Instability Effects in Diffusion Analyses of Antimicrobial Activity

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Diameter reductions accompanying preincubation, and funnel-shaped diffusion zones from agar wells, readily demonstrate chemical and physical instability of preparations in antimicrobial analyses.

Methods for determining chemotherapeutic and antibiotic activity began with the agar well assay in the early 1940's (4, 5, 7) and later included cylinder plate (1, 6) and disc techniques (3). With the exception of general discussions, such as those by Cooper (2) or Schmidt and Moyer (8), few authors have remarked on specific effects of manner of application or preincubation on inhibition zones in these studies, and variation reflecting preparation instability may be an important consideration in all diffusion analyses. While developing sensitive microbiologic assays for buffer- and milk-diluted nitrofurans, several formulas were found to produce these variations, and two of the more active compounds (A and B, differing in hydroxyl group positions on a benzene ring) are the subject of this report.

Standard agar well methods (e.g., a uniformly seeded, 40 ml or 7.5 mm deep layer) or cylinder-plate techniques of the type described by Kirshbaum et al. (6), with 6-ml base and 4-ml seed layers in each plate, were employed for the analyses. Stock solutions prepared in dimethyl formamide were diluted further in milk or phosphate buffer (pH 8.0) to give final concentrations of 0.003 to 1.6 μg/ml for standard line determinations. All tests were performed in a Trypticase Soy Agar (BBL) medium against a 1.25 × 10⁻⁸ cfu/ml concentration of Difco Bacillus subtilis spores (ATCC no. 6633) by using two or three plates for each sample assay. Each plate contained three samples and three reference reservoirs (alternating), and zones were read after 18 to 24 hr incubation at 28 C.

The possibility of preparation instability was indicated by reduction in reference concentration zone diameters on replicate plates when cylinder filling time was extensive. To determine the source of the reduction, assays were performed on samples prepared over five time intervals by using (i) standard concentrations (0.2 and 1.0 μg/ml), pre-

incubated (at a room temperature of 22 to 25 C or under refrigeration at 4 C) on freshly seeded plates, and (ii) freshly prepared standard concentrations placed in cylinder reservoirs on similarly

| Table 1. Average inhibition zone diameters of standard, stored, and room temperature samples from compound A |
|---------------------------------------------------------------|
| Sample                          | Time | Assay concentration | Average zone diameter |
|---------------------------------|------|---------------------|-----------------------|
|                                 | hr   | μg/ml               | mm                    |
| Standard line concentrations a  |      |                     |                       |
|                                 | 0.25 | 0.025               | 9.4                   |
|                                 | 0.05 | 0.05                | 11.6                  |
|                                 | 0.10 | 0.10                | 14.2                  |
|                                 | 0.20 | 0.20                | 16.9                  |
|                                 | 0.40 | 0.40                | 20.6                  |
|                                 | 0.80 | 0.80                | 22.0                  |
|                                 | 1.60 | 1.60                | 23.6                  |
| Stored standards (fresh seed assays) b | 1.50 | 1.0                 | 21.8                  |
|                                 | 0.2  | 0.2                 | 16.4                  |
|                                 | 2.25 | 1.0                 | 21.3                  |
|                                 | 0.2  | 0.2                 | 16.2                  |
|                                 | 2.75 | 1.0                 | 21.1                  |
|                                 | 0.2  | 0.2                 | 15.6                  |
|                                 | 3.75 | 1.0                 | 20.3                  |
|                                 | 0.2  | 0.2                 | 15.2                  |
|                                 | 4.75 | 1.0                 | 20.0                  |
|                                 | 0.2  | 0.2                 | 15.2                  |
| Fresh standards (stored seed assays) b | 2.00 | 1.0                 | 19.8                  |
|                                 | 0.2  | 0.2                 | 15.1                  |
|                                 | 2.75 | 1.0                 | 19.2                  |
|                                 | 0.2  | 0.2                 | 15.1                  |
|                                 | 3.75 | 1.0                 | 17.6                  |
|                                 | 0.2  | 0.2                 | 13.8                  |
|                                 | 4.25 | 1.0                 | 14.2                  |
|                                 | 0.2  | 0.2                 | 11.3                  |
|                                 | 5.25 | 1.0                 | 13.4                  |
|                                 | 0.2  | 0.2                 | 8.8                   |

* Nine determinations per sample.

b Six determinations per sample.

a Reference concentration.

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TABLE 2. Average inhibition zone diameters of standard, stored, and room temperature samples from compound B

| Sample                        | Assay concentration | Average zone diameter |
|-------------------------------|---------------------|-----------------------|
| Standard line concentrations* | µg/ml                | mm                    |
|                               | 0.025               | 0                     |
|                               | 0.05                | 0                     |
|                               | 0.10                | 11.3                  |
|                               | 0.20*               | 14.0                  |
|                               | 0.40                | 15.9                  |
|                               | 0.80                | 18.2                  |
|                               | 1.60                | 20.1                  |
| Stored standards (fresh seed assays)* | 0.50 | 1.0 | 18.9 |
|                               | 1.00                | 1.0 | 19.0 |
|                               | 1.50                | 0.2 | 13.8 |
|                               | 2.25                | 0.2 | 14.0 |
|                               | 2.75                | 0.2 | 12.5 |
| Fresh standards (stored seed assays)* | 1.00 | 1.0 | 18.2 |
|                               | 1.50                | 0.2 | 16.8 |
|                               | 2.00                | 0.2 | 12.6 |
|                               | 2.75                | 0.2 | 13.5 |
|                               | 3.25                | 0.2 | 13.2 |

* Nine determinations per sample.

† Six determinations per sample.

‡ Reference concentration.

preincubated seeded plates. Further experiments demonstrating instability of formulations were performed initially to compare inhibition zone diameters developed from compound preparations as a function of reservoir application method. Test compounds were diluted in buffer or milk (with or without a 3% peanut oil-aluminum monostearate vehicle), and 25 µg/ml concentrations of 2,3,5-triphenyltetrazolium chloride were included in the media to enhance zone clarity.

Typical zone diameter readings and standard lines from preincubated inocula and milk-diluted compound preparations are shown in Tables 1 and 2, and in Fig. 1. Sensitivities of 0.015 to 0.035 µg/ml were routinely obtained in this medium, and these were increased 80 to 90% in the buffer diluent with little or no change in linearity or slope of the standard lines. Figures 2 and 3 demonstrate the reductions in zone diameter found with 1.0 and 0.2 µg/ml concentrations of compound A and compound B, also in milk, with solution or

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**Fig. 1.** Standard line response of compound concentrations in milk by using cylinder plate assay method (T for storage study). Reference concentration was 0.2 µg/ml.

**Fig. 2.** Zone diameter reduction with time by using freshly seeded assay media and compound concentrations prepared in milk stored at room temperature.
TABLE 3. Average inhibition zone diameters from 1 μg/ml compound concentrations by reservoir application method

| Reservoir        | Compound A          | Compound B          |
|------------------|---------------------|---------------------|
|                  | Buffer   | Milk    | Gel-milk | Buffer   | Milk    | Gel-milk |
| Cylinder         | 22.8     | 15.9    | 7.9      | 21.8     | 15.5    | 7.5      |
| Wellb            | 20.4     | 15.2    | 9.8, 8.5c| 19.3     | 14.6    | 10.6, 8.1c|
| Modified welld   | 22.8     | 18.0    | 11.1     | 23.4     | 18.3    | 12.7     |

*Expressed as millimeters.
*b Single seeded layer.
*c Top surface, bottom surface.
*d Seeded on unseeded layer.

![Graph](image)

**FIG. 3. Zone diameter reduction with time by using freshly prepared compound concentrations (in milk) and seeded assay media stored at room temperature.**

seeded plate preincubation. Zone reduction, although more pronounced with seeded plate storage (reflecting organism growth), resulted similarly with preincubation of both compounds at each test concentration, particularly beyond a 1- to 1.5-hr preparation time. Subsequent studies with refrigerated preparations in phosphate buffer gave comparable results, the delay in zone reductions being extended by storage at lower temperatures.

The average zone diameters obtained from 1 μg/ml concentrations of each compound are presented for comparison in Table 3. With reference to zones obtained in buffer preparations, the results indicate that diameters are reduced somewhat in corresponding milk dilutions, and to a greater degree where the gel vehicle was present. The reductions were detected with both methods and occurred similarly with each compound. When gel-milk preparations were assayed by the single-layer well method, funnel-shaped zones were made readily apparent with the 2,3,5-triphenyltetrazolium indicator, and diameters were recorded for both agar surfaces (Fig. 4). Relatively large zones were obtained from the milk and gel-milk samples with a modified well method by using unseeded and seeded agar media layers of 36 ml and 4 ml, respectively, as compared with the cylinder application. These findings indicate that concentrations of active material were greater in the upper part of the reservoirs. Presumably, inverted cone-shaped zones would be obtained similarly with the method if precipitation or settling were to concentrate activity at the well base.

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