

# UNIT-IV

## Methods for standardization of antibiotics, vitamins and amino acids

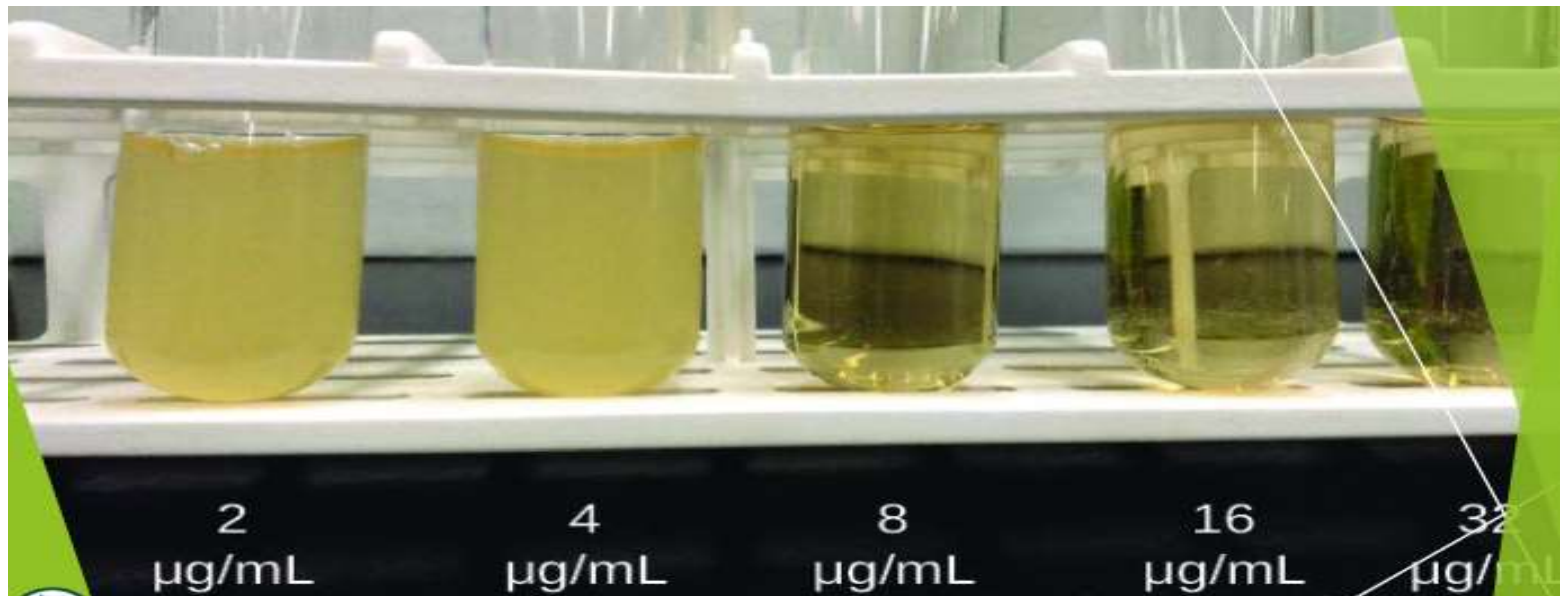
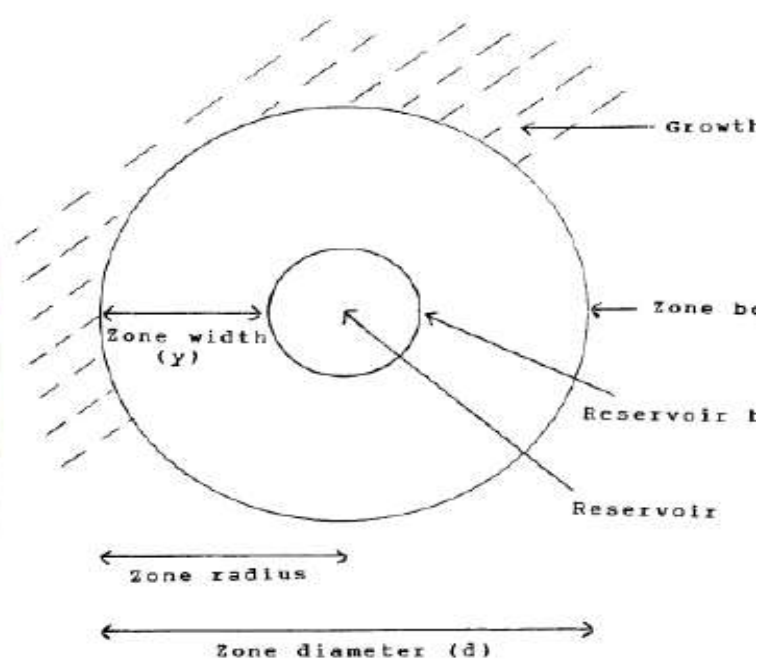


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**RASMITA JENA**  
**ASSISTANT PROFESSOR**  
**SoPLS, CUTM, BBSR**

## **Standardization of Antibiotics/ Microbiological Assay of Antibiotics**

- The microbiological assay of an antibiotic is based upon a **comparison of the inhibition of growth of micro-organisms by measured concentrations of the antibiotics under examination with that produced by known concentrations of a standard preparation of the antibiotic having a known activity.**
- Two general methods are usually employed, i.e.,
  - **Cylinder-plate (or cup plate) method**
  - **Turbidometric (or tube assay) method.**



- The **cylinder-plate method** (Method A) depends upon **diffusion of the antibiotic from a vertical cylinder through a solidified agar layer** in a Petri dish or plate to an extent such that **growth of the added micro-organism is prevented entirely in a zone around the cylinder containing a solution of the antibiotic.**
- The **Turbidometric method** (Method B) depends upon the **inhibition of growth of a microbial culture in a uniform solution of the antibiotic in a fluid medium that is favorable to its rapid growth in the absence of the antibiotic.**

- The assay is designed in such a way that the mathematical model on which the potency equation is based can be proved to be valid.
- If a parallel-line model is chosen, the two log dose response lines of the preparation under examination and the standard preparation should be parallel; they should be rectilinear over the range of doses used in the calculation.
- These conditions should be verified by validity tests for a given probability. Other mathematical models, such as the slope ratio method, may be used provided that proof of validity is demonstrated.

# Methods for Standardization of Vitamins

- Vitamins are essentially required for the growth and multiplication of microorganisms (which are highly sensitive even to small amounts of growth factors).
- These test microorganisms can synthesize the factor being assayed which forms the basis of microbiological assay of vitamins and amino acids.
- The test microorganisms used for the standardization of water-soluble vitamins are given in table 4.12.

**Table 4.12: Test Microorganisms and Conditions for the Microbial Assay of Vitamins**

Vitamins	Test Microorganisms	Incubation Temperature (°C)	Assay pH
Vitamin B <sub>12</sub>	<i>Lactobacillus leichamannii</i> ATCC 7830	37	6.1
	<i>Poteriochromonas stipitata</i> ATCC 11532	30	5.5
Vitamin B <sub>6</sub>	<i>Saccharomyces uvarum</i> ATCC 9080	30	4.5
	<i>Tetrahymena thermophila</i> ATCC 30008	30	6.1
Riboflavin	<i>Lactobacillus casei</i> ATCC 7469	37	6.8
	<i>Tetrahymena thermophila</i> ATCC 30008	30	6.1
Thiamine	<i>Lactobacillus viridescens</i> ATCC 12706	30	6.0
	<i>Ochromonas danica</i> ATCC 30004	30	5.5
Biotin	<i>Lactobacillus plantarum</i> ATCC 8014	37	6.8
	<i>Ochromonas danica</i> ATCC 30004	30	5.5
Niacin	<i>Lactobacillus plantarum</i> ATCC 8014	37	6.8
	<i>Tetrahymena thermophila</i> ATCC 30008	30	6.1
Pantothenate	<i>Lactobacillus plantarum</i> ATCC 8014	37	6.7
	<i>Tetrahymena thermophila</i> ATCC30008	30	6.1

## Principle:

- Vitamins are essential growth factor for microorganisms. In the standardization of vitamins, the ability of test organisms to use the substance being standardized under a proper nutritional condition is measured.
- These growth factors (vitamins) are required by the organisms in micro or nano grams.
- The growth response produced by the test organism is proportional to the amount of growth factor added to the medium.



# Standardization of Niacin

- The selected organism should have the ability to utilize free niacin, niacinamide, nicotinic acid, cozymase, and niacinamide nucleoside. This requirement is satisfied by *Lactobacillus plantarum* (an acid forming organism), cannot synthesize niacin for its metabolic processes.
- This bacterium is also non-pathogenic, can be easily cultured, and is negligibly affected by stimulatory or inhibitory substances present in niacin-containing pharmaceutical preparations.
- This organism is grown on a simple stab culture containing gelatin, yeast extract, and glucose.
- For assay it is cultured in assay tubes by transferring to the liquid medium containing the basic medium having an optimum amount of added niacin. A measurable response can range from 0.05-0.5 $\mu$ g of niacin per tube.
- For preparing the niacin test solution, sample is extracted, either in acid or alkali medium by adding such amount of alkali or acid that each ml of final volume contains 0.1gm niacin.

## Reagents

- 1) Standard Niacin Stock Solution I: It contains 100ug/ml of U.S.P. Niacin RS.
- 2) Standard Niacin Stock Solution II: It contains 10ug/ml of U.S.P. Niacin RS, and is prepared by diluting solution I with water.
- 3) Standard Niacin Solution: It contains 10-40ng of niacin per ml and is prepared by diluting solution with water.
- 4) Basal Medium Stock Solution: It contains the following ingredients:
  - The medium is prepared by dissolving anhydrous dextrose and sodium acetate in the previously mixed solutions. The pH is adjusted to 6.8 using 1N NaOH solution, and volume is adjusted to 250ml by adding water.

<b>Ingredients</b>	<b>Amounts</b>
Acid-hydrolysed casein solution	25ml
Cystine-tryptophan solution	25ml
Dextrose (anhydrous)	10gm
Sodium acetate (anhydrous)	5gm
Adenine-guanine-uracil solution	5ml
Riboflavin-thiamine hydrochloride-biotin solution	5ml
Aminobenzoic acid-calcium pantothenate-pyridoxine Hydrochloride solution	5ml
Salt Solution A	5ml
Salt Solution B	5ml

## **Culture Medium:**

- The culture medium is prepared in a series of test tubes each containing 5ml of basal medium stock solution. Further, each tube is added with 5ml water containing 1.0ug of niacin, plugged with cotton, autoclaved at 121°C for 15 minutes, and cooled

## **Preparation of Inoculum:**

- The inoculum is prepared by transferring cells from the stock culture of *Lactobacillus plantarum* to 10ml culture medium in a sterile tube, and then incubating for 16-24 hours at 30- 37°C.

## **Principle:**

- Niacin assay medium (a dehydrated medium) is free from nicotinic acid and its analogues, but contains other essential nutrients and vitamins used for preparing the culture of *L. plantarum*. Addition of nicotinic acid or its analogues in increasing concentrations as specified yields a growth response which is either turbidometrically or titrimetrically measured.

## Procedure

- 1) The spectrophotometer is calibrated as per the Pharmacopoeia.
- 2) Standard niacin solution in 0.0, 0.5, 1.0, 1.5, 2.0, 5.0ml is added to test tubes in duplicate.
- 3) Each tube is added with 5ml of basal medium stock solution and volume is adjusted to 10ml with water.
- 4) Each tube is added with 5ml medium solution and volume is adjusted up to 10ml with water.
- 5) Tubes containing the material to be assayed are prepared by taking in duplicate, 1.0, 2.0, 3.0, and 4.0ml, respectively of the test solution of the material to be assayed.
- 6) Again each tube is added with 5ml basal medium stock solution and volume is adjusted up to 10ml with water.
- 7) The contents in tubes are mixed, plugged with cotton, and autoclaved.
- 8) After cooling, each tube is aseptically inoculated with a drop of inoculum and incubated for 16-24 hours at 30-37°C.
- 9) The transmittance for the un-inoculated blank is adjusted at 1.0, using which the transmittance of inoculated blank is read.
- 10) Finally, using the transmittance of the inoculated blank set at 1.0, the transmittance for the remaining tubes is measured.

## Calculation:

- A standard curve of niacin standard transmittances for each level of standard niacin solution is plotted against  $\mu\text{g}$  of niacin present in the respective tubes. By interpolating this standard curve, the niacin content of test solution in each tube is determined.
- The niacin content of the test material is calculated from the average values obtained from at least 6 tubes not varying by more than 10% from the average.
- For expressing the results as niacinamide, the obtained values are multiplied by 0.992.

# Standardization of Vitamin B12

## Assay Preparation

- 1) An accurately weighed quantity of the material to be assayed is taken in the form of fine powder in a vessel.
- 2) An aqueous extracting solution is prepared containing 1.29gm disodium phosphate 1.1gm anhydrous citric acid, and 1.0gm sodium meta bisulphate in each 100ml,
- 3) For each gm or ml of material taken, 25ml of this freshly prepared aqueous extracting solution is added and mixed.
- 4) The resultant mixture is autoclaved at 121°C for 10 minutes.
- 5) The undissolved particles of the extract are allowed to settle, and later filtered or centrifuged.
- 6) An aliquot of this so obtained clear solution is diluted with water so that the final test solution contains vitamin B12 having activity equivalent to the standard cyanocobalamin solution added to the assay tubes.

## Basal Medium Stock Solutions

The basal medium utilised contains the following ingredients given in table 4.13:

**Table 4.13: Basal Medium Ingredients**

<b>Ingredients</b>	<b>Amounts</b>
L-Cystine	0.1 gm
L-Tryptophan	0.05 gm
1N Hydrochloric acid	5 ml
Adenine-guanine-uracil solution	5 ml
Xanthine solution	5 ml
Vitamin solution I	10 ml
Vitamin solution II	10 ml
Salt solution A	5 ml
Salt solution B	5 ml
Asparagine solution	5 ml
Acid-hydrolysed casein solution	25 ml
Dextrose (anhydrous)	10 gm
Sodium Acetate (anhydrous)	5 gm
Ascorbic Acid	1 gm
Polysorbate 80 Solution	5 ml



- Cysteine and tryptophan are dissolved in hydrochloric acid and then the next 8 solutions are added.
- To the resultant solution, 100ml water is added and mixed. To this mixture, dextrose, sodium acetate, and ascorbic acid are added.
- The solution is filtered and polysorbate 80 solution is added.
- The pH is adjusted between 5.5 and 6.0 using 1 N sodium hydroxide, and volume is adjusted to 250ml with purified water.

## Standard Cyanocobalamin Stock Solution

- The standard stock solution is prepared by adding sufficient amount of 25% alcohol to an accurately weighed quantity of U.S.P. cyanocobalamin RS. The solution obtained having a known concentration of 1.0 $\mu$ gm of cyanocobalamin per ml is stored in a refrigerator.
- A fresh standard cyanocobalamin solution is prepared for each assay. It is prepared by diluting a suitable volume of standard cyanocobalamin stock solution with water up to a measured volume such that after incubation the difference in transmittance between the inoculated blank and 5.0ml of the standard cyanocobalamin solution is not less than that which corresponds to a difference of 1.25mg in a dried cell weight. This concentration falls between 0.01-0.04 $\mu$ g/ml of standard cyanocobalamin solution.

## **Culture Medium**

- The culture medium is prepared by dissolving 0.75gm water-soluble yeast extract, 0.75gm dried peptone, 1.0gm of anhydrous dextrose, and 0.20gm of potassium biphosphate in 60-70ml of water.
- To the resultant solution, 10ml tomato juice and 1ml polysorbate 80 solution are added.
- The pH of the solution is adjusted to 6.8 using 1N NaOH solution, and volume is adjusted to 100ml with water.
- 10ml portions of the solution are taken in test tubes, plugged with cotton, and autoclaved at 121°C for 15 minutes. The solution is immediately cooled to avoid colour formation which may result due to overheating of the medium.

## **Suspension Medium**

- The suspension medium is prepared by diluting a measured volume of basal medium stock solution using an equal volume of water. 10ml portions of this diluted medium are taken in test tubes, sterilized, and cooled same as for the culture medium.

## **Stock Culture of *Lactobacillus Leichmannii***

- 1) The stock culture of the organism is prepared by heating with continuous stirring of 100ml of culture media with 1.0-1.5gm of agar medium on a steam bath till the agar dissolves.
- 2) Approximately, 10ml portions of the hot solution are taken in test tubes, plugged with cotton, and autoclaved at 121°C for 15 minutes.
- 3) After cooling the tubes in a vertical position, 3 or more tubes are inoculated by transferring stab of a pure culture of *Lactobacillus Leichmannii*.

- 4) The inoculum is incubated for 16-24 hours at  $30-40 \pm 0.5^{\circ}\text{C}$  temperature, and stored in a refrigerator.
- 5) Fresh stab cultures are prepared at least thrice a week, and are not used for inoculum preparation if more than four days old.
- 6) The microorganism activity can be increased by transferring daily or twice-daily the stab culture to the point where definite turbidity in the liquid inoculum can be observed after 2-4 hours of inoculation.
- 7) A Slow-growing culture rarely yields a suitable response curve, resulting in irregular results.

## **Preparation of Inoculum:**

- 1) The inoculum is prepared by transferring cells from the stock culture of organism to two sterile tubes, each containing 10ml culture medium.
- 2) These culture tubes are then incubated for 16-24 hours at  $30-40 \pm 0.5^{\circ}\text{C}$  temperature.
- 3) The incubated tubes are aseptically centrifuged, and the supernatant is decanted.

- 4) The cells from the culture are suspended and mixed with 5ml sterile suspension medium.
- 5) The volume is adjusted using sterile suspension medium so that 1 in 20 dilutions in saline TS produces 70% transmittance on a spectrophotometer at 530nm wavelength, and read against saline TS set at 100% transmittance.
- 6) 1 in 400 dilution of the adjusted suspension is prepared using basal medium stock solution to use for the test inoculum.

## Principle

- Vitamin B12 assay medium is free from vitamin B12, but contains other essential nutrients and vitamins used for the culture of *Lactobacillus leichmannii*.
- Addition of U.S.P. cyanocobalamin reference in increasing concentrations as specified yields a standard curve providing growth response which is either titrimetrically or turbidimetrically measured.



## Procedure

- 1) Standard cyanocobalamin solution in 1.0ml, 1.5ml, 2.0ml, 3.0 ml, 4.0ml, and 5.0ml is added to thoroughly cleaned hard-glass test tubes in duplicate.
- 2) These tubes and other 4 identical empty tubes are added with 5.0 ml of basal medium stock solution and volume is adjusted to 10ml with water.
- 3) Assay preparation in 1.0ml, 1.5ml, 2.0ml, 3.0ml, and 4.0ml is added to similar tubes in duplicate.
- 4) Each of these tubes is added with 5ml basal medium solution and volume is adjusted to 10ml with water.

- 5) A complete set of standard and assay tubes is placed in a tube rack and the duplicate set is placed in the other rack in random order.
- 6) The tubes are covered to prevent bacterial contamination and autoclaved for 5 minutes at 121°C.
- 7) The tubes are immediately cooled to prevent colour formation which may result due to overheating of the medium.
- 8) Each tube [except 2 of the 4 without standard cyanocobalamin solution (the un-inoculated blanks)] are added with 0.5ml inoculum.

- 9) These tubes are incubated for 16-24 hours at  $30-40\pm 0.5^{\circ}\text{C}$  temperature. Bacterial growth is terminated by heating for 5 minutes at temperature not lower than  $80^{\circ}\text{C}$ .
- 10) After cooling to room temperature, the contents are agitated, and when a steady state is reached, transmittance is recorded in a spectrophotometer at the wavelength of 30nm. This steady state is observed after a few seconds of agitation when the reading remains unchanged for 30 seconds or more.
- 11) Reading for each tube is recorded after the same time interval.

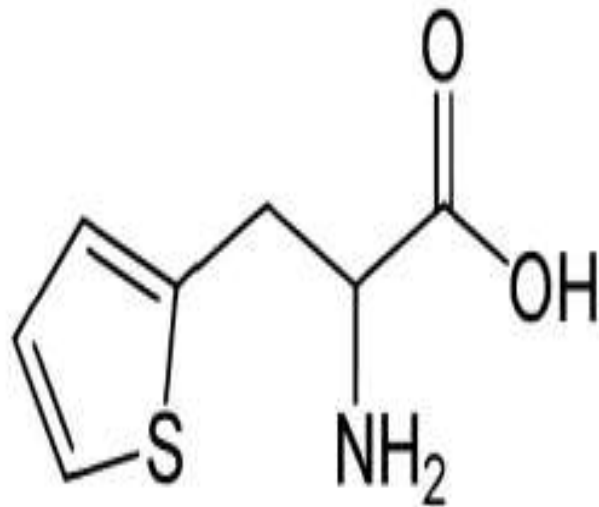
- 12) With the transmittance set at 100% for the uninoculated blank, the transmittance of the inoculated blank is recorded. If the difference is greater than 5% or if Contamination with a foreign microorganism is observed, the assay results are ignored.
- 13) With the transmittance set at 100% for the uninoculated blank, the transmittance of the remaining tubes is recorded. If the slope of the standard curve indicates a sensitivity problem, the assay results are ignored.

# Standardization of Amino Acids

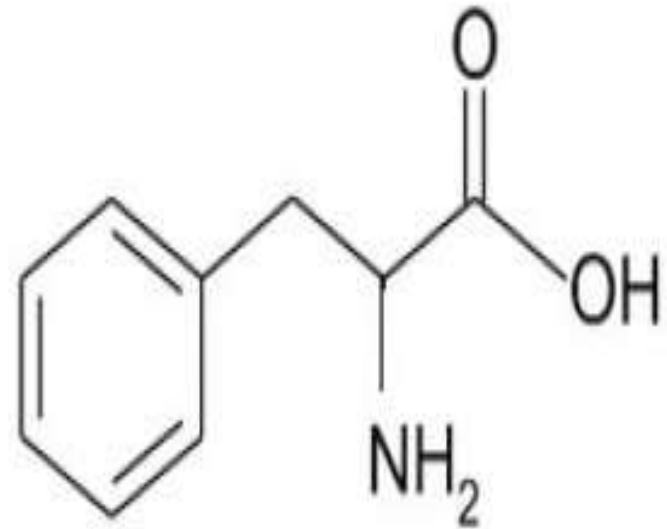
- Microbiological assays were used for quantization of amino acids since many years as they were the most reliable, sensitive, and specific available tests.
- They can be used for any type of biological material, however sometimes they are affected by the activators or inhibitors present in the material being standardized.
- These inexpensive methods are used for analyzing large batches of samples, but are time-consuming and also not suitable for all amino acids.

- Amino acids are essential for the growth and replication of some microorganisms.
- Many strains of such microbes depend on a particular amino acid.
- Thus, if such microorganisms are cultured using a small amount of that particular amino acid, a limited degree of growth will be observed, which is measured by turbidometry or by measuring the increased lactic acid production by either micro titration or pH change.

- Guthrie and Susi introduced a modified version of microbiological assay utilizing diffusion in gels in clinical biochemistry laboratories for screening blood Samples having increased phenylalanine levels.
- This test was named as the Guthrie test (after its inventors) and is the most widely used microbiological assay method of an amino acid.
- This bacterial inhibition assay is based on the ability of phenylalanine to counteract the effects of  $\beta$ -2-thienylalanine (a competitive metabolic antagonist on the growth of a special strain of *Bacillus subtilis* which depends on phenylalanine for its growth (figure 4.5).



$\beta$ -2-Thienylalanine



Phenylalanine

**Figure 4.5:** Guthrie Test: The similarity in structure between phenylalanine and its metabolic antagonist  $\beta$ -2-thienylalanine provides the basis for a microbiological assay for phenylalanine.



- The test is carried out on an agar layer in which a mixture of the suspension of *B. subtilis* spores, minimum amount of growth nutrients, and a fixed amount of  $\beta$ -2 thienylalanine is added.
- Filter paper discs of 4 mm diameter are soaked in blood and placed on the agar surface along with the blood soaked discs of phenylalanine standards.
- These agar plates are incubated at 37°C overnight.
- Bacterial growth is observed only when phenylalanine concentration in the blood discs is sufficient to overcome the effects of  $\beta$ -2 thienylalanine.
- Growth is observed in zones of growth around each disc.
- The next day diameter of each zone of growth is measured and related to phenylalanine concentration.