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 250-350 g were used to study degradation of valproyl hydroxamic acid. All of experimental animals were obtained from National Laboratory Animal Center , Mahidol University, Salaya, Nakornpathom.

The animals were acclimatized in the laboratory for at least 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 standard food (C.P. mice food) and tab water. In anticonvulsant studies, the experiments with 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 a.m. - 6.00 p.m. except for the sleeping time and locomotor experiments which were carried out between 8.00 - 12.00 a.m. Each animal was used only once.

Equipments

1. Electroshock apparatus with corneal etectrode (King Mongkut Institute of Technology, North Bangkok, Thailand)
2. Rotorod (King Mongkut Institute of Technology, North Bangkok, Thailand)
3. Activity cage (UGO Basile, Comerico, Italy)
4. Microliter syring (Hamilton, Nevada, U.S.A.)
5. Hypodermic needle (Trade mark, Japan)
6. pH meter (Suntex, Japan)

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

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

9. HPLC system (Shimatzu, Japan)

10. 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. Valproyl hydroxamic acid (VHA) was supplied by Assist. Prof. Dr. Chamnan Patarapanich (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand)
2. Acetonitrile (Sigma, U.S.A.)
3. 9– anthryldiazomethane (ADAM; Funakoshi , Japan)
4. Bicuculline (Sigma, U.S.A.)
5. Methanol HPLC grade (Merck, U.S.A.)
6. Pentobarbital sodium (PB; Nembutal, Sanofi (France) Ltd.)
7. Pentylenetetrazole (PTZ, Sigma, U.S.A.)
8. Polyethylene glycol 400 (PEG, Witayasom, Thailand.)
9. SKF–525A (Sigma, U.S.A.)
10. Sodium dihydrogen phosphate-2-hydrate (Reidel de Haen, Germany)
11. di-Sodium hydrogen phosphate–2-hydrate (Reidel de Haen, Germany)
12. Strychnine sulfate (Sigma, U.S.A.)
13. Valproic acid (VPA; Sigma, U.S.A.)

Experimental methods

1. Preparation and administration of the test substances

The test substances being solubilized in water were PTZ, strychnine, bicuculline and pentobarbital. VPA and VHA which are insoluble in water were

dissolved in PEG 400. 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.

In degradation studies, the stock solution of test substance was prepared in methanol at concentration of 0.1 M and kept at $0^{o}C$ in freezer of refrigerator.

2. Anticonvulsant efficacy

2.1 Maximal Electroshock Seizure (MES)

The MES, convulsion induced by electric current, was elicited by passage of an alternating electric current (current = 55 mA., frequency = 50 Hertz., duration = 0.2 sec.) from electroshock apparatus through the brain via corneal electrods 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 seizure with tonic hindlimb extension (Thomson, 1990; Loscher and Nolting, 1991).

2.1.1. Determination of anticonvulsant activity and the optimal pretreated time of test substances given by an intraperitoneal route

The peak time of maximal anticonvulsant activity which would be subsequently used as the optimal pretreated time was performed on mice. They were divided into 3 groups according to pretreated times (15, 30 and 60 min). Each group of mice was then divided into 9 subgroups of 10 animals each. One subgroup (PEG 400, 0.1 ml/25 g B.W., i.p.) was used as a control group. The other 8 subgroups were used for determination of anticonvulsant activity of test substances, expressed as the median effective dose (ED_{50}) against MES. VPA (150, 200, 300, 400 mg/kg B.W., i.p. at 15, 30, and 60 min pretreated time) and VHA (95, 110, 125, 140 mg/kg B.W., i.p. at 15 min pretreated time ; dose 130, 150, 160, 170 mg/kg B.W., i.p. at 30 min pretreated time; dose 180, 190, 200, 220 mg/kg B.W., i.p. at 60

min pretreated time) were intraperitoneally injected into the animals. When pretreated time was due, MES was performed as previously described in 2.1. The minimum pretreated time that gives the maximal anticonvulsant activity of each test substance was selected and used as optimal pretreated time in other experiments.

2.1.2. Anticonvulsant activity of test substances at 3 and 6 hrs

For determining the duration of action of the test substances (VPA and VHA), mice were divided into 2 groups according to pretreated time (3 and 6 hr). Each group was then divided into 8 subgroup of 10 animals each, for the determination of the ED_{50} of test solution at each pretreated time. VPA (dose 150, 200, 300, 400 mg/kg B.W., i.p. at 3 hr pretreated time and dose 200, 300, 400, 500 mg/kg B.W., i.p. at 6 hr pretreated time) and VHA (dose 200, 300, 400, 500 mg/kg B.W., i.p. at 3 hr pretreated time and dose 500, 700, 800, 900 mg/kg B.W .,i.p. at 6 hr. pretreated time) were given intraperitoneally to respective groups of animal. When pretreated time was due, MES was performed as previously described in 2.1.

2.1.3 Anticonvulsant activity of test substances given by an oral route (p.o.)

Anticonvulsant activity of test substances against MES was performed on 9 groups of 10 mice each. One group (PEG 400, 0.3 ml/25 g B.W., p.o.) was used as a control group. The other 8 groups were used for the determination of the ED_{50} of the test substances. VPA (300, 400, 500, 600 mg/kg B.W.) and VHA (150, 200, 250, 300 mg/kg B.W.) were given orally by gavage tube. After the optimal pretreated time selected according to 2.1.1 MES was performed as previously described in 2.1.

2.1.4 Anticonvulsant activity of test substances given by intracerebroventricular route (i.c.v.)

The test substances were administered intracerebroventricular (i.c.v.) in a volume of 4 μl by the method of Haley and McCormick (1957). The injection was made by a hypodermic needle, 3-3.5 mm. long, attached to a 0.25 ml Hamilton microliter syringe. For this experiment, VHA and VPA (sodium valproate) were dissolved in NSS due to irritating effect of PEG 400 on brain tissue (Brittain and Handley, 1967).

Anticonvulsant activity of test substances against MES was performed on 9 groups of 10 mice each. One group (NSS) was used as a control group. The other 8 groups were used for the determination of the ED_{50} of the test substances. VPA (sodium valproate ;100, 150, 200, 250 μM) and VHA (50, 100, 150, 200 μM) were intracerebroventricularly given at the optimal pretreated time from 2.1.1. When pretreated times was due, MES was performed as previously described in 2.1.

2.1.5 Tolerance

The test substances were orally given (p.o.) to mice by gavage tube (once a day) at the same time of the day for 5 days successively prior to MES test. In MES test, male Swiss albino mice were divided into 2 groups. Each group was then divided into 4 subgroups of 10 animals each, for the determination of the ED_{50} of test substances.

Group 1 received PEG 400 (0.3 ml / 25 g B.W., p.o.) for 5 days. On day 6 VHA (150, 200, 250, 300 mg/kg B.W.) were given orally at the optimal pretreated time from 2.1.1.

Group 2 received VHA (ED_{50} from 2.1.3) for 5 days. On day 6 VHA (200, 250, 300, 350 mg/kg B.W.) were given orally at the optimal pretreated time from 2.1.1.

When pretreated time was due, MES was performed as previously described in 2.1.

2.1.6 Effects of SKF-525A on anticonvulsant activity of test substances

To determine if the test substances or its metabolite is the active anticonvulsant agent, animals were pretreated with SKF-525A, a P_{450} enzyme inhibitor, Then anticonvulsant activity was tested using the MES test. SKF-525A (50 mg/kg p.o.) was administered 1 hr before the test substances (Shank et al., 1994), which were intraperitoneally injected at the optimal pretreated time from 2.1.1.

After pretreatment with SKF-525A, mice were divided into 9 groups of 10 animals. One group was used as a control (PEG 400, 0.1 ml/25 g B.W., i.p.). The other 8 groups were used for the test substances, VPA (50, 150, 200, 250 mg/kg B.W., i.p.) and VHA (50, 100, 150, 200 mg/kg B.W., i.p.). MES test as previously described in 2.1 was then performed.

2.2 Chemically induced seizure tests

Chemoshock was induced by an injection of either pentlyenentetrazole or bicuculline or strychnine into the experimental animals. Abolition of seizures by pretreatment of test substances indicated their anticonvulsant activity.

2.2.1 Anticonvulsant activity against Pentylentetrazole Seizure Test (PTZ)

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

Male Swiss albino mice were divided into 10 groups of 10 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.,i.p.). The other 9 groups were used for the test substances, VPA (50, 75, 100, 150, 200 mg/kg B.W.) and VHA (75, 110, 145, 180 mg/kg B.W.), which were intraperitoneally injected at the optimal pretreated time from 2.1.1.

2.2.2 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 test substances. The end point of strychnine convulsion was generalized seizure 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 10 animals each for the determination of the ED_{50} against strychnine seizures. One group was used as a control (PEG 400, 0.1ml/25 g B.W., i.p.). The other 8 groups were used for the test substances, VPA (200, 300, 400, 500 mg/kg B.W.) and VHA (200, 300, 400, 500 mg/kg B.W.), which were given intraperitoneally at the optimal pretreated time from 2.1.1.

2.2.3 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 test substances. The end point 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 8 groups of 10 animals each for the determination of the ED_{50} against bicuculline seizure. One group was used as a control (PEG 400, 0.1 ml/25 g B.W., i.p.). The other 7 groups were used for the substances, VPA (200, 300, 400, 500 mg/kg B.W.) and VHA (100, 150, 200

mg/kg B.W.), which were given intraperitoneally at the optimal pretreated time from 2.1.1.

3. Toxicity test

3.1 Acute toxicity

Mice were divided into 7 groups of 10 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 (500, 600, 800, 1000 mg/kg B.W.) and VHA (600, 800, 1000 mg/kg B.W.). The test compounds were intraperitoneally injected and the observation for lethality was made within 72 hrs.

3.2 Neurotoxicity test

3.2.1 Rotorod test

The rotorod test was modified from the one previously described by Dunham and Miya (1957), carried out with a rod of 3.5 cm diameter, rotated at 13 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 balances 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.

3.2.1.1 Determination of median neurotoxic dose (TD_{50})

Neurotoxicity of test substances was performed on 9 groups of 10 mice each. One group (PEG 400, 0.1 ml/25 g B.W., i.p.) was used as a control group. The other 8 groups were used for the determination of the median

neurotoxic dose (TD_{50}) of the test substances. VPA (150, 200, 300, 400 mg/kg B.W.) and VHA (100, 200, 300, 400 mg/kg B.W.) were intraperitoneally injected. After the optimal pretreated time selected according to 2.1.1, rotorod test was performed as previously described.

3.2.1.2 Duration of neurotoxic effect

For determining the duration of neurotoxic effect, mice were divided into 3 groups according to pretreated time (1, 3 and 6 hr). Each group was then divided into 8 subgroups of 10 mice each, for the determination of the TD_{50} of test substances. VPA (does 150, 200, 300, 400 mg/kg B.W. at 1 hr pretreated time and does 200, 300, 400, 500 mg/kg B.W. at 3 and 6 hr pretreated time) and VHA (dose 200, 300, 400, 500 mg/kg B.W. at 1 hr pretreated time and dose 500, 700, 800, 900 mg/kg B.W. at 3 and 6 hr pretreated time) were given intraperitoneally to respective groups of animal. When pretreated time was due, rotorod test was performed as previously described in 3.2.1.

3.2.2 Locomotor activity test

Activity cage (UGO Basile, Comerico - Italy) was used to access the locomotor activity of mouse which was placed in a box (length 35 cm; width 23 cm; height 20 cm). The cage floor is made of evenly spaced stainless steel bars which have 3 mm. diameter and spaced 11 mm. apart, connected to the circuit and, optional, horizontal and vertical infra - red sensors. The counters were read at 15 min intervals. Motor activity was measured in an animal cage for a period of 90 min. A basal locomotor activity of each mouse was establish allowing a control period of 45 min before the administration of the test substances. Immediately after administration of the test substance, the animals were placed in the individual cages. The experiments were always carried out between 8.00 - 11.00 a.m.

Male Swiss albino mice were divided into 6 groups of 10 animals each. Two groups were used as the control groups (PEG 400 and NSS, 0.1 ml/25 g B.W., i.p.). The other 4 groups were used for the test substances in 2 dose levels of VHA (90, 120 mg/kg B.W., i.p.) and VPA (100, 200 mg/kg B.W., i.p.).

3.2.3 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 B.W. 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.)

Mice were divided into 6 groups of 10 animals each. Two groups (NSS and PEG 400, 0.1 ml/25 g B.W., i.p.) were used as control groups. The other 4 groups (VPA 100, 200 mg/kg B.W. and VHA 90, 120 mg/kg B.W., i.p.) were used to test for the potentiation of barbiturate sleeping time.

4. The degradation of VHA

4.1 Degradation experiment using brain and liver homogenates

Wistar albino rats were used to prepare brain and liver homogenates. They were sacrificed by decapitation. Brain and liver were immediately removed and homogenized at 0-5 $^{\circ}$C in glass – Teflon homogenizer to prepare 40% homogenate in pH 7.4 isotonic phosphate buffer, then centrifuged at 2500 g for 15 min, and the supernatant was used for the experiment. Degradation experiment were performed at 37 $\pm$ 0.2 $^{\circ}$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

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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-anthydiazomethane (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 $-10^{\circ}C$ 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 hours. Then a portion of solution was injected to 50 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 were converted from analog data 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.

Calculation and Statistical Analysis

1. For the determination of ED_{50}, TD_{50} and LD_{50} groups of 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% response or non response and transformed to probit unit by transformation table of Fisher and Yates (Diem and Lentrer, 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 Cricket graph program (Macintosh® computer). The 95 percent confidence interval was calculated by the method of Litchfield and Wilcoxon (1949).

2. 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 ($p < 0.05$).