Section-II

Chapter-3

MATERIALS & METHODS

3. MATERIALS AND METHODS

3.1. Animals: Sprague-Dawley rats (200–250 g) of either sex or Swiss albino mice (25–35 g) of either sex were used for these studies. Animals were housed in a temperature- and humidity-controlled facility with a 12-h light/dark cycle and free access to food and water throughout the study. All animal procedures were reviewed and approved by Institutional Animal Ethical Committee, Pharmacy Department, The M. S. University of Baroda. Samples of all test compounds were prepared using 2% DMSO and distilled water.

3.2. Methods

3.2.1. *In vitro* 5-HT assay using isolated rat fundus preparation

Male Sprague-Dawley rats (200-250 g) were used for the study. After 48 hrs fasting, the animals were sacrificed by overdose of anaesthetic agent. Fundus was exposed and washed, slit along the greater curvature and spread out after opening the abdomen. Some of the strips were obtained by cutting the fundus horizontally while certain others were obtained by cutting it longitudinally. The cut strips thus obtained were of 4 cm in length. 20 ml Tyrode solution was filled in aerated bath and kept at 37° C. The cut strip was then suspended in Tyrode solution. Assay was commenced after 30 min. Minimum of 6 horizontal and 6 longitudinal strips were used to screen the compounds. Contractile responses of the synthesized compounds were then recorded using a 2-channel recorder with an isotonic transducer (UGO Basile, Italy). Compounds were exposed for 60 sec in a 3 min time cycle in the assay. Solutions of varying concentrations of 5-hydroxytryptamine-creatinine sulphate complex (Sigma) were used as standard [110].

3.2.2. *In vitro* 5-HT assay using isolated rat thoracic aorta strip

Male Sprague-Dawley rats weighing 200-250 g were used in the experiment. The animals were sacrificed by cervical dislocation; descending thoracic aortae were removed immediately and placed in ice-cold Kreb's bicarbonate solution of the following composition (mM): NaCl (112), $NaHCO_3$ (12), glucose (11.1), KCl (5.0), $MgSO_4$ (1.2), KH_2PO_4 (1.0) and $CaCl_2$ (2.5). The aeration was given to the tissue using 95% O_2 and 5% CO_2. Peri-adventitious tissue was removed taking care not to stretch the tissue. A needle was inserted in the tissue and rotated gently to denude the endothelium. Following this, the tissue was cut spirally into a helical strip (20 mm × 3 mm) using a surgical blade. The strip was tied at both ends using a cotton thread and suspended in a 25 ml organ tube under an initial resting tension of 2 g. The *p*H of the Kreb's solution was 7.4. Thermostat was used to maintain the temperature at 37°C. The Kreb's solution in the organ tube was changed every 10 mins during an equilibration period of about 90 mins. Isometric contractions were recorded for the

synthesized compounds using a force transducer (UGO BASILE, Italy) coupled to a Gemini 7070 recorder (UGO BASILE, Italy) [111].

3.2.3. Tail suspension test (TST) in mice

3.2.3.1. Principle

The tail suspension test (TST) has become one of the most widely used models for assessing antidepressant-like activity in mice. The test is based on the fact that animals subjected to the short-term inescapable stress of being suspended by their tail, will develop an immobile posture. Immobility is defined as the absence of initiated movements and includes passive swaying. The main advantages of this procedure are:

1. The use of a simple, objective test situation.
2. The concordance of the results with the validated "behavioral despair" test from Porsolt.
3. The sensitivity to a wide range of drug doses.

3.2.3.2. Procedure

The TST [112] in mice is a substantiation of the despair-swim test. TST is considered to be more sensitive. This test is used to screen a wide series of antidepressants. Animals were pre-treated (30 min prior) with fluoxetine (10 mg/kg) as a positive control, test compounds (**7, 10-14, 18-20, 23, 26-29, 32-41, 45, 46, 48, 51, 57** and **58**) or saline. Individual animals were suspended (35 cm above the floor) by their tails. An adhesive tape was used (1 cm from the tip of the tail) to suspend the animals. 'Immobility' and 'mobility' time was considered as a behavioural score for the animal throughout the assessment period of 5 min. The animals were considered 'immobile' only when the animals remain suspended inactively and totally motionless [113].

3.2.4. Acute oral toxicity study

See `Appendix III`

3.2.5. Elevated plus-maze (EPM) test

3.2.5.1. Principle

The elevated plus maze has been described as a simple method for assessing anxiety responses of rodents by File and co-workers [114]. A task, using a plus-shaped apparatus that included an elevated open alley which produced a strong approach–avoidance conflict, and an enclosed path which was first described by Montgomery [115]. This task was modified into an elevated maze with four arms (two open and two enclosed) that are arranged to form a plus shape and was described by Handley and Mithani [116]. These authors described the assessment of anxiety behavior of rodents by using the ratio of time spent in the open arms to

the time spent in the closed arms. Unlike other behavioral assays used to assess anxiety responses that rely upon the presentation of noxious stimuli (i.e., electric shock, food/water deprivation, loud noises, exposure to predator's odor, etc.) that typically produce a conditioned response, the elevated plus maze relies upon rodents' proclivity toward dark, enclosed spaces (approach) and an unconditioned fear of heights/open spaces (avoidance) [117].

There is great diversity in possible applications of the elevated plus maze. Pre-screening of newly developed pharmacological agents for treatment of anxiety-related disorders can be carried out. The anxiolytic and anxiogenic effects of pharmacological agents, drugs of abuse and hormones can be investigated by elevated plus maze. The effects of reproductive senescence/aging and/or pre- or postnatal exposure to various stressors can be assessed. Furthermore, beyond its utility as a model to detect anxiolytic effects of benzodiazepine-related compounds, the elevated plus maze can be used as a behavioral assay to study the brain sites (e.g., limbic regions, hippocampus, amygdala, dorsal raphe nucleus, etc) [118, 119] and mechanisms (e.g., GABA, glutamate, serotonin, hypothalamic–pituitary–adrenal axis neuromodulators, etc.) [114-116, 120-125] underlying anxiety behavior. Indeed, the elevated plus maze has been used as a model of state of unconditioned anxiety for over two decades, and there are now over 2,000 papers related to this topic [126-134] . Behavioral responses in the elevated plus maze are easily accessed and quantified by an observer. Briefly, rodents are placed in the intersection of the four arms of the elevated plus maze and their behavior is typically recorded for 5 min.

3.2.5.2. Procedure

The plus-maze apparatus consists of two open arms (15 cm×5 cm) and two enclosed arms (15cm×5cm×5cm), extending from the middle platform (5 cm×5 cm) and elevated 50 cm above floor level. Mice were randomly assigned to drug treatment groups. The animals (mice) were treated with the test compounds (**7**, **19** and **28**) or the positive control, 30 min prior to the beginning of the experiment. Individual animal was positioned at the middle of the EPM in a way that the animal faces the open arm first. The animals were allowed to explore the EPM freely for 5 min. Behaviour of the animal was assessed by recording the number of entries in the enclosed or open arms (fore limbs on enclosed or open arms) and the time spent on enclosed or open arms [135].

3.2.6. Hypophagic response in rats

3.2.6.1. Procedure

Food deprived rats were housed individually on day 1. Later (after 23 h), they were treated with ***m*CPP**, test compounds (**7, 19** and **28**), the antagonist **SB206553** [136] and saline intraperitoneally. The animals were treated with the antagonist (**SB206553)** intraperitoneally, fifteen minutes prior to the test compounds (**7, 19** and **28)** in the antagonist treated group. The animals were then placed in their cages. After twenty minutes' time period, food pellets of weighed amount were placed in the food hoppers of all the animals for consumption and the amount of the food pellets remaining after 2 h was measured [137].

3.2.7. Penile erection rat model

3.2.7.1. Procedure

The whole study was conducted during 08:30 to 14:30 h. One rat from each group was placed in individual cage and observed concurrently. Animals were observed for two days to collect more than a few reproducible data. Animal cages (7.5 × 18 × 30 cm) were made of transparent perspex. Following treatment with the compounds (**7, 19** and **28)**, animals were positioned in the individual cages. Behavior of the animals was studied and made easy by placing a mirror behind the row of observation cages. After the treatment, the animals were observed for 1 h and the number of yawns and penile erections were counted. The behaviour parameters for a penile erection are given below: repeated pelvic thrusts instantly pursued by an upright position, a rising, engorged penis which the animal keep on licking while consuming the ejaculate [138]. The animals in antagonist treated group were treated with **SB206553** [30 min prior to the agonists (**7, 19** and **28**)].

3.2.8. Estimation of dopamine (DA) and 5-HT levels using HPLC technique

3.2.8.1. Procedure

Levels of DA and 5-HT were estimated in compounds (**19** and **28**) or standard (***m*-CPP)** treated rat brains using high performance liquid chromatography (HPLC) with electrochemical detector. All the brains were weighed and homogenized in 10% w/v 0.17M perchloric acid at 4^{o}C for 30 sec to extract the monoamines. Homogenates were allowed to stand for 15 min and the supernatants were decanted in separate eppendrof tubes and were centrifuged at 4°C for 20 min at 15,000 rpm. After centrifugation, supernatants were separated and immediately analysed or stored at -70°C until assayed. Brain concentrations of DA and 5-HT were assessed by RP-HPLC coupled with electrochemical detector (Model no. Waters 2465) (Waters Corporation, Milford, USA). Estimation was performed as previously described method with minor modifications. Briefly, a Sunfire® C18 column

(4.6mm×150mm, particle size 5µm) was used and separation was carried out using mobile phase containing methanol (15%V/V) in a solution of (*p*H 4.2) 32mM citric acid, 12.5mM disodium hydrogen orthophosphate, 0.5mM octyl sodium sulphate, 0.5mM EDTA and 2mM KCl at flow rate of 1.2 ml/min, at an operating pressure of 3000 psi and an operating potential of 0.61V.The internal standards were prepared by spiking known amount of mixed standard (10, 20, 40, 80 and 160 ng/ml of each DA and 5-HT) in 1 ml of pooled brain homogenates. The levels of endogenous DA and 5-HT were assessed in the aliquots of the homogenates by subtracting their amounts from those of the corresponding standard samples prepared in the same aliquots [139, 140].

3.3. Statistical analysis

Data was statistically analysed using GraphPad InStat software (version 5.00, San Diego, CA). All data was presented as mean ± SEM. The mean significant difference in the experimental groups was determined using one way ANOVA followed by Bonferroni test. Values of $p<0.05$ were considered statistically significant.