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●*A REVIEW*●

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Animal models for screening anxiolytic agents

MILIND PARLE, SUMAN DEVI AND SURESH KUMAR

ABSTRACT

The Animal models contribute to reveal the underlying pathophysiology of various psychiatric disorders. Furthermore, these models are useful not only in screening new compounds but also help in discovering new medicines based on reversing the underlying pathological deficiency. Since human life is precious, it becomes necessary to test the new medicines in small animals before applying to human beings. It is often difficult to produce psychiatric disorders in laboratory animals similar to human beings. Anxiety is a condition of persistent and uncontrollable nervousness, stress, and worry, which is characterized by feelings of apprehension, insecurity, uncertainty or fear. The animal models employed for screening anti-anxiety agents are basically of two types. First type of models are based upon spontaneous (unconditioned) responses and second type are based upon learned (conditioned) tasks. In the present review article, the authors have attempted to describe the principle, end point and rationale of each model employed for screening of anti-anxiety agents.

Key words : Anxiety, Models, Screening, Rodents

INTRODUCTION

Anxiety is a condition of persistent and uncontrollable nervousness, stress, and worry that is triggered by anticipation of future events, memories of past events, or ruminations over day-to-day events, with disproportionate fears of catastrophic consequences. It is characterized by feelings of apprehension, insecurity, uncertainty or fear. Anxiety is a term used to describe both a normal emotional state associated with stressful or difficult events and a pathological condition. When anxiety is chronic and is not clearly linked to well-defined events, it is generally considered abnormal and appropriate for psychological or psychiatric intervention. There are various types of anxiety disorders such as Generalized Anxiety Disorder (GAD)(Excessive, unrealistic worry that lasts six months or more), Obsessive-Compulsive Disorder (OCD) (Persistent, recurring thoughts or obsessions that reflect exaggerated anxiety or fears) Post-Traumatic Stress Disorder (PTSD) (Exposure to a traumatic event), Panic Disorders (Severe attacks of panic for no apparent reason) and Phobias (Extreme anxiety about being judged by others, *or* intense fear reaction to a specific object or situation such as spiders, dogs, or heights). Although many kinds of strategies have been applied, anxiety is currently most frequently treated with Anxiolytic medicines. Animal models largely contribute to reveal the underlying mechanisms of anxiety disorders and help in screening and developing new medications. Animal models for psychopathology have become an indispensible tool in the analysis of the multitude of causes whether genetic, environmental or pharmacological, which bring about symptoms analogous to those of patients with a specific disorder. However, there are traditional difficulties in accepting these models because there is no direct evidence for concluding that what occurs in the brain of an animal is equivalent to what occurs in the brain of a human being. Often researchers fail to specify, whether they are looking for a correlation model. (e.g. predictive validity, a model that is selectivity sensitive to therapeutic agents), an isomorphic model (face validity, a model that implies that the behavioral response in humans and animals is the same) or a analogous model (true construct validity, a model that implies that the 'cause' of the behavioral response in animals is sufficient to provoke the same response in humans).

Experimental models:

The animal models employed for screening anti-anxiety agents can be broadly classified into two types:

– Models based upon spontaneous (unconditioned)

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responses: These models involve true comprehensive behavioral profile of experimental animals without any interference with learning/memory, hunger/ thirst.

– Models based upon learned (conditioned) tasks: These models involve precise control over behavioral baselines. They often necessitate food or water deprivation, the use of electric shock and considerable time investment in the training of subjects by experimenter.

Models based upon spontaneous (unconditioned) responses can be further sub-classified as under:

A_1 Exploratory behaviors:
- A_{1a} Elevated plus maze (other Mazes such as Y, X, T, Radial and Zero Maze)
- A_{1b} Light-dark model
- A_{1c} Open field/Closed field
- A_{1d} Staircase test
- A_{1e} Hole board test
- A_{1f} Mirror chamber test

A_2 Social behaviors:
- A_{2a} Social interaction
- A_{2b} Social competition
- A_{2c} Ultrasonic distress vocalization

A_3 Predator:
- A_{3a} Defense test battery in rats
- A_{3b} Human threat (Primates)
- A_{3c} Odor associated avoidance behavior

A_4 Others:
- A_{4a} Novelty suppressed feeding
- A_{4b} Schedule induced polydipsia in rats
- A_{4c} Marble burying
- A_{4d} Cork gnawing
- A_{4e} Stress induced hyperthermia

Models based upon learned (conditioned) tasks can be further sub-classified as under:

B_1 Punishment models:
- B_{1a} Four plate test
- B_{1b} Punishment induced operant behaviour
- B_{1c} Conditioned emotional response

B_2 Conflict models:
- B_{2a} Vogel punished drinking
- B_{2b} Geller seifter conflict (marmoset, pigeon conflict)
- B_{2c} Shock probe conflict procedure

B_3 Respondent conditioned with aversion stimuli:
- B_{3a} Conditioned suppression
- B_{3b} Potentiated startle response
- B_{3c} Electric brain stimulation

B_4 Frustration (non-reward) test:
- Shock probe- burying test

A_1 Exploratory behavior:

Natural exploration in rodents has been widely used for assessing the effects of psychotropic agents. Exploratory behavior is based upon two competing drives *i.e.* rodents are driven to approach and investigate novel objects and places, but the novelty also induces fear (Montgomery, 1955). This fear would tend to suppress exploration or produce active avoidance of the novel situation. Observed exploratory behavior is thus the result of these two competing drives and will depend to a large extent on the degree of novelty of the situation, as well as on other aspects of the animals past experience.

A_{1a} Elevated plus maze:

Out of many possibilities to modify maze tests e.g. water maze, the Y-maze, the radial maze, and the elevated plus maze (Pellow *et al.,* 1985) have found acceptance in many laboratories. The test has been proposed for selective identification of anxiolytic and anxiogenic drugs. Anxiolytic compounds, by decreasing anxiety, increase the open arm exploration time; anxiogenic compounds have the opposite effect.

Principle:

One of the most popular behavioral model used for testing anxiety in rodents is the elevated plus maze (EPM). The EPM test was developed by Pellow *et al*. (1985) and Pellow and File (1986) to assess anxiolytic and anxiogenic drugs effects on transition activity in rodents between open arms and closed arms. Initially developed for rats (Pellow *et al*., 1985) and more recently, for other species such as guinea pigs, voles, hamsters and gerbils. There has also been the development of several derivatives of the EPM including the elevated T maze, zero maze and the unstable elevated exposed plus maze, UEEPM, (Jones and King, 2001). The EPM (Fig.1) is in the form of a 'plus sign' with two open arms facing each other and separated by a central square and two closed arms of the same dimensions, but enclosed by walls. The maze is raised from the ground to a height that the open arms combine elements of unfamiliarity, fear, openness and height. The EPM is based on the natural aversion of rodents for open spaces and uses conflict between exploration and aversion to elevated open places. Provoked behaviour profiles in the EPM appear to include elements of neophobia,

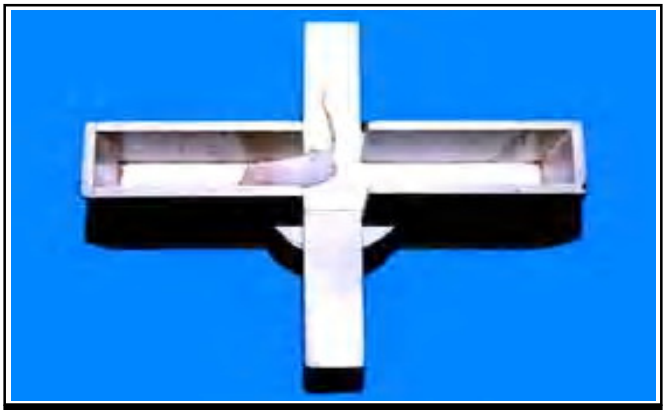

Fig. 1 : Fig.1. Shows elevated plus maze for mice comprising of two open and two closed arms

Fig. 2 : Shows zero model with two open and two closed arms

exploration and approach/avoidance conflict; thus, the apparatus is often referred to as an unconditioned spontaneous behavioural conflict model.

Mice generally taken from their home cages will show a pattern of behaviour characterized by open-arm avoidance with a consistent preference for the closed arms. The rank order preference profile is closed > centre > open, indicative of a penchant for relatively secured portion of the maze. This tendency is suppressed by anxiolytics and potientiated by anxiogenic agents (Lister, 1987). The typical endpoints measured include the number of open and closed arm entries and the time spent in each arm. Occasionally, other endpoints are measured, including frequency and duration of "scanning", which is the protruding of the head over the edge of an open arm and fanning with the vibrissae (*i.e.* hair growth at the nares) in any direction, "risk assessment", which is the protruding from an enclosed arm with the fore paws and head only, and "end activity", which is the amount of time spent at the end of an open arm. This model is also used for testing learning ability and memory, where in transfer latency from open arm to closed arm is measured on Ist day and again after 24 h. Sometimes animal spends most of the time on the central platform of the maze. This limitation is overcome by zero maze by removing the central platform as shown in Fig.2.

A_{1b} Light – dark test:

Crawley and Goodwin (1980) described a simple behavior model in mice to detect compounds with Anxiolytic effects. Mice and rats tend to explore a novel environment, but retreat from the aversive properties of a brightly-lit open field. In a two chambered system, where the animals can freely move between a brightly-lit open field and a dark corner, they show more crossings between the two chambers and more locomotor activity after treatment with anxiolytics. The numbers of crossings between the light and dark sites are recorded.

Principle:

This test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behaviour of rodents in response to mild stressors, *i.e.* novel environment and light. This model permits mice to freely explore two inter-connected compartments that vary in size (2:1), colour (white:black) and illumination (bright : dim). Thus, control mice, when placed into the brightly lit section will rapidly move into the dark area. After anxiolytic drug treatment, the apparent apprehension of remaining in or moving to the light area is abolished. Since then the L-D test has been widely adopted as an anxiolytic screening test in mice (Costall *et al.*, 1989) extended for use in rats and has been subject to several modifications. The size of the box and compartments has been adjusted.

Fig. 3 : Shows Light-Dark model with light and dark compartment

Five main parameters are now available to assess the anxiolytic profile of drug treatment: the latency time for the first passage from the light compartment to the dark one, the number of transitions between the two compartments, the movement in each compartment and the time spent in each compartment. Sometimes rearing and grooming are also measured.

A_{1c} Open field:

This test, originally designed by Hall on rats, consists in placing an animal in an unknown environment with surrounding walls, so as to observe a number of behavior patterns, including the tendency to stay on the periphery of the field without entering the centre (called thigmotaxis and often interpreted as anxious behavior), levels of defecation and urination (Hall, 1934).

Fig. 4 : Shows spontaneous activity in open field model

Principle:

In this model, the open field floor is often divided into squares. Animals are tested individually; always being placed in the same position. Anxiety behavior in the open field is triggered by two factors: individual testing and agoraphobia. Anxiety state is indicated by diminished ratio of 'number of squares visited in centre to number of squares visited on periphery'. In experiments involving rodents, observers do not measure the effects of treatments on exploration, but the reaction to a stressful event. Therefore, anxiolytic treatments do not by themselves increase exploration in the open field but they decrease the stress-induced inhibition of exploration behavior. Open-field test may be a rodent model of normal anxiety sensitive to the anxiolytic-like effects of benzodiazepines and 5-HT_{1A} receptor agonists but not to the effects of compounds displaying anxiolytic-like effects in the clinical entity termed 'anxiety disorders' (Prut and Belzung, 2003). Observations are recorded generally for duration of 5 min.

A_{1d} Hole – board test:

The hole-board test, which was developed by Boissier and Simon (1962) nearly a half century ago, provides a simple means to assess the response of an animal (mouse) to an unfamiliar setting. A typical hole board uses the 40 cm square and 1.8 cm thick board with equally spaced holes [3 cm in diameter]. It is mounted on four 2.5 cm legs and placed on a gray floor in a cubicle with three gray painted walls with the observer seated near the floor at the window side. Each mouse is assessed by placing it singly in the centre of the board facing away from the observer for 5 min. A mouse is said to have made a head dip, if both ears go below the top of the hole. Each hole is numbered so that the number of times a mouse explores a specific hole may be counted.

A_{1e} Staircase test:

Principle:

The staircase test for evaluating anxiolytic activity was originally described for rats by Thiebot *et al.* (1973). When introduced into a novel environment, rodents experience a conflict between anxiety and exploratory behavior manifested by increased vigilance and behavioral activity. In the staircase paradigm, step-climbing is purported to reflect exploratory or locomotor activity, while rearing behavior is an index of anxiety state. The number of rearings and steps climbed are recorded in a 5 min period. The dissociation of these parameters is considered to be characteristic for anxiolytic drugs. The test was modified for rapid screening of anxiolytic activity in mice.

The staircase test is a relatively rapid and simple procedure involving easily quantified aspects of behavior. A rat or mouse is placed in a test box containing a five-step staircase. During a 3-min test, the activity of the animal is assessed as the number of steps climbed up/down (both ascents and descents) and the number of times the animal rears on its hind paws. In the initial descriptions of this procedure (Thiebot *et al.*, 1973), it was proposed that rearing was a sign of anxiety and the steps climbing was considered as an index of exploration. Later on, it was revealed that rearings were directly related to locomotion rather than exploration. In open field test, it was observed that in first few min of the testing procedure, the locomotion response is an expression of anxiety in which escape activity predominates and only later does the exploration component occur. It was proposed that rearing was decreased by anxiolytic drugs, which might also increase the number of steps climbed. An alternative parameter (Steru *et al.*, 1987) is that increased number of steps climbed indicates anxiolytic activity, whereas decreases in rearing provide a measure

of sedation.

A_{1f} Mirror chamber test:

Principle:

The mirror chamber, designed to detect anxiolytic agents, is based on the principle that many species show approach-avoidance conflict behavior when faced with a mirror image (Kulkarniand Reddy, 1996). The outer box containing the chamber is constructed of black plastic, 40 cm × 40 cm × 30.5 cm high. Located within this box is a black 30.5 cm cube, open on one end. The three inner walls, ceiling, and floor of the cube are mirrored. The illuminance within the mirrored chamber was approximately 10 W. The space between the inner cube and outer box provides the animal with a 4.6 cm darkwalled dim (1-2 W) alley surrounding the cube. The mouse is placed in the narrow alley at the farthest point from the opening to mirrored chamber. Infrared emitter detectors monitored alley-to-alley transitions, rearing and latency to enter the mirrored chamber. Total time of the test is 5 min, in which time taken (in sec) for first entry into the mirror chamber, number of entries in 5 min and total time spent in mirror chamber were calculated. Anxiolytic drugs increase the total number of entries and time spent in mirror chamber.

A_2 Social behavior:

A_{2a} Social interaction:

Principle:

In an unfamiliar and brightly lit environment, the normal social interaction of rats (e.g. sniffing, nipping, grooming) is suppressed. Anxiolytics counteract this suppression. Social interaction test (Fig.5) involves responses to novel, unfamiliar and brightly lit environment. Anxious rats show suppression of their normal social interactive behaviour (such as sniffing, nipping, and grooming) when exposed to this novel environment. Anxiolytic agents counteract this suppression. File (1980) has designed a procedure that does not involve electric shock punishment or deprivation of food or water, and is, therefore, more relevant and analogous to human anxiety. Pairs of rats, unfamiliar with each other, are placed in an arena, and their behavior is assessed for a period of 10 min. The extent to which the arena was assumed to be fear-inducing was manipulated by changing the level of illumination and whether or not rats had previously been exposed, singly, to the arena. This method provided four test conditions-dim light/familiar arena, bright light/ unfamiliar arena, dim light/unfamiliar arena, and bright light/ familiar arena, which were assumed to vary in the extent to which they induced fear, anxiety, or uncertainty in the rats. The amount of time for which the animals were in social contact differed in these different conditions, with the smallest time being observed in the bright light/ unfamiliar condition. Photocells are also used to quantify locomotion, and it was proposed that manipulations that increased social interaction without increasing locomotion might be doing so by reducing anxiety.

Fig. 5 : Shows presence of unfamiliar social partner

A_{2b} Social competition:

This test was first described by Woodall *et al*. (1996) is carried out twice a week for a period of 5 weeks. In the first week, animals are familiarized to test box and sweetened milk from a drinking spout located on the end wall. The drinking spout is surrounded by a Perspex tube (4.5 cm diameter), which ensured that only one animal was able to drink at a time. All the animals were, deprived of water overnight and next day free access was given to the sweetened milk for 15 min. In the second week, animals were no longer deprived of water and free access was given to sweetened milk for 5 min. During the test, rats were observed for every 5 sec. and the animal drinking milk was noted down. Anxiolytic drugs are given to either to dominant or subordinate rat. Anxiolytic drugs increase the access of subordinate member to sweetened milk.

A_{2c} Ultrasonic distress vocalization:

Rats and mice produce ultrasonic vocalizations in a variety of situations, for instance in response to stress, anxiety and pain (22 kHz), or during social interaction such as sexual behavior (50 kHz). Small rat pups emit ultrasounds in response to separation from their mother and littermates (40 kHz). These ultrasonic vocalizations (USV) can be used as an indicator of emotional and motivational status.

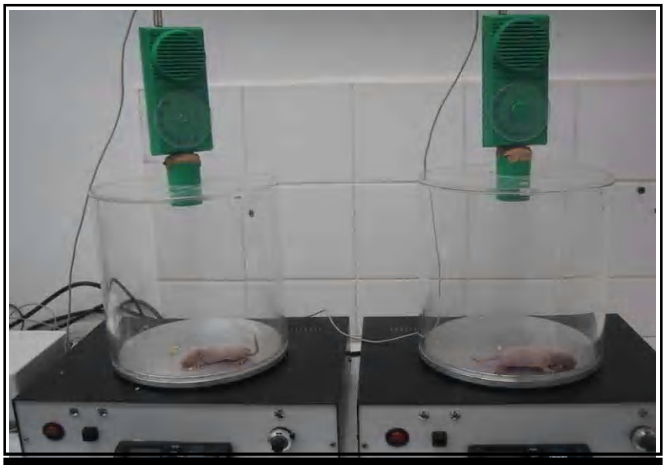

Fig. 6 : Shows isolation testing chamber to induce distress

A_{2ci} Isolation (maternal separation) distress calls:

Isolation of rat pups leads to a short bout of distress calls. The isolation testing chamber (Fig.6), which is located in a testing room separate from the housing room, consisted of a 500 ml glass beaker with an ultrasonic microphone suspended approximately 25 cm above the base of the beaker. USV are recorded using a high frequency bat detector, Pettersen D980 ultrasonic detector (Uppsala, Sweden), which digitally recorded 196 kHz, and USV are analyzed offline *via* sonograph (Avisoft Bioacoustics). The pups are habituated for 1 minute to the testing chamber on postnatal day nine. On postnatal day ten, the pups are removed from the colony room and were placed individually in the isolation testing apparatus for 2 minutes with USV being recorded. There were no other animals present in the testing room during the testing session. After testing, animals were transported back to the colony room and were returned to their home cages and mother. Data are manually scored offline for total number of 40 kHz distress vocalizations (Winslow and Insel,1991).

A_{2cii} Stress induced distress vocalization in rat pups: Principle:

Measurement of ultrasonic vocalization induced by tail holding in rat pups was proposed as a simple screening method for anxiolytic drugs by Gardner (1985). Parameters such as time spent with ultrasonic vocalization or total number of ultrasonic vocalizations were measured as end points. In this test pups are subjected to handling stress and the magnitude of their ultrasound emission is observed. The stress consists of first holding the pups gently into the hands of the experimenter, whereby the pups emits only a few ultrasounds in 30 sec. Secondly, stress induced by holding the pups by the base of the tail and suspending it 5 cm above the bench for 30 sec. Responses when held by the tail are 10 times higher. Ultrasounds are recorded with suitable detectors with 42 kHz as the centre of a 10 kHz recording range. Only pups, which produce more than 50 ultrasounds in first test, were selected for test drugs. After 3-4 hours of first test, animals are subjected to test drugs. Anxiolytic drugs inhibit vocalization.

A_{2ciii} Social interaction induced vocalization:

Rats produce a 50 kHz vocalization under non-aversive conditions, and these vocalizations reflect a positive affective state of the animals, which include the potential rewards such as play, sex or winning a fight. Rats emit short, chirping 50 kHz vocalization during sexual behaviors, male agonistic behaviors during fighting, juvenile play, and manual tactile stimulation ('tickling') by experimenters. As in case of ultrasonic vocalizations emitted by an approaching male mouse (Fig.7) indicate to the female mouse that the male mouse is sexually motivated rather than aggressively motivated (Sales and Pye, 1974). Male song also may be an indicator of the male mouse's fitness, and the female mouse may use this index to determine whether she will allow mating to occur or not.

Fig. 7 : Shows socially and environmentally enriched cage housing multiple male and female mice

A_3 Predator:

This includes induction of defensive behaviour in animals on exposure to predator, such as exposure of rats to natural predator threat stimulus (cat) or cat odor or it may be human threat for primates.

A_{3a} Defence test battery in rats: Principle:

Blanchard *et al.* (1989, 1992) described a set of procedures designed to assess the defensive reactions of rats to a natural predator, the cat. These tests involve a brief confrontation of laboratory rats with an unconditioned

threat stimulus (cat) with which physical contact, is avoided by a wire mesh barrier. The primary measures, taken both during and after cat presentation, include movement arrest and risk assessment (proxemics/activity test) and the inhibition of non-defensive behaviors (eat/drink or freezing test).

The test apparatus for both procedures consists of two parallel subject chambers (53 cm x 20 cm x 25 cm). The inside walls of each chamber were constructed of opaque black Plexiglas, while outer walls and lid were clear Plexiglas to allow video recording from lateral and overhead views. The end wall of each chamber, constructed of wire mesh, adjoins a separate cat compartment. Five photocells were attached to each chamber for monitoring subject movement, and a food hopper and drinkometer are positioned 2.5 cm to each side of the central photocell. Access to the food hopper/ drinkometer can be prevented by insertion of Plexiglas gates. Initially the effects of cat exposure on proxemic/ activity was accessed followed by 7 days on eat/drink behaviour during and after cat exposure under dim red light.

A_{3b} Human threat (primates):

Suarez and Gallup (1982) first demonstrated human being as a predator. It can be studied in various ways of avoidance of experimenter. One method involves an experimenter sitting in a chair in the center of a floor containing chicks. The proximity of the chicks to the human is then determined usually by numbering imaginary zones around the occupied chair. The chicks are then given an 'avoidance score' of 1-5 (either in ascending or descending proximity to the experimenter) based on a total of their positions over a certain time frame. Another method is called the 'box plus experimenter' method. This test uses the same premise as the above method; however, during the box plus experimenter test, the human is seated behind a wire mesh wall at the end of an arena. The chick is scored on its approach or avoidance to the experimenter behaviors. Again, higher avoidance suggests higher fear levels.

A_{3c} Odour associated avoidance behaviour:

The predatory odour avoidance model relies upon the apparently innate fear that rodents have for the odor of their natural predators, such as cats and foxes. Rats tend to avoid such odors and engage in a variety of defensive behaviours in their presence. Novel methodology for testing is given by Dielenberg and McGregor (1999). Testing chambers comprised of a rectangular arena with perspex walls (60 cm x 26 cm x 36 cm) and a metal grid floor which was raised 2 cm above a tray containing wood shavings. At one end of the chamber was a small wooden box (21 cm x 24 cm x 22 cm) termed the 'hide box'. On the front wall of the hide box was a small square hole (6 cm x 6 cm) that allowed rats to enter the box. On the opposite wall of the apparatus to the hide box was an alligator clip positioned 4 cm above the metal grid floor. During testing, a piece of cat collar was attached to the clip as shown in Fig.8. A domestic cat wore this cat collar for a period of three weeks before the start of the experiment. Photocell detectors were placed at opposite ends of the chamber. The placement of the photocells allowed determination of: i) the amount of time the rats spent in close vicinity to the cat collar (approach time); and ii) the amount of time spent in the hide box (hide time). During testing, the room in which the chambers were situated was illuminated by a 40 W red-light suspended 1.5 m above the apparatus.

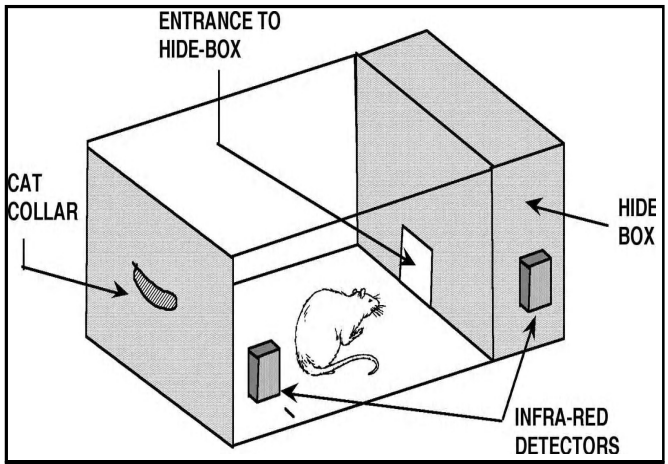

Fig. 8 : Shows predatory odour avoidance apparatus

A_4 Others:

A_{4a} Novelty suppressed feeding:

Porschel in 1971 described that conflict could be induced from the situation of fear, novel environment and food. As placing a hungry rat (Fig.9) into an unfamiliar environment with access to food resulted in a suppression of feeding behaviour relative to the condition when the test environment was familiar. Test apparatus consists of plexiglass open fields, 76 cm x 76 cm x 46 cm. Thirty feed pellets are placed in a pile directly in the centre of the open field. Animals are deprived of food 48 h before the testing. Total time of testing is taken as 720 sec. If the animal has not eaten within 720 sec, the test is terminated and the animal is assigned a latency score of 720 sec.

Fig. 9 : Shows novelty-suppressed feeding

A_{4b} Schedule induced polydipsia in rats:

Principle:

Food deprived rats exposed to a procedure in which food is delivered intermittently will drink large amounts of water if given the opportunity to do so. This behavioral phenomenon is termed schedule-induced polydipsia and is an example of a more general class of behaviors termed adjunctive behaviors. Adjunctive behaviors have been cited as potential animal models of human obsessive-compulsive disorders (Pitman 1989). Male Wistar rats weighing 180–250 g are individually housed at a 12 h/12 h light/dark cycle for 1 week acclimatization period with free access to food and water. Then, they are placed on a restricted diet, which maintains 80% of their free feeding body weight. To induce polydipsia, rats are placed in test chambers housed in sound attenuated boxes, where a pellet dispenser automatically dispenses two 45 mg pellets on a fixed time 60-s (FT-60s) feeding schedule over a 150 min test session. Water is available at all times in the test chambers. After 4 weeks exposure to the FT-60s feeding schedule, approximately 80% of the rats meet the pre-determined criterion for water consumption (greater than 60 ml water per session) and are considered to have polydipsic behavior. Rats receive the test compounds in various doses daily or the vehicle intraperitoneally 60 min prior to testing. They are tested once a week to assess schedule induced polydipsia. Water bottles are weighed before and after the 150-min test sessions.

A_{4c} Marble burying:

Marble-burying behavior is considered to be a potential model of obsessive-compulsive disorder, on the basis of behavioral similarity (Ichimaru *et al.*,1995). Presence of unfamiliar object is the potential source of danger in this test. In this model (Fig.10.) mice are placed individually in clear plastic boxes (30 cm × 30 cm × 28 cm), containing 25 glass marbles (1.5 cm in diameter) evenly spaced on sawdust 5 cm deep, without food and water. At the same time, the locomotor activity of the mice is measured using an automated activity counter. The activity is measured with the illumination of a 100 W bulb. The results of marble-burying behavior are expressed as the number of marbles buried to at least two-thirds of the depth, within 30 min. Anxiolytic drugs decrease the number of buried marbles (Matsushita *et al.*, 2005).

Fig. 10 : Shows marble burying model

A_{4d} Cork gnawing:

Principle:

Cork gnawing behavior in the rat has been proposed as a screening method for buspirone-like Anxiolytics by Pollard and Howard (1991).

Test apparatus consists of a stainless steel cage with wire mesh bottom. A session consists of placing the subject in the test cage with a cork stopper weighing between 2-3 gm for 30 min. Initially, the amount gnawed is relatively high and variable within and between subjects. After 30 training sessions, the amount gnawed is low and stabilized. Buspirone like compounds as well as benzodiazepines and meprobamate show a dose dependent increase of cork gnawing.

A_{4e} Stress induced hyperthermia:

When group housed mice are removed one by one from their home cage, the last mouse removed have always higher rectal temperature than those removed first (Borsini *et al.*, 1989). This phenomenon is interpreted as being caused by anticipatory fear for an aversive event (Fig.11). The anticipatory increase in temperature is prevented by prior treatment with diazepam and buspirone. Rectal

Fig. 11 : Shows stress induced (handling order) hyperthermia

temperature is recorded by inserting a silicon lubricated thermistor probe for 2.5 cm into the rectum.

B_1 Punishment procedures:

Punishment procedures or conflict tests as they are often called have been very widely used with great success for assessing the effects of anxiolytic drugs. Punishment refers to the presentation of an aversive stimulus, generally a brief, mild electric shock, to an animal, contingent upon the emission of a particular behavior. It is this contingent relationship between a response, such as pressing a lever or licking a tube, and an aversive event that has apparently been of great importance in establishing the sensitivity of these methods to the effects of anti-anxiety drugs. It is often assumed that the behavior of animals tested in punishment procedures is under the control of two motivations, a positive motivation, such as hunger, thirst, or the need to explore, which tends to induce the animal to emit a response, and a negative motivation, presumably fear of the impending punishment, which decreases response output. The term conflict refers to the opposing nature of these two motivations.

B_{1a} Four plate test:

Principle:

The four plate test in mice has been described by Boissier *et al.* (1968) as a method for the rapid screening of minor tranquilizers. It is based on the suppression of a simple innate ongoing behavior, *i.e.* the exploration of novel surroundings, by the mouse. The apparatus consists of a floor made of four identical rectangular metal plates. This exploration behavior is suppressed by the delivery of mild electric foot shock contingent on quadrant crossings. Every time the mouse crosses from one plate to another, the experimenter electrifies the whole floor evoking a clear flight-reaction of the animal. Benzodiazepines increase the number of punished crossings accepted by the animal. In the original technique, mice were tested for a very short period (1 min following 15 s of exploration without shock), which makes the four-plate test very rapid and thus suitable for screening large number of compounds (Bourin *et al.*, 2007).

B_{1b} Punishment induced operant behavior:

This is the most traditional punishment procedure using operant behavior for analysis of anxiolytic drugs. Experimental animals are trained to emit a response, such as a lever press, that results in the delivery of food or water reinforcement according to the schedule in operation. Geller and Seifter (1960) presented data using rats trained to lever press for sweetened condensed milk on a variable-interval schedule, so that lever presses are reinforced every 2 min. Every 15 min, a tone is presented for 3 min. During the periods of tone presentation, the schedule changed, and every lever press produced both milk delivery and electric foot shock. This had the effect of suppressing responding during the tone with the degree of suppression dependent upon the level of shock. Drugs such as benzodiazepines, barbiturates, and carbamates could greatly increase rates of lever pressing during the punishment period.

B_{1c} Conditioned emotional response:

Ogawa *et al.* (1993) designed a communication box to induce experimental anxiety in mice by employing interspecies emotional communication. The inside of the communication box was divided into foot shock and non-foot-shock compartments by transparent plastic boards. The animals, which were individually placed into each compartment, were unable to make physical contact with each other, but were able to receive other cues such as visual, auditory and olfactory sensations. During the foot shock period, the animal placed in the non-foot-shock compartment [Responder animal] was exposed to the emotional cues from foot-shocked animals, such as shrieks, smell of feces or urine, and jumping response. The floor of the communication box is equipped with grid for electric shock. Electric shock for the duration of 10 sec at an intervals of 50 sec for 3 h was given to animals placed in foot shock compartment (sender animals). Current for the shock increased stepwise from 1.6 mA to 2.0 mA at a rate of 0.2 mA per 1 hr. Responder animals are exposed daily to the emotional responses of sender animals, 3 h per day for 3 days.

On the third day responder animals are sacrificed and examined for gastric lesions and bleeding. Anxiolytic drugs decrease the incidence of gastric ulcers in food-deprived animals.

B_2 Conflict models:

B_{2a} Vogel's punished drinking:

Principle:

Vogel *et al.* (1971) described a simple and reliable conflict procedure for testing anti-anxiety agents. Thirsty, naive rats were administered shocks while licking water. It involves a stressful situation (48 h water deprivation), which produces a conflict between thirst and punishment after drinking. After 48 h, water deprived rats were placed in a test chamber containing a drinking spout. Whenever an animal made contact with the spout, a pulse generator sent out pulses at a rate of 7/s (to imitate the natural rate of licking of rats), and with every 20 pulses (*i.e.*, just under 3 s of contact with the tube or 20 notional licks), the animal received a shock through the tube. In control animals, this procedure resulted in very low rates of drinking, which were substantially increased by the anxiolytic drugs. Diazepam and pentobarbital produced a significant anti-conflict effect, which means that these drugs increased the number of electric shocks mice received during the test session.

Fig. 12 : Shows Vogel's punished drinking model

B_{2b} Geller Seifter conflict:

In this model (Geller and Seifter, 1960), rats were trained in operant chambers to operate a lever to obtain food. Presentation of an auditory cue signaled a change in the reinforcement contingencies. Further responding resulted in both an increased availability of food, and foot-shock. In other words, this procedure involves a multiple schedule of reinforcement. In the first segment of the schedule (signaled by an auditory or visual cue), response is reinforced at irregular intervals. In the second segment (the conflict component), every response is simultaneously reinforced (signaled by a different signal); and punished (by the delivery of a brief, inescapable electro-shock). The suppression of the response in the conflict component can be specifically attenuated through the administration of anxiolytics; their potency in the experiment being proportional to their clinical potency. However, the response to the simple food-rewarded component without punishment is not enhanced by the anxiolytics. The disadvantages of this classical procedure include a long period of training (one to several weeks) until the animals reach a stable base-line response to the conflict component as well as the necessity for long-term food restriction. Once the subjects have learned the tasks in the Geller-Seifter paradigm response rates in all operant components remain relatively stable over long periods. This makes the Geller-Seifter conflict a suitable test for repeated drug-testing in order to demonstrate reliable and repeatable responses to anxiolytics over time in individual subjects.

B_{2c} Shock probe conflict procedure:

Principle:

The shock probe conflict procedure, an assay responsive to benzodiazepines, barbiturates and related compounds, was described by Meert and Colpaert (1986). Rats being placed in a novel test environment containing a probe, explore the environment and also the probe. The exploration of the probe, quantified as the number of times that the animal makes physical contact with it, is reduced when the probe is electrified. Rats treated with anxiolytics continue to touch the electrified probe. Animals are placed in novel environment for exploration of the environment as well as probe. The number of times the animal makes physical contact with probe is reduced when the probe is electrified. Test apparatus used for this test consists of a Plexiglas chamber with metal grid floor. A shock intensity of 0.9 mA is applied with a Teflon probe provided in the test apparatus, 3 cm above the floor of the chamber. Responses are recorded for a total period of 5 min. Animals treated with anxiolytic drugs continues to touch the electrified probe.

B_3 Respondent conditioned with aversion stimuli:

Punishment techniques are often referred to as conflict procedures on the assumption that the animal's behavior is under the control of competing drives: for example, to approach and respond on the manipulandum to obtain reinforcement and to avoid responding in order not to experience the aversive stimulus. However, it has often been noted that animals do not appear particularly emotional when well trained on punishment procedures, and the type of behavior seen under such conditions seems essentially appropriate and adaptive rather than inappropriate as is the case with pathological anxiety. The essential difference between conditioned suppression and punishment procedures is, of course, that the aversive

stimuli are unavoidable during conditioned suppression. Thus, even if the animal stops responding completely during the shock-associated stimulus, this will have no influence on whether the shock is presented (Gray, 1977). There has, however, been discussion as to whether the animal is in fact able to modify the aversiveness of the stimulus by suppressing operant responding and what, exactly; the nature of this response suppression is.

B_{3a} Conditioned suppression:

A method that has certain similarities with punishment procedures, but with at least one fundamental difference is that described as conditioned suppression or the conditioned emotional response (CER). This method involves the pairing of a neutral stimulus, such as a light or noise, with an aversive stimulus, usually a brief, but unavoidable electric shock. After a number of pairings, which thus involve a process of respondent conditioning, the previously neutral stimulus itself induces behavioral changes. Usually the stimulus is presented to animals responding on an operant schedule for food or water reinforcement, and responding is found to be depressed in rate or completely suppressed during the stimulus. The most frequently used method is that first described by Estes and Skinner (1941), in which the stimulus-shock pairings are presented during regular sessions in which subjects work for positive reinforcement (on the baseline). Alternatively, the respondent conditioning can be carried out in a different environment, and only the stimulus (CS) can be presented during regular operant sessions (off the baseline conditioning). There were several reports that benzodiazepines and barbiturates attenuated conditioned suppression, although meprobamate apparently exerted less clear effects (Lauener, 1963).

B_{3b} Potentiated startle response:

It was originally designed by Brown *et al.* (1951). This pavlovian fear conditioning procedure involves two different steps. First, the animals are trained to associate a neutral stimulus, generally a light, with an aversive stimulus such as an electric foot-shock. After training, animals are submitted to an intense sound. The startle response to this unconditioned stimulus is potentiated by simultaneous presentation of the previously conditioned light stimulus. This potentiation can be found even one month after the training. Anxiolytics produce a dose-dependent reduction of the startle amplitude with no change in the baseline level of the startle (observed in the absence of the conditioned stimulus). A decrease in the baseline would reveal a non-specific locomotor impairment. Main results of this model have been published by Davis and Whalen (2001). Benzodiazepines, as well as buspirone-like drugs, decrease fear-potentiated startle, often without any change in the baseline response.

B_{3c} Electric brain stimulation:

Aversive effects can be produced by direct electrical stimulation of the periaqueductal grey area of the brain via chronically implanted microelectrodes (Bovier *et al.*, 1982), which induces defensive reaction and/or flight behaviour in several species. Animals are placed in a rectangular cage with a grid floor and a 2 cm high barrier dividing the cage in half. Strain stimulation of 0.1 ms is given with neurostimulator. Stimulation frequency is fixed *i.e.* 50 Hz. Aversive behavioral signs are observed, which are characterized by autonomic reactions. Animals in this test are trained to stop the stimulation by escaping from one compartment to another.

B_4 Frustration (Non-Reward) test:
Shock probe-burying test:

Shock probe burying test was first introduced by Pinel and Treit (1978). Defensive burying is regarded as a species specific active avoidance strategy and a coping response directed towards proximal, immediate threat (DeBoer *et al.*, 2003). In this test, rats (or mice) are individually placed in a plexiglass test chamber that contains 5 cm of bedding material (e.g., wood chips) spread evenly across the chamber's floor. An electrified probe is inserted into the middle of one side of the chamber 4 cm above the bedding. When the animals make contact with the electrified probe they receive a brief 2.5 mV shock and subsequently display a natural tendency to bury the shock probe by using their forepaws and snouts to shovel the bedding material towards and over the probe. Typically, the test is video recorded and the duration and frequency of behaviors such as burying, rearing, immobility; grooming and risk assessment is scored (DeBoer *et al.*, 1990). The duration of time spent burying the electric probe is used as the primary measure of anxiety in this test. Pharmacological validation studies demonstrated that animals given anxiolytic drugs (e.g. diazepam) prior to testing show dose dependent decreases in burying behavior, whereas administration of an anxiogenic (e.g. yohimbine) prior to testing yields dose dependent increases in burying behavior (Treit *et al.*, 1981).

Concluding remarks:

Anxiety is a term used to describe both, a normal emotional state associated with stressful or difficult events and a pathological condition. When anxiety is chronic and is not clearly linked to well-defined events, it is generally

considered abnormal and appropriate for psychological or psychiatric intervention. The Animal models contribute to reveal the underlying pathophysiology of various psychiatric disorders. Furthermore, these models are useful not only in screening new compounds but also help in discovering new medicines based on repairing the underlying pathological deficiency. It is often difficult to produce psychiatric, disorders in laboratory animals similar to human beings. Since human life is precious, it becomes necessary to test the new medicines in small animals before applying to human beings. In this direction, the authors have attempted to describe the principle and brief procedure of each model employed for screening of anti-anxiety agents. However, the young scientists may like to modify slightly the specified procedure depending on their laboratory conditions, while adhering to the basic features of the models.

REFERENCES

Blanchard, D.C., Shepherd, J.K., Rodgers, R.J. and Blanchard, R.J. (1992). Evidence for differential effects of 8-OH-DPAT on male and female rats in the anxiety/defense test battery. *Psychopharmacology*, **106**: 531–539.

Blanchard, R.J. and Blanchard, D.C. (1989). Antipredator defensive behaviors in a visible burrow system. *J. Comp. Physiol.,* **103**: 70–82.

Boissier, J.R., Simon, P. and Aron, C. (1968). A new method for rapid screening of minor tranquillizers in mice. *European J. Pharmacol.,* **4** : 145–151.

Borsini, F., Lecci, A., Volterra, G. and Meli, A. (1989). A model to measure anticipatory anxiety in mice? *Psychopharmacology*, **98**: 207-211.

Bourin, M., Petit-Demoulie"re b, Dhonnchadha, B.N. and Hascoet, M. (2007). Animal models of anxiety in mice. *Fundamental & Clinical Pharmacology*, **21**: 567–574.

Bovier, P., Broekkamp, C.L.E. and Lloyd, K.G. (1982). Enhancing GABAergic transmission reverses the aversive state in rats induced by electrical stimulation of the periaqueductal grey region. *Brain Res.,* **248**: 331–320.

Brown, J. S., Kalish, H.I. and Farber, I. E. (1951). Conditioned fear as revealed by magnitude of startle response to an auditory stimulus. *Exp. Psychol.,* **41**: 317-328.

Costall, B., Jones, B.J., Kelly, M.E., Naylor, R.J. and Tomkins D.M. (1989). Exploration of mice in a black and white test box: validation as a model of anxiety. *Pharmacol. Biochem. Behav.,* **32**: 777–785.

Crawley, J.N. and Goodwin, F.K. (1980). Preliminary report of a simple animal behaviour for the anxiolytic effects of benzodiazepines. *Pharmacol. Biochem. Behav.*, **13**: 167 – 170.

Davis, M. and Whalen, P.J. (2001). The amygdala: Vigilance and emotion. *Mol. Psychiatry*., **6** : 13-34.

DeBoer, S.F. and Koolhaas, J.M. (2003). Defensive burying in rodents: ethology, neurobiology and psychopathology. *European J. Pharmacology,* **463**: 145-161.

DeBoer, S.F., Slangen, J.L. and Van der Gugten, J. (1990). Plasma catecholamine and corticosterone levels during active and passive shock-probe avoidance behavior in rats: effects of chlordiazepoxide. *Physiology & Behavior.,* **47**: 1089-1098.

Dielenberg and McGregor (1999). Differential anxiolytic efficacy of a benzodiazepine on first versus second exposure to a predatory odor in rats. *Psychopharmacology*, **147**:174–181.

Estes, W. K. and Skinner, B.F. (1941). Some quantitative properties of anxiety. *J. Exp. Psychol.,* **29**: 390-400.

File, S.E. (1980). The use of social interaction as a method for detecting anxiolytic activity of chlordiazepoxide-like drugs. *Neurosci. Methods*, **2**:219-238.

Gardner, C.R. (1985). Distress vocalisation in rat pups: A simple screening method for anxiolytic drugs. *J. Pharmacol. Meth.,* **14**: 181–187.

Geller, I. and Seifter, J. (1960). The effects of meprobamate, barbiturates, D-amphetamine and promazine on experimentally induced conflict in the rat. *Psychopharmacologia*, **1**: 482-492.

Hall, C.S. (1934). Emotional behavior in the rat. I. Defecation and urination as measures of individual differences in emotionality. *J. Comp. Psychol.,* **18**: 385–403.

Ichimaru, Y., Egawa, T. and Sawa, A. (1995). 5-HT1A-receptor subtype mediates the effect of fluvoxamine, a selective serotonin reuptake inhibitor, on marble-burying behavior in mice. *Japan J. Pharmacol.,* **68**: 65–70.

Jones, N. and King, S.M. (2001). Influence of circadian phase and test illumination on pre-clinical models of anxiety. *Physiol. Behav.,* **72**: 99–106.

Kulkarni, S.K. and Reddy D.S. (1996). Animal behavioral models for testing antianxiety agents. *Clin Pharmacol.,* **18**: 219-30.

Lauener, H. (1963). Conditioned suppression in rats and the effect of pharmacological agents thereon. *Psychoyharmacologia,* **4**: 311-325.

Lister, R.G. (1987). The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology*, **92**: 180–185.

Matsushita, M., Egashira, N., Harada, S., Okuno, R., Mishima, K., Iwasaki, K., Nishimura, R. and Fujiwara, M. (2005). Perospirone, novel antipsychotic drugs, inhibits marbleburying behavior via 5-HT1A receptor in mice: implications for obsessive– compulsive disorder. *J. Pharmacol. Sci.,* **99**: 154–159.

Meert, T.F. and Colpaert, F.C. (1986). The shock probe conflict procedure. A new assay responsive to benzodiazepines, barbiturates and related compounds. *Psychopharmacol*, **88**: 445–450.

Montgomery, K.C. (1955). The relation between fear induced by novel stimulation and exploratory behavior. *J. Comp. Physiol.,* **48** : 254-260.

Ogawa, N., Hara, C. and Takaki, S. (1993). Anxiolytic activity of SC- 48274 compared with those of buspirone and diazepam in experimental anxiety models. *Japan J. Pharmacol.,* **61**: 115–121.

PeIIow, S. and File, S.E. (1986). Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat. *Pharmacol. Biochem. Behav.,* **24**: 525-529.

Pellow, S., Chopin, P., File, S.E. and Briley, M. (1985). Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J. Neurosci. Methods,* **14**: 149–167.

Pinel, J.P.J. and Treit, D. (1978). Burying as a defensive response in rats. *J. Comparative & Physiological Psychol.*, **92**: 708-712.

Pitman, R.K. (1989). Animal models of compulsive behavior. *Biol. Psychiatry*, **26**: 189–198.

Pollard, G.T. and Howard, J.L. (1991). Cork gnawing in the rat as a screening method for buspirone-like anxiolytics. *Drug Dev. Res.,* **22**: 179–187.

Prut, L. and Belzung, C. (2003). The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *European J. Pharmacol.,* **463**: 3–33.

Sales, G.D. and Pye, D. (1974). Ultrasonic communication by animals. *New York: Wiley.,* **79**: 533-47.

Steru, L, Thierry, B., Chermat, R., Millet, B., Simon, P. and Porsolt R.D. (1987). Comparing benzodiazepines using the staircase test in mice. *Psychopharmacol.*, **92**: 106-109.

Suarez, S.D. and Gallup, Jr., G.G. (1982). Open-field behavior in chickens: the experimenter is a predator. *J. Comp. Physiol. Psych.,* **96**: 432-439.

Thiebot, M.H., Soubrie, P., Simon, P. and Boissier, J.R. (1973). Dissociation de deux composantes du comportement chez le rat sous l'effet de psychotropes. Application an I'etude des anxiolytiques. *Psychopharmacologia*, **31**: 77-90.

Treit, D., Pinel, J.P. and Fibiger, H.C. (1981). Conditioned defensive burying: a new paradigm for the study of anxiolytic agents. *Pharmacology, Biochemistry & Behavior.,* **15**: 619-626.

Vogel, J.R., Beer, B. and Clody, D.E. (1971). A simple and reliable conflict procedure for testing anti-anxiety agents. *Psychopharmacologia.*, **21**: 1–7.

Winslow, J.T.and Insel, T.R. (1991). Serotonergic modulation of the rat pup ultrasonic isolation call: studies with 5HT1 and 5HT2 subtype-selective agonists and antagonists. *Psychopharmacology*, **105**: 513-520.

Woodall, K.L., Domeney, A.M. and Kelly, M.E. (1996). Selective effects of 8-OH-DPAT on social competition in the rat. *Pharmacol. Biochem. Behav.,* **54**: 169–173.

Address for correspondence :
MILIND PARLE
Pharmacology Division, Department of Pharmaceutical Sciences,
Guru Jambheshwar University of Science and Technology,
HISAR (HARYANA) INDIA
E-mail. : mparle@rediffmail.com

Authors' affiliations :
SUMAN DEVIAND SURESH KUMAR
Pharmacology Division, Department of Pharmaceutical Sciences,
Guru Jambheshwar University of Science and Technology,
HISAR (HARYANA) INDIA