UNIT IX

MICROBIOLOGICAL SENSITIVITY TESTING

TOPIC: MICROBIOLOGICAL ASSAY OF VITAMINS

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INTRODUCTION:

- Microbiological assay is a class of biological assay based on the usage of micro organism for the estimation of the potency of a preparation by means of its effect on same type of living organism.

- It is done when the potency cannot be adequately determined by chemical or physical type of assays.

- The assay depends on comparing the potencies of preparation on an agreed scale, by determining how much of the sample being tested produces the same biological effects as a given quantity of the standard preparation.

- The microbiological assay of vitamins involves a stimulus applied to a subject i.e, bacterial culture. The intensity of the stimulus applied to a subject is known as the dose and is measured by weight, a volume or concentration of the preparation.

SELECTION OF MICRO ORGANISMS:

- The organism like bacteria, yeast, fungi and some protozoan like euglena gracillis are used for this purpose.
- The organisms should be genetically stable so that undesirable changes in response to the test compound do not occur.
- It should be respond only to the test compound.
- It should grow relatively quick on simple media.
- The fashion of growth should facilitate the reading of the assay.
- It should not clump for turbidimetric assay.
- It should possibly be aerobic. Since anaerobic process are more difficult.
- It should grow well at a pH that does not affect the stability of the material.

METHODS OF MICROBIOLOGICAL ASSAY:

- DILUTION METHOD.
- TURBIDIMETRIC METHOD.
- TITRIMETRIC METHOD.

A. DILUTION METHOD:

1. Diffusion assay are carried out on a solid media usually agar media.
2. The compound to be assayed is allowed to diffuse through the medium in a radial fashion from a pad or cup which results in the stimulation of growth of micro organisms.
3. The diameter of this area will give the concentration of the compound being assayed.
4. It is compared with similar zones produced by various known concentration of standard or reference compound.
5. The zone diameter of the standard are plotted against logarithms of the concentration used.
6. The linear portion of the standard curve is used for determining the actual concentration of the sample being assayed.

B. TURBIDIMETRIC AND GROWTH ASSAY:

1. Turbidimetric method of assay is done in liquid media.
2. Micro organisms in liquid media will produce an increase in turbidity.
3. A serious of tubes containing liquid media is taken and adds a graded amount of vitamin to be assayed.
4. The above tubes and their contents are sterilized and cooled. Inoculate a pure 24 hours culture and incubate for the specified period of time at constant temperature.
5. After incubation period the turbidity produced is employed for spectrophotometric analysis.
6. The selection of wavelength depends upon the colour of the medium used.
7. The percentage absorbances are found out and are plotted against the concentration of standard to obtain standard curve.

MICROBIOLOGICAL ASSAY OF VITAMIN B-12 or CYANOCOBALAMINE

Cyanocobalamine can be assayed by the following two methods:

- TITRIMETRIC METHOD.
- TURBIDIMETRIC METHOD.

1.TITRIMETRIC METHOD:

The following reagents are prepared before starting the assay experiment.

1.ACID HYDROLYSED CASEIN SOLUTION:

Method of preparation:

- Mix 100gms of vitamin free casein with 500 ml of 6N HCL in a flask and sterilized for 5 hours at 121^0C and 15 lbs pressure.
- Remove the acid by distillation to form a thick paste.
- Add 400ml of water and remove water by distillation to form a thick paste.
- Again paste is dissolved in 800ml of water and pH is adjusted with 40% sodium hydroxide to 3.5.
- Make up the volume up to one litre with water.
- Decolorize the solution with 20gms of decolorizing charcoal for 1 hour.
- Filter with Buckner funnel.
- Place the clear solution in a cool place.

2. TOMATO JUICE FILTERATE:

- Clarify 1000ml of canned tomato juice by filtration in a Buckner funnel with the help of 8gm of analytical grade filter aid.
- Straw colored filtrate is stored under a thin layer of toluene in a refrigerator.

3. ASPARAGINE SOLUTION:

- Dissolve 1gm of L-asparagine in water and make up the volume to 100ml.
- Store under suitable condition.

4. ADENINE GUANINE URACIL SOLUTION:

- Suspend 100mg each of adenine hydrochloride, Guanine hydrochloride and Uracil hydrochloride in 5ml of 6N HCL and dissolved by heating gently.
- Stored in a suitable container.

5. XANTHINE SOLUTION:

- Suspend 100mg of xanthine in 20ml of water. Heat to 60^0C.
- Add 3ml of 110% w/v ammonia solution.
- Make up the volume with 100ml of water and store under suitable condition.

6. BIOTIN STOCK SOLUTION:

- Dissolve 10mg of D-biotin in 200 ml of 50% alcohol.
- Store under suitable condition.

7. RIBOFLAVIN –THIAMINE-BIOTIN-NICOTINIC ACID SOLUTION:

- 5mg of riboflavin.
- 5mg of thiamine hydrochloride,
- 10mg of nicotinic acid and
- 1ml Biotin stock solution

Dissolved in 150ml of 0.02N Acetic acid and store under suitable condition.

8. PARA AMINO BENZOIC ACID-PYRIDAZINE-PYRIDOXAL- PYRIDOXAMINE SOLUTION:

- 10mg of Para amino benzoic acid,
- 20mg of pyridoxine,
- 20mg of pyridoxal hydrochloride and
- 4mg of pyridoxamine di-hydro chloride

Dissolve in 200ml of 25% alcohol.

Store under suitable condition not more than two weeks.

9. CALCIUM PANTOTHENATE-FOLIC ACID SOLUTION:

- 5mg of folic acid,
- 25mg of calcium pantothenate,
- Small amount of dilute ammonia solution

Dissolve in 500ml of 25% of alcohol.

10. SALT SOLUTION A:

- Dissolve 5gm of potassium phosphate and 5gm of potassium di-hydrogen phosphate in water.
- Add a drop of hydrochloric acid and make up to 100ml of water.

11. SALT SOLUTION B:

Dissolve the following in water

- 2gm of magnesium sulphate,
- 0.1gm of sodium chloride,
- 0.1gm of ferrous sulphate and
- 0.1gm of manganese sulphate.
- Add a drop of HCL and make the volume up to 100ml with water.

12. SORBITON MONE-OLEATE DERIVATIVE SOLUTION:

- Dissolve 10gm of poly-oxy ethylene derivative of sorbiton mono-oleate in alcohol.
- Make up the volume to 100ml with alcohol.
- Store in cold condition.

CULTURE MEDIUM

Constituents of the medium

- Dried yeast extract - 0.75gm
- Peptone – 0.75gm.
- Anhydrous dextrose – 1gm
- Potassium dihydrogen phosphate – 0.20gm
- Tomato juice filtrate – 10ml
- Sorbiton mono oleate solution – 1ml
- Distilled water up to 100ml.

Dissolve all the ingredients in water and adjust the pH to 6.8 with sodium hydroxide.

Place 10ml portion of the solution in test tube, plug with cotton.

Sterilized for 15minutes at 121^0C and at 15 lbs pressure.

ORGANISMS USED:

Lactobacillus leichmannii 313

STOCK CULTURE:

- Prepare stock culture of lactobacillus leichmannii in two or more tubes.
- Incubate at 37^0C for 16-24 hours.
- Mark one tube as stock culture and use other for transfer into inoculum medium.
- From stock culture prepare fresh sub-culture every 2-3 days.

BASAL MEDIUM STOCK SOLUTION:

- First dissolve L-cysteine and dL-tryptophan in HCL and add other reagents in the following order.
- Dissolve dextrose, sodium acetate and ascorbic acid separately in 100ml.
- Adjust the pH to 6.0 with 1N NaOH.
- Add sorbiton mono oleate derivative solution and
- Make up the volume to 250ml with water.

REAGENTS	QUANTITY
L-cysteine	0.1gm
dL-tryptophan	0.1gm
1N HCL	10ml
Adenine-Guanine-Uracil solution	5ml
Xanthine solution	5ml
Riboflavin-Thiamine-Biotin-Nicotinic acid solution	10ml
Calcium pantothenate-Folic acid solution	5ml
Salt solution A	5ml
Salt solution B	5ml
Asparagines solution	5ml
Acid hydrolysed casein solution	25ml
Dextrose	10gm
Sorbiton mono-oleate derivative	5ml

SUSPENSION MEDIUM:

- Dilute 25ml of the basal medium stock solution with 25ml of water.
- Place 10ml of the dilute solution in 5 test tubes and sterilize.
- Cool and store in a refrigerator.

INOCULUM:

- Inoculate a loop full of a sub culture of lactobacillus lechmannii into tube containing 10ml of culture medium.
- Incubate for 10-24 hours at a temperature of 37^0C.
- Centrifuge the culture till the cells have settled and decant the supernated liquid.
- Repeat this process till all cells have been settled.
- Finally suspend the cells uniformly in 10ml of suspension.
- The resulting cell suspension is the inoculum.

STANDARD CYANOCOBALAMINE STOCK SOLUTION:

Accurately weigh the Cyanocobalamine reference standard and dissolve in 25% of alcohol to yield a solution containing 0.1µg /ml of Cyanocobalamine.

STANDARD CYANOCOBALAMINE SOLUTION:

Dilute the stock solution with water to yield a standard Cyanocobalamine solution contains 0.01 – 0.04 µg/ml.

TEST SOLUTION:

- Weigh accurately a suitable amount of the material to be assayed and dissolved in water.
- Adjust the pH to 6 with dilute HCL or NaOH solution.

ASSAY PROCEDURE:

1. To triplicate test tubes add 0.1ml, 0.5ml, 1ml, 2ml, 2.5ml, and 3ml up to 5ml respectively of the standard Cyanocobalamine solution.
2. Similarly to the tube a triplicate test tube add 1,2,3,4 and 5ml respectively of the test solution to be assayed.
3. To each tube add 5ml of basal medium stock solution and sufficient water to make 10ml.
4. Mix the contents and plug with non-absorbent cotton and sterilize for 5min at 121^0C and 15 lbs pressure in autoclave.
5. Cool the tubes and inoculate one drop of inoculum.
6. Incubate for 64 – 72 hours at 37^0C.
7. Finally titrate the contents of each tube with 0.05N NaOH to pH 7.
8. This may also do in another method: to the test tubes add 0.1% w/v solution of bromo thymol blue as an internal indicator to give green colour.
9. Determine the average of the triplicate titration volumes for each level of standard and test sample yield.
10. Plot a graph taking titration values in Y axis and amount of the standard solution in X axis.
11. A smooth standard calibration is obtained.
12. By interpolation determine the potency of the test sample.

2. TURBIDIMETRIC ANALYSIS

REAGENTS AND APPARATUS:

All the reagents and apparatus are the same under Titrimetric analysis.

ASSAY PROCEDURE:

Proceeds as described under titrimetric method except the following deviations.

1. Include also two tubes as Uninoculated blank (to which neither standard Cyanocobalamine solution nor test solution, nor inoculum is added).
2. Incubate all the tubes for only 16-24 hours.
3. Transfer the contents of Uninoculate blank into colorimeter and adjust the transmittance at 640nm to 100%.
4. Record the transmittance of each sample and plot a graph taking transmittance at Y axis and standard Cyanocobalamine solution in X axis.
5. Draw a smooth curve and by interpolation method determine the potency of the test sample.

MICROBIOLOGICAL ASSAY FOR OTHER VITAMINS:

Reagents and Apparatus:

1. All the apparatus and reagents are the same as described for vitamin B12.
2. Except on preparing the basal medium stock solution, we have to create a deficiency of that particular vitamin in the individual assay.
3. Example: in the assay of Cyanocobalamine we have to create a deficiency of vitamin B12 in the preparation of basal medium stock solution.

VITAMINS	ORGANISMS
Riboflavin (Vitamin B2)	Lactobacillus casei
Niacin (Vitamin B3)	L. arabinosus
Pantothenic acid (Vitamin B5)	L. prantarum
Folic acid (Vitamin B9)	Streptococcus facalis or L. casei

THANK YOU