

A DIRECT BIOAUTOGRAPHIC TLC ASSAY FOR COMPOUNDS POSSESSING ANTIBACTERIAL ACTIVITY¹

MATTHIAS O. HAMBURGER and GEOFFREY A. CORDELL*²

*Program for Collaborative Research in the Pharmaceutical Sciences, College of Pharmacy,
University of Illinois at Chicago, Chicago, Illinois, 60612*

ABSTRACT.—A simple bioassay for the direct detection of antibacterial compounds on tlc plates has been developed. A series of natural products and different stationary phases were tested in order to establish the utility of the assay for the isolation of antibacterial compounds from higher plants.

If one defines pharmacognosy as that science concerned in the broadest sense with the study of biologically active compounds from natural sources, it is readily apparent that the rapid and efficient detection of such compounds is a critically important aspect of the discovery process. Because of the complexity of plant extracts, there has been relatively limited work conducted on the isolation of antibacterial agents from plants. The principal problem has been that of interference in the *in vitro* assays by the structurally diverse, sometimes highly colored compounds.

Bioautography, as a method to localize antibacterial activity on a chromatogram, has found widespread application in the search for new antibiotics (1-4). However, almost all of the published procedures are based on the so-called agar-diffusion technique, whereby the antibacterial compound is transferred from the chromatographic layer to an inoculated agar plate through a diffusion process. Zones of inhibition are then visualized by appropriate vital stains. The procedure has several disadvantages and requires the use of suitable microbiological equipment. Considering the problems that arise due to the differential diffusion of compounds from the chromatogram to the agar plate, it is surprising that, to date, only one attempt to simplify the procedure through direct bioautographic detection on the chromatographic layer has been published (5). We describe here a simple, direct bioautographic assay on tlc for compounds with activity against gram-positive and gram-negative bacteria.

The principle of the assay is the following: A suspension of a microorganism in a suitable broth is applied to a developed tlc plate. Incubation in a humid atmosphere permits growth of the bacteria. Zones of inhibition are then visualized by a dehydrogenase-activity-detecting reagent, *i.e.*, a tetrazolium salt. Metabolically active bacteria convert the tetrazolium salt into the corresponding intensely colored formazan. Thus, antibacterial compounds appear as clear spots against a colored background.

Bacillus subtilis and *Escherichia coli* were chosen as initial representatives for gram-positive and gram-negative bacteria, respectively. These particular strains are used in recognized antimicrobial test protocols (6). In order to determine the optimal conditions for bacterial growth on silica gel plates, various parameters were modified, such as the volume of bacterial suspension and reagent solution and the incubation period for bacterial growth and enzymatic reaction. The bacterial suspension was applied with a roller device. This technique allowed an even distribution of the bacteria while minimizing airborne microorganisms, as will be discussed later. The results of the optimization of these various parameters can be summarized as follows: Nutrient broth was found to be a better medium than trypticase-soy broth. A volume of 5-6 ml of

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bacterial suspension per plate (20×20 cm) was found to be optimal. This amount could be readily absorbed by the plate. Smaller or larger volumes resulted in weak or irregular growth and poorly defined zones of inhibition, possibly due to an inoculum effect from the excessive number of bacteria. Maximal bacterial growth was realized by an incubation overnight. Apparently, the bacteria enter a stationary growth phase at this point. Prolonged incubation led generally to poorly defined zones of inhibition. A number of tetrazolium salts were evaluated as potential substrates for the enzymatic reaction. In accordance with the literature (7), *p*-iodonitrotetrazolium violet (INT), tetranitro blue tetrazolium (TNBT), and methyl thiazolyl tetrazolium (MTT) were found to be good substrates. Preference was given to INT, since this tetrazolium salt, in contrast to the two other compounds mentioned above, is colorless, thereby facilitating evaluation of the bioautogram.

The amount of formazan produced by the enzymatic reaction increased over a period of several hours. Incubation for 4 h, however, produced very good results and was chosen for practical reasons.

Under the above-mentioned conditions, the assay showed good reproducibility, and we, therefore, examined the sensitivity of the assay. In the ideal test, ubiquitous or widespread inactive or weakly active compounds should not interfere with the detection of minor compounds with good antibacterial activity. We, therefore, determined the approximate detection limits of some common natural compounds and two reference antibiotics. The results are listed in Table 1. No growth inhibition was observed for β -sitosterol, stigmasterol, palmitic acid, and sucrose even at 20 μ g/spot. The polyphenols and *p*-coumaric acid showed activity down to 5 μ g, and 2.5 μ g, respectively, which is still somewhat more than usually applied on an analytical tlc plate. We can, therefore, presume that weakly active compounds would only be detected in an extract if present in high amounts.

TABLE 1. Sensitivity of the Bioautographic Assay Against Some Natural Compounds

Compounds	Bacterial System	
	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>
β -Sitosterol	>20 ^a	>20
Stigmasterol	>20	>20
Palmitic acid	>20	>20
Sucrose	>20	>20
Quercetin	5	5
Tannic acid	5	5
<i>p</i> -Coumaric acid	2.5	5
Pencillin V	0.005	> 2
Chloramphenicol	0.01	0.02

^aMinimal amount (in μ g) of compound spotted on a silica gel tlc plate that produces detectable growth inhibition.

The assay proved to be insensitive against various cytotoxic agents belonging to the camptothecin (camptothecin, 9-methoxycamptothecin, 10-methoxycamptothecin, 10-hydroxycamptothecin), the quassinoid (6 α -senecierylchapparione, chaperinone, 6 α -senecierylchapparione, holacanthone, glaucarubolone, isobrucein A), and the lignan (β -peltatin, 4'-demethyldeoxy-podophyllotoxin) series when tested at 5 μ g. Podophyllotoxin and α -peltatin were the only cytotoxic compounds tested to show inhibition.

The suitability of stationary phases other than silica gel was also investigated. *B. subtilis* and *E. coli* grew on plates coated with cellulose, but poor results were obtained on polyamide 11 and neutral aluminum oxide. With respect to the choice of eluents, preference should be given to volatile solvents. Mobile phases containing solvents of low volatility such as *n*-BuOH can be used, if they are completely removed prior to the assay. This is, however, not possible in the case of most acids or bases. We observed that traces of HOAc remaining even after prolonged drying completely inhibited bacterial growth.

A major concern with any direct bioautographic assay is the dispersal of the bacteria and the safe disposal of the contaminated material. A contained system was used for the application of the bacterial suspension to the tlc plate. We found that a glove bag is an inexpensive alternative to the glove box or safety hood. The equipment, such as glassware, a device for application of the bacteria, and boxes for incubation were all autoclavable and were collected in autoclave bags immediately after use. The efficiency of the safety precautions, especially the number of airborne bacteria was monitored throughout the different steps of the assay (for details see Experimental). A small number of bacteria were released into the air inside the containment during the application of the bacterial suspension. No airborne colony forming units could be detected outside of the glove bag or during the other steps of the assay. Smears taken from different places inside the containment bag after disinfection showed no outgrowing bacteria.

So far, agar streaking (8,9), disc assay (10,11), in vitro dilutions (11), and agar-layer techniques (12) have been used for the detection of antibacterial agents in higher plants. Bioautography permits the facile localization of an activity even in complex mixtures. The main feature of the direct assay is the elimination of the diffusion step. Well-defined zones of inhibition observed directly on the tlc plate improve the localization of the activity and enhance the sensitivity of the assay. Comparison with a chromatogram developed under identical conditions and visualized with an appropriate chromogenic reagent may give extremely useful information about the nature of the active principles. However, some restrictions in the choice of stationary phases and eluents must be taken into account.

In subsequent publications we will describe the successful use of this assay for the activity-directed isolation of antibacterial compounds from higher plants.

EXPERIMENTAL

CHEMICALS.—All solvents were of analytical grade. Quercetin, *p*-coumaric acid, tannic acid, sucrose, palmitic acid, penicillin V, chloramphenicol, 2,3,5-triphenyltetrazolium chloride (TTC), *p*-iodonitrotetrazolium violet (INT), tetranitro blue tetrazolium (TNBT), and methyl thiazolyl thiazolium (MTT) were purchased from Sigma Chemicals, St. Louis, Missouri. 9-Methoxycamptothecin, 10-methoxycamptothecin and 10-hydroxycamptothecin were obtained from the National Cancer Institute. All other compounds were isolated in these laboratories.

THIN-LAYER CHROMATOGRAPHY.—Silica gel G60 F254 A1 sheets, neutral aluminum oxide F254 A1 sheets, polyamide 11 F254 A1 sheets, and cellulose A1 sheets (Merck, Darmstadt, West Germany) were used. Dilution series of the test-compounds were spotted onto the tlc plate with graduated micropipettes (Drummond Scientific, Broomall, Pennsylvania). The following solvent systems were used for the development of the silica gel tlc plates: CHCl₃-MeOH (98:2) for stigmaterol, sitosterol, and palmitic acid; CHCl₃-MeOH (80:20) for quercetin and *p*-coumaric acid; *i*PrOH-EtOAc-H₂O (70:20:10) for tannic acid and sucrose; MeOH-Me₂CO (50:50) for penicillin V; CHCl₃-MeOH (90:10) for chloramphenicol; CHCl₃-EtOH-EtOAc-hexane (18:6:34:42) for the lignans; CHCl₃-EtOH-EtOAc-hexane (70:10:17:3) for the quassinoids; and THF-CH₂Cl₂ (25:75) for the camptothecines. The chromatograms were developed in standard tanks lined with filter paper.

BACTERIA.—*B. subtilis* (ATCC 6633) and *E. coli* (ATCC 25922) were purchased from the American Type Culture Collection, Rockville, Maryland. The test organisms were maintained on trypticase-soy agar or nutrient agar slants (BBL, Lockeyville, Maryland).

CULTURE MEDIA.—Cultures of *B. subtilis* and *E. coli* were grown in nutrient broth (BBL, Lockesville, Maryland) at 37° for 48 h on a shaker at a speed of 80 rpm. The cultures were centrifuged (1500×g for 10 min), the supernatant liquid was discarded, and the bacteria suspended in a small volume of fresh nutrient broth. Volumes, equivalent to ca. 10⁹ bacteria/ml after dilution to 20 ml (turbidity at 560 nm: 0,84 A) were dispensed in cryotubes and stored in liquid N₂ until used.

MATERIAL FOR BIOAUTOGRAPHY.—A polyethylene glove bag (Aldrich Chemicals, Milwaukee, Wisconsin), polyethylene boxes (32×25×13 cm) (Ace Glass, Vineland, New Jersey), polypropylene autoclave bags (Fisher Scientific, Pittsburgh, Pennsylvania), and pyrex glass dishes were used. A roller device made from autoclavable materials was purchased from a local artist supply store.

BIOAUTOGRAPHY.—The developed chromatograms were carefully dried with a hair-dryer for complete removal of the solvents. Uv-active compounds were detected at 254 nm and 366 nm and marked on the tlc plate. The bacterial suspension of a cryovial was diluted to 20 ml with nutrient broth. A sample (5-6 ml) of this suspension was dispersed evenly over a tlc plate (20×20 cm) using a roller device covered with chromatography paper (Whatman, Clifton, New Jersey). The tlc plates were incubated overnight in plastic boxes lined with wet chromatography paper. The plates were then sprayed with ca. 5 ml of an aqueous solution of TTC (20 mg/ml), INT (5 mg/ml), INTB (5 mg/ml), or MTT (2.5 mg/ml). The tlc plates were again incubated in the plastic boxes at 37° for 4 h. The tlc plates were treated with EtOH (70%) before evaluation of the bioautograms.

MICROBIOLOGICAL CONTROL.—The contaminated material was collected in polypropylene bags and autoclaved. The airborne bacteria were monitored by exposing open nutrient agar and trypticase soy agar petri dishes during the different steps of the assay. Exposure time was 5 min. The petri dishes were incubated at 37° for 48 h, and the number of CFU determined. The monitoring was performed at least in duplicate, with sterile and growth control. The following average numbers of CFU per petri dish were found: 21 CFU inside, 0 CFU outside the glove bag during application of the bacterial suspension; 0 CFU during spraying of tlc plate with the tetrazolium salt solution. After use, the closed glove bag was sprayed inside with EtOH (70%). The bag was left overnight, and no outgrowing bacteria were found in smears obtained from different locations inside the bag.

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