FOOD & PHARMACEUTICAL MICROBILOGY
(PHARMACEUTICS-VII)

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Sterility Testing as per I.P.

Sterility testing is designed to demonstrate the presence or absence of extraneous viable contaminating microorganisms in biological parenterals designed for human use. The sterility test is applied to substances, preparations or articles, which, according to the United States Pharmacopoeia (USP) and European Pharmacopoeia (EP), are required to be sterile. Sterility testing is used for bulk and final drug products, cell banks and raw materials.

Charles River provides sterility testing that is compliant with both USP and EP regulations. Sterility testing is conducted on bulk or final products by direct inoculation or membrane filtration methods and can be performed in an isolator or cleanroom environment. A bacteriostasis/fungistasis test is also performed to assess whether or not the test article inhibits the growth of microorganisms. The performance of bacteriostasis and fungistasis testing is necessary in order to validate the sterility result and confirm that there are no antimicrobial properties within the test article that would inhibit the detection of microbial organisms during the sterility assay.

Medium used for sterility testing:-

Fluid thioglycollate medium:-aerobic culture media

Pancreatic digest of casein 15.000

Yeast extract - 5.000

Dextrose (Glucose) - 5.500

Sodium chloride - 2.500

L-Cystine - 0.500

Sodium thioglycollate - 0.500

Resazurin sodium - 0.001

Agar - 0.750

Final p (at 25°C) - 7.1±0.2

Alternative thioglycollate medium:-anaerobic culture media

Pancreatic digest of casein - 15.000

Yeast extract - 5.000

Dextrose (Glucose) - 5.500

Sodium chloride - 2.500

L-Cystine - 0.500

Sodium thioglycollate - 0.500

Final pH (at 25°C) - 7.1±0.2

Soybean casein digests media: - aerobic culture media

Pancreatic digest of casein - 17.000

Papaic digest of soyabean meal - 3.000

Sodium chloride - 5.000

Dextrose (Glucose) - 2.500

Dipotassium hydrogen phosphate - 2.500

Final pH (at 25°C) - 7.3±0.2

Table 2: Minimum Quantity to be Used for Each Medium

Quantity per Container	Minimum Quantity to be Used *(unless otherwise justified and authorized)*
Liquids (other than antibiotics)	
Less than 1 mL	The whole contents of each container
1-40 mL	Half the contents of each container,but not less than 1 mL
Greater than 40 mL,and not greater than 100 mL	20 mL
Greater than 100 mL	10% of the contents of the container, but not less than 20 mL
Antibiotic liquids	1 mL
Other preparations soluble in water or in isopropyl myristate	The whole contents of each container to provide not less than 200 mg
Insoluble preparations, creams, and ointments be suspended or emulsified	Use the contents of each container to provide not less to than 200 mg
Solids	
Less than 50 mg	The whole contents of each container
50 mg or more, but less than 300 mg	Half the contents of each container, but not less than 50 mg
300 mg-5 g	150 mg
Greater than 5 g	500 mg
Devices *[Omitted for this article]*	

source: USP (Volume 30)[2]

Method:-

Direct Inoculation Sterility Testing

- The test article is directly inoculated into two types of media to allow for the detection of both aerobic and anaerobic microorganisms.

- After inoculation, the media is incubated for 14 days with intermittent observations as well as a final observation at the end of the testing period to detect evidence of microbial contamination.

Membrane Filtration Sterility Testing

- Sterile, enclosed units allow for the simultaneous filtration of the equal volumes of test samples through two membrane filters.
- Samples are incubated in two types of media for 14 days, facilitating the detection of both aerobic and anaerobic microorganisms.

Preservative efficacy:-

A **preservative** is a substance or a chemical that is added to products such as food, beverages, pharmaceutical drugs, paints, biological samples, cosmetics, wood, and many other products to prevent decomposition by microbial growth or by undesirable chemical changes. In general, preservation is implemented in two modes, chemical and physical. Chemical preservation entails adding chemical compounds to the product. Physical preservation entails processes such as refrigeration or drying.[1] Preservative food additivesreduce the risk of foodborne infections, decrease microbial spoilage, and preserve fresh attributes and nutritional quality. Some physical techniques for food preservation include dehydration, UV-C radiation, freeze-drying, and refrigeration. Chemical preservation and physical preservation techniques are sometimes combined

pharmaceutical preparations for preservative efficacy (PET), including parenteral, ophthalmic, oral liquid, ear preparations and topical preparations, performing methods of assessment to the EP, BP, USP and the JP.

Laboratory testing for Efficacy of Antimicrobial Preservation (PET) is performed to determine the type and minimum effective concentration of preservative required for satisfactory multi-dose pharmaceutical formulation.

The test consists of challenging the preparation with live organisms at a series of time points over an in use period of up to 28 days. At each time point analysis is performed to establish if organisms are present or not and therefore if the preservative is effective.

Microbial assay of antibiotics:-

Microbial assay of antibiotics:-

An antibiotic assay is used to determine the potency of the antibiotics using an assay technique. The potency of the antibiotic can be examined by creating a suitable environment. Its inhibitory effects are then studied on the respective microorganisms.

An antibiotic sensitivity assay tests the efficacy of a drug in inhibiting the growth of microorganisms that causes a disease. Antibiotic assays are carried out on the infecting organism that is found in clinical specimens of body fluids such as blood and urine collected from patients seeking treatment. Isolation and identification of the pathogens responsible for the disease is necessary before selecting the appropriate antibiotic for testing. Once isolated from the patient's specimen, the organism is cultured in a medium with a nutrient broth at pH 7.0, in most cases. Then, the culture is inoculated with a suitable solution of the antibiotic to be used to fight the disease, and its effectiveness in inhibiting the growth of the microorganism is recorded.

Antibiotic Assay In Body Fluid

The Stokes method tests sensitivity to an antibiotic by using an inoculation of a controlled organism on a part of the plate, while the rest of the plate is coated with the test organism. Disks placed at the interface allow the comparison of the zones of inhibition.

Cylinder Plate Method:-

This test is used to determine the sensitivity of an organism. This organism is usually isolated from a patient before the tests are begun. This is a method in which the antibiotic solution is diffused from a cylinder which is placed on an inoculated surface. After incubation, the diameter of the inhibition zone is measured. This diameter depends on the concentration of the antibiotic used and its specific activity. This method is popular and is therefore used in the commercial preparation of antibiotics.

The result of such an assay can help a physician determine the kind of drugs that need to be prescribed. If the organism is susceptible to the antibiotic in this test, it is highly likely that it will also be killed off when the antibiotics interact with the microbe in the blood stream.

Turbidimetric Tube Method:-

In this method, a uniform solution of an antibiotic is made. The microbial culture is added to the fluid. The biggest advantage of this method is that it requires a relatively shorter incubation period. However, there is also a big disadvantage. The presence of foreign material that may be inhibitory to the growth of microbes may influence the results of this assay. This method is therefore appropriate when the samples are clear.

Antibiotic protection assay is another assay that might be done in order to check the ability of the microbe to invade the epithelial cells and cause harm. Such a test can help the doctors determine the course that will be taken by a disease that is caused by microbes. It can also help the doctors determine the kind of treatment that should be given to the patient for the particular microbe. Additional tests may be required with the antibiotic protection test.

MEDIA:-

Prepare the media required for the preparation of test organism inocula from the ingredients listed in Table 1. 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 1 M sodium hydroxideor 1 M hydrochloric acid, as required so that after sterilization the pH is as given.

Media used in oxetetracycline:-	**test organism**
Peptone-6 gm	staphylococcus aureus
Yeast extract-1.5gm	
Beef extract -1.5gm	
Dextrose-1gm	
Nacl – 3.5 gm	
Dipotassium hydrogen phosphate-3.68	
Potassium di hydrogen phosphate-1.32	

PH - 6.9 to 7.1

Media used in erythromycin: - **test organism**

Peptone-6 gm micrococcus luteus

Pancreatic digest of casein-4.o gm

Yeast extract-3.0gm

Beef extract -1.5gm

Dextrose- 1gm

PH - 7.8 to 8.0

STANDARD PREPARATION AND UNITS OF ACTIVITY

A Standard Preparation is an authentic sample of the appropriate antibiotic for which the potency has been precisely determined by reference to the appropriate international standard. The Potency of the standard preparation may be expressed in International Units or in µg per mg of the pure antibiotic.

The Standard Preparations for India are certified by the laboratory of the Indian Pharmacopoeia Commission or by any other notified laboratory(ies) and are maintained and distributed by the agency(ies) notified for the purpose.

A Standard Preparation may be replaced by a working standard prepared by any laboratory which should be compared at definite intervals under varying conditions with the standard.

Buffersolution:-

Prepare by dissolving the following quantities given in Table 2 of dipotassium hydrogen phosphate and potassium dihydrogen phosphatein sufficient water to produce 1000 ml after sterilisation, adjusting the pH with 8 M phosphoric acidor 10 M potassium hydroxide.

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 3, in the solvent specified in the table, and then dilute to the required concentration as indicated. Store in a refrigerator and use within the period indicated. On the day of assay, prepare from the stock solution five or more test dilutions, the successive solutions increasing stepwise in concentration, usually in the ratio 1:1.25 for Method A or smaller for Method B. Use the final diluent specified and a sequence such that the middle or median has the concentration specified in Table 3

Preparation of the sample solution

From the information available for the substance under examination (the "unknown"), assign to it an assumed potency per unit weight or volume, and on this assumption prepare on the day of the assay a stock solution and test dilution as specified for each antibiotic in Table 3 but with the same final diluent as used for the Standard Preparation. The assay with 5 levels of the Standard requires only one level of the unknown at a concentration assumed equal to the median level of the standard.

Assay method:-

Two general methods are usually employed, the cylinder-plate (or cup-plate) method and the turbidimetric (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

turbidimetric 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 favourable 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 doseresponse 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.

Microbial assay of Vitamin B-12

Method: - Agar cup method
Culture maintenance medium: - Nutrient agar
Assay Medium: -

Ingredients	Quantity / 100 ml.
1 Dipotassium Hydrogen Phosphate	- 0.7 g
2 Potassium Dihydrogen Phosphates	- 0.3 g
3 Sodium Citrate A.R	-0.05 g
4 Magnesium Sulphate	- 0.01 g
5 Ammonium Sulphate	- 0.1 g
6 Sodium Chloride	- 0.005 g
7 Distilled Water	- 100 ml

Dissolve the ingredients separately to avoid precipitation & shake and mix the solution. If necessary, adjust the pH of the solution to 7.0 ± 0.1. Add 1.5 gmof agar powders and sterilize at 15 lbs pressure for 20 minutes.
Dextrose Solution: Prepare 40% w/v solution of Dextrose (A.R.) using distilled water. Sterilize at 15 lbs pressure for 15 minutes. Cool and store in the refrigerator.

Inoculums medium:

Nutrient broth – 1.3 gm
Distilled water - 100 ml
Adjust the pH of the solution to 7.0 ± 0.1. Dispense in screw capped test tubes and autoclave at 15 lbs pressure for 15 minutes. Cool & store at 4°C.

Preparation of Seeding Inoculum :-

Using a bacteriological loop inoculate a test tube of inoculum medium or NB with growth from a fresh agar slope & incubate at 37°C. For 16-18 hours. Store
at 4°C. Centrifuge required amount of inoculum medium for 20 min at 2200 rpm & Suspend the cells in 5 ml of sterile saline centrifuge for 10 min at 2200
rpm and decant the supernatant. Repeat the process. Finally, suspend the cells in 1 ml of sterile saline and mix well and use it as inoculum.

Preparation of Assay Plates:

To 100 ml of sterile and cooled at 45°C. Vitamin B12 assay medium, add 1.0 ml of 40% sterile dextrose solution and shake well. Add 1 ml of inoculum of

E.coli M200 of required thickness. Mix gently but thoroughly. Distribute 25 to 30 ml of the inoculated medium in sterile Petri plates. Allow to set the medium
& store in refrigerator until use. Plates should be used on the same day or within one day. (*Addition from the inoculum suspension will depend upon
Culture activity that should be validated by the Microbiologist.)

Preparation of Agar Cups:-

A standard 8.0 mm diameter borer is taken. Dip the borer in Isopropyl alcohol and burn the remaining Isopropyl alcohol from borer on flame, cool the borer
Properly and bore cups in preseeded agar plates. Bore four cups per plates.
Preparation of Standard Stock Solution:
Weigh accurately 10 mg of crystalline Cyanocobalmin powder (dark red coloured). Transfer it to 10 ml volumetric flask and make up the volume to 10 ml.
Take 1 ml and dilute it to 100 ml with water. Take the reading of this solution at 361 nm and calculate the conc. taking E1 % as 0.207. Calculate exactly the
Conc. of vitamin B12/ml.
Calculation:
O.D. at 361
---------- X 100 = X mcg/ml.
0.207
Accordingly calculate X ml [about 10 ml of this solution] dilute to 100 ml to get [1 mcg/ml]. This is one mcg /ml stock, use within one month.
Preparation of Standard Dilutions :-
Take 5 ml of Std. stock solution & make up the volume to 50 ml, treat it as Standard High (SH).Take 5 ml. of Std. High dilution and make up the volume to
50 ml. Treat it as Standard Low (SL).
Preparation of Test Dilution:
Weigh and transfer a sample quantity eq. to 5 mcg. to 50 ml volumetric flask. Add distilled water, shake vigorously and dilute to 50 ml with distilled
Water(TH). Further dilute 5 ml of the TH to 50 ml with distilled water [TL]. Application of standard and Test Dilutions: With the help sterile micropipette tips
Apply 100 µl of different dilution to different cups. Mark every cup with proper dilution and keep the plates at low temperature (around 10°C) for 10-20
Minutes for diffusion.
Incubation of Plates: Incubate the plates at 30-37°C for overnight (18-24 hours).
Measurement of Diameter:
Measure the diameter of every zone of growth from three different sides. Note down every reading.
Calculations:
Sum up three readings of individual dilution.
Formula -
Assay % = Antilog (2 ± a log I)
Where,
(TH + TL) - (SH + SL)