PHARMACOLOGY

Principles and Methods of Bioassay

Prof. Ramesh K. Goyal
L. M. College of Pharmacy
Navrangpura
Ahmedabad- 380 009

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CONTENTS

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Bioassay is defined as the estimation of the potency of an active principle in a unit quantity of preparation or detection and measurement of the concentration of the substance in a preparation using biological methods (i.e. observation of pharmacological effects on living tissues, microorganisms or immune cells or animal). Hence micro bioassay, radioimmunoassay are also regarded as `bioassay'. Recently `biotechnology' has also been considered for bioassay. Bioassay of the products like erythropoietin, hepatitis-B vaccine etc. is being done through biotechnology.

Importance of Bioassay

Bioassays, as compared to other methods of assays (e.g. chemical or physical assay) are less accurate, less elaborate, more laborious, more troublesome and more expensive. However, bioassay is the only method of assay if (1) Active principle of drug is unknown or cannot be isolated, e.g. insulin, posterior pituitary extract etc. (2) Chemical method is either not available or if available, it is too complex and insensitive or requires higher dose e.g. insulin, acetylcholine. (3) Chemical composition is not known, e.g. long acting thyroid stimulants. (4) Chemical composition of drug differs but have the same pharmacological action and vice-versa, e.g. cardiac glycosides, catecholamines etc. Moreover, even if chemical methods are available and the results of bioassay conflict with those of the chemical assay, the bioassay is relied upon and not the chemical assay, since it is the assessment on living organism.

The purpose of bioassay is to ascertain the potency of a drug and hence it serves as the quantitative part of any screening procedure *(Research)*. Other purpose of bioassay is to standardize the preparation so that each contains the uniform specified pharmacological activity. In this way, it serves as a pointer in the *Commercial Production of drugs* when chemical assays are not available or do not suffice. From the *clinical* point of view, bioassay may help in the diagnosis of various conditions, e.g. gonadotrophins for pregnancy.

Principle of Bioassay

The basic principle of bioassay is to compare the test substance with the International Standard preparation of the same and to find out how much test substance is required to produce the same biological effect, as produced by the standard. The standards are internationally accepted samples of drugs maintained and recommended by the Expert Committee of the Biological Standardization of W.H.O. They represent the fixed units of activity (definite weight of preparation) for drugs. In India, standard drugs are maintained in Government institutions like Central Drug Research Institute, Lucknow, Central Drug Laboratory, Calcutta, etc.

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.

Methods of Bioassay for Agonists

An agonist may produce graded response or quantal response. Graded response means that the response is proportional to the dose and response may lie between no response and the maximum response. By quantal, it is meant 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 bioassayed by end point method. The drugs producing graded responses can be bioassayed by (1) Matching or bracketing method or (2) Graphical method.

1. 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 (one receiving standard and other the test) is done. e.g. bioassay of digitalis in cats. Here the cat is anaesthetized with chloralose and its blood pressure is recorded. The drug is 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 the other using test preparation of digitalis is done and then potency is calculated as follows:

$$\text{Conc. of Unknown} = \frac{\text{Threshold dose of the Standard}}{\text{Threshold dose of the Test}} \text{ X Conc. of Std.}$$

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. 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 doses are adjusted such that one is giving response of approximately 20% and other 70% of the maximum. The response of unknown which lies between two responses of standard dose is taken. The panel is repeated by increasing or decreasing the dose s of standard till all three equal responses are obtained. The dose of test sample is kept constant. At the end, a response of the double dose of the standard and test which match each other are taken. These should give equal responses. Concentration of the test sample can be determined as follows:

$$\text{Conc. of Unknown} = \frac{\text{Dose of the Standard}}{\text{Dose of the Test}} \text{ X Conc. of Std.}$$

This method has following limitations:

1. It occupies a larger area of the drum as far as tracings are concerned.
2. The match is purely subjective, so chances of error are there and one cannot determine them.

3. It does not give any idea of dose-response relationship.

However, this method is particularly useful if the sensitivity of the preparation is not stable. Bioassay of histamine, on guinea pig ileum is preferably carried out by this method.

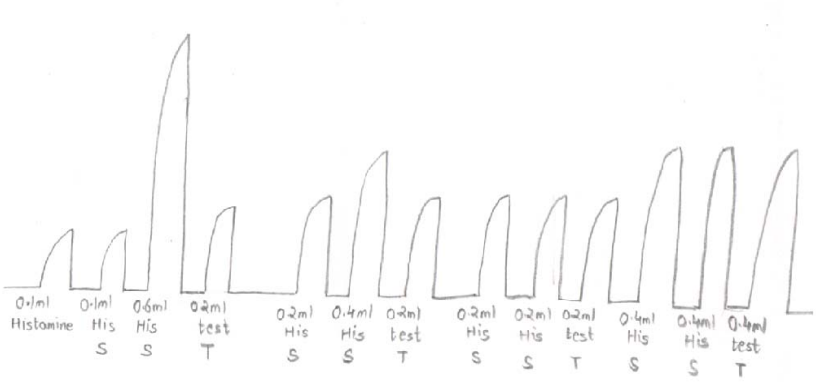


Fig.1: Bioassay of histamine by the matching method

3. 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 contraction 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 same formula as mentioned before.

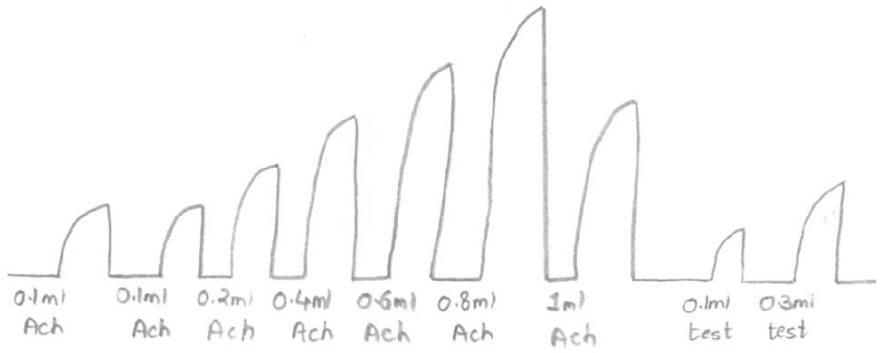


Fig.2: Graphical method of bioassay

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.

Advantage of this method is that, it is a simple method and chances of errors are less if the sensitivity of the preparation is not changed. Other methods which are based on the dose-response relationship 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. In 4 point method 2 doses of standard and 2 doses of the test are used. In 6 point method 3 doses of standard and 3 doses of the test are used. Similarly one can design 8 point method also. The sequence of responses is followed as per the Latin square method of randomization in order to avoid any bias.

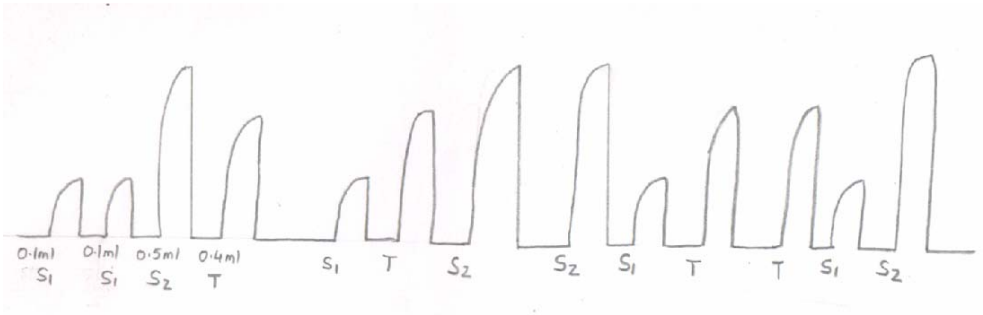


Fig.3: Bioassay of histamine by three point method.

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 graphically as well as mathematically:

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

n_1 = Lower Standard dose

n_2 = Higher Standard dose

t = Test dose

S_1 = Response of n_1

S_2 = Response of n_2

T = Response of test (t)

Cs = Concentration of standard

Similarly, in 4 point method, amount of standard producing the same response as produced by the test can be determined by graphical method. It is determined mathematically as follows:

$$\frac{n_1}{t_1} \text{ X antilog } \left\{ \frac{(S_1 + S_2) - (T_1 + T_2)}{(S_2 + T_2) - (S_1 + T_1)} \text{X log } n_1 \frac{n_2}{} \right\}$$

t_1 = lower dose of test; t_2 = higher dose of test; T_1 = response of t_1; T_2 = response of t_2.

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 responses of agonist are 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 are used and then one to two doses of test sample of the antagonist is used similarly. The percentage inhibition is calculated, plotted against log dose of antagonist and the concentration of unknown is determined as usual.

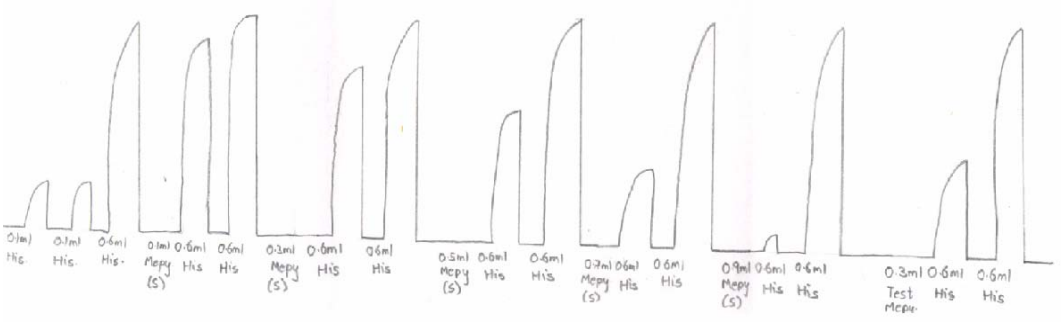


Fig.4: Bioassay of mepyramine

Bioassay of 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:

Drug	Preparation	Activity Assayed
Digitalis	Cat blood pressure	Fall in B.P. and death
	Guinea pig blood pressure	Stopage of heart and death.
	Pigeon	Emesis
Adrenaline	Blood pressure of the spinal cat	Rise in B.P.
	Isolated rabbit duodenum	Inhibition of the tone
	Isolated caecum of fowl	Inhibition of the tone
	Isolated rat uterus	Inhibition of the tone
Noradrenaline	Blood pressure of the pithed cat	Rise in B.P.
Acetylcholine	Isolated rectums	Contractile effect.

	abdominis muscle of frog	
	Rat ileum	Contractile effect
	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
5 Hydroxy-tryptamine	Isolated atropinized rat uterus,	Contractile effect
	Isolated terminal colon of rat,	"
	Isolated fundus strip of rat stomach,	"
	Perfused rabbit ear	Constriction of blood vessels
Curariform drugs	Rabbit	Dropping of head
e.g. d-tubo-curarine	Rat diaphragm with phrenic nerve or Cat gastrocnemius muscle with sciatic nerve.	Inhibition of the contractile effect
Heparin	Whole blood of ox with thrombokinase extract and acetone dried ox brain	Prolongation of blood clotting time
Antibiotics	Suitable micro-organism grown on suitable nutrient agar medium	Inhibition of growth of micro-organism
Vitamin D	Rats maintained on richetogenic diet	Alleviation of rachetic stage.
Insulin	Rabbits	Lowering of blood-sugar Level.
	Mice	Convulsions and/or death due to hypoglycaemia

	Isolated rat diaphragm	Increase in glycogen content.
	Rat's epididymal fat	Increased metabolism of glucose, indicated by increased in 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 epiphysea cartilage
Gonadotrophin (FSH)	Hypophysectomized male rats	Increase in testicular weight.
	Hypophysectomized female rats	Increase in weight of ovaries.
Gonadotrophin (L.H.)	Immature male rats	Enlargement of prostate gland.
Gonadotrophin (FSH and LH)	Immature female rats	Increase in weight of uterus.
Prolactin	Cloves of pigeons	Increase in weight of crop sac.
	Female g. pig or rabbit.	Secretory changes in mammary gland.
	Female rats	Lengthening of estrous cycle and function of corpus luteum
	Hypophysectomized rat	Inhibition of estrogen upon vaginal smear.
* Corticortrophin	Hypophysectomized rats	Depletion of ascorbic acid from adrenal gland.
* Thyrotropin	Mice or rats	Release of previously

		administered ^{131}I (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 immatur rabbits	Proliferative changes in endometrium of uterus
		Increase in carbonic anhydrase-activity in uterus

*Radioimmunoassay or radio receptor assay methods are also available.

(i). Bioassay of acetylcholine

***Principle*:** Potency of the test sample is compared with that of the standard preparation. There are several biological methods for its assay.

1. Rectus Abdominis Muscle of Frog: Dissect the rectus muscle and arrange the assembly as per assay of d-tubocurarine. Plot log dose-response curve and find out the potency of the sample of acetylcholine.

2. Cat's Blood Pressure: A cat is anaesthetized with suitable anaesthetic. The carotid artery is cannulated for recording BP. Femoral vein is cannulated for injecting acetylcholine. Trachea is cannulated for giving artificial respiration. Acetylcholine produces a fall in BP by dilating peripheral blood vessels. This principle is utilised for its bioassay. The extent to which BP falls due to the test sample is compared with the fall by the standard preparation.

3. Guinea-pig Ileum: Guinea–pig is killed by a blow on the head and bled to death. The abdominal wall is dissected out so as to isolate the ileum, the faecal matter, mesentery and bood vessels are removed from the piece of ileum. It is ligated on both sides and suspended in mammalian organ bath containing Tyrode solution maintained at 37.0^{o}C and oxygenated continuously. Acetylcholine contracts the ileum. This principle is utilised for its bioassay. The extent of contraction produced by the test sample is compared with the standard preparation of acetylcholine.

4. Anaesthetised Rats: Compare the extent of fall in BP of the test sample with that produced by the standard preparation.

5. Leech Muscle: Compare the contractions produced by the standard and test samples on eserinised dorsal muscle of the leech. This muscle is highly sensitive (picograms) to acetylcholine.

6. Isolate Heart Preparations: Rabbit's auricle, frog's heart, rabbit's heart or venous merceneria's heart is used. Ach decreases the force and rate of the heart.

7. Rabbit's Intestine and Tracheal Chain: Ach contracts these tissues.

(ii). Bioassay of adrenaline

Principle: Potency of the test sample is estimated by comparing the rise in blood pressure of the test sample with the produced by the standard preparation of adrenaline.

Standard Preparation: It should fulfill all the tests of purity as per adrenaline monograph in the Indian Pharmacopoeia. The specific rotation of 4% w/v solution of the standard adrenaline in 0.1 N HCl should lie between –5 ^{O}C to 5 ^{O}C dilutions of adrenaline are prepared by diluting it with saline. A suitable concentration is 1: 10,000. Test sample solution is also prepared in saline.

1. Method using Anaesthetised Dog: A medium size dog (10-13 kg.) is anaesthetised with pentobarbitone sodium in a dose of 25 mg/kg, i.v. Artificial respiration is given through tracheal tube, if necessary. BP is recorded on a kymograph after cannulating carotid artery with arterial cannula and connecting the same with a mercury manometer.

The animal should be given 0.001-0.002 mg. of atropine sulphate to paralyse the vagi. This paralysis is confirmed by stimulating vagi by electrical stimulation. There will be no fall in BP in atropinised animal after electrical stimulation.

Adrenaline is injected into the femoral vein through venous cannula. Two successive responses of the same dose of adrenaline are noted. The similar responses after the same doses of adrenaline, is an indication that there is no variation in B.P., and that the animal is now ready for the assay. Then the test and the standard samples are given in alternate till both produce similar rise in BP. Injections are given at intervals of five minutes. Potency of the test sample is compared with that of the standard sample.

2. On Spinal Cat: Refer pressor activity of posterior pitutary extract and apply the same method.

3. Method Using Rabbit's Duodenum: A rabbit weighing 2-3 kg. is killed by headblow method and bled to death. The animal is dissected out, the abdominal organs are exposed to isolate the duodenum. The duodenum is suspended in the inner bath of the mammalian organ bath.

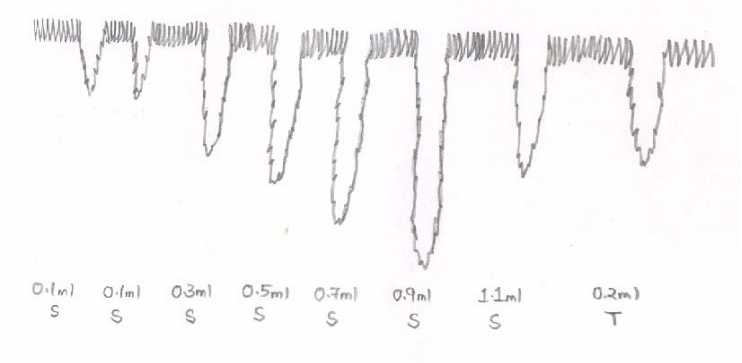


Fig.5: Bioassay of adrenaline

Inner bath is filled with Tyrode solution which is maintained at 37.5 ^{o}C. The muscle is allowed to stabilize for 30 min. Rhythmic contractions are recorded by means of the isotonic frontal writing lever on the kymograph. Adrenaline relaxes the duodenum and the same property is taken upto consideration for finding out the potency of the test sample. Adrenaline is bioassayed by graphical method as shown in Fig. 5.

4. De Jalon's Method on Rat's Uterus: This method was first demonstrated by De Jalon. A virgin female rat, weighing about 100-150 g. is used for the experiment. The rat is killed by the headblow method and bled to death. The abdomen is opened and uterine horns are isolated and placed in a petri dish containing de Jalon solution. Uterine horns are ligated and suspended in a mammalian organ bath containing modified Ringer solution having the following composition is used:

NaCl 9.0 gm; $CaCl_2$ 0.06 gm; KCl. 0.45 gm; Dextrose 0.5 gm $NaHCO_3$ 0.5 gm. and distilled water 1000 ml. This solution abolishes the rhythmic contraction of the uterus. It is maintained at 37^{o}C and bubbled continuously with air.

Two identical contractions for 30 sec. with carbachol are recorded (0.75 mcg/ml). Then selecting three different doses of standard adrenaline and one intermediate dose test adrenaline the percentage reduction of carbachol induced contractions are recorded.

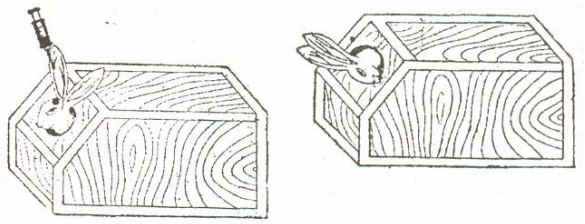
5. Straub's Method: In this method, a glass cannula is passed through the aorta and valves into the ventricle of heart so that the solution is pumped up and down in the cannula with every beat. A graph paper attached behind the cannula to give an estimale of the force of contraction of systole of the heart at every beat. The beat is recorded by attaching the tip of the ventricle through a thread to a lever. This preparation has been widely use for the detection of small quantity of adrenaline. Adrenaline increases the force of contraction of the ventricle. The increased force produced by the test sample is compared with that of the standard preparation.

(iii). Bioassay of D-Tubocurarine

1. Rabbit Head-drop Method : Principle: d-Tubocararine hydrochloride is injected into the marginal vein of a rabbit's ear till the rabbit's neck muscles are relaxed such that the animal cannot hold its head up. The total amount of test sample required to produce the endpoint is compared with the total amount of the standard sample required to produce similar endpoint.

Selection of Rabbits: Rabbits weighing 2 kg. are used. Animals should be free from disease, obtained from a healthy colony and should be accustomed with the experimental procedure.

Experimental Procedure: Each rabbit is placed in a holder with its head protruding outside. The head should be freely movable. Minimum 8 rabbits are used. They are divided into two groups each containing 4 rabbits. First group will receive standard sample and the second group will receive the sample under test. d-Tubocurarine solution is injected at a constant speed by infusion apparatus through the marginal vein.



(i) (ii)

Fig. 6: Rabbit head drop method for the bioassay of d-tubocurarine.
(i) : i.v. inj. of d-tubocurarine. (ii) : Head drop after injection.

Injection should be given at a rate of 0.4 ml/min and should take about 10 min. Infusion is continued till the rabbit will not be in a position to hold its head erect or there will be no response by focussing light on the eyes and the neck gets elongated and toneless.

Suitable dose of d-tubocurarine is 0.012% w/v in saline. Rabbits recover immediately from the effect of curarization. During the expt. there is a possibility or respiratory embarrassment which is treated by injecting neostigmine methyl sulphate (0.05 mg.) and atropine sulphate immediately through the marginal ear vein.

Cross-over test is carried out to minimise biological error due to animal variation. Those rabbits which received the standard sample on the first day will be given test sample on the second day of expt. and vice versa. Mean dose which produces head drop of the test sample is compared with the mean dose of standard preparation.

2. Frog's Rectus Abdominis muscle Preparation: A frog is pithed and laid on its back on a cork covered board to which it is pinned. The skin covering the abdomen is cut away and the rectus abdominis muscle of one side is dissected from the pelvic girdle to its insertion in the cartilage of the pectoral girdle. The muscle is then pinned to the cork by four pins to keep its normal length while a thread is sewn through each end. It is then mounted in the organ bath containing frog's Ringer solution which contains : NaCl, 6.5

gm.; KCl, 0.29 gm.; $CaCl_2$, 0.24 gm.; $NaHCO_3$, 0.4 gm.; glucose, 1.5 gm. and distilled water 2000 ml. Oxygenation is carried out to keep the tissue alive. The muscle is stabilized for 30-45 min. in order to get critical quantitative response. The responses are recorded using isotonic frontal writing lever with 1 G. tension.

Two similar contractions with the same concentration of acetylcholine are obtained. Three doses of the standard sample and one intermediate dose of the test sample are selected and the reduction in height of contraction induced by acetylcholine is noted down.

Acetylcholine contraction is recorded on slow moving drum for 90 sec. d-Tubocurarine is allowed to act for 30 sec. The percentage reduction at each dose levels is calculated and log dose response curve of the standard drug is plotted. A linear response will be obtained. The potency of test sample is calculated from the standard curve.

(iv). Bioassay of digitalis

Principle: Potency of the test sample is compared with that of the standard preparation by determining the action on the cardiac muscle. Any other equivalent method, which gives results similar to those obtained by this method as also valid.

Standard Preparation and Units: The standard preparation is a mixure of dried and powdered digitalis leaves (1 unit = 76 mg.)

Preparation of Extracts: Exact amount of the powder is extracted with dehydrated alcohol in a continuous extraction apparatus for six hours. The final extract should contain 10 ml. (5 ml. alcohol + 5 ml. water) per 10 g. of digitalis powder. It should be stored in between 5 ^{o}C and –5 ^{o}C.

1. Guinea–pig Method (End point method) : Standard and test sample extracts are diluted with normal saline in such a way that 1 g of digitalis powder is diluted to 80 ml. A guinea pig is anaesthetized with a suitable anaesthetic. It is dissected on the operation table. The jugular vein is traced out by removing adhering tissues and cannulated by means of venous cannula. A pin is inserted in the heart, such that it gets inserted in the apex of the heart. In this way, we can observe the heart beats by up and down movements of the pin. The injection is continued through venous cannula untill the heart is arrested in systole. The amount of extract required to produce this effect is taken as the lethal dose of the extract. Another set of 19 animals of the same species are used for this experiment and the average lethal dose is determined. It is not necessary to determine the lethal dose of the std. during each time of the experiment. But it should be occasionally checked.

The lethal dose of the test sample is determined in a similar way using minimum 6 guinea–pigs of the same strain.

The potency of the test sample is calculated in relation to that of the std. preparation by dividing the average lethal dose of the sample to the test and expressed as units per gram.

2. Pigeon Method: Minimum 6 pigeons are used for testing each sample. They should be free from gross evidence of disease or emaciation. The weight of the heaviest pigeon should not exceed twice the weight of the lightest pigeon. Food is withheld 16-28 hours before the experiment. Pigeons are divided on the basis of their sex, weight and breed, into two groups. They are anaesthetized with anaesthetic ether.

One side of the wing is dissected and the alar vein is cannulated by means of a venous cannula. Dilutions are made with normal saline. Average lethal dose of each sample is determined; results are tabulated and calculated as per guinea pig method. The lethal dose per kg. of body weight is determined for each pigeon. The potency of the test sample is determined by dividing the mean lethal dose of standard by the mean lethal dose of the test sample.

In pigeons, stoppage of heart is associated with a characteristic vomiting response called 'emesis'. The milk from the crop sac of pigeons is being ejected out. This may be taken as the end point response of digitalis.

(v). Bioassay of insulin

Standard preparation and unit: It is pure, dry and crystalline insulin. One unit contains 0.04082 mg. This unit is specified by Ministry of Health, Government of India and is equivalent to international unit.

Preparation of standard solution: Accurately weigh 20 units of insulin and dissolve it in normal saline. Acidify it with HCl to pH 2.5. Add 0.5% phenol as preservative. Add 1.4% to 1.8% glycerin. Final volume should contain 20 units/ml. Store the solution in a cool place and use it within six months.

Preparation of test sample solution: The solution of the test sample is prepared in the same way as the standard solution described above.

1. Rabbit Method: ***Selection of rabbits:*** They should be healthy, weighing about 1800-3000 gms. They should then be maintained on uniform diet but are fasted for 18 hrs. before assay. Water is withdrawn during the experiment.

Standard and Sample Dilutions*:* These are freshly prepared by diluting with normal NaCl solution so as to contain 1 unit/ml. and 2 units/ml.

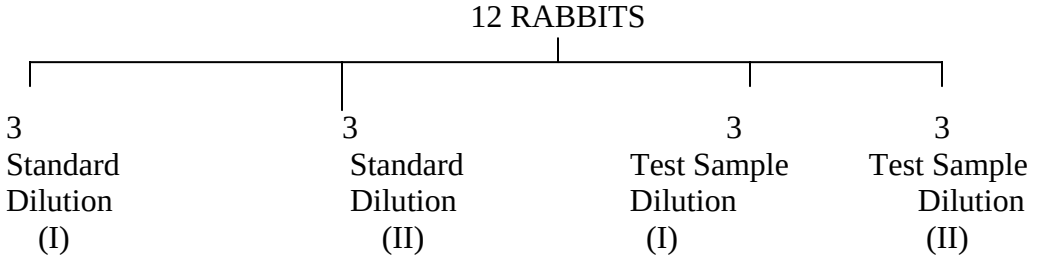
Doses*:* The dose which can produce suitable fall in blood sugar level is calculated for the standard.

Principle: The potency of a test sample is estimated by comparing the hypoglycemic effect of the sample with that of the std. preparation of insulin. Any other suitable method can also be used.

Experimental Procedure: Animals are divided into 4 groups of 3 rabbits each. The rabbits are then put into an animal holder. They should be handled with care to avoid excitement.

First part of the Test: A sample of blood is taken from the marginal ear vein of each rabbit. Presence of reducing sugar is estimated per 100 ml. of blood by a suitable chemical method. This concentration is called 'Initial Blood Sugar Level'.

The four groups of rabbits are then given sc. injections of insulin as follows:



From each rabbit, a sample of blood is withdrawn up to 5 hrs. at the interval of 1 hr. each. Blood sugar is determined again. This is known as 'Final Blood Sugar Level'.

Second part of the test **(Cross over test) :** The same animals are used for the second part. The experiment can be carried out after one week. Again they are fasted and initial blood sugar is determined. The grouping is reversed, that is to say, those animals which received the standard are given the test and those which received the test are now given the standard. Those animals which received the less dose of the standard are given the higher dose of the test sample and vice-versa. This test is known as 'Twin Cross Over Test'.

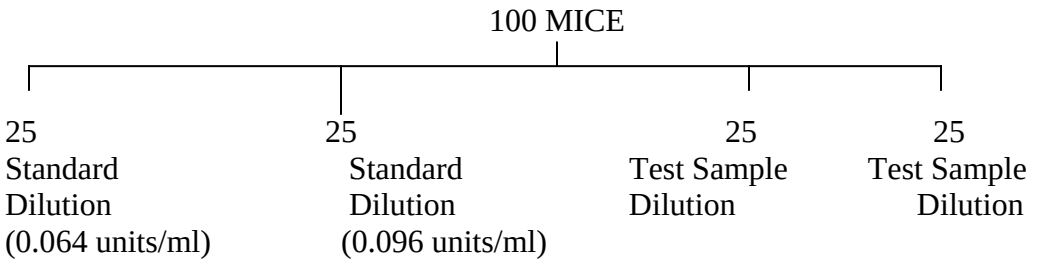
Mean percentage decrease in blood sugar of the first and second part is calculated.

2. Mouse Method: Mice show characteristic convulsions after s.c. inj. of insulin at elevated temperatures. The percentage convulsions produced by the test and standard preparations are compared.

Experimental procedure: Minimum 100 mice weighing between 18-22 gms. of the same strain are used. They should be maintained on constant diet. They should be fasted 18 hrs. prior to the experiment.

Standard and sample dilutions: Dilutions are prepared with sterile saline solution, so as to contain 0.064 units/ml. (std dilution I) and 0.096 untis/ml. (std. dilution II). Similarly, test sample solutions are also prepared.

Mice are divided into 4 groups each containing 25 mice and insulin is injected s.c. as follows:



Mice are put in an air incubator at 33^{o}C and observed for one and a half hr. An air incubator with a glass front provided with six shelves is used. The temperature is thermostatically controlled. Two mice are kept in each of the boxes made up of perforated sheets of metal.

The mice which convulse or die are taken out of the incubator and observed. These mice usually convulse severely but failure of the animal to upright itself when placed on its back, should as well be considered as convulsion. Convulsive mice may be saved by an inj. of 0.5 ml. of 5% dextrose solution.

Percentage convulsions produced by the test sample are compared with those of the standard sample. Those animals which survive may be used again for another expt. after an interval of one week.

Dose	S1 0.25 ml	S2 0.25 ml	T1 0.25 ml	T2 0.25 ml
Convulsions				
Percentage Convulsions				

3. Rat diaphragm method: In this method increase in glycogen content of the muscle or increase in glucose uptake by muscle in response to insulin is taken as the index of potency of insulin.

4. Rat epididymal fat-pad method: Here, the ability of insulin to increase CO_2 production by the fat-pad is taken as the parameter for the measurement of potency of the insulin preparation.

5. Radioimmunoassay: It is the estimation of the concentration of the substance in a unit quantity of preparation using radiolabelled antigens. A number of drugs are estimated now days by radioimmunoassy methods because these methods are highly specific and highly sensitive.

The radioimmunoassay of insulin is based on the ability of human insulin (unlabelled) to displace beef's insulin (which may be labelled) from the binding sites (i.e. antibodies). The method involves the following steps:

I. Bovine insulin is injected into the sheep. After a week the serum containing antibodies produced against bovine insulin is collected form the blood of the sheep.

II. The serum containing antibodies is exposed to radiolabelled insulin and the bound vs free ratio is determined.
III. The mixture of labelled antigen-antibodies is then added in different test-tubes labelled as standard and test. About 6 concentrations of the standards are taken. They are then added to different tubes and the bound vs free ratio is again determined using gamma-counter.
IV. Standard curves are determined and the concentration of test insulin is determinded using this standard curve.

(vi). Biological Assay of Heparin Sodium

Principle: The potency of heparin sodium is determined by comparing the concentration necessary to prevent the clotting of sheep or goat or human plasma with the concentration of the Standard Preparation of heparin sodium necessary to give the same effect under the conditions of the following method of assay.

Standard Preparation and Unit*:* The Standard Preparation is the freeze-dried sodium salt of the purified active principle from bovine intestinal mucous membranes or any other suitable preparation, the potency of which has been determined in relation to the International Standard. The Unit is the specific activity contained in 7.7 µ g of the Standard Preparation and is the same as the International Unit; 1 mg contains 130 Units.

Special Reagents: Prepared Plasma**:** Collect blood from sheep or goats or human volunteers directly into a vessel containing 8% w/v solution of *sodium citrate* in the proportion of 1 volume to each 19 volumes of blood to be collected. Mix immediately by gentle agitation and inversion of the vessel. Immediately centrifuge and pool the separated plasma. To 1 ml of the pooled plasma in a clean test-tube add 0.2 ml of a 1% w/v solution of *calcium chloride* and mix. The plasma is suitable if a solid clot forms within 5 minutes.

Solution of standard preparation: Determine by preliminary trial, if necessary, approximately the minimum quantity of the Standard Preparation of heparin sodium which, when added in 0.8 ml of saline solution, maintains fluidity in 1 ml of *prepared plasma* for 1 hour after the addition of 0.2 ml of a 1% w/v solution of *calcium chloride*. On the day of the assay prepare a solution of the Standard Preparation such that it contains in each 0.8 ml of *saline solution* the above-determined quantity of the Standard Preparation.

Test solution**:** Weigh accurately about 25 mg of the preparation being examined and dissolve in sufficient saline solution to give a concentration of 1 mg per ml and dilute to a concentration estimated to correspond to that of the solution of the Standard Preparation.

Method: To very clean test-tubes (150 mm x 16 mm) add graded amounts of the *solution of standard preparation*, selecting the amounts so that the largest dose does not exceed 0.8 ml and so that they correspond roughly to a geometric series in which each step is approximately 5% greater than the next lower. To each tube add sufficient saline solution to make the total volume 0.8 ml. Add 1.0 ml of *prepared plasma* to each tube. Then add

0.2 ml of a 1% w/v solution of *calcium chloride*, note the time, immediately stopper each tube with a suitable stopper and mix the contents by inverting three times in such a way that the entire inner surface of the tube is wet.

In the same manner set up a series using the *test solution*, completing the entire process of preparing and mixing the tubes of both the *solution of standard preparation* and the *test solution* within 20 minutes after the addition of the *prepared plasma*. Exactly one hour after the addition of the calcium chloride solution, determine the extent of clotting in each tube, recognizing three grades between zero and full clotting.

The dilution of the *test solution* which contains heparin sodium in the same concentration as the dilution of the *solution of standard preparation* is that contained in the series of dilutions which show the same degree of clotting as the series of dilutions of the *solution of standard preparation*. If the degree of clotting observed in the series of dilutions of the *solution of standard preparation* lies between that observed in two of the series of dilutions of the sample being examined, the potency of the latter is estimated. If there is no such correspondence between the degrees of clotting produced by the *solution of standard preparation* and any of the dilutions of the sample being examined, new dilutions of the latter are prepared and assay is repeated.

Carry out no fewer than three independent assays. Calculate the estimated potency of the preparation being examined by combining the results of these assays by standard statistical methods.

Limits of error - The limits of error (P = 0.99) attainable with the test are:

90 and 110%, with three determinations;
92 and 108%, with four determinations.

Heparin Sodium intended for use in the manufacture of injectable preparations without a further procedure for the removal of pyrogens complies with the following additional requirement.

(vii). Biological Assay of Oxytocin
Principle: The potency of oxytocin is determined by comparing its activity with that of the Standard Preparation of oxytocin under the conditions of a suitable method of assay.

Standard Preparation: The Standard Preparation is the 4th International Standard for Oxytocin, established in 1978, consisting of freeze-dried synthetic oxytocin peptide with human albumin and citric acid (supplied in ampoules containing 12.5 Units), or another suitable preparation the potency of which has been determined in relation to the International Standard.

Suggested methods
Method A: ***By depression of the blood pressure in chicken —*** Anaesthetise a young healthy adult cockerel weighing 1.2 to 2.3 kg with an anaesthetic that will maintain a

prolonged and constant high blood pressure. Expose the gluteus primus muscle in one thigh and cut and retract it to reveal the popliteal artery and crural vein. Cannulate the popliteal artery and record the blood pressure on a suitable recorder calibrated for use over a linear range. Cannulate the crural or brachial vein. Immediately before use prepare a solution of the Standard Preparation in saline solution so that the volume to be injected is between 0.1 ml and 0.5 ml. Record the blood pressure responses to the injection into the cannulated vein of two doses of this solution; the doses should be such as to produce clearly discriminated, precipitous, submaximal decreases in blood pressure; the required doses normally lie between 20 and 100 milliUnits. The interval between injections should be constant and lie between 3 and 10 minutes depending on the rate at which the blood pressure returns to normal. Immediately before use dilute the preparation being examined with *saline solution* so as to obtain responses similar to those obtained with the Standard Preparation. The ratio between the two doses of the preparation being examined should be the same as that between the two doses of the Standard Preparation and this ratio should be kept constant throughout the assay.

The two doses of the Standard Preparation and the two doses of the preparation being examined should be given according to a randomised block or a Latin square design and at least six responses to each should be recorded.

If the animal rapidly becomes insensitive to the repeated injections of the solutions another animal must be used. Measure all the responses and calculate the result of the assay by standard statistical methods.

Method B: ***By contraction of the rat uterus —*** Inject 100 mcg of *oestradiol benzoate* intramuscularly into a female rat weighing 120 to 200 g 18 to 24 hours before the assay. Immediately before the assay confirm by vaginal smear that the rat is in oestrus or preoestrus. Kill the rat and suspend one horn of the uterus in a bath containing a solution of the following composition:

	Composition (% w/v)
Sodium chloride	0.662
Potassium chloride	0.045
Calcium chloride	0.007
Sodium bicarbonate	0.256
Disodium hydrogen phosphate	0.029
Sodium dihydrogen phosphate	0.003
Magnesium chloride	0.010
Dextrose	0.050

Maintain the bath at a temperature of 32^{o} or at some other suitable temperature at which spontaneous contractions of the uterus are abolished and the preparation maintains its sensitivity. Oxygenate the solution with a mixture of 95% of oxygen and 5% of *carbon dioxide* and record the contractions of the muscle using a suitable instrument giving a linear response (for example an isotonic lever with a load not exceeding 2 g). Record the

contractions produced by the addition to the bath of two doses of the Standard Preparation suitably diluted with the above solution. The doses should be such as to produce clearly discriminated, submaximal contractions; the required doses normally lie between 10 and 50 micro Units per ml of bath liquid. When maximal contraction has been reached, replace the bath liquid by a fresh solution. The doses should be added at regular intervals of 3 to 5 minutes depending upon the rate of recovery of the muscle. Dilute the preparation being examined so as to obtain responses on the addition of two doses similar to those obtained with the Standard Preparation. The ratio between the two doses of the preparation being examined should be the same as that between the two doses of the Standard Preparation and this ratio should be kept constant throughout the assay.

The two doses of Standard Preparation and the two doses of the preparation being examined should be given according to a randomized block or a Latin square design and at least six responses to each should be recorded.

Measure all the responses and calculate the result of the assay by standard statistical methods.

Method C: ***By measurement of milk-ejection pressure in a lactating rat*** — Select a lactating rat, in the third to twenty-first day after parturition and weighing about 300 g, separate it from the litter and 30 to 60 minutes later anaesthetise (for example, by the intraperitoneal injection of a solution of Pentobarbitone Sodium). Tie the rat to an operating table, maintained at 37^{o}, by its hind legs leaving the front legs free. Cannulate the trachea with a short polyethylene tube of internal diameter about 2.5 mm in such a manner so as to ensure a free airway; apply artificial respiration only if necessary. Cannulate an external jugular or femoral vein with a polyethylene tube of internal diameter about 0.4 mm which is filled with saline solution and closed with a pin.

Shave the skin surrounding the inguinal and abdominal teats and excise the tip of one teat, preferably the lower inguinal teat. Insert a polyethylene tube of internal diameter about 0.3 mm and external diameter about 0.6 mm, to a depth sufficient to obtain appropriate measurement of pressure (3 to 10 mm depth), into the primary teat duct which opens onto the cut surface and tie firmly in place with a ligature. Connect this cannula with a suitable strain gauge transducer (such as that used for recording arterial blood pressure in the rat) and fill the whole system with a 3.8% w/v solution of *sodium citrate* or *saline solution* containing 50 Units of *heparin sodium* per ml to prevent clotting of milk. After cannulation, inject a small volume (0.05 to 0.2 ml) of this solution into the teat duct through the transducer to clear the milk from the tip of the cannula. (This procedure may be repeated during the assay should obstruction arise from milk ejected into the cannula). Clamp the strain gauge so that a slight tension is applied to the teat and its natural alignment is preserved and connect the gauge to a potentiometric recorder adjusted to give full-scale deflection for an increase in milk-ejection pressure of about 5.3 kPa. Inject all solutions through the venous cannula using a 1-ml syringe graduated in 0.01 ml and wash them in with 0.2 ml of *saline solution*.

Prepare a solution of the Standard Preparation and a solution of the preparation being examined in *saline solution* so that the volume to be injected is between 0.1 ml and 0.4 ml. Choose two doses of the Standard Preparation such that the increase in milk-ejection pressure is about 1.35 kPa for the lower dose and about 2.7 kPa for the higher dose. As an initial approximation, a lower dose of between 0.1 and 0.4 milliUnit and an upper dose of 1.5 to 2 times this amount may be tried. Choose two doses of the preparation being examined with the same inter-dose ratio, matching the effects of the doses of the Standard Preparation as closely as possible. Inject the four doses (two doses of the Standard Preparation and two doses of the preparation being examined) at intervals of 3 to 5 minutes. The two doses of Standard Preparation and the two doses of the preparation being examined should be given according to a randomised block or a Latin square design and at least four responses to each should be recorded. Measure all the responses and calculate the result of the assay by standard statistical methods.

(viii). Biological Assay for Vasopressor Activity

Principle*:* The vasopressor activity is estimated by comparing the activity of the preparation being examined with that of the Standard Preparation of arginine vasopressin under the conditions of a suitable method of assay.

Standard Preparation: The Standard Preparation is the Ist International Standard for Arginine vasopressin, established in 1978, consisting of freeze-dried synthetic arginine vasopressin peptide acetate with human albumin and citric acid (supplied in ampoules containing 8.20 Units), or another suitable preparation the potency of which has been determined in relation to that of the International Standard.

Suggested Method: Inject slowly into the tail vein of a male albino rat weighing about 300 g a solution of a suitable alpha -adrenoceptor blocking agent, for example 10 ml per kg of body weight of a solution prepared by dissolving 5 mg of *phenoxybenzamine hydrochloride* in 0.1 ml of *ethanol (95%)*, adding 0.05 ml of 1M hydrochloride acid and diluting to 5 ml with saline solution. After 18 hours, anaesthetise the rat with an anaesthetic that will maintain a prolonged and uniform blood pressure. After 45 to 60 minutes, tie the rat on its back to the operating table by its hind legs. Cannulate the trachea with a short polyethylene tube of external diameter about 2.5 mm and dissect a carotid artery ready for cannulation. Then cannulate the femoral vein close to the inguinal ligament. Retract the abdominal muscles to expose the inguinal ligament. Retract the superficial pudendal vein to one side and dissect the femoral vein towards the inguinal ligament from the corresponding artery. When dissecting, a deep branch reaching the femoral vein must be found and tied off to prevent bleeding during cannulation. Tie a short polyethylene cannula of external diameter about 1 mm into the femoral vein by two ligatures and join by a short piece of flexible tubing to a 1-ml burette with an attached thistle funnel containing *saline solution* at about 37^{o}. Firmly fix a wet absorbent cotton swab to the thigh so as to cover the incision and cannula. At this stage inject through the venous cannula 200 Units of heparin, dissolved in saline solution, per 100 g of body weight. Then tie in a carotid cannula of external diameter about 1 mm and connect by a column of *saline solution* containing *heparin* with a suitable pressure measuring device such as a mercury manometer of internal diameter about 2 to 3 mm.

The central and peripheral nervous system including both vagus and associated sympathetic nerves is left intact. No artificial respiration is necessary. Taking care that no air is injected, inject all solutions through the venous cannula by means of a 1-ml syringe graduated in 0.01 ml and wash in with 0.2 ml of *saline solution* from the burette.

Dilute the extract of the Standard Preparation and the preparation being examined with *saline solution* so that the volume to be injected is between 0.1 ml and 0.5 ml. Choose two doses of the Standard Preparation such that the elevation of the blood pressure is about 4 kPa for the lower dose and about 7 kPa but always submaximal for the higher dose, the ratio of low to high dose being determined by the response and usually being 3 to 5. As an initial approximation doses of 3 and 5 milliUnits may be tried. Choose two doses of the preparation being examined with the same inter-dose ratio, matching the effects of the dose of the Standard Preparation as closely as possible. Inject doses at intervals of 10 to 15 minutes. The two doses of the Standard Preparation and the two doses of the preparation being examined should be given in a randomised block or a Latin square design and four to five responses to each should be recorded. Measure all the responses and calculate the result of the assay by standard statistical methods.

(ix). Biological Assay of Chorionic Gonadotrophin

Principle*:* The potency of chorionic gonadotrophin is determined by comparing its effect in increasing the weight of the seminal vesicles or the prostate glands of immature rats with that of the Standard Preparation of chorionic gonadotrophin under the conditions of the following method of assay.

Standard Preparation*:* The Standard Preparation is the 3rd International Standard for Chorionic Gonadotrophin, human, established in 1986, consisting of a freeze-dried extract of human chorionic gonadotrophin with human albumin (supplied in ampoules containing 650 Units), or another suitable preparation the potency of which has been determined in relation to the International Standard.

Suggested Method*:* Use immature male rats of the same strain, approximately 21 days old and of approximately equal weight within the range 25 to 35 g. Assign the rats at random to four equal groups of atleast eight animals. If sets of four litter mates are available, allot one litter mate from each set at random to each group and mark according to the litter.

Choose two doses of the Standard Preparation and two of the preparation being examined such that the smaller dose is sufficient to produce a positive response in some of the rats and the larger dose does not produce a maximum response in all of the rats. As an initial approximation, doses of 7.5 and 15 Units may be tried although the dose will depend on the sensitivity of the animals used which may vary widely. Dissolve separately the total quantities of the preparation being examined and of the Standard Preparation corresponding to the daily doses to be used in sufficient albumin-phosphate buffer pH 7.2 so that the daily dose is about 0.2 ml. Add a suitable antimicrobial preservative such as 0.4% w/v of *phenol* or 0.002% w/v of *thiomersal*. Store the solutions at a temperature of 2^{o} to 8^{o}. Inject subcutaneously into each rat the daily dose allocated to its group on 4

consecutive days at the same time each day. On the fifth day, about 24 hours after the last injection, kill the rats and remove the seminal vesicles or the prostate glands from each animal. Remove any extraneous fluid and tissue from the vesicles or glands and weigh them immediately. Calculate the result of the *assay* by standard statistical methods using the weight of the vesicles or prostate glands as the response.

(x). Biological Assay of Urokinase

Principle:The potency of urokinase is determined by comparing its ability to activate human plasminogen to form plasmin with that of the Standard Preparation. The plasmin generated is determined by measurement of the time taken to lyse a fibrin clot under the conditions of a suitable method of assay.

Standard Preparation: The Standard Preparation is the 1st International Reference Preparation for Urokinase, human, established in 1968, consisting of partially purified freeze-dried urokinase from human urine with lactose (supplied in ampoules containing 4800 Units of urokinase activity) or another suitable preparation the activity of which has been determined in relation to the International Reference Preparation.

Method: Prepare a solution of the Standard Preparation containing 1000 Units of urokinase activity per ml and prepare a solution of the preparation being examined expected to have the same concentration; keep the solutions in ice and use within 6 hours. Prepare three 1.5-fold serial dilutions of the solution of the Standard Preparation so that the longest clot-lysis time is less than 20 minutes and the shortest clot-lysis time is greater than 3 minutes. Prepare three similar dilutions of the solution of the preparation being examined. Keep the solutions in ice and use within 1 hour. Using 24 tubes 8 mm in diameter, label the tubes S_1, S_2, S_3 for the dilutions of the Standard Preparation and T_1, T_2, T_3 for the dilutions of the preparation being examined, allocating four tubes to each dilution. Place the tubes in ice. Into each tube introduce 0.2 ml of the appropriate dilution, 0.2 ml of *phosphate buffer pH 7.4* containing 3% w/v of *bovine albumin* and 0.1 ml of a solution containing 20 Units of *thrombin* per ml. Place the tubes in a water-bath at 37^o and allow to stand for 2 minutes to attain temperature equilibrium. Using an automatic pipette, introduce into the bottom of the first tube 0.5 ml of a 1.0% w/v solution of *bovine euglobulin fraction* ensuring mixing. At 5-second intervals introduce successively into the remaining tubes 0.5 ml of a 1.0% w/v solution of *bovine euglobulin fraction*. Using a stop-watch, measure for each tube the time in seconds that elapses between the addition of the euglobulin and the lysis of the clot.

Using the logarithms of the lysis times, calculate the result of the assay by standard statistical methods.

(xi). Biological Assay of Streptokinase

The potency of streptokinase is determined by comparing its ability to activate human plasminogen to form plasmin with that of the Standard Preparation. The plasmin generated is determined by measurement of the time taken to lyse a fibrin clot under the conditions of a suitable method of assay.

Standard Preparation: The Standard Preparation is the 2nd International Standard for Streptokinase, established in 1989, consisting of freeze-dried streptokinase (supplied in ampoules containing 700 Units of streptokinase activity), or another suitable preparation the activity of which has been determined in relation to the International reference preparation.

***Suggested Method* :** Prepare a solution of the Standard Preparation to contain 1000 Units of streptokinase activity per ml and prepare a solution of the preparation being examined expected to have the same concentration; keep the solutions in ice and use within 6 hours. Prepare three 1.5-fold serial dilutions of the solution of the Standard Preparation so that the longest clot-lysis time is less than 20 minutes and prepare three similar dilutions of the solution of the preparation being examined. Keep the solutions in ice and use within 1 hour. Using 24 tubes, 8 mm in diameter, label the tubes S_1, S_2, S_3 for the dilutions of the Standard Preparation and T_1, T_2, T_3 for the dilutions of the Standard Preparation being examined, allocating four tubes to each dilution. Place the tubes in ice. Into each tube introduce 0.2 ml of the appropriate dilution, 0.2 ml of *citro-phosphate buffer pH 7.2* containing 3% w/v of *bovine serum albumin* and 0.1 ml of a solution containing 20 Units of *thrombin* per ml. Place the tubes in a water-bath at 37^o and allow to stand for 2 minutes to attain temperature equilibrium. Using an automatic pipette, introduce into the bottom of the first tube 0.5 ml of a 1% w/v solution to *human euglobulins* ensuring mixing. At 5-second intervals introduce successively into the remaining tubes 0.5 ml of a 1% w/v solution of human euglobulins. Using a stop-watch, measure for each tube the time in seconds that elapses between the addition of the euglobulin and the lysis of the clot.

Using the logarithms of the lysis times, calculate the result of the assay by standard statistical methods.

Current Status of Bioassay in Different Pharmacopoeia

The above mentioned bioassay have been routinely used in out of various bioassays mentioned above, some are used in academic institutions whereas official methods utilized in pharmaceutical industries. With the advent of technology, availability of advanced, sophisticated and more reliable analytical methods the scenario for bioassay has changed dramatically.

Pharmacopoeia, the official publication by competent government authorities of various countries, gives recommendations for analytical methods for various drugs to have assurance of quality of drugs. If one review the emphasis of bioassay in pharmacopeias published before 1980 as compared to those published recently, it will be clear that

(a) There are very few drugs which are now recommended to be assayed by biological methods.
(b) Most of the drugs which were assayed by biological methods are now being recommended to be assayed by chemical methods. (Table 2)
(c) Newer drugs have been included for which bioassay recommended.

Table 2: List of drugs for which bioassay was recommended in earlier pharmacopoea,* now replaced by other methods:

Names of Drugs	Assay method Recommended
Acetylcholine	Titrimetric method
Histamine	Titrimetric method
Heparin	Titrimetric method
Thyroxine	Titrimetric method
Adrenaline	UV absorbance
Progesterone	UV absorbance
Digoxin	UV absorbance
Estradiol	Chromatography
Penicillin	Chromatography
Insulin	HPLC

In the Pharmacopea (USP-95, EP-97 and IP-96) oxytocin, heparin and chorionic gonadotrophins are included for bioassay. Corticotrophin and glucagen are also recommended to be assayed by biological method. In addition to these calcitonin, pancreatin and menotrophin are to be analyzed by bioassay according to BP 93 and Vitamin D, FSH and LH are to be bioassayed as per USP-95, IP-96 has included several vaccines, streptolkinase and prokinase in the list of bioassay

Other Official Bioassays (As per Indian Pharmacopoeia 1996)

1. Biological Assay of Diphtheria Antitoxin

The potency of diphtheria antitoxin is determined by comparing the dose necessary to protect guinea-pigs or rabbits against the erythrogenic effects of a fixed dose of the Standard Preparation of diphtheria antitoxin necessary to give the same protection. For this purpose, a suitable preparation of diphtheria toxin is required to be used as a test toxin. The test dose of the toxin is determined in relation to the Standard Preparation. The potency of the preparation being examined is then determined in relation to the Standard Preparation using the test toxin.

Standard Preparation: The Standard Preparation is the Ist International Standard for Diphtheria antitoxin, equine, established in 1934, consisting of the dried hyperimmune horse serum and glycerin, or another suitable preparation the potency of which has been determined in relation to the International Standard.

Method

Test toxin: Prepare diphtheria toxin by filtering through bacteria-proof filter the medium in which a toxigenic strain of *C. diphtheriae* has grown. Store at a temperature of 2^{o} and 8^{o}.

Selection of test toxin: In selecting a toxin for use as the test toxin determine the following:

Lr/100 dose — This is the smallest quantity of the toxin which, when mixed with 0.01 Unit of antitoxin and injected intracutaneously into guinea-pigs or rabbits causes a characteristic reaction at the site of the injection within 48 hours.

Minimal reacting dose — This is the smallest quantity of toxin which, when injected intracutaneously into guinea-pigs or rabbits, causes a characteristic reaction at the site of injection within 48 hours.

A suitable toxin is one which contains at least 200 minimal reacting doses in the Lr/100 dose. The test toxin is allowed to stand for some months before being used for the **assay** of samples of antitoxin. During this time its toxicity declines and the Lr/100 dose may be slightly increased. When experiment shows that the Lr/100 dose is constant, the test toxin is ready for use and may be used for a long period. Determine the minimal reacting dose and the Lr/100 dose at frequent intervals. Store the test toxin in the dark at a temperature between 0^{o} and 5^{o}. Maintain its sterility by the addition of *toluene* or other antimicrobial preservative which does not cause a rapid decline in specific toxicity.

Determination of test dose of toxin (*Lr/100 dose)*: Prepare a solution of the Standard Preparation with *saline solution* such that 1 ml contains 0.1 Unit. Prepare mixtures such that 2.0 ml of each mixture contains 1.0 ml of the dilution of the Standard Preparation (0.1 Unit) and one of a series of graded volumes of the test toxin. Dilute each mixture with *saline solution* to the same final volume (2.0 ml). Allow the mixtures to stand at room temperature, protected from light, for 15 to 60 minutes and inject intracutaneously 0.2 ml of each mixture at suitably spaced sites into the shaven or depilated flanks of two animals. Observe the animals for 48 hours.

The test dose (Lr/100) of the toxin is the amount present in 0.2 ml of that mixture which causes at the site of injection a small, characteristic reaction in the skin of the guinea-pig or rabbit. Mixtures containing larger amounts of toxin cause larger reaction and necrosis and mixtures containing smaller amount of toxin cause no reaction.

Determination of potency of the antitoxin: Dilute the test toxin with *saline solution* so that 1.0 ml contains 10 times the test dose. Prepare mixtures such that 2.0 ml of each mixture contains 1.0 ml of the dilution of the toxin and one of a series of graded volumes of the preparation being examined. Prepare further mixtures such that 2.0 ml of each contains 1.0 ml of the solution of the test toxin and 0.1 Unit of antitoxin. Dilute each mixture with *saline solution* to the same final volume (2.0 ml). Allow the mixtures to stand at room temperature, protected from light, for 15 to 60 minutes. Inject a dose of 0.2 ml of each mixture into the animals under the conditions described in the determination of the Lr/100 dose of the toxin.

The mixture of the preparation being examined that contains 0.01 Unit of antitoxin in 0.2 ml is the mixture that produces the same degree of local reaction as that produced by the injection into the same animals of the mixture of the Standard Preparation that contains in 0.2 ml the test dose (Lr/100) of the toxin and 0.01 Unit of antitoxin.

When at least four distinct tests are carried out by this method, the limits of error have been estimated to be between 90% and 111%.

2. Biological Assay of Plague Vaccine

The potency of plague vaccine is estimated by determining the dose necessary to protect mice against a lethal dose of a virulent strain of *Yersinia pestis.*

Test Animals: Use white mice, 6 to 7 weeks old, each weighing between 20 and 28 g and of a strain susceptible to plague infection. The selected strain of mice should be such that an infective dose of 6 to 12 organisms of a virulent strain of *Y. pestis* per mouse given subcutaneously produces a mortality of not less than 80% of the animals used. The animals should be healthy and free from intercurrent infection with organisms such as Salmonella.

Suggested Method

Selection of suitable virulent strain: A freeze-dried virulent culture of *Y. pestis*, established to be suitable for challenge, is revived by sub-culturing 0.5 ml in 9.5 ml of nutrient broth contained in another test-tube and incubating at 28^{o} for exactly 48 hours. Such a culture should contain 300 to 600 million organisms per ml. Make 10-fold dilutions in *nutrient broth* and test for virulence. Use a 10^{-7} dilution containing 6 to 12 organisms in 0.2 ml as the test infective dose per animal. Those strains which produce a mortality of 80% among the animals tested with this dose are considered to be virulent.

Standard challenge dose: Freshly reconstitute the freeze-dried culture and dilute with nutrient broth to a strength such that 0.2 ml contains 60 to 120 organisms.

Measurement of protective power: Prepare a series of five graded doses of the preparation being examined arranged in such a manner that the 50% protective dose (ED_{50}) lies about the middle of the selected series. For each dose a batch of 16 mice is used. Inject subcutaneously the selected dose in two equal parts with an interval of 7 days between them. Seven days after the second half of the dose, inject subcutaneously into each group of mice the standard challenge dose. At the same time inject into 10 control mice, 8 to 9 weeks old and weighing between 28 g and 30 g, the standard challenge dose.

Observe the animals for 15 days and record the number of deaths in each group. Carry out a post-mortem on the dead animals and look for signs of plague in them. If plague organisms are not seen, such deaths are excluded from the calculation. The test is not valid unless the number of such deaths is not more than 1. At the end of the period of observation kill all the surviving animals and examine for signs of plague. Calculate the median effective immunizing dose, ED_{50}, by standard statistical methods. The vaccine passes the test if it has an ED_{50} of 0.004 ml or less per mouse.

Suggested Reading

Indian Pharmacopoeia, 1996