Chapter II
Materials and Methods

Experimental animals

All experiments were performed on male swiss albino mice weighing 18-25 g except for the study of anticonvulsant effects and effect on some cortical amino acid levels, relating to convulsion, which were performed on male albino rats (Wistar strain) weighing 200-350 g. Both of them were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom.

The experimental animals were maintained on 12 : 12 light-dark cycle at controlled temperature (25°C) in a group of 10 and were allowed free access to both food (F.E. Zeulig (Thailand) Co.,Ltd.) and water. In behavioral studies, the animals were acclimatized in the laboratory for a week before the experiments were started and all experiments with the respective of animals were then completed within the week to minimize the effect of increasing age on seizure susceptibility (Loscher, W., and Nolting, B., 1991). All experiments were carried out between 8 a.m. and 6 p.m. Each animal was used for only one experiment.

Chemicals

1. N-(2-Propylpentanoyl) Urea (VPU was synthesized by Assist. Prof. Dr.Chamnan Patarapanich et al. at Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.)
2. γ-Amino-n-butylic acid (SIGMA, U.S.A.)
3. l-Aspartic acid (SIGMA, U.S.A.)
4. Bicuculline (SIGMA, U.S.A.)
5. Boric acid (MERCK, Germany)
6. Calcium chloride-2-hydrate (Reidel de Haen, Germany)
7. Ethanol absolute (MERCK, Germany)

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8. D(+)-Glucose monohydrate (Reidel de Haen, Germany)
9. l-Glutamic acid (SIGMA, U.S.A.)
10. Glycine (SIGMA, U.S.A.)
11. l-Homoserine (SIGMA, U.S.A.)
12. Magnesium sulfate-6-hydrate (Reidel de Haen, Germany)
13. 2-Mercaptoethanol (MERCK, U.S.A.)
14. Methanol HPLC grade (MERCK, U.S.A.)
15. Pentobarbital Sodium Injection (Nembutal®, Sanofi (France) Ltd.)
16. Pentylenetetrazole (SIGMA, U.S.A.)
17. *o*-Phthaldialdehyde (SIGMA, U.S.A.)
18. Polyethyleneglycol 400 (PEG 400, T.Chemical Ltd., Thailand)
19. Potassium chloride (Reidel de Haen, Germany)
20. Sodium dihydrogen phosphate-2-hydrate (Reidel de Haen,Germany)
21. Sodium hydrogen carbonate (Reidel de Haen, Germany)
22. di-Sodium hydrogen phosphate-2-hydrate (Reidel de Haen, Germany)
23. Sodium hydroxide (Reidel de Haen, Germany)
24. Strychnine Sulfate (SIGMA, U.S.A.)
25. Valproic Acid (Aldrich, U.S.A.)

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Drug administration

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PEG 400 was used as a vehicle for VPA, VPU, and bicuculline. 0.9% Sodium chloride solution was used as a vehicle for strychnine sulfate, PTZ, and pentobarbital sodium. The dose levels of tested substances were expressed as milligram of substance/kilogram of body weight (mg/kg B.W.), and they were injected intraperitoneally (i.p.) except for PTZ which was given by a subcutaneous injection (s.c.). The volumns of injection were 0.1-0.2 ml in mice and 0.4-0.8 ml in rats.

Experimental instruments

1. Analog Digital Instruments (MacLab™/4, ADInstruments, Australia)
2. Animal activity cage (OPTO-VARIMEX, Columbus Instruments, U.S.A.)
3. Automatic micropipette (Pipetman®, Gilson, France)
4. Electroshock Apparatus with Corneal electrodes (King Mongkut Institue of Technology, North Bangkok Campus, Thailand)
5. HPLC Column; C18-Reverse phase, 250 x 4.60 mm., particle size 5 µm. (SPHERISORB 5 µm. 10 ODS (2), Phenominex®, U.S.A.)
6. HPLC Column Oven (Model 2155, LKB, Sweden)
7. HPLC Pump (Model 2150, LKB, Sweden)
8. LC Controller (Model 2152, LKB, Sweden)
9. Laser printer (Laser writter select 360, Apple computer, Inc., U.S.A.)
10. Macintosh® computer (Model LC 475, Apple computer, Inc., U.S.A.) with software programs ; Chart™ v. 3.2.8 for data recording system (MacLab™, ADInstruments, Australia) and Peaks™ v. 1.3 for data processing system (MacLab™, ADInstruments, Australia)
11. Microdialysis probe; horizontal type, molecular weigh cut off at 50,000 (Homofilter PNF-140, Asahi Medical Co., Japan)
12. Microinjection Pump (CMA/100, Carnegie, Sweden)
13. Microsyringes ; 100, 250 µl, and 1 ml (Exmire microsyringe, Ito Corporation, Japan)
14. Personal computer (IBM compatible, 486DX, Acermate 433, Acer peripherals Inc., Taiwan)
15. pH meter (Suntex, Japan)
16. Spectrofluorometric Detector (Jasco Model FP-210, Spectroscopic Co., Ltd., Japan)
17. Stereotaxic Instruments (NARISHIGE, Japan)
18. Ultrasonic cleaner (Branson, USA.)

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Experimental methods

1. Anticonvulsant activity

1.1 Anticonvulsant activity against Maximal Electroshock Seizure (MES)

The MES was elicited by the passage of an alternating electric current (50 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 either 55 mA., 0.2 second duration in mice or 155 mA., 0.2 second duration in rats. The endpoint of MES test was generalized seizure with tonic hindlimb extention in both species (Thompson, 1990; Loscher and Nolting, 1991).

1.1.1 Determination of the optimal pretreated time and anticonvulsant activity against MES in mice

The peak time of maximal anticonvulsant activity which would be subsequently used as the optimal pretreated time were performed on male swiss albino mice. They were divided into 3 groups according to pretreated time (15, 30, and 60 minutes) for determining the median effective dose (ED_{50}) against MES. Each group of mice was then divided into 11 subgroups of 8 animals each. One subgroup was used as a control (PEG 400, 0.1 ml i.p.). The others 10 subgroups were used for the test of anticonvulsant activity of VPA and VPU in 5 dose levels of 50, 100, 200, 300, and 400 mg/kg B.W. i.p. for each tested substance.

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1.1.2 Anticonvulsant activity against MES in rats

Male albino rats (Wistar strain) were divided into 11 groups of 8 animals each for the determination of the ED_{50} against MES. One group was used as a control (PEG 400, 0.4 ml i.p.). The others 10 groups were used for the test of anticonvulsant activity of VPA and VPU in 5 dose levels of 50, 100, 200, 300, and 400 mg/kg B.W. i.p. for each tested substance using the optimal pretreated time from 1.1.1.

1.2 Anticonvulsant activity against Pentylenetetrazole Seizure (PTZ seizure)

After the optimal pretreated time of tested substances (see 1.1.1), PTZ seizure was induced by subcutaneous injection of PTZ 70 mg/kg B.W. to the animals. The endpoint of PTZ seizure test was generalized clonic seizure with loss of righting reflex in 60 minutes after injection of PTZ (Loscher et al, 1991).

1.2.1 Anticonvulsant activity against PTZ seizure in mice

Male swiss albino mice were divided into 11 groups of 8 animals each for determination of the ED_{50} against PTZ seizure. One group was used as a control (PEG 400, 0.1 ml i.p.). The others 10 groups were used for the test of anticonvulsant activity of VPA and VPU in 5 dose levels of 50, 75, 100, 200, and 250 mg/kg B.W. i.p. for each tested substance using the optimal pretreated time from 1.1.1 .

1.2.2 Anticonvulsant activity against PTZ seizure in rats

Male albino rats (Wistar strain) were divided into 11 groups of 8 animals each for determination of the ED_{50} against PTZ seizure. One group was used as a control (PEG 400, 0.4 ml i.p.). The others 10 groups were used for the test of anticonvulsant activity of VPA and VPU in 5 dose levels of 50, 75, 100, 200, and 250 mg/kg B.W. i.p. for each tested substance using the optimal pretreated time from 1.1.1 .

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1.3 Anticonvulsant activity against Strychnine convulsion

Strychnine convulsion was induced by an intraperitoneal injection of strychnine sulfate 2 mg/kg B.W. after the pretreated time of tested substances. The endpoint of stychnine convulsion was generalized seizure with tonic extension in 30 minutes after the injection of strychnine (Ticku and Rastogi, 1986).

Male swiss albino mice were divided into 9 groups of 8 animals each for determination of the ED_{50} against strychnine seizure. One group was used as a control (PEG 400, 0.1 ml i.p.). The others 10 groups were used for the test of anticonvulsant activity of VPA and VPU in 5 dose levels of 100, 200, 300, and 400 mg/kg B.W. i.p. for each tested substance using the optimal pretreated time from 1.1.1 .

1.4 Anticonvulsant activity against Bicuculline convulsion

Bicuculline convulsion was induced by an intraperitoneal injection of bicuculline 8 mg/kg B.W. after the pretreated time of tested substances. The endpoint of bicuculline convulsion was generalized seizure with tonic extension of the hindlimbs in 30 minutes after the injection of bicuculline (Ticku and Rastogi, 1986).

Male swiss albino mice were divided into 11 groups of 8 animals each for determination of the ED_{50} against bicuculline seizure. One group was used as a control (PEG 400, 0.1 ml i.p.). The others 10 groups were used for the test of anticonvulsant activity of VPA and VPU in 5 dose levels of 100, 200, 300,400, and 500 mg/kg B.W. i.p. for each tested substance using the optimal pretreated time from 1.1.1 .

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2. Toxicity

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2.1 Acute toxicity

Male swiss albino mice were divided into 9 groups of 8 animals each for determination of the median lethal dose (LD_{50}) and other observing effects, such as ataxia, sedation, hypnotic, respiratory secretion, etc., in 72 hours of VPA (4 dose levels; 500, 750, 1000, and 1250 mg/kg B.W. i.p.) and VPU (5 dose levels; 750, 1000, 1250, 1500, 2000, and 2500 mg/kg B.W. i.p.).

2.2 Effect on locomotor activity

Activity cage (OPTO-VARIMEX) was used to access the locomotor activity of mice placed in a plastic box (17 x 17 x 8 inches) equipped with infra-red photobeams. Signals generated by an interuption of the beams were registered and processed by a personal computer (IBM compatible, 486 DX, Acermate, Acer peripherals Inc., Taiwan) for a period of 180 minutes. A basal locomotor activity of each mouse was established by allowing a control period of 40 minutes before the administration of the tested substances.

The experiment was carried out between 0.05-3.05 p.m. Male swiss albino mice were divided into 5 groups of 8 animals each. One group was used as a control (PEG 400, 0.1 ml i.p.). The others 4 groups were used for the test of effect on the locomotor activity of VPA (2 dose levels; 100 and 200 mg/kg B.W. i.p.) and VPU (2 dose levels; 50 and 70 mg/kg B.W. i.p.).

2.3 Effect on barbiturate sleeping time

Effect on barbiturate sleeping time was used to evaluate the depressing effect of substances on the CNS. In this study, pentobarbital sodium 60 mg/kg B.W. was intraperitoneally injected immediately to the animals after the administration of the tested substances. The sleeping time was measured as the time between the loss and the recovery of righting reflex (McLeod, et al., 1970).

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Male swiss albino mice were divided into 5 groups of 8 animals each. One group was used as a control (PEG 400, 0.1 ml i.p.). The others 4 groups were used for the test of effect on the barbiturate sleeping time of VPA and VPU in 2 dose levels of 100 and 200 mg/kg B.W. i.p. for each tested substance.

3. Effect on some cortical amino acid neurotransmitter levels relating to convulsion in anesthetized rats by microdialysis technique

3.1 Experimental animals

Male albino rats (Wistar strain) weighing 250-350 g were divided into 5 groups of 5 animals each for determining the effect of tested substances on the levels of glutamate, aspartate, glycine and GABA in rat cerebral cortex. One group was used as a control (PEG 400, 0.4 ml i.p.). The others 4 groups were used for testing the effect of tested substances. 2 Dose levels of VPA and VPU, 200 and 400 mg/kg B.W. i.p., were used. (one group for each dose level)

3.2 Microdialysis technique

This technique was modified from those described by Ungerstedt (1984) and Benveniste and Huttemeier (1990).

These investigations were conducted on rats anaesthetised with pentobarbital sodium 40 mg/kg B.W. i.p. The anesthetized animal was then placed in a stereotaxic head holder (Narishige, Japan). After the appropriate area of the skull was exposed, a horizontal microdialysis probe (0.2 mm outer diameter, acrylic polymer with 50,000 molecular weight cut off, Asahi Medical Co., Japan) 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), and then was fixed to the skull by epoxy resin. The outer surface of microdialysis probe was totally covered with epoxy resin except the area of 5 mm in length that is in contact with the cortex of rat. After microdialysis probe implantation, one side of microdialysis probe was connected to a constant-flow perfusion pump by polyethylene tube, and the other side was placed into a collecting tube. Artificial CSF (120 mM NaCl, 15 mM $NaHCO_3$,

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5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgSO_4$, 6 mM Glucose , pH 7.4; Benveniste and Huttemeier, 1990) was perfused into the microdialysis probe by a perfusion pump at the rate of 2 µl/min. The dialysate emerging from the other end was collected every 20 minutes. The first dialysate sample was collected at 60 minutes after the perfusion was begun, and was determined for the glutamate, aspartate, glycine and GABA levels. At the equilibrium state of amino acid levels, the tested substances, VPA or VPU, were intraperitoneally injected to the animals and the dialysate samples were continuously collected for another three hours. Determination of amino acid levels was done by means of high performance liquid chromatography (HPLC) technique.

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.

3.3 The HPLC determination of rat cortical amino acid levels

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 Lindorth 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 degased with continuous helium gas. The gradient run was started at 20 % methanol content of the mobile phases and increasing to 60 % at the rate of 2 %/min for 20 minutes. At the end of the run, initial condition was then restored by the reversed methanol gradient run form 60 % to 20 % at the rate of 10 %/min. A delay period of abount 10 minutes was required for column equilibration. The flow rate used was 0.8 ml/min.

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The buffer reagent solution of OPA/2-mercaptoethanol was prepared by dissolving 50 mg of OPA in 1 ml absolute ethanol and adding 40 µl of 2-mercaptoethanol and then adjusting the volume to 10 ml with borate buffer pH 9.5. The reagent mixture was allowed to age for at least 24 hours before use. The reagent strength was maintained by an addition of 4 µl of 2-mercaptoethanol every 4 days.

The derivatization 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 (about 25 °C). Then 50 µl injection to HPLC was made after a precise 2-min incubation period.

The spectofluorometric detector (Jasco Model FP-210, Japan) was set emission wavelength to 418 nm and excitation wavelength to 330 nm. Output data from detector were changed from analog to digital by MacLab™ (ADInstruments, Australia), recorded by Chart™ v. 3.2.8 (ADInstruments, Australia), and analysed by Peaks™ v. 1.3 (ADInstruments, Australia) in Macintosh® computer (Model LC 475, U.S.A.)

4 Statistical analysis

Data presented are expressed as mean ± standard error of the mean (S.E.M.). The ED_{50} and the LD_{50} were calcurated by probit analysis (Burn, Finney, and Goodwin, 1952; McLeod et al., 1970). For all numerical data generate statistically significants between treated and control groups were determined by Student's *t*-test at $P < 0.05$.

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