Analysis of Rifampin Disk Diffusion and Stability in 7H10 Agar

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Rifampin was incorporated into Middlebrook 7H10 medium either by adding an aliquot of the antibiotic into melted agar (final concentration 1.0 and 3.0 μg/ml) or by submerging a 5 or 15 μg of rifampin paper disk into 5 ml of melted agar contained in one quadrant of a Felson "X" plate. At intervals, plugs of agar were removed from the stored plates and assayed. Plates stored at 5 C for 28 days showed no loss of potency; at 37 C, the half-life of rifampin was 9 days. Stability of rifampin at these concentrations in 7H10 medium was independent of the method used for incorporation. Using the disk method, uniform rifampin concentrations of 0.75 μg/ml on day 5 for the 5-μg disk and 2.7 μg/ml on day 6 for the 15-μg disk were observed. Results indicated that the rifampin concentrations within the agar dilution and disk diffusion plates were equivalent at these times.

The agar dilution method routinely used for determining the susceptibility of Mycobacterium tuberculosis to rifampin is a multisteped, time-consuming procedure in which the probability of manipulative errors is increased. A simplified susceptibility test was described by Wayne and Krasnow (7), in which paper disks containing various drugs were submersed into 7H10 agar. Other investigators (2, 3) have reported good agreement in mycobacterium drug susceptibility profiles when comparing results obtained from the agar dilution to the disk diffusion method. It was noted by these workers that disk placement, temperature, and storage time had an effect on drug concentration in the agar medium although no attempt was made to determine these concentrations. A subsequent rifampin stability study (5) reported a reduction in the number of test strains inhibited as the 37 C incubation time increased.

In this investigation, we have used a modified cylinder-plate assay technique to study the stability of rifampin at 5 and 37 C in 7H10 agar. In addition, a study was made to quantify the diffusion pattern of this antibiotic in the same media when submersed paper disks were used as a means of antibiotic incorporation.

MATERIALS AND METHODS

A sample of rifampin powder was solubilized with a minimum quantity of absolute methanol, diluted with 1% phosphate buffer, pH 6.0, and sterilized by membrane (0.22 μm) filtration. The filtrate was assayed microbiologically to determine the potency. Appropriate amounts of this stock solution were diluted with melted (48 C) 7H10 agar base which contained 10% Middlebrook OADC Enrichment, to yield final concentrations of 1.0 and 3.0 μg of rifampin per ml. A 5-ml aliquot of each agar concentration was dispensed into quadrants of Felson "X" plates (Falcon Plastics No. 1009, Cockeysville, Md.) and allowed to solidify; the plates were sealed with Parafilm (Scientific Glass Apparatus, Bloomfield, N. J.). One set of plates (consisting of both rifampin concentrations) was stored at 5 C while a duplicate set was incubated at 37 C. At predetermined time intervals, plates were removed from their respective storage conditions and assayed for rifampin content.

Assay of rifampin in agar. The microbiological potency of the rifampin-agar mixtures was determined by the method of Ryan et al. (4), wherein agar plugs were removed from the plates with a 9-mm cork borer. The excised agar plugs were placed directly onto the seeded agar surface of an assay plate. The assay plates were incubated at 31 C for 16 to 18 h. The resulting zones of inhibition were measured and the rifampin concentrations were then calculated from a standard curve. The standard curve for this assay was prepared daily as follows: specific amounts of rifampin standard solution were added to melted complete 7H10 medium to obtain final concentrations of 0.5, 1.0, 2.0, 4.0, and 8.0 μg/ml. A 5-ml volume of each concentration was dispensed into quadrants of Felson "X" plates and allowed to solidify. Excised agar plugs of these standard curve concentrations were placed directly onto the seeded agar surface of an assay plate. Assay plates, using Bacillus subtilis as the test organism, were prepared in accordance with the CFR monograph (1). For samples in which the test concentrations fell below 0.5 μg/ml, a more sensitive assay method using plates with a 10-ml base layer and Sarcina lutea ATCC no. 9341 as the test organism was used. The standard curve concentrations were reduced to 0.03, 0.06, 0.12, 0.24, and 0.48 μg/ml. Calculations were based on the modified least square method.
Diffusion study: incorporation of rifampin-imregnated disks into agar. Each quadrant of a Felson "X" plate received 5 ml of complete 7H10 agar. Before solidification, antibiotic susceptibility disks containing either 5 or 15 μg of rifampin were submerged into the agar with a sterile needle and centrally located within the quadrant. Samples of these antibiotic disks, prepared by Baltimore Biological Laboratories (Cockeysville, Md.), were assayed and found to have values of 5.2 and 14.4 μg for the 5- and 15-μg concentrations. Duplicate sets of plates consisting of both 5- and 15-μg disks were placed into sealed plastic bags to prevent dehydration and stored at either 5 or 37 C. At predetermined time intervals, plates were removed from their respective storage conditions. Agar plugs from selected areas of the quadrant were removed and assayed for rifampin content. The diagram presented in Fig. 1 shows the location of the sampling sites and the distances from the disk to each site.

RESULTS

rifampin stability. When rifampin was incorporated into 7H10 agar using the agar dilution method and stored at 5 C, no loss of potency could be detected after 28 days of incubation. The graphs in Fig. 2a and b indicate that this was true for both the 1.0- and 3.0-μg concentrations. When similarly prepared plates were incubated at 37 C, rifampin degradation occurred from day 1. Applying the least square method to the regression lines (6), the half-life at 37 C of both the 1.0- and 3.0-μg rifampin concentrations were calculated to be 10 and 9 days, respectively.

rifampin diffusion patterns. When rifampin was incorporated into 7H10 agar by means of impregnated 5- or 15-μg paper disks, the antibiotic was detected at sampling sites A and B after 24 h of storage at 5 C. Sampling sites C and D demonstrated the presence of rifampin after 48 h of storage (Fig. 3a, b). The theoretical, uniform rifampin concentration for a 5- or 15-μg disk deposited in 5 ml of 7H10 agar was 1 μg/ml and 3 μg/ml, respectively. These levels were approached on day 9 for the 5-μg disk and day 12 for the 15-μg disk. After 20 days, the average rifampin level at all four sampling sites within the quadrant was 1.4 μg/ml for the 5-μg disk and 3.4 μg/ml for the 15-μg disk. As with the agar dilution method, no loss of antibiotic potency was observed at 5 C when the disks were the source of rifampin.

In contrast to the slow diffusion-stable potency characteristic of the 5 C plates, the 37 C plates contained detectable levels of rifampin at all sample sites within 24 h (Figs. 4 and 5). Theoretical rifampin levels of 1.0 μg/ml of agar for the 5-μg disk and 3.0 μg/ml of agar for the 15-μg disk were not achieved because antibiotic degradation was concurrent with diffusion. The highest average agar concentration for the 5-μg disk was 0.75 μg/ml on day 5; the highest average agar concentration for the 15-μg disk was 2.7 μg/ml on day 6. Least square method calculation of these regression lines indicated a t1/2 of 9 days for both the 5- and 15-μg disks.

DISCUSSION

Previous reports have expressed the in vitro stability of rifampin as a function of the number of bacterial strains inhibited versus storage time at a given temperature. In this communication the stability of rifampin in 7H10 agar is reported as micrograms of microbiologically active rifampin remaining at different time and temperature conditions.

Plates prepared by either the agar dilution or disk diffusion method can be stored up to 28 days at 5 C with no loss in potency. This allows a prediffusion period for disk diffusion plates which eliminates zoning. Incubation at 37 C causes rifampin to degrade at the rate t1/2 = 9 days; the half-life value was the same for both the agar dilution and disk diffusion plates. In the agar plate susceptibility test, the inhibition of microbial growth is dependent upon a minimal inhibitory concentration of antibiotic being in contact with a critical population of bacteria which are in a susceptible phase of growth.

Fig. 1. Diagram of a Felson "X" plate. The letters A, B, C, and D represent the sites within each quadrant that were monitored for rifampin concentrations. A 9-mm agar plug was removed at each site and assayed as described. Symbols: ○ indicates the location of the rifampin disk; numbers represent distance of sampling site from disk in millimeters.
Fig. 2. (a, b) Stability of rifampin (1.0 μg/ml and 3.0 μg/ml) in 7H10 agar when incubated at 5 and 37°C.
FIG. 3. (a, b) Diffusion pattern of rifampin when 5- and 15-µg rifampin disks were placed in 7H10 agar and incubated at 5 C. The letters A, B, C, and D represent sampling sites (see Fig. 1).
FIG. 4. Diffusion pattern of rifampin when 5-μg rifampin disks were placed in 7H10 agar and incubated at 37 C. The letters A, B, C, and D represent sampling sites (see Fig. 1).
Quantitative analysis for rifampin content of the agar at various distances from either the 5- or 15-µg disk showed the presence of the drug in the extreme sections of the quadrant within 24 h and uniform drug distribution within 6 days when exposed to 37°C; this is well within the usual 14- to 21-day mycobacterium test period.

The data presented here assures that equivalent antibiotic concentrations exist within the quadrant plates when they are prepared by either the agar dilution or disk method for rifampin incorporation into 7H10 medium. Convenience of preparation would make the disk method of mycobacterium susceptibility...
testing preferential especially in laboratories where small numbers of strains are tested at infrequent intervals.

LITERATURE CITED


