CHAPTER III

MATERIALS & METHODS

1. Animals, Chemicals and Reagents

Animal Subjects

Male ICR mice weighing 15-20 grams were obtained from National Laboratory Animal Center, Salaya Campus, Mahidol University, Nakornprathom Province. All animals were housed in a climate-controlled facility with 12-hour alternating light and dark cycles and acclimated to the research facility for at least 1 week. All animals had *ad libitum* access to food (CP Food) and tap water. This study was in accordance with the guidelines for animal use which approved by the Ethical Committee of Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Chemicals and Reagents

1. (3-Aminopropyl)(diethoxymethyl) phosphinic acid (CGP-35348, Sigma, USA, National Institutes of Drug Abuse, National Institues of Health, USA)
2. Butylated hydroxytoluene (Sigma, USA)
3. Corn oil
4. Diazepam (Roche, Thailand)
5. Flumazenil (Sigma, USA)
6. Gamma-butyrolactone (99%, Fluka)
7. Imipramine (Sigma, USA)
8. Picrotoxin (Sigma, USA)
9. 6,7,8,9-tetrahydro-5-hydroxy-5*H*-benzocyclohept-6-ylideneacetic acid (NCS-382, National Institutes of Drug Abuse, National Institues Of Health, USA)
10. Tetrahydrofuran preserved with < 0.025% butylated hydroxytoluene (99%) (Sigma, USA)
11. Artificial Cerebrospinal Fluid pH 7.40 : 124 mM NaCl, 5 mM KCl, 0.1mM $CaCl_2$, 3.2 mM $MgCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose
12. 0.9% w/v Sodium Chloride Solution

Handling and Storage of Some Chemicals

The observation was taken for the physical properties of tetrahydrofuran, a peroxide forming compound. Tetrahydrofuran was stored in the refrigerator, protected against light and kept away from sources of ignition. Care of abuse had been taken to the handling and storage of gamma-butyrolactone (GBL), a GHB-related chemical.

2. Experimental Methods

Righting Reflex Test (Swinyard, Brown, and Goodman, 1952)

The righting reflex test was used to test the ability of the animal to regain an upright posture within 10 seconds of being placed supine. This method was used to test drugs that cause significant CNS depression or impairment. Animals were given two consecutive attempts to demonstrate the righting reflex before which time failure was documented.

Rotarod Test (Dunham and Miya, 1957)

The rotarod test was used to assess the ability of the animal to log roll for 10 seconds on a 1-inch diameter rod revolving at 15 revolutions per minute. Animals were given two consecutive attempts to perform the rotarod test before which time failure was documented. This test required a high degree of sensorimotor coordination and was therefore used to test more subtle neurological deficits.

Technique of Injection into Cerebral Ventricles (Brittain and Handley, 1967)

To immobilize the head of the mouse, the loose skin behind the head was grasped between thumb and forefinger and the animal held firmly onto the bench by extending the skin on either side of the neck. The site of injection was within 1 mm of the midline and on a line joining the anterior bases of the ears. The injection was made with a 50 μl Hamilton microsyringe fitted with a 26-gauge needle that was inserted to a depth of 2.4 mm. The vehicle for injection was artificial cerebrospinal fluid pH 7.40 which composed of 124 mM NaCl, 5 mM KCl, 0.1mM $CaCl_2$, 3.2 mM $MgCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose

Locomotor Activity Test (de Fiebre et al., 2004)

Locomotor activity was conducted with an activity cage consisting of plexiglass chamber (23 x 35 x 20 cm) and grid floor detecting arrangement which made of stainless steel insulated bars. The odd bars were grounded and the even ones were wired in four sets. The bridges which the animals made or broke with their paws produce a random configurations were converted to pulses. A counter sumed up the pulses which were displayed and printed at preset time. After vehicle or drug administration, the locomotor activity was continuously recorded in the activity cage at 10-min interval for a total period of 150 min.

Open-Field Test (Leppänen, Ewalds-Kvist, and Selander, 2005)

This method was used to evaluate exploratory activity of animals. Each animal was placed into the center of the apparatus, which consisted of a circular (45 cm in diameter) with a 20 cm high wall of metallic plate. The field was marked with thin black lines to delineate three concentric circles. The outer circle was divided into 12 partitions and the middle circle (24 cm in diameter) into 6 partitions. The center circle (8 cm in diameter) was not divided. That is, the floor of arena was subdivided into a total of 19 partitions to aid the recording of the ambulation of the animal, thereby providing the raw scores for computation. The center circle and the 6 partitions of the middle circle were defined as 7 inner units, and the 12 partitions of the peripheral circles as outer units. The floor of the arena was cleaned between successive recordings. Animals were individually recorded by video camera for the analysis of open-field activity. At the beginning of the test, the animal was placed in the center unit of the open-field. The ambulation of animal was manually recorded when the mouse moved from one floor unit to another during 10-min period. The parameters were ambulation, rearing, grooming, defecation, urination and the thigmotactic ratio. The ambulation denoted unprovoked motor activity in the form of spontaneous whole body movements from one open-field unit to another. Rearing was registered by counting the number of times the animal rose onto its hind legs with the front limbs either against the wall or freely in the air. Animal defecation and urination were recorded by counting the animal's fecal boli and the number of urinary spots void in the open-field, respectively. The thigmotactic ratio was computed on the basis of ambulation scores by dividing the number of inner units the mouse entered by the total

sum of units it visited. Hence the smaller ratio, the more prone the mouse was to keeping close to the open-field wall. The thigmotactic ratio signified the animal's orientation toward the open-field peripheral units but did not necessarily imply bodily contact with the wall. The thigmotactic ratio ranged from 0.00 to 1.00. Inner ambulation and outer ambulation were recored by counting the number of inner units and outer units entered during a 10-min open-field test.

Elevated Plus Maze Test (Redrobe, Dumont, Herzog, and Quirion, 2003)

The elevated plus-maze test was used as a model of anxiolytic activity testing. The maze consisted of two open (30 cm × 5 cm × 0.2 cm) and two closed (30 cm × 5 cm × 15 cm) arms, extending from a central platform (5 cm × 5 cm) and elevated to a height of 45 cm above the floor. The entire maze was made of black Plexiglas. Mice were individually placed on the center of the maze facing an open arm under a light intensity of 200 lux for 5 minutes, and the number of entries and the time spent in closed and open arms were recorded during a 5-min observation period. The time spent in the open arms was expressed as a percentage of the total time spent in the arms (% time), and the number of entries in the open arms as a percentage of the total number of entries (% entry). The apparatus was cleaned with 30% ethanol solution between subjects. Anxiolytic-like effects were indicated by increases in open arm time or in number of open-arm entries.

Y-Maze Task (Yamada et al., 1996)

The apparatus was made with black plastic. Each arm of Y-maze was 25 cm long, 14 cm high and 5 cm wide and positioned at an equal angle. The mouse was placed at the end of one arm and allowed to move freely for 8 min without reinforcers in the maze. An arm entry was defined as complete entry of the body of a mouse, except for its tail, in an arm compartment. The sequence of arm entries was recorded manually. Spontaneous alternation behavior was defined as the entry into all three arms on consecutive choices in overlapping triplet sets. The percentage of the spontaneous alternation behavior was calculated as the ratio of actual to possible alternation (defined as the total number of arm entries minus 2) and multiplied by 100. Spontaneous alternation behavior is considered to reflect a primitive form of spatial working memory.

Morris Water Maze Test (Jang et al., 2003)

The principle of the Morris water maze involves the ability of mice to escape from a water-filled compartment by swimming randomly, or in unsystematic search paths throughout the pool; but in practice, normal mice are learning quickly to swim directly towards the submerged platform from any starting position at the circumference of the pool. The accurate directionality of their escape behavior provides evidence that the animal escapes by learning the spatial position of the submerged platform relative to distal extra-maze cues. Thus, this test offers a way to examine the neurobiology of spatial learning and test drugs that cause amnesia and affect cognition and memory.

The maze consisted of a round pool (80 cm in diameter and 30 cm high), containing water (ambient room temperature) to a depth of 14.5 cm. A platform (7 cm in diameter and 14 cm high) submerged 0.5 cm below the water surface and placed at the midpoint of one quadrant. The pool was located in a test room, which contained various prominent extra-maze visual cues (for example: square and triangle). Each mouse was trained for 5 consecutive training days and tested for 6 consecutive reference memory testing days. For each trial, the mouse was placed in the water facing the pool wall at one of three non-platform containing quadrant starting positions, and the time required for the released mouse to find the hidden platform was recorded. A mouse that found the platform was allowed to remain on the platform for 10 seconds and then returned to its cage for the 15 seconds intertrial interval. A mouse which could not find the platform within 120 seconds was placed on the platform for the 10 seconds at the end of the trial. On day 6 of the reference memory testing day, the probe test was carried out, which involved removing the platform from the pool. The trial was performed with the cut-off time of 90 seconds. The time (seconds) spent in the quadrant that previously contained the platform was recorded as a percentage of the total time in the pool.

Open-Space Swimming Test (Sun and Alkon, 2003)

The open-space swimming test was used as an animal model of depression. The model consisted of a large pool, with the induced depressive behavior resulting from a lack of motivation (opportunities or hope). When mice were free to swim in an open-space in 15-min session, they would eventually stop attempting to escape and become immobile. Mice were placed individually in a circular black pool

(75 x 30 cm) containing fresh water up to a height of 20 cm at a temperature of 25 ± 2°C. The animals were treated either with a drug (test group) or vehicle (control group) during the swimming trial sessions at 23, 3, and 1 hour before the 2nd, 3rd, and 4th trial sessions, respectively. The mobility time included all times during the entire 15 minutes that a mouse showed active swimming was recorded. The mouse was judged to be immobile if it ceased struggling and remained floating motionless in water making only those movements necessary to keep its head above water. All test sessions were recorded with a video camera.

Conditioned Place Preference Test (Roux, Froger, Porsolt, Valverde, and Maldonado, 2002)

The conditioned place preference procedure has been widely used in rodents for assessing drug effects on motivational processes. A two-compartment rectangular plastic box (25×33×36 cm^3) connected to an intermediate area (25x14x36 cm^3) by guillotine doors and covered with a transparent plastic cover to prevent mice from escaping the box was used in this conditioned place preference procedure. The compartments were different from each other in interior texture and visual cues. One compartment was painted white with a wood floor, and the second was painted black with a steel grid floor. The middle compartment was painted gray. Animals were conditioned in a two-compartment box in which they were allowed to explore one compartment under the influence of the test substance, while the other compartment was always explored after administration of vehicle. A preference for the drug-paired compartment (drug-induced place preference) was indicated if the animal spent significantly more time in the drug-pair compartment. The place preference scores (PPS) were calculated by using data obtained during the preconditioning and testing phase as follows: multiply the time spent in the conditioned compartment by the total time in second and divide by the total time minus the time spent in the neutral area. A positive score indicated a preference for the compartment associated with the drug, which revealed a rewarding effect of the drug. A negative result indicates an aversive effect of the drug. Conditioned place preference consisted of three phases: preconditioning, conditioning, and postconditioning.

Preconditioning Phase (day 1 to 3)

Three days were dedicated to accustoming the animals to the conditioned place preference boxes. The guillotine doors were raised, each mouse was placed in the middle of the neutral area and allowed to explore both sides for 1200 seconds (20 min) per day. Time spent on each compartment was recorded on the third day in order to ensure there was no strong spontaneous preference expressed by mice. Entry into a given compartment was defined as being when the head and the two forepaws were inside of the compartment. The preconditioning score was measured as the subtraction of the preferred compartment staying time from the nonpreferred compartment staying time. After the test, the animals were grouped randomly (six per cage).

Conditioning Phase (day 4 to 9)

This phase consisted of six consecutive days of alternate drug or vehicle injection. The duration of each session was 30 min and the mice were confined to the considered compartment, by isolating the compartment using a removable partition. Administered test compound to the mouse on day 1, 3, and 5 of the conditioning phase and immediately after injection, placed the mouse in the nonpreferred compartment (defined in the preconditioning phase, which should be the white compartment). Administered the vehicle to the mouse and placed the mouse in the preferred compartment on day 2, 4, and 6 of the conditioning phase. Control mice were given vehicle in both boxes similarly.

Postconditioning Phase (day 10)

This phase was conducted exactly as the preconditioning phase. On test day, 24 h after the final conditioning day, all animals were in a drug-free state. Removed the guillotine doors allowed the mouse free access to each compartment for 20 min. The time spent in each compartment was recorded.

3. Experimental Designs

3.1 Determining the Dose-Response of THF and GBL for the Loss of Righting Reflex and Failure in the Rotarod Test (intraperitoneal route and intracerebroventricular route)

Initially, the relative potency of THF and GBL in inducing sedation and motor-impairment were tested. THF and GBL were acutely administered at the incremental doses, beginning at 0, 1, 3, 5, 10, 15, 30 mmol/kg, i.p. (n=10 per group). In all experiments, after injection, each mouse was placed on its back once and observed for its ability to regain an upright posture within 10 seconds. Animals were given two consecutive attempts to demonstrate the righting reflex before which time failure was documented every 15 min until animals recovered. At every 15 min test, the animal which demonstrated the righting reflex was performed on the rotarod test. Animals were also given two consecutive attempts to successfully perform the rotarod. The percentage of animals that failed the rotarod test was recorded every 15 min until animals recovered.

For the intracerebroventricular injection study, mice were divided into 5 groups of 10 animals each. One group was used as a control (artificial CSF, i.c.v.). The other groups were used to test the effect on the loss of righting reflex and the failure in the rotarod test for THF (10-200 μmol/10μl, i.c.v.). Each mouse was injected with a 50 μl Hamilton microsyringe fitted with a 26-gauge needle that was inserted to a depth of 2.4 mm. In all groups, mice were evaluated with the righting reflex and rotarod test every 15 min until animals recovered.

The percentage of animals that lost the righting reflex and failed the rotarod test at 30 min at that dose were recorded. The complete dose-response curves were generated from the data and the TD_{50} was calculated using the Litchfield-Wilcoxon test.

3.2 Investigating the Effects of THF and GBL on the Locomotor activity

Mice were divided into 7 groups of 10 animals each. One group was used as a control (normal saline, 10 ml/kg, i.p.). The other groups were used for testing the effect on the locomotor activity of THF (1, 3, 5, 10 mmol/kg, i.p.). Each mouse was placed in the chamber and the activity was recorded by electronic counting machine at 10-min interval for 150 min after injection. This study was performed in the same fashion by substitute GBL for THF.

3.3 Investigating the Effects of THF and GBL on the Open-Field Study

Mice were divided into 5 groups of 8 animals each. One group was used as a control (normal saline, 10 ml/kg, i.p.). The other groups were used for testing the effect of THF (0.1 and 0.3 mmol/kg, i.p.) and GBL (0.1 and 0.3 mmol/kg, i.p.) on the open-field study. After administration of the drugs for 30 min, each mouse was placed in the open-field. During a 10-min test period, the inner ambulation and outer ambulation, rearing, grooming, defecation and urination were measured and the thigmotactic ratio were computed.

3.4 Investigating the Effects of THF and GBL on the Elevated Plus Maze Test

Mice were divided into 6 groups of 10 animals each. One group was used as a control (normal saline, 10 ml/kg, i.p.). The other groups were used for testing the effect of THF (0.1 and 0.3 mmol/kg, i.p.) and GBL (0.1 and 0.3 mmol/kg, i.p.) on anxiolytic activity. After administration of the drugs for 30 min, each mouse was placed in the elevated plus maze apparatus. Mice were individually videotaped during an 5-min test period and the following measures were taken: the number of entries and time spent in the open arms.

3.5 Investigating the Effects of THF and GBL on the Spontaneous Alternation Behavior

Mice were divided into 5 groups of 8 animals each. One group was used as a control (normal saline, 10 ml/kg, i.p.). The other groups were used for testing the effects of diazepam (DZP 0.007 mmol/kg which equating 2mg/kg), THF (0.1 and 0.3 mmol/kg, i.p.) and GBL (0.1 and 0.3 mmol/kg, i.p.) on the Y-maze task. After administration of the drugs for 30 min, each mouse was placed in the Y-maze. During

an 8-min test period, arm entries were recorded manually. The percentage of spontaneous alternation behavior was calculated.

3.6 Investigating the Effects of THF and GBL on the Spatial Memory

Mice were divided into 5 groups of 8 animals each. One group was used as a control (normal saline, 10 ml/kg, i.p. daily for 6 days). The other groups (THF 2 low-dose levels, GBL 2 low-dose levels, i.p. daily for 6 days) were used to test the effect on memory. After administration of the drugs, mice were subjected to Morris water maze testing daily for six days. Parameters such as latency (sec) to reach the platform, and time spent in the quadrant that previously contained the platform were recorded.

3.7 Investigating the Effects of THF on the Open-Space Swimming Test

Mice were randomly assigned to six different groups (n=8). After administration of the drugs, mice were free to swim (or not to swim) for 15 min and the duration of mobility were measured. The same procedure (15 minute sessions per day) was followed 24 h later for 3 more days. One group was used as a control (normal saline, 10 ml/kg, 3 injections per day, i.p.). The other groups were imipramine (15 mg/kg, 3 injections per day, i.p.), THF (0.1 and 0.3 mmol/kg, 3 injections day, i.p.), and GBL (0.1 and 0.3 mmol/kg, 3 injections per day, i.p.). The percentage of mobility time was calculated.

3.8 Investigating the Tolerance to Chronic THF Treatment on the Loss of Righting Reflex Test

Mice were divided into 2 groups of 20 animals in each group. Group 1, received normal saline, once daily, i.p. (THF naïve group; n=10) or THF 5 mmol/kg, once daily per day, i.p. for a total period of 14 consecutive days. Tolerance was studied by comparing the treated group with a fresh set of animals, which was administered THF 15 mmol /kg, i.p. on day 15. Mice were evaluated by the righting reflex test every 15 min until animals recovered. The percentage of animals that lost the righting reflex test was recorded.

3.9 Investigating the Effects of THF and GBL on the Conditioned Place Preference Test

Four different doses of THF and GBL (THF 3 and 5 mmol/kg, i.p. and GBL 0.5 and 1 mmol/kg, i.p.) and morphine (5 mg/kg, s.c.) were tested for producing place preference. Control group that received saline (10 ml/kg, i.p.) was included in order to confirm that the injection and conditioning schedule did not affect the time spent in the compartments. The time spent in each compartment was recorded on the third day of the preconditioning phase and day 10 of the study and the place preference score was calculated.

3.10 Clarifying whether the Motor Impairment Induced by THF Is Mediated by Activation of $GABA_B$ Receptor, GHB Receptor , and/or $GABA_A$ Receptor by Pretreatment with Receptor Antagonists

The blockade tests on the motor-impairment were conducted. Mice were divided into 5 groups of 20 animals each. Group 1 was administered normal saline, group 2 was administered a $GABA_B$ antagonist, CGP-35348 200 mg/kg, i.p, group 3 was administered a GHB receptor antagonist, NCS-382, 250 mg/kg, i.p., group 4 was administered a $GABA_A$ antagonist, picrotoxin, 2 mg/kg, i.p. (n=20), and group 5 was administered a $GABA_A$ antagonist, flumazenil 10 mg/kg, i.p. (n=20). Fifteen minutes later, 10 mice from each group were administered THF at the TD_{50} dose for the loss of righting reflex, i.p. and another 10 mice from each group were administered normal saline i.p.. In all groups, mice were evaluated by the rotarod test every 15 min until animals recovered.

4. Data Analysis

a. For THF and GBL TD_{50} for the rotarod and righting reflex test. The TD_{50} was calculated by the Litchfield-Wilcoxon Test using Pharmacological Calculation System (Tallarida, Pharmacological Calculation Systems, Springer Verlag, NY, 1987).

b. Values from the locomotor activity test, the open-field study, the elevated plus maze test, the Y-maze study, the Morris water maze study, the open-space swimming test, and the conditioned place preference study were analyzed using an analysis of variance (ANOVA) followed by Duncan *post hoc* test. Differences with $p<0.05$ were considered statistically significant.

c. Data from the blockade test with receptor antagonists and the tolerance to chronic THF study were analysed by the Fisher's exact test for a 2X2 table [treatment (control, drug) X loss of righting reflex (presence, absence)], [treatment (control, drug) X fail to perform the rotarod test (presence, absence)].