MICROBIOLOGICAL ASSAY OF ANTIBIOTICS, VITAMINS & AMINO ACIDS
Introduction

- Microbiological assay is a technique in which the potency or concentration of a compound is assessed by determining its effect on micro-organisms.

- The principles are discussed by Roberts and Boyce. Microbiological assay required for the assay of a number of antibiotics, in both the British Pharmacopoeia (BP) and United States Pharmacopoeia (USP).

- Bioassay compares a reference standard and an unknown sample, the two preparations being measured simultaneously.
A lot of preliminary work is necessary for microbiological assays, and if only a small number of samples are expected irregularly, the method is inefficient. Two assay methods are normally used, agar diffusion and tube assays. They have several common features:

- The compound being assayed must influence the growth of the test organism.
- A varying response in growth must be produced by addition of varying quantities of the test material.
- The growth medium must contain an excess of all the compounds required by the test organism for growth. The exception to this is the compound being assayed which should be totally absent from the basic medium.
- The assumption is made that the compound being assayed is the only growth promoting or inhibiting compound present.
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:

- The cylinder-plate or (cup-plate) method.
- The turbidimetric or (tube assay) method.
Cylinder-plate or (cup-plate) method

1. Prepare Nutrient Agar plate inoculated with test organism, with a depth of 4-5mm and then allow it to solidify. Divide the NA plate into four equal portions. Then with the help of a sterile borer make four cavities one in each portion. Then in three the cavity fills antibiotic solution and in one fill the standard solution.

2. Incubate the plates at 37°C for 24 hours.

3. Measure the zone of inhibition.

Microbiological assay
- Cup plate method
- Disc diffusion method

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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 OF 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 adjusting the pH with 8M phosphoric acid or 10 M potassium hydroxide.

<table>
<thead>
<tr>
<th>Buffer No.</th>
<th>Dipotassium Hydrogen Phosphate, $K_2HPO_4$ (g)</th>
<th>Potassium Dihydrogen Phosphate, $KH_2PO_4$ (g)</th>
<th>pH adjusted after sterilisation to:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.0</td>
<td>8.0</td>
<td>6.0 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>16.73</td>
<td>0.532</td>
<td>8.0 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>13.61</td>
<td>4.5 ± 0.1</td>
</tr>
<tr>
<td>4</td>
<td>20.0</td>
<td>80.00</td>
<td>6.0 ± 0.1</td>
</tr>
<tr>
<td>5</td>
<td>35.0</td>
<td>-</td>
<td>10.5 ± 0.1*</td>
</tr>
<tr>
<td>6</td>
<td>13.6</td>
<td>4.0</td>
<td>7.0 ± 0.2</td>
</tr>
</tbody>
</table>
**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.
### Stock solutions and test dilutions of Standard Preparation

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Assay</th>
<th>Prior Initial Fin</th>
<th>Use Diluent before</th>
<th>Final Median Incubation</th>
<th>Temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>B</td>
<td>No Water 1 mg</td>
<td>14 Water 10 μg</td>
<td>32 - 35</td>
<td></td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>A</td>
<td>Yes DMF 1 mg</td>
<td>Same day B5 1.0 μg</td>
<td>29 - 31</td>
<td></td>
</tr>
<tr>
<td>Bacitracin</td>
<td>A</td>
<td>Yes 0.01M HCl 100</td>
<td>Same day B1 1.0 unit</td>
<td>32 - 35</td>
<td></td>
</tr>
<tr>
<td>Bleomycin</td>
<td>A</td>
<td>Yes B6 2 14</td>
<td>B6 0.04 unit</td>
<td>32 - 35</td>
<td></td>
</tr>
</tbody>
</table>
PREPARATION OF TEST ORGANISMS

- The test organism for each antibiotics 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 (NCIB) & Microbial Type Culture Collection and Gene Bank (MTCC).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Test Organism</th>
<th>ATCC No.</th>
<th>NCTC² No. (NCIB³ No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>Staphylococcus aureus</td>
<td>29737</td>
<td>7447</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>Saccharomyces cerevisiae</td>
<td>9763</td>
<td>10716</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>Micrococcus luteus</td>
<td>10240</td>
<td>7743</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>Mycobacterium smegmatis</td>
<td>607</td>
<td>_</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>Pseudomonas aeruginosa</td>
<td>25619</td>
<td>_</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>Staphylococcus aureus</td>
<td>29737</td>
<td>7447</td>
</tr>
</tbody>
</table>
Inoculate a previously liquified medium appropriate to the assay, with the requisite quantity of suspension of the microorganism, 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.
PROCEDURE FOR CUP PLATE METHOD

1. Microbial growth
2. Molten agar at 50 °C
3. Agitate to disperse organisms
4. Pour plates and allow to solidify
5. Place sterile wells on agar
   - Make appropriate dilutions reference material
   - Make appropriate dilutions test material
6. Add dilutions to wells
7. Incubate overnight at appropriate temperature
8. Measure diameter of zone of inhibition or exhibition

Procedure for agar diffusion
Diagram showing a zone.
\[ L = 3a + 2b + c - e : H = 3e + 2d + c - a \]

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 ratio 4 : 5.
- Select the media and concentration & dilute the solution of the substance being examined (unknown) to obtain approximately this concentration.
- Place 1ml of each concentration of the standard solution and of the sample solution in each of the tubes in duplicate.
To each tube add 9ml. 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 at third containing un inoculated 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 530 nm of each of the solutions in the tubes against the blank.
PROCEDURE FOR TURBIDIMETRIC ASSAY

Reaction tube made from glass or plastic with same height. Spectrophotometric tube has to be sterile.

All residues are removed and sterilized before and after use.

1 ml of test solution and standard solution of each doses placed in 3 tubes (triplicate) randomly

Make 2 control tubes

Add 9 ml of inoculation into each tubes

Place tubes in water bath or incubator at (36-37.5)°C for 2 hr.

After incubation, add 0.5 ml of dilute formaldehyde

Transmittance or Absorbance at 530 nm

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\[ L = 3a + 2b + c - e \quad : \quad H = 3e + 2d + c - a \]

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.
Antibiotic concentration
MICROBIOLOGICAL ASSAY OF VITAMINS:

VITAMIN $B_{12}$:

- **About Vit. $B_{12}$:**
  - Also known as cyanocobalamin. It’s 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.
  - Vit. $B_{12}$ deficiency causes Macrolytic anaemia, Pernicious anaemia.
  - National Research Council, USA recommends a daily intake of about 5mg of vit$B_{12}$.
DEFINITION:

✓ Principles of microbial assay were developed in 1920s.

✓ 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.

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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

- A stock solution.
- An inoculum media.
- Assay medium.
- A standard curve.
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°C for at least 1 hr. 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 10 ml of sterile culture medium.
# BASAL MEDIUM STOCK SOLUTION

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Cystine</td>
<td>0.1 gm</td>
</tr>
<tr>
<td>DL-Tryptophan</td>
<td>0.1 gm</td>
</tr>
<tr>
<td>1N HCl</td>
<td>10 ml</td>
</tr>
<tr>
<td>Adenine-Guanine-Uracil Solution</td>
<td>5 ml</td>
</tr>
<tr>
<td>Xanthine Solution</td>
<td>5 ml</td>
</tr>
<tr>
<td>Riboflavin-Thiamine-Biotin-Nicotinic Acid Solution</td>
<td>10 ml</td>
</tr>
<tr>
<td>p-Aminobenzoic Acid - Pyridoxine</td>
<td></td>
</tr>
<tr>
<td>Pyridoxal-Pyridoxamine Solution</td>
<td>10 ml</td>
</tr>
<tr>
<td>Calcium Pantothenate-Folic Acid Solution</td>
<td>5 ml</td>
</tr>
<tr>
<td>Salt Solution A</td>
<td>5 ml</td>
</tr>
<tr>
<td>Salt Solution B</td>
<td>5 ml</td>
</tr>
<tr>
<td>Asparagine Solution</td>
<td>5 ml</td>
</tr>
<tr>
<td>Acid-Hydrolysed Casein Solution</td>
<td>25 ml</td>
</tr>
<tr>
<td>Dextrose</td>
<td>10 gm</td>
</tr>
<tr>
<td>Sodium Acetate</td>
<td>5 gm</td>
</tr>
<tr>
<td>Ascorbic Acid</td>
<td>1 gm</td>
</tr>
<tr>
<td>Sorbitan Mono-Oleate Derivative Solution</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

## Table 16.4: Procedure for microbiological assay of Vitamin B₁₂

<table>
<thead>
<tr>
<th>Tube number</th>
<th>Std. cyanocobalamin solution (0.01 to 0.04 μg/ml) (ml)</th>
<th>Basal medium stock solution (ml)</th>
<th>Volume of sterile water (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
<td>5</td>
<td>5.0</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>5</td>
<td>4.5</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>5</td>
<td>4.0</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
<td>5</td>
<td>3.5</td>
</tr>
<tr>
<td>5</td>
<td>2.0</td>
<td>5</td>
<td>3.0</td>
</tr>
<tr>
<td>6</td>
<td>2.5</td>
<td>5</td>
<td>2.5</td>
</tr>
<tr>
<td>7</td>
<td>3.0</td>
<td>5</td>
<td>2.0</td>
</tr>
<tr>
<td>8</td>
<td>4.0</td>
<td>5</td>
<td>1.0</td>
</tr>
<tr>
<td>9</td>
<td>4.5</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>10</td>
<td>5.0</td>
<td>5</td>
<td>0.0</td>
</tr>
<tr>
<td>1'</td>
<td>1.0 (Test solution)</td>
<td>5</td>
<td>4.0</td>
</tr>
<tr>
<td>2'</td>
<td>2.0 (Test solution)</td>
<td>5</td>
<td>3.0</td>
</tr>
<tr>
<td>3'</td>
<td>3.0 (Test solution)</td>
<td>5</td>
<td>2.0</td>
</tr>
<tr>
<td>4'</td>
<td>4.0 (Test solution)</td>
<td>5</td>
<td>1.0</td>
</tr>
</tbody>
</table>
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
Procedure for assay:

Titrimetric Assay

- Clean ten test tubes & add to it 0, 0.5, 1.0, 2.0, 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°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 72 hr at temperature range of 30 to 37°C. After incubation period titrate contents of each test tube with 0.05 N 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. B12. From the graph the concentration of test solution of cyanocobalamin is found & reported.
Minimum inhibitory concentration

- **minimum inhibitory concentration (MIC)** is the lowest concentration of a chemical, usually a drug, which prevents visible growth of bacterium. MIC depends on the microorganism, the affected human being (in vivo only) or the environment in general and the antibiotic.

- The MIC is determined by preparing solutions of the chemical in vitro at increasing concentrations, incubating the solutions with the separate batches of cultured bacteria, and measuring the results using agar dilution or broth dilution.
Agar dilution

The antibiotic to be tested is diluted with water to produce a series of concentrations. An appropriate volume is then combined with melted agar to produce plates in which the final antibiotic concentrations represent a 2-fold dilution series. After this, bacteria prepared to a standard concentration are added as a spot to each plate, with $10^4$ colony forming units (CFU) per spot. This technique allows for replicate spots of one bacterial type to be tested or spots of different bacteria so that the MIC of the antibiotic against multiple types of bacteria can be tested.

Necessary controls include a control plate that does not receive any antibiotics and bacterial spread plates demonstrating that the bacterial inoculation are in the correct concentration range.

The dilution plates are then incubated at a temperature of 37 degrees Celsius. The plates are then incubated for sixteen to eighteen hours, although incubation time may be less for bacteria populations that divide quickly.

After incubation, the plates are examined to determine if bacterial growth has occurred in the inoculated spots. The lowest concentration of antibiotics that prevented bacterial growth is considered to be the minimum inhibitory concentration of that antibiotic against that bacterium.
Broth dilution assay

or

Turbidimetric Method

There are three main reagents necessary to run this assay which are the media, antimicrobial and the microbe being tested.

Most commonly used media is Cation adjusted-Mueller Hinton Broth. This is due to its ability to support growth of most pathogens and the lack of inhibitors towards common antibiotics.

Depending on the pathogen and antibiotics being tested, the media can be changed and/or adjusted.

The antimicrobial concentration is adjusted into the correct concentration by mixing stock antimicrobial with media.

The adjusted antimicrobial is serially diluted into multiple tubes (or wells) to get a gradient. The dilution rate can be adjusted depending on the breakpoint and the practitioner's needs.

The microbe, or the inoculating agent, needs to come from the same colony forming unit per sample, and needs to be at the correct concentration.

This can be adjusted by incubation time and dilution. For verification, the positive control is plated in a hundred fold dilution to count colony forming units.

The microbes inoculate the tubes (or plate) and are incubated for 16-20 hours. The MIC is determined by turbidity.

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STEP 1: TUBE DILUTION

<table>
<thead>
<tr>
<th>volume of microbial rich broth (mL)</th>
<th>120</th>
<th>100</th>
<th>80</th>
<th>60</th>
<th>40</th>
<th>20</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>volume antimicrobial agent (mL)</td>
<td>0</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
<td>100</td>
<td>120</td>
</tr>
</tbody>
</table>

inoculating tubes – each with same amount bacteria

STEP 2: INCUBATE FOR 24 HOURS

percent concentration of antimicrobial agent

0% 16.7% 33.3% 50% 66.7% 83.3% 100%

Minimum Inhibitory Concentration

STEP 3: DETERMINE MIC BASED ON TURBIDITY

high microbial growth no microbial growth

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ASSAY OF AMINO ACIDS

- There are 20 types of different amino acids which helps in synthesis of protein.
- Different types of microorganisms like bacteria and fungus are used according to the amino acid.
- Microorganisms grown in specific media then add a freshly prepared amino acid solution on the same day a standard solution containing 200µg/ml.
- With an appropriately diluted solution, duplicate tubes were prepared using 0 to 1.0 ml per tube in graduation of 0.1 ml.
- Two tubes were also prepared using 1.0 ml of the original at levels of 0.2, 0.4, 0.6, 0.8 and 1.0 ml of amino acid.
- Add the amino acid into the media and autoclave it.
- When the media becomes cool, inoculate all the test tubes with microorganisms and leave some tubes for blank.
- Incubate all test tubes at 37º C for 15 to 20 hrs.
- After incubation compare all the test tubes with blanks (having no microorganisms).
- Finally determine the potency of the amino acid.
THANK YOU