A Simple Biological Method for Determination of Small Amounts of Tuberculostatic Agents in Fluids*

K. D. STOTTMIEER,¹ C. L. WOODLEY, G. P. KUBICA & R. E. BEAM

The authors describe the use of the vertical diffusion test for determining the quantity of certain tuberculostatic drugs in body fluids and the degree of mycobacterial susceptibility to these drugs, using tubercle bacilli as test organisms. It is found to be a reliable assay method for isoniazid, para-aminosalicylic acid, ethionamide and ethambutol when carried out on a modification of Middlebrook 7H-10 solid medium containing 1.5% Oxoid Ion-Agar No. 2.

As a drug-susceptibility test, it yields results expressed qualitatively in degrees of sensitivity or resistance and is therefore recommended for use only in laboratories not equipped for more comprehensive testing. The method may also be used for ascertaining the level of tuberculostatic activity of a patient's serum against his own mycobacteria.

The determination of drug levels in serum or other body fluids is not done routinely in most laboratories. A major deterrent to routine serum level determinations is that different tests have to be employed for the assay of different tuberculostatic drugs (Russell & Middlebrook, 1961; Lorian, 1966). The purpose of this paper is to describe a simple biological test which allows quantitative determination of tuberculostatic drugs in fluids as well as a qualitative determination of drug-susceptibility of mycobacteria.

The vertical diffusion method has already been described and employed for the assay of drugs and for drug-susceptibility tests by Schmiedel (1958) and Bönicke (1953), using media other than the synthetic Middlebrook 7H-10 agar. Preliminary studies indicated that the use of Middlebrook 7H-10 agar increased the diffusibility of drugs in the vertical diffusion method. The method here recommended is most suitable for the assay of isoniazid, ethionamide, para-aminosalicylic acid (PAS) and ethambutol. Antibiotics such as streptomycin, kanamycin, viomycin, capreomycin, and cycloserine can also be tested by this method; however, other bacteriological methods employing Bacillus subtilis or Staphylococcus albus provide results within 24 hours and thus are much more rapid than the method to be described, which employs slowly growing tubercle bacilli as test organisms. Determination of pyrazinamide drug levels is done most successfully in this laboratory by a chemical method (Allen et al., 1953).

MATERIAL AND METHODS

Löwenstein-Jensen egg medium, Middlebrook 7H-10 agar (Difco), and a "home-made" modification of the latter were tried initially to determine their suitability for use in the vertical diffusion method.

The modification of 7H-10 agar involved a series of stock solutions (US Veterans Administration, 1966) and 1.5% Oxoid Ion-Agar No. 2 instead of the usual Bacto agar. Commercially available oleic-acid-albumin--dextrose (OADC) enrichment was added to the autoclave-sterilized basal medium previously cooled to 56°C. With an automatic syringe, 5 ml of the completed medium were placed into a series of sterile 16 mm × 125 mm screw-capped tubes and allowed to solidify in a slanting position. To

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* From the Tuberculosis Unit, Laboratory Program, National Communicable Disease Center, Atlanta, Ga., USA. Reprints may be ordered from this address.
¹ Present address: Forschungsinstitut, 2061 Borstel, Germany.
² Also H. H. Kleeberg—personal communication, 1965.
³ The use of trade names is for identification only and does not constitute endorsement by the Public Health Service, or by the US Department of Health, Education, and Welfare.
ensure uniformity of the slants, tubes of the same brand were used and each series of tubes was solidified at the same angle in a slanting rack. The medium should be kept overnight in this position and may then be kept for as long as several months at 4°C before use. Slants of commercial 7H-10 medium (Difco) and Löwenstein-Jensen medium were prepared as usual and placed in tubes of the same size.

Method for determining drug level in various fluids

The prepared slants were inoculated with 0.1 ml of a 1-week-old culture of Mycobacterium tuberculosis strain H37Rv grown in 7H-9 broth (Difco) standardized to approximate that of a McFarland No. 1 turbidity standard. Excess inoculum or fluid was removed from the bottom of the tube. The fluid to be tested was set up in serial 2-fold dilutions ranging from undiluted to 1:32; dilutions were made in sterile, distilled water. Using a sterile 1.0-ml pipette, 0.5 ml of the fluid to be tested was placed in the bottom of the tube without making contact with the already inoculated slant. Different drugs contained in one fluid specimen may be assayed by using suitable drug-resistant strains of Myco. tuberculosis. The isoniazid concentration of serum may be determined in the presence of PAS, if a PAS-resistant strain (50–100 μg/ml resistant) of tubercle bacilli is employed, whereas the assay of PAS will not be influenced if an isoniazid-resistant strain (10 μg/ml resistant) is used. Furthermore, the total non-specific antimycobacterial titre of serum or other fluid may be determined by using drug-sensitive strains of tubercle bacilli; this might be particularly valuable in studies of drug synergism. As a control in every test an inoculated slant with 0.5 ml of sterile distilled water was used. Optimal results were obtained by using 2–5 tubes for every dilution being tested and averaging the results. The number of tubes used for each serial dilution of fluid to be tested is limited, of course, by the quantity of fluid available.

Prior to drug-level testing, standard curves for each drug were established by adding to previously inoculated slants the following concentrations of drugs, each contained in 0.5 ml of sterile distilled water:

(1) isoniazid: 0.125 μg, 0.25 μg, 0.5 μg, 1.0 μg and 2.0 μg
(2) PAS: 0.5 μg, 1.0 μg, 2.0 μg, 3.0 μg and 4.0 μg
(3) ethionamide: 2.0 μg, 6.0 μg, 8.0 μg, 10.0 μg and 12.0 μg
(4) ethambutol: 6.0 μg, 12.0 μg, 16.0 μg, 18.0 μg and 20.0 μg

After inoculation and addition of the standard or test fluid, the tubes were incubated in an upright position at 37°C for 10 days.

Growth-inhibition zones were determined by measuring the distance (in mm) from the fluid level (which should be marked prior to incubation) to the observed growth line on the slant. The size of the inhibition zone is a function of the drug concentration employed; this function can be plotted in a standard curve (Fig. 1 and 2). Serial dilutions of any unknown fluid to be tested usually ensure measurable inhibition zones; high drug concentrations in undiluted test fluids may result in growth inhibition on the entire slant, making measurement impossible.

Comparative tests were done to determine the tuberculostatic activity of the different drugs when dissolved in distilled water and normal 7H-10 medium (without agar). These tests were undertaken to investigate whether an alteration of isoniazid, ethionamide, PAS, and ethambutol occurred if these drugs were incorporated in normal 7H-10 medium for routine drug-susceptibility testing.

A simplified method of drug susceptibility testing

By reversing the assay method, a simple drug-susceptibility test can be performed in which unknown strains of mycobacteria are tested against known drug concentrations deposited in the bottom of the tube. Inoculation of the slants with a 1-week-old liquid subculture of the test organism is done as described above for the H37Rv culture. Known concentrations of drug, placed in the tube after inoculation, produced a zone of growth inhibition which was related to the degree of drug resistance of the strain. The following drug concentrations were employed and dispensed in 0.5-ml amounts:

(1) isoniazid: 2.5 μg/tube
(2) streptomycin: 40 μg/tube
(3) PAS: 2.5 μg/tube
(4) ethionamide: 8.0 μg/tube
(5) cycloserine: 100 μg/tube
(6) viomycin: 100 μg/tube
(7) kanamycin: 100 μg/tube
(8) ethambutol: 20 μg/tube
Using routine drug-susceptibility tests in 7H-10 agar (Kubica & Dye, 1967), the minimal inhibitory concentrations of isoniazid, streptomycin, PAS, ethionamide, cycloserine, viomycin, kanamycin, and ethambutol were determined for 40 drug-sensitive and drug-resistant strains of tubercle bacilli. These results were then correlated with the vertical diffusion susceptibility test as described above.

RESULTS

The diffusion of isoniazid in slants of Löwenstein-Jensen egg medium, normal 7H-10 medium and 7H-10 medium with ion agar is shown in Fig. 1. The superiority of the ion-agar medium for vertical diffusion of drugs is readily apparent. For example, growth-inhibition zones with 0.5 \( \mu g \) isoniazid were 50 mm in ion-agar and 20 mm–30 mm in Löwenstein-Jensen medium and normal 7H-10 medium. The concentration of 0.125 \( \mu g \) of isoniazid is not detectable by diffusion on Löwenstein-Jensen slants, but shows an inhibition zone of 5 mm–10 mm in normal 7H-10 medium and of 20 mm on the 7H-10 ion-agar slant. Concentrations of 1 \( \mu g \) or more of isoniazid inhibit growth on the entire 7H-10 ion-agar slant. Hence, to determine concentrations greater than 1 \( \mu g \) of isoniazid, serial dilutions of the test fluid have to be made; the results obtained must then be multiplied by the appropriate dilution factor.

The diffusion of PAS, ethionamide, and ethambutol in 7H-10 ion-agar slants is shown in Fig. 2. The diffusion of PAS in a concentration of 0.5 \( \mu g \) resulted in a growth inhibition zone of 20 mm; a concentration of 2 \( \mu g \) resulted in an inhibition zone of 55 mm. The vertical diffusion method on 7H-10 ion-agar slants was less sensitive in determining concentrations of ethionamide and ethambutol. Concentrations of 3 \( \mu g \)–4 \( \mu g \) of ethionamide or 5 \( \mu g \)–6 \( \mu g \) of ethambutol were necessary to obtain a growth-inhibition zone of 10 mm. Drug concentrations of 16 \( \mu g \) of ethionamide and ethambutol inhibited the growth of the H37Rv with a zone of 40 mm–60 mm.

Vertical diffusion tests were done to compare the activity of the different drugs when dissolved in water and in 7H-10 medium without agar. Growth-inhibition curves for isoniazid, PAS, and ethambutol dissolved in 7H-10 medium (without agar) were identical to the standard curves of these drugs dissolved in water (Fig. 1 and 2). Growth-inhibition zones for ethionamide dissolved in 7H-10 medium (without agar) were 5 mm–8 mm smaller than those obtained by the same concentrations in water. A loss of 1 \( \mu g \) of ethionamide per concentration tested was observed for this drug when dissolved in 7H-10 medium (Fig. 3).
Using vertical diffusion methods, Schmiedel (1958) and Kleeberg (personal communication, 1965) have demonstrated that the size of the growth-inhibition zone is a function of the degree of resistance of the mycobacterial strain. The inverse relationship between isoniazid- or streptomycin-susceptibility of tubercle bacilli and the size of the growth-inhibition zones is shown in Tables 1 and 2. Strains of tubercle bacilli susceptible to 0.05 \( \mu \text{g/ml} \) to 5.0 \( \mu \text{g/ml} \) of isoniazid or to 1.0 \( \mu \text{g/ml} \) to 10.0 \( \mu \text{g/ml} \) of strepto-
mycin were tested in the vertical diffusion test against 2.5 μg/tube of isoniazid or 40.0 μg/tube of streptomycin.

Twenty-two strains of tubercle bacilli susceptible to 0.05 μg/ml to 0.1 μg/ml of isoniazid showed growth inhibition zones of 45 mm–69 mm; 5 strains susceptible to 1.0 μg/ml showed inhibition zones of 20 mm–49 mm; and 13 strains susceptible to 5.0 μg/ml showed growth-inhibition zones of 0–39 mm (Table 1).

Three strains of tubercle bacilli susceptible to 0.5 μg/ml of streptomycin showed more than 60 mm of inhibition when tested against 40 μg streptomycin in the vertical diffusion test; 26 strains susceptible to 1.0 μg/ml to 2.0 μg/ml of streptomycin showed inhibition zones of 35 mm–49 mm; and 11 strains highly resistant to streptomycin (5.0 μg/ml or more) were inhibited by the diffusing streptomycin in zones measuring only 15 mm–29 mm.

The growth-inhibition zones obtained from 40 strains of tubercle bacilli sensitive or resistant to different concentrations of PAS, ethionamide, kanamycin, viomycin, ethambutol, and cycloserine are given in Table 3.

DISCUSSION

Determination of drug levels in body fluids can be done by either chemical or biological tests (Lorian, 1966). The chemical methods so far described for this purpose have proved to be neither sensitive enough nor specific enough for the detection of low concentrations of such tuberculostatic drugs as isoniazid, PAS, ethionamide, and ethambutol. Another objection to chemical tests is that chemotherapeutically inactive breakdown products may give a false-positive indication of tuberculostatic drug level in serum (Björnesjö, 1961). However, chemical tests deliver results within hours, whereas biological tests using mycobacteria as test organisms can be read only after 10–14 days.

Drug-level tests performed in a biological system on solid medium, as in the vertical diffusion method, have an advantage because the fluid to be tested does not have to be diluted into a liquid medium. Furthermore, the test results can be expressed in μg/ml rather than in titres of the dilution made. By the vertical diffusion method every sterile fluid may be assayed for its antituberculosis activity without altering or diluting the test medium itself; in the liquid tube-dilution method the test organism grows under different conditions in each tube of the dilution series.

As in the method of Dye, Kass & Gill (1961), the vertical diffusion method can be used to test the tuberculostatic activity of the serum against the patient's own mycobacteria. When specific drug levels are desired, the interference of other drugs in the treatment regimen can be avoided by using drug-resistant strains of tubercle bacilli. This, however, necessitates the preparation, by selection, of a number of single and multiple drug-resistant strains of tubercle bacilli. The vertical diffusion test in 7H-10 ion-agar slants constitutes a simple, reliable assay of tuberculostatic drugs which can be employed routinely in laboratories once the standard curves for the different drugs have been accurately determined.

In laboratories with limited technical and financial facilities, the same medium may be used for drug-susceptibility tests. The information presented in Tables 1–3 indicates that the results of drug-susceptibility tests obtained in the vertical diffusion method cannot be expressed in absolute concentrations; i.e., strains of tubercle bacilli resistant to 0.1 μg/ml to 1.0 μg/ml of isoniazid sometimes show the same growth-inhibition zones in the vertical diffusion test (40 mm–49 mm). Using the vertical diffusion method, however, results of susceptibility tests should be recorded and reported only as "sensitive", "moderately resistant", and "totally resistant". The sizes of the inhibition zones, of course, vary for different drugs (Tables 1–3). The relationship between drug concentrations and size of inhibition zones may be influenced by technical factors such as inoculum size, inoculation technique, and length of slant of the medium. Therefore, every laboratory should test a number of mycobacterial strains with known drug susceptibility patterns before routinely using this test.

The vertical diffusion test on 7H-10 ion-agar slant is recommended as a reliable assay method for isoniazid, PAS, ethionamide, and ethambutol; as a drug-susceptibility test this method should be limited to laboratories where drug tests in normal 7H-10 agar plates or other acceptable media cannot be done satisfactorily owing to limited technical facilities.
RÉSUMÉ

Les auteurs décrivent une épreuve biologique simple pour déterminer la concentration des tuberculostatiques dans le sérum ou d'autres liquides organiques, le niveau de sensibilité à ces médicaments de souches mycobactériennes inconnues, et l'activité tuberculostatique du sérum d'un malade envers la souche mycobactérienne responsable de son affection.

On a utilisé pour cette épreuve le milieu de Löwenstein-Jensen, le milieu original 7 H-10 de Middlebrook et ce dernier milieu modifié et enrichi, en tubes inclinés. Pour déterminer la concentration des tuberculostatiques dans un liquide organique, on inocule les milieux au moyen de 0,1 ml d'une culture de Mycobacterium tuberculosis souche H37 RV. Le liquide à analyser (0,5 ml de dilutions en série) est introduit dans les tubes qui sont ensuite placés en position verticale à l'étuve à 37°C pendant 10 jours. Le degré d'inhibition de la croissance est mesuré par la distance en mm séparant le niveau du liquide à examiner et le point limite atteint par la culture. Les chiffres obtenus sont rapportés à des courbes de référence établies au préalable pour des concentrations variables de divers tuberculostatiques.

Pour la recherche de la sensibilité de souches mycobactériennes inconnues, on inocule les milieux au moyen de la culture à examiner, puis on introduit dans les tubes 0,5 ml d'une série de tuberculostatiques à des concentrations variables. La concentration inhibitrice minimale des médicaments a été déterminée sur 40 souches sensibles ou résistantes de Myco. tuberculosis.

Lorsqu'il s'agit d'évaluer la concentration d'un tuberculostatique dans un liquide organique, le milieu de Middlebrook modifié se montre supérieur. Sur ce milieu, une concentration de 0,5 µg d'isoniazide inhibe la croissance de Mycobacterium sur une distance de 50 mm, alors qu'avec les autres milieux, la zone d'inhibition n'atteint que 20-30 mm. Les résultats sont cependant moins favorables avec l'éthionamide et l'éthambutol.

Par la méthode décrite, les résultats ne peuvent être exprimés en concentrations absolues, mais doivent être enregistrés sous les mentions "souche sensible", "souche moyennement résistante" ou "souche complètement résistante". Son emploi sera donc réservé aux laboratoires qui ne disposent pas d'un équipement permettant d'effec- tuer des dosages plus précis.

REFERENCES

US Veterans Administration (1966) Handbook of tuberculosis laboratory methods, Washington, D.C., US Veterans Administration, Department of Medicine and Surgery