EXPERIMENT NO:-08 **DATE**

AIM:- Introduction to general principles of bioassay.

THEORY

DEFINATION:

It is an estimation of the potency of an active principle in a unite quantity of preparation and measurement of the concentration of the substance in a preparation using biological method (i.e. observation of pharmacological effect on living tissues, microorganisms or immune cells or animal) is known as biological assay or bioassay.

IMPORTANCE OF BIOASSAYS:

Bioassays are essential in the development of new drugs. In the preclinical assessment of a new compound, the biological activity is compared with that of known compounds using appropriate test systems. The precision, reliability and reproducibility of bioassay depend on the proper selection of the tissue or method with highest selectivity and sensitivity for the drug. In spite of the tremendous advancement in the analytical chemistry and modern instrumentations, bioassay procedures continue to be used as successful tools not only in the estimation of bioactive substances but also for the discovery of biologically active substances.

Bioassays are generally employed

- When active principle of drug is unknown or cannot be isolated.
- When a chemical assay for the substance is not available or interacting with chemicals as the case with hormones inactivates the substance. Chemical method is too complex, insensitive or requires higher dose.
- When the quantity of the sample is too small. In such situation a matching type of bioassay is conveniently done to compare the biological response with the standard drug.
- To estimate the concentrations of active principles present in the tissue extracts, the endogenous mediators like acetyl choline, 5-HT, prostaglandins
- To measure the pharmacological activity of new or chemically unidentified substances
- To measure drug toxicity and
- When bioassay is more sensitive than chemical assay.

The purpose of bioassay is to ascertain the potency of a drug and hence serves as the quantitative part of any screening procedure. Other purpose of bioassay is to standardize the preparation so that each contains the uniform specified pharmacological activity, serve as pointer for the commercial production of drugs, and help diagnosis of various conditions.

PRINCIPLE OF BIOASSAY:

The basic principle of bioassay is to compare the substance with the international preparation of the same and to find out how much test substance is required to produce the same biological effect, as produced by standard. The problem of biological variation must be minimized as far as possible. For that one should keep uniform experimental conditions and assure the reproducibility of the responses.

USE OF STANDARDS:

Bioassays are designed to measure relative potency of two preparations, usually a standard and an unknown. It is unsatisfactory to designate a unit of particular drug at that amount and causes a particular effect because biological effects vary from animal to animal, time to time & from lab to lab. Use of standard substance for comparison also helps in solving problems arising from biological variations. The observed response/effect of the unknown would be always relative to the effect that produced by a standard substance. The standard substance is a pure substance and in official bioassays it refers to pharmacopoeial standards. In case of hormones, biological products and vaccines it is often necessary to establish the standard response of the standard substances against which unknown samples can be calibrated.

DISADVANTAGES OF BIOASSAY:

- Less accurate
- Less elaborate
- More laborious
- More troublesome
- *More expensive*

PRECAUTIONS IN BIOASSAYS TO MINIMIZE BIOLOGICAL VARIATIONS:

- All the experimental conditions should be constant.
- The response studied should be reproducible.
- The biological response being studied should be sensitive to the drug.
- The animals should be of same species, strain, approximate of same age and weight and sex. Also should be kept on a similar diet and housed under similar conditions.

METHODS OF BIOASSAY FOR AGONISTS:

An agonist may produce two types of response.

1. Quantal response:

Quantal means that the response is in the form of "all or none" i.e. either no response or maximum response. The drugs producing quantal effect can be bioassay by end point method.

End Point Method:

Here the threshold dose producing a positive effect is measured on each animal and the comparison between the average results of two groups of animals is done eq. Bioassay of digitalis in cats.

Here the cat is anaesthetized with chloralase and its blood pressure is recorded. The drug is taken slowly infused into the animal and the moment the heart stops beating and blood pressure falls to zero, the volume of fluid infused is noted down. Two series of such experiments- one using standard digitalis and other using test preparation of digitalis is done and then potency is calculated as follows:

Conc. of Unknown = (Threshold dose of std./Threshold dose of test) X Conc. of std.

In case, if it is not possible to measure individual effective dose or if animals are not available, fixed doses are injected into groups of animals and the percentage of mortality at each dose level is determined. The percentage of mortality is taken as the response and then the comparison is done in the same way as done for graded response.

2. Graded response:

Graded response means that the response is proportional to the dose and response may lie between no response and the maximum response. Graded response assays are based on the proportionate increase in the response in the response observed with an increase in the concentration or the dose of the drug. The parameters employed in such bioassays are based on the nature of the effect, the drug or substance is expected to produce. eq. Contraction of smooth muscle of rat ileum for bioassay of acetylcholine, Relaxation of smooth muscle of rabbit ileum for bioassay of Adrenaline. The drugs producing graded responses can be bioassay by

(A) Graphical method or interpolation method

(B) Matching or bracketing method

(C) Multiple point method.

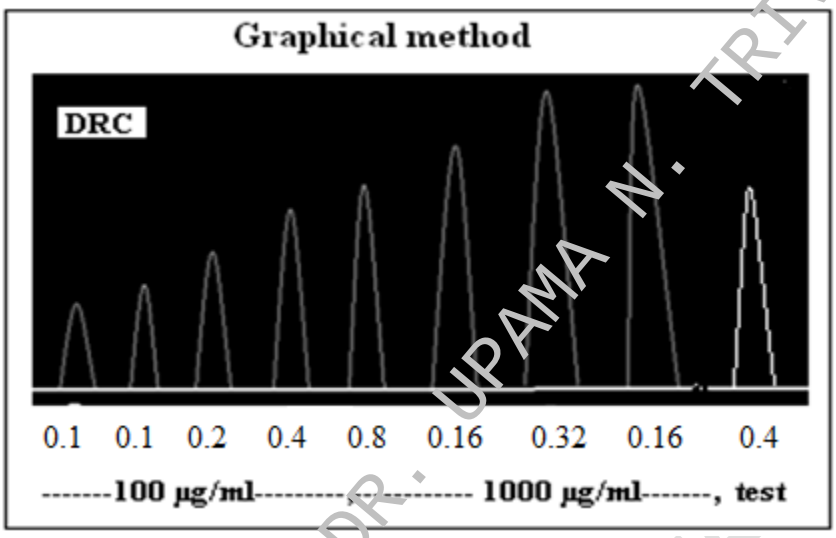
The choice of the procedure or method depends upon precision or accuracy of assay, the quantity of test sample available, the availability of experimental animals.

(A) Graphical method:

This method is based on the assumption of the dose-response relationship. Log-dose- response curve is plotted and the dose of standard producing the same response as produced by the test sample is directly read from the graph. In simpler design, 5-6 responses of the graded doses of the standard are taken and then two equiactive responses of the test sample are taken. The height of concentration is measured and plotted against the log-dose. The dose of standard producing the same response as produced by the test is read directly from the graph and the concentration of test sample is determined by the following formula:

Conc. of Unknown = (Dose of Std. / Dose of Test) X Conc. of std.

The characteristic of log-dose response curve is that it is linear in the middle (20-80%). Thus, the comparison should be done within this range only. In other words, the response of test sample must lie within this range.



Advantages:

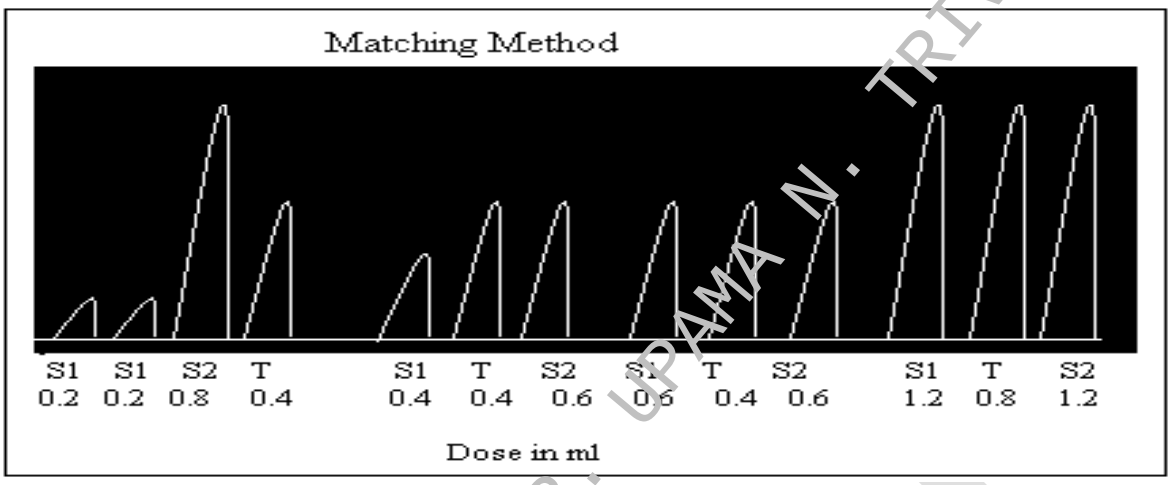
- It is a simple method
- Chances of errors are less if the sensitivity of the preparation is not changed.

(B) **Matching Method:**

In this method a constant dose of the test is bracketed by varying doses of standard till the exact match is obtained between test dose and the standard dose. Initially, two responses of the standard are taken. The does are adjusted such that one is giving response of approximately 20% and other 70% of the maximum. The response of unknown that lies between two responses of standard dose is taken. The panel is repeated by increasing or decreasing the doses of standard till all three equal responses are obtained. The dose of test sample is kept constant.

In the end, a response of the double dose of the standard and test that match each other are taken. These should give equal responses. Concentration of the test sample can be determined as follows:

Conc. of Unknown = (Dose of Std. / Dose of Test) X Conc. of std.



Advantage:

Useful when sensitivity is not stable

Limitations:

It occupies a larger area of the drum as far as tracings are concerned. The match is purely subjective, so chances of error are there and one cannot determine them. It does not give any idea of dose-response relationship. Method is not accurate and not reliable. Eq. Bioassay of histamine on guinea pig ileum is preferably carried out by this method.

(C)Multiple point bioassays:

These methods include 3 point, 4 point, 5 point and 6-point methods. In these methods, the responses are repeated several times and the mean of each is taken. Thus, chances of error are minimized in these methods.

In 3-point assay method, 2 doses of the standard and one dose of the test are used. Initially a graded dose response curve for the standard drug is taken. From this response two doses of the standard drug S1 & S2 are selected. The two doses should preferably be in the ration of 1:2. The test dose is fixed in such a way that it gives the response between the responses produced by S1 & S2. These three selected doses are repeated by the Latin Square design method i.e. S1, S2, T – S2, T, S1 – T, S1, S2. in order to avoid bias. The mean responses are calculated and plotted against log-dose and amount of standard producing the same response as produced by the test is determined mathematically:

$$\text{Conc. of Unknown} = \frac{n_1}{t} \times \text{antilog} \left\{ \frac{T - S_1}{S_2 - S_1} \times \log \frac{n_2}{n_1} \right\} \times Cs \times \text{dil. factor}$$

n_1= Lower std. dose

n_2= higher std. dose

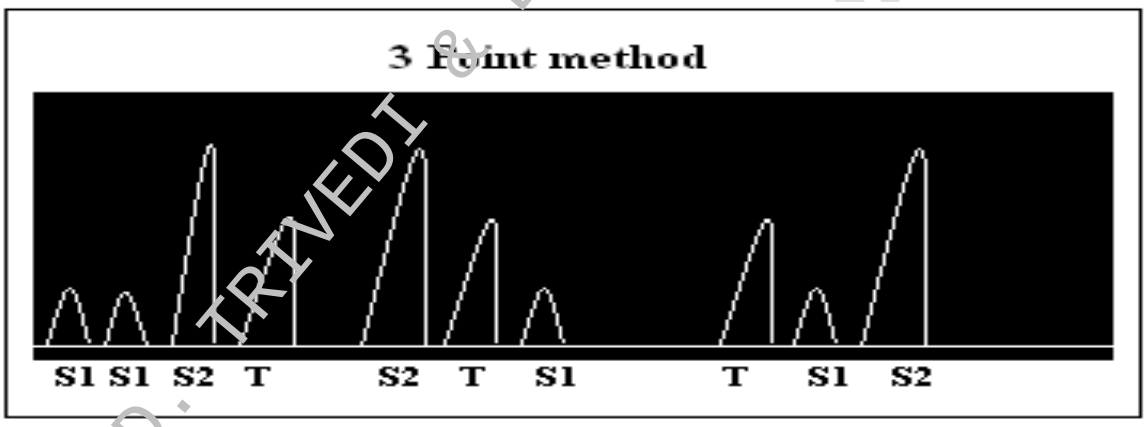
t = test dose

S_1= response of n_1

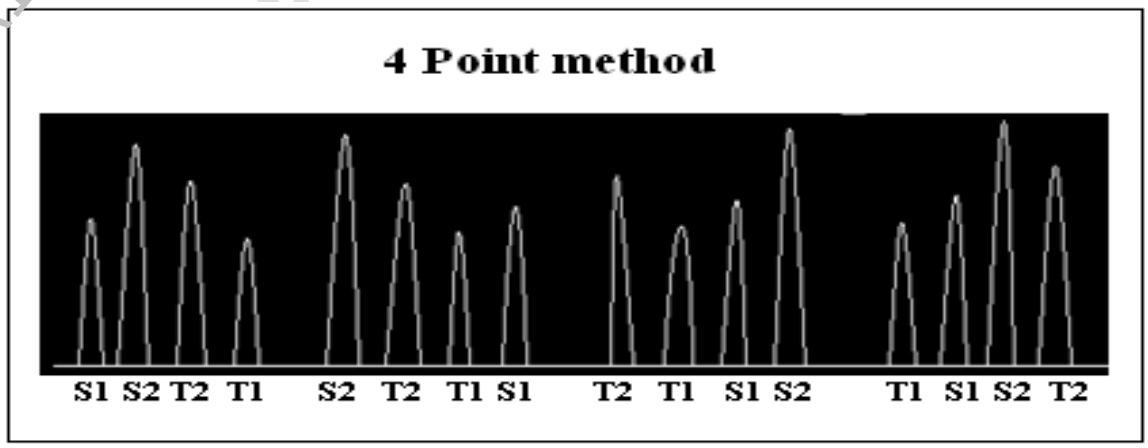
S_2= response of n_2

T= response of Test

Cs = conc. of std.



In 4 point method two doses of standard and two doses of test ,in 5 point method three doses of standard and two doses of test, in 6 point method three doses of standard and three doses of test are used. Similarly one can design 8-point method also.



$$\text{Concentration of unknown} = n_1/t_1 \times \text{anti log}\left\{\frac{(S_1+S_2)-(T_1+T_2)}{(S_2+T_2)-(S_1+T_1)}\right\}\log n_2/n_1 \times Cs$$

Where,

n1 = lower standard dose

n2 = Higher standard dose

t1 = Lower test dose

t2 = Higher test dose

S1= response of n1

S2 = response of n2

T1 = response of t1

T2 = response of t2

Cs= Concentration of standard

BIOASSAY OF ANTAGONISTS:

Commonly used method for the bioassay of antagonist is simple graphical method. The responses are determined in the form of the percentage inhibition of the fixed dose of agonist. These are then plotted against the log dose of the antagonist and the concentration of unknown is determined by finding out the amount of standard producing the same effect as produced by the test.

In this method, two responses of the same dose of agonist (sub maximal giving approximately 80% of the maximum response) are taken. The minimum dose of standard, antagonist is added in the bath and then the response of the same dose of agonist is taken in presence of antagonist. The response of agonist is repeated every ten min. till recovery is obtained. The higher dose of standard, antagonist is added and responses are taken as before, three to four doses of the standard. Antagonist is used and than one to two doses of test sample of the antagonist is used similarly. The percentage inhibition is calculated, plotted against log-dose and the concentration of unknown is determined as usual.

BIOASSAY ON SOME IMPORTANT DRUGS:

Depending upon pharmacological action of various drugs, different, preparations may be used. Following chart gives different preparations and the pharmacological activity for which a particular drug is assayed:

DRUGS	PREPARATION	ACTIVITY ASSAYED
Digitalis	Cat blood pressure Guinea pig Blood pressure	Fall in B. P. and death or stoppage of Heart & death.
Adrenaline	Blood pressure of the spinal cat	Rise of B. P.
	Isolated rabbit duodenum, Isolated	Inhibition of the tone
Nor adrenaline	Blood pressure of the pitched cat.	Rise of B. P.
Acetylcholine	Isolated rectus abdominus of frog, rat ileum and leech dorsal muscle	Contractile effect.
	Isolated mouse heart	Inhibition of cardiac contractions.
	Rat / Cat blood pressure	Fall in blood pressure.
Histamine	Isolated, atropinized terminal ileum of guinea – pig.	Contractile effect.
	Anaesthetized and atropinized cat.	Fall in blood pressure.
5Hydroxy-tryptamine	Isolated atropinized rat uterus, Isolated Terminal colon of rat, Isolated fundus Strip of rat stomach, Isolated heart of cat	Contractile effect
	Perfused rabbit ear	Constriction of blood vessels
Curariform drugs e.g. d-tubo-curarine	Rabbit	Dropping of head
	Rat diaphragm with phrenic nerve, Cat Gastrocnemius muscle with sciatic nerve	Inhibition of the contractile effect
Heparin	Sulfated whole blood of ox with thrombokinase extract and acetone	Prolongation of blood clotting time.
Antibiotics	Suitable micro-organism grown on suitable nutrient agar	Inhibition of growth of microorganism.
Vitamin D.	Rats maintained on rich etogenic diet.	Alleviation of rachitic stage
Insulin	Rabbits	Lowering of blood-sugar level
	Mice	Convulsions and/or death due to hypoglycemia

	Isolated rat diaphragm	Increase in glycogen content.
	Rat's epididymal fat	Increased metabolism of glucose, indicated by increase CO_2 production.
Oxytocin	Adult cockerel.	Vasodepressor activity.
	Isolated rat uterus.	Contractile effect
	Rabbits(female)	Ejection of milk from mammary duct.
Vasopressin	Rat blood-pressure	Vasopressor activity.
Growth hormone	Hypophysectomized rats.	Gain in weight, Increase in width of epiphyseal cartilage
Gonadotrophin (FSH)	Hypophysectomized male rats	Increase in testicular weight.
	Hypophysectomized female	Increase in weight of ovaries.
Gonadotrophin (L.H.)	Immature male rats.	Enlargement of prostate gland.
Gonadotrophin (FSH and LH)	Immature female rate.	Increase in weight of uterus.
***Prolactin**	Cloves of pigeons.	Increase in weight of crop sac.
	Female rats.	Lengthening of estrous cycle and function of corpus luteum.
	Hypophysectomized rat.	Inhibition of estrogen upon vaginal smear.
***Corticotrophin**	Hypophosectomized rats.	Depletion of ascorbic acid from adrenal gland.
***Thyrotropin**	Mice or rats.	Release of previously administered 131I (Iodine) from thyroid gland.
***Androgen**	Castrated capon	Increase in size of comb
	Castrated male rat.	Increase in weight of prostate gland and seminal vesicles.
	Castrated male rats.	Increase in weight of levator- ani muscles.
Estrogen	Rat or mouse(Female)	Increase in weight of uterus.
Progesterone	Sexual immature rabbits	Increase In Carbonic anhydrase-activity in uterus.

*Radioimmunoassay or radio receptor assay methods are also available.

CURRENT STATUS OF BIOASSAY:

Above-mentioned discussion is an overview of bioassay, which is prevailing, in various academic institutions. However, with advent of technology, availability of advanced

sophisticated and more reliable analytical method the scenario for bioassay has changed dramatically. If one reviews the emphasis of bioassay in pharmacopoeia published before 1980 as compared to those published recently. It will be clear that:

- There are very few drugs which are now recommended to be assayed b6 biological method.
- Most of drugs, which were assayed by biological methods, are now being recommended to be assayed by chemical methods.
- Newer drugs have been included for which bioassay recommended.

QUESTIONS

1. Define Bioassay. Give principle of bioassay.
2. Enlist the methods of bioassay.
3. Write down formulas of all bioassay method.