

PHARMACOLOGY

Evaluation of New Drugs (Pre-clinical and Clinical Toxicity Studies)

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Keywords

toxicity

Discovery of a new drug that is therapeutically useful and goes in to clinical setting is a life time dream for a medicinal chemist, pharmacologist and pharmacist. Currently, new drug discovery process involves combinatorial chemistry, computer aided drug design (CADD), quantitative structure activity relationship (QSAR), absorption, distribution, metabolism, excretion and toxicological studies (ADME-T) using in vitro methodology and bioinformatics. Biotechnological tools and molecular biology techniques are applied, in addition to in-vivo and in-vitro methods of drug screening, for pharmacological evaluation of drugs. At the end of the pharmacological testing and molecular or biotechnological evaluation of a compound one may get the lead molecule i.e. a leading candidate for a successful new drug. However, the real challenge for the scientist starts now when one has to carry out animal toxicity testing (**Pre-clinical/Non-clinical toxicity study**) and the study in human beings (**Clinical study/Trial**). All these studies are to be carried out as per the regulatory requirements. In India they are governed by Drugs Controller General of India (DCGI), New Delhi, Director-General of Indian Council of Medical Research, New Delhi. Drugs and Cosmetics Act of 1988 (Schedule 'Y') narrates the guidelines for carrying out not only the animal studies but also the clinical trials.

Pre-Clinical/Non-Clinical Toxicity Studies

Various the safety tests or toxicity tests carried out in animals before a drug is administered to human beings (clinical trial), can be grouped into two:

1. General Toxicity Study
2. Specialized Toxicity Study.

In General toxicity study, the compound is given to evaluate systemic effects and practically all the organs are examined. Specialized toxicity studies are for particular organ or system toxicity.

General Toxicity Study

Acute Toxicity

In this study a drug is tested to determine LD₅₀, i.e. Lethal dose for 50% of mortality (death) in a group of animals. Various doses of the drug; estimated to cover the range from 0 to 100% mortality are administered to a group of 10 animals. The mortality in each group within a fixed period of time (eg. one day or two days etc.) is determined. The data are plotted as % mortality against the dose of drug and LD₅₀ is determined using standard statistical procedures such as 'probits'. Acute toxicity is carried out in two species-preferably one rodent and another non-rodent. Acute toxicity test is a crude and relatively uninformative test. For example, it does not measure sub-lethal toxicity, long term effects or idiosyncratic reactions.

Single-dose Toxicity Studies: As per Schedule Y, single dose toxicity studies are to be carried out in 2 rodent species (mice and rats) using the same route as intended for humans. In addition, unless the intended route of administration in humans is only intravenous, at least one more route is used in one of the species to ensure systemic absorption of the drug. This route should depend on the nature of the drug. A limit of

2g/kg (or 10 times the normal dose that is intended in humans, whichever is higher) is recommended for oral dosing. Animals are observed for 14 days after the drug administration, and minimum lethal dose (MLD) and maximum tolerated dose (MTD) are established. If possible, the target organ of toxicity should also be determined. Mortality is observed for up to 7 days after par-enteral administration and up to 14 days after oral administration. Symptoms, signs and mode of death are reported, with appropriate macroscopic and microscopic findings where necessary. LD₁₀ and LD₅₀ are reported preferably with 95 percent confidence limits. If LD₅₀ cannot be determined, reasons for the same are stated.

In single Dose Toxicity Study each group should contain at least 5 animals of either sex. At least four graded doses should be given. Animals should be exposed to the test substance in a single bolus or by continuous infusion or several doses within 24 hours. Animals should be observed for 14 days. Signs of intoxication, effect on body weight, gross pathological changes should be reported. It is desirable to include histo-pathology of grossly affected organs, if any.

The dose causing severe toxic manifestations or death has to be defined in the case of cytotoxic anticancer agents, and the post-dosing observation period should be 14 days. Mice should first be used for determination of median toxic dose (MTD). Findings should then be confirmed in rat for establishing linear relationship between toxicity and body surface area. In case of nonlinearity, data of the more sensitive species should be used to determine the Phase I starting dose. Where rodents are known to be poor predictors of human toxicity (e.g., antifolates), or where the cytotoxic drug acts by a novel mechanism of action, MTD is established in non-rodent species.

Sub acute Toxicity

In this test, three doses of drug are administered for a period of one month to 6 months in two species of animals and the effect on various organs are studied. One should carry out clinical chemistry, physiological signs, autopsy studies, hematology, histology and electron microscopy studies particularly of the target organs of drug producing toxicity. Sub-acute toxicity is now a pre-requisite for advancing a drug for clinical trial. List of parameters to be included in such studies are given (Table 1).

Table 1: Laboratory parameters in sub-acute and chronic toxicity studies

1. Haematological Parameters:	2. Urinalysis:
(a) Haemoglobin	(a) Colour
(b) Total RBC Count	(b) Appearance
(c) Total WBC Count	(c) Specific Gravity
(d) Haematocrit	(d) 24-hour urinary output
(e) Differential WBC Count	(e) Reaction (pH)
(f) Platelet Count	(f) Albumin
(g) Terminal Bone Marrow Examination	(g) Sugar
(h) ESR (Non-rodents only)	(h) Acetone
(i) General Blood Picture: A special	(i) Bile pigments

mention of abnormal and immature cells should be made (j) Coagulation Parameters (Non-rodents only): Bleeding Time, Coagulation Time, Prothrombin Time, Activated Partial Thromboplastin Time	(j) Urobilinogen (k) Occult Blood (l) Microscopic examination of urinary sediment
3. Blood BiochemicalParameters: (a) Glucose (b) Cholesterol (c) Triglycerides (d) HDL Cholesterol (Non-rodents only) (e) LDL Cholesterol (Non-rodents only) (f) Bilirubin (g) SGPT (ALT) (h) SGOT (AST) (i) Alkaline Phosphatase (ALP) (j) GTT (Non-rodents only) (k) Blood Urea Nitrogen (l) Creatinine (m) Total Protein (n) Albumin (o) Globulin (Calculated values) (p) Sodium (q) Potassium (r) Phosphorus (s) Calcium	4. Gross and Microscopic Pathology (a) Brain*: Cerebrum, cerebellum, Midbrain (b) (Spinal Cord) (c) Eye (d) (Middle Ear) (e) Thyroid (f) (Parathyroid) (g) Spleen* (h) Thymus (i) Adrenal* (j) (Pancreas) (k) (Trachea) (l) Lung* (m) Heart* (n) Aorta (o) Oesophagus (p) Stomach (q) Duodenum (r) Jejunum (s) Terminal ileum (t) Colon (u) (Rectum) (v) Liver* (w) Kidney* (x) Urinary bladder (y) Epididymis (z) Testis* (aa) Ovary (bb) Uterus* (cc) Skin (dd) Mammary gland (ee) Mesenteric lymph node (ff) Skeletal muscle

****Organs marked with an asterisk should be weighed.***

() Organs listed in parenthesis should be examined if indicated by the nature of the drug or observed effects

Chronic Toxicity

These studies are required when the drug is intended to be used in humans for prolonged periods. It is usually carried out concurrently with clinical trials. The study is carried out in different animal species and the drug is administered for a period of 1-2 years. The goal is to find out if any organs are susceptible to drug toxicity. In this study clinical chemistry, physiological signs, autopsy studies, hematology, histology etc. are also carried out in animals as mentioned above.

As per Schedule Y, sub-acute and chronic toxicity studies are the *Repeated-dose Systemic Toxicity Studies* carried out in at least two mammalian species, of which has to be a non-rodent. Dose ranging studies should precede the 14-, 28-, 90- or 180- day toxicity studies. Duration of the final systemic toxicity study will depend on the duration, therapeutic indication and scale of the proposed clinical trial (Table 2). If a species is known to metabolize the drug in the same way as humans, it should be preferred for toxicity studies.

Table 2: Animal toxicity requirements for clinical trials and marketing of a new drug

Systemic Toxicity Studies			
Route of administration	Duration of proposed human administration	Human Phase(s) for which study is proposed to be conducted	Long term toxicity requirements
Oral or Parenteral or Transdermal	Single dose or several doses in one day, Upto 1wk	I,II,III	2sp,2wk
	> 1 wk but upto 2wk	I,II,III	2sp;4wk
	> 2 wk but upto 4wk	I,II,III	2sp;12wk
	Over 1mo	I,II,III	2sp;24wk
Inhalation (general anaesthetics, aerosols)	Upto 2 wk	I,II,III	2sp;1mo; (Exposure time 3h/d, 5d/wk)
	Upto 4wk	I,II,III	2sp;12wk, (Exposure time 6h/d, 5d/wk)
	> 1 4wk	I,II,III	2sp;24wk, (Exposure time 6h/d, 5d/wk)
Local Toxicity Studies			
Dermal	Upto 2 wk	I,II	1sp;4wk
		III	2sp;4wk
	> 2 wk	I,II,III	2sp;12wk
Ocular or Otic or Nasal	Upto 2 wk	I,II	1sp;4wk
		III	2sp;4wk
	> 2 wk	I,II,III	2sp;12wk
Vaginal or Rectal	Upto 2 wk	I,II	1sp;4wk
		III	2sp;4wk
	> 2 wk	I,II,III	2sp;12wk

In repeated-dose toxicity studies, four groups of each gender (total eight groups are required). One of the 4 groups has to be control receiving either a vehical or distilled water. The other three groups have to be low dose (human equivalent dose), high dose (the dose of the drug that produces observable toxicity but no mortality). The third dose has to be intermediate of the low and high dose. Many a time the dose 10 or 20 time may be considered as intermediate or high dose if permissible by the DCGI. The drug is administered 7 days a week by the route intended for clinical use. To make allowance for the sensitivity of the species the intermediate dose should cause some symptoms, but not gross toxicity or death, and should be placed logarithmically between the other two doses. The number of animals required for these studies, are given in the following Table 3.

Table 3: Number of animals required for repeated-dose toxicity studies

<i>Duration of treatment</i>	<i>14-28 days Study</i>				<i>84-182 days Study</i>			
Group	Rodent (Rat)		Non-rodent (Dog or Monkey)		Rodent (Rat)		Non-rodent (Dog or Monkey)	
	<i>M</i>	<i>F</i>	<i>M</i>	<i>F</i>	<i>M</i>	<i>F</i>	<i>M</i>	<i>F</i>
Control	6-10	6-10	2-3	2-3	15-30	15-30	4-6	4-6
Low dose	6-10	6-10	2-3	2-3	15-30	15-30	4-6	4-6
Intermediate dose	6-10	6-10	2-3	2-3	15-30	15-30	4-6	4-6
High dose	6-10	6-10	2-3	2-3	15-30	15-30	4-6	4-6

The parameters to be monitored and recorded in long-term toxicity studies include behavioral, physiological, biochemical and microscopic observations. In case of parenteral drug administration, the sites of injection are also to be examined grossly as well as microscopically. Initial and final electrocardiogram and fundus examination may also be carried out in the non-rodent species.

Various repeat Dose Toxicity studies (As per schedule Y)

(i) 14-28 Day repeated-dose toxicity studies: One rodent (6-10/sex/group) and one non-rodent (2-3/sex/group) species are needed. Daily dosing by proposed clinical route at three dose levels should be done with highest dose having observable toxicity, mid-dose between high and low dose, and low dose. The doses should preferably be multiples of the effective dose and free from toxicity. Observation parameters should include cage-side observations, body weight changes, food/water intake, blood biochemistry, haematology, and gross and microscopic studies of all viscera and tissues.

(ii) 90-Day repeated-dose toxicity studies: One rodent (15-30/sex/group) and one non-rodent (4-6/sex/group) species are needed. Daily dosing by proposed clinical route at

three graded dose levels should be done. In addition to the control a “high-dose-reversal” group and its control group should be also included. Parameters should include signs of intoxication (general appearance, activity and behavior etc), body weight, food intake, blood biochemical parameters, hematological values, urine analysis, organ weights, gross and microscopic study of viscera and tissues. Half the animals in “reversal” groups (treated and control) should be sacrificed after 14 days of stopping the treatment. The remaining animals should be sacrificed after 28 days of stopping the treatment or after the recovery of signs and/or clinical pathological changes – whichever comes later, and evaluated for the parameters used for the main study.

(iii) 180-Day repeated-dose toxicity studies: One rodent (15-30/sex/group) and one non-rodent (4-6/sex/group) species are needed. At least 4 groups, including control, should be taken. Daily dosing by proposed clinical route at three graded dose levels should be done. Parameters should include signs of intoxication, body weight, food intake, blood biochemistry, hematology, urine analysis, organ weights, gross and microscopic examination of organs and tissues.

In the case of cytotoxic anticancer agents dosing and study design should be in accordance with the proposed clinical schedule in terms of days of exposure and number of cycles. Two rodent species may be tested for initiating Phase I trials. A non-rodent species should be added if the drug has a novel mechanism of action, or if permission for Phase II, III or marketing is being sought.

(iv) Dose-ranging Study: Some times it is essential to carry out the dose ranging study in the animals. The objective of this study includes the identification of target organ of toxicity and establishment of MTD for subsequent studies. It is carried out in rodents as well as non-rodents.

Rodents: Study is performed in one rodent species (preferably rat) by the proposed clinical route of administration. At least four graded doses including control are given, and each dose group as well as the vehicle control should consist of a minimum of 5 animals of each sex. Animals are to be exposed to the test substance daily for 10 consecutive days. Highest dose is the maximum tolerated dose of single-dose study. Animals are observed daily for signs of intoxication (general appearance, activity and behaviour etc), and periodically for the body weight and laboratory parameters. Gross examination of viscera and microscopic examination of affected organs should be done.

Non-rodents: One male and one female are to be taken for ascending Phase MTD study. Dosing should start after initial recording of cage-side and laboratory parameters. Starting dose may be 3 to 5 times the extrapolated effective dose or MTD (whichever is less), and dose escalation in suitable steps should be done every third day after drawing the samples for laboratory parameters. Dose should be lowered appropriately when clinical or laboratory evidence of toxicity is observed. Administration of test substance should then continue for 10 days at the well-tolerated dose level following which, samples for laboratory parameters should be taken. Sacrifice, autopsy and microscopic examination of affected tissues should be performed as in the case of rodents.

Specialized Toxicity Study

These include fertility study, carcinogenicity, genotoxicity and teratogenicity etc.

Reproductive Toxicity

This includes male fertility, female fertility and developmental toxicity studies.

(i) Male fertility study: It is carried in one rodent species (preferably rat). Dose selection is done from the results of the 14 or 28-day toxicity study in rat. Three dose groups, the highest one showing minimal toxicity in systemic studies, and a control group are taken. Each group consists of 6 adult male animals. Animals should be treated with the test substance by the intended route of clinical use for minimum 28 days and maximum 70 days before they are paired with female animals of proven fertility in a ratio of 1:2 for mating.

Drug treatment of the male animals should continue during pairing. Pairing should be continued till the detection of vaginal plug or 10 days, whichever is earlier. Females getting thus pregnant are to be examined for their fertility index after day 13 of gestation. All the male animals should be sacrificed at the end of the study. Weights of each testis and epididymis are separately recorded. Sperms from one epididymis are examined for their motility and morphology. The other epididymis and both testes are examined for their histology.

(ii) Female Reproduction and Developmental Toxicity Studies: These studies are to be carried out for all drugs proposed to be used in women of child bearing age. Female fertility study is to be performed in albino mice or rats (segment I) and developmental toxicity study is performed albino rabbits.

On the occasion, when the test compound is not compatible with the rabbit (e.g. antibiotics which are effective against gram positive, anaerobic organisms and protozoas) the Segment II data in the mouse may be substituted.

(a) Female Fertility Study (Segment I): The study is done in one rodent species, preferably rat. The drug should be administered to both males and females, beginning a sufficient number of days (28 days in males and 14 days in females) before mating. Drug treatment should continue during mating and, subsequently, during the gestation period. Three graded doses are used. The highest dose (usually the MTD obtained from previous systemic toxicity studies) should not affect general health of the parent animals. At least 15 males and 15 females should be used per dose group. Control and the treated groups should be of similar size. The route of administration should be the same as intended for therapeutic use.

The animals are allowed to litter and their medication is continued till the weaning of pups. Observations on body weight, food intake, clinical signs of intoxication, mating behaviour, progress of gestation/ parturition periods, length of gestation, parturition, post-partum health and gross pathology (and histopathology of affected organs) of animals are recorded. The pups from both treated and control groups are observed for general signs

of intoxication, sex-wise distribution in different treatment groups, body weight, growth parameters, survival, gross examination, and autopsy. Histopathology of affected organs should be done.

(b) Developmental toxicity (Teratogenicity Study): This may be performed in one rodent (preferably rat) and one non rodent (rabbit). The drug should be administered throughout the period of organogenesis, using three dose levels as described above. The highest dose should cause minimum maternal toxicity and the lowest one should be proportional to the proposed dose for clinical use in humans or a multiple of it. The route of administration should be the same as intended for human therapeutic use.

The control and the treated groups should consist of at least 20 pregnant rats (or mice) and 12 rabbits, on each dose level. All foetuses should to be subjected to gross examination of the foetuses should be examined for skeletal abnormalities and the other half for visceral abnormalities. Observation parameters should include: signs of intoxication, effect on body weight, effect on food intake, examination of uterus, ovaries and uterine contents, number of corpora lutea, implantation sites, resorptions (if any); and for the foetuses, the total number, gender, body length, weight and gross/ visceral/ skeletal abnormalities.

(c) Developmental Toxicity Study (Perinatal Study): This study is specially recommended if the drug is to be given to pregnant or nursing mothers for long periods or where there are indications of possible adverse effects on foetal development. One rodent species (preferably rat) is needed. Dosing at levels comparable to multiples of human dose should be done by the intended clinical route. At least 4 groups (including control), each consisting of 15 pregnant females are used. The drug is administered throughout the last trimester of pregnancy (from day 15 of gestation) and then the dose that causes low foetal loss is continued throughout lactation and weaning. The animals are then sacrificed and examined for various organs.

One male and one female from each litter of F₁ generation (total 15 males and 15 females in each group) are selected at weaning and treated with vehicle or test compound (at the dose levels described above) throughout their periods of growth to sexual maturity, pairing, gestation, parturition and lactation. Mating performance and fertility of F₁ generation should thus be evaluated to obtain the F₂ generation whose growth parameters should be monitored till weaning. The criteria of evaluation should be the same as described earlier.

Animals are sacrificed at the end of the study and the observation parameters should include body weight, food intake, general signs of intoxication, progress of gestation/ parturition periods and gross pathology of the animal. For pups, the clinical signs, sex-wise distribution in dose groups, body weight, growth parameters, gross examination, survival and autopsy (if needed) and where necessary, histopathology are required to be done.

Local Toxicity

These studies are required when the new drug is proposed to be used by some special route (other than oral) in humans. The drug is applied to an appropriate site like skin or vaginal mucous membrane etc. to determine local effects in a suitable species. Typical study designs for these studies also include three dose levels and untreated and/ or vehicle control. Usually 2 species are to be used and the group size should be increased with increase in duration of treatment. If the drug is absorbed from the site of application, appropriate systemic toxicity studies should also be carried out.

(i) Dermal toxicity study: The study is done in rabbits and rats. Daily topical (dermal) application of the test compound in its clinical dosage form should be done. Test material should be applied on shaved skin covering not less than 10% of the total body surface area. Porous gauze dressing should be used to hold liquid material in place. Formulations with different concentrations (at least 3) of the test compound that are several fold higher than the clinical dosage form are used. Period of application may vary from 7 to 90 days depending on the intended duration of use in humans. If skin irritation is grossly visible in the initial studies, a recovery group should be included in the subsequent repeated-dose study. Local signs (erythema, oedema and eschar formation) as well as histological examination of sites of application are carried out to evaluate the results.

(ii) Photo-allergy or dermal photo-toxicity: This test is done in guinea pigs if the drug or a metabolite is related to an agent causing photosensitivity or the nature of action suggests such a potential (e.g., drugs is to be used in treatment of leucoderma). Pretest in 8 animals may be done to evaluate 4 concentrations. The patch is applied for 2 hours $\pm$ 15 min. with and without UV exposure (10 J/cm^2). After 24 and 48 hours, the observation is done to ascertain the highest nonirritant dose. Main test is performed with 10 test animals (given the highest non-irritant dose) and 5 controls (given the solvent /diluent used in the formulation). Induction with the dose selected from pretest is 0.3 ml/patch for 2 hour $\pm$ 15 min. followed by 10 J/cm^2 of UV exposure. This is repeated on day 0, 2, 4, 7, 9 and 11 of the test. Animals are then challenged with the same concentration of test substance between day 20 to 24 of the test with a similar 2-hour application followed by exposure to 10 J/cm^2 of UV light. Examination and grading of erythema and oedema formation at the sites of application of the test compound is done 24 and 48 hours after the challenge. A positive control like musk ambrett or psoralin can also be used.

(iii) Vaginal Toxicity Test: This test is to be done in rabbit or dog. Test substance is applied topically at vaginal mucosa in the form of pessary, cream or ointment. Six to ten animals per dose group are taken. Higher concentrations or several daily applications of test substance is done to achieve multiples of daily human dose. The minimum duration of drug treatment is 7 days and to a maximum of 30 days. Observation parameters should include swelling, closure of introitus and histopathology of vaginal wall.

(iv) Rectal Tolerance Test: Preparations meant for rectal administration are tested in rabbits or dogs. Six to ten animals per dose group are taken. Formulation in volume comparable to human dose (or the maximum possible volume) is applied once or several times daily, per rectally, to achieve administration of multiples of daily human dose. The minimum duration of application is 7 days and to a maximum of 30 days. Size of suppositories may be smaller, but the drug content in the formulation should be several fold higher than the proposed human dose. Observation parameters should include clinical signs (sliding on backside), signs of pain, blood and/or mucus in faeces, condition of anal region/sphincter, gross and (if required) histological examination of rectal mucosa.

(v) Parenteral Drugs: For products meant for intravenous or intramuscular or subcutaneous or intradermal injection the sites of injection in systemic toxicity studies should be specially examined grossly and microscopically. If needed, reversibility of adverse effects is also determined.

(vi) Ocular toxicity studies (for products meant for ocular instillation): These studies should be carried out in two species, one of which should be the albino rabbit which has a sufficiently large conjunctival sac. Direct delivery of drug onto the cornea in case of animals having small conjunctival sacs should be ensured. Liquids, ointments, gels or soft contact lenses (saturated with drug) should be used. Initial single dose application should be done to decide the exposure concentrations for repeated-dose studies and if needed one should include a recovery group. Duration of the final study depends on the proposed length of human exposure Subject to a maximum of 90 days. At least two different concentrations exceeding the human dose should be used for demonstrating the margin of safety. In acute studies, one eye is used as control administration and the other kept as control. A separate control group should be included in repeated-dose studies.

Slit-lamp examination should be done to detect the changes in cornea, iris and aqueous humor. Fluorescent dyes (sodium fluorescein, 0.25 to 1.0%) should be used to detect the defects in surface epithelium of cornea and conjunctiva. Changes in intra-ocular tension should be monitored by a tonometer. Histological examination of eyes is done at the end of the study after fixation in Davidson's or Zenker's fluid.

(vii) Inhalation toxicity studies: The studies are to be undertaken in one rodent and one non-rodent species using the formulation. Acute, subacute and chronic toxicity studies should be performed according to the intended duration of human exposure. Standard systemic toxicity study designs as described above are normally be used. Gases and vapors should be given in whole body exposure chambers; aerosols are to be given by nose-only method. Exposure time and concentrations of test substance (limit dose of 5mg/l) is adjusted to ensure exposure at levels comparable to multiples of intended human exposure. Three dose groups and a control (plus vehicle control, if needed) are required. Duration of exposure may vary Subject to a maximum of 6 hours per day and five days a week. Food and water are withdrawn during the period of exposure to test substance.

Temperature, humidity and flow rate of exposure chamber are recorded and reported. Evidence of exposure with test substance of particle size of 4 micron (especially for aerosols) with not less than 25% being 1 micron is provided. Effects on respiratory rate, findings of bronchial lavage fluid examination, histological examination of respiratory passages and lung tissue are included along with the regular parameters of systemic toxicity studies or assessment of margin of safety.

Allergenicity/ Hypersensitivity

Standard tests include guinea pig maximization test (GPMT) and local lymph node assay (LLNA) in mouse.

Guinea Pig Maximization Test: The test is performed in two steps; first, determination of maximum nonirritant and minimum irritant doses, and second, the main test. The initial study also has two components. First, to determine the intradermal induction dose, 4 dose levels are tested by the same route in a batch of 4 male and 4 female animals (2 of each sex should be given Freund's adjuvant). The minimum irritant dose is then used for induction. Similarly, a topical minimum irritant dose is determined for challenge. This is established in 2 males and 2 females. A minimum of 6 male and 6 female animals per group should be used in the main study. There has to be one test and one control group. It is preferable to have one more positive control group. Intradermal induction (day 1) coupled with topical challenge (day 21) should be done. If there is no response, re-challenge is done 7-30 days after the primary challenge. Erythema and oedema (individual animal scores as well as maximization grading) should be used as evaluation criteria.

Local Lymph Node Assay: Mice used in this test should be of the same sex, either only males or only females. Drug treatment is given on ear skin. Three graded doses, the highest being maximum nonirritant dose plus vehicle control are used. A minimum of 6 mice per group are used. Test material is applied on ear skin on three consecutive days and on day 5, the draining auricular lymph nodes should be dissected out 5 hours after i.v. ^3H -thymidine or bromo-deoxy-uridine (BrdU). Increase in ^3H -thymidine or BrdU incorporation should be used as the criterion for evaluation of results.

Genotoxicity

Genotoxic compounds, in the absence of other data, are presumed to be trans-species carcinogens, implying a hazard to humans. Such compounds need not be subjected to long-term carcinogenicity studies. However, if such a drug is intended to be administered for chronic illnesses or otherwise over a long period of time - a chronic toxicity study (up to one year) may be necessary to detect early tumorigenic effects. Genotoxicity tests are *in vitro* and *in vivo* tests conducted to detect compounds which induce genetic damage directly or indirectly. These tests should enable hazard identification with respect to damage to DNA and its fixation.

The various standard tests that are generally conducted include test for gene mutation bacteria, *in vitro* tests and *in vivo* tests. Other genotoxicity tests e.g. tests for

measurement of DNA adducts, DNA strand breaks, DNA repair or recombination serve as options in addition to the standard battery for further investigation of genotoxicity test results obtained in the standard battery. Only under extreme conditions in which one or more tests comprising the standard battery cannot be employed for technical reasons, alternative validated tests can serve as substitutes provided sufficient scientific justification should be provided to support the argument that a given standard battery test is not appropriate.

In-vitro tests: In-vitro studies include Ames' Salmonella assay and chromosomal aberrations (CA) in cultured cells. In-vivo studies should include micronucleus assay (MNA) or CA in rodent bone marrow. Data analysis of CA should include analysis of 'gaps.'

Ames' Test (Reverse mutation assay in Salmonella): *S. typhimurium* tester strains such as TA98, TA100, TA102, TA1535, TA97 or *Escherichia coli* WP2 *uvrA* or *Escherichia coli* WP2 *uvrA* (pKM101) are used. In-vitro exposure (with and without metabolic activation, S9 mix) is done at a minimum of 5 log dose levels. "Solvent" and "positive" control should be used. Positive control may include 9-amino-acridine, 2-nitrofluorine, sodium azide and mitomycin C, respectively, in the tester strains mentioned above. Each set should consist of at least three replicates. A 2.5 fold (or more) increase in number of revertants in comparison to spontaneous revertants would be considered positive.

In-vitro cytogenetic assay : The desired level of toxicity for *in vitro* cytogenetic tests using cell lines should be greater than 50% reduction in cell number or culture confluency. For lymphocyte cultures, an inhibition of mitotic index by greater than 50% is considered sufficient. It is performed in CHO cells or on human lymphocyte in culture. In-vitro exposure (with and without metabolic activation, S9 mix) is done using a minimum of 3 log doses. "Solvent" and "positive" control should be included. A positive control like Cyclophosphamide with metabolic activation and Mitomycin C for without metabolic activation is used to give a reproducible and detectable increase clastogenic effect over the background which demonstrates the sensitivity of the test system. Each set consists of at least three replicates. Increased number of aberrations in metaPhase chromosomes should be used as the criteria for evaluation.

In-vivo micronucleus assay: This is done on one rodent species (preferably mouse). Route of administration of test substance should be the same as intended for humans. Five animals per sex per dose groups are used. At least three dose levels, plus "solvent" and "positive" control are tested. A positive control like mitomycin C or cyclophosphamide may also be used. Dosing is done on day 1 and 2 of study followed by sacrifice of animals 6 hours after the last injection. Bone marrow from both the femora should be taken out, flushed with fetal bovine serum (20 min.), pelleted and smeared on glass slides. Giemsa-MayGruenwald staining is done and increased number of micronuclei in polychromatic erythrocytes (minimum 1000) should be used as the evaluation criteria.

In-vivo cytogenetic assay: In this method one rodent species (preferably rat) is used. Route of administration of test substance should be the same as intended for humans. Five animals/sex/dose groups should be used. At least three dose levels, plus “solvent” and “positive” control are tested. Positive control may include cyclophosphamide. Dosing is done on day 1 followed by intra-peritoneal colchicine administration at 22 hours. Animals should be sacrificed 2 hours after colchicine administration. Bone marrow from both the femora should be taken out, flushed with hypotonic saline (20 min.), pelleted and resuspended in Carnoy’s fluid. Once again the cells are pelleted and dropped on clean glass slides with a Pasteur pipette. Giemsa staining should be done and increased number of aberrations in metaPhase chromosomes (minimum 100) should be used as the evaluation criteria.

Carcinogenicity

Carcinogenicity studies are performed for

- a. All drugs that are expected to be clinically used for more than 6 months as well as for drugs used frequently in an intermittent manner in the treatment of chronic or recurrent conditions.
- b. For the drugs if there is concern about their carcinogenic potential emanating from previous demonstration of carcinogenic potential in the product class that is considered relevant to humans.
- c. If structure-activity relationship suggests carcinogenic risk.
- d. If there is evidence of preneoplastic lesions in repeated dose toxicity studies.
- e. If long-term tissue retention of parent compound or metabolite(s) results in local tissue reactions or other pathophysiological responses.

For pharmaceuticals developed to treat certain serious diseases, Licensing Authority may allow carcinogenicity testing to be conducted after marketing permission has been granted.

In instances where the life-expectancy in the indicated population is short (i.e., less than 2 - 3 years) - no long-term carcinogenicity studies may be required. In cases where the therapeutic agent for cancer is generally successful and life is significantly prolonged there may be later concerns regarding secondary cancers. When such drugs are intended for adjuvant therapy in tumour free patients or for prolonged use in non-cancer indications, carcinogenicity studies may be / are needed. Completed rodent carcinogenicity studies are not needed in advance of the conduct of large scale clinical trials, unless there is special concern for the patient population.

Carcinogenicity studies should be done in a rodent species (preferably rat). Mouse may be employed only with proper scientific justification. The selected strain of animals should not have a very high or very low incidence of spontaneous tumors.

At least three dose levels are used. The highest dose should be sub-lethal, and it should not reduce the life span of animals by more than 10% of expected normal. The lowest dose should be comparable to the intended human therapeutic dose or a multiple of it, e.g. 2.5x; to make allowance for the sensitivity of the species. The intermediate dose is to be placed logarithmically between the other two doses. An untreated control and (if indicated) a vehicle control group should be included. The drug is administered 7 days a week for a fraction of the life span comparable to the fraction of human life span over which the drug is likely to be used therapeutically. Generally, the period of dosing should be 24 months for rats and 18 months for mice.

Observations should include macroscopic changes observed at autopsy and detailed histopathology of organs and tissues. Additional tests for carcinogenicity (short-term bioassays, neonatal mouse assay or tests employing transgenic animals) may also be done depending on their applicability on a case to case basis.

Each dose group and concurrent control group not intended to be sacrificed early should contain at least 50 animals of each sex. A high dose group for evaluation of pathology other than neoplasia contains 20 animals of each sex while the control group contains 10 animals of each sex. Observation parameters include signs of intoxication, effect on body weight, food intake, clinical chemistry parameters, hematology parameters, urine analysis, organ weights, gross pathology and detailed histopathology. Comprehensive descriptions of benign and malignant tumour development, time of their detection, site, dimensions, histological typing etc. should be given.

Clinical Evaluation of Drugs

Clinical trials is a name given to a disciplined organized research, conducted in human beings and is intended to shed light on the drug as a therapeutic agent, for its efficacy and safety. Science concerned with scientific studies of drugs in man is called **Clinical Pharmacology** and the essential feature of this branch is Clinical trials of drugs. Perhaps the first ever clinical trial was James Lind's demonstration that citrus juice cures scurvy. He compared the effects of various different acidic substances, ranging from vinegar to cider, on groups of afflicted sailors, and found that the group who were given oranges and lemons had largely recovered from scurvy after 6 days.

A new drug with a completely new structure and ideal pharmacological action is rarely born. Many new drugs are being synthesized and studied for their therapeutic efficacy. From extensive studies on animals, they may be called as therapeutically effective but unless they are studied in human beings, they cannot be marketed. Most of the time, new drugs are similar to the known drugs. In all cases prior to clinical trials, the investigator must obtain reasonable answer for the following questions:

(a) Are the data from animal studies adequate?

(b) Is there any need for a new drug for a particular disease under consideration and if so, is the new remedy promising ?

(c) Which are the probable risks involved in giving drugs to humans?

(d) What is its therapeutic index in animals?

Thus, before one can think about clinical trials, one should have thoroughly investigated the actions of the new drug in animals and their acute, sub-acute and chronic toxicity in various species of animals. Although animal studies will tell us much about the efficacy and toxicity of the drug, there are a number of limitations as well. Many common unwanted effects such as malaise, allergy neurological disorders, blood-dyscrasias etc. cannot be predicted from the animal studies. Metabolism of drugs may differ qualitatively and quantitatively in different species. Also the subjective responses like nausea, vomiting, headache, weakness, loss of libido etc. are difficult to be discovered in animals. Thus, no matter how much we know of structure-activity relationship, or how much we have studied in animals, ultimate evaluation of safety, effectiveness and therapeutic efficacy has to be determined in human beings, since drugs are to be finally used on the human.

The most commonly performed clinical trials evaluate new drugs, medical devices, biologics or other interventions on patients in strictly scientifically controlled settings, and are required for regulatory authority approval of new therapies. Trials may be designed to assess the safety and efficacy of an experimental therapy, to assess whether the new intervention is better than standard therapy, or to compare the efficacy of two standard or marketed interventions. The trial objectives and design are usually documented in a clinical trial protocol.

To be ethical, they must evolve the **Informed Consent Form** of participating human subjects. They are closely supervised by appropriate regulatory authorities. All interventional studies must be approved by an Ethical **Committee** or the **Institutional Review Board** before permission is granted to run the trial.

Informed Consent Form is a Legal condition whereby a person can be said to have given consent based upon an appreciation and understanding of the facts and implications of an action. The individual needs to be in possession of relevant facts and also of his reasoning faculties, such as not being mentally retarded or ill and without an impairment of judgment at the time of consenting. Such impairments might include illness, intoxication, insufficient sleep, and other health problems.

The **Ethics Committee** or the **Institutional Review Board**, is an independent body in an institute carrying out the clinical trial consisting of healthcare professionals and non-medical members, whose responsibility it is to protect the rights, safety and well being of human subjects involved in the trial and to provide public assurance of that protection, by, among other things, expressing an opinion on the clinical trial protocol, the suitability of the investigators involved in the trial and the adequacy of facilities, and on the methods and documents to be used to inform trial subjects and obtain their informed consent.

Clinical trial of a drug can be divided into four phases :

- Phase I : First in the human study,
- Phase II : First in the patient study,
- Phase III : Statistical evaluation,
- Phase IV : Field trials and comparative trials.

Phase I : First in the Human study

Phase I trials are the first-stage of testing in human subjects. Normally a small (20-48) group of healthy volunteers will be selected. The aim of this phase is to obtain the precise information for the following, from the smallest possible number of the patients, in a minimum possible time :

- (a) Initial efficacy,
- (b) Initial safety and maximum tolerance
- (c) Pharmacokinetics profile of the drug.

The trial is carried out usually in healthy human volunteers or patient volunteers. Phase I trials normally include dose-ranging studies so that doses for clinical use can be refined. The tested range of doses will usually be a small fraction of the dose that causes harm in animal testing. Phase I trials most often include healthy volunteers, however there are some circumstances when patients are used, such as with oncology (cancer) and HIV drug trials. In Phase I trials of new cancer drugs, for example, patients with advanced (metastatic) cancer are used. These trials are usually offered to patients who have had other types of therapy and who have few, if any, other treatment choices.

There are different kinds of Phase I trials:

Single Ascending Dose (SAD): These studies are those in which small groups of patients are given a single dose of the drug while they are observed and tested for a period of time. If they do not exhibit any adverse side effects, and the pharmacokinetic data is roughly in line with predicted safe values, the dose is escalated, and a new group of patients is then given a higher dose. This is continued until pre-calculated pharmacokinetic safety levels are reached, or intolerable side effects start showing up (at which point the drug is said to have reached the Maximum tolerated dose (MTD)).

Multiple Ascending Dose studies(MAD): They are conducted to better understand the pharmacokinetics & pharmacodynamics of multiple doses of the drug. In these studies, a group of patients receives multiple low doses of the drug, whilst samples (of blood, and other fluids) are collected at various time points and analyzed to understand how the drug is processed within the body. The dose is subsequently escalated for further groups, up to a predetermined level.

Food effect: It is a short trial designed to investigate any differences in absorption caused by eating pre-dose, and its effect on the pharmacokinetic profile. These studies are

usually run as a cross over study, with volunteers given two identical doses of the drug on different occasions; one while fasted, and one after being fed.

The prime requisite for such studies are pointed diagnosis and uniformity of patient with respect to age, sex, weight and severity of the disease as well as sensitivity of the patient.

The first dose which is to be administered is usually $1/6^{\text{th}}$ to $1/8^{\text{th}}$ of the predicted effective dose. Once this minimum dose is tolerated, the dose is increased gradually to establish the maximum tolerated dose at which it has greater effect but devoid of toxicity or the undesirable side effect. No individual is exposed to more than single dose in a short period of time and a rest period, of at least one week interval is given to prevent the cumulative effect. If other known drugs are also to be administered, it is necessary to watch for the abnormal reaction, if any.

These trials are almost always conducted in an inpatient clinic, where the subject can be observed by full-time medical staff. The bioavailability and bioequivalence studies are also considered to be the Phase I study. The subject is usually observed until several half-lives of the drug have passed.

Phase II: First in the Patient study

Once the initial safety of the therapy has been confirmed in Phase I trials, Phase II trials are performed on larger groups (20-300) and are designed to assess clinical efficacy of the therapy; as well as to continue Phase I safety assessments in a larger group of volunteers and patients. The development process for a new drug commonly fails during Phase II trials due to the discovery of poor efficacy or toxic effects.

Phase II studies are sometimes divided into Phase IIA and Phase IIB. Phase IIA is specifically designed to assess dosing requirements, whereas Phase IIB is specifically designed to study efficacy.

The aim of this phase is to know whether or not the drug is to be developed as a therapeutic agent. This phase is also termed as '*controlled clinical trial phase*' and is universally accepted as a standard requirement for the evaluation of comparative efficacy and safety of the drug in clinical pharmacology. It is a carefully and ethically designed experiment with the sole aim of obtaining answers to some of the precisely framed questions in equivalent groups of patients, concurrently treated with different drugs.

The Phase II is carried out in two ways :

- (a) *Between subject comparison*, i.e, where the drug is given to one group and results are compared with those of the other group. It is widely used and here one group of the patients gets the new drug whereas, the other gets either a previously established drug or the placebo.

- (b) *Within subject comparison*, i.e. where the drug is given alternatively with the control drug therapy either placebo or previously established drug to the same patient.

In order to avoid the 'bias' factor in results, these trials are carried out under so called blind conditions. The word bias implies a mental tendency for strong likes and dislikes. The nature of medication is concealed from the patient in the *single blind study*. In the other type of study, the drug is given a code number and its nature is concealed from the patient as well as the attending physician. This is called the *double blind study*.

Placebo : In medical term, placebo means 'dummy medication' it is a pharmacologically inert substance, e.g. lactose, that resembles the actual medicament in physical properties like size, shape, color, smell and taste. In clinical trials, it acts as the unknown control and is useful for the following :

- (a) To distinguish the pharmacodynamic effect of the drug from the psychological factors such as softness in medication, increased interest of the physician such as frequent visits, mode of approach, his personality etc. affecting the patient.
- (b) To distinguish real drug effect from the fluctuations in the illness that follow in course of time and other extraneous factors.
- (c) To avoid false negative conclusions.

Careful observations are made during the acute and sub-acute administration of the drug to determine the therapeutic index, dose and adverse reactions.

Some phase II trials are designed as case series, demonstrating safety and efficacy in a selected group of patients. Other phase II trials are designed as randomized clinical trials, complete with a treatment arm and a comparison arm. Randomized phase II trials have far fewer patients than randomized phase III trials.

Phase III: Statistical Evaluation

Here, the results obtained in Phase II at limited centers are confirmed at more centers and are subjected to the statistical analysis. If the results are in accordance with each other, the new drug is released for the market.

Phase III studies are randomized controlled trials on large patient groups (300–3,000 or more depending upon the condition) and are aimed at being the definitive assessment of the efficacy of the new therapy, in comparison with current 'Gold Standard' treatment. Phase III trials are the most expensive, time-consuming and difficult trials to design and run; especially in therapies for chronic conditions. Once a drug has proven satisfactory over Phase III trials, the trial results are usually combined into a large document containing a comprehensive description of the methods and results of human and animal studies, manufacturing procedures, formulation details, and shelf life. This collection of information makes up the "regulatory submission" that is provided for review to various regulatory authorities in different countries for marketing approval.

It is also common practice with many drugs whose approval is pending, that certain phase III trials will continue. This typically serves to provide lifesaving products after involvement in a clinical trial until the marketed product can be obtained. Other reasons for performing trials at this stage include attempts at "label expansion" to prove additional efficacy for uses beyond the original use for which the drug was designed, to obtain additional safety data, or to support marketing claims. Studies in this phase are by some companies categorised as "Phase IIIB studies."

While not required in all studies, it is typically expected that there be at least two successful phase III trials, proving a drug's safety and efficacy, for approval from the standard regulatory agencies (FDA, DCGI etc.).

As mentioned above, Phase II and Phase III trials are normally Randomized trials. A **randomized controlled trial** (RCT) is a scientific procedure most commonly used in testing medicines or medical procedure. It is a trial that uses randomized control. This is considered the most reliable form of scientific evidence because it eliminates all forms of spurious causality (Some people call it a **randomized control trial**.) The term **RCT** is also sometimes use to abbreviate **randomized clinical trial**, which is a form of clinical trial. However, randomized control trials are used in other sectors (e.g. judicial, educational, social), so the clinical sector does not have a monopoly of this technique. The basic idea is that treatments are allocated to subjects at random. This ensures that the different treatment groups are 'statistically equivalent'. Trials are used to establish average efficacy of a treatment as well as learn about its most frequently occurring side effects. This is meant to address the following concerns. First, effects of a treatment may be small and therefore undetectable except when studied systematically on a large population. Second, biological organisms (including humans) are complex, and do not react to the same stimulus in the same way, which makes inference from single clinical reports very unreliable and generally unacceptable as scientific evidence. Third, some conditions will spontaneously go into remission, with many extant reports of miraculous cures for no discernible reason. Finally, it is well-known and has been proven that the simple process of administering the treatment may have direct psychological effects on the patient, sometimes very powerful, what is known as the placebo effect.

Randomized trials are employed to test efficacy while avoiding these factors. Trials may be *open*, *blind* or *double-blind*.

Open trial: In an open trial, the researcher knows the full details of the treatment and so does the patient. These trials are open to challenge for bias, and they do nothing to reduce the placebo effect. However, sometimes they are unavoidable, particularly in relation to surgical techniques, where it may not be possible or ethical to hide from the patient which treatment he or she received. Usually this kind of study design is used in bioequivalence studies.

Single-blind trial: Here the researcher knows the details of the treatment but the patient does not. Because the patient does not know which treatment is being administered (the

new treatment or another treatment) there should be no placebo effect. In practice, since the researcher knows, it is possible for them to treat the patient differently or to subconsciously hint to the patient important treatment-related details, thus influencing the outcome of the study.

Double-blind trial: Here trial, one researcher allocates a series of numbers to 'new treatment' or 'old treatment'. The second researcher is told the numbers, but not what they have been allocated to. Since the second researcher does not know, they cannot possibly tell the patient, directly or otherwise, and cannot give in to patient pressure to give them the new treatment. In this system, there is also often a more realistic distribution of sexes and ages of patients. Therefore double-blind (or randomized) trials are preferred, as they tend to give the most accurate results..

Triple-blind trial: Some randomized controlled trials are considered triple-blinded, although the meaning of this may vary according to the exact study design. The most common meaning is that the subject, researcher and person administering the treatment (often a pharmacist) are blinded to what is being given. Alternately, it may mean that the patient, researcher and statistician are blinded. These additional precautions are often in place with the more commonly accepted term "double blind trials", and thus the term "triple-blinded" is infrequently used. However, it connotes an additional layer of security to prevent undue influence of study results by anyone directly involved with the study.

Aspects of 'control' in clinical trials:

Traditionally the *control* in randomized controlled trials refers to the group of treated patients not in isolation but in comparison to other groups of patients, who do not receive the treatment under study. The use of control gives the investigator important clues to the effectiveness of the treatment, its side effects, and the parameters that modify these effects. Other aspects of control include having other members of the research team, who will typically review the test to try to remove any factors which might skew the results. For example, it is important to have a test group which is reasonably balanced for ages and sexes of the subjects (unless this is a treatment which will never be used on a particular sex or age group). Additionally, peer review and/or review by government regulators can be seen as another source of control. These bodies examine the trial results when they are presented for publication or when the drug manufacturer applies for a licence for the drug.

The importance of having a control group is to avoid bias of getting a miraculous cure to a patient—even if the pill contains nothing more than sugar. Further, some times the procedure or interventions like needle prick while giving injection can itself produce ill effects. For example, in one study on rabbits where these subjects were receiving daily injections of a drug, it was found that they were developing cancer. If this was a result of the treatment, it would obviously be unsuitable for testing in humans. Because this result was reflected equally between the control and test groups, the source of the problem was investigated and it was shown in this case that the administration of daily injections was the cancer risk—not the drug itself.

The analysis of the clinical trial results requires knowledge of medicine, epidemiology, and in particular statistics. The branch of statistics that deals specifically with biomedical research is biostatistics. Pharmaceutical firms employ groups of biostatisticians to try to make sense of the data. Likewise, regulators pay keen attention to the appropriateness of statistical methods used to analyze trial results.

Phase IV : Field trials and Post Marketing Surveillance

Once the drug is released for general use, it will appear in different environment and different clinical conditions. Studies under such circumstances may reveal unexpected observations and also drug-interactions. This phase is thus an unending phase. Phase IV trials involve the post-marketing safety surveillance and ongoing technical support of a drug. Phase IV studies may be a mandate by the regulatory authorities to evaluate safety of existing drug. Some times Phase IV study may be undertaken by the sponsoring company for getting own data and compare with the for competitive drugs. The drug may also be tested for interactions with other drugs, or on certain ethnic group of population or groups such as pregnant women, children etc. who are unlikely to be the subject themselves to trials as per the law. Post-marketing safety surveillance is designed to detect any rare or long-term adverse effects over a much larger patient population and timescale than was possible during the initial clinical trials. Such adverse effects detected by Phase IV trials may result in the withdrawal or restriction of a drug - recent examples include troglitazone, celecoxib etc.