Stability of Antibiotics and Chemotherapeutics in Agar Plates

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The stability of chemotherapeutic agents incorporated into agar plates was studied by comparison of minimum inhibitory concentrations on fresh and stored plates and by direct bioassay of the chemotherapeutic agar plates. Plates were stored in sealed bags at 4 C. No loss of bioactivity was demonstrated after 30 days of storage in plates containing methicillin, erythromycin, cephalothin, tetracycline, chloramphenicol, kanamycin, streptomycin, polymyxin B, or nalidixic acid. Penicillin G, ampicillin, and nitrofurantoin showed statistically significant losses of activity after 4 weeks. None of the chemotherapeutics tested showed significant loss in activity after 1 week.

The stability of many antibiotics and chemotherapeutics in the dry state and in aqueous solution has been established (6). Less is known about their stability in nutrient agar. This is important in the use of the agar dilution susceptibility test method, because its practicability as a routine method depends partly on how long plates containing clinically relevant concentrations of chemotherapeutics can be stored without deterioration. (The term "chemotherapeutics" is used throughout to include antibiotics.) The two studies reported here were designed to provide this information. The first involved agar dilution susceptibility tests of 29 bacterial strains by using both freshly prepared and stored plates to determine any changes of inhibitory end points. The second used a more sensitive direct agar diffusion bioassay technique to determine the amounts of active chemotherapeutic in agar plates after various periods of storage. These studies were done as part of an international collaborative study on antibiotic susceptibility testing.

MATERIALS AND METHODS

Chemotherapeutics. The 13 agents listed in Tables 1 and 2 were tested. Standard powders of known activity were obtained from the manufacturers. Stock solutions containing 1,280 µg/ml or units/ml (polymyxin) of the chemotherapeutic were prepared by solubilizing them in sterile water or saline. Erythromycin and chloramphenicol were first dissolved in a minimum amount of absolute ethyl alcohol, and nalidixic acid was similarly solubilized with a minimum amount of 0.1 N NaOH. The chemotherapeutic stock solutions were stored at —20 C or below in small portions. They were discarded after use and never refrozen.

Media. Nutrient agar (Difco) and Trypticase Soy Agar (BBL) were used for the first part of the study, and Mueller-Hinton agar (Difco) was used for the second part. They were prepared from the dehydrated media as directed by the manufacturer.

Organisms. The 29 organisms used in the first part of the study were recent isolates from the clinical microbiology laboratories at the Mayo Clinic. They comprised six isolates each of Staphylococcus aureus, Streptococcus faecalis, Escherichia coli, and Enterobacter sp. and five isolates of Pseudomonas aeruginosa. They were purified by two successive single-colony isolations and stored at room temperature on nutrient agar slants under sterile mineral oil.

The assay strains used in the second part of the study were Sarcina lutea ATCC 9341 (ampicillin, methicillin, penicillin G, erythromycin, chloramphenicol, and tetracycline), Bacillus subtilis (kanamycin), S. aureus (cephalothin and streptomycin), Bordetella bronchiseptica ATCC 4617 (polymyxin B), and Shigella C (nalidixic acid and nitrofurantoin). The S. aureus and Shigella C strains were isolated in the clinical microbiology laboratories of the University of Washington Hospital. They were known to be consistently susceptible to the agents assayed through previous testing. The B. subtilis strain was the same one used by Bennett et al. (1).

Preparation of chemotherapeutic-containing plates. For the first part of the study, each chemotherapeutic was added to 100-ml volumes of melted agar at 48 C to give twofold dilutions of chemotherapeutics over a clinically significant range. Three sets of plates were poured for each agent. One was used immediately and
the other two sets were stored in sealed plastic bags for 30 and 60 days, respectively.

For the second part of the study, chemotherapeutics from the frozen stock solutions were incorporated in Mueller-Hinton agar plates to give the concentration shown in Tables 2 and 3. Sets of plates were prepared at various intervals of as long as 4 weeks before the assays were to be performed and again sealed in Mylar bags and held at 4°C. One set was prepared immediately before each assay as a standard.

**Agar dilution susceptibility tests.** For the first part of the study, the test organisms were transferred to tubes of nutrient broth 24 hr before the tests were to be performed. After overnight incubation, one loopful of growth was transferred to another tube of broth and incubated for 6 hr. The resulting growth served as the inoculum and was applied to the surface of the agar plates with a Steers imprinter (5). This apparatus permitted all 29 strains to be tested simultaneously. The plates were incubated for 18 hr at 37°C. The lowest concentration of chemotherapeutic inhibiting growth completely as judged by the naked eye was taken as the minimum inhibitory concentration (MIC) of the chemotherapeutic for the organisms. Simultaneous tests were always done on freshly prepared plates when stored plates were being tested.

**Assay of antibiotic in agar.** Large assay plates (9 by 9 inches (22.9 by 22.9 cm)) were prepared containing Trypticase Soy Agar (BBL) with 1% yeast extract (Difco) seeded with the appropriate assay strain for the agent under investigation. The method followed that previously described by Dunsmoor, Pim, and Sherris (2). A 75-ml amount of agar was used for the cephalothin, chloramphenicol, streptomycin, and tetracycline assays, and 150 ml was used for the other agents. Preliminary experiments had shown these to be the optimal conditions with the chemotherapeutic concentrations and assay strains used. To assay the residual antibiotic in the stored plates, 9-mm plugs of agar were taken from their centers by using a cork borer. The plugs were then carefully placed on the surface of the assay plate. Plugs from each freshly prepared standard plate were similarly treated. In the case of cephalothin, chloramphenicol, tetracycline, kanamycin, nalidixic acid, and nitrofurantoin, the sensitivity of the assay was slightly improved by placing two plugs one on top of the other, and this procedure was used for the unknowns and standards with these agents only. After placing all of the plugs, the assay plates were covered with a glass plate and incubated for 18 hr at 37°C. Zones of inhibition around the plugs were measured with calipers and recorded. Duplicate plugs from the test plates and quadruplicate plugs from the standards were used for the experiment summarized in Table 2. Six replicates were used in all cases for that shown in Table 3. The assay values were calculated from the standard curve by the methods of Bennett et al. (1).

**RESULTS**

The results of the comparative agar dilution susceptibility tests performed at the Mayo Clinic are shown in Table 1. The denominators indicate the number of strains tested with each chemotherapeutic, and the numerators indicate the number of strains which required higher concentrations of chemotherapeutic for inhibition on the stored plates than on the control plates. This was interpreted as evidence of drug deterioration.

With plates stored for 30 days, 2 of 167 MIC determinations showed evidence of loss of chemotherapeutic activity. In both instances, the changes were with penicillin G plates used to test the sensitivity of *S. aureus* and involved one dilution change in MIC. The plates stored for 60 days showed similar changes in 5 of 167 MIC.

| Chemical therapeutic | Thirty-day-old media | Sixty-day-old media |
|----------------------|----------------------|----------------------|
|                      | Staphylococcus aureus | Streptococcus faecalis | Escherichia coli | Enterobacter aerogenes | Pseudomonas aeruginosa | S. aureus | S. faecalis | E. coli | E. aerogenes | P. aeruginosa |
| Penicillin G         | 2/6                  | 0/6                  | 0/6               | 0/6                  | 0/6                  | 2/6       | 0/6       | 0/6       | 0/6       | 0/6       |
| Erythromycin        | 0/6                  | 0/6                  | 0/6               | 0/6                  | 0/6                  | 0/6       | 0/6       | 0/6       | 0/6       | 0/6       |
| Methicillin         | 0/6                  | 0/6                  | 0/6               | 0/6                  | 0/6                  | 1/6       | 0/6       | 0/6       | 0/6       | 0/6       |
| Tetracycline        | 0/6                  | 0/6                  | 0/6               | 0/6                  | 0/6                  | 0/6       | 0/6       | 0/6       | 0/6       | 0/6       |
| Streptomycin        | 0/6                  | 0/6                  | 0/6               | 0/6                  | 0/6                  | 0/6       | 0/6       | 0/6       | 0/6       | 0/6       |
| Amoxicillin         | 0/6                  | 0/6                  | 0/6               | 0/6                  | 0/6                  | 1/6       | 1/6       | 0/6       | 0/6       | 0/6       |
| Bacitracin           | 0/6                  | 0/6                  | 0/6               | 0/6                  | 0/6                  | 0/6       | 0/6       | 0/6       | 0/6       | 0/6       |
| Chloramphenicol     | 0/6                  | 0/6                  | 0/6               | 0/6                  | 0/6                  | 0/6       | 0/6       | 0/6       | 0/6       | 0/6       |
| Kanamycin           | 0/6                  | 0/6                  | 0/6               | 0/6                  | 0/6                  | 0/6       | 0/6       | 0/6       | 0/6       | 0/6       |
| Polymyxin B         | 0/6                  | 0/6                  | 0/6               | 0/6                  | 0/5                  | 0/6       | 0/6       | 0/6       | 0/6       | 0/6       |
| Nalidixic acid      | 0/6                  | 0/6                  | 0/6               | 0/6                  | 0/6                  | 0/6       | 0/6       | 0/6       | 0/6       | 0/6       |

* Numerators indicate the number of strains which required higher concentrations of chemotherapeutic for inhibition on the stored plates than on the control plates. Denominators indicate the number of strains tested with each chemotherapeutic.
TABLE 2. Agar bioassay of fresh and stored chemotherapeutic agar plates

| Chemotherapeutic | Conc (μg/ml) | Standard (mean zone size) | 1 week at 4 °C | 2 weeks at 4 °C | 4 weeks at 4 °C |
|------------------|-------------|--------------------------|--------------|--------------|--------------|
|                  | Mean zone size | Assay concn (μg/ml) | Mean zone size | Assay concn (μg/ml) | Mean zone size | Assay concn (μg/ml) |
| Penicillin G     |             |                         |              |              |              |
| 0.125            | 18          | 0.13                    | 16.5          | 0.11         | 16.0          | 0.10         |
| 0.50             | 27          | 0.46                    | 25.0          | 0.34         | 26.5          | 0.42         |
| Ampicillin       |             |                         |              |              |              |
| 0.03             | 15.8        | 0.027                   | 14.0          | 0.022        | 13.5          | 0.020        |
| 0.125            | 23.8        | 0.09                    | 24.5          | 0.105        | 22.0          | 0.068        |
| Methicillin      |             |                         |              |              |              |
| 0.5              | 14.5        | 0.5                     | 13.5          | 0.42         | 13.0          | 0.41         |
| 2.0              | 22.3        | 2.1                     | 22.0          | 2.0          | 22.0          | 2.0          |
| Cephalothin      |             |                         |              |              |              |
| 0.125            | 10          | 0.11                    | 10.5          | 0.12         | 11.3          | 0.13         |
| 0.5              | 20          | 0.42                    | 21.3          | 0.54         | 20.5          | 0.46         |
| 2.0              | 28          | 1.8                     | 28.5          | 2.0          | 27.8          | 1.8          |
| Chloramphenicol  |             |                         |              |              |              |
| 2.0              | 12.8        | 1.7                     | 13.3          | 1.9          | 13.5          | 1.9          |
| 8.0              | 19.8        | 5.6                     | 21.8          | 8.0          | 19.8          | 5.6          |
| 32.0             | 28.3        | 39.0                    | 28.0          | 36.0         | 26.3          | 26.0         |
| Tetracycline     |             |                         |              |              |              |
| 0.5              | 11.5        | 0.45                    | 12.0          | 0.5          | 11.8          | 0.48         |
| 2.0              | 18.8        | 2.5                     | 18.5          | 2.4          | 18.0          | 2.1          |
| 8.0              | 24.5        | 9.9                     | 24.0          | 9.0          | 22.3          | 6.0          |
| Erythromycin     |             |                         |              |              |              |
| 0.125            | 14.5        | 0.10                    | 15.0          | 0.11         | 15.0          | 0.11         |
| 0.5              | 22          | 0.50                    | 22.5          | 0.56         | 23.0          | 0.64         |
| 2.0              | 28          | 2.0                     | 28.5          | 2.4          | 27.9          | 2.0          |
| Nitrofurantoin   |             |                         |              |              |              |
| 32               | 12          | 24                      | 12            | 32           | 12.5          | 38           |
| 128              | 18.5        | 124                     | 18.8          | 132          | 19            | 134          |
| Nalidixic acid   |             |                         |              |              |              |
| 8.0              | 10          | 8.0                     | 10.5          | 9.2          | 11            | 10           |
| 32.0             | 15.8        | 30                      | 16.8          | 50           | 16.3          | 44           |
| Kanamycin        |             |                         |              |              |              |
| 2.0              | 11.5        | 1.6                     | 11            | 1.6          | 11            | 1.6          |
| 8.0              | 14.5        | 4.0                     | 14.5          | 8.0          | 13.5          | 5.0          |
| 32.0             | 17.5        | 26.0                    | 17            | 26.0         | 17            | 26.0         |
| Polymyxin B (units/ml) | 2.0 | 10          | 2.0          | 10           | 2.0          | 9.5          | 1.7          |
|                 |             |                         |              |              |              |
|                 | 8.0         | 11.8                    | 11.3          | 4.6          | 12.0          | 8.0          |
|                 | 32.0        | 14.5                    | 14.5          | 32.0         | 14.0          | 25.0         |
| Streptomycin     |             |                         |              |              |              |
| 8.0              | 9.0         | 8.0                     | 9.0           | 8.0          | 9.0           | 8.0          |
| 32.0             | 12.0        | 32.0                    | 12.0          | 32.0         | 12.0          | 32.0         |

* All test assays done in duplicate and standard plate assays in quadruplicate.

b Inhibition zone diameter measured in millimeters.

determinations, all among the penicillin group of antibiotics. In addition to the two changes with penicillin G seen at 30 days, there was evidence of methicillin deterioration in one test and ampicillin deterioration in two tests. None of the other comparative MIC determinations showed any evidence of loss of chemotherapeutic activity.

The results of the first direct bioassays performed at the University of Washington are shown in Table 2. This gives the means of the diameters of zones of inhibition around plugs removed from the standard plates and from test plates stored for 1, 2, and 4 weeks. Assay values for the test plates are also shown. With the exception of ampicillin, there was little or no difference between the standard (unstored) plates and those stored for as long as 4 weeks. Assays made from ampicillin plates showed zones of inhibition more than 3 mm smaller than the
calculated for either the significant assays. Kanamycin, ampicillin, and tetracycline showed losses of 23%, 10%, and 17% respectively.

| Chemotherapeutic | Concentration (µg/ml) | Statistically significant reduction in zone size compared with standards | Percent loss at 4 weeks |
|------------------|-----------------------|-------------------------------------------------|------------------------|
| Penicillin G     | 0.125                 | NS                  | P < 0.01               | 23                     |
| Ampicillin       | 0.03                  | NS                  | P < 0.01               | 10                     |
| Methicillin      | 2                     | NS                  | NS                     |                        |
| Cephalothin      | 0.5                   | NS                  | NS                     |                        |
| Chloramphenicol  | 8                     | NS                  | NS                     |                        |
| Tetracycline     | 2                     | NS                  | NS                     |                        |
| Erythromycin     | 0.5                   | NS                  | NS                     |                        |
| Nitrofurantoin   | 128                   | NS                  | P < 0.01               | 17                     |
| Kanamycin        | 32                    | NS                  | NS                     |                        |
| Polymyxin B      | (units/ml)            | NS                  | NS                     |                        |
| Streptomycin     | 32                    | NS                  | NS                     |                        |

* Analysis of variance on six parallel assays at 0 time, 1 week, and 4 weeks.

<sup>a</sup> From calculated assay curves (only for agents with significant loss).

<sup>b</sup> No significant difference at 5 or 1% level.

<sup>c</sup> Significant reduction at confidence limit given.

<sup>d</sup> Not done.

diffused poorly, producing a very flat concentration-zone size curve; thus very small changes in zone size (0.5 to 1.5 mm) gave apparently large differences in assay values. Because for any one concentration only duplicate assays were made, meaningful statistical analysis of these data was not possible. The data were internally consistent, however, with duplicate zone measurements never differing by more than 1 mm.

To facilitate statistical analysis, a second set of assays was made by using six replicates at a single concentration for each chemotherapeutic. The plates were stored for 1 and 4 weeks. The results are shown in Table 3. An analysis of variance and studentized range test (3) were performed to compare the zone size measurements from unstored plates (0 time) with those stored for 1 and 4 weeks. The only significant differences were with the 4-week-old penicillin G, ampicillin, and nitrofurantoin plates. These showed differences significant at the 1% level. None of the plates stored for 1 week showed significant differences from the standards at either the 1 or 5% levels. Standard curves were calculated for the antibiotics showing statistically significant deterioration. These indicated a loss of bioactivity after 4 weeks of storage of 23% for penicillin G, 10% for ampicillin, and 17% for nitrofurantoin.

### DISCUSSION

The two methods presented in this paper both show that the chemotherapeutics studied are remarkably stable when incorporated in nutrient agar media. Comparative agar dilution tests showed some evidence of deterioration with the penicillins after 30 or 60 days of storage but no deterioration with the other chemotherapeutics. Because a twofold dilution series was used to determine the MIC values, it is possible that up to 50% deterioration could occur without changing the end point. For example, a strain susceptible to 11 µg/ml of kanamycin when tested in a dilution series including 5, 10, and 20 µg/ml plates would have an MIC reading of 20 µg/ml as evidenced by growth at 10 but not 20 µg/ml. The kanamycin concentration would have to fall below 11 µg/ml in the 20 µg/ml plate (almost 50%) before the organisms could grow and thus give a different end point reading. For this reason, the more sensitive agar bioassay method was used to determine whether smaller losses were escaping detection. The assays confirmed the general stability demonstrated in the comparative MIC studies but again showed evidence of deterioration with the penicillins on prolonged storage. These agents are known to be unstable under other conditions (4, 6). The statistical analysis of repeat bioassays confirmed significant deterioration of ampicillin and penicillin G after 4 weeks of storage but not deterioration of methicillin. In addition, nitrofurantoin activity was significantly less after 4 weeks of storage. Loss of activity in each of these cases was less than 25%. None of the chemotherapeutics tested showed significant loss of bioactivity after 1 week of storage.

For practical purposes, it appears safe, with any of the chemotherapeutics tested here, to prepare chemotherapeutic-agar plates on a weekly schedule, storing them in sealed bags at 4°C until used. It also appears possible to hold some antibiotics in this form for longer periods if small losses of activity can be accepted and if other changes which may take place in the medium are acceptable.

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