DIFFUSION PLATE ASSAY FOR CHLORAMPHENICOL AND AUREOMYCIN [1]

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In the development of bioassay methods for antibiotics a certain standard pattern is usually followed. First, a wide variety of microorganisms are tested for their sensitivity to the new drug and then sensitive strains are selected and tested for their suitability for use in a plate assay, turbidimetric assay and serial dilution assay. Since the Food and Drug Administration tests a number of antibiotics, every effort is made to adapt test procedures for new antibiotics to those already in use. Thus, in the two plate assay methods to be described, it has been found possible to utilize the same test organism; the same medium as that used in the seed layer for the penicillin plate assay; and the general procedure for determining the potency of an unknown which is the same as that described for penicillin in the Federal Register of June 3, 1948.

The percentage error of the assay may be determined by the double dose technic described by Knudsen and Randall (1). A 1:4 ratio is used for chloramphenicol (20 μg. and 80 μg.), and a 1:10 ratio for aureomycin (3 μg. and 30 μg.). The chart and nomograph for the 1:10 ratio are not given in the above publication but may be obtained on request from the Food and Drug Administration. The percentage error of the chloramphenicol plate assay has averaged 2.23% on about 21 determinations with a range of from 0.7% to 4.58%. The error of the aureomycin assay is somewhat higher. In 24 determinations the percentage error ranged from 2.5% to 14.8% with an average of 7.4%. In our experience the cup-plate method for chloramphenicol is the most accurate and reproducible one yet encountered. A composite curve was prepared from ten daily chloramphenicol curves and the potency of samples previously run recalculated. Since no significant differences in potency were noted, a daily curve is no longer necessary; however, the composite curve is checked against a new standard curve once every week. A description of the methods follows.

Culture media. Use ingredients that conform to the standards prescribed by the U.S.P. or N.F. Make nutrient agar for the seed and base layer and for carrying the test organism as follows:

Peptone	6.0 gm.
Pancreatic digest of casein	4.0 gm.
Yeast extract	3.0 gm.
Beef extract	1.5 gm.
Glucose	1.0 gm.
Agar	15.0 gm.
Distilled water, q.s.	1000.0 ml.

pH 6.5 to 6.6 after sterilization

Make nutrient broth, for preparing a suspension of the test organism, as follows:

Peptone	5.0 gm.
Yeast extract	1.5 gm.
Beef extract	1.5 gm.
Sodium chloride	3.5 gm.
Glucose	1.0 gm.
Dipotassium phosphate	3.68 gm.
Potassium dihydrogen phosphate	1.32 gm.
Distilled water, q.s.	1000.0 ml.

pH 7.0 after sterilization

In lieu of preparing the media from the individual ingredients specified above, they may be made from a dehydrated mixture which, when reconstituted with distilled water, has the same composition as such media.

Working standard

A. Chloramphenicol. Weigh out carefully appropriate amounts of the standard which is crystalline synthetic chloramphenicol and dilute in 1% phosphate buffer pH 6 to give a solution containing 100 μg. per ml. Keep this stock solution at a temperature of 15° C. or less, and use for only one week. From this stock solution make appropriate working dilutions in the above buffer. To facilitate solutions, the standard may be first dissolved in a small amount of ethyl alcohol.

B. Aureomycin. Proceed as above except dilute the aureomycin standard which is crystalline aureomycin to 1000 μg. per ml. in 1% potassium acid phthalate-tartaric acid buffer pH 3.0 and use for only three days.

Preparation of sample.

A. Chloramphenicol. Dissolve the sample to be tested in a small amount of ethyl alcohol and then further dilute

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in 1% phosphate buffer pH 6 to make an appropriate stock solution.

B. Aureomycin. Dissolve the sample to be tested in sterile distilled water to make an appropriate stock solution.

Preparation of plates

I. Base layer. Add 21 ml. of agar to each Petri dish (20 × 100 mm.). Distribute the agar evenly in the plates and allow it to harden. Use the plates the same day they are prepared.

II. Seed layer. The test organism is *Sarcina lutea* (P.C.I. 1001). Maintain the test organism on slants of nutrient agar prepared as described above, and transfer to a fresh agar slant once a week. Prepare a suspension of the test organisms as follows: Streak an agar slant heavily with the test organism. Incubate 24 hours at 26° C. Wash the growth off in about 3 ml. of nutrient broth. Use the suspension so obtained to inoculate the surface of a Roux bottle containing 300 ml. of the above agar. Spread the suspension over the entire surface with the aid of sterile glass beads. Incubate 24 hours at 26° C.

A. Chloramphenicol. Wash the resulting growth from the agar surface with about 20 ml. of nutrient broth. If an aliquot of this bulk suspension, when diluted with nutrient broth 1 : 10, gives a 10% light transmission, the bulk suspension is satisfactory for use in the test. It may be necessary to dilute the bulk suspension so that a 1 : 10 dilution of an aliquot gives a light transmission of 10%. A photoelectric colorimeter equipped with a filter having a wave length of 6500 Angstrom units is used in the standardization. The bulk suspension may be used in the test for one week or longer. Add 1.5 ml. of the adjusted bulk suspension to 100 ml. of agar which has been melted and cooled to 48° C. Use 4 ml. per plate of this inoculum for the seed layer.

B. Aureomycin. Wash the growth from the agar surface with 50 ml. of nutrient broth. If an aliquot of this bulk suspension when diluted 1 : 50 in saline gives 75% light transmission (filter same as above), the bulk suspension is satisfactory for use in the test. As above, it may be necessary to dilute the bulk suspension so that an aliquot gives 75% light transmission. The bulk suspension may be used in the test for at least one week. Add 0.3 ml. of the adjusted bulk suspension to 100 ml. of agar which has been melted and cooled at 48° C. Use 4 ml. per plate of this inoculum for the seed layer.

Assay

A. Chloramphenicol. The potency of the sample is determined by the standard curve technic, using a single dose of standard and unknown.

Dilute the sample to be tested to an estimated 50 μg. per ml. in 1% phosphate buffer pH 6. Place six cylinders on the inoculated agar surface so that they are at approximately 60° intervals on a 2.8 cm. radius. Use three plates for each sample. Fill three cylinders on each plate with the 50 μg. per ml. standard and three cylinders with the 50 μg. per ml. (estimated) sample, alternating standard and sample. At the same time prepare a standard curve, using concentrations of the standard of 30.0, 35.0, 40.0, 45.0, 50.0, 55.0, 60.0, 65.0, and 70.0 μg. per ml. A total of 24 plates is used in the preparation of the standard curve, three plates for each solution except the 50 μg. per ml. solution. The latter concentration is used as the reference point and is included on each plate. On each of three plates fill three cylinders with the 50 μg. per ml. standard and the other three cylinders with the concentration of the standard under test. Thus, there will be seventy-two 50-μg. determinations and nine determinations for each of the other points on the curve. Incubate the plates for 16 to 18 hours at 37° C. and measure the diameter of each circle of inhibition. Average the readings of the 50 μg. per ml. concentration and the readings of the point tested for each set of three plates, and average also all 72 readings of the 50 μg. per ml. concentration. The average of the 72 readings of the 50 μg. per ml. concentration is the correction point for the curve. Correct the average value obtained for each point to the figure it would be if the 50 μg. per ml. reading for that set of three plates were the same as the correction point. Thus, if in correcting the 40 μg. per ml. concentration the average of the 72 readings of the 50 μg. per ml. concentration is 18.0 mm., and the average of the 50 μg. per ml. concentration of this set of three plates is 17.8 mm., the correction is .2 mm. If the average reading of the 40 μg. per ml. concentration of these same three plates is 17.0 mm., the corrected value is then 17.2 mm.

Plot these corrected values including the average of the 50 μg. per ml. concentrations on two-cycle semi-log paper, using the concentration in μg. per ml. as the ordinate (the logarithmic scale) and the diameter of the zone of inhibition as the abscissa. Draw the standard curve through these points.

To estimate the potency of the sample, average the zone readings of the standard and the zone readings of the sample on the three plates used. If the sample gives a larger zone size than the average of the standard, add the difference between them to the 50 μg. per ml. unit zone on the stand-

ard curve. If the average value is lower than the standard value, subtract the difference between them from the 50 μg. per ml. unit value on the curve. From the curves read the potencies corresponding to these corrected values of zone sizes.

B. Aureomycin. The assay for aureomycin follows very closely that described for chloramphenicol. The following exceptions are noted.

1. Dilute the stock solution of the sample to be tested to 10.0 μg. per ml. (estimated) in 1% potassium acid phthalate-tartaric acid buffer (pH 3.0).

2. Prepare a standard curve, using concentrations of the standard of 4.0, 6.0, 8.0, 10.0, 13.0, 17.0, 22.0, and 29.0 μg. per ml. in 1% potassium acid phthalate-tartaric acid buffer pH 3.0. The 10 μg. per ml. concentration is the reference point for the aureomycin curve, and the correction point is obtained by averaging the readings of all of the 10 μg. per ml. concentrations.

SUMMARY

1. A cup-plate assay for chloramphenicol and aureomycin is described.

2. The organism, medium and the general method for estimating the potency are the same for both antibiotics.

BIBLIOGRAPHY

1. Knudsen, L. F., and Randall, W. A., Penicillin assay and its control chart analysis. J. Bact., 1945, **50**, 187.