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Rapid Quantitative Microbiological Assay of Antibiotics and Chemical Preservatives of a Nonantibiotic Nature

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A rapid quantitative assay of 14 antibiotics and 6 chemical preservatives by the cylinder-plate diffusion method is described, in which *Bacillus stearothermophilus* ATCC 12980 was used as the test organism. After 4 hr of incubation at 65 C, the zones of inhibition can be read off easily with the naked eye. The 4-hr assay was compared under identical conditions with the conventional 16- to 18-hr agar diffusion assay, i.e., by using the test organisms and media proposed by the *Code of Federal Regulations*, and no difference in accuracy between the two methods was detected. In both cases, the coefficient of variation for replicate tests was less than 2%. The test strain is highly sensitive to penicillins, cephalosporins, and bacitracin and is consequently also particularly suitable for the determination of low-level concentrations of these substances in aqueous solutions and in urine.

The many variants of the diffusion test (5, 6, 12) differ considerably from one another in their accuracy and in the amount of time and effort they involve, depending upon whether the test solution is placed in porcelain cylinders or wells punched in the agar or on paper discs, and also upon whether the results are determined by the standard-curve technique, the parallel-lines technique, or simultaneous comparison of two, three, or more concentrations each of the standard and the test samples (4). One characteristic feature common to all these tests, however, is that they are performed with sensitive but relatively slow-growing organisms, requiring incubation for 16 to 18 hr at 30 to 37 C (2, 8).

Quick tests carried out according to the microcolony method (1, 11) of determining the sensitivity of clinical strains are unsuitable for quantitative assays because, although zones of inhibition are discernible after 4 hr of incubation, they can only be measured under a binocular microscope at a magnification of 100. Other quick tests, in which the inoculated medium is preincubated before the impregnated discs are applied and the inhibition zones can be measured directly, have been proposed for the qualitative determination of antibiotic residues in meat and meat products (10) and in milk (9).

This communication describes a quantitative plate diffusion method that makes it possible to assess the potency of 14 clinically active antibiotics under standardized experimental conditions after incubation for 4 hr at 65 C, the inhibition zones being measured by the usual laboratory techniques, e.g., with an optical projector.

The method can also be used for determining the antimicrobial concentration of chemical preservatives in aqueous pharmaceutical formulations because, like antibiotics, chemical preservatives also meet the theoretical requirements with regard to dose-response relationship. The method is, however, of practical value only in the case of preservatives that are employed in concentrations not lower than the range of concentrations of the standard curve.

The test is carried out with the same strain, *Bacillus stearothermophilus* ATCC 12980, for all substances.

The limits of error of the 4-hr and the conventional 16- to 18-hr diffusion test assays were calculated on the basis of 20 parallel determinations with 10 antibiotics.

MATERIALS AND METHODS

Test strain and inoculum. The endospore suspension of the test strain *B. stearothermophilus* ATCC 12980 was prepared according to the sporulation method of Kim and Naylor (7) and adjusted with physiological saline to a concentration of 5 to 6 $\times$ 10^8 spores per ml. Phenol red agar (100 ml) was inoculated with 1 ml of this suspension, giving a final concentration of 5–6 $\times$ 10^6 spores per ml of the medium to be used as seed layer. The endospore suspension may be stored refrigerated for 3 weeks.

Nutrient medium. All 20 substances were tested on phenol red dextrose agar of the following composition: 1% Trypticase, 1% dextrose, 0.5% NaCl, 0.0018% phenol red, 1.5% agar (*p*H 7.3).

Buffer solutions. Three different buffers were used: A, phosphate buffer at *p*H 6.0, containing 2 g of K_2HPO_4 and 8 g of KH_2PO_4 in a 1,000-ml volume of distilled water; B, phosphate buffer at *p*H 7.0, containing 13.6 g of K_2HPO_4 and 4.0 g of KH_2PO_4 in a 1,000-ml volume of distilled water; and C, phosphate buffer at *p*H 7.9, containing 16.73 g of K_2HPO_4 and 0.523 g of KH_2PO_4 in a 1,000-ml volume of distilled water.

Antibiotics and preservatives. All of the active substances (Table 1) were obtained in powder form from commercial sources, and only antibiotics of known activity were used. If necessary, the antibiotics were predried according to the recommendations of the *Code of Federal Regulations* (3). They were then diluted with sterile distilled water or phosphate buffer (Table 1) to an initial concentration of 100 or 1,000 μg or units/ml. From this starting solution, the final standard-curve concentrations (Table 1) were prepared in sterile volumetric flasks.

Preparation of the plates. Petri dishes (22 by 97 mm) with flat bottoms and well-fitting porcelain covers (to prevent interference due to condensation) were used. Spore-free phenol red dextrose agar (15 ml) was first pipetted into each dish. After this layer had solidified, 6 ml of agar inoculated with the endospore suspension was added and evenly dispersed. After solidification of the seed layer, four porcelain cylinders, 10 mm in height with an outer diameter of 8 mm and an inner diameter of 6 mm, were placed at regular intervals on each plate.

Standard curve. The linear concentration ranges for all 20 active substances were determined with the test strain, *B. stearothermophilus* ATCC 12980, according to the standard-curve procedure, which is described in and recommended by authoritative textbooks and publications (2, 6, 8, 12). The linearity of the standard curve in the concentration ranges shown in Table 1 was tested statistically at the 95% probability level after three replications (13).

Assay procedure. Four plates each were used for the 1st, 2nd, 4th, and 5th point of the standard response line, i.e., a total of 16 plates with 4 test cylinders each. On each of four plates, two cylinders were

TABLE 1. *Standard curve concentrations for assays with 14 antibiotics and 6 preservatives of nonantibiotic nature*

Active substances	Initial solution of standard[a]	Diluent[a]	Standard curve concn (μg or units/ml)
Antibiotics			
Ampicillin	100 μg/ml in distilled water	Buffer C	0.05; 0.071; 0.1; 0.143; 0.203
Bacitracin	100 units/ml in A	Buffer A	0.1; 0.15; 0.225; 0.337; 0.506
Cephaloridine	1,000 μg/ml in A	Buffer A	0.064; 0.08; 0.1; 0.125; 0.156
Cephalothin	1,000 μg/ml in A	Buffer A	0.064; 0.08; 0.1; 0.125; 0.156
Dicloxacillin	1,000 μg/ml in A	Buffer A	0.064; 0.08; 0.1; 0.125; 0.156
Dihydrostreptomycin	1,000 μg/ml in C	Buffer C	7.0; 10.0; 15.7; 23.6; 35.4
Gentamicin	1,000 μg/ml in C	Buffer C	4.0; 7.2; 13.0; 23.3; 42.0
Methicillin	1,000 μg/ml in A	Buffer A	0.05; 0.07; 0.1; 0.143; 0.203
Oxacillin	1,000 μg/ml in A	Buffer A	0.064; 0.08; 0.1; 0.125; 0.156
Potassium penicillin G	1,000 μg/ml in A	Buffer A	0.02; 0.028; 0.039; 0.055; 0.077
Phenethicillin	1,000 μg/ml in C	Buffer C	0.064; 0.08; 0.1; 0.125; 0.156
Phenoxymethylpenicillin	1,000 μg/ml in methanol and in A	Buffer A	0.064; 0.08; 0.1; 0.125; 0.156
Rimactane[b]	1,000 μg/ml in methanol and in B	Buffer B	0.5; 0.71; 1.0; 1.43; 2.03
Streptomycin	1,000 μg/ml in C	Buffer C	7.0; 10.0; 15.7; 23.6; 35.4
Preservatives			
Benzalkonium chloride	1,000 μg/ml in distilled water	Distilled water	100; 141; 200; 283; 401
Bradosol[c]	1,000 μg/ml in distilled water	Distilled water	80; 120; 180; 270; 405
Bronopol[c]	1,000 μg/ml in distilled water	Distilled water	64; 80; 100; 125; 156
Cialit[c]	100 μg/ml in distilled water	Distilled water	3.2; 4.0; 5.0; 6.25; 7.81
Merthiolate	100 μg/ml in distilled water	Distilled water	3.2; 4.0; 5.0; 6.25; 7.81
Phenylmercuric borate	100 μg/ml in distilled water	Distilled water	0.50; 0.71; 1.0; 1.43; 2.03

[a] Buffers A, B, and C are described in Materials and Methods.
[b] Trademark. Generic name in the U.S.A. and in Canada: rifampin.
[c] Trademark.

TABLE 2. *Comparison of the 4-hr and 16- to 18-hr assay methods by parallel determinations of 10 antibiotic substances*

Antibiotic	Antibiotic potency							
	Assay by 4-hr method				Assay by 16-to 18-hr method			
	Frequency	Per cent of standard	95% Confidence limits	Coefficient of variation (%)	Frequency	Per cent of standard	95% Confidence limits	Coefficient of variation (%)
Ampicillin	10	100.2	99.3–101.2	1.3	10	99.4	98.9– 99.9	0.5
Bacitracin	10	100.6	100.0–101.1	0.6	10	100.6	100.1–101.1	0.7
Cephaloridine	10	100.2	99.0–101.4	1.6	10	98.8	98.3– 99.4	0.7
Cephalothin	10	100.3	99.1–101.6	1.8	10	101.7	100.2–103.2	1.5
Dicloxacillin	10	100.3	99.2–101.4	1.6	10	99.4	98.4–100.3	1.8
Methicillin	10	99.6	99.4– 99.9	0.6	10	100.0	99.0–100.9	1.4
Oxacillin	10	100.1	99.0–101.2	1.4	10	99.9	99.6–100.1	1.2
Potassium penicillin G	10	98.7	97.4–100.0	1.8	10	99.6	99.1–100.0	0.6
Phenethicillin	10	100.1	98.8–101.1	1.6	10	100.1	99.6–100.6	0.7
Phenoxymethylpenicillin	10	98.8	98.2– 99.3	0.8	10	98.8	97.8– 99.8	1.4

filled with 0.1 ml of the reference concentration solution, and the other two cylinders were filled with the concentration under test. Thus, there were 32 readings for the reference concentration and 8 readings for each of the other points of the curve.

For each sample tested, four plates were used, two cylinders being filled with the standard reference point solution and two cylinders with the sample, which had been diluted to the reference concentration. Thus, eight readings were obtained for the sample and eight readings for the reference concentration of the standard.

The plates were incubated for 4 hr at 65 ± 1 C, the cylinders were removed, and the inhibition zones were measured by using an optical projector.

Estimation of potency. The potency of the sample was estimated according to the directions of the *Code of Federal Regulations* (2).

Comparison of the 4-hr assay and the conventional 16- to 18-hr agar diffusion assay. To test the accuracy of the 4-hr assay with *B. stearothermophilus*, it was compared with the conventional agar diffusion assay by applying the test organism, volume of standardized inoculum, standard response line concentrations, test medium, incubation time, and temperature proposed by the *Code of Federal Regulations* (2). The assay procedure was the same for both methods, as described in this report.

In this way, the activity of each of the 10 antibiotics listed in Table 2 was determined, each preparation being tested simultaneously with both methods by two different persons on the same day; all tests were repeated 10 times. The same initial concentration (100 or 1000 μg or units/ml) of the antibiotics was used to prepare all final concentrations of the standard curve and of each sample. Theoretically, therefore, each sample could be expected to exhibit an activity of 100%. To minimize any individual error, i.e., any error introduced by the person performing the assay in favor of one or the other method, each person made five determinations by each method starting with the initial concentration.

TABLE 3. *Minimal concentrations required to give measurable inhibition zones on Bacillus stearothermophilus plates*

Antibiotic	Buffer solution		Urine	
	Concn (μg or units/ml)	Zone size (mm)[a]	Concn (μg or units/ml)	Zone size (mm)[a]
Ampicillin	0.02	13.3	0.02	11.4
Bacitracin	0.1	10.2	0.2	9.8
Cephaloridine	0.02	12.7	0.02	12.8
Cephalothin	0.03	10.0	0.03	11.1
Dicloxacillin	0.04	12.9	0.04	10.3
Methicillin	0.03	12.5	0.03	10.1
Oxacillin	0.04	11.8	0.04	13.2
Potassium penicillin G	0.01	12.3	0.01	12.4
Phenethicillin	0.03	11.3	0.03	12.9
Phenoxymethylpenicillin	0.02	12.2	0.02	11.5

[a] Calculated (x = avg) on the basis of 20 plates. Cylinders with outside diameter of 8.0 mm and inside diameter of 6.0 mm were used, and any zones less than 9.0 mm were considered negative.

Determination of the minimum accurately measurable concentration in buffer solutions and urine. Starting with the initial standard-curve concentration (100 or 1000 μg or units/ml), the minimum accurately measurable concentrations of the 10 antibiotics listed in Table 3 were determined on 20 *B. stearothermophilus* plates. The concentration yielding an average zone of inhibition exceeding the diameter of the porcelain

cylinder by at least 1 mm was taken as being the effective minimum concentration. Urine was obtained from a patient who had not received antibiotics during the previous month.

RESULTS AND DISCUSSION

The advantages of the 4-hr assay described here may be summed up as follows. All 14 clinically active antibiotics and the 6 chemical preservatives can be tested with a single rapid-growing assay organism and on a single test medium. The resultant inhibition zones are readily measurable with the naked eye after 4 hr of incubation. The selective incubation temperature of 65 C eliminates the risk of contamination, which very much simplifies the experimental procedure.

The microbiological assay is of particular value in testing the antimicrobial activity of chemical preservatives in aqueous pharmaceutical preparations. It makes it possible to determine in a simple way the activity decrease of the preservative agents caused, for instance, by interaction with nonionic surfactants or pharmaceutical container materials.

The linear concentration ranges for the standard curves, statistically verified at the 95% probability level, are shown in Table 1 for all 20 substances. The linearity of the regression lines was verified in replicate tests carried out on three different days by two persons, and no significant deviation from linearity was found. Under the experimental conditions described, the standard curves are fully reproducible.

The 20 determinations performed with each of 10 antibiotics (Table 2) listed in the *Code of Federal Regulations* to compare the 4-hr and 16- to 18-hr assays revealed no important deviations, either in or between the two methods. Because all samples were further diluted from the same initial quantity and starting concentration as the standard, each could theoretically be expected to exhibit 100% potency. The deviations noted in the 20 replicate tests carried out with each antibiotic, i.e., all discrepancies resulting from inadvertant errors in the use of the pipette, dilution, the preparation of the plates, and the measurement of the inhibition zones, amounted to at most 5% in isolated cases. The coefficient of variation (s/x) did not exceed 2%, which is a thoroughly acceptable limit for microbiological determinations.

The 4-hr assay thus entails no loss of accuracy in comparison with the conventional method and offers a rapid and economical procedure for use in the analytical laboratory, permitting analyses to be completed within one working day.

Because *B. stearothermophilus* ATCC 12980 is highly sensitive to bacitracin, cephalosporins, and penicillins, this test is also particularly suitable for detecting low-level concentrations of these antibiotics in aqueous solutions, e.g., for the rapid detection of cross-contamination in drug containers and the demonstration of low concentrations of antibiotics in the urine. The organisms frequently present in urine samples, which if resistant can impede the analyses to a considerable extent, are eliminated by the selective incubation temperature and hence do not interfere in any way with the measurement of the inhibition zone.

The minimum accurately measurable concentration in buffer solution and urine lies between 0.01 and 0.3 μg or units/ml (Table 3), depending upon the antibiotic in question.

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