UNIT IV

4.1: Designing of aseptic area, Laminar flow equipment's

4.2: Study of different sources of contamination in an aseptic area and methods of prevention

4.3: Clean area classification

4.4: Principles and methods of different microbiological assay

4.5: Methods for standardization of antibiotics, vitamins and amino acids

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4.1. ASEPTIC AREA

4.1.1. Introduction

Aseptic techniques are employed to provide protection to ophthalmic and parenteral products by preventing the entry of microbial and particulate contamination. Prevention of microbial contamination is also required to remove pyrogens and toxic bacterial products. The terminally sterilised products (product sealed in container and then sterilised) are prepared in clean areas: while products not terminally sterilised are prepared under aseptic conditions using sterile materials or are sterilised by filtration before being packed in sterile containers. Such aseptic products are formulated or prepared in an aseptic area, which is a room within a clean area designed, constructed, serviced, and used for controlling and preventing microbial contamination of the product.

Like sterile medicinal products, vaccines containing dead microorganisms, microbial extracts, or inactivated viruses are also filled in aseptic areas; while live or attenuated vaccines are filled in separate areas.

The goal of any facility is to design such an aseptic or sterile environment which provides a controlled environment so that the entrance of viable (microbial) and non-viable (particles) contaminants can be minimised. A controlled environment prevents cross- contamination of Compounded Sterile Preparations (CSPs). To reduce the contamination-related risk, all compounding should be done within primary engineering controls, like a Laminar Airflow Hood (LAFH), also known as workbenches, Biological Safety Cabinet (BSC), or a Compounding Aseptic Isolator (CA).

4.1.2. Designing of Aseptic Area

The sterile production unit and the general manufacturing area within the hospital pharmacy or factory should be located separately. The sterile production unit should be buffered, i.e., unauthorised personnel should not gain access to this unit.

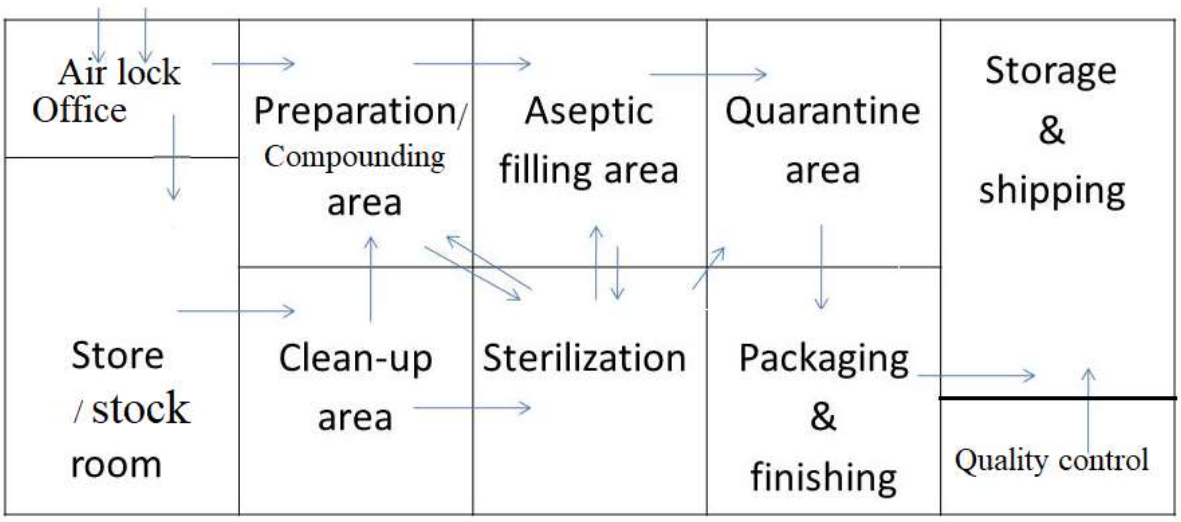

Figure 4.1: Flow Diagram of Aseptic Area

The aseptic unit is designed to carry out each stage of production separately. The unit should also ensure a safe and organised workflow so that the need for personnel to move around the clean rooms is minimised. The unit is built and the equipment is positioned in such a manner that the product remains protected from contamination.

The layout of aseptic area should be such that cleaning can be done easily and dust accumulation can also be reduced. Arrangement should be such that the risk of cross-contamination (contamination of one product or material with another) is reduced.

The filling area should be adjacent to the compounding area where the personnel assemble and prepare materials utilised by the staff in the filling area. In figure 4.1 the layout of areas and rooms for preparing terminally sterilised products (such as small or large volume injections) is shown.

4.1.2.1. Design and Construction

Only authorised personnel can gain access to the clean and aseptic filling areas. The personnel enter the clean rooms by passing through the changing rooms where they put on and remove their clean room uniform.

A pass-Over (or cross-over) bench extends across the changing room to form a physical barrier for separating the different areas for changing by the personnel.

Special precautions are taken for preventing clean and aseptic filling areas from getting contaminated while materials are being passed through airlocks or hatchways. Thus, sterilisers and entry ports are fitted with double-sided doors, which are interlocked to prevent simultaneous opening of both the doors.

4.1.2.2. Surfacing Materials

The floor, wall, and ceiling surface of clean rooms should be smooth, impervious, and unbroken to reduce the release and accumulation of contaminating particles and organisms. The surface material should be such that they can withstand the effects of cleaning agents and disinfectants. The ceilings are scaled so that the contaminants do not enter from the space above them.

Uncleanable recesses should be avoided within the clean rooms to minimise the accumulation of contaminating particles. Thus, the wall and floor junction should be covered. Minimum shelves, ledges, cupboards, and equipment should be present. Non opening and sealed windows should be present to prevent the entry of contaminants.

4.1.2.3. Services

The piped liquids and gases entering the clean rooms should be filtered first, thus ensuring that the liquid or gas at the work position is as clean as the air in the clean room. The position of pipes and ducts should be such that they can be easily cleaned. Other fittings, like fuse boxes and switch panels should be placed outside the clean rooms.

Sinks and drains should not be present in areas where aseptic procedures are carried out within the clean room areas. They should nowhere be present in the complete unit. The areas having sinks and drains should be designed, positioned, and maintained such that the risk of microbial contamination is reduced; therefore, they are fitted with easily cleanable traps, installed with electrically heated disinfection devices.

A limited number of doors and ports should be present for entry of personnel and materials, respectively. The entry doors should be self-closing to allow easy movement of the personnel.

Airlock doors, wall ports, through-the-wall autoclaves, and dry heat sterilisers should have interlocked doors to prevent simultaneous opening of both the doors. All the doors should have an alarm system which should ring when more than one door are being opened.

Lights in clean rooms should be fitted with the ceiling so that dust accumulation reduces and also the airflow pattern within the room is not disturbed. The equipment in clean rooms should be positioned such that accumulation of particles and microbial contaminants does not Occur.

4.1.3. Laminar Flow Equipment (Laminar Airflow Hood)

A Laminar Airflow Hood (LAFH)/ Laminar Aseptic Hoods, or a workbench, is a primary engineering control device which provides the following services during aseptic compounding

1) Clean air to the critical sites (immediate aseptic compounding area),

2) Constant flow of air out of the work area to prevent the entry of room air, and

3) Outward flow of air from the hood that suspends and removes contaminants which have been introduced in the work area by personnel.

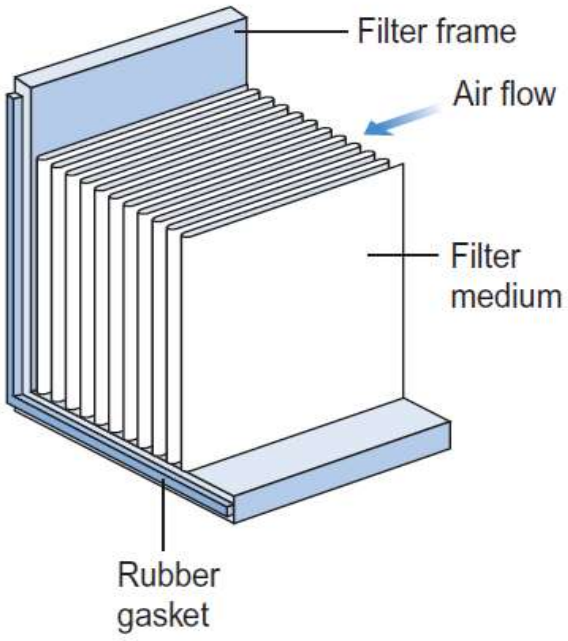

Figure 4.2: Section through a Mini-Pleat High-Efficiency Filter

A High Efficiency Particulate Air (HEPA) Filter is the most important part of a LAFH. The air within the room is taken into this filter and passed through a pre-filter which removes the gross contaminants (lint, dust, etc.). The air is then blown at a uniform velocity through the hood and HEPA filter in a unidirectional (laminar flow) manner over the critical sites (immediate aseptic compounding area). HEPA filter is a particulate filter which traps the airborne particles and microbes; but allows the gases to pass through.

HEPA filter should be fitted either at or near to the clean room inlet. A pre-filter is fitted upstream of the HEPA filter, thus extending the final filter's life. A fan is also fitted which pumps the air through the filter.

The filter medium used in HEPA filters 1S made up of pleated fiberglass paper. Parallelly arranged pleats not only increase the filter surface area but also the air flow through the filter (figure 10.2). This parallel arrangement of filter medium also allows the filter to retain a compact volume. In the traditional type of HEPA filters, aluminium foil was used as spacers, which are no longer used in the modern mini-pleat type of filter (now widely used). The mini-pleat filters have a shallower depth in construction than the traditional HEPA filter. The filter material is sealed to an aluminium frame within the filter (figure l0.2). One side of the filter is protected with a coated mild steel mesh. HEPA filters provide:

1) A high air flow rate,

2) A high particulate holding capacity, and

3) A low-pressure drop across the filter.

HEPA filters remove larger, medium, and smaller particles from the air by inertial impaction, direct interception, and by Brownian diffusion, respectively. The HEPA filters are least

efficient in removing particles of about 0.3um size. However, its efficiency of removing particles is affected by air velocity and filter packing. It removes larger and smaller particles with more efficiency.

The LAFH works on the principle that twice-filtered laminar layers of aseptic air continuously sweep the work area within the hood to prevent the entry of contaminated room air.

4.1.3.1. Types

A laminar air flow hood is of two types:

1) Horizontal Airflow Hoods: These hoods (figure 10.3) sweep the filtered air from the back to the front of the hood. An electrical blower draws the contaminated room air using a pre-filter (similar to a furnace filter), to remove gross contaminants. The pre-filter demands timely cleanliness and replacement. The air after passing through the pre-filter is accelerated (but not pressurised) so that a consistent airflow distribution reaches the final filter (HEPA filter), present at the back portion of the hood's working area.

HEPA filter removes 99.97% of 0.3µ or larger sized particles, thus removing most of the 0.5µ or larger sized airborne microorganisms.

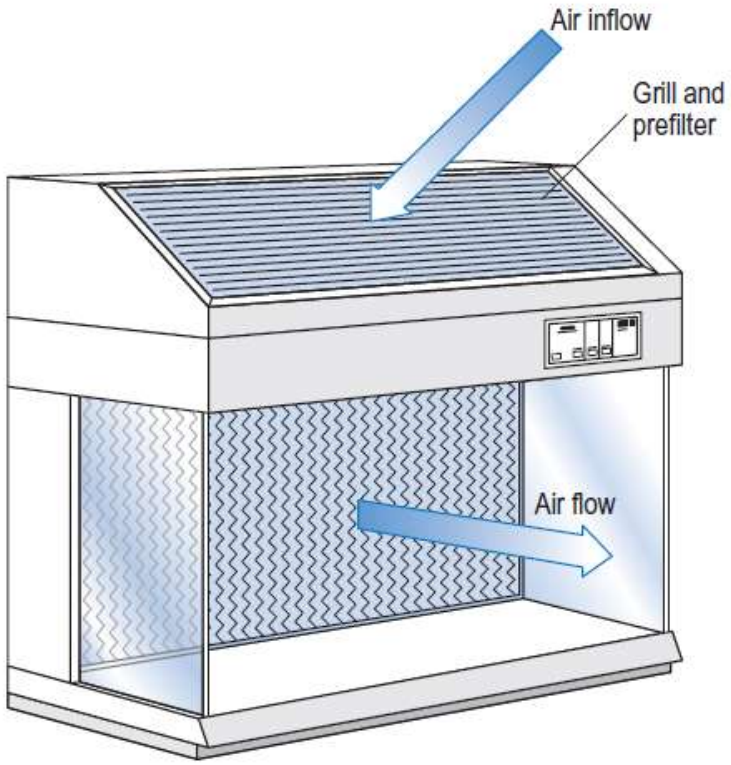

Figure 4.3: Horizontal Laminar

2) Vertical Airflow Hoods: These hoods (figure 4.4) sweep the filtered air vertically. The air passing through HEPA filter merges from the top and passes downward through the working area. The vertical airflow hoods work on the principle that the air flow between the HEPA filter and the component used for CSPs preparation should not be interrupted. When a foreign object comes in between the sterile object and the HEPA filter, the wind turbulence (zone of

turbulence) in the critical area increases, and foreign contaminants are carried to the sterile work surface, thus contaminating the injection port, needle, or syringe.

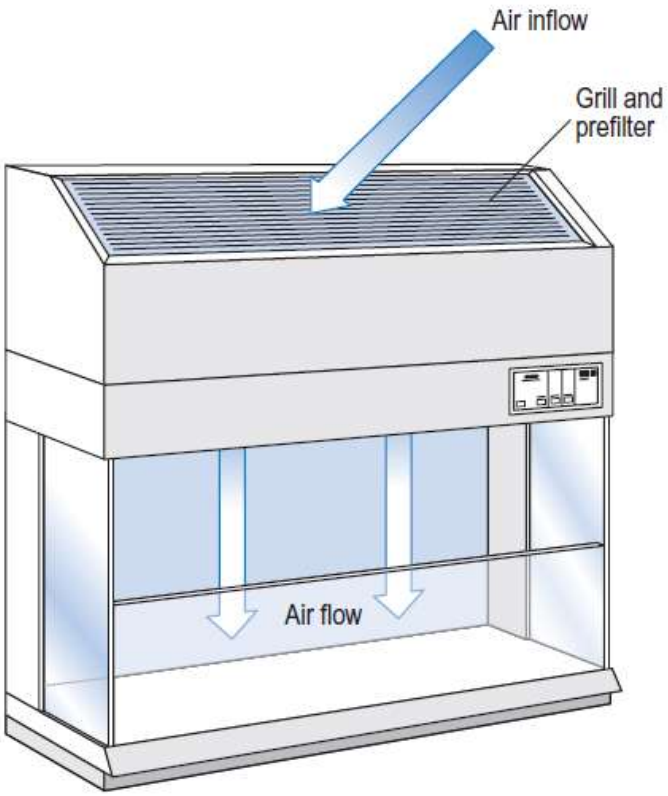

Figure 4.4: Vertical Laminar

For ensuring complete sterility, nothing should pass behind a sterile object in a horizontal airflow hood; and in case of vertical airflow hood, nothing should pass above the sterile object. The materials within the compounding work area interrupt the pattern of air blowing from the HEPA filter.

4.1.3.2. Operating Principles

A Laminar Air Flow Hood (LAFH) operates on the following general principles:

1) It should be placed away from traffic, doors, air vents, or anything that can produce air currents, to avoid entrance of contaminants.

2) It should remain on for 24 hours otherwise, non-filtered, non-sterile air occupies the compounding work area. Thus, when again it is turned on, it should be left for 30 minutes for disinfection and then used. This also allows the hood to eliminate the non-sterile air from the compounding work area.

3) Before using the LAFH, its interior working surfaces should be disinfected using 70% isopropyl alcohol (or other suitable disinfecting agents) and a clean, lint-free cloth. Cleaning should be done efficiently beginning from the HEPA filter in a top-to-bottom and back-to-front motion, to completely remove the contaminants.

4) Cleaning should be done after every compounding batch and also when the work surface becomes dirty. Some materials on the surface are insoluble in alcohol (e.g., dextrose and amino

acids), thus sterile water is used initially. After wiping the water from the surface, it is then cleaned using alcohol. Plexiglas sides present in some types of LAFHs, should not be cleaned using alcohol but with warm water and a germicidal detergent (e.-g., Lysol IC, Vesphene, or LpH).

5) HEPA filter grill (protective grill) should be cleaned in such a way that it does not get wet.

6) Objects essential for aseptic compounding should be placed in the hood. Paper, pens, labels, or trays should not be placed in this area.

7) HEPA filter should not be brought into contact with cleaning solution, aspirate from syringes, and glass from ampoules (should not be opened towards the filter)

8) The operators during compounding should not wear any jewellery on hands or wrists since they affect the integrity of personnel clothing (gowns and gloves).

9) The operators should minimise talking and should cough away from the compounding work area.

10) The operators should avoid or reduce the unnecessary movement within or around the LAFH to minimise the airflow turbulence.

11) Smoking, eating, gum chewing, and drinking are strictly prohibited in the aseptic environment.

12) The objects should be arranged to get full benefit of the laminar air flow. Critical items should be placed close to the air source. The objects in horizontal hood should not be placed closer than 3 inches from the back of the hood (ensuring that nothing should be in direct contact with the filter). The pharmacist in some cases, may stack a few objects (such as IVPBs), but from lower to higher starting from the back of the hood. Also, the stack should contain only 3-4 objects.

13) The aseptic procedures should be carried out 6 inches away from the sides and front edge of the hood to prevent contamination.

14) Testing of primary engineering controls (e.g., LAFHs, BSCs, and CAIs) should be carried every 6 months by qualified personnel. Testing is also required whenever the device 1s moved, or the filter is damaged. Specific tests are used for the certification of integrity of airflow velocity and HEPA filter.

4.1.3.3. Uses

Given below are the uses of a laminar air flow hood:

1) It has various applications, especially where an individual clean air environment is required for smaller items, e.g., particle sensitive electronic devices.

2) It is used for special operations in laboratories.

3) It can be tailor made to the specific requirements of laboratories and are also used for general lab work, especially in medical, pharmaceutical, electronic, and industrial sectors.

4.1.3.4. Advantages

Given below are the advantages of a laminar air flow hood:

1) The sterile area is spacious.

2) Since the sterile area does not become hot, the plant material can stay for a longer duration.

3) Bigger flasks having wide lids can be used.

4.1.3.5. Limitations

The airflow velocity can be overcome easily without any strict work practices and aseptic technique, thus an area of turbulence is created which can introduce room contaminants particles and microorganisms) into the critical work area. A laminar air flow hood Should remain on for 24 hours. lf it is turned off; it should be left on for atleast 30 minutes for its disinfection before use.

4.2. Different Sources of Contamination in an Aseptic Area

There are various contamination sources in an aseptic area, which are discussed below:

1) Personnel: Those supervising, performing, or controlling drug manufacturing are a potential source of microbial contamination, due to the following reasons:

i) Inadequate training,

ii) Direct contact between the hands and starting materials, primary packaging materials, and intermediate or bulk products,

iii) Improper hygiene,

iv) Unauthorised personnel entering the production, storage, and product control areas,

V) Insufficient gowning and protective equipment, and

vi) Eating, drinking, or smoking within the storage and processing areas.

2) Buildings and Facilities: These are also important contributors to microbial contamination due to the following reasons:

i) Inadequate size and organisation of the space, which lead to selection errors (such as mix-ups or cross-contamination between consumables, raw materials, in-process materials, and finished products),

ii) Poor filth and pest controls,

ii) Rough floors, walls, and ceilings,

iv) Absence of air filtration systems,

v) Inadequate lighting and ventilation systems,

vi) Poorly located vents, ledges, and drains, and

vii) Improper washing, cleaning, toilet, locker facilities, sanitary operation, and personal cleanliness.

3) Equipment and Utensils: These are used during processing, holding, transferring and packaging. They are the common sources of microbial contamination due to the following reasons

i) Unsuitable design, size, corrosion-causing materials, static material accumulation, and/or adulteration with lubricants, coolants, dirt, and sanitising agents,

ii) Inadequate cleaning and sanitisation,

iii) Inefficient cleaning and maintenance due to their designing

iv) Inappropriate calibration and irregular service, and

v) Using defective equipment.

4) Raw Materials: These are used during production and are considered a potential source of contamination due to the following reasons:

i) Improper storage and handling, which leads to mix-ups or selection errors,

ii) Microbial or chemical contamination,

ii) Degradation due to extreme environmental conditions (like heat, cold, sunlight, moisture, etc.),

iv) Wrong labelling.

v) Incorrect sampling and testing, and

vi) Using materials not meeting the acceptance criteria.

5)Manufacturing Process: During the manufacturing process, microbial contamination of raw materials, intermediates or packaging materials can widely occur due to the following reasons:

i) Absence of facilities required for manufacturing of a single product,

ii) Improper cleaning between batches for minimising the amount of product changeovers,

ii) Use of an open manufacturing system for exposing the product to the room environment,

iv) Improper zoning,

v) Lacking an area line clearance (as per the approved procedures) after each cleaning process and between each batch, and

vi) Lack of cleaning status labelling on all equipment and materials used within the manufacturing facility.

AHVAC (Heating, Ventilation, and Air Conditioning) System: An inappropriate HVAC system is a possible source of microbial growth and also disperses the contaminants throughout the manufacturing unit. This occurs due to the follow1ng reasons:

i) Organic materials accumulate in or near HVAC air intakes,

ii) Inadequate air filtration system,

iii) Inadequate magnitude of pressure differentials, which causes flow of reversal,

iv) Incorrect ratio of fresh air to re-circulated air,

v)Incapability of accessing ventilation dampers and filtering from outside the manufacturing areas, and

vi) Non-directional airflow within production or primary packing areas.

4.2.1. Methods of Prevention of Contamination

The steps by which microbial contamination of products can be prevented in aseptic areas are discussed below:

1) Personnel: They can take the following measures:

i) Access to production areas by the unauthorised personnel should be restricted, and only trained ones should be allowed to enter.

ii) Adequate personnel hygiene should be maintained.

iii) The personnel should be given proper and regular training with respect to hygiene as well to ensure that their activities do not hamper the product quality.

iv) Personnel entering the manufacturing area should wear protective clothing (over-garments, hair cover, beard or moustache cover, and overshoes).

V) Personnel should avoid touching with naked hands the exposed products or any part of equipment in contact with the product.

2) Facility Design: This includes the following measures:

i) In aseptic rooms, the differential air pressures should be higher than the adjacent controlled areas.

ii) Air filtration and air change rates should be set to attain the defined clean room class.

iii) Unidirectional (laminar flow) air flow should be maintained over critical areas at sufficient velocity to sweep particles away from filling/closing area.

iv) Ambient temperature and humidity should not be very high.

v)The ventilated cabinets, RABS (Restricted Access Barrier System), isolators systems, etc. should be used depending on the facility/product risk assessment results in order to achieve an absolute or partial barrier to contain microorganisms at their point of use

vi) The areas of unequal risk are separated by UV air locks and door barriers.

vii) All open-container processing should be performed within the isolator/RABS.

viii) The air pressure of the changing room should be negative with respect to the manufacturing area corridor, but positive with respect to the external adjacent areas.

ix) The ventilation dampers, specially designed filters, and other services should be positioned such that they can be accessed from outside the manufacturing areas (service voids or service corridors) for maintenance purposes.

X) An impermeable barrier should be used in appropriate areas to prevent cross- contamination between two zones, such as closed systems, pumped, or vacuum transfer of materials.

xi) HVAC air distribution components should be used for preventing the spreading of contaminants generated within the room.

xii) All rooms and surfaces should be maintained and monitored for viable and non-viable particulates and this facility should be re-certified on a semi-annual basis.

Facility and equipment design cannot eliminate microbial contamination completely but good risk assessment and consequent hygienic design can reduce these risks several times. For example,

i) Flow of man and material should be uniform,

ii) Equipment having smooth surfaces of appropriate material can facilitate effective cleaning, and

iii) Closed Material Transfer Systems.

3) Access to Areas: This includes the following measures:

i) Access to production, packaging, and QC areas by unauthorised personnel should be restricted.

ii) Personnel should gain access to these areas only via changıng rooms.

ii) Materials should be accessed via specific routes (generally air locks).

4) Building Requirements: This includes the following measures:

i) Smooth, crack-free, and easily cleanable floors, walls, and ceilings should be used as they facilitate easy and effective cleaning.

i) Windows or viewing panels should be closed (non-opening), fixed with wall panels, and sealed to prevent accumulation of dust and microbial contaminants.

iii) The designing of pipe work, ventilation, and light points should be such that Creation of recesses which are not easily cleanable is avoided.

iv) Sinks of stainless steel should be present within the production areas.

5) Cleaning and Disinfection: This includes the following measures:

i) Time-to-time cleanliness and disinfection of the areas should be done.

ii) Adequate hygiene should be maintained in every aspect of the drug product manufacturing.

iii) Cleaning agents of proper grades should be used for minimising health risks.

iv) Contact time, application, temperature, mechanical action, and the chemistry of cleaning agents should be considered during the cleaning process.

v)The cleaning agents should not be directly applied on the product.

vi) The cleaning practices should be validated to prove that the process effectively controls microbial contamination.

6) Utilities: This includes the following measures:

i) Water of pharmaceutical grade, microbiologically controlled and monitored should be used in the manufacturing of products.

ii) Clean and additive-free steam should be used for cleaning and sanitisation of production tools and equipment, and to supply for autoclaves and humidification.

4.3. Clean Area Classification

The clean or buffer room is the secondary engineering control. It houses the primary engineering controls (the LAFH, BSC, or CA) where the aseptic compounding is actually performed. Clean room is a specially constructed enclosed area, which contains one or more clean zones, where the concentration of airborne particles is controlled using HEPA filters, continuous air circulation, and a physical barrier to non-filtered (or outside) air. Clean rooms establish appropriate environmental levels for airborne particulates, temperature, humidity, air pressure, and airflow patterns. Clean rooms are categorised by their constant air quality or class. Clean rooms are rated as given in table 4.1:

Table 4.1: Classes of Clean Rooms and their Properties

Clean Room	Properties
Class 10,000 (ISO class 7)	10,000 or less particles of 0.5 μ and larger size exist in a given cubic foot of air.
Class 1,000 (ISO class 6)	1,000 or less particles of 0.5 μ and larger size exist in a given cubic foot of air.
Class 100 (ISO class 5)	100 or less particles of 0.5 μ and larger size exist in a given cubic foot of air.

Lower the classification number, cleaner is the air.

Apart from the above mentioned basic components, a clean room also includes:

1) UV irradiation,

2) Air-lock entry portals,

3) Special filtration or treatment systems for incoming air,

4) Sticky mats for removing particulate matter from shoes, and

5) Positive room air pressure for controlling entry of contaminants from adjacent rooms.

Clean rooms are divided into different classes in standards. Table 4.2 lists the equivalence of classes from different international standards. For manufacturing sterile products, a certain classification (table 4.3) with grades A to D are characterised to activity category (tables 4.3 and 4.4) is used.

Table 4.2: Class Limits for Federal 209D and ISO Standards

Class	Measured Particle Size (Micro meter)						
Federal 209 D	ISO	0.1	0.2	0.3	0.5(a)	1.0	5.01
1	3	1000	237	102	(1)	8	
10	4	10000	2370	1020	(10)	83	
100	5	100000	23370	10200	(100)	832	29
1000	6	1000000	237000	102000	(1000)	8320	293
10000	7				(10000)	832000	2930
100000	8				(10000)	8320000	293000

(a) - Particle count for this particular size is per ft while others are per m.

Table 4.3: Air Particle Classification System for the Manufacturing of Sterile Products

GRADE	Maximum Permitted Number of Particles per m Equal to or Above			
	At Rest (b)		In Operation (b)	
	0.5µm (d)	5µm	0.5µm (d)	5µm
A	3500	0	3500	0
B(a)	3500	0	350000	2000
C(b)	350000	2000	3500000	20000
D(c)	3500000	20000	Not defined (c)	Not defined (d)

Remarks

1) In order to reach the air grades B, C, and D, the number of air changes should be related to the room size, and the equipment and personnel in the room. The air system should be provided with HEPA filters for grades A, B, and C.

2) After 15-20 minutes of "'clean up" period, an unmanned state (no manual activity) should be received.

3) Appropriate alert and action limits should be fixed for particulate and microbiological monitoring results. A corrective action in the operating procedures should be taken in case these limits are exceeded.

The need for other parameters (like temperature, relative humidity, etc.) depends on the nature of product and manufacturing procedure. These parameters do not have any relation with the purifying classes.

Table 4.4: Terminally Sterilised Products

Grade	Examples of Operation
A	Filling of products, when unusually at risk.
C	Preparation of solutions, when unusually at risk. Filling of product.
D	Preparation of solutions and components for subsequent filling.

Most products should be prepared in grade D environment; while grade C environment should be used in case of an unusual risk.

Table 4.5: Aseptic Parameters

Grade	Examples of Operation
A	Aseptic preparation and filling
C	Preparation of solutions to be filtered.
D	Handling of components after washing.

The components after washing should be handled in grade D environment. The sterile starting material should be handled in grade A environment with grade B background. Solutions to be sterile filtered should be prepared in grade C environment; while if not filtered, the preparation should be done in grade A environment with grade B background. The aseptically prepared products should be handled and filled in grade A environment with grade B background. The sterile ointments, creams, suspensions, and emulsions should be prepared and filled in grade A environment with grade B background, when the product is exposed and is not subsequently filtered. Control of purity of zones by the particles in operation state and microbial control at an aseptic production area is required; and the recommended levels are listed in table 4.6:

Table 4.6: Recommended Limits for Microbial Contamination in the Operation State (Average Values)

GRADE	Air Sample, cfu/m^3	Settle Plates (Diameter 90mm), cfu/4 hours (a)	Contact Plates (Diameter 55mm), cfu/plate	Glove Print, 5 Fingers cfu/glove
A	, 1	<1	<1	<1
B	10	5	5	5
C	100	50	25	-
D	200	100	50	-

(a) - Individual settle plates are exposed for 4 hours or less; cfu - colony-forming unit.

Warning limitation and action for contamination by particles and microbes depend on the controlling results. Also corrective action should be provided in case of exceeding the above limits.

4.4. MICROBIOLOGICAL ASSAY

4.4.1. Introduction

The microbiological or microbial assay is a type of biological assay in which the relative potency of activity of a compound is determined by measuring the amount required for producing the predicted effect on a suitable test organism under standard conditions.

The principles involved in microbial assays are similar to those applied to assays or higher plants or animals. An existing difference is the relative size of experimental population. In bioassays, the response each test animal produces is observed and results are obtained when a few animals have gone through statistical analysis to calculate mean activity, standard error, etc. In microbial assays, each evaluation is performed using a microbial culture, and the results obtained indicate the average response produced by large number of test organisms. Often in bioassays, the log dose and the response have a linear relationship; while in microbial assays; the dose and the response (within certain limits) have a linear relationship.

The microbiological assay is a biological assay performed using microorganisms, e-g. bacteria, yeast, and moulds. These assays are much easier than those performed using mammals for isolating essential vitamin; for example, a growth factor for Lactobacillus proved to be similar to a haematopoietic factor in pernicious anaemic patients.

Many therapeutic agents, such as antibiotics inhibiting microbial growth or the essential growth factors (vitamins and amino acids), are standardised by microbiological assays. The activity of antibiotics (extent of inhibiting the microbial growth), vitamins, or amino acids (extent of supporting the microbial growth) is determined by microbiological assays; while the potency (concentration or amount) of such substances is determined by chemical assays. Thus, microbiological assays of antibiotics, vitamins, and amino acids are significantly important.

4.4.2. Principles

A microbiological assay relies on the principle that when certain compounds are present in limited amounts, the amount of microbial growth corresponds to the amount of these compounds. The basic procedure of most of the microbial assays is same; however, the test conditions vary. The test substance is added to a liquid or gel medium, test microorganism is inoculated on the medium, and the resultant response (which depends on the substance's biochemical effect on the test organism) is observed. It may be a growth response (positive in the assay of nutrients and negative in the assay of antibiotics), which is determined by counting, optical density, weight, or area; the growth response may be a definite end-point, or an all-or-none response.

In case of either a positive or negative metabolic response, the metabolic products or Changes in some function may be measured. Examples of some measurable metabolic responses are acid production, CO_2 production, oxygen uptake, nitrate reduction, haemolysis of RBCs, anti-luminescent activity, or inhibition of spore germination. In 1962, **Cancellien and Morpurgo** proposed a determination method of amino acids vitamins, and purine and pyrimidine bases. Substances analogous and antagonistic to regular metabolites inhibit the growth of wild, prototrophic strains of bacteria. The nutrilite content can be assayed by adding graded amounts of nutrilite for re-activating the growth.

4.4.3. Merits

A microbiological assay has the following merits:

1)It is suitably used for compounds which cannot be assayed by either physical or chemical methods.

2) It is used for the assay of naturally occurring therapeutic agents.

3) It minimises the mortality rate of animals.

4) It is a simple and rapid method as compared to bioassays.

5) It is used for accurate standardisation of medicinal compounds.

6) Tt determines the concentration as well as activity of compounds.

7) It does not require large amount of sample and instruments.

8) Its complete procedure can be automated to minimise the duration.

4.4.4. Demerits

A microbiological assay has the following demerits:

1) lt requires a specific test organism for the assay of a particular compound.

2) It demands maintenance of sterile conditions within the laboratory.

3) It may give invalid results due to a slight variation in incubation temperature.

4) It is a time-consuming method.

5) It requires well trained and expert individuals.

4.5. Standardisation 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, 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 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.

4.5.1. Media. Prepare the media required for the preparation of test organism inocula from the ingredients listed in Table 4.7. 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 hydroxide or 1 M hydrochloric acid, as required so that after sterilization the pH is as given in Table 4.7.

Table 4.7– Media: Quantities in g of ingredients per 1000 ml

Ingredient	Medium									
	A	B	C	D	E	F	G	H	I	J
Peptone	6.0	6.0	5.0	6.0	6.0	6.0	9.4	-	10.0	-
Pancreatic digest of casein	4.0	-	-	4.0	-	-	-	17.0	-	15.0
Yeast extract	3.0	3.0	1.5	3.0	3.0	3.0	4.7	-	-	-
Beef extract	1.5	1.5	1.5	1.5	1.5	1.5	2.4	-	10.0	-
Dextrose	1.0	-	1.0	1.0	-	-	10.0	2.5	-	-
Papaic digest of soyabean	-	-	-	-	-	-	-	3.0	-	5.0
Agar	15.0	15.0	-	15.0	15.0	15.0	23.5	12.0	17.0	15.0
Glycerin	-	-	-	-	-	-	-	-	10.0	-
Polysorbate 80	-	-	-	-	-	-	-	10.0*	-	-
Sodium chloride	-	-	3.5	-	-	-	10.0	5.0	3.0	5.0
Dipotassium Hydrogen Phosphate	-	-	3.68	-	-	-	-	2.5	-	-
Potassium dihydrogen Phosphate	-	-	1.32	-	-	-	-	-	-	-
Final pH (after sterilisation)	6.5-6.6	6.5-6.6	6.95-7.05	7.8-8.0	7.8-8.0	5.8-6.0	6.0-6.2	7.1-7.3	6.9-7.1	7.2-7.4

* Quantity in ml, to be added after boiling the media to dissolve the agar.

4.5.2. 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.

4.5.3. Buffer Solutions. Prepare by dissolving the following quantities given in Table 4.8 of dipotassium hydrogen phosphate and potassium dihydrogen phosphate in sufficient water to produce 1000 ml after sterilisation, adjusting the pH with 8 M phosphoric acid or 10 M potassium hydroxide.

Table 4.8 – Buffer Solutions

Buffer number	Dipotassium Hydrogen Phosphate 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	0.523	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

* After addition of 2 ml of 10M potassium hydroxide

4.5.4. 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 4.9, 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 4.9.

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4.5.5. 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 4.9 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.

4.5.6. Test Organisms. The test organism for each antibiotic is listed in Table 4.10, together with its identification number in the American Type Culture Collection (ATCC). Maintain a culture on slants of the medium and under the incubation conditions specified in Table 4.11, and transfer weekly to fresh slants.

4.5.7. Preparation of inoculum. Prepare the microbial suspensions for the inoculum for the assay as given in Table 4.11. If the suspensions are prepared by these methods, growth characteristics are sufficiently uniform so that the inoculum can be adequately determined by the trials given below.

Table 4.9 - Stock solutions and test dilutions of Standard Preparation

Antibiotic	Standard Stock Solution					Test Dilution		
	Assay Method	Prior Drying	Initial solvent (further diluent, if different)	Final Stock Concentration per ml	Use before (number of days)	Final diluent	Median dose µg or units per ml	Incubation temp (ºC)
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	0.0lM HCl	100 units	Same day	B1	1.0 unit	32-35
Bleomycin	A	Yes	B6[8]	2 units	14	B6	0.04 units	32-35
Carbenicillin	A	No	Bl	1 mg	14	B6	20 µg	36-37.5
Chlortetracycline	A[1]	No	0.lM HCl	1 mg	4	Water	2-5 µg	37-39
	B[10]	No	0.lM HCl	1 mg	4	Water	0.24µg	35-37
Erythromycin	A	Yes	Methanol (l0 mg/ml)[9] (B2)	1 mg	14	B2	1-0 µg	35-37
Framycetin	A	Yes	B2	1 mg	14	B2	1.0 µg	30-35
Gentamicin	A	Yes	B2	1 mg	30	B2	0.1 µg	36-37.5
Kanamycin sulphate	A[1]	No	B2	800 units	30	B2	0.8 µnits	37-39
	B[2]	No	Water	1000 units	30	Water	10 units	32-35
Neomycin	A	Yes	B2	1 mg	14	B2	1.0 µg	36-37.5
Novobiocin	A	Yes	Ethanol (10 mg/ml)9 (B2)	1 mg	5	B4	0.5 µg	32-35
Nystatin	A	Yes	DMF7	1000 units	Same day	B4	20 units	29-31
Oxytetracycline	A[3]	No	0.lM HCl	1 mg	4	B3	2.5 µg	32-35
	B[2]	No	0.lM HCl	1 mg	4	Water	0.24 µg	35-37
Polymyxin B	A	Yes	Water (B4)	10,000 units	14	B4	10 µnits	35-39
Spiramycin	A[4]	No	Methanol	1mg	1	B2	12-50 units	30-32
Streptomycin	A[4]	Yes	Water	1mg	30	Water	1-0 µg	32-35
	B[5]	Yes	Water	1mg	30	Water	30 µg	35-37
Tetracycline	A[3]	No	0.lM HCl	1mg	1	Water	2-5 µg	32-35
	B[6]	No	0.lM HCl	1mg	4	Water	0.24 µg	35-37
Tobramycin	B	Yes	Water	1mg	14	Water	2-5 µg	32-35
Tylosin	B[10]	No	*	1mg	Same day	*	0.05 - 0.25 units	37

1. With Bacillus pumilus ATCC 14884 as test organism; 2. With Staphylococcus aureus ATCC 29737 as test organism;3. With Bacillus cereus var mycoides ATCC 11778 as test organism;4. With Bacillus subtilis ATCC 6633 as test organism; 5. With Klebsiella pneumoniae ATCC 10031 as test organism;6. With Staphylococcus aureus ATCC 29737 as test organism; 7. DMF = Dimethyl formamide 8. In columns 4 & 7, B denotes buffer solution and the number following refers to the buffer number in Table 4.8; 9. Initial concentration of stock solution, 10. With Staphylococcus aureus ATCC 9144 as test organism.

NOTES — For Amphotericin B and Nystatin, prepare the standard solutions and the sample test solution simultaneously.

For Amphotericin B, further dilute the stock solution with dimethyl formamide to give concentrations of 12.8,16,20,25, & 31.2µg per ml prior to making the test solutions. The test dilution of the sample prepared from the solution of the substance under examination should contain the same amount of dimethyl formamide as the test dilutions of the Standard preparation.

For Bacitracin, each of the standard test dilutions should contain the same amount of hydrochloric acid as the test dilution of the sample.

For Nystatin, further dilute the stock solution with dimethyl formamide to give concentrations of 64.0, 80.0, 100.0,125.0,156.0 µg per ml prior to making the test dilutions. Prepare the standard response line solutions simultaneously with dilution of the sample being examined. The test dilution of the sample prepared from the solution of the substance being examined should contain the same amount of dimethyl formamide as test dilutions of the Standard Preparation. Protect the solutions from light.

When making the stock solution of Polymyxin B, add 2 ml of water for each 5 mg of the weighted Standard preparation material.

Where indicated, dry about 100 mg of the Standard Preparation before use in an oven at a pressure not exceeding 0.7 kPa at 60º for 3 hours, except in the case of Bleomycin (dry at 25º for 4 hours), Novobiocin (dry at 100º for 4 hours), Gentamicin (dry at 110º for 3hours) and Nystatin (dry at 40º for 2 hours),

Where two level factorial assays are performed use the following test doses per ml: Amphotericin B, 1.0 to 4.0 µg; Bacitracin, 1.0 to 4.0 units; Kanamycin Sulphate, 5.0 to 20.0 units; Streptomycin, 5.0 o 20.0 µg

Table 4.10 - Test Organisms for Microbiological Assay of Antibiotics

Antibiotic	Test Organism	ATCC[1] No.
Amikacin	*Staphylococcus aureus*	29737
Amphotericin B	*Saccharomyces cerevisiae*	9763
Bacitracin	*Micrococcus luteus*	10240
Bleomycin	*Mycobacterium smegmatis*	607
Carbenicillin	*Pseudomonas aeruginosa*	25619
Chlortetracycline	*Bacillus pumilus*	14884
Erythromycin	*Micrococcus luteus*	9341
Framycetin	*Bacillus pumilus*	14884
	Bacillus subtilis	6633
Gentamicin	*Staphylococcus epidermidis*	12228
Kanamycin sulphate	*Bacillus pumilus*	14884
	Staphylococcus aureus	29737
Neomycin	*Staphylococcus epidermidis*	12228
Novobiocin	*Staphylococcus epidermidis*	12228
Nystatin	*Saccharomyces cerevisiae*	2601
Oxytetracycline	*Bacillus cereus var mycoides*	11778
	Staphylococcus aureus	29737
Polymyxin B	*Bordetella bronchiseptica*	4617
Spiramycin	*Bacillus pumilus*	6633
Streptomycin	*Bacillus subtilis*	6633
	Klebsiella pnumoniae	10031
Tetracycline	*Bacillus cereus*	11778
	Staphylococcus aureus	29737
Tobramycin	*Staphylococcus aureus*	29737
Tylosin	*Staphylococcus aureus*	9144

1. American Type Culture Collection, 21301 Park Lawn Drive, Rockville, MD20852, USA

4.5.8. Determination of inoculum

For Method A. After the suspension is prepared as given under Table 4.11, add different volumes of it to each of several different flasks containing 100 ml of the medium specified in Table 4.9 (the volume of suspension suggested in Table 4.9 may be used as a guide). Using these inocula, prepare inoculated plates as described for the specific antibiotic assay. While conducting cylinder-plate assays, double-layer plates may be prepared by pouring a seed layer (inoculated with the desired micro-organism) over a solidified uninoculated base layer. For each Petri dish, 21 ml of base layer and 4 ml of the seed layer may be generally suitable. Fill each cylinder with the median concentration of the antibiotic (Table 4.9) and then incubate the plates. After incubation, examine and measure the zones of inhibition. The volume of suspension that produces the optimum zones of inhibition with respect to both clarity and diameter determines the inoculum to be used for the assay.

For Method B. Proceed as described for Method A and, using the several inocula, carry out the procedure as described for the specific antibiotic assay running only the high and low concentrations of the standard response curve. After incubation, read the absorbances of the appropriate tubes. Determine which inoculum produces the best response between the low and high antibiotic concentrations and use this inoculum for the assay.

Table 4.11 – Preparation of inoculum

Test organism	Incubation conditions			Suggested dilution factor	Suggested inoculum composition		
	Medium/ Method of Prepa-ration	Temp. (°C°)	Time		Med ium	Amount (ml per 100 ml)	Antibiotics assayed
Bacillus cereus var. mycoides	A [1/2]	32-35	5 days	-	F	As required	Oxytetracycline Tetracycline
Bacillus pumilus	A [1/2]	32-35	5 days	-	D	As required	Chlortetracycline Framycetin Kanamycin sulphate
Bacillus subtilis	A [1/2]	32-35	5 days	-	E E B A	As required As required As required As required	Framycetin Kanamycin B Spiramycin Streptomycin
Bordetella bronchiseptica	A/1	32-35	24 hr	1:20	H	0.1	Polymyxin B
Klebsiella pneumoniae	A/1	36-37	24 hr	1:25	C	0.1	Streptomycin
Micrococcus luteus (9341)	A/1	32-35	24 hr	1:40	D	1.5	Erythromycin
Micrococcus luteus (10240)	A/1	32-35	24 hr	1:35	A	0.3	Bacitracin
Mycobacterium smegmatis	J/4	36-37.5	48 hr	As determined	I	1.0	Bleomycin
Pseudomonas aeruginosa[2]	A/1	36-37.5	24 hr	1:25	H	0.5	Carbenicillin
Saccharomyces cerevisiae (9763)	G/3	29-31	48 hr	As determined	G	1.0	Amphotericin B
Saccharomyces cerevisiae (2601)	G/3	29-31	48 hr	As determined	G	1.0	Nystatin
Staphylococcus aureus (29737)	A/1	32-35	24 hr	1:20	C C	0.1 0.2	Amikacin Doxycycline Oxytetracycline Tetracycline Tobramycin Tylosin Kanamycin sulphate
Staphylococcus epidermidis	A/1	32-35	24 hr	1:40	D D A	0.03 0.4 4.0	Gentamicin Neomycin Novobiocin

1. Use Medium A containing 300 mg of manganese sulphate per litre.

2. For Pseudomonas aeruginosa in the assay of Carbenicillin, use the dilution yielding 25 per cent light transmission, rather than the stock suspension, for preparing the inoculum suspension.

Methods of preparation of test organism suspension:

1. Maintain the test organism on slants of Medium A and transfer to a fresh slant once a week. Incubate the slants at the temperature indicated above for 24 hours. Using 3 ml of saline solution, wash the organism from the agar slant onto a large agar surface of Medium A such as a Roux bottle containing 250 ml of agar. Incubate for 24 hours at the appropriate temperature. Wash the growth from the nutrient surface using 50 ml of saline solution. Store the test organism under refrigeration. Determine the dilution factor which will give 25 per cent light transmission at about 530 nm. Determine the amount of suspensions to be added to each 100 ml of agar of nutrient broth by use of test plates or test broth. Store the suspension under refrigeration.

2. Proceed as described in Method 1 but incubate the Roux bottle for 5 days. Centrifuge and decant the supernatant liquid. Resuspend the sediment with 50 to 70 ml of saline solution and heat the suspension for 30 minutes at 70º. Wash the spore suspension three times with 50 to 70 ml of saline solution. Resuspend in 50 to 70 ml of saline solution and heat- shock again for 30 minutes. Use test plates to determine the amount of the suspension required for 100 ml of agar. Store the suspension under refrigeration.

3. Maintain the test organism on 10 ml agar slants of Medium G. Incubate at 32º to 35º for 24 hours. Inoculate 100 ml of nutrient broth. Incubate for 16 to 18 hours at 37º and proceed as described in Method I.

4. Proceed as described in Method 1 but wash the growth from the nutrient surface using 50 ml of Medium 1 (prepared without agar) in place of saline solution.

4.5.9. Apparatus

All equipment is to be thoroughly cleaned before and after each use. Glassware for holding and transferring test organisms is sterilised by dry heat or by steam.

Temperature Control. Thermostatic control is required at several stages of a microbial assay, when culturing a microorganism and preparing its inoculum and during incubation in a plate assay. Closer control of the temperature is imperative during incubation in a tube assay which may be achieved by either circulated air or water, the greater heat capacity of water lending it some advantage over circulating air.

Spectrophotometer. Measuring transmittance within a fairly narrow frequency band requires a suitable spectrophotometer in which the wavelength of the light source can be varied or restricted by the use of a 580-nm filter for preparing inocula of the required density or with a 530-nm filter for reading a absorbance in a tube assay. For the latter purpose, the instrument may be arranged to accept the tube in which incubation takes place, to accept a modified cell fitted with a drain that facilitates rapid change of contents, or preferably fixed with a flow-through cell for a continuous flow-through analysis. Set the instrument at zero absorbance with clear, uninoculated broth prepared as specified for the particular antibiotic, including the same amount of test solution and formaldehyde as found in each sample.

Cylinder-plate assay receptacles. Use rectangular glass trays or glass or plastic Petri dishes (approximately 20 x 100 mm) having covers of suitable material and assay cylinders made of glass, porcelain, aluminium or stainless steel with outside diameter 8 mm ± 0.1 mm, inside diameter 6mm ± 0.1mm and length 10 mm ± 0.1 mm. Instead of cylinders, holes 5 to 8 mm in diameter may be bored in the medium with a sterile borer, or paper discs of suitable quality paper may be used. Carefully clean the cylinder to remove all residues. An occasional acid bath, e.g. with about 2 M nitric acid or with chromic acid solution is needed.

Turbidimetric assay receptacles. For assay tubes, use glass or plastic test-tubes, e.g. 16 mm x 125 mm or 18 mm x 150 mm that are relatively uniform in length, diameter, and thickness and substantially free from surface blemishes and scratches. Cleanse thoroughly to remove all antibiotic residues and traces of cleaning solution and sterilise tubes that have been used previously before subsequent use.

4.5.10. Assay Designs

Microbial assays gain markedly in precision by the segregation of relatively large sources of potential error and bias through suitable experimental designs. In a cylinder plate assay, the essential comparisons are restricted to relationships between zone diameter measurements within plates, exclusive of the variation between plates in their preparation and subsequent handling. To conduct a turbidimetric assay so that the difference in observed turbidity will reflect the differences in the antibiotic concentration requires both greater uniformity in the environment created for the tubes through closer thermostatic control of the incubator and the avoidance of systematic bias by a random placement of replicate tubes in separate tube racks, each rack containing one complete set of treatments. The essential comparisons are then restricted to relationships between the observed turbidities within racks. Within these restrictions, two alternative designs are recommended; i.e. a 3-level (or 2-level) factorial assay,

or a 1- level assay with a standard curve. For a factorial assay, prepare solutions of 3 or 2 corresponding test dilutions for both the standard and the unknowns on the day of the assay, as described under Preparation of the Standard and Preparation of the samples. For a 1-level assay with a standard curve, prepare instead solutions of five test dilutions of the standard and a solution of a single median test level of the unknown as described in the same sections. Consider an assay as preliminary if its computed potency with either design is less than 60 per cent or more than 150 per cent of that assumed in preparing the stock solution of the unknown. In such a case, adjust its assumed potency accordingly and repeat the assay.

Microbial determinations of potency are subject to inter-assay variables as well as intra-assay variables, so that two or more independent assays are required for a reliable estimate of the potency of a given assay preparation or unknown. Starting with separately prepared stock solutions and test dilutions of both the standard and unknown, repeat the assay of a given unknown on a different day. If the estimated potency of the second assay differs significantly, as indicated by the calculated standard error, from that of the first, conduct one or more additional assays. The combined result of a series of smaller, independent assays spread over a number of days is a more reliable estimate of potency than that from a single large assay with the same total number of plates or tubes.

4.5.11. Methods: Carry out the microbiological assay by Method A or Method B.

A. Cylinder-plate or Cup-plate method

Inoculate a previously liquefied medium appropriate to the assay (Tables 1 and 3) with the requisite quantity of suspension of the micro-organism, add the suspension to the medium at a temperature between 40º and 50º and immediately pour the inoculated medium into the petri dishes or large rectangular plates to give a depth of 3 to 4 mm (1 to 2mm for nystatin). Ensure that the layers of medium are uniform in thickness, by placing the dishes or plates on a level surface.

Store the prepared dishes or plates in a manner so as to ensure that no significant growth or death of the test organism occurs before the dishes or plates are used and that the surface of the agar layer is dry at the time of use.

Using the appropriate buffer solutions indicated in Tables 4.8 and 4.9, prepare solutions of known concentrations of the standard preparation and solutions of the corresponding assumed of concentrations the antibiotic to be examined. Where directions have been given in the individual monograph for preparing the solutions, these should be followed and further dilutions made with buffer solution as indicated in Table 4.9. 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. When paper discs are used these should be sterilised by exposure of both sides under a sterilising lamp and then impregnated with the standard solutions or the test solutions and placed on the surface of the medium. When Petri dishes are used, arrange the solutions of the Standard Preparation and the antibiotic under examination on each dish so that, they alternate around the dish and so that the highest concentrations of standard and test preparations are not adjacent. When plates are used, place the solutions in a Latin square design, if the plate is a square, or if it is not, in a randomised block design. The same random design should not be used repeatedly.

Leave the dishes or plates standing for 1 to 4 hours at room temperature or at 4º, as appropriate, as a period of preincubation diffusion to minimise the effects of variation in time between the application of the different solutions. Incubate them for about 18 hours at the temperature indicated in Table 4.9. Accurately measure the diameters or areas of the circular inhibition zones and calculate the results. Selection of the assay design should be based on the requirements stated in the individual monograph. Some of the usual assay designs are as follows.

(a) One-level assay with standard curve

Standard Solution. Dissolve an accurately weighed quantity of the Standard Preparation of the antibiotic, previously dried where necessary, in the solvent specified in Table 4.9, and then dilute to the required concentration, as indicated, to give the stock solution. Store in a refrigerator and use within the period indicated. On the day of the assay prepare from the stock solution, 5 dilutions (solutions S1 to S5) representing 5 test levels of the standard and increasing stepwise in the ratio of 4:5. Use the diluent specified in Table 4.9 and a sequence such that the middle or median has the concentration given in the table.

Sample Solution. From the information available for the antibiotic preparation which is being examined (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 with same solvent as used for the standard. Prepare from this stock solution a dilution to a concentration equal to the median level of the standard to give the sample solution.

Method. For preparing the standard curve, use a total of 12 Petri dishes or plates to accommodate 72 cylinders or cavities. A set of 3 plates (18 cylinders or cavities) is used for each dilution. On each of the three plates of a set fill alternate cylinders or cavities with solution S3 (representing the median concentration of the standard solution) and each of the remaining 9 cylinders or cavities with one of the other 4 dilutions of the standard solution. Repeat the process for the other 3 dilutions of the standard solution. For each unknown preparation use a set of 3 plates (18 cylinders or cavities) and fill alternate cylinders or cavities with the sample solution and each of the remaining 9 cylinders of cavities with solution S3. Incubate the plates for about 18 hours at the specified temperature and measure the diameters or the zones of inhibition.

Estimation of potency. Average the readings of solution S3 and the readings of the concentration tested on each sets of three plates, and average also all 36 readings of solution S3. The average of the 36 readings of solution S3 is the correction point for the curve. Correct the average value obtained for each concentration (S1, S2, S4 and S5) to the figure it would be if the readings for solution S3 for that set of three plates were the same as the correction point. Thus, in correcting the value obtained with any concentration, say S1, if the average of 36 readings of S3 is, for example, 18.0 mm and the average of the S3 concentrations on one set of three plates is 17.8 mm, the correction is + 0.2 mm. If the average reading of S1 is 16.0 mm the corrected reading of S1 is 16.2 mm. Plot these corrected values including the average of the 36 readings for solutions S3 on two-cycle semilog paper, using the concentrations in

Units or µg per ml (as the ordinate logarithmic scale) and the diameter of the zones of inhibition as the abscissa. Draw the straight response line either through these points by inspection or

through the points plotted for highest and lowest zone diameters obtained by means of the following expressions:

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

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.

Average the zone diameters for the sample solution and for solutions S3 on the plates used for the sample solution. If sample gives a large average zone size than the average of the standard (solution S3), add the difference between them to the zone size of solution S3 of the standard response line. If the average sample zone size is smaller than the standard values, subtract the difference between them from the zone size of solution S3 of the standard response line. From the response line read the concentration corresponding to these corrected values of zone sizes. From the dilution factors the potency of the sample may be calculated.

(b) Two-level factorial assay

Prepare parallel dilutions containing 2 levels of both the standard (S1 and S2) and the unknown (U1and U2). On each of four or more plates, fill each of its four cylinders or cavities with a different test dilution, alternating standard and unknown. Keep the plates at room temperature and measure the diameters of the zones of inhibition.

Estimation of potency. Sum the diameters of the zones of each dilution and calculate the percentage potency of the sample (in terms of the standard) from the following equation:

$$\textbf{Per cent potency} = \textbf{Antilog}\,(\mathbf{2.0} + \mathbf{a\ log\ I})$$

wherein 'a' may have a positive or negative value and should be used algebraically and

$$\mathbf{a} = \frac{(\mathbf{U_1} + \mathbf{U_2}) - (\mathbf{S_1} + \mathbf{S_2})}{(\mathbf{U_1} - \mathbf{U_2}) + (\mathbf{S_1} - \mathbf{S_2})}$$

U1 and U2 are the sums of the zone diameters with solutions of the unknown of high and low levels.

S1 and S2 are the sums of the zone diameters with solutions of the standard of high and low levels.

I = ratio of dilutions.

If the potency of the sample is lower than 60 per cent or greater that 150 per cent of the standard, the assay is invalid and should be repeated using higher or lower dilutions of the same solution.

The potency of the sample may be calculated from the expression 100

$$\textbf{Potency of the sample} = \frac{\textbf{per cent potency} \times \textbf{assumed potency of the sample}}{\textbf{100}}$$

(c) Other designs

1. Factorial assay containing parallel dilution of three test levels of standard and the unknown.

2. Factorial assay using two test levels of standard and two test levels of two different unknowns.

B. 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 and care should be taken to ensure freedom from such substances in the final test solutions. This method is not recommended for cloudy or turbid preparations.

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 (Table 4.9) and increasing stepwise in the ration 4:5. Select the median concentration (Table 4.9) and 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 (Table 4.9) previously seeded with the appropriate test organism (Table 4.9).

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 (Table 4.9) 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.

Estimation of potency. Plot the average absorbances for each concentration of the standard on semi-logarithmic paper with the absorbances on the arithmetic scale and concentrations on the logarithmic scale. Construct the best straight response line through the points either by inspection or by means of the following expressions:

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

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

Plot the values obtained for L and H and connect the points. Average the absorbances for the sample and read the antibiotic concentration from the standard response line. Multiply the concentration by the appropriate dilution factors to obtain the antibiotic content of the sample.

4.5.12. Precision of Microbiological Assays

The fiducial limits of error of the estimated potency should be not less than 95 per cent and not more than 105 per cent of the estimated potency unless otherwise stated in the individual monograph.

This degree of precision is the minimum acceptable for determining that the final product complies with the official requirements and may be inadequate for those deciding, for example, the potency which should be stated on the label or used as the basis for calculating the quantity of an antibiotic to be incorporated in a preparation. In such circumstances, assays of greater precision may be desirable with, for instance, fiducial limits of error of the order of 98 per cent to 102 per cent. With this degree of precision, the lower fiducial limit lies close to the estimated potency. By using this limit, instead of the estimated potency, to assign a potency to the antibiotic either for labelling or for calculating the quantity to be included in a preparation, there is less likelihood of the final preparation subsequently failing to comply with the official requirements for potency.

4.5.13. Methods for Standardisation 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 synthesise the factor being assayed which forms the basis of microbiological assay of vitamins and amino acids. The test microorganisms used for the standardisation 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 (^{0}C)	Assay pH
Vitamin B_{12}	*Lactobacillus leichamannii ATCC 7830* *Poteriochromonas stipitata ATCC 11532*	37 30	6.1 5.5
Vitamin B_6	*Saccharomyces uvarum ATCC 9080* *Tetrahymena thermophila ATCC 30008*	30 30	4.5 6.1
Riboflavin	*Lactobacillus casei ATCC 7469* *Tetrahymena thermophila ATCC 30008*	37 30	6.8 6.1
Thiamine	*Lactobacillus viridescens ATCC 12706* *Ochromonas danica ATCC 30004*	30 30	6.0 5.5
Biotin	*Lactobacillus plantarum ATCC 8014* *Ochromonas danica ATCC 30004*	37 30	6.8 5.5
Niacin	*Lactobacillus plantarum ATCC 8014* *Tetrahymena thermophila ATCC 30008*	37 30	6.8 6.1
Pantothenate	*Lactobacillus plantarum ATCC 8014* *Tetrahymena thermophila ATCC30008*	37 30	6.7 6.1

4.5.13.1. Principle

Vitamins are essential growth factor for microorganisms. In the standardisation of vitamins, the ability of test organisms to use the substance being standardised 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.

4.5.13.2. Standardisation of Calcium Pantothenate

The reagents and the methods utilised for the standardisation of calcium pantothenate are discussed below:

Reagents

Standard Stock Solution of Calcium Pantothenate

The stock solution is prepared by dissolving 50mg calcium pantothenate in 500ml water. To the resultant solution, 10ml of 0.2N acetic acid and 100ml of 1.6%w/v sodium acetate solution are added. Volume is adjusted up to 1000ml by adding water. This solution is stored in a refrigerator under toluene. Each ml of this resultant stock solution contains 50ug of calcium pantothenate.

Standard Solution

Each ml of standard solution contains 0.04ug of calcium pantothenate. This solution is prepared by diluting the standard stock solution with water.

Test Solution

The concentration of calcium pantothenate in test solution is approximately equivalent to that in the standard solution prepared in water.

Medium

The medium utilised contains the following ingredients:

Ingredients	Amounts
Acid-hydrolysed casein solution	25 ml
Cystine- tryptophan solution	25 ml
polysorbate 80 solution	0.25 ml
Dextrose (anhydrous)	10gm
Sodium acetate (anhydrous)	5gm
Adenine-guanine-uracil solution	5ml
Riboflavin-thiamine HCI-biotin solution	5ml
P-amino benzoic acid-niacin-pyridoxine HCl solution	5ml
Salt solution A	5ml
Salt solution B	5ml

The medium is prepared by dissolving the anhydrous dextrose and sodium acetate in previously mixed solutions. The pH of solution is adjusted to 6.8 using 1 N NaOH. Ultimately, the solution is diluted to 250ml with water and mixed.

Stock Culture of Organism

The stock culture is prepared by dissolving 2gm water-soluble yeast extract in 100ml water; 500mg each of anhydrous dextrose and sodium acetate, and 1.5gm agar is also added. The resultant mixture is heated to dissolve the agar. 10ml of this hot solution is added to test tubes and sterilised at 121°C by mounting in vertical position. Stab culture of Lactobacillus plantarum is prepared in 3 tubes, incubated for 16-24 hours at 30-37 ^{0}C and stored in a refrigerator.

Preparation of Inoculum

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

Principle

Pantothenate assay medium (a dehydrated medium) is free from pantothenic acid or pantothenate, but contains other essential nutrients and vitamins used for preparing the culture of L. plantarum. Addition of calcium pantothenate in increasing concentrations as specified yields a growth response which is either turbidimetrically or titrimetrically measured.

Procedure

1) Standard solution in 1, 2, 3, 4, and 5ml is added to test tubes in duplicate.

2) Each of these tubes and another four similar tubes containing no standard solution are added with 5ml medium solution and volume is adjusted to 10ml with water.

3) Volumes of test solution corresponding to 3 or more of the levels as taken above are added to similar test tubes, in duplicate.

4) Each tube is added with 5ml medium solution and volume is adjusted up to 10ml with water.

5) A complete set of standard and assay tubes is placed in a tube rack and the duplicate set is kept in the other rack.

6) Tubes of both the series are autoclaved for 5 minutes at 121°C. After cooling, a drop of inoculum is added to each tube except 2 of the 4 tubes which do not have standard solution (un-inoculated blanks), mixed, and incubated for 16-24 hours at 30-37° C.

7) Spectrophotometer is used to measure the transmittance of the tubes at a specific wavelength lying between 540-660nm.

Calculation

A standard concentration-response curve 1s obtained by plotting the transmittance against the logarithm of the ml of standard solution per tube. The response is calculated by summing the two transmittances for each level of the test solution. This curve helps in determining the concentration of calcium pantothenate in the test sample.

4.5.13.3. Standardisation of Niacin

The selected organism should have the ability to utilise free niacin, niacinamide, nicotinuric acid, cozymase, and niacinamide nucleoside. This requirement is satisfied by Lactobacillus plantarum (an acid forming organism), cannot synthesise 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 0.05-0.5µg 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:

Ingredients	Amounts
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

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.

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 turbidimetrically 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 µg 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 tl0% from the average. For expressing the results as niacinamide, the obtained values are multiplied by 0.992.

4.5.13.4. Standardisation of Vitamin B_{12}

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 bisulphite 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 B_{12} 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

Ingredients	Amounts
L-Cystine	0.1gm
L-Tryptophan	0.05gm
1N Hydrochloric acid	5 ml
Adenine-guanine-uracil solution	5ml
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 1.0µgm of cyanocobalamin per ml is stored in a refrigerator.

Standard Cyanocobalamin Solution

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 0.01-0.04µg/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, sterilised, 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 ± 0.5°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

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 ± 0.5°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 B_{12} assay medium is free from vitamin B_{12}, 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 1dentical 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±0.5°C temperature. Bacterial growth is terminated by heating for 5 minutes at temperature not lower than 80°C.

10) After cooling to room temperature, the contents are agitated, and when a steady state 1s 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 un-inoculated 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 un-inoculated 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.

Calculation

1) For preparing a standard concentration-response curve, any abnormal individual transmittance is prevented.

2) For each level of the standard, the response is calculated by adding the duplicate values of the transmittances ($\sum$) as the difference, $y = 2.00 - \sum$.

3) On plotting this response against the logarithm of the ml of standard cyanocobalamin solution per tube, a straight line or a smooth curve is produced.

4) The response (y) is calculated by adding the two transmittances for each level of the assay preparation.

5) From the standard curve the logarithm of the volume of standard preparation corresponding to each value of y falling within the range of the lowest and highest points plotted for the standard is determined.

6) From this logarithm value the logarithm of the volume (ml) of assay preparation is subtracted to obtain the difference (x) for each dosage level.

7) Average the values of x for each of three or more dosage levels to obtain x = M (the log-relative potency of assay preparation).

8) The quantity of U.S.P. Cyanocobalamin RS (µg) corresponding to the cyanocobalamin in the material being standardised is determined by the equation:

$$\text{Antilog } M = \text{antilog } (M + \log R)$$

Where, R Number of µg of cyanocobalamin present in each mg of the material standardised.

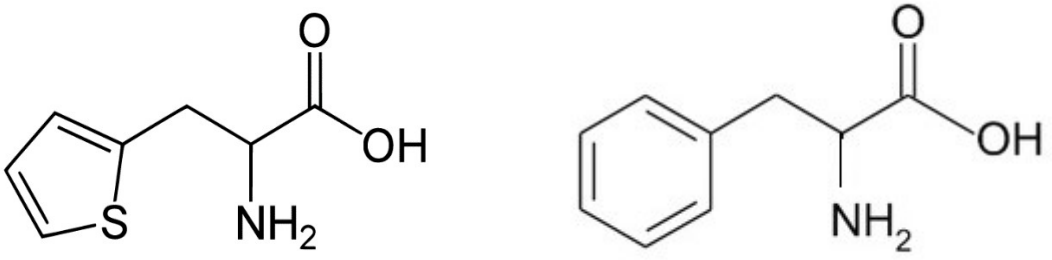
4.5.14. Standardisation of Amino Acids

Microbiological assays were used for quantitation 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 standardised. These inexpensive methods are used for analysing 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 turbidimetry or by measuring the increased lactic acid production by either microtitration or pH change.

Guthrie and Susi introduced a modified version of microbiological assay utilising 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 β-2-theienylalanine (a competitive metabolic antagonist on the growth of a special strain of Bacillus subtilis which de pends on phenylalanine for its growth (figure 4.5).



β-2-Thienylalanine Phenylalanine

Figure 4.5: Guthrie Test: The similarity in structure between phenylalanine and its metabolic antagonist β-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 β-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 β-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.

4.6. Assessment of a New Antibiotic

Antimicrobial Resistance (AMR) occurs when microbes (bacteria, viruses, fungi, and parasites) change in ways making the medications used for curing the infections ineffective. If new antib1otics are not introduced, the patients will die due to the previously treatable infections.

However, a major issue is adequate evaluation or the antibiotics, particularly by payers and/or Health Technology Assessment (HTA) Bodies, to take account of AMR and reflect the full benefit provided to patients and society.

Two Key Challenges

1) Clinical trials are designed for establishing non-inferiority, while HTA bodies require demonstration of clinical superiority.

2) HTA bodies do not have a mechanism to evaluate the health benefits of antibiotics, and control the rise in AMR.

Elements of Value Relevant to Antibiotics: these are given in the table below 4.14 and explained as follows.

Table 4.14: Additional Elements of Value Relevant to Antibiotics

Relevant Benefits included in Traditional HTA	Other Types of Benefit of Possible relevance to Antibiotics
Health gain Unmet need Cost offsets Productivity benefits	Insurance value Diversity value Diagnostic value Uniqueness or innovation value Enablement value Spectrum value

Relevant Benefits included in Traditional HTA: are

Health Gain

1) It includes life extension and quality of life gains.

2) It is accepted as a criterion for positive HTA recommendation.

3) Evidence required by HTA bodies is often inaccessible for antibiotics (superiority trials).

Unmet Need

1) It includes severity of disease and current availability of alternative treatments.

2) It involves use of priority pathogen lists.

Cost Offsets

1) It includes the reduction in costs in other areas as a result of use of new medicine.

2) It involves use of modelling studies and/or evidence from clinical trials.

Productivity Benefits

1) It involves profits or losses related to value of patient's time, receiving medical care or out of work.

2) It involves use of modelling studies and/or evidence from clinical trials/observational studies.

Other Types of Benefit of Possible relevance to Antibiotics: are

Insurance Value

1) It is the value of treatment available in case of catastrophic health events, e.g, outbreak of MDR infections which cannot be controlled by existing last-line antibiotics.

2) It is analogous to availability of a fire engine.

3) It also needs to add in the "precautionary principle".

4) It involves use of modelling studies.

Diversity Value

1) It is the selection pressure, i.e., the antibiotic is able to eliminate susceptible species of bacteria but no other resistant pathogens; thus these pathogens survive and multiply, making the antibiotic ineffective.

2) It involves evidence that reducing selection pressure by withdrawing antibiotic for a period of time may result in restoration of susceptibilities.

3) It involves use of modelling studies.

Diagnostic Value

1) If infection is diagnosed at an early stage, appropriate antibiotic therapy can be started earlier.

2) It needs evidence of test accuracy.

Uniqueness or Innovation Value

1) It is the potential value associated with new or unique mechanism of action.

2) Antibiotics with novel mechanism of action may avoid problems of cross-resistance occurring in many existing classes.

3) Discovery of new mechanism of action of an antibiotic makes it easier for' follow on' products to enter market.

4) It needs evidence of new mechanism of action.

Enablement Value

1) It is the availability of effective antibiotics supporting many surgical procedures and treatments for people having compromised immune systems.

2) It involves use of modelling studies.

Spectrum Value

1) Narrow spectrum antibiotics may be more valuable than broad spectrum ones as the former can reduce spread of AMR by preventing 'collateral damage' to the microbiome.

2) It depends on the antibiotic.

Reasons for which additional elements of value for antibiotics are required:

Given below are the reasons for which additional elements of value for antibiotics are required:

1) AMR is a Public Health Priority: The rise of AMR is a serious threat, control of which is a priority for the national and international health organisations. The current HTA methods do not account for the value of reducing this public health threat, e.g., of the insurance value of having a treatment available in case of a future major or rapidly intensifying resistance problem.

2) Diverse Set of Non-Inferior Antibiotics is Valuable to Society: Since AMR is increasing widely, it has become valuable developing a new antibiotic for MDR pathogens, even if it is ineffective than the existing antibiotics in treating susceptible (non-resistant) pathogens. This concept is unique to antibiotics and is not considered by HTA bodies.

3)Non-Clinical and Microbiology Data are important for Demonstrating the Value of Antibiotics: Non-clinical and microbiology data for antibiotics can predict their results. Due to the difficulties in conducting clinical trials for antibiotics for MDR pathogens, regulators accept these alternative types of evidence as part of the approval process in areas of high unmet need.

4) Antibiotics have Benefits that go Beyond the Patient Treated: When one patient is treated with an antibiotic, the spread of the infectious disease is reduced thus benefitting the population.

5) Antibiotics Enable Other Types of Treatment and Procedures: Apart from treating infections, antibiotics also reduce the risk associated with other types of treatment such as surgery and chemotherapy.

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