Pharmaceutical Quality Management

BIOLOGICAL ASSAY

By:

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ASSAY:

An assay is an investigative (analytic) procedure in laboratory medicine, pharmacology, environmental biology, and molecular biology for qualitatively assessing or quantitatively measuring the presence or amount or the functional activity of a target entity (the analyte) which can be a drug or biochemical substance or organic sample.

Types Of Assay

1. Chemical Assay
2. Immunoassay
3. Bioassay

Chemical Assay:

It is the study of the separation, identification and quantification of the chemical components of natural and artificial materials.

Immunoassay

A technique that makes use of the binding between an antigen and its homologous antibody in order to identify and quantify the specific antigen or antibody in a sample

Biological Assay

- Biological testing procedure for estimating the concentration of a pharmaceutical drug substance in a formulated drug product or bulk material

- The specific potency of drug is given to animal or human volunteers and then drug response is compared with the standards.

➢ Comparison of Chemical & Bioassay

BIOASSAY	CHEMICAL ASSAY
➢Less Precise	➢More Precise
➢More time consuming	➢Less time consuming
➢More expensive	➢Less expensive
➢Active constituent & structure not known.	➢Active constituent & structure fully established.
➢More sensitive	➢Less sensitive
➢More men power Required	➢Less men power required
➢Difficult to handle	➢Easy to handle

➢ Indications of Bioassay

1. Chemical method is either
   - Not available
   - If available, too complex,
   - Insensitive to low doses e.g. Histamine
2. If active principle of drug is not known e.g. insulin
3. Unknown Chemical composition, e.g. long acting thyroid stimulator
4. Chemical composition of drug variable but has same pharmacological action e.g. cardiac glycosides.
5. Active principle cannot be isolated e.g. posterior pituitary extract, insulin etc.
6. Biological activity of drug cannot defined by a chemical assay e.g. Cis and Trans form of methyl phenidate.

7. Not possible to separate interfering substance e.g. Vitamin D.

➢ Principles of Bioassay

- All bioassays should be comparative against a standard drug
- Standard & new drug should be as far as possible identical to each other
- Activity assayed should be the activity of interest
- The degree of pharmacological response produced should be reproducible under identical conditions. e.g. Adrenaline.
- Method of comparison preferably (not essentially) test therapeutic property of drug.
- Individual variations must be minimised.

➢ Classification of Bioassay

There are three types of bioassay:

1. Quantal
2. Graded
3. Effect produced in confined period

1. QUANTAL

- All or none response in all individuals,

  e.g. Digitalis induced cardiac arrest in guinea pigs
  hypoglycemic convulsions in mice by insulin and
  Calculation of LD50 in mice or rats

- Not précised

  Employed for:

- Comparison of LD50 and ED50
- Comparison of Threshold response

2. GRADED BIOASSAY

- Effect is produced gradually depending on dose.
- E.g. Contraction of smooth muscle preparation

➢ Accuracy Limits of Bioassay

"Accuracy improves the efficiency of bioassay for pharmaceutical biological products."

- An accuracy within ± 20 % of true value is good.
- An accuracy within ± 10 % of true value is excellent.

➢ Dependency Of Biological Testing Procedure

Biological assay depends on:

- size of the dose
- intensity of drug

— It is not necessary that the effect produced in animals would be same for that produced in humans. But few exceptions are there this is the main draw back of bioassay.

➢ Partial List of Official Quantitative Biological Tests

Drug and Dosage form	Test Animal (s)
Antibiotics	Suitable microorganism
Insulin	Rabbit & Mice
Digitalis & other glycosides	Pigeon & Guinea Pig
Vitamin A & D	Rat
Parathyroid drugs	Dog
Posterior pituitary	Rat
Tubocurarine Chloride	Rabbit

➢ Purpose of Bioassay

1. Compare test sample with standard substance to determine quantity of test sample required to produce an equivalent biological response to that of the standard substance.
2. Measuring pharmacological activity of new or chemically undefined substance.
3. Test method employed in measuring the response of living animals to toxicity of chemical contaminant. Certain no. of individuals of sensitive specie are exposed to specific conc. of contaminant for specific period to examine toxic effects.
4. Investigating function of endogenous mediators.
5. Determine concentration as well as potency of unknown substance.
6. Improving and maintaining standards of basic environmental conditions affecting well-being of people e.g. pollutants released by particular source
7. To determine specificity of compounds to be used e.g.

   penicillin's are effective against G+ve but not on G-ve.

➢ Preparation of Standard

A selective representative sample of a substance for which it is to serve as a basis of the measurement is called standard preparation.

- Uniform quality
- Stable

Types of Standard Preparation

Two type of standard preparation:

1) *International standard and reference standard*

- USP units(highly recognized-able and authorized standard)

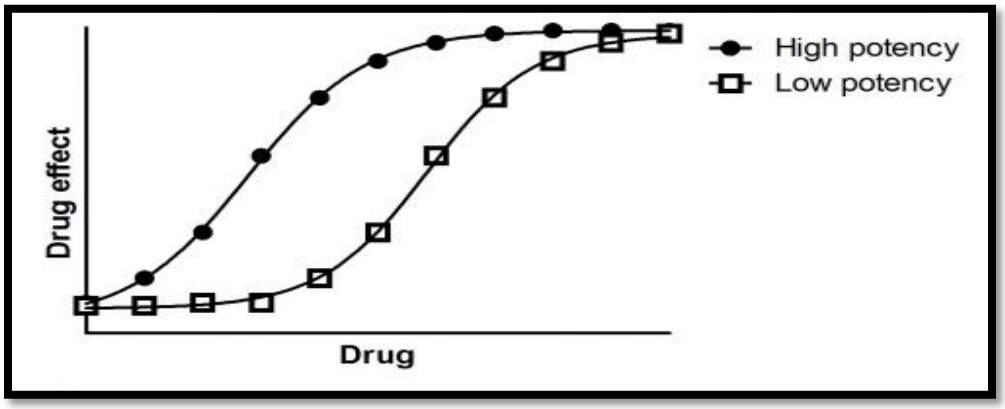
2) *British standard and reference standard*

➢ Potency

Measurement of drug activity expressed in terms of amount required to produce an effect of given intensity.

EXAMPLES:

Highly potent drugs like morphine, alprazolam, chlorpromazine etc produced high response at low conc. And low potent drugs like ibuprofen and acetylsalicyclic acid produced low response at low conc.



➢ Advantages

- Bioassays are procedures that can determine the concentration of purity or biological activity of a substance such as vitamin, hormone, and plant growth factor.
- While measuring the effect on an organism, tissue cells, enzymes or the receptor is preparing to be compared to a standard preparation.
- Bioassays may be qualitative or quantitative. Qualitative bioassays are used for assessing the physical effects of a substance that may not be quantified, such as abnormal development or deformity.

- Quantitative bioassays involve estimation of the concentration or potency of a substance by measurement of the biological response that it produces. Quantitative bioassays are typically analyzed using the methods of biostatistics.
- They not only help to determine the concentration but also the potency of the sample.
- It is especially used to standardize drugs, vaccine, toxins or poisons, disinfectants, antiseptics etc. as these are all used over biological system in some or other form.
- These also help determine the specificity of a compound to be used ex: Penicillin's are effective against Gram+ve but not on Gram-ve. Testing of infected patients sputum helps determine which anti-biotic be given for quick recovery.
- Certain complex compounds like Vitamin B-12 which can't be analyzed by simple assay techniques can be effectively estimated by Bioassays.
- Sometimes the chemical composition of samples are different but have same biological activity.
- For samples where no other methods of assays are available.
- Biological products like toxin, anti-toxin, sera can be conveniently assayed. Measure minute (Nano mole & Pico mole) quantities of active substances can detect active substance without prior extraction or other treatment.

➢ Disadvantages

- Key problem is variability in response
- Large number of animal to be used
- Expertise in experimental design, execution of assay & analysis of data required

- Leads to expensive & time consuming
- Time related changes ins sensitivity of test organ.
- Tachyphylactic responses of substance being assayed.

1. BIOASSAY OF ANTIBIOTICS

- The activity (potency) of antibiotics can be demonstrated by their inhibitory effect on microorganisms under suitable conditions. A reduction in antimicrobial activity may not be adequately demonstrated by chemical methods. This chapter summarizes procedures for the antibiotics recognized in the United States Pharmacopeia (USP) for which the microbiological assay is the standard analytical method.
- Two general techniques are employed, the cylinder plate (or plate) assay and the turbidimetric (or tube) assay.

Table 1

Antibiotic	Type of Assay
Amphotericin B	Cylinder-plate
Bacitracin	Cylinder-plate
Bleomycin	Cylinder-plate
Capreomycin	Turbidimetric
Carbenicillin	Cylinder-plate
Chloramphenicol	Turbidimetric
Chlortetracycline	Turbidimetric
Cloxacillin	Cylinder-plate
Colistemethate	Cylinder-plate
Colistin	Cylinder-plate
Dihydrostreptomycin	Cylinder-plate
	Turbidimetric

➢ Techniques:

1.1. Cylinder-plate assay

The cylinder-plate assay depends on diffusion of the antibiotic from a vertical cylinder through a solidified agar layer in a Petri dish or plate. The growth of the specific microorganisms inoculated into the agar is prevented in a circular area or *zone around* the cylinder containing the solution of the antibiotic.

1.2. Turbidimetric assay

The turbidimetric assay depends on the inhibition of growth of a microorganism in a uniform solution of the antibiotic in a fluid medium that is favorable to the growth of the microorganism in the absence of the antibiotic.

1.1. CYLINDER-PLATE METHOD

➢ **Temperature control:**

Use appropriately qualified and calibrated equipment to obtain the temperature ranges specified.

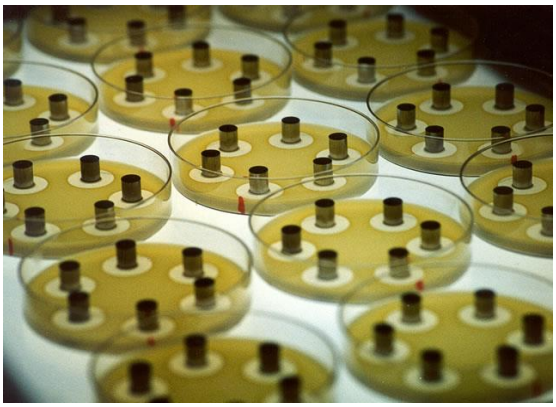
➢ **Apparatus**

- Plates:

  Glass or disposable plastic Petri dishes (approximately 20 x 100 mm) with lids

- Cylinders:

  Stainless steel or porcelain cylinders; 8 mm + 0.1 mm o.d.; 6 mm + 0.1 mm i.d.; 10 mm+0.1 mm high



➢ Standard solutions

- To prepare a stock solution, dissolve a suitable quantity of the USP Reference Standard of a given antibiotic, or the entire contents of a vial of USP Reference Standard, where appropriate, in the solvent specified in and dilute to the specified concentration. Store at 2–8, and use within the period indicated. On the day of the assay, prepare from the stock solution five or more test dilutions, in which the successive solutions increase stepwise in concentration, usually in the ratio of 1:1.25.

➢ Sample solutions

- Assign an assumed potency per unit weight or volume to the sample. On the day of the assay prepare a stock solution in the same manner specified for the USP Reference Standard. Dilute the sample stock solution in the specified final diluent to obtain a nominal concentration equal to the median concentration of the standard (S3).

➢ Inocula:

- Suspend the test organism from a freshly grown slant or culture in 3 mL of sterile saline TS. Glass beads can be used to facilitate the suspension. Spread the saline suspension onto the surface of two or more agar plates (covering the entire surface) or onto the surface of a Roux bottle containing 250 mL of

the specified medium. Incubate for the specified time and at the temperature as specified, or until growth is apparent.

- Prepare the inoculum by adding a portion of stock suspension to a sufficient amount of agar medium that has been melted and cooled to 45–50 ºC. Swirl the mixture without creating bubbles in order to obtain a homogeneous suspension.

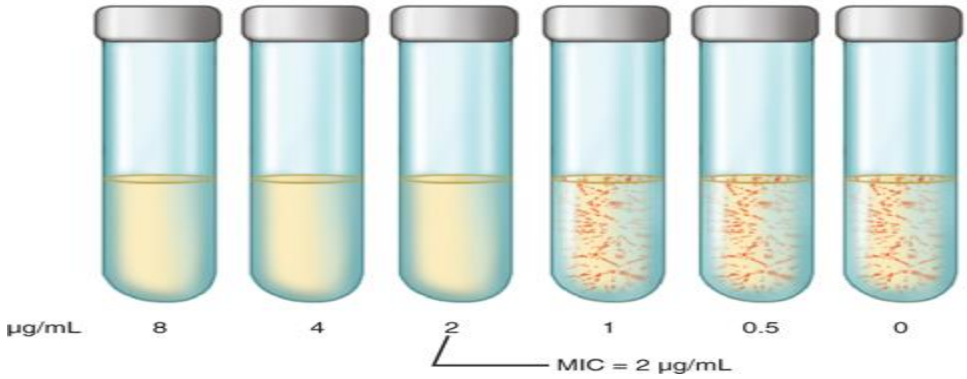
➢ **Analysis:**

- Prepare the base layer for the required number of assay Petri plates, using the medium and volume. Allow it to harden into a smooth base layer of uniform depth. Prepare the appropriate amount of seed layer inoculum as directed for the given antibiotic with any adjustments made based on the preparatory trial analysis. Tilt the plate back and forth to spread the inoculum evenly over the base layer surface, and allow it to harden.
- Incubate the plates for 16–18 h, and remove the cylinders. Measure and record the diameter of each zone of growth inhibition to the nearest 0.1 mm.

1.2. TURBIDIMETRIC METHOD

➢ **Apparatus:**

- Glass or plastic test tubes, e.g., 16 × 125 mm or 18 × 150 mm.
- Spectrophotometer: Measuring absorbance or transmittance within a fairly narrow frequency band requires a suitable spectrophotometer in which the wavelength can be varied or restricted by use of 580-nm or 530-nm filters. Alternatively, a variable-wavelength spectrophotometer can be used and set to a wavelength of 580 nm or 530 nm.



➢ Standard solutions:

- To prepare a stock solution, dissolve a quantity of the USP Reference Standard of a given antibiotic or the entire contents of a vial of USP Reference Standard, where appropriate, in the solvent specified, and dilute to the required concentration. Store at 2–8, and use within the period indicated. On the day of the assay, prepare from the stock solution five or more test dilutions, the successive solutions increasing stepwise in concentration, usually in the ratio of 1:1.25. Use the final diluent specified such that the median level of the standard (S3)

➢ Sample solutions:

- Assign an assumed potency per unit weight or volume to the unknown, and on the day of the assay prepare a stock solution in the same manner specified for the USP Reference Standard. Dilute the sample stock solution in the specified final diluent at a nominal concentration equal to the median concentration of the standard (S3)

➢ Inocula:

- Suspend the test organism from a freshly grown slant or culture in 3 mL of sterile saline TS. Glass beads can be used to facilitate the suspension. Enterococcus hirae (ATCC 10541) and Staphylococcus aureus (ATCC

9144) are grown in a liquid medium, not on agar. Spread the saline suspension onto the surface of two or more agar plates (covering the entire surface) or onto the surface of a Roux bottle containing 250 mL of the specified medium. Incubate at the time and temperature specified, or until growth is apparent. After incubation, harvest the organism from the plates or Roux bottle with approximately 50 mL of sterile saline TS, using a sterile bent glass rod or sterile glass beads. Pipet the suspension into a sterile glass bottle. This is the harvest suspension.

- **Analysis:**
  - On the day of the assay, prepare the necessary concentration of antibiotic by dilution of stock solutions of the standard and of each sample as specified under Standard solutions and Sample solutions. Prepare five test levels, each in triplicate, of the standard (S1–S5) and a single test level (U3), also in triplicate, of up to 20 samples corresponding to S3 (median concentration) of the standard.
  - Place the tubes in test tube racks or other carriers. Include in each rack 1–2 control tubes containing 1 mL of the inoculum medium but no antibiotic. Add the volumes of the standard and sample test dilutions as indicated. Randomly distribute one complete set, including the controls, in a tube rack. Add the volume of inoculum specified to each tube in the rack in turn, and place the completed rack immediately in an incubator or a water bath maintained at the temperature specified and for the time specified.

2. 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.)

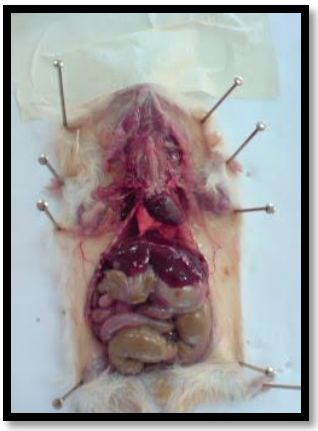
➢ Preparation of Extracts

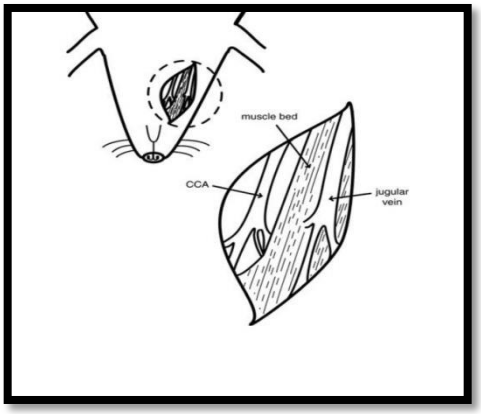
- Exact amount of the powder is extracted with dehydrated alcohol in a continuous extraction apparatus for six hours. The final extract should contain 10 ml. (5 ml. alcohol + 5 ml. water) per 10 g. of digitalis powder. It should be stored in between 5 ºC and –5 ºC.

2.1. 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 standard. during each time of the experiment. But it should be occasionally checked.





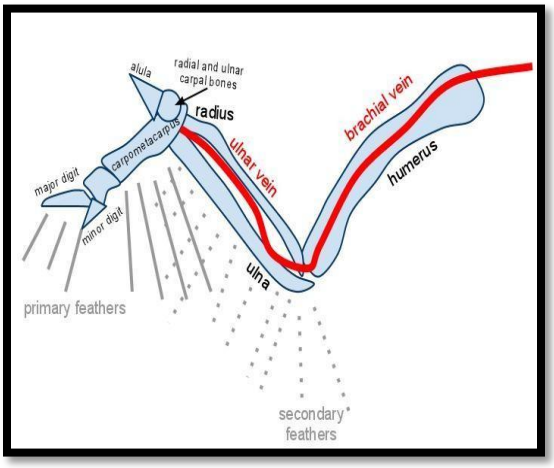
- The lethal dose of the test sample is determined in a similar way using minimum 6 guinea–pigs of the same strain.

  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.

2.2. 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 ulnar 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.

3. BIO-ASSAYS OF INSULINE

➢ Mechanism of action

- Every pancreatic islet contains ~1,000 endocrine cells of which 75% are insulin-producing beta-cells.
- Insulin is synthesised as pro-insulin in the endoplasmic reticulum and is processed to the biologically active form inside the secretory granules.

- The beta-cell is electrically excitable and uses changes in membrane potential to couple variations in blood glucose to trigger insulin secretion.
- The beta-cell contains about 20 different ion channels proteins
- Two types of ion channels are particularly important for the initiation of insulin secretion. The KATP-channels are active at low glucose concentrations, because of the high intracellular ADP levels.

➢ Bioassay of insulin

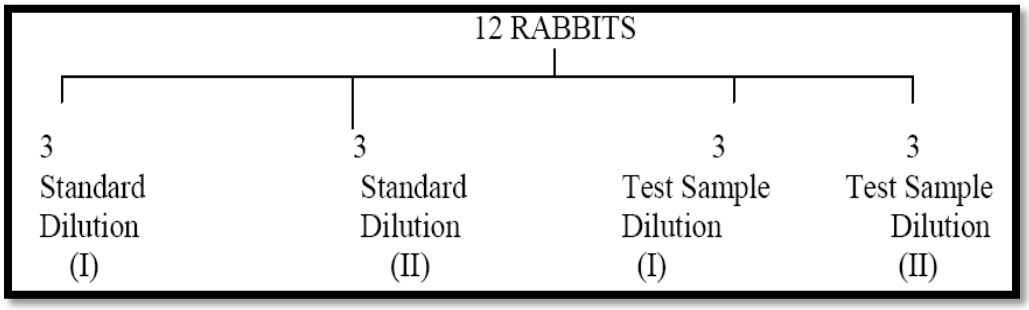
- Standard preparation and unit: It is pure, dry and crystalline insulin. One unit contains 0.04082 mg. This unit is equivalent to international unit.
- Preparation of standard solution: Accurately weigh 20 units of insulin and dissolve it in normal saline. Acidify it with HCl to pH 2.5. Add 0.5% phenol 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.

3.1. 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'.

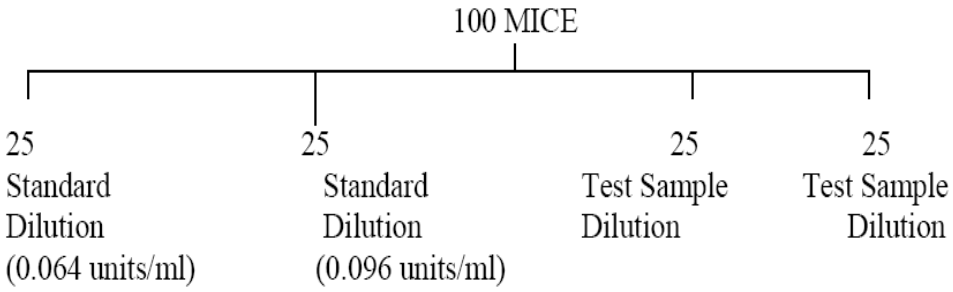
3.2. Mouse Method

- 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 33ºC 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 as well be considered as convulsion.

3.3. Rat diaphragm method

- Sprague Dawley rats weighing 70–100 g are used. The animals are sacrificed during anesthesia and the diaphragms still attached to the rib cages are carefully removed, released from the rib cages and adhering connective and fat tissues, washed in PBS, spread out and divided into two equal pieces as described by Müller and coworkers (1994). For assaying the effects of insulin/compounds/drugs, the hemidiaphragms are incubated in KRH buffer gassed with carbogen (95% O2/5% CO2) in the presence of 5 mM glucose

➢ **Epididymal fat pad of rats:**

- Insulin-like activity can be measured by the uptake of glucose into 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 or measured oxygen consumption in Warburg vessels, Radiolabelled 14C glucose, the 14CO2 is trapped and counted.
- The concentration is determined by immuno-assay.

4. BIOASSAY OF VITAMIN D

- The biological assay of vitamin D comprises the recording and interpretation of observations on groups of rats maintained on specified dietary regimens throughout specified periods of their lives whereby the biological response to the preparation under assay is compared with the response to USP Vitamin D Capsules RS.

➢ Preliminary Period

- Throughout the preliminary period in the life of a rat, which is not longer than 30 days and extends from birth to the first day of the depletion period, maintain litters of rats under the immediate supervision of, or according to the directions of, the individual responsible for the assay.
- During the preliminary period, use a dietary regimen that provides for normal development but is limited in its content of vitamin D, so that when placed upon the Rachitogenic Diet in the depletion period the rats develop rickets. At the end of the preliminary period, reject any rat that weighs less than 44 g or more than 60 g, or that shows evidence of injury, disease, or anatomical abnormality.

➢ Depletion Period

- Through the depletion period, which extends from the end of the preliminary period to the first day of the assay period, provide each rat ad libitum with the Rachitogenic Diet and water, and allow access to no other food or dietary supplement.

➢ Rachitogenic Diet

Ingredient	Parts by weight
Whole yellow corn, ground	76
Wheat gluten, ground	20
Calcium carbonate	3
Sodium chloride	1

➢ Assigning Rats to Groups

- Consider a litter suitable for the assay period when individual rats in the litter show evidence of rickets such as enlarged joints and a distinctive wobbly, rachitic gait, provided that the depletion period is not less than 19 or

more than 25 days. The presence of rickets may be established also from the width of the rachitic metaphysis upon X-ray examination or by applying the Line Test to a leg bone of one member of each litter.

- Record the weight of each rat, and assign it to a group, in which each rat will be fed a specified dose of the Reference Standard or of an assay sample that is under examination for its vitamin D potency.
- The two standard groups may be used for the concurrent assay of more than one assay sample.
- At Analysis for complete balance, whereby each litter is represented equally in every group, use 7 or more litters containing at least as many depleted rats as there are groups.

➢ Assay Doses

- Select two dosage levels of the USP Cholecalciferol RS, spaced so that the ratio of the larger to the smaller dose is not less than 1.5 or more than 2.5. The dosage levels of the sample are equivalent to those of the standard or to a mid-level equal to the square root of the product of the two dosage levels of the standard.
- Select dosage levels such that, when fed to rachitic rats, they are expected to produce degrees of calcification within the range specified under the test of data acceptability.
- Before feeding, the Reference Standard and/or sample may be diluted with cottonseed oil, provided that not more than 0.2 mL is fed on any one day. Store the oil solutions in well-closed bottles, protected from light, at a temperature not exceeding 10 , and use within 5 weeks.
- Assign one group of rats to each dosage level of the standard and of the one or more samples

➢ Assay Period

- During the assay period, which extends from the end of the depletion period for a fixed interval of 7 to 10 days, cage each rat individually and provide it ad libitum with the Rachitogenic Diet and water. Supply a Rachitogenic Diet prepared from the same lots of ingredients to all rats. On the first and on the third (or fourth) day of the assay period, feed each rat one-half of its total assigned dose.
- Throughout the assay period, maintain as uniform environmental conditions as possible for all rats, and exclude exposure to antirachitic radiations. At the end of a fixed period of 7 to 10 days, weigh and kill each rat. From those rats that do not weigh less at the end than at the start of the assay period and that have consumed each assigned dose within 24 hours of the time it was fed, dissect out one or more leg bones for examination by the Line Test.

➢ Line Test

Remove the proximal end of a tibia or the distal end of a radius, and clean adhering tissue from it, in any one assay using the same bone from all animals. With a clean, sharp blade cut a median, longitudinal section through the juncture of the epiphysis and diaphysis at the same place on each bone. Rinse both sections in purified water, immerse immediately in silver nitrate solution (1 in 50) for 1 minute, and rinse again in purified water. Expose the cut surface of bone, in water, to daylight or another source of actinic light until the calcified areas develop a clearly defined stain without marked discoloration of the uncalcified areas. The staining procedure may be modified to differentiate more clearly between calcified and uncalcified areas.

➢ Acceptability

- If the average score of the standard group on the high dosage level is not greater than the average score of the standard group on the low dosage level, discard the results, and repeat the assay.
- Assay groups that are not acceptable for measuring vitamin D potency, in each of which the average score is less than the average score of the standard group on the low dosage level or more than the average score of the standard group on the high dosage level, its assayed content of vitamin D is respectively less than that represented by the low dose or more than that represented by the high dose of the Reference Standard.