CHAPTER III

MATERIALS AND METHODS

1. Test Substances

CU 763-14-07 and CU 763-14-10 were synthesized in Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University. They were kindly provided by Assistant Professor Dr. Chamnan Patarapanich.

2. Animals

Behavioural experiments were performed by using male Swiss albino mice, weighing 20-25 g. Male Sprague-Dawley rats, weighing 150-250 g, were used in the *ex vivo* experiments for studying effects of test substances on brain monoamine levels. All animals were obtained from the National Animal Center, Mahidol University, Nakornpathom. The animals were kept in cages containing 4-6 rats or 10-12 mice per cage for at least 7 days before the experiment. Animals were fed with food pellets and water *ad libitum* under natural light/dark condition. In all experiments, each animal was used only once and the experiments were carried out between 7.00 a.m.-4.00 p.m. Except the measurement of locomotor activity which was performed between 7.30-11.00 a.m.

3. Solutions and administration of drugs

Normal saline solution (NSS) was used as the vehicle for all standard antidepressants and CU 763-14-07. In the case of CU 763-14-10, 2% tween 80 in normal saline solution was used as the vehicle. The doses of test substances were expressed as milligram of substance/kilogram of body weight (mg/kg BW), and they

were injected intraperitoneally (i.p.). The volumes of injection were 10 ml/kg in mice and 2 ml/kg in rats.

4. Behavioural despair (forced swimming) test

Standard antidepressants

Amitriptyline hydrochloride

Pargyline hydrochloride

Instruments

1. Glass cylinder vessels (height: 19 cm; diameter: 10.5 cm) containing tap water (23 ± 1°C, 10 cm deep) was used as a forced swimming arena. The glass cylinder was positioned beneath a video camera recorder so that the animal behavior could be recorded for later analyses.
2. Video camera (Sony, Japan)

Procedure

The duration of immobility in the forced swimming test was measured in mice. The general procedure was a modification of that described by Persolt, Bertin, Blavet, Deniel, and Jalfre (1977). Mice were plunged individually into a transparent cylinder vessel, containing 10 cm of water maintained at 23 ± 1°C for 15 min on the first day of each experiment, after that they were removed and dried with a towel before being returned to their home cages. The following day (24 h after a pre-exposure to forced swimming), the animals were exposed to the experimental conditions outlined above for only 6 min and the duration of immobility was scored for the last 5 min of a six min test. A mouse was judged to be immobile when it was floating in the water almost

motionless, in an upright position, making only the necessary movements to keep its head above water.

The behavior of the mice was recorded on videotape and subsequently quantified by measuring the total immobility time. Each experimental group consisted of 10-12 animals. The dose responsiveness of mice to the treatment was assessed 30 min after drug administration. Control animals received vehicle only.

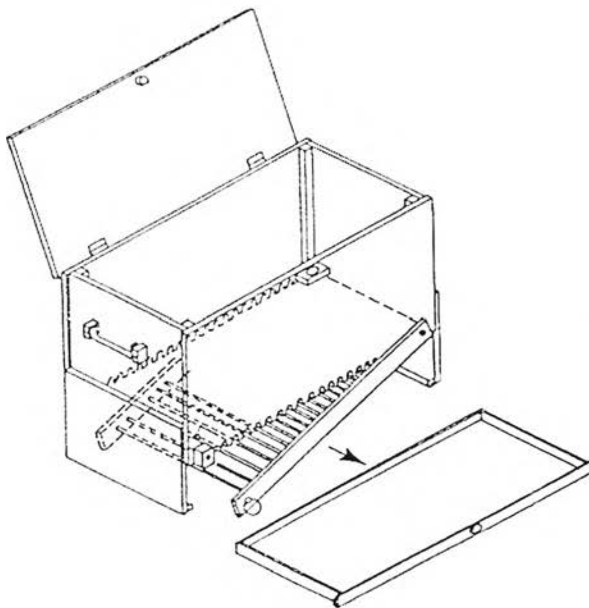
Figure 10. Immobility posture of mice in the behavioral despair (forced swimming) test

5. Measurement of locomotor activity

Instrument

Activity cage (UGO Basile, Comerio-Italy)

Inside dimensions of the activity cage are, length 35 cm; width 23 cm; and height 20 cm. The cage floor is made of evenly spaced stainless steel bars (3 mm. Diameter) that are spaced 11 mm. apart., connected to the circuit of horizontal and vertical Infra-red sensors. Whenever the animal move, these changes are converted into pulse by detectors. A counter sums up the pulses which are displayed and printed at preset time.



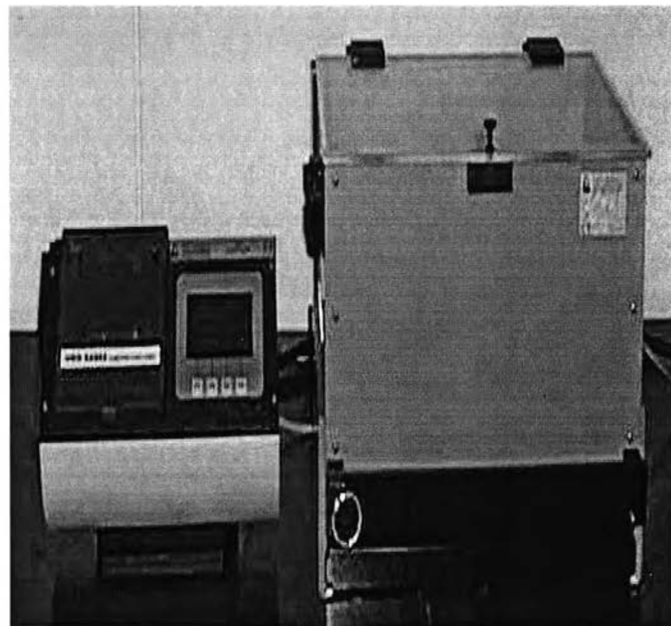


Figure 11 The activity cage

Procedure

These measurements served to assess whether changes in immobility time were associated with changes in motor behavior, as previously suggested by Persolt Bertin, Blavet, Deniel, and Jalfre (1977). Motor activity was measured in the activity cage for a total period of 105 min. A basal locomotor activity of each mouse was established by allowing a control period of 45 min before removing it for the administration of test substance. Immediately after the administration of test substance, the animals were returned to their activity cages.

The experiment was carried out between 7.30-11.00 a.m.. Male Swiss albino mice were divided into 8 groups of 8-10 animals each. Two groups were used as control groups (NSS and 2 % tween 80 in NSS). The other 6 groups were used as the treatment groups of 2 test substances (dose level each; 10, 20 and 40 mg/kg B.W. i.p)

6. Rota-rod test

Instrument

Rotarod tredmills for mice (UGO Basile, Comerio-Italy)

The apparatus consists of five 3-cm diameter drums. Six flanges divide the drums, enabling five mice to be tested on the treadmill simultaneously.

The rotors are driven by a heavy-duty, induction-geared, motor that sets the rotors in motion via the belt gear and ball bearing.

When a mouse falls off to the plate below, the plate trips and the corresponding counter is disconnected, thereby recording the animal's endurance time in seconds. (Figure 12)

Procedure

The rota-rod technique was originated by Dunham and Miya (1957) for screening of drugs which are potentially active on motor coordination or fatigue resistance

This experiment using the procedure that was modified by Assoc. Prof. Dr. Boonyong and co-worker (1995) in the following:

1. Selecting animals before testing

Selecting mice in criteria that they were able to maintain their equilibrium on the rota-rod apparatus which rotated at 20 rev/min for at least 2 min in each of two trials. Mice which were selected would be tested in 1 hr later.

2. Administration of compounds

CU 763-14-07 or CU 763-14-10 or vehicle was given intraperitoneal 30 min before testing

3. Testing

Neurological deficit or motor incoordination was indicated by inability of mice to maintain the body on the rotating rod for at least 1 min in each of three trials. Mice which were able to maintain the body on the rod for 1 min were put back on the rod twice. Mice that could maintain their equilibrium on the rod in all of the three trials would be decided that they did not relate with neurological deficit especially motor incoordination or muscle relaxation

Mice would be counted and motor deliberation would be considered.

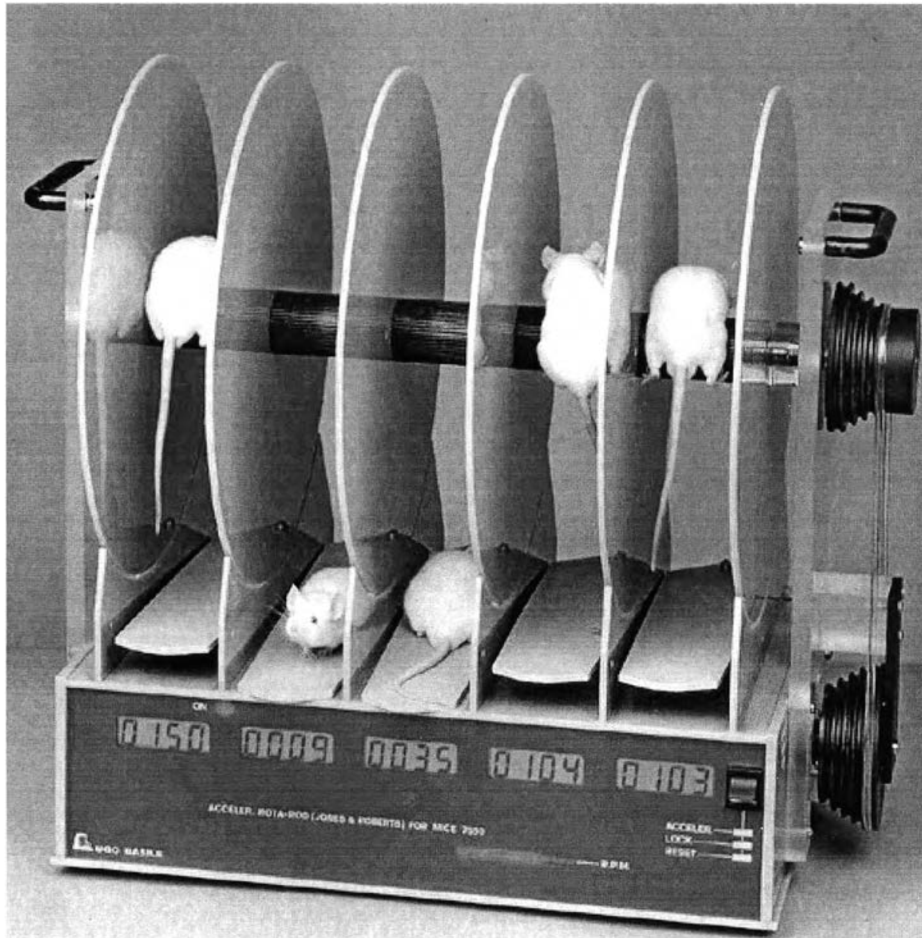


Figure 12. The Rotarod Apparatus

7. Measurement of monoamine levels in the rat brains

Chemicals

1. Dopamine hydrochloride (Sigma, U.S.A)
2. Norepinephine bitartrate
3. Serotonin creatinine complex
4. Isoproterenol hydrochloride
5. Methanol HPLC grade
6. Perchloric acid
7. Potassium phosphate monobasic anhydrous (Sigma Chemical, USA.)
8. Sodium metabisulphite (May & Baker LTD, England)

Instrument

1. pH-meter (Suntex, Japan)
2. Heidolph homogenizer (Type S0203 RZR 2)
3. Refrigerated centrifuge Himac SCR 20 B, Rotor model RPR 18-3
4. HPLC Column; C18-Reverse phase, 25 x 4.60 mm., particle size 5 μm. (TSP, USA)
5. HPLC pump Model P4000 Quat Gradient (TSP, USA)
6. Injection Device Model AS 3000 (TSP, USA)
7. Spectrofluorometric Detector Model FL 3000 (TSP, USA)
8. Laser printer 6P (Hewlett Packard)

Procedure

Monoamine (norepinephrine, dopamine, and 5-hydroxytryptamine) levels in the whole rat brains were determined using a modification of the procedure describe by Krstulovic and Powell (1978). The mobile phase used, consisting of gradient run between 0.02 M phosphate buffer pH 3.5 and methanol, was delivered at a flow rate of 0.8 ml/min. The gradient run was started at 5% methanol and 95% potassium phosphate buffer

Excitation and emission wavelengths were set at 286 and 346 nm, respectively. The sample in the volume of 100 μl was injected directly into the chromatograph.

Rats were killed by decapitation. The brains were removed quickly and then weighed as soon as possible. The whole brains were homogenized with a homogenizer in chilled 0.4 M perchloric acid containing 1 μg/ml of isoproterenol as an internal standard and 1% sodium metabisulfite. The ratio of acid to tissue was 6 ml of

perchloric solution : g of tissue. The homogenizer was washed with perchloric solution and the washings added to the homogenate. The homogenates were centrifuged at 14,000 rpm (4°C) for 15 min and the supernatants were collected and passed through 0.2 μm nylon filter before storage in the freezer. Standard curves for each monoamine were constructed and were linear between 0.2 and 15 μg/ml

8. Statistics

Data were presented as mean ± S.E.M. Significant differences between treated and control groups were statistically determined by one way analysis of variance (ANOVA) followed by Fisher's PLSD test. In case of the rotarod experiment, Pearson Chi-square test was used to analyze the difference in the ability of mice which maintained the equilibrium on the rotating rod of the rotarod test. The p-value of less than 0.05 was chosen as a significant level for all comparisons.