MICROBIOLOGICAL ASSAY OF ANIBIOTICS

The inhibition of growth under standardized conditions may be utilized for demonstrating the therapeutic efficacy of antibiotics.

Any subtle change in the antibiotic molecule which may not be detected by chemical methods will be revealed by a change in the antimicrobial activity and hence microbiological assays are very useful for resolving doubts regarding possible change in potency of antibiotics and their preparations.

PRINCIPLE

The microbiological assay is based upon a comparison of the inhibition of growth of micro-organisms by measured concentration of the antibiotics to be examined with that produced by known concentrations of a standard preparation of the antibiotic having a known activity.

Two general methods are usually employed:

1. The cylinder-plate (or cup-plate) method.

2. The turbidimetric (or tube assay) method.

PREPARATION OF MEDIA:

The Media required for the preparation of test organism are made from the ingredients.

Minor modifications of the individual ingredients may be made, or reconstituted dehydrated media may be used provided the resulting media have equal or better growth-promoting properties and give a similar standard curve response.

Dissolve the ingredients in sufficient water to produce 1000 ml and add sufficient 1M Sodium hydroxide or 1M Hydrochloride acid, as required so that after sterilization the PH is b\w 6.5 to 7.5.

PREPARATION BUFFER SOLUTIONS

- Buffer solutions are prepared by dissolving the following quantities of dipotassium hydrogen phosphate and potassium dihydrogen phosphate in sufficient water to produce 1000 ml after adjustive the pH with 8 M phosphoric acid or 10M potassium hydroxide.

Buffer No.	Dipotassium Hydrogen Phospahate, K_2HPO_4 (g)	Potassium Dihydrogen Phosphate, KH_2PO_4 (g)	pH adjusted after sterilisation to :
1	2.0	8.0	6.0 ± 0.1
2	16.73	0.532	8.0 ± 0.1
3	-	13.61	4.5 ± 0.1
4	20.0	80.00	6.0 ± 0.1
5	35.0	-	10.5 ± 0.1*
6	13.6	4.0	7.0 ± 0.2

PREPARATION OF THE STANDARD SOLUTION

- To prepare a stock solution, dissolve a quantity of the Standard Preparation of a given antibiotic, accurately weighed and previously dried where so indicated in Table, in the solvent specified, and then dilute to the required concentration as indicated. Store in a refrigerator and use within the period indicated.

TABLE – Stock solutions and test dilutions of Standard Preparation

	Standard Stock Solution					Test Dilution		
Antibiotic (1)	**Assay Method (2)**	**Prior Drying (3)**	**Initial solvent (further diluent, if different) (4)**	**Final Stock Con per ml (5)**	**Use before (number of days) (6)**	**Final diluent (7)**	**Median dose µg or units per ml (8)**	**Incubation temp. (*C) (9)**
Amikacin	B	No	Water	1 mg	14	Water	10 µg	32 - 35
Amphotericin B	A	Yes	DMF[7]	1 mg	Same day	B5	1.0 µg	29 - 31
Bacitracin	A	Yes	0.01M HCl	100 units	Same day	B1	1.0 unit	32 - 35
Bleomycin	A	Yes	B6[8]	2	14	B6	0.04 unit	32 - 35

PREPARETION TEST ORGANISMS

- The test organism for each antibiotic is listed in Table, together with its identification number in the American Type Culture Collection (ATCC) and the National Collection of Type Cultures (NCTC) or the National Collection of Industrial Bacteria (VCIB).

TABLE – Test Organisms for Microbiological Assay of Antibiotics

Antibiotic	Test Organism	ATCC[1] No.	NCTC[2] No. (NCIB[3] No.)
Amikacin	Staphylococcus aureus	29737	7447
Amphotericin B	Saccharomyces cerevisiae	9763	10716
Bacitracin	Micrococcus luteus	10240	7743
Bleomycin	Mycobacterium smegmatis	607	–
Carbenicillin	Pseudomonas aeruginosa	25619	–
Doxycycline	Staphylococcus aureus	29737	7447

CYLINDER-PLATE OR CUP-PLATE METHOD

- Inoculate a previously liquified medium appropriate to the assay, with the requsite quantity of suspension of the micro organism, add the suspension to the medium at a temperature between 40 and 50 and immediately pour the inoculated medium into the petri dishes or large rectangular plates to give a depth of 3 to 4 mm.

- Ensure that the layers of medium are uniform in thickness, by placing the dishes or plates on a level surface.

- Using the appropriate buffer solutions, prepare solutions of known concentrations of the antibiotic to be examined

- Apply the solutions to the surface of the solid medium in sterile cylinders or in cavities prepared in the agar. The volume of solution added to each cylinder or cavity must be uniform and sufficient almost to fill the holes when these are used.

- Leave the dishes or plates standing for 1 to 4 hours at room temperature or at 4 , as appropriate, as a period of pre-incubation diffusion to minimise the effects of variation in time between the application of the different solutions. Incubate them for about 18 hours at the particular temperature.

- Accurately measure the diameters or areas of the circular inhibition zones and calculate the results.

$$L = \frac{3a + 2b + c - e}{5} \quad H = \frac{3e + 2d + c - a}{5}$$

Where

L = the calculated zone diameter for the lowest concentration of the standard curve response line.

H = the calculated zone diameter for the highest concentration of the standard curve response line.

c = average zone diameter of 36 readings of the reference point standard solution.

a, b, d, e = corrected average values for the other standard solutions, lowest to highest concentrations, respectively.

TURBIDIMETRIC OR TUBE ASSAY METHOD

- The method has the advantage of a shorter incubation period for the growth of the test organism (usually 3 to 4 hours) but the presence of solvent residues or other inhibitory substances affects this assay more than the cylinder plates assay.
- Prepare five different concentrations of the standard solution for preparing the standard curve by diluting the stock solution of the Standard Preparation of the antibiotic & increasing stepwise in the ration 4:5.
- Select the median concentration & dilute the solution of the substance being examined (unknown) to obtain approximately this concentration.
- Place 1 ml of each concentration of the standard solution and of the sample solution in each of the tubes in duplicate.

- To each tube add 9 ml. of nutrient medium, previously seeded with the appropriate test organism.

- At the same time prepare three control tubes, one containing the inoculated culture medium (culture control), another identical with it but treated immediately with 0.5 ml of *dilute formaldehyde solution* (blank) and a third containing uninoculated culture medium.

- Place all the tubes, randomly distributed or in a randomized block arrangement, in an incubator or water-bath and maintain them at the specified temperature, for 3 to 4 hours. After incubation add 0.5 ml of *dilute formaldehyde solution* to each tube. Measure the growth of the test organism by determining the *absorbance* at about 530nm of each of the solutions in the tubes against the blank.

$$L = \frac{3a + 2b + c - e}{5} \quad H = \frac{3e + 2d + c - a}{5}$$

Where

L = the calculated absorbance for the lowest concentration of the standard response line.

H = the calculated absorbance for the highest concentration of the standard response line.

a, c, b, d, e = average absorbance values for each concentration of the standard response line lowest to highest respectively.

MICROBIOLOGICAL ASSAY OF VITAMINS

DEFINITION:

- Microbiological assay of vitamins is a type of biological assay performed with the aid of microorganisms.
- Many therapeutic agents, which either inhibit the growth of microorganisms or are essential for the growth of them are standardized by microbial assay.
- Principles of microbial assay were developed in 1920s.

MICROBIOLOGICAL ASSAY OF VITAMINS:

PRINCIPLE:

- Vitamins and amino acids are essential for the growth of microorganisms.
- The basis of this assay is to measure the ability of test organism to utilize the substance being assayed under a proper nutritional condition.
- The organisms require these growth factors (vitamins & amino acids) in micro or nano grams.

- The response (growth of test organism) is proportional to the dose (amount of factor) added to medium.

Materials required for Microbial assay of vitamins & amino acids:

- A stock solution.
- A inoculum media.
- Assay medium.
- A standard curve.

MICROBIAL ASSAY OF VITAMIN B_{12}:

About VitB_{12}:

- Also known as cyanocobalamin. Its a water soluble vitamin.
- Structure is similar to that of heme where the iron is replaced with cobalt as a centre of molecule.
- Its main sources are liver, eggs , milk, meat & fish.
- VitB_{12} deficiency causes Macrolytic anemia, pernicious anemia.
- National Research Council, USA recommends a daily intake of about 5mg of vitB_{12}

PRINCIPLE OF ASSAY:

- The test organism selected must be capable of utilizing free cyanocobalamin.
- Lactobacillus Liechmannii is found to satisfy the requirements.
- Gram negative bacilli, non-pathogenic, easy to culture & easily available.
- Isolated from milk, cheese, & other dairy products.
- Assay is performed by using either titrimetric or turbidimetric method.

PRECAUTIONS:

- Great care must be taken to avoid contamination
- All the glass wares must be free from detergents and other chemicals.
- Glass wares must be heated to 250^0C for atleast 1hr before use.
- The whole experiment must be carried out under proper aseptic condition.

Reagents Required :

1) Standard stock solution:

- An accurately weighed amount of Cyanocobalamin reference Standard is added to sufficient 25%ethanol (resulting in a solution containing 1.0 µg of cyanocobalamin per ml).
- Stored in refrigerator. It should be used within 2 months.
- Further dilutions of this stock solution (1 µg/ ml) are made as follows:
- Add 1 ml stock solution to 99 ml purified water (1 ml =10 ng).
- Add 1 mL of the above solution to 199 ml purified water (1 ml = 0.05 ng).

2) Test solution to be assayed:-

- Accurate amount of Vitamin to be assayed is taken & dissolved in water, Dil HCl or NaOH is added to adjust pH at 6.0.
- Make up to volume with water.

3) Preparation of Inoculum:-

- Transfer a loop full of Lactobacillus Liechmannii from a recent sub-culture into two tubes each containing 10ml of sterile culture medium.

Composition of culture medium :(pH 6.8)

Yeast extract	- 0.75gm
Peptone	- 0.75gm
Dextrose	- 1gm
Pot dihydrogen phosphate	- 0.2gm
Tomato juice filtrate	- 10ml
Sorbitan mono oleate solution	- 1ml
Water up to	- 100ml

- Incubate for 18 to 24hrs at 37^{o}C.
- Centrifuge the culture .
- Decant the supernatent fluid, under aseptic condition.
- Suspend these cultured cells into 10ml of sterile suspension of Basal medium stock solution.
- Again centrifuge & decant off supernatent fluid.
- Repeat this for atleast 3times to avoid contamination.
- Finally suspend the cells uniformly in 10ml of sterile medium.
- Aseptically transfer 1ml of the suspension of cells to 10ml of sterile medium & mix.
- This resulting cell suspension is taken as inoculum.

Procedure for assay :-

- Clean ten test tubes & add to it 0, 0.5, 1.0, 2.0, 2.5, 2.5, 3.0, 3.5, 4.0, 4.5, & 5ml of standard cyanocobalamin solution.
- To each tube add 5ml of Basal medium solution
- Volume of each is adjusted to 10ml by water
- In another 4 test tubes add 1, 2, 3, 4ml of test solution which is to be assayed .
- To each of this also add 5ml of Basal medium stock solution & adjust volume to 10ml with water.

- Sterilize all test tubes in autoclave at 121^{o}C for 15mins.
- Cool the test tubes at room temperature.
- Inoculate a drop of inoculum prepared of lactobacillus liechmannii.
- Incubate the test tubes for 64 to 72hrs at temperature range of 30 to 37^{o}C.
- After incubation period titrate contents of each test tube with 0.05N NaOH using bromothymol blue as indicator until green colour.
- Record all the titre readings clearly.

Interpretation of results:-

- Determine the average of titration values of each level of both standard & test solutions.
- Plot a graph considering average titration values(in ml) of 0.05N NaOH against concentration of standard cyanocobalamin solution.
- A linear graph is obtained.
- By interpolating the standard curve determine the concentration as activity per ml of vit B_{12}.
- From the graph the concentration of test solution of cyanocobalamin is found & reported.