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Anti-Microbiological Assay Test or Antibiotic Assay Test of Pharmaceutical Preparation Containing Antibiotics using 'Cylinder Plate Method'

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ABSTRACT

In this paper, we are going to discuss the anti-microbiological assay of the antibiotics. Aim of this paper is to predict the potency of the antibiotic preparation in reference with the working standard of the antibiotic and using the mathematical model in order to obtain the potency of the preparation in regards to its claim.

KEYWORDS: *Buffer solution, stock solution, standard solution, microbial culture selection, inoculum preparation, media preparation, mathematical model.*

INTRODUCTION

There are generally two methods to perform the anti-microbiological assay test for antibiotics. Those are: (a) cylinder plate method, and (b) turbidimetric method. The cylinder plate method (method A) is a method of diffusion of the antibiotic solution through a solidified agar layer. 90mm petri plate is used for this test. A zone of growth inhibition is produced due to the diffusion of antibiotic through the agar layer. Turbidimetric method (method B) is depends on the growth inhibition of microbial culture in a fluid medium (rapid growth supporting medium) in a uniform antibiotic solution.

Further mathematical calculations are being carried out giving out the result that the antibiotic preparation is valid.

REQUIREMENTS

1. Antibiotic test sample.
2. Antibiotic working standard.
3. Weighing balance.
4. Butter paper.
5. Volumetric flask 6 pieces.
6. Dipotassium hydrogen phosphate (K_2HPO_4)
7. Potassium dihydrogen phosphate (KH_2PO_4).
8. Chloroform (for liquid 2-phase separation techniques in case of ointments).
9. Extractor (for liquid 2-phase separation techniques in case of ointments).
10. 2 reagent bottle.
11. Test organisms for microbiological assay according to ATCC Number.
12. Media according to the test sample and test organism.
13. Autoclave.
14. Beaker.
15. Lintfree cloth.
16. Sonicator.
17. Laminar air flow.
18. Marker pen.
19. 90mm petriplates.
20. Borer.
21. 200µl micropipette and sterilized tips.
22. Bacteriological Incubator.
23. Antibiotic zone reader.

TEST PROCEDURE

1. Preparation of the buffer solution:

Buffer is prepared by dissolving the following quantities of Dipotassium hydrogen phosphate K_2HPO_4 and Potassium dihydrogen phosphate KH_2PO_4 in water in order to obtain 1000ml after sterilization. The pH has to be adjusted using 8M Phosphoric acid and 10M Potassium hydroxide. The buffer is then used to prepare the dilutions.

Table: 01

Buffer number	Dipotassium hydrogen phosphate K_2HPO_4 (gram)	Potassium dihydrogen phosphate KH_2PO_4 (gram)	Ph after sterilization (adjusted)
B1	2.0	8.0	6.0 ± 0.1
B2	16.73	0.523	8.0 ± 0.1
B3	-	13.61	4.5± 0.1
B4	20.0	80.00	6.0 ± 0.1
B5	35.0	-	10.5 ± 0.1
B6	13.6	4.0	7.0 ± 0.1

Note: For some antibiotics, some other solvent can be used in the place of buffers.

2. Preparation of stock solution and test dilution of standard preparation:

Stock solution of working standard is being prepared according to the potency of the antibiotic and the required volume. While the stock solution for test sample is prepared according to the label claim and the required volume.

After preparation of stock, for both the solutions, it is needed to prepare higher concentration solution and lower concentration solution by a serial dilution technique.

Table: 02

Stock solution and Test dilution 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 (no. of days)	Final diluent	Median dose µg or units per ml	Incubation temp. ºC
Amikacin	B	No	Water	1mg	14	Water	10 µg	32-35
Amphotericin B	A	Yes	DMF[7]	1mg	Same day	B5	1.0 µg	29-31
Bacitracin	A	Yes	0.01M HCl	100units	Same day	B1	1.0unit	32-35
Bleomycin	A	Yes	B6[8]	2units	14	B6	0.04unit	32-35
Carbenicillin	A	No	B1	1mg	14	B6	20 µg	36-37.5
Chlortetracycline	A[1]	No	0.1M HCl	1mg	4	Water	2.5 µg	37-39
	B[20]	No	0.1M HCl	1mg	4	Water	0.24 µg	35-37
Erythromycin	A	Yes	Methanol (10mg/ml)[9], (B2)	1mg	14	B2	1.0 µg	35-37

3. Selection of the microbial culture:

The test organism for each of the antibiotic is listed along with its ATCC identification numbers. ATCC stands for the American Type of Culture Collection. Culture of the medium is to be maintained and under the incubation condition Table 04.

Table: 03

Antibiotic	Test Organism	ATCC 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
	Staphylococcus epidermidis	12228
Neomycin	*Staphylococcus epidermidis*	12228
Novobiocin	*Saccharomyces cerevisiae*	2601
Nystatin	*Bacillus cereus var, mycoides*	11778
Oxytetracycline	*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

**ATCC: American Type Culture Collection, 21301 Park Lawn Drive, Rockville, MD20852, USA

4. Preparation of inoculum:

Table: 04

Preparation of inoculum							
Test organism	Inoculum conditions			Suggested dilution factor	Suggested inoculum composition		
	Medium/ method of preparation	Temp. (ºC)	Time		Medium	Amount ml per 100ml	Antibiotics assayed
Bacillus cereus var. mycoides	A ½	32-35	5 days	-	F	As required	Oxytetracycline
							Tetracycline
Bacillus pumilus	A ½	32-35	5 days	-	D	As required	Chlortetracycline
							Framycetin
							Kanamycin sulphate
Bacillus subtilis	A ½	32-35	5 days	-	E	As required	Framycetin
					E	As required	Kanamycin B
					B	As required	Spiramycin
					A	As required	Streptomycin
Staphylococcus aureus	A/1	32-35	24hr	1:20	C	0.1	Amikacin

5. Preparation of the medium:

Ingredients are dissolved in the sufficient amount of water to produce 1000 ml, and later add sufficient amount of 1 M sodium hydroxide or 1 M hydrochloric acid after sterilization to maintain the pH of the medium.

Table: 05

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 soybean	-	-	-	-	-	-	-	3.0	-	5.0
Agar	15.0	15.0	-	15.0	15.0	15.0	23.5	12.0	17.0	15.0
Glycerine	-	-	-	-	-	-	-	-	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 sterilization)	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

CALCULATION

1. Solution associated to Antibiotic working standard:

1.1 Weight calculation for antibiotic working standard (mg.):

$$\text{Working Standard weight (mg)} = \frac{1}{\text{Potency of salt}} \times \text{Volume of volumetric choosen} \times 1000$$

1.2 Preparation of the stock solution:

$$\text{Stock Solution} = \frac{\text{Working Standard Weight}}{\text{Total Solution Volume equals to the Volumetric choosen}}$$

Note: *Stock contains 1mg of antibiotic salt per ml of solution.*

1.3 Preparation of the Standard High solution by diluting stock solution with buffer:

$$\text{Standard High Dilution} = \text{Stock Solution} \times \frac{1}{50}$$

1.4 Preparation of the Standard Low solution by diluting Standard High solution with buffer:

$$\text{Standard Low Dilution} = \text{Standard High Dilution} \times \frac{25}{100}$$

Note: *Dilution ratio in between High and Low conc. solution =*

2. Solution associated to Antibiotic Test Sample:

2.1 Weight calculation for Antibiotic Test Sample:

$$\text{Test Sample weight (gram)} = \frac{1}{\frac{\text{Label Claim}}{100} \times 1000} \times \text{Volume of volumetric choosen}$$

2.2 Preparation of the stock solution:

$$\text{Stock Solution} = \frac{\text{Test Sample weight (gram)}}{\text{Total Solution Volume equals to the Volumetric choosen}}$$

Note: *Stock contains 1mg of antibiotic salt per ml of solution.*

2.3 Preparation of the Standard High solution by diluting stock solution with buffer:

$$\text{Test High Dilution} = \text{Stock Solution} \times \frac{1}{50}$$

2.4 Preparation of the Standard Low solution by diluting Standard High solution with buffer:

$$\text{Test Low Dilution} = \text{Test High Dilution} \times \frac{25}{100}$$

Note: *Dilution ratio in between High and Low conc. solution = 4: 1*

3. Observation table enlisted by the different diameters of zones as recorded by the antibiotic zone reader for all concordant readings:

S. No.	TH	TL	SH	SL
01.				
02.				
03.				
04.				
Average				

Average is to be taken from all the concordant readings.
Where,
TH: Test High,
TL: Test Low,
SH: Standard High,
SL: Standard Low.

4. Percentage of Potency:

$$\%\text{Potency} = \text{Antilog}\,(2 + a\,\text{Log I}) = 10^{(2+ a\,\text{Log I})}$$

Where,

$$a = \frac{(TH + TL) - (SH + SL)}{(TH - TL) + (SH + SL)}$$

Note: *'I' is the dilution ration between Low conc. and High conc.*

Here, (I = 4).

5. Assay obtained:

$$\text{Assay} = \frac{\%\text{Potency}}{100} \times \frac{\text{Standard Low Dilution}}{\text{Test Low Dilution}} \times \frac{\text{Potency of Salt}}{1000} \times \frac{100}{1000}$$

6. Effective Percentage of Assay:

$$\%\text{Assay} = \frac{\text{Assay}}{\text{Label Claim}} \times 100$$

CONCLUSION

%Assay when reaches 100%, signifies Assay to Label Claim ratio to be ≤1, signifies that the pharmaceutical product contains sufficient amount of antibiotics and which satisfies label claim and the product in context with the antibiotic assay is said to be *PASS.*

If %Assay is less than the label claim and does not satisfies the criteria, are considered as *FAIL.*

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