A simple technique in developing thin-layer bioautographs

The literature includes several reports on bioautographic techniques for antibiotic identifications. NICHOLAUS *et al.*[1] described a method utilizing tetrazolium chloride for zone definition and sensitivity. A combined procedure utilizing paper and silica gel was described by MEYERS AND SMITH[2].

It has been possible to greatly simplify these methods, as well as including other antibiotics not previously tested.

Materials and methods

This simplified method included the use of silica gel plates prepared by the standard procedure of STAHL[3] and developed in chromatographic chambers. For practical purposes drying periods extended for one to two hours at room temperature. This period of time was altered depending upon the solvents used. An agar medium was sprayed onto the dried plates with a Devilbiss paint spray gun attached to the laboratory air line. To standardize the pressure for more uniform results, a pressure reducing valve adjusted to 27 lb./sq.in. pressure was added to the line. Agar at 100° gave satisfactory results, in fact, the mixture of agar and air passing through the nozzle of the spray gun was sufficiently cool to allow solidification immediately on the upright plate.

An agar medium, cooled to 48° and inoculated with a suitable microbiological assay organism, was poured directly over the surface of the prepared plate. To insure even distribution of the medium, a box, 20 cm × 20 cm, was constructed of plexiglass to hold the plate. Volumes of agar ranging between 60–100 ml were sufficient to produce the desired effects over the surface of the described area. Lids, also prepared from plexiglass, were used to cover the plates during incubation.

Zones of inhibition were identified after incubation by directly viewing the opaque plates.

Results and discussion

The method of developing thin-layer plates described by NICHOLAUS *et al.*[1] includes a rather complicated procedure of adding tetrazolium chloride to the inoculated medium to protect the reagent from the atmosphere. They also used a preincubation period of 1 h at 0° to allow diffusion of the antibiotic into the inoculated agar while deterring growth of the organism. Variations in the percent inoculum and the agar depth were used to increase the sensitivity as well as zone definition.

Several attempts were made to apply the inoculated medium directly to the silica gel but the antibiotics were spread over the plate surface producing vast areas of inhibition. Since using the spraying device no malconfigurations of inhibitory zones were noticed.

Pressurized cans containing liquid propellants were also tried and worked satisfactorily but more uniform results were obtained with the present method.

Antibiotics tested in our laboratories include sodium penicillin G, tylosin base, erythromycin base, streptomycin sulfate, and several derivatives of tylosin.

A system using methanol–acetone (60:40) was satisfactory to differentiate penicillin, tylosin, streptomycin and erythromycin. Table I includes the R_F values for these antibiotics.

Only streptomycin failed to move from the original spot. Many different solvent systems were tried but all failed to produce the desired effect. This may be associated with the strong binding capacity of streptomycin and the solid substrate and the relative insolubility of the antibiotic in organic solvents. Although the literature contained many systems for separating streptomycin on paper, none were found for thin-layer methods.

TABLE I

R_F VALUES OF SOME ANTIBIOTICS

Antibiotic	*R_F value*
Sodium penicillin G	0.75
Erythromycin base	0.20
Streptomycin sulfate	Not mobile
Tylosin base	0.83

It was possible, however, to move streptomycin in an aqueous system. An R_F value of 0.27 was obtained under these conditions. Erythromycin and tylosin failed to move with water and an R_F value of 0.63 was determined for penicillin. With these two systems, it would be possible to identify each of the components. In fact, a two-dimensional system might be used.

It was also noted that higher concentrations of antibiotics applied to silica gel and located with a 1:1 solution of 2 % sodium carbonate and 1 % potassium permanganate could also be detected by microbiological methods. In this instance the plates were sprayed with a laboratory atomizer containing the reagent. After marking the yellow spots, the plates were dried at room temperature for 1 h and developed by microbiological methods.

It is assumed that the surface antibiotic is oxidized allowing for chemical detection and the sub-surface antibiotic diffused into the medium allowing for microbiological activity. This technique offers a unique advantage of qualitatively assaying a sample by both means showing biologically inactive degradation products as well as active components.

Thus far, three adsorbents have been tested: Silica Gel G, Silica Gel GF 254 and Kieselguhr G. All have proved satisfactory for our purposes. The added indicator in Silica Gel GF 254 allows detection of spots by U.V. light. But like the permanganate systems, larger quantities of antibiotics are required.

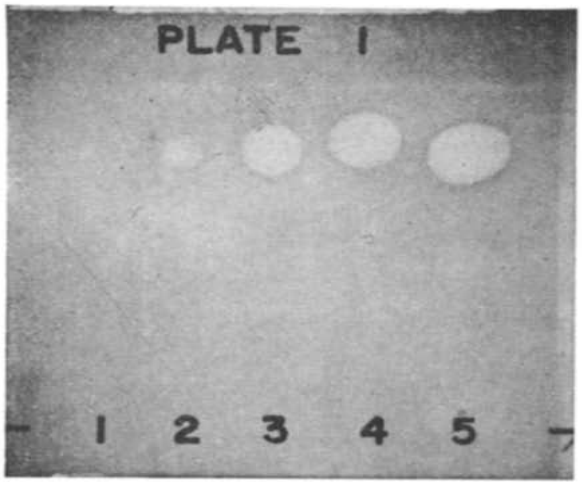
Two organisms, *Bacillus subtilis* A.T.C.C. 6633 and *Sarcina lutea* A.T.C.C. 9341, have been used for developing the bioautographs. *Bacillus subtilis* is routinely used for developing the paper chromatograms. Both of these organisms are used for standard microbiological assays and both were suitable for the detection of the antibiotics used in these studies. *Bacillus subtilis*, however, is more susceptible to streptomycin than *Sarcina lutea* and is recommended for this purpose.

Sensitivity, as defined for these procedures, is the smallest detectable quantity of antibiotic. This would vary according to the quantity of agar used, the concentration of inoculum and the antibiotic–organism relationship.

For the penicillin–*Sarcina lutea* combination on medium No. 1 (GROVE AND RANDALL[4]) the sensitivity was 0.005 units.

Bacillus subtilis' susceptibility to streptomycin on medium No. 5 (GROVE AND RANDALL[4]) approaches 0.1 μg. Tylosin against *Sarcina lutea* on penicillin seed agar was effective at a level of 0.025 μg. The smallest detectable level of activity for erythromycin against *Sarcina lutea* was 0.025 μg.

Two plates (Figs. 1 and 2) are included to illustrate the results of this technique. In certain instances, the poor zone definition is a result of the photographic equipment. Agar color and organism pigment create no problem in measuring mobilities directly on the plate surface.



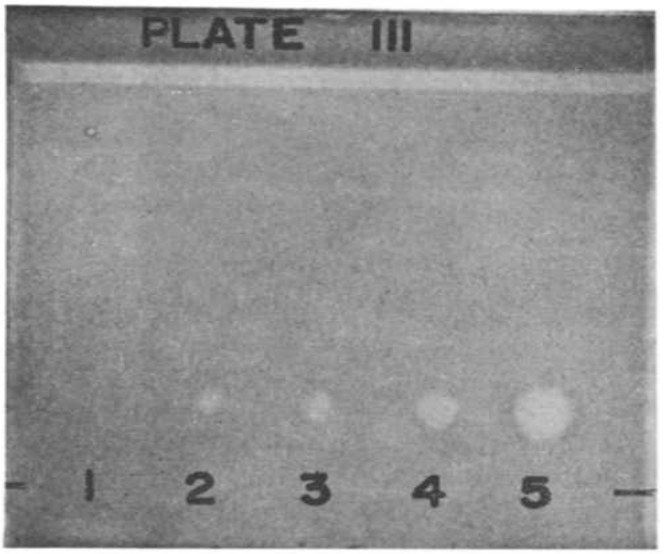


Fig. 1. Sodium penicillin G developed on Silica Gel G with *Sarcina lutea*. 1 = 0.005 units; 2 = 0.01 units; 3 = 0.025 units; 4 = 0.05 units; 5 = 0.1 units.

Fig. 2. Erythromycin base developed on Silica Gel G with *Sarcina lutea*. 1 = 0.025 μg; 2 = 0.05 μg; 3 = 0.1 μg; 4 = 0.2 μg; 5 = 0.25 μg.

For these reasons it appears that this method might be routinely used for the identification of minute quantities of antibiotic. Prior to this time, only one antibiotic could be unequivocally identified at these concentrations. The antibiotic, of course, is penicillin and the reagent penicillinase. Microbiological spectra could be used but it could not be recommended for routine use.

Additional systems are being screened as well as different absorbents and other antibiotics to find the ideal combinations for complete separation.

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