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

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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 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 pH 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 mg 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 bac-
terial 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 be-
tween 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 dilu-
tion made to obtain the concentration of antibiotic
in the original specimen.

RESULTS

Zones surrounding discs in Fig. 1 and 2 re-
sulted 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 cephalori-
dine. The antibiotic within the zone prevented
growth of the streptococci and lysis of the RBC.
Thus, the zone remained red and the area be-
tween zones was cleared by lysis of the RBC by
viable bacteria. Diameters of the zones were de-
pendent 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 genta-
micin, 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 anti-
biotics that were used as "reference" standards on
assay plates. Zone diameters for these antibiotic
concentrations were close to those at the mid-
points of the linear ranges of the curves. For as-
say 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 refer-
ence standard antibiotic solutions were employed.

Disc-plate assay procedures usually require
overnight incubation. Incubation time was short-
ened by preincubating the assay plates. These
preincubated plates were refrigerated and were
used after storage for as long as 5 days. Photo-
graphs 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.

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 pre-incubated 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**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Antibiotic</th>
<th>Zone diameter (mm) for standard curves</th>
<th>Reference standard concn (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>Cephalaridine</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Cephalaridine</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Gentamicin</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Kanamycin</td>
<td>22.5</td>
<td>21.5</td>
</tr>
<tr>
<td></td>
<td>Streptomycin</td>
<td>18.5</td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td>Vancomycin</td>
<td>14.0</td>
<td>12.5</td>
</tr>
</tbody>
</table>

* Zone diameters measured after 5 to 6 hr of incubation.
* Suggested concentration of antibiotic for use as a reference standard on assay plates.
* Concentrations of antibiotic in micrograms per milliliter in human serum.
* Not determined.
Table 2. Approximation of antibiotic concentrations in a human serum specimen

<table>
<thead>
<tr>
<th>Assay</th>
<th>Antibiotic</th>
<th>Assay value (µg/ml) of 100-µg/ml specimen</th>
<th>Approx amt of antibiotic in specimen (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Undiluted</td>
<td>1:10 Dilution</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>Cephaloridine</td>
<td>&gt;12.2</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>Cephaloridine</td>
<td>&gt;6.2</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>Gentamicin</td>
<td>&gt;6.2</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>Kanamycin</td>
<td>95</td>
<td>10c</td>
</tr>
<tr>
<td></td>
<td>Streptomycin</td>
<td>100</td>
<td>10c</td>
</tr>
<tr>
<td></td>
<td>Vancomycin</td>
<td>81</td>
<td>9.0c</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Usual daily dose</th>
<th>Dosagea</th>
<th>Route</th>
<th>Conc of antibiotic in serum (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephaloridine</td>
<td>4 g</td>
<td>1 g, Q6h</td>
<td>IMb</td>
<td>15–50 (median)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>NDc</td>
<td>30–60 mg, Q8h</td>
<td>IM</td>
<td>5–10 (range)</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>15 mg/kg</td>
<td>0.25 g, Q6h</td>
<td>IM</td>
<td>13.5 (avg)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 g, Q6h</td>
<td>IM</td>
<td>15.4 (avg)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0 g, Q12h</td>
<td>IM</td>
<td>25–30</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>1–2 g</td>
<td>0.5 g, Q6h</td>
<td>IV</td>
<td>10 (peak)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>1–2 g</td>
<td>1.0 g, Q12h</td>
<td>IV</td>
<td>25 (peak)</td>
</tr>
</tbody>
</table>

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

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

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