Chapter II

Material and Methods

Experimental animals

The experiments were performed on either male Swiss albino mice or male Wistar albino rats weighing 18-25 g and 250-350 g, respectively. Both of them were obtained from the 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 the animals were maintained under natural light/dark cycle at control temperature (25°c) and were allowed free access to standard food (C.P. food, Thailand) and water. In behavioral studies, the experiments with respective groups of mice were then completed within a week to minimize the effect of increasing age on seizure susceptibility (Lösher and Nolting, 1991). All the experiments were carried out between 8.00a.m. - 6.00 p.m. Each animal was used only once. All animal care and handling was approved by the Ethical Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Chemicals

1. Ametolide and N- (*P*-aminobenzoly)-1,2,3,4-tetrahydro-4-methylquinoline(CU-17-06) were kindly supplied by Assist. Prof. Chamnan Patarapanich and co worker (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Science, Chulalongkorn University, Thailand)
2. γ-Amino-n-butylic acid (GABA ; Sigma, U.S.A.)
3. L-Aspartic acid (Sigma, U.S.A.)
4. Calcium chloride 2 hydrate ($CaCL_2.2H_2O$; Riedel de Häen, Germany)
5. Chloral hydrate (Witayasom, Thailand)
6. D-Glucose monohydrate (Riedel de Häen, Germany)
7. L-Glutamic acid (Sigma, U.S.A.)

8. Glycine (Sigma, U.S.A.)
9. L-Homoserine (Sigma, U.S.A.)
10. Magnesium sulfate 6 hydrate ($MgSO_4$. 6 H_2O ; Riedel de Häen, Germany)
11. 2- Mercaptoethanol (Merck, Germany)
12. Methanol, HPLC grade (Merck, Germany)
13. Pentylenetetrazole (PTZ ; Sigma, U.S.A.)
14. O- Phathaldialdehyde (Sigma, U.S.A.)
15. Polyethylene glycol 400 (PEG 400 ; Witayasom, Thailand)
16. Potassium chloride (KCL ; Riedel de Häen, Germany)
17. Sodium chloride (NaCL ; Riedel de Häen, Germany)
18. Sodium dihydrogen phosphate 2 hydrate ($Na_2H_2PO_4$. 2 H_2O ; Riedel de Häen, Germany)
19. Sodium hydrogen carbonate ($NaHCO_3$; Riedel de Häen, Germany)
20. di-Sodium hydrogen phosphate 2 hydrate (Na_2HPO_4. 2 H_2O ; Riedel de Häen, Germany)
21. Sodium hydroxide (NaOH ; Riedel de Häen, Germany)

Drug preparations and administrations

The test substances (ameltolide and CU-17-06) which are insoluble in water were dissolve in PEG 400 (which was used as a vehicle) and the others, which soluble in water (Chloral hydrate and PTZ) were dissolved in 0.95% 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.) except PTZ, which was given by subcutaneous (s.c.). The volumes of injection were 0.1 ml / 25 g B.W. in mice and 0.4-0.8 ml in rat.

Equipments

1. Electroshock apparatus with corneal electrode (King Mongkut Institute of Technology, North Bangkok, Thailand)
2. Rotarod (UGO Basile, Comerico, Italy)
3. Autonomic infusion pump (CMA/100, Carnegie, Sweden)
4. Stereotaxic Instruments (Narishige, Japan)

5. Automatic mixer (Vortex, U.S.A.)
6. Microsyringe pump (Hamilton, U.S.A.)
7. System for freely moving animal (CMA/120, Carnegie, Sweden)
8. HPLC system
   - C_{18} Reverse phase, 250×4.6 mm, particle size 5 μm, spherisorp ODS(2) (Hicrome®, England)
   - Guard column with packing material, particle size 5 μm, spherisorp ODS(2) (Phenominex®, 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} V 3.2.8 for data recording system and peak^{TM} V 1.3 for data processing system.
   - Laser printer (Laser writer select 360, Apple Computer, Inc; U.S.A.)
9. Microdialysis probe; Horizontal type, molecule weight cut off. 50,000 (Homofilter PNF-140, Asahi Medical Co., Tokyo Japan)
10. pH meter (Suntex, Japan)

Experimental methods

1. Anticonvulsant activity

1.1 Anticonvulsant activity against Maximal Electroshock Seizure (MES)

The MES was elicited by a passage of an alternating current (55 mA, 50 Hz, 0.2 sec) from electroshock apparatus through the brain via corneal electrodes after pretreated with tested substances. The endpoint of MES test was generalized seizure with tonic hindlimb extention (Thompson, 1990; Lösher and Nolting, 1991).

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

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

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 time (15, 30, and 60 min.). Each group of mice was divided into 12 subgroups of 8 animals each. Two subgroups (NSS and PEG 400, 0.1 ml/25 g B.W., i.p.) were used as control group (3 animal each). The other 10 subgroups were used for determination of anticonvulsant activity, expressed as the median effective (ED_{50}) against MES. The test substances, ameltolide (0.5, 0.75, 1.0, 1.25, and 1.5 mg/kg B.W.) and CU-17-06 (50, 75, 100, 125, and 150 mg/kg B.W.) were intraperitoneally injected. When pretreated time was due, MES was performed as described in 1.1. 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.

1.1.2 Duration of protection against MES

For determining the duration of action of the test substances (ameltolide and CU-17-06), mice were divided into 2 groups according to pretreated time (3,and 6 hr). Each group was then divided into 8 subgroups of 8 animals each, for the determination of the ED_{50} of test solution at each pretreated time. Ameltolide (dose2, 3,3.5, and 4 mg/kg B.W. i.p. at 3 hr pretreated time and dose 6, 8, 10, and 12 mg/kg B.W. i.p. at 6 hr pretreated time) and CU-17-06 (dose 150, 300, 450,and 600 mg/kg B.W. i.p. at 3 hr pretreated time and dose 300, 450, 600,and 800, 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 1.1.

1.1.3 Anticonvulsant activity against Pentylenetetrazole (PTZ) seizure Test

The PTZ seizure was elicited by a subcutaneous injection of PTZ 70 mg/kg B.W. to the animals after the pretreated time of the test substances. The endpoint of this test was generalized clonic seizure with loss of righting reflex within 60 min after the injection of PTZ (Löscher and Nolting, 1991).

Male Swiss albino mice were divided into 8 groups of 8 animals each for determination of the ED_{50} against PTZ seizures. Two groups were used as a control (PEG 400 and NSS, 0.1 ml/25 g B.W. i.p.). The other 6 groups were used for the test substances, Ameltolide (3, 6, and 8 mg/kg B.W. i.p.) and CU-17-06 (150, 300, and 600 mg/kg B.W. i.p.). Which were intraperitoneally injected at the optimal pretreated time from 1.1.1.

2. Toxicity

2.1 Acute toxicity test

Male Swiss albino mice were divided into 6 groups of 8 animals each for determination of the median lethal dose (LD_{50}) and other effects such as ataxia, sedation, hypnosis, respiratory secretion, etc., of ameltolide (60,65,and 70mg/kg B.W. i.p.) and CU-17-06 (600,800,and1,000 mg/kg B.W. i.p.). The test compounds were intraperitoneally injected and the observation for lethality was made within 72 hrs.

2.2 Neurotoxicity test

2.2.1 Rotarod test

The rotarod test was modified from the one previously described by Dunham and Miya (1957), was carried at with a rod of 3.5 cm diameter, rotating at 18 rev/min. Neurological deficit was indicated by inability of the animals to maintain their equilibrium for at least 1 minute on the rotating rod in each of three successive trials. Untreated mice were able to maintain their balances on the rod for several minutes. Substances or vehicle-treated mice, which were not able to maintain their equilibrium on the rod for 1 minute, 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.

Neurotoxicity of test substances was performed on 10 groups of 8 mice each. Two groups (PEG 400 and NSS, 0.1 ml/25 g B.W. i.p.) were used as control

group. The other 8 groups used for the determination of the median neurotoxic dose (TD_{50}) of the test substances. Ameltolide (6, 8, 10,and 12 mg/kg B.W. i.p.) and CU-17-06 (150, 300, 450,and 600 mg/kg B.W. i.p.) were intraperitoneally injected. After the optimal pretreated time selected according to 1.1.1 rotarod test was performed as previously described.

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

3.1 Experimental animal

Male Wistar rats weighing 250-350 g were divided into 6 groups of 5 animals each for determination of the effect of test substances on the levels of aspartate, glutarmate, glycine and GABA in rat cerebral cortex. Two groups were used as control (PEG 400 and NSS, 0.4 ml/kg B.W. i.p.). The others 4 groups were used for testing the effect of tested substances (ameltolide 1.1, 2.2 mg/kg B.W. i.p. and CU-17-06 77.62, 155.2mg/kg B.W. i.p.).

3.2 Microdialysis technique

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

3.2.1 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 (0.2mm outer diameter, acrylic polymer with 50,000 molecular weight cut off) was covered with epoxy resin totally 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 rostal 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 hrs for recovery before the experiment was started.

3.2.2 Collection of cerebrospinal fluid (CSF) samples

The rat was place 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/120, 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 $CaCL_2$, 1.0 mM $MgSO_4$, and 6 mM glucose, pH 7.4 (Benveniste and Huttermeier (1990). The aCSF was continuously perfused at the rate of 2 μl/min. The Dialysate collected during the equilibration period of 60 min was discarded. After the equilibration period of 60 min the time first sample was collected.

Basal amino acid levels were determined from the 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 specimen with a sharp blade and the inspected visually. The data was valid only when the right of microdialysis probe was confirmed.

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3.3 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 7.4 in triple distilled water and methanol (HPLC grade). Both of the mobile phase gradients 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 20%/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 prepared by mixing of 5-10μl of 2-mercaptoethanol and 1ml of O-Phthaldialdehyde,then maintained by an addition of 4 μl 2-mercaptoethanol every 4 days. The derivatization procedure was performed by mixing 10 μl of dialysate sample with 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 minute incubation period.

4. Calculation and statistical analysis

4.1 The ED_{50}, TD_{50}, and LD_{50}, were 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 Instrument, Australia). The 95 percent confidence interval was calculated by the method of Litchfield and Wilcoxon(1949).

4.2 Statistical analysis was carried out using SPSS/PC+(1991) software Data presented is expressed as mean±standard error of the mean (S.E.M). Analysis of variance (oneway ANOVA) followed by Duncan's Multiple rang Test was used to compare the data between various groups ($p<0.05$).

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