**Bioassay Criteria for Experimental Pharmacology**

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**Introduction**

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 is also regarded as `bioassay'. Recently `biotechnology' has also been considered for bioassay. Bioassay of the products like erythropoietin, hepatitis 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 bioassay by end point method.

The drugs producing graded responses can be bioassay 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 experimentsone using standard digitalis and the other using test preparation of digitalis is done and then potency is calculated as follows:

**Conc. of Unknown = Threshold dose of the Standard X Conc. of Std.**

**Threshold dose of the Test**

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:

**Conc. of Unknown = Dose of the Standard X Conc. of Std.**

**Dose of the Test**

**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.

**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 equative 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.

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 3point, 4point, 5 point and 6point 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 8point method also. The sequence of responses is followed as per the Latin square method of randomization in order to avoid any bias.

**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 are used similarly. The percentage inhibition is calculated, plotted against log dose of antagonist and the concentration of unknown is determined as usual.

**Bioassay of Some Important Drugs** Depending upon pharmacological action of various drugs, different preparations may be used.

**BIOASSAY OF ACTH**

ACTH (Adrenocorticotropic hormone, corticotropin) is polypeptide tropic hormone (39 amino acids) secreted by the anterior pituitary gland.

ACTH stimulates the production of cortisol, a steroid hormone important for regulating glucose, protein and lipid metabolism, suppressing the immune system response, and helping to maintainblood pressure.

**Official Preparations**

**Corticotropin injection**: Is a sterile solution, in a suitable diluent, of the polypeptide from the pituitary glands of mammals. Potency range should be 80.0 – 120.0 % of USP cardiotropin units.

Corticotropin for injection, antimicrobial agent. Repository corticotropin injection is corticotropin in a sterile solution of partially hydrolyzedgelatin and is intended for subcutaneous and intramuscular use. This solution hasbeenadopted as the reference standard for the bioassay.

**Packing**: Preserve in single-dose or multiple-dose containers of Type-1glass.

**Storage:**Store in cold place.

**Labeling**:Injection recommends intravenous administration

**Purpose and rationale**

This is a historical assay method. Administration of pituitary ACTH decrease the ascorbic acid present in the adrenals. The depletion of adrenal ascorbic acid is a function of the dose of ACTH administered. This relationship has been used for a quantitative assay ofACTH.

**Solutions:**

**Solution A:**

Five units of test or standard dissolved in 0.25 ml of 0.5% phenol solution and diluted with 8.1 ml of 15% gelatin solution (Now 0.5ml contain 300 mU ACTH).

**solution B:**

Three ml of solution A diluted with 6 ml gelatin solution. Nowconcentration reduced to 100 mU ACTH/ 0.5 ml.

**solution C:**

Again 3 ml of solution B diluted with 6 ml of gelatin solution, theresalting solution contains 33 mU ACTH/ 0.5 ml.

**Procedure**

Male Wistar rat (100-200 g) are hypophysectomized (pituitary gland removed by surgery) one day prior to the test.

For one test with 3 dose of test preparation and standard solution used for the study.

Number of hypophysectomized rats required: at least 36(preferably 60).

The hypophysectomized rats are randomly distributed in to sixgroups. Each rat receives subcutaneous 0.5 ml of the variousconcentrations of test or standard.

Three hours after injection, the animals are anesthetized andboth adrenals removed, freed from extraneous tissue andweighed. The rats are sacrificed and the scull opened to verifycompleteness of hypophysectomy.

The adrenals are homogenized in glass tubes contains 200 mgpure sand and 8.0 ml of 4% trichloroacetic acid and the ascorbicacid determined. (Roe and Kuether 1943).

The potency ratio including confidence limits is calculated withthe 3 + 3point assay.

**Ascorbic acid determination:**

**Reagents**

• 0.02% ascorbic acid solution

• 85 % sulfuric acid (9N H2SO4)

• 0.02 g/ml of dinitrophenylhydrazine in 9N H2SO4

• 0.06 g/ml of thiourea are dissolved in distilled water

• Charcoal

**Preparation of 0.02% ascorbic acid solution**

100 mg L-ascorbic acid are dissolved in 100 ml of 4% trichloroacetic acid (1mg/ml solution) (Solution A= 1 % solution)

2 ml of Solution A diluted in 10 ml of 4% trichloroacetic acid to achieve a 0.2% ascorbic acid solution (solution B)

1 ml of solution B diluted in 10 ml of 4% trichloroacetic acid to achieve a 0.02% ascorbic acid solution (solution C)

**Preparation of other solutions**

Sulfuric acid (85%) is obtained by adding 900 ml concentrated sulfuric acid to 100 ml distilled water.

Two g dinitrophenylhydrazine are dissolved in 100 ml 9 N H2SO4 (75 ml distilled water and 25 ml concentrated sulfuric acid).

Six g thiourea are dissolved in 100 ml distilled water.

**Calibration**

Trichloroacetic acid (4%) is added to 0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0 ml of the 0.02% ascorbic acid solution (solution C) and 1.0, 1,5 and 2.0 ml of the 0.2% ascorbic acid solution to reach a final volumeof 8.0 ml (Solution B).

100 mg charcoal is added to each sample and thoroughly mixed byshaking for 1 min.

After 5 min the solutions are filtered.

An aliquot of 0.1 ml of the 6% thiourea solution is added to 2.0 mlof the filtrate followed by 0.5 ml dinitrophenylhydrazine solution.

The mixture is shaken and heated for 45 min at 57°C in a waterbath.

The solutions are placed in an ice-cold water bath and withfurther cooling 2.5 ml of the 85% sulfuric acid are added.

The calibration curve is established at a wave length of 540 mmusing the solutions without ascorbic acid as blank.

**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.

**Standard Preparation and Units:**

The standardpreparation is a mixture of dried and powdereddigitalis leaves (1 unit = 76 mg.)

**Preparation of Extracts:**

Exact amount of thepowder is extracted with dehydrated alcohol in acontinuous extraction apparatus for six hours. Thefinal extract should contain 10 ml(5 ml. alcohol +5 ml. water) per 10 g. of digitalis powder. It shouldbe stored in between 5oC and – 5oC.

1. **Guinea-pig Method (Endpoint method):**

Standard and test sample extracts are diluted with normal saline in such a way that 1gof 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 movementsof the pin

The injection is continued through venous cannula until the heart is arrested in systole. The amount of extract required to produce this effect is taken as the lethal dose ofthe 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 doseof the standard 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 6guinea-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.

1. **Pigeon Method**

Minimum 6 pigeons are used for testing eachsample. The weight of the heaviest pigeon should notexceed twice the weight of the lightest pigeon.Food is withheld 16- 28 hours before theexperiment.

Pigeons are divided on the basis of their sex,weight and breed, into two groups.They are anaesthetized with anesthetic ether.

One side of the wing is dissected and the alevin is cannulated by means of a venous

cannula. Dilutions are made with normalsaline.

The test sample and standard sample isinfused through cannula.In pigeons, stoppage of heart is associated witha characteristic vomiting response called ‘emesis’.

The milk from the crop sac of pigeons is beingejected out. This may be taken as the end pointresponse of digitalis.

The lethal dose per kg. of body weight isdetermined for each pigeon.

The potency of the test sample is determinedby dividing the mean lethal dose ofstandard bythe mean lethal dose of the test sample.

**BIOASSAY OF HISTAMINE**

Histamine is present in almost all the animal tissues mostly within mast cell and basophil granules. Tissues rich in histamine are skin, gastric and intestinal mucosa, lungs, liver and placenta.

Non-mast cell histamine is present in brain, epidermis, gastric mucosa and growing regions.

Histamine is also present in blood, most body secretions, venoms and pathological fluids. It is now known to play important physiological roles. Histamine produces effects by acting on the histamine receptors (H1, H2, H3 and H4) present on target cells.

Bioassay of histamine on isolated guinea pig ileum can be determined by graded bioassay procedure i.e.

1. **Matching bioassay**
2. **Interpolation bioassay**
3. **Bracketing assay**
4. **Multiple point assays.**

Bioassay of histamine in biological samples can be studied by using different bioassay methods.

Depending upon pharmacological action, histamine can be assayed by:

Contractile effect of isolated ileum of guinea pig,

Contractile effect of uterusof guinea pigand

Fall in blood pressure of anaesthetized and atropinized cat. Guinea pig's uterus

**Bioassay of histamine using guinea pig ileum:**

**Principle:**

Guinea pig ileum is very useful preparation for the bioassay methods. It is more sensitive to histamine. Contractile response of histamine to ileum is due to presence of H1 receptor.

**Preparation of Standard and other solution:**

Prepare the stock of Tyrode solution. Also prepare the standard stock solution of histamine (1 mg/ml) and then different concentrations of histamine by serial dilution method.

**Procedure:**

Sacrifice the 24 hr fasted guinea pig by stunning on the head and carotid bleeding. Fix the animal on the dissecting board by tying its legs. Open the abdominal cavity by a small horizontal cut followed by vertical midline incision and expose the abdominal organs.

Trace the ileocecal junction by lifting the caecum, then go upwards up to 8 cm from the junction and cut the 2-3 cm long segment of ileum muscle (excluding the terminal 5-8 cm, contains excess of excitatory α-adrenergic receptor, which may interfere in the study).

Immediately place it in a petri dish containing aerated warm Tyrode solution. Slowly remove the mesentery attached to the muscle and then gently clean the lumen of ileum by passing Luke warm Tyrode solution through it with a pipette or syringe.

Mount the preparation in the inner organ bath containing Tyrode solution (20 ml) maintained at 37ºC. Tie the bottom end of the muscle to the hook of aeration tube and the upper end to the isotonic frontal lever by a thread without closing the lumen.

Adjust the magnification of response to 7-10-fold.

Aerate the tissue with O2 or carbogen slowly (40-60 bubbles per minutes).

Stabilize the tissue for 30 min by applying a tension of 0.5 g weight attached to the lever, during which wash the tissue with fresh Tyrode solution once in every 10 min.

Use 5 min time cycle with contact time of 30s for recording the contraction dependent responses of tissue due to histamine on the smoked drums.

Record the responses of standard and test compounds.

Record the responses of test compound i.e., unknown concentration of histamine with gradually increasing volume, till obtaining a response (T) which would lie on the linear portion (30-70%) of the CRC.

Fix the response obtained due to volume of T.

Record the graded responses of standard solution and test solutionof histamine.

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

**Bioassay of Insulin**

In 1921 Banting and Best first obtained insulin in the form of pancreatic extract. In 1922 the extract containing insulin was first used on a 14 years old boy suffering from severe diabetes mellitus with excellent response. Insulin was then purified in a few years.

**Chemistry, synthesis and secretion** Natural insulin is a polypeptide synthesized from the precursor proinsulin. It has two peptide chains - A chain (21 amino acids) and B chain (30 amino acids) linked by disulphide bridges. Human insulin differs from bovine insulin by 3 amino acids and from porcine insulin by 1 amino acid. Hence porcine insulin is closer to humaninsulin.

Insulin is stored in granules in the β islet cells of the pancreas. Normal pancreas releases about 20-40 units of insulin every day. The secretion is regulated by factors like food, hormones and autonomic nervous system. Blood glucose concentration is the main factor. The islets of Langerhans are composed of 4 types of cells–β cells secrete insulin, α (A) cells glucagon, δ (D) cells somatostatin and P cells secrete pancreatic polypeptide. Insulin is metabolized in the liver, kidney and muscle.

**Mechanism of action** Insulin acts by binding to specific receptors. Insulin receptor is made up of two α and two β subunits. Insulin receptors are present on almost all cells in the body. Insulin binds to these receptors present on the surface of target cells. This binding stimulates tyrosine kinase activity in the β subunit. This in turn activates a cascade of phosphorylation and dephosphorylation reactions which stimulate or inhibit the enzymes involved in the metabolic actions of insulin.

**Standard preparation and unit:**

It is a 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 HCI to ph. 2.5

Add 0.5%phenol as a preservative

Add 1.4% to 1.8%glycerine

Final volume should contain 20 unit/ml

Store the solution in a cool place and use it within six months.

**Preparation of test sample solution:**

The solution of test sample is prepared in the same way as the standard solution.

**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 18hrs. Before assay water is withdrawn during the experiment.

**Standard and sample Dilution:**

These are freshly prepared by diluting with normal NaCL solution so as to contain 1 unit/ml and 2unit/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.

**Experimental Procedure:**

Animals are divided in to 4 groups of 3 rabbits each. The rabbits are then put into a 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. Injection of insulin as follows:

3 std dilution I

3 std dilution II

3 test sample dilution I

3 test sample dilution II

From each rabbit, a sample of blood is with drawn 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 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 receive the less dose of standard are given the higher dose of the test sample and vice-versa. **This test is known as Twin cross over test.**

**MOUSE METHOD:**

Mice show characteristic convulsion after s.c. injection of insulin at elevated temperature. The percentage convulsions produced by the test and standard preparations are compared.

**Experimental Procedures:**

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 18hrs. prior to the experiment.

**Standard and sample dilution:**

Dilutions are prepared with sterile saline solution so as to contain 0.064 units/ml (std dilution 1) and 0.096 units/ml (std dilution 2). Similarly, test sample solution is also prepared.

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

25 standard dilution 0.064units/ml

25 standard dilution 0.096 units/ml

25 test sample dilution

25 test sample dilution

Mice are put in an air incubator at 33c and observed for one and a half hr. 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 ae well be considered as convulsion.

**RAT DIAPHRAGM METHOD:**

Sprague Dawley rats weighing 70-100g are used. The animals are sacrificed during anesthesia and the diaphragm still attached to the rib cage are carefully removed, released from the rib cages and adhering connective and fat tissues. Washed spread out and divided in to two equal pieces as described by muller and coworkers 1994.

For assaying the effect of insulin /compounds/drugs the hemidiaphragms are incubated vin KRH buffer gassed with carbogen 95%O2/5%CO2 in the presence of 5mM glucose.

**EPIDIDYMAL FAT PAD OF RAT:**

Insulin like activity can be measured by the uptake of glucose in to fat cells. Adipose tissue from the epididymal fat pad of rats has been found to very suitable.

The difference of glucose concentration in the medium after incubation of pieces of epididymal rat adipose tissue measured oxygen consumption in Warburg vessels, Radiolabeled 14C glucose the 14CO2 is trapped and counted.

The concentration is determined by immunoassay.

**BIOASSAY OF OXYTOCIN**

Oxytocin is a peptide hormones and Neuropeptide. Oxytocin is normally produced by the paraventricular nucleus of the hypothalamus and released by the posterior pituitary. Oxytocin is a natural hormone that causes the uterus to contract. Oxytocin is used to induce labor or strengthen labor contractions during childbirth, and to control bleeding after childbirth. Oxytocin is also used to stimulate uterine contractions in a woman with an incomplete or threatenedmiscarriage.

* **Different bioassay methods used for oxytocin are**

**By contraction of the rat uterus**

**By depression of the blood pressure in chicken**

**By measurement of milk ejection pressure in a lactating rat etc.**

**Principle:**

The potency of oxytocin injection is determined by comparing its activity with that of the standard preparation of oxytocin under the conditions of the following method of assay.

**Standard preparation:**

The standard preparation is consisting of a freeze-dried preparation of oxytocin with human albumin and citric acid (supplied in ampoules containing 12.5 units) or any other suitable preparation, the potency of which had been determined in relation to the International standard.

The Unit is the specific oxytocin activity corresponding to that yielded by 0.0005 g of the Standard preparation.

The standard preparation is as per the 4th international standard for Oxytocin, established in 1978.

**Experimental Methods:**

**1) By contraction of the rat uterus:**

Use female rats weighingbetween 120 and 200 g. Immediately before the assay confirm the rat is in oestrous or proestrus( A recurring period of sexual receptivity and fertility) by vaginal smear.

Inject 100 microgram of oestradiol benzoate intramuscularly 18-24 hours before the assay. Kill the rat and suspend one horn of the uterus in a bath containing a solution of the

**followingcomposition:**

|  |  |
| --- | --- |
| **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 32º C so that spontaneous contraction of the uterus is abolished and preparation maintain its sensitivity.

Oxygenate the solution with a mixture of 95% of oxygen and 5% of carbon dioxide.

Record the contractions of the muscles 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 (being less than the maximum) contractions. The required doses normally lie between 10 and 50 microUnitsper ml of bath liquid.

When maximal contraction has reached replace the bath liquid by a fresh solution.

The doses should be added at regular intervals of 3 to 5minutes depending upon the rate of recovery of the muscle.

Dilute the preparation being examined so as to obtain the responses on the addition of two doses similar to thoseobtained with the Standardpreparation.

The ratio between the two doses of the preparation being examined should be the same as that between the twodoses of the Standard Preparation and this ratio should keptconstant throughout the assay

The two doses of Standard preparation and the two doses of the test preparation should be given according to a randomized order or Latin square design and at least six responses toeach should be recorded.

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

**2) By depression of the blood pressure in chicken:**

Anaesthetize a young healthy adult cockerel weighing 1.2 to 2.3 kg with an anesthetic 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 cruralvein.

Cannulate the popliteal artery and record the bloodpressure.Cannulate the crural or brachial vein.

Prepare standard solution with saline so that the volume to be injected is between 0.1- 0.5 ml.

Inject 2 doses of standard solution into cannulated vein and record blood pressure. The doses should be such that as to produce clearly discriminated, precipitous, submaximal decreases in B.P.

The required doses normally lie between 20 and 100 milli units.

The interval between injections should be constant and lie between 3-10 minutes depending on the rate at which B.P. returnstonormal.

Dilute test sample before use with saline solution so as to obtain responses similar to those obtained with the standard preparation.

The ratio between the two doses of the test preparation being examined should be same as that between the two doses of the standard preparation and the ratio should be kept constant throughout the assay.

The two doses of Standard preparation and the two doses of the test preparation should be given according to a randomized order or Latin square design and at least six responses toeach should be recorded.

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

**3) By measurement of milk-ejection pressure in a lactating rat:**

Select alactating rat, in the third to twenty-first day after parturition and weighing about 300 gseparate it from the litter and 30 to 60 minutes later anaesthetize (for example, bytheintraperitoneal injection of a solution of Pentobarbitone Sodium).

Tie the rat to anoperating table, maintained at 37, by its hind legs leaving the front legs free. Cannulatethe trachea with a short polyethylene tube of internal diameter about 2.5 mm in such amanner 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 internaldiameter 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 oneteat, preferably the lower inguinal teat. Insert a polyethylene tube of internal diameterabout 0.3 mm and external diameter about 0.6 mm, to a depth sufficient to obtainappropriate measurement of pressure (3 to 10 mm depth), into the primary teat ductwhich opens onto the cut surface and tie firmly in place with a ligature.

Connect thiscannula with a suitable strain gauge transducer (such as that used for recording arterialblood pressure in the rat) and fill the whole system with a 3.8% w/v solution of sodiumcitrate or saline solution containing 50 Units of heparin sodium per ml to prevent clottingof milk.

After cannulation, inject a small volume (0.05 to 0.2 ml) of this solution into theteat duct through the transducer to clear the milk from the tip of the cannula. (Thisprocedure may be repeated during the assay should obstruction arise from milk ejectedinto the cannula).

Clamp the strain gauge so that a slight tension is applied to the teat andits natural alignment is preserved and connect the gauge to a potentiometric recorderadjusted to give full-scale deflection for an increase in milk-ejection pressure of about 5.3kPa.

Inject all solutions through the venous cannula using a 1-ml syringe graduated in0.01 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 beingexamined in saline so that the volume to be injected is between 0.1 ml and 0.4ml.

Choose two doses of the Standard Preparation such that the increase in milkejectionpressure is about 1.35 kPa for the lower dose and about 2.7 kPa for the higher dose.

As aninitial approximation, a lower dose of between 0.1 and 0.4 milliUnit and an upper dose of1.5 to 2 times this amount may be tried.

Choose two doses of the preparation beingexamined with the same inter-dose ratio, matching the effects of the doses of theStandard Preparation as closely as possible.

Inject the four doses (two doses of theStandard Preparation and two doses of the preparation being examined) at intervals of 3to 5 minutes.

The two doses of Standard Preparation and the two doses of the preparationbeing examined should be given according to a randomized block or a Latin squaredesignand at least four responses to each should be recorded. Measure all the responsesand calculate the result of the assay by standard statistical methods.

**BIOASSAY OF d-TUBOCURARINE**

1. **Rabbit Head-drop Method**

**Principle:** d-Tubocurarine 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 producesimilar 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:**

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 eachcontaining 4 rabbits.

First group will receive standard sample andthe second group will receive the sampleunder test.d-Tubocurarine solution is injected at aconstant speed by infusion apparatus throughthe marginal vein.

Injection should be given at a rate of 0.4ml/min and should take about 10 min. Dose0.012% w/v in saline.

Infusion is continued till the rabbit will not bein a position to hold its head erect or therewill be no response by focusing light on theeyes.Rabbits recover immediately from the effectof curarization.

During the experiment there is a possibility ofrespiratory embarrassment which is treatedby injecting neostigmine methyl sulphate(0.05 mg.) and atropine sulphate immediatelythrough the marginal ear vein.

Cross-over test is carried out to minimizebiological error due to animal variation.

Those rabbits which received the standardsample on the first day will be given testsample on the second day of experiment andvice versa.

Mean dose which produces head drop of thetest sample is compared with the mean doseof standard preparation.

1. **Frogs 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 in the cartilage of the pectoral girdle.

The muscle is then pinned to the cork byfour 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, 6.5gm.;KCl, 0.29 gm.; CaCl2, 0.24 gm.; NaHCO3, 0.4 gm.; glucose, 1.5 gm. and distilledwater 2000 ml.

Oxygenation is carried out to keep the tissue alive. The muscle isstabilized 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 noteddown.

Acetylcholine contraction is recorded on slow moving drum for 90 second. DTubocurarine 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 beobtained. The potency of test sample is calculated from the standard curve.

**BIOASSAY OF VASOPRESSIN**

**OR**

**Biological Assay for Vasopressor Activity**

**Principle:**

The vasopressor activity is estimated by comparing the activity of thepreparation 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 forArginine vasopressin,established in 1978,consisting of freeze-dried synthetic argininevasopressin peptide acetate with human albumin and citric acid(supplied in ampoulescontaining 8.20 Units),or another suitable preparation the potency of which has beendetermined in relation to that of the International Standard.

**Method:**

* Inject slowly into the tail vein of a male albino rat weighing about300 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 phenoxy benzamine 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, anaesthetize the rat with 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 in genial 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 in genial ligament rom 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.
* 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 bodyweight.
* 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 3to 5.
* As an initial approximation doses of 3 and 5 milli Units 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 randomized 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.**