Review on: A Systemic Review on Preclinical Experimental Studies

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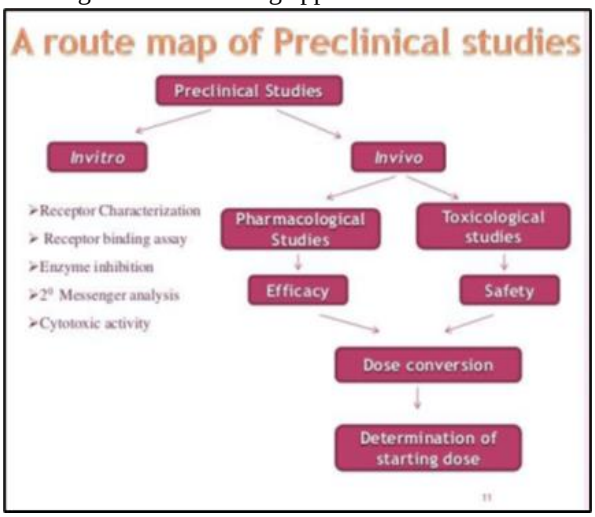
Abstract-Preclinical studies using animals to study the potential of a therapeutic drug or strategy are important steps before translation to clinical trials. However, evidence has shown that poor quality in the design and conduct of these studies has not only impeded clinical translation but also led to significant waste of valuable research resources. It is clear that experimental biases are related to the poor quality seen with preclinical studies. In this chapter, we will focus on hypothesis testing type of preclinical studies and explain general concepts and principles in relation to the design of in vivo experiments, provide definitions of experimental biases and how to avoid them, and discuss major sources contributing to experimental biases and how to mitigate these sources. We will also explore the differences between confirmatory and exploratory studies and discuss available guidelines on preclinical studies and how to use them. Preclinical testing is the link between drug discovery and availability to the patient. It takes at least 12–15 years for a drug to get from the lab to clinical use.

Keywords: experimental bias, invivo studies, Preclinical studies

INTRODUCTION

Preclinical trials or Non clinical trials are laboratory test of a new drug substance or medical devices, usually done on animal, to see whether the treatment really works and if it is safe to test on humans. The main goals of pre-clinical studies are to determine a product's ultimate safety Profile. Products may include new medical devices, drugs, gene therapy solutions, etc. After identifying a compound, it is tested on animals to expose the whole pharmacological profile. The experiment generally performed on rodent like mouse, guinea pig, hamster, rabbit. Preclinical trials to be conducted and synthesized to improve that the predictability of animal study for the human condition, idea for implementation of practice and outcomes pertaining to clinical translation are not clearly understood that can provide more comprehensive transparent, evidence based and theoretically informed rationale for analysis of preclinical studies. The drug development process is typically divided into three major steps: discovery, preclinical development, and clinical trial. selection. The boundary between preclinical development and clinical trial is sharply defined by the filing of an Investigational New Drug application. [1]



A route map of preclinical studies [2]

TYPES OF SCREENING

1. Simple Screening: It involves the use of one or two simple tests to find substances having a particular property. For example, a single test for conc. of glucose in blood can be used to screen compound for hypoglycemic activity.
2. Blind Screening: It is used to detect the pharmacological activities of new drugs whose pharmacological activity is unknown. The chief purposes is to demonstrate whether these new drugs are worthy of further attention or not.

3. Programmed Screening: It is used when a new drug of specific type is to be screened for some pharmacological effects. Examples are screening of certain drugs on the CVS, CNS, kidney, blood etc. It includes the use of quantitative assay of the compounds and their comparison with standard drugs that are quite active representative members of their pharmacological class. It also provides indications of potential side effects.[3]

BEHAVIOURAL ANIMAL MODEL

The animal model is living organism in which normative biology or behaviour can be studied, or in which a spontaneous or induced pathological processes can be investigated.

1. Elevated Plus Maze :

Animal Used: Mice/Rat

Principle:

The elevated plus maze task is a simple method to assess anxiety-like behaviours in rodents. This version describes the procedure used in mice.

The animal is allowed to freely explore the maze for 5 min while the duration and frequency of entries into open and closed arms is recorded.

Elevated plus maze[5]

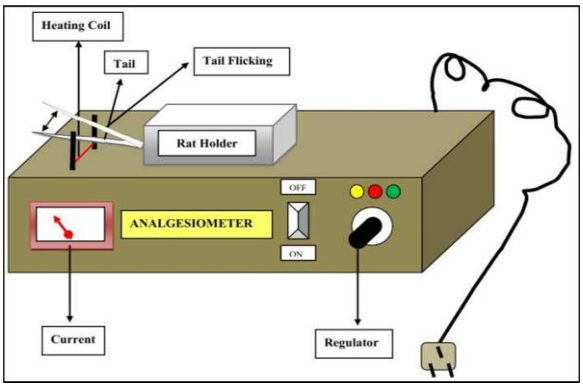
Application: Screening of Anxiolytic drugs [4]

2. Eddy's Hot Plate (Analgesiometer)

Animal Used: Mice

Principle:

They used a behavioural model of nociception where behaviours such as jumping and hind paw-licking are elicited following a noxious thermal stimulus. Licking is a rapid response to painful thermal stimuli that is a direct indicator of nociceptive threshold. The apparatus is used to testing the effectiveness of analgesics by reaction to pain caused by heat[6]



Analgesiometer[7]

Biochemical Assayy

Biochemical assays are analytical methods used to measure and quantify metabolic reactions and cellular processes such as cell signalling and apoptosis.

- Multiple techniques, including ELISA and Western blotting, exist for quantifying metabolic activity and measuring the functional behaviour of biomolecules such as enzymes, proteins, cofactors, and small molecules.
- Biochemical assays are used in drug discovery as targeted assays using well established reaction components.
- Similarly, cell-based assays can be used to determine target binding through biophysical or biochemical assay development.
- Biochemical assays can be used to assess cell membrane permeability and the capability of modulating the drug's target, when inside components.
- Furthermore, biochemical assays can identify protein-protein, protein-RNA, and protein-DNA interactions.
- Biochemical methods are mostly commonly applicable in the field of Membranes and protein purification. Immunoassays, Cell biology, General cell and organ culture, Pharmacological and toxicological research techniques.[8]

Types of Biochemical Analysis

1.Enzymatic Assay

The enzymes or proteins present in the human body exhibit catalytic activity, the ability to perform tasks resulting in reactions. Hence, it is necessary for enzymes synthesized industrially to exhibit the correct functions.

The enzymatic assay divide into two types according to their sampling method:

- ❖ Continues assay:- Where the assay gives a continuous reading of activity.
  - Multiple measurements, usually of absorbance, change are made during the reaction.
  - These assays are advantageous over fixed time methods because the linearity of the reaction may be more adequately verified.
- ❖ Discontinues assay:- Where sample are taken, the reaction stopped and then concentration of substrate/products determined.
  - The reaction proceed for designated time
  - The reaction is stopped (usually by inactive the enzyme with weak acid)
  - The measurement is made of the amount of reaction that has occurred.[9]

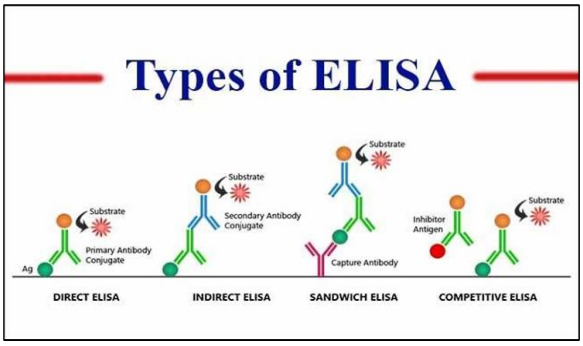
1. Enzyme linked immuno sorbent assay (ELISA)

Principle:

Enzyme-linked Immuno sorbent Assays (ELISAs) combine the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily assayed enzyme. ELISAs can provide a useful measurement of antigen or antibody concentration.

There are 4 main types of ELISA.

1. Direct ELISA: - In a direct ELISA, an antigen or sample is immobilized directly on the plate and a conjugated detection antibody binds to the target protein.
2. Indirect ELISA: - An indirect ELISA is similar to a direct ELISA in that an antigen is immobilized on a plate, but it includes an additional amplification detection step
3. Sandwich ELISA: - In this technique two specific antibodies are used to sandwich the antigen, commonly referred to as matched antibody pairs. Capture antibody is coated on a microplate, sample is added, and the protein of interest binds and is immobilized on the plate.
4. Competitive ELISA: - Competitive ELISAs are commonly used for small molecules, when the protein of interest is too small to efficiently sandwich with two antibodies. Similar to a sandwich ELISA, a capture antibody is coated on a microplate. Instead of using a conjugated detection antibody, a conjugated antigen is used to complete for binding with the antigen present in the sample. The more antigen present in the sample, the less conjugated antigen will bind to the capture antibody. Substrate is added and the signal produced is inversely proportional to the amount of protein present in the sample.



Types of Elisa[11]

Applications of ELISA:

- Presence of antigen or the presence of antibody in a sample can be detected by ELISA.
- ELISA can be used to determination of serum antibody concentrations in a virus test (such as HIV test).
- ELISA can also be used in toxicology as a rapid presumptive screen for certain classes of drugs
- It is used in food industry to detect potential food allergens such as milk, peanuts, walnuts, almond[10]

Protein Estimation

A *protein* is a naturally occurring, extremely complex substance that consists of amino acid residues joined by peptide bonds.

Plasma normally contains about 6.5 to 8.5 g/dL protein, and serum about 4% less.

Methods of protein estimation:

- Biuret Method
- Lowry Method
- Bicinchonic acid Method

1.Biuret Method

Principle: A violet-purplish colour is produced when cupric ion is complexed with peptide bond under alkaline conditions. The absorbance of colour produced is read at 540 nm. The colour intensity is proportional to protein content of sample. The reagent used in the method is the Biuret reagent which contains the sodium hydroxide, copper sulphate and potassium sodium tartrate. Advantages: Very few

substance other than protein in food interfere with Biuret reactions[12]

2. Lowry Method

Principle: This method combines the Biuret reaction with the Folin-Coicalteau phenol reagent (phosphomolibdic - phosphotungstic acid) by tyrosine and tryptophan residues in the protein. The bluish colour developed is read at 750 nm (high sensitivity for low protein concentration) or at 500 nm (low sensitivity for high protein concentration).[13]

3. Bicinchonic acid Method (BCA)

Principle: Protein and peptide reduce cupric ion to cuprous ion under alkaline conditions. The cuprous ion then react with apple-greenish bicinchonic acid to form purple complex. The colour measured at 562 nm is near linearly proportional to protein concentration over a wide range of concentration from micrograms upto 2mg/ml.

Advantages: The reagent is more stable than Lowry reagent.[14]

Nucleic Acid Estimation

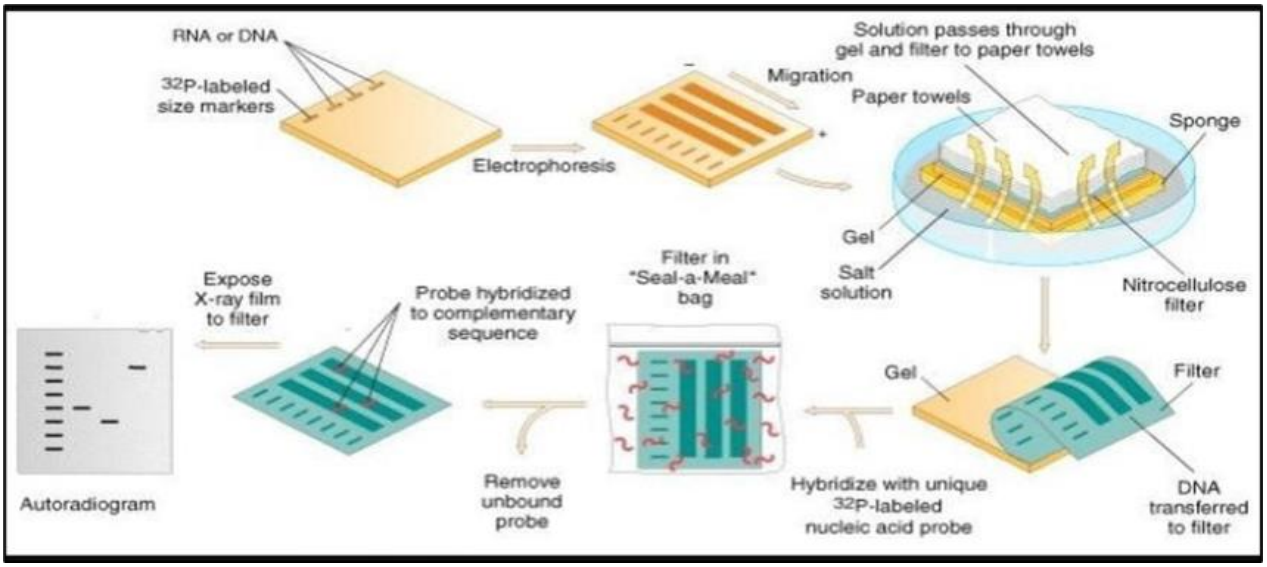
In nucleic acid estimation blotting techniques are very widely used as analytical tools for the specific identification of desire DNA and RNA fragments from thousands of molecule. Blotting refers to process of immobilization of sample nucleic acid on solid support. The blotted nucleic acid then used as target in the hybridization experiments for their specific detection.

Types of blotting:

1.Southern blotting:

Principle:

It is based on the principle of transfer of separated DNA fragments to a carrier membrane (usually nitrocellulose) using gel electrophoresis and subsequent identification of specific DNA fragments by abeled probe hybridization. Hybridization is a technique in which a double stranded DNA molecule is formed in between a single stranded DNA probe and a target single stranded DNA. The probes are labeled with a marker and hence detected. The principle is based on separation DNA fragments by gel electrophoresis followed by the identification by labelled probe hybridization. The DNA fragments are separated based in their size and charge during electrophoresis. Restriction endonuclease, which is an enzyme, is used to break the DNA into small fragments. These fragments are then separated using electrophoresis. The fragments achieved are then classified according to their size (kD). Thus, DNA fragments are transferred to the blotting paper where it is incubated with probes. Probes used in the Southern blotting can be highly selective. They can selectively bind with a resolution of 1 in a million and the characteristics to bind to the intended target fragments.[15]



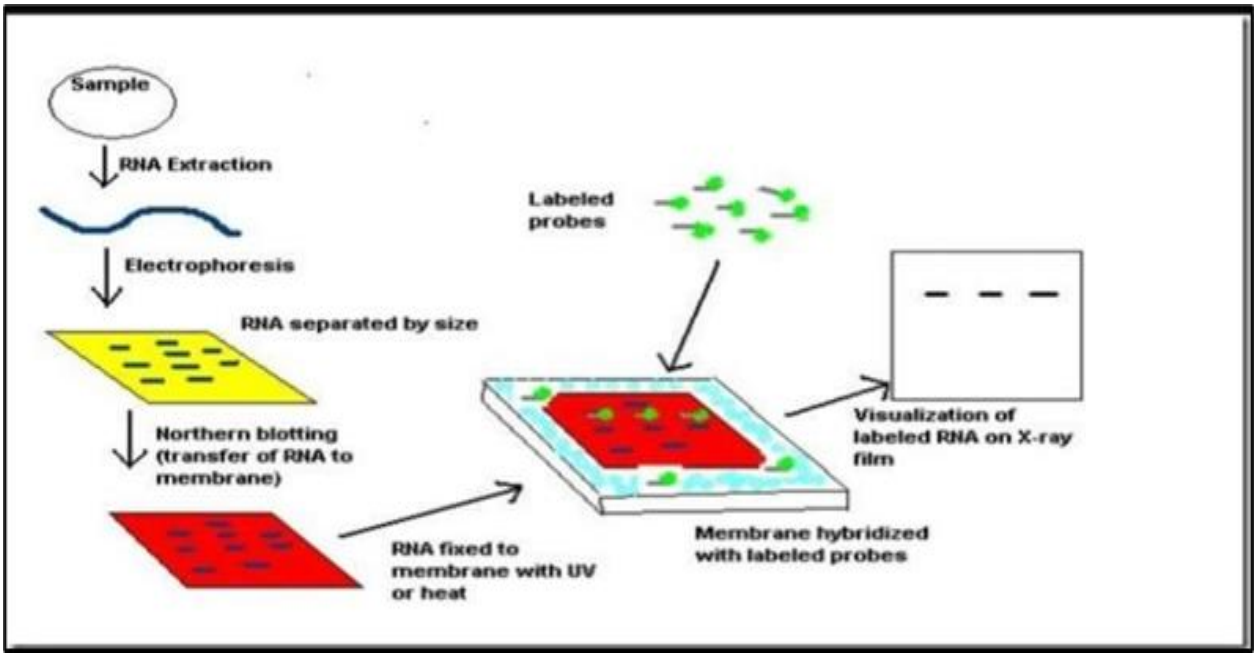
Southern blotting[16]

Applications; It is an invaluable method in gene analysis.

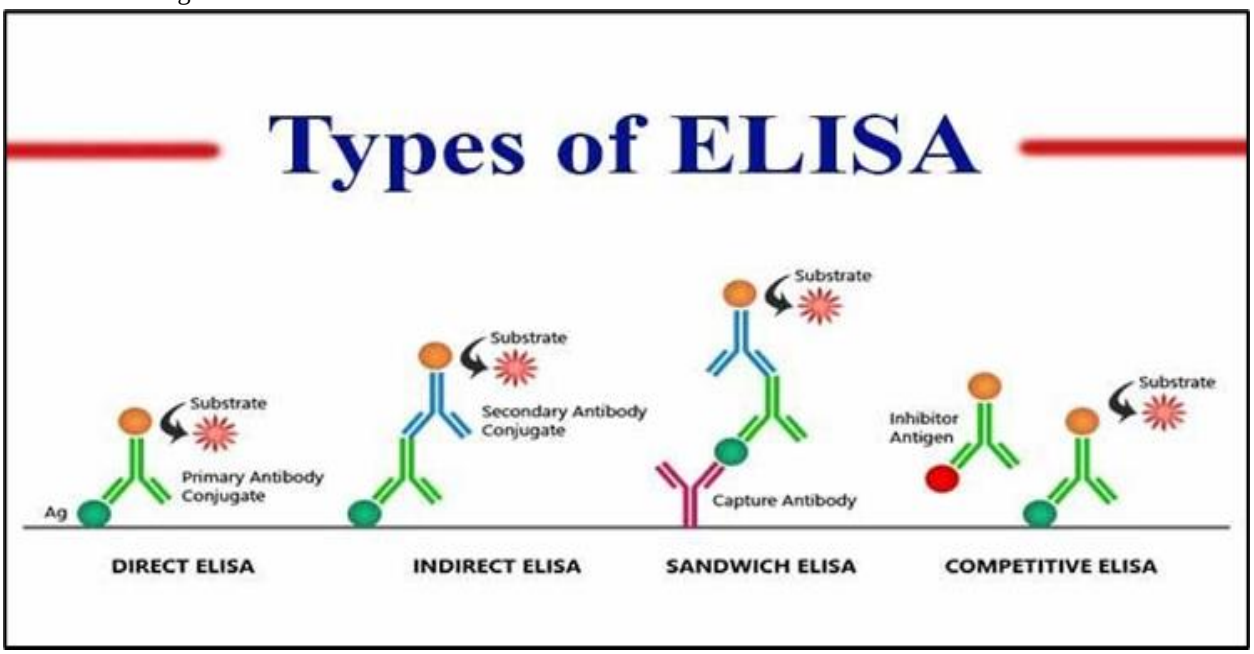
2, Nouthern blotting

Principle

Northern blotting can be used to identify novel splice variants, pre-processed RNAs, and non- coding RNAs, as well as their relative abundances. The underlying principle of Northern blotting is that RNA are separated by size and detected on a membrane using a hybridization probe with a base sequence complementary to all, or a part, of the sequence of the target mRNA. RNA molecules are subjected to electrophoresis, followed by blot transfer, hybridization and autoradiography. RNA molecules do not easily bind to nitrocellulose paper or nylon membranes. Transfer of RNA molecules is carried out by using a chemically reactive paper prepared by diazotization of aminobenzyloxymethyl to create diazobenzyloxymethyl (DBM) paper.[17]



Northern blotting[18]



3.Western Blot Analysis

Principle: -

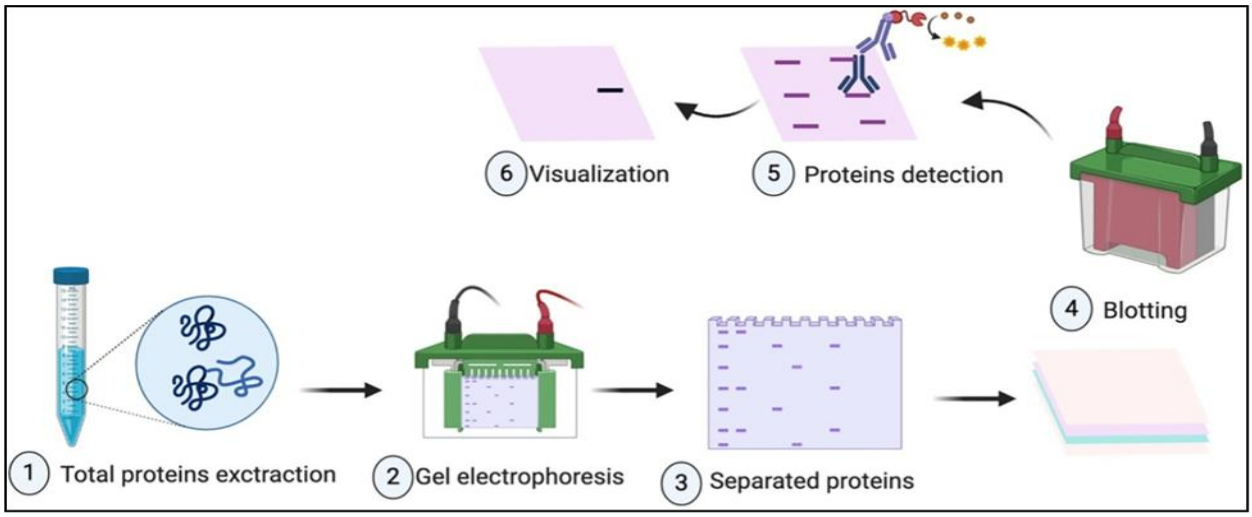
In western blotting (WB), target proteins are transferred to a hydrophobic membrane after SDS-PAGE and detected using specific antibodies. After SDS-PAGE, a membrane is placed on the gel, to which the separated proteins in the gel are electrophoretically transferred.

Types of western blot:

a) Gel blot:- A native gel separates proteins based on their size and charge. The proteins remain in their native state and are not denatured during the process. Since proteins are not reduced or denatured in this assay, native gels are typically used to investigate protein complexes or enzymatic activity.
b) SDS-PAGE gel:- An SDS-PAGE gel separates proteins based on their size. The proteins are denatured during the process by a detergent called sodium dodecyl sulphate (SDS). This type of assay is more commonly selected than native gels due to its simplicity. SDS-PAGE gels can be used when the estimated molecular weight of a denatured protein together with primary antibody affinity is sufficient for protein detection.

Applications of Western Blotting

- Diagnosis of HIV by ELISA involves the western blotting technique.
- Western blotting technique is also used to detect some forms of Lyme Diseases.
- Confirmatory test for Hepatitis-B involves western blotting technique.
- Western blotting test is used in the analysis of Biomarkers such as hormones, growth factors and cytokines.
- This technique is also employed in The Gene expression studies. [19]]



Western blotting[20]

Battery Test (Irwin Test)

The Irwin test is an observational screening paradigm that is comprised of a battery of tests used to assess a mouse or rat's neurobiological and physiological state.

The Irwin Test is use to evaluate the qualitative effects of the NCE (New Chemical Entities) on behaviour and physiological function, from the first dose that has observable effects up to doses that induce clear behavioural toxicity or even death.

- This test also provides an initial estimate of the duration of action of the NCE on the different end-points.
- The test is performed in a highly standardized manner by experienced bend point
- Specific items recorded as part of the Irwin test include death, convulsions, tremor, Straub tail, sedation, excitation, jumping, motor incoordination, altered muscle tone, loss of grasping, akinesia, catalepsy, loss of balance, fore-paw treading, piloerection, stereotypic behaviours, head-twitches, altered respiration, aggression, altered fear, altered reactivity to touch, ptosis, exophthalmia, loss of corneal reflex, analgesia, defecation/diarrhoea, salivation, lacrimation, rectal temperature, and pupil diameter.

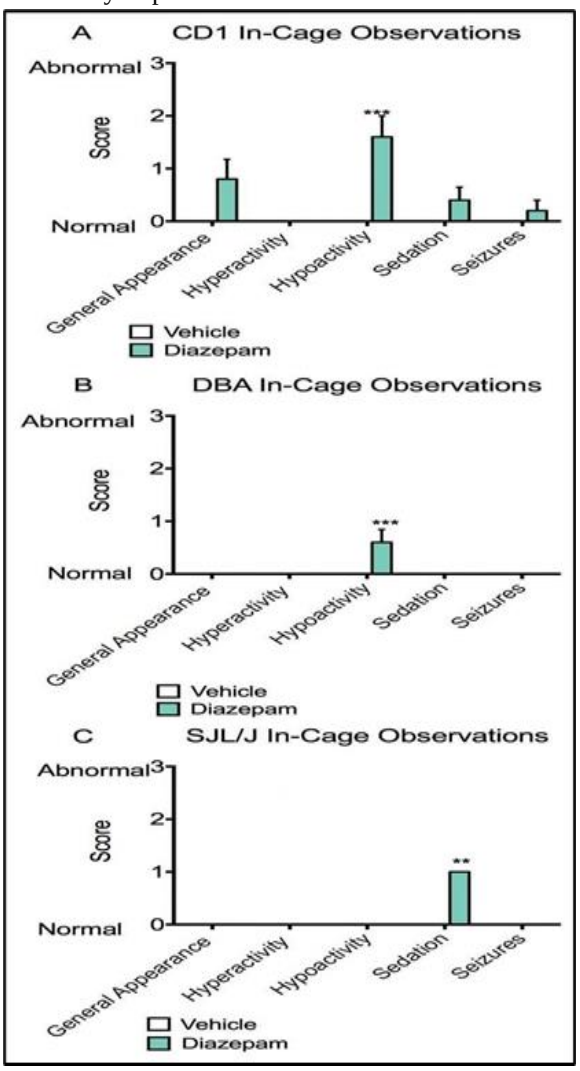
- The test substance is usually evaluated at 6 different doses administered p.o. immediately before the test.
  Species: Rattus Rorvegicus (Sprague Dawley or Wistar Hannover)

  Number of animals/groups: 8 animals
  Route of administration: upon request
  Treatment mode: acute
  Main read-outs: Excitation, sedation, stereotypy, motor, pain and autonomic parameters.

  This test estimates the following parameters:
- Minimum lethal dose of a test substance
- Dose range for CNS responses
- Primary effects on behavior and physiological functions

  Parameters that are evaluated include:
- Autonomic and sensory motor functions
- Convulsive behavior
- Other activities produced by a drug after administration.
  When conducted by an experienced tester the Irwin test can provide insight into a drug's activity including potential molecular targets, therapeutic benefits and deleterious side-effects.

  Applications of Irwin Test:
- To identify subtle neurological perturbations produced by a drug.
- To control for other behavioral and locomotor assays.
- Although a behavioral test of sorts, it is not directed towards a specific indication.
  The Irwin test is principally used by Melior to provide a context for other assays.
  This assay system will also be used during the pharmacokinetic and maximal tolerated dose preliminary studies to establish dose-response ranges for subsequent efficacy studies.
  In the study summarized below, we assessed Diazepam, a centrally acting anxiolytic with some sedative activities in three different strains of mice: male CD1, male DBA1 and female SJL/J mice.
- Mice assessed for the features described below by two observers blinded to the treatment, 30 minutes after vehicle or Diazepam administration. Each parameter was scored on a 0 to 5 scale, with 0 representing the response in a normal animal and 5 representing a maximally impaired animal.[22]



IMPORTANCE OF PRECLINICAL EXPERIMENTS IN DRUG DISCOVERY PROCESS

- The main goals of preclinical studies are to determine a starting, safe dose for first-in-human study and assess potential toxicity of the product, which typically include new medical devices, prescription drugs, and diagnostics
- Preclinical development encompasses the activities that link drug discovery in the laboratory to initiation of human clinical trials.
- Toxicology and safety studies identify potential target organs for adverse effects and define the therapeutic Index to set the initial starting doses in clinical trials.

- Rodent and no rodent mammalian models are used to delineate the pharmacokinetic profile and general safety, as well as to identify toxicity patterns
- One or more species may be used to determine the drug's mean residence time in the body, which depends on inherent absorption, distribution, metabolism, and excretion properties.
- Preclinical studies can be designed to identify a lead candidate from several hits; develop the best procedure for new drug scale-up, select the best formulation, determine the route, frequency, and duration of exposure, and ultimately support the intended clinical trial design.[31]

CONCLUSION

Pre-clinical studies constitute an important segment in the drug development process. A successful pre-clinical trial provides extensive information for perfection of the drug development and subsequent trial in human beings. It is a pre-requisite for investigational new drug application. However, preclinical drug trial has mainly relied on animal species for testing prior to application on humans. This is greatly being challenged and hampered by the animal right activists who are advocating for total ban on the use of animals for research and experimentation. Though efforts are ongoing to find alternatives to animal testing of drugs prior to clinical trial, the use of animals may still go on for some time to come.

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