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# Four-Hour Microbiological Assay of Gentamicin in Serum[1]

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Since the microbiological assay of the antibiotic content of serum generally requires 18 to 24 hr of incubation, results of such procedures may not become available in time to make appropriate adjustments in subsequent dosages of antibiotic. A 4-hr bioassay for determining concentrations of gentamicin in serum has been developed in which *Staphylococcus aureus* ATCC 6538P is used as the test organism. Poured plates have yielded satisfactory results after storage at 4 C for 5 days. Results of the 4-hr procedure agree closely with those of a conventional 18-hr disc-plate assay performed with the same test organism.

In the treatment of serious infections, the administration of potentially toxic antibiotics at sufficiently frequent intervals to maintain therapeutic concentrations in the blood requires careful monitoring and adjustment of antibiotic dosage. Since microbiological assay of the antibiotic content of serum generally requires 18 to 24 hr of incubation, results of such procedures may not be available in time to enable appropriate adjustments in subsequent dosages of the antibiotic. This report describes a 4-hr assay method for determining concentrations of gentamicin with the use of *Staphylococcus aureus* ATCC 6538P as the test organism.

## MATERIALS AND METHODS

**Preparation of assay plates.** Antibictic medium no. 11 (Difco control no. 544244) was autoclaved and adjusted to *p*H 7.9. To bottles containing 98 ml of the melted agar cooled to 50 C was added 2.0 ml of a well-mixed 18-hr Trypticase Soy Broth (BBL) culture of *S. aureus* ATCC 6538P. Viable-colony counts of this inoculum, determined on 10 separate occasions, ranged from $2.4 \times 10^8$ to $1.8 \times 10^9$ colony-forming units (CFU)/ml (mean, $6.6 \times 10^8$ CFU/ml). Portions of 9 ml of the seeded agar, containing an average of $1.2 \times 10^7$ CFU/ml, were pipetted into plastic petri dishes (100 by 15 mm) and allowed to harden; the plates then were stored at 5 C for a maximum of 5 days.

**Preparation of stock antibiotic solutions.** A stock solution (1,000 μg/ml) of gentamicin (donated by Schering Corp.) in 0.1 M phosphate buffer, *p*H 8.0, was prepared and diluted in sterile pooled human serum to concentrations of 5.0, 4.0, 2.0, and 1.0 μg/ml.

**Standard curve.** Paper discs, 12.7 mm in diameter (no. 740E, Schleicher & Schuell Co.), were saturated with each of the dilutions of gentamicin and placed on the surface of the seeded agar (each disc absorbs approximately 0.08 ml of solution). The solution containing 2.0 μg/ml was selected as the reference standard. Two discs saturated with the reference standard were placed on each assay plate opposite each other, and two other discs saturated with 1, 4, or 5 μg/ml were placed in the other two quadrants. These concentrations were replicated 20 times. All plates were incubated for 4 hr at 37 C. The diameters of the zones of inhibition of the reference standard discs were measured by use of a dark-field Quebec bacterial colony counter with a millimeter scale on the glass; the mean of these values was taken as the correction point. Mean diameters of inhibition zones at 1, 4, and 5 μg/ml were corrected according to the deviation from the correction point of the reference standard tested on the same plate. The corrected zone diameters of 5.0, 4.0, and 1.0 μg/ml with the correction point of 2.0 μg/ml were plotted against concentration on semilogarithmic paper.

**Assay procedure.** Serum samples containing unknown concentrations of antibiotic were diluted 1:2, 1:4, and 1:5 in sterile pooled human serum (previously tested for antibacterial activity against *S. aureus* ATCC 6538P). Four seeded agar plates were used, each with two discs saturated with the reference standard and two discs saturated with the patient's undiluted or diluted serum. For maximal accuracy, duplicate plates were set up with discs saturated with diluted serum. The plates were incubated for 4 hr at 37 C. Zone diameters of the reference standards and of the patient's serum were measured and averaged. Correction of the zone diameter was accomplished by adding or subtracting the difference between the mean zone diameter of the reference standard obtained in the assay plate and the zone diameter of the correction point of 2.0 μg/ml in the standard curve. The final result, in micrograms per milliliter,

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was extrapolated from the standard curve and multiplied by the dilution of the original specimen.

**Comparison of conventional 18-hr method with 4-hr method.** Patients' sera submitted to the bacteriology laboratory for assay of gentamicin were analyzed in parallel by use of an 18-hr technique with *S. aureus* ATCC 6538P as described by Oden et al. (8) and by use of the 4-hr technique. Penicillinase (BBL) or cephalosporinase (generously provided by J. L. Ott, Lilly Research Laboratories) was added to inactivate penicillins or cephalosporins, respectively, being administered concurrently (11).

## RESULTS

A linear standard curve was obtained (Fig. 1) with the 4-hr assay procedure. The standard error of the final estimate of concentration at any point on the curve was calculated to be in the range of ± 6%. Recovery of at least 90% of gentamicin added to serum in known concentrations of 5 to 15 μg/ml was accomplished by diluting the serum so that final concentrations fell within the standard curve.

The correction point of 2.0 μg/ml on the standard curve gave a zone diameter of inhibition of 21.7 mm. The mean of 259 subsequent determinations of this point carried out in the course of patients' serum assay procedures was 21.5 mm (range, 20.5 to 22.5 mm). Variation of the zone diameter of the correction point within any given lot of plates over a 5-day period of storage at 5 C was 6% or less, and lot-to-lot differences in the zone diameters of the correction points were not statistically significant.

Reduction of the volume of seeded agar to 5 ml per plate increased the zone diameter of the correction point of 2.0 μg/ml to 22.5 mm; however, it was our opinion that a uniformly distributed seed layer could not be obtained as readily as with 9 ml.

Serum samples from 35 patients receiving gentamicin alone or in combination with penicillins or cephalosporins were assayed for gentamicin activity by both the 18- and the 4-hr procedures (Fig. 2). Excluding those sera with no detectable activity by either method, we found that in 23 instances (72%) the results of the two procedures agreed within 0.5 μg/ml and in all instances the results agreed within 1.0 μg/ml.

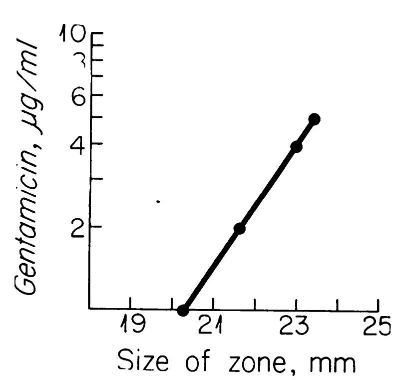


FIG. 1. *Standard dose-response curve of gentamicin tested against S. aureus ATCC 6538P.*

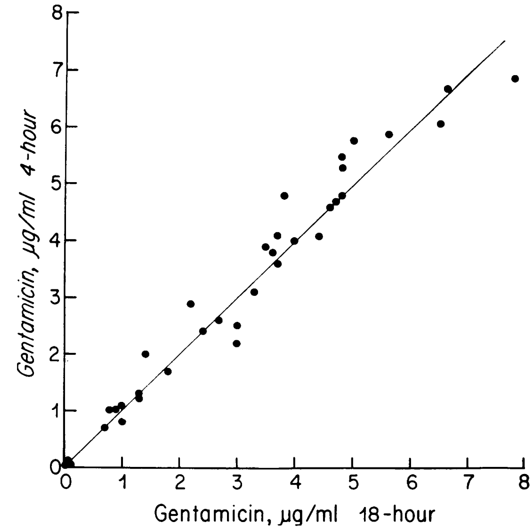


FIG. 2. *Comparison of results of 18-hr and 4-hr disc-plate bioassays of gentamicin concentration in serum samples from 35 patients.*

## DISCUSSION

Oden and co-workers (8) described two methods for microbiological assay of gentamicin, based on those described by Grove and Randall (5) for assay of neomycin. One of these methods was a cylinder-plate assay with *Bacillus subtilis* ATCC 6633 for determination of levels between 0.07 and 0.4 μg/ml. The other method was a disc-plate assay with *S. aureus* ATCC 6538P for determination of levels between 1.0 and 10.0 μg/ml. Both methods required overnight incubation and have been described in detail elsewhere (Laboratory Reference Manual, Garamycin, Schering Corp., 1969). Both methods have been used in our laboratory; however, since therapeutic serum levels of gentamicin usually are in the range of 1 to 10 μg/ml, the disc-plate assay with *S. aureus* ATCC 6538P was adopted for routine purposes.

The substitution of paper discs for stainless-steel, glass, or porcelain cylinders has simplified microbiological assay procedures (1, 4, 7, 12). In addition, the use of larger inocula in agar-diffusion or disc-plate assay procedures has decreased the incubation time of assays to 3 to 6 hr (6, 9–11).

TABLE 1. *Cation content (micrograms per milliliter) of various susceptibility test media*[a]

| Cation | Mueller-Hinton | | Trypticase Soy | | Plain | | Brain-heart infusion | | Nutrient agar | Antibiotic medium no. 11 |
|---|---|---|---|---|---|---|---|---|---|---|
| | Broth | Agar | Broth | Agar | Broth | Agar | Broth | Agar | | |
| Ca.... | 7.5 | 33.5 | 22.4 | 82.5 | 7.9 | 22.1 | 3.5 | 20.4 | 27.7 | 96.0 |
| Mg.... | 4.9 | 28.0 | 38.5 | 54.5 | 7.6 | 28.0 | 6.6 | 18.9 | 22.4 | 52.5 |
| Cu.... | 0.19 | 0.30 | 0.16 | 0.30 | 0.13 | 0.13 | 0.23 | 0.14 | 0.04 | 0.06 |
| Zn.... | 0.54 | 1.50 | 1.50 | 2.40 | 0.22 | 0.12 | 0.13 | 0.13 | 0.17 | 1.4 |
| Fe.... | 0.65 | 2.50 | 0.80 | 2.50 | 0.15 | 0.18 | 0.44 | 0.35 | 0.17 | 1.82 |

[a] Determined by atomic absorption spectrophotometry through the courtesy of John T. McCall, Section of Clinical Chemistry, Department of Laboratory Medicine, Mayo Clinic.

Rapid assay procedures for gentamicin reported to date have used *B. stearothermophilus* with incubation at 56 C (6) and *B. subtilis* spores in small volumes of agar (9, 11). Although recovery rates of known concentrations of gentamicin added to serum are reported in these studies and appear to be reasonably accurate, there are no data on clinical specimens comparing the results of any of these methods with results obtained by conventional methods of assay. Because the disc-plate method with *S. aureus* ATCC 6538P was recommended for determination of serum levels between 1.0 and 10.0 μg/ml, the range of concentrations usually encountered clinically, and because *S. aureus* appeared to provide more clear-cut zones of inhibition than *B. subtilis*, it seemed appropriate to try to decrease the time required for this procedure.

Sabath et al. (9) studied the effects of type of medium (including *p*H), salt concentration, size of sample, and depth of agar on the sensitivity and accuracy of assays of aminoglycosidic antibiotics. They also found that crude β-lactamase II did not alter the quantitative results of gentamicin assays, a finding substantiated by Stroy and Preston (11).

An additional factor affecting gentamicin activity in vitro is the cation content of the medium (3, 13). There are appreciable differences in cation content among the various media used for susceptibility testing (Table 1); agar contributes the major share of cation, and antibiotic medium no. 11 has the highest cation content of any of the media tested. There are also batch-to-batch differences in given types of media, as has been discussed by Ericsson and Sherris (2). Assays in an agar medium of relatively low cation content, Mueller-Hinton agar, were attempted in our laboratory; however, indistinct zone diameters were obtained.

The method described in this paper provides a rapid means of bioassay of gentamicin in serum of patients being treated with this antibiotic alone or in combination with penicillins or cephalosporins.

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