APPLIED MICROBIOLOGY, July 1969, p. 31-34
Vol. 18, No. 1
Copyright © 1969 American Society for Microbiology
Printed in U.S.A.

Modified Microbiological Assay for Rapid Estimation of Antibiotic Concentrations in Human Sera

S. ANN STROY

The Lilly Research Laboratories, Eli Lilly & Company, Indianapolis, Indiana 46206

Received for publication 31 January 1969

Antibiotic concentrations in human sera were estimated in 5 to 6 hr by a modified microbiological assay. By using *Staphylococcus aureus* and *Streptococcus pyogenes* as the assay organisms, the seeded assay plates were preincubated for 2 to 6 hr and then were stored at 4 C until used for assay. Paper discs saturated with the specimen were placed on the preincubated assay plates with reference discs saturated with known concentrations of antibiotic. After 5 to 6 hr of incubation, zones of antibacterial activity were measured and compared with a standard curve for estimation of antibiotic concentration. Results from this rapid assay method compared favorably with those from the commonly used 24-hr assay.

Marked differences in the rate of renal clearance for some antibiotics could lead to increasing concentrations in blood and tissue that may be related to serious toxic reactions. Therefore, it would be advantageous to know the antibiotic concentration in serum before subsequent dosage. Chemical assay procedures are available for many of these antibiotics, but some small laboratories may not possess the necessary reagents or equipment to perform them. Microbiological procedures used for assaying antibiotic concentrations in human serum usually require an incubation period of 16 to 24 hr. This communication describes data obtained from a modification of the disc-plate assay method which permits approximation of antibiotic concentration in serum after 5 to 6 hr of incubation.

MATERIALS AND METHODS

Streptococcus pyogenes assay plates. Trypticase Soy Blood Agar Base (BBL) was prepared and maintained in a liquid state at 45 to 50 C. A 5-ml amount of defibrinated human blood was added to each 100 ml of melted agar to support the growth of the assay organism, *S. pyogenes* strain C-203. Each 100-ml flask of blood-agar was then inoculated with 1 ml of a 1:10 dilution from an overnight broth culture. Samples (6 ml) of the seeded agar were pipetted to 9-cm plastic petri plates. The inoculated plates were incubated at 37 C for 6 hr and then stored at 4 C, for not longer than 5 days, until used for assay. This preincubation of the plates was the basis for the rapidity of the assay described below.

Staphylococcus aureus assay plates. Penassay Seed Agar (Difco), adjusted to *p*H 8.0 before autoclaving, was prepared and maintained in a liquid state at 45 to 50 C. Each 100-ml flask of agar was then inoculated with 1 ml of a 1:10 dilution from an overnight broth culture of *S. aureus* strain 3055. Plates were prepared and incubated as above with *S. pyogenes*, except that the preincubation time was only 2 hr.

Standard antibiotic solutions. Standard reference solutions were prepared by dissolving 10 mg of antibiotic standard powder in 10 ml of sterile distilled water, and making the necessary twofold serial dilutions in human serum to have the final concentrations of the antibiotics shown in Table 1.

Standard curves. Paper discs (6 mm) were saturated with antibiotic standard solutions and were placed on the surface of the preincubated agar plates. The number of discs placed on each plate was varied to avoid overlapping zones of inhibition of red blood cell (RBC) lysis with streptococcal plates or inhibition of growth of *S. aureus*. Plates for standard curves were replicated four times. All plates were then incubated for an additional 5 to 6 hr at 37 C. Zones of cell lysis or bacterial growth inhibition were distinguishable after 4 hr of incubation; however, edges of zones were easier to determine after 5 to 6 hr. Zone diameters were measured, and a standard curve was plotted. Once a standard curve was established for each antibiotic, it was not necessary to repeat this procedure. Reference to this established standard curve for assay of specimens was accomplished by using discs saturated in only one standard solution on each assay plate.

Assay procedure. To illustrate the reliability of the assay procedures, human serum was prepared with 100 μg of an antibiotic per ml and diluted in serum to concentrations that were within the linear range of the standard curve. Paper discs (6 mm) were saturated with specimens or dilutions thereof, and two discs were placed on each assay plate. Two discs, saturated with the proper reference standard solution, were placed on the same plate, directly opposite each other and at right angles with the specimen discs. The plates

were incubated at 37 C for an additional 5 to 6 hr, and diameters of the resulting zones of cell lysis or bacterial growth inhibition were measured. Both zone diameters, surrounding either the standard or sample discs, were averaged. Reference was made to the zone for the same standard solution as was plotted on the standard curve. The average zone for the sample was corrected by adding or subtracting the difference between the average standard zone diameter from the assay plates and the zone for the same concentration of antibiotic from the standard curve. The result, in micrograms per milliliter, for the corrected average sample zone diameter was then extrapolated from the standard curve. This result was multiplied by the dilution made to obtain the concentration of antibiotic in the original specimen.

RESULTS

Zones surrounding discs in Fig. 1 and 2 resulted from the interference of lysis of the RBC in the agar by the assay organism, *S. pyogenes*. Edges of the zones marked the maximal diffusion of the antibiotic, which in this case was cephaloridine. The antibiotic within the zone prevented growth of the streptococci and lysis of the RBC. Thus, the zone remained red and the area between zones was cleared by lysis of the RBC by viable bacteria. Diameters of the zones were dependent on the antibiotic content of the discs. On the other hand, the zones shown in Fig. 3 resulted from direct inhibition of *S. aureus* by kanamycin.

Photographs of plates used to plot standard curves are shown for cephaloridine (Fig. 1) and kanamycin (Fig. 3). Two plates (replicated four times) were utilized for cephaloridine because of the large zone diameters. Smaller zones for gentamicin, kanamycin, streptomycin, and vancomycin permitted the use of only one plate (replicated four times), as illustrated in Fig. 3 for kanamycin.

A typical curve is shown in Fig. 4. The zone diameters obtained for various concentrations of five antibiotics are presented (Table 1). Also shown in Table 1 are concentrations of the antibiotics that were used as "reference" standards on assay plates. Zone diameters for these antibiotic concentrations were close to those at the midpoints of the linear ranges of the curves. For assay of specimens, discs saturated with a reference standard solution were utilized on each assay plate. Replication of a standard curve was not necessary for each assay performed when reference standard antibiotic solutions were employed.

Disc-plate assay procedures usually require overnight incubation. Incubation time was shortened by preincubating the assay plates. These preincubated plates were refrigerated and were used after storage for as long as 5 days. Photographs of assay plates (Fig. 2 and 3) show the arrangement of discs saturated from specimen or reference standard solutions of cephaloridine or kanamycin. It is obvious that zone diameters for the specimen were approximately equal to those of the reference standard on only one of three assay plates of each antibiotic. Corrected zone diameters for the specimen from this plate were utilized to estimate the antibiotic concentration in the original specimen. This was accomplished by

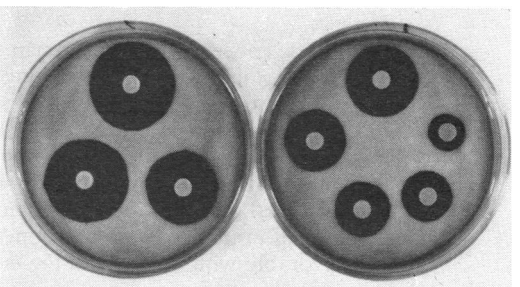

FIG. 1. *Streptococcus pyogenes assay plates utilized for the standard curve for cephaloridine. The plate on the left represents cephaloridine at 12.5, 6.25, and 3.12 μg/ml, and the plate on the right represents 1.6, 0.8, 0.4, 0.2, and 0.1 μg/ml. Circles of black paper, the same diameter as the zones of inhibition, were used on the bottoms of the plates for photographic purposes.*

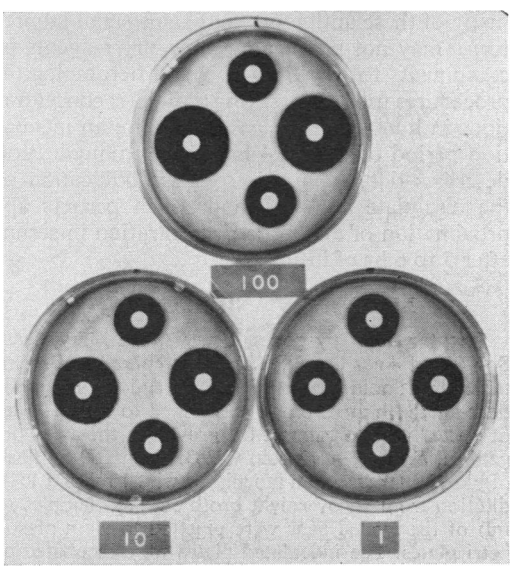

FIG. 2. *Streptococcus pyogenes assay plates utilized for a human serum specimen containing 100 μg of cephaloridine per ml. The upper plate represents the undiluted specimen or 100 μg/ml, the plate on the lower left represents a 1:10 dilution or 10 μg/ml, and the plate on the lower right represents a 1:100 dilution or 1 μg/ml.*

extrapolation from the standard curve. Results tabulated in Table 2 illustrate the reliability of the assay procedures. Concentrations of the antibiotics in human serum could be estimated with reasonable accuracy.

DISCUSSION

Disc-plate assay procedures described above may be useful in determining serum concentrations before subsequent dosage with certain antibiotics. Other procedures, such as chemical analysis or turbidimetric assay methods, could be used but are more difficult to perform.

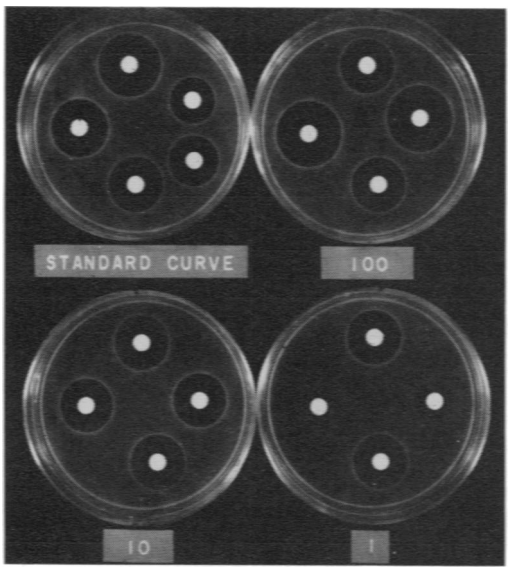

FIG. 3. *Staphylococcus aureus disc-plate assay for kanamycin. Zones on the plate at the upper left represent the standard curve with discs saturated from solutions of 100, 50, 25, 12.5, and 6.25 μg/ml. The other three plates are assay plates for a human serum specimen containing 100 μg of kanamycin per ml. The plates represent the undiluted specimen (100) and a 1:10 (10) or 1:100 (1) dilution thereof.*

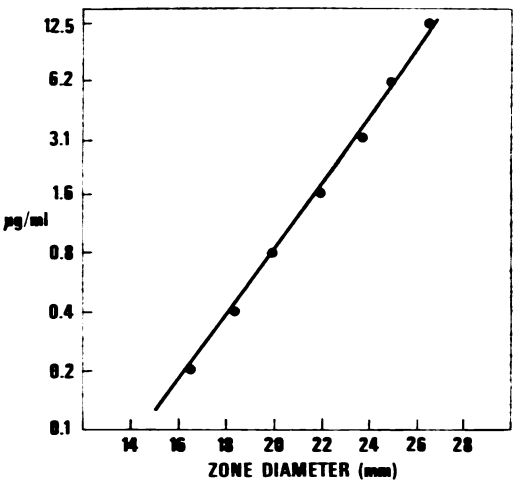

FIG. 4. *Streptococcus pyogenes standard curve with cephaloridine.*

The estimated concentrations of five different antibiotics in human serum specimens (Table 2) were obtained after only 5 to 6 hr of incubation. Assay within this time period was made possible by simple modification of disc-plate assay procedures. Agar plates seeded with controlled inocula of either *S. aureus* or *S. pyogenes* were preincubated for 2 and 5 hr, respectively. Zones of cell lysis or bacterial growth inhibition were sufficiently visible after 5 to 6 hr of additional incubation to estimate approximate concentration of antibiotics in serum specimens. Accuracy of the assay increased with incubation time.

Marked differences in the rates of renal clear-

TABLE 1. *Zone diameters and reference standard concentrations for assays with five antibiotics*

Assay	Antibiotic	Zone diameter (mm) for standard curves[a]											Reference[b] standard concn (μg/ml)
		100[c]	50	25	12.5	6.2	3.1	1.6	0.8	0.4	0.2	0.1	
Streptococcus pyrogenes	Cephaloridine	ND[d]	ND	ND	26.7	25.0	23.7	22.0	20.0	18.2	16.4	14.3	0.8
Staphylococcus aureus	Cephaloridine	ND	ND	ND	ND	21.7	19.7	17.7	15.7	13.7	11.7	ND	0.8
	Gentamicin	ND	ND	ND	ND	20.5	19.1	17.7	15.9	13.5			1.6
	Kanamycin	22.5	21.5	20.0	19.0	17.3	ND	ND	ND	ND	ND	ND	12.5
	Streptomycin	18.5	17.5	16.0	15.0	14.0	ND	ND	ND	ND	ND	ND	12.5
	Vancomycin	14.0	12.5	11.5	10.0	8.0	ND	ND	ND	ND	ND	ND	25.0

[a] Zone diameters measured after 5 to 6 hr of incubation.
[b] Suggested concentration of antibiotic for use as a reference standard on assay plates.
[c] Concentrations of antibiotic in micrograms per milliliter in human serum.
[d] Not determined.

TABLE 2. *Approximation of antibiotic concentrations in a human serum specimen*[a]

Assay	Antibiotic	Assay value (μg/ml) of 100-μg/ml specimen			Approx amt of antibiotic in specimen (μg/ml)[b]
		Undiluted	1:10 Dilution	1:100 Dilution	
Streptococcus pyogenes	Cephaloridine	>12.2	8.6	0.98[c]	98
Staphylococcus aureus	Cephaloridine	>6.2	>6.2	0.96[c]	96
	Gentamicin	>6.2	>6.2	1.02	102
	Kanamycin	95	10[c]	<6.2	100
	Streptomycin	100	10[c]	<6.2	100
	Vancomycin	81	9.0[c]	<6.2	90

[a] A 100-μg amount of antibiotic was added to 1 ml of human serum.
[b] Accepted assay value multiplied by the dilution made.
[c] Assay value most accurately fitting the linear range of the standard curve.

TABLE 3. *Usual serum concentrations obtained in humans with five antibiotics*

Antibiotic	Usual daily dose	Concn of antibiotic in serum		
		Dosage[a]	Route	Concn (μg/ml)
Cephaloridine	4 g	1 g, Q6h	IM[b]	15–50 (median) 40–140 (range)
Gentamicin	ND[c]	30–60 mg, Q8h	JM	5–10
Kanamycin	15 mg/kg	0.25 g, Q6h	IM	13.5 (avg)
		0.5 g, Q6h	IM	15.4 (avg)
Streptomycin	1–2 g	0.5 g, Q6h	IM	7–15
		1.0 g, Q12h	IM	25–30
Vancomycin	1–2 g	0.5 g, Q6h	IV	10 (peak)
		1.0 g, Q12h	IV	25 (peak)

[a] Q6h, every 6th hr; Q8h, every 8th hr; Q12h, every 12th hr.
[b] IM, intramuscular; IV, intravenous.
[c] Not well defined.

ance of the five antibiotics used could lead to increasing concentration in blood and tissue that may be related to serious toxic reactions. Serum concentrations for the antibiotics that would indicate occurrence of the above phenomenon have not been well defined; however, usual serum concentrations obtained in humans, after administration of five antibiotics, are shown in Table 3. The assay procedures described provide a rapid method of determining whether serum concentrations for the antibiotics studied are within normal ranges. If not, subsequent dosage could be withheld until sufficient excretion had occurred. This procedure could increase safety during therapy with these potent antimicrobial compounds.

ACKNOWLEDGMENTS

I thank W. E. Wick for advice on the assay procedures and for assistance with the manuscript. I also thank T. Markowitz, H. V. Kuder, and L. D. Bechtol for supplying information about usual antibiotic serum concentrations.