreatment with test substances. Observation was then made of the course of seizure within 0.2 -0.5 sec. The end point of the test was generalized seizures with tonic hindlimb extension (Thomson, 1990 ;Loscher and Nolting, 1991).

1.2 Determination of the optimal pretreated time and anticonvulsant activity against MES

1.2.1 Intraperitoneal route

Anticonvulsant activity of test substance was performed on male Swiss albino mice which were divided into 3 groups. Each group of them was then divided into 11 subgroups of 8 animals each. One subgroup (PEG 400, 0.1 ml/25 g BW.) was used as a control group. The others 10 subgroups were used for the test substances, VPA (100, 150, 200, 300 and 400 mg /kg BW.) and Amide 1C (50, 75, 90, 110 and 150 mg /kg BW.), which were intraperitoneally injected at various pretreated times (15, 30 and 60 min.) for determining the median effective dose (ED_{50}) against MES.

1.2.2 Oral route

Anticonvulsant activity of test substances, orally given (p.o), against MES was performed on 3 groups of 4 mice each. One group (PEG 400, 0.3 ml/25g BW.) was used as a control group. The other two groups were used for the test substances, Amide 1C 200 and 400 mg/kg BW. were given orally by gavage tube at optimal pretreated times from 1.2.1

1.3 Pentylenetetrazol seizure test (PTZ)

The PTZ seizures were elicited by a subcutaneous injection of PTZ 70 mg/kg BW. to the animals. The end point of this chemoshock test was a generalized clonic seizures with loss of righting reflex within 60 min after the injection of PTZ (Loscher et al, 1991)

Male Swiss albino mice were divided into 11 groups of 8 animals each for determination of the ED_{50} against PTZ seizures. One group was used as a control (PEG 400, 0.1 ml /25 g B W.). The other 10 groups were used for the test substances, VPA (50, 75, 100, 150 and 200 mg/kg BW.) and Amide 1C (15, 25, 50, 75 and 100 mg/kg BW.), which were intraperitoneally injected at the optimal pretreated time from 1.2.1

1.4 Bicuculline seizure test

Bicuculline convulsion was induced by an intraperitoneal injection of bicuculline 8 mg/kg after the pretreated time of test substances. The endpoint of bicuculline convulsion was generalized seizures with tonic extension of the hindlimb in 30 min. after the injection of bicuculline (Ticku and Rastogi, 1986).

Males Swiss albino mice were divided into 9 grops of 8 animals each for the determination of the ED_{50} against bicuculline seizures. One group was used as a control (PEG 400, 0.1 ml /25 g BW.). The other 8 groups were used for the test substances, VPA (200, 300, 400 and 500 mg /kg BW.) and Amide 1C (100, 200, 300 and 400 mg/kg BW.), which were given intraperitoneally at the optimal pretreated time from 1.2.1

1.5 Strychnine seizure test.

Strychnine convulsion was induced by an intraperitoneal injection of strychnine sulfate 2 mg /kg BW. after the pretreated time of test substances. The endpoint of strychnine convulsion was generalized seizures 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. One group was used as a control (PEG 400, 0.1 ml /25g BW.). The other 8 groups were used for the test substances,VPA (200, 300, 400 and 500 mg /kg BW.) and Amide 1C (100, 200,300 and 400 mg/kg BW.), which were given intraperitoneally at the optimal pretreated time from 1.2.1.

2. Toxicity test.

2.1 Acute toxicity.

Male Swiss albino mice were divided into 9 groups of 8 animals each for the determination of the median lethal dose (LD_{50}) and other effects, such as ataxia, sedation, hypnosis, respiratory secretion, etc., of VPA (600, 800 and 1000 mg /kg BW.) and Amide 1C (550, 600, and 700 mg/kg BW.). The test compounds were intraperitoneally injected and the observation for lethality was made within 72 hrs.

2.2 Neurotoxicity test.

2.2.1 Rotorod test.

The Rotorod test was used to evaluate the neurological deficit. In this study, the rotorod test was carried out by a rod of 3.5 cm. diameter, rotated at 13 rev/min. Neurological deficit was indicated by an inability of the animals to maintain their equilibrium for at least 1 min on the rotating rod in each of three successive trials.

Neurotoxicity of test substances was tested on 10 groups of 8 mice each for the determination of the median neurotoxic dose (TD_{50}).

One group was used as a control (PEG 400, 0.1 ml/25 g BW.). The other 8 groups were used for the test substances, VPA (200, 300, 400, 500 and 600 mg /kg BW.) and Amide 1C (100, 200, 300 and 400 mg /kg BW.), which were intraperitoneally injected. After the pretreated time was due, rotorod test was performed immediately.

2.2.2 Locomotor activity test.

Activity cage (Opto-varimex) was used to access the locomotor activity of mouse which was placed in a plastic box (17 x 17x 8 inches). A locomotor activity of each mouse was established by means of infra-red beams. Signals generated by an interuption of the beams were registered and processed by personal computer for 45 min. After that each mouse was removed from the box for the administration of the test substances and then returned to the box for further registration of 90 min. The experiments were always carried out between 8.00 - 11.00 a.m.

Male Swiss albino mice were divided into 6 groups of 8 animals each. Two groups were used as the control groups (PEG 400 and NSS, 0.1 ml./25 g. BW. i.p.). The other 4 groups were used for the test substances in 2 dose levels of VPA (100, 200 mg/kg i.p.) and Amide 1C (50, 100 mg /kg i.p.)

2.2.3 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 BW. was intraperitoneally injected immediately to the animal after the administration of the test substances. 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).

Male swiss albino mice were divided into 6 groups of 8 animals each. Two groups were used as the control groups (PEG 400 and NSS, 0.1 ml/25 g BW. i.p.). The other 4 groups were used for the test substances of VPA (100, 200 mg /kg BW. i.p.) and Amide 1C (50, 100 mg /kg BW. i.p.).

3. The measurement of amino acid neurotransmitters in cerebral cortex of anesthetized rats by microdialysis technique and HPLC.

3.1 Apparatus.

The HPLC system was consisted of many components, but the three main parts of this instrument are pump, column and detector. The solvent delivery pump (LC-10 AD Shimatzu, Japan) was used for the delivery of the mixture of two solvents (phosphate buffer and methanol). HPLC controller, a central processing unit, was used for a precise and accurate gradient elution. The column oven composed of an injection valve with a 50-µl sample loop capacity and a column (C-18 reverse phase, 250 x 4.60 mm., particle size 5 µm, Spherisorb ODS (2) Phenominex, U.S.A.) which was protected by a guard column. The Fluorescence detector operating at 330 nm. excitation wavelength was equipped with a 418 nm. cut-off emission filter. The area of each chromatographic peak was automatically determined by a computing integrator (Macintosh computer, Model LC 630, Apple computer, Inc., U.S.A.). The diagram of the HPLC system was shown in Figure 6.

3.2 Chromatography

3.2.1 Chromatographic conditions.

The separation in this HPLC analysis was gradient elution. The mobile phase was made freshly on a daily basis and composed of methanol (HPLC grade) and 0.05 M. phosphate buffer. A phosphate buffer was prepared from Na_2HPO_4. The pH was adjusted to 7.3 with 0.05 M. NaH_2PO_4. This mixture was filtered through a 0.22 µm. membrane filtered (Whatman, U.S.A.) under vacuum and degased with continuous helium gas until saturation prior to used.

For gradient run, the mobile phase was increased from 26 % to 76 % methanol in one convex curve step in a period of 14 min. Then, it was decreased from 76% to 26% methanol within 5 min. with equilibration time of about 10 min.

3.2.2 Preparation of OPA derivatizing reagent.

The OPA reagent was prepared by dissolving 54 mg of OPA in 1 ml of absolute ethanol and 40 µl. of 2 - mercaptoethanol was added then the volume was adjusted to 10 ml. with 0.4 M. borate buffer (H_3BO_3, NaOH) pH 9.5. The reagent mixture was allowed to age for at least 24 hrs, prior being use. The reagent strength was maintained by an addition of 4 µl. of 2 - mercaptoethanol every 4 days.

3.2.3 Preparation of artificial cerebrospinal fluid.

The artificial cerebrospinal fluid (aCSF) was consisted of 120 mM. NaCl, 15 mM. $NaHCO_3$, 5 mM. KCL, 1.5 mM. $CaCl_2$, 1 mM. $MgSO_4$ and 6 mM. glucose. The mixture was adjusted to pH 7.3 with 1 M. HCl.

3.3 Microdialysis Experiment.

3.3.1 Microdialysis probe.

The probe was purchased from Asahi Medical Co, Tokyo, Japan. The dialysis membranes were constructed from polyacrylonitrite. The molecular weight cut off is 55,000 and the diameter is 0.3 mm. The outer surface of the dialysis tube was covered with epoxy resin except the area 5 mm. of center portion that contacted the surface of rat cerebral cortex. Then, the dry probe was put into the chamber of aCSF for 24 hours prior to being used.

3.3.2 *In vivo* experiment.

a : The experimental animals.

Male albino rats (Wistar strain) weighing 330 - 360 g were divided into 6 groups of 5 animals each. The effect of test substances on the levels of glutamate, aspartate, glycine and GABA in rat cerebral cortex was assessed in comparison to PEG 400 and NSS. Two groups of animals were used as control (PEG 400 and NSS) while the other 4 groups were used for two dose levels of test substances, VPA (200 and 400 mg/kg. BW. i.p) and Amide 1C (100 and 200 mg/kg BW. i.p).

b : Implantation procedure.

The rat was anesthetized with pentobarbital 50 mg /kg BW. i.p. followed by supplymentary doses as required to maintain surgical anesthesia. After complete anesthesia, the rat was fixed in the stereotaxic apparatus. The appropriate area of the skull was exposed and a horizontal microdialysis 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 (Collins, 1978) according to a stereotaxic atlas of the rat brain (Pelligrino, Pelligrino and Cushman, 1979). The probe was fixed to the skull by epoxy resin.

c : Sample collection.

After microdialysis probe implantation, one side of microdialysis probe was connected to a constant - flow microinjection pump and the other side was placed into a collecting tube. Artificial cerebrospinal fluid (aCSF) was perfused into the microdialysis probe by a microinjection pump at the rate of 2 µl./min. After the perfusion period of 60 min. The test substances was intraperitoneally injected to the animals. The sample was collected continually every 20 min. for 3 hours. and then immediately placed into ice-box and kept frozen until being analyzed.

At the end of the experiment, the brain was exposed and checked for the appropriate position of microdialysis probe. The data was valid only when the right position of microdialysis probe was confirmed.

d : Analysis of Amino acid.

The measurement of the amount of amino acids was performed on HPLC with fluorescence detector. According to the method of Lindroth & Mopper (1979), the precolumn fluorescence derivatization with OPA was used.

The derivertization procedure was performed by mixing 10 µl of dialysate sample with 10 µl of 10 µM. Homoserine solution (internal standard) and adding 50 µl. of buffer reagent of OPA.

Then 50 µl. of mixing solution was injected to HPLC after a precise 2 min. incubation period.

Data were collected on a computing integrator. Peak was identified by comparing the retention time of each peak in the sample to that of individual peak in standard solution. The peak area of each amino acids and the internal standard were used for quantification.

Calculation and Statistical Analysis.

1. To determine either anticonvulsant activity (ED_{50}) or toxicity (LD_{50}, TD_{50}), groups of at least 8 mice were tested for the effect of various dose of test substance until at least three points were established between the limit of 0-100% protection or toxicity and transform to probit unit and log dose scale by transformatiom table of Fisher and Yates (Diem and Lentner, 1972). The 95% confidence interval was then calculated by the method of Litfield and Wilcoxom (1949).

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

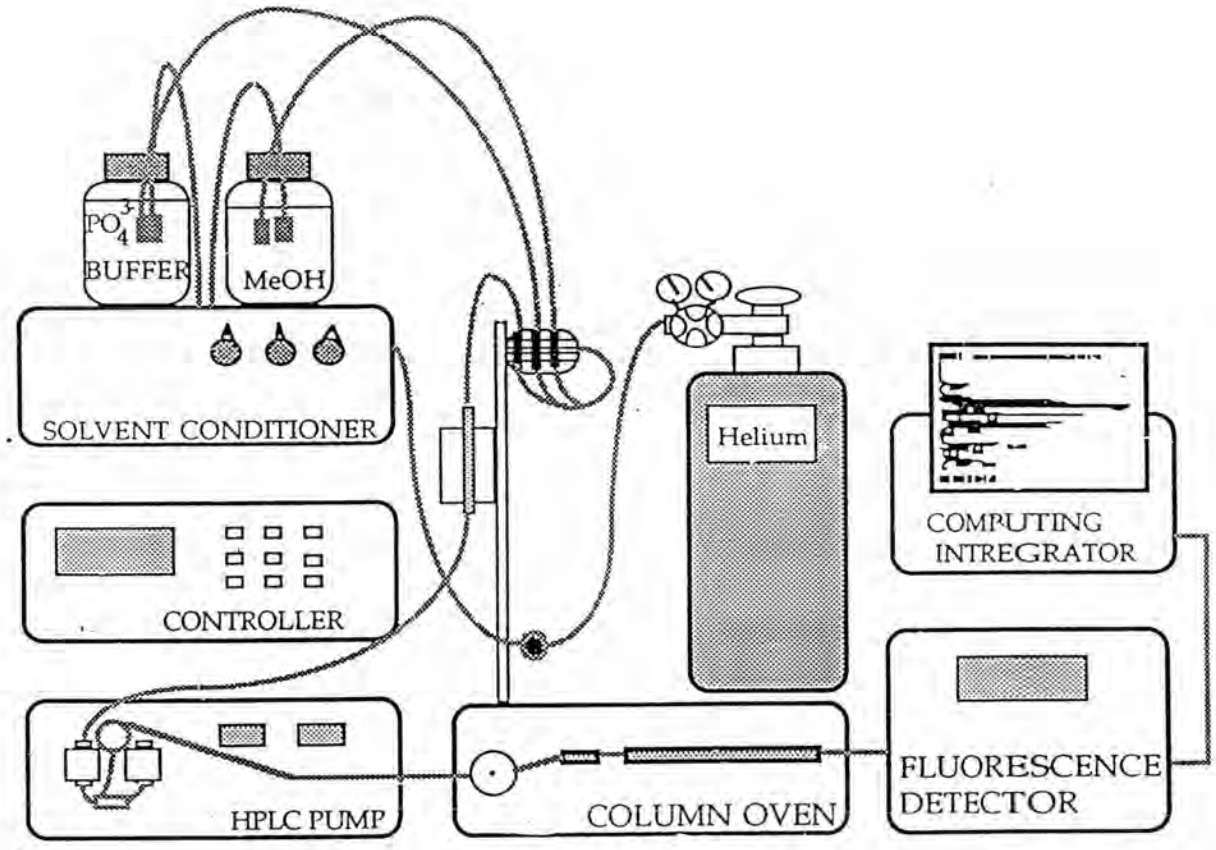

Figure 6. The diagram of HPLC system.