Specific Assay of Aminoglycosidic- or Polymyxin-Type Antibiotics Present in Human Sera in Combination with Cephalosporins

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Monitoring of serum concentrations of aminoglycosidic or polymyxin antibiotics when administered concurrently with cephalosporins or penicillins requires a special assay technique. Selective enzymatic degradation of the β-lactam antibiotic from the serum specimen allows subsequent assay of the antibiotic being monitored. This report gives details of a simple procedure for laboratory production of a crude enzyme capable of degrading cephalosporins or penicillins. An assay procedure for quantitating aminoglycosidic or polymyxin antibiotics after enzymatic degradation of a coexisting β-lactam antibiotic is described.

Antibiotic combinations that show additive or synergistic activity are sometimes used for the treatment of serious bacterial infections. β-Lactam antibiotics (penicillins and cephalosporins) are often administered concurrently with aminoglycosidic (gentamicin, kanamycin, streptomycin) or polymyxin antibiotics (colistin and polymyxin B; C. M. Martin et al., Abstr. 8th Intersci. Conf. Antimicrob. Ag. Chemother., p. 31–32, 1968). The aminoglycosidic or polymyxin antibiotics may cause ototoxicity and renal impairment. If renal insufficiency exists, abnormally high serum antibiotic concentrations could result. A rapid assay method for monitoring the concentrations of these antibiotics in human sera would be a valuable adjunct to other laboratory tests for maintaining safety during therapy with antibiotic combinations.

Although an assay method for the rapid estimation of singly administered antibiotics in human sera has been reported (7), methodology related to cephalosporins in combination with other types of antibiotics has not been described. The present investigation proposes procedures for the specific determination of aminoglycosidic and polymyxin antibiotics administered in combination with β-lactamase-sensitive antibiotics. Since a β-lactamase enzyme active against cephalosporins is not commercially available, a simple procedure was developed for the laboratory preparation of such a substance.

MATERIALS AND METHODS

Preparation of crude β-lactamase enzyme. A clinical isolate of Enterobacter cloacae, for which the minimal inhibitory concentration (MIC) of cephalothin by the broth dilution method was >1,000 μg/ml, was chosen for enzyme preparation. A 1-ml amount from a 1:100 dilution of an overnight Trypticase Soy Broth (BBL) culture of E. cloacae was used to inoculate a flask containing 100 ml of Trypticase Soy Broth. Induction of β-lactamase enzyme production was accomplished during incubation at 37 C by the addition of 50 mg of cephalothin at 2, 4, 6, 8, 24, 26, 28, 30, and 32 hr. At 16 hr after the last addition of cephalothin, most of the bacterial cells were removed from the broth by centrifuging (International Clinical) for 20 min. The broth was first clarified through an RA (1.2 μm) membrane filter (Millipore Corp., Bedford, Mass.) and then was sterilized with an HA (0.45 μm) filter membrane. The resulting broth filtrate (pH 6.8) contained enzymes that destroyed the biological activity of β-lactam antibiotics. However, in addition to the β-lactamase, other enzymes, such as amidases or esterases, which may also degrade these antibiotics, were probably present. No studies were undertaken to identify chemically the degradation products to prove that the destruction of the biological activity was due specifically to β-lactamase activity. Therefore, for the sake of nomenclature, broth containing these enzymes was designated as the enzyme preparation (EP). The EP was stored at 4 C for as long as 6 months without loss of activity.

Estimation of the potency of the enzyme preparation. Potency of EP was determined by a modification of a previously described assay method (9). A serial two-fold dilution of EP was made in 0.1-ml amounts of Trypticase Soy Broth in tubes (10 by 100 mm). A control was prepared by substituting 0.1 ml of broth in an extra tube. A 0.3-ml amount of saline (0.85%) containing 2.5 μg of a β-lactam antibiotic was added to each tube. After incubation at 37 C for 1 hr, the mixture in each tube was assayed for antibiotic activity. Paper discs (6.3 mm in diameter, Schleicher
and Schuell, Inc.), saturated by dipping into each tube, were placed on seeded agar assay plates containing *Sarcina lutea* PCI-1001-FDA. (See preparation of assay plates, discussed below.) Additional reaction between residual antibiotic and β-lactamase after 1 hr was interrupted by adsorption of the enzyme on the paper discs (1), allowing only the remaining antibiotic to diffuse into the agar to inhibit the growth of *S. lutea*.

Zones of inhibition were observed surrounding discs from the control tube and those dilutions of EP in which there was insufficient enzyme to degrade the antibiotic. The highest dilution of EP that prevented formation of a zone of inhibition was used to calculate potency of the enzyme preparation. For example, if 0.1 ml of a 1:64 dilution of EP degraded the 2.5 μg of cephalothin added to the tube, then 0.1 ml of undiluted EP would be expected to degrade 160 μg of the antibiotic.

**Preparation of assay plates.** Disc-plate assays of aminoglycosidic antibiotics were performed against Bacillus subtilis ATCC 6633 obtained commercially (Difco) as a spore suspension. Antibiotic Medium No. 5 (Difco) in 100-ml volumes was prepared, adjusted to pH 8.0, sterilized, and maintained in a liquid state at 50°C. The melted agar was inoculated by the addition of 0.25 ml of the *B. subtilis* spore suspension. Volumes (6 ml) of the inoculated agar was pipetted to 9-cm plastic petri plates. The agar plates were allowed to solidify and were stored at 4°C for as long as 5 days.

Colistin and polymyxin B were assayed against Bordetella bronchiseptica ATCC 4617. A 1-ml amount solution concentrations for preparation of the assay organism was added to 100 ml of melted Antibiotic Medium No. 1 (Difco) modified to contain 0.5% NaCl. The 6 ml *B. bronchiseptica* plates, poured as described above for *B. subtilis*, were stored at 4°C and used within 2 days.

Activity of cephalosporin antibiotics used in the potency assay of EP was detected by use of plates seeded with *Sarcina lutea* PCI-1001-FDA. (See preparation of assay plates, discussed below.) Additional reaction between residual antibiotic and β-lactamase activity of cephalosporin antibiotics used in the potency assay of EP was monitored by use of plates seeded with *Sarcina lutea* PCI-1001-FDA. (See preparation of assay plates, discussed below.) The addition of 2 ml from an overnight Trypticase Soy Broth culture to 100 ml of melted agar was found to be an acceptable alternate procedure.

**Assay procedures.** Antibiotic assay procedures of Grove and Randall (2) were modified as described. Aminoglycosidic or polymyxin antibiotics were dissolved in pH 8.0 phosphate buffer (1% solution of Harleco Na+, K+ phosphate salts, pH 7.0). The suspension was adjusted to 80% light transmittance at 660 nm in a Coleman Jr. spectrophotometer. A 1.25-ml volume of the *S. lutea* suspension was added to 100 ml of the melted agar. Plates containing 6 ml of the seeded agar were prepared and stored at 4°C for as long as 5 days. The addition of 2 ml from an overnight Trypticase Soy Broth culture to 100 ml of melted agar was found to be an acceptable alternate procedure.

**FIG. 1. Assay of the potency of an enzyme preparation (EP) against five β-lactam antibiotics.** Paper discs contained samples of antibiotic solutions previously incubated with various dilutions of the enzyme. The vertical columns represent dilutions of the EP. A control solution of each antibiotic was incubated without enzyme. Each horizontal row represents a different antibiotic: A, cephalaxin; B, cephaloglycin; C, cephaloridine; D, cephalothin; E, benzyl penicillin. Absence of a zone of inhibition demonstrated degradation of the antibiotic by the indicated dilution of the enzyme.

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5, 2.5, 1.25, and 0.63 μg of antibiotic per ml. A greater volume of the 5 μg/ml solution was prepared for use as a standard on the assay plates. Solutions for the reference curve preparation for the polymyxin-type antibiotics were 80, 60, 40, 20, 10, and 5 μg of antibiotic per ml, employing the 20 μg/ml solution as the standard for the assay plates.

Samples in human sera were prepared to contain 10 and 30 μg of non-β-lactam antibiotics per ml, concentrations that might be found in patients receiving the antibiotics. These samples also contained a constant concentration of 50 μg of cephalaxin, cephaloridine, cephaloglycin, or cephalothin per ml. A 0.1-ml amount of EP was added to 0.3 ml of each serum specimen. A specimen of each antibiotic without the EP was prepared as a control as indicated in Table 1. All specimens, reference solutions, and controls were incubated for 1 hr at 37°C. After incubation of the solutions, paper discs (6.3 mm in diameter) were saturated (0.02 ml) with specimen or appropriate standard and were placed on seeded agar plates in duplicate. Samples containing 30 μg of the non-β-lactam antibiotics per ml were diluted 1:10 in the serum mixture to be read within the linear range of the standard curve. Assay plates for the aminoglycosidic antibiotics were incubated for 6 hr at 37°C, whereas those with colistin or polymyxin B required 16 hr of incubation. Diameters of the resulting zones of bacterial growth inhibition were measured with a Fisher-Lilly zone reader and recorded. To eliminate variation between assay plates, a correction value for each plate was determined by comparing the averaged zone diameters of the two reference standards with the corresponding point on the previously plotted reference standard curve. Application of the correction value to the averaged zone diameters of the two samples on the plate gave a corrected sample zone diameter. Direct reading of the assay value was then obtained from the reference standard curve. Assayed antibiotic activity was divided by 0.75 to compensate for the dilution factor introduced by addition of the EP solution to the serum specimen.

RESULTS

A cell-free culture fluid (EP) containing enzymes produced by E. cloacae effectively destroyed the antibacterial activity of β-lactam antibiotics. Figure 1 illustrates the relative enzymatic activity of EP against five β-lactam antibiotics. A measure of the activity of EP against an antibiotic is shown by the dilution at which the enzyme concentration is no longer sufficient to destroy all antibiotic activity of the mixture. The order of stability of the antibiotics to degradation by EP was found to be cephaloglycin > cephalaxin > benzyl penicillin > cephaloridine > cephalothin.

Figure 2 illustrates the specificity of EP for β-lactam antibiotics and its lack of interference with the activity of the aminoglycosidic antibiotics. In all cases, 100 μg/ml specimens of the β-lactam antibiotics were degraded completely by the undiluted EP. The effects of decimal dilutions of EP illustrate the relative susceptibility of the cephalosporins to the enzymes. By contrast, activities of the aminoglycosidic antibiotics were unaffected by undiluted EP.

Serum specimens containing known mixtures of cephalosporin and aminoglycosidic or polymyxin antibiotics were subjected to the enzymatic action of EP. Assay of the treated specimen for the remaining antibiotic yielded values close to

![Fig. 2. Demonstration of specificity of an enzyme preparation (EP) for β-lactam antibiotics. The discs contained samples of antibiotic specimens previously incubated with the enzyme preparation. The horizontal rows A through C represent 100 μg/ml serum specimens of cephalaxin, cephaloridine, and cephalothin, respectively. Rows D through G represent 20 μg/ml serum specimens of aminoglycosidic antibiotics, gentamicin, nebramycin (factor 6), kanamycin, and streptomycin, respectively. The vertical columns indicate the dilution of the enzyme preparation made before incubation.](http://aem.asm.org/)
those obtained by assay of an identical amount of that antibiotic without the cephalosporin or the EP added. Table I indicates the assay values obtained for each of five antibiotics in specimens containing the antibiotic in combination with each of four cephalosporins. The assay values obtained for the non-β-lactam antibiotics after destruction of the cephalosporins compared favorably with those observed by assay of the antibiotic alone.

**TABLE 1. Assay of non-β-lactam antibiotics after enzymatic degradation of cephalosporins**

<table>
<thead>
<tr>
<th>Enzyme added to degrade</th>
<th>Gentamicin 10 µg/ml</th>
<th>Gentamicin 30 µg/ml</th>
<th>Kanamycin 10 µg/ml</th>
<th>Kanamycin 30 µg/ml</th>
<th>Nebamycin factor 6 10 µg/ml</th>
<th>Nebamycin factor 6 30 µg/ml</th>
<th>Streptomycin 10 µg/ml</th>
<th>Streptomycin 30 µg/ml</th>
<th>Colistin 10 µg/ml</th>
<th>Colistin 30 µg/ml</th>
<th>Polymyxin B 10 µg/ml</th>
<th>Polymyxin B 30 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephalaxin</td>
<td>9.0</td>
<td>27.0</td>
<td>11.2</td>
<td>30.0</td>
<td>9.8</td>
<td>28.8</td>
<td>10.8</td>
<td>32.0</td>
<td>11.4</td>
<td>30.0</td>
<td>9.7</td>
<td>29.2</td>
</tr>
<tr>
<td>Cephaloglycin</td>
<td>8.8</td>
<td>28.0</td>
<td>10.8</td>
<td>36.0</td>
<td>8.9</td>
<td>34.0</td>
<td>10.6</td>
<td>32.0</td>
<td>11.4</td>
<td>32.0</td>
<td>12.6</td>
<td>28.0</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>10.0</td>
<td>34.0</td>
<td>10.8</td>
<td>34.0</td>
<td>9.6</td>
<td>29.6</td>
<td>10.4</td>
<td>36.4</td>
<td>11.6</td>
<td>36.0</td>
<td>9.6</td>
<td>34.0</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>10.4</td>
<td>32.0</td>
<td>10.4</td>
<td>28.0</td>
<td>9.0</td>
<td>28.8</td>
<td>9.8</td>
<td>36.0</td>
<td>11.6</td>
<td>29.2</td>
<td>10.6</td>
<td>26.0</td>
</tr>
<tr>
<td>Control(^a)</td>
<td>10.1</td>
<td>30.5</td>
<td>11.0</td>
<td>32.7</td>
<td>9.7</td>
<td>31.5</td>
<td>10.4</td>
<td>34.5</td>
<td>10.1</td>
<td>30.6</td>
<td>10.9</td>
<td>30.7</td>
</tr>
</tbody>
</table>

\(^a\) Control specimens containing each cephalosporin alone showed no activity after incubation with enzyme preparation.

\(^b\) Specimens with no cephalosporins or enzyme preparation added were assayed for the indicated antibiotic.

\(^c\) Values expressed as micrograms per milliliter.

**DISCUSSION**

Combination therapy with cephalosporins and aminoglycosidic- or polymyxin-type antibiotics creates a problem in proper monitoring of the serum concentrations of the latter two potentially toxic families of antibiotics. Enzymatic degradation of the cephalosporin antibiotic from the mixture allowing subsequent assay of the aminoglycosidic- or polymyxin-type antibiotic is a logical approach. A simple laboratory procedure for the preparation of a suitable enzyme was developed. This procedure was designed to be within the capabilities of a clinical laboratory with minimal equipment and materials.

Selection of a bacterial culture suitable for production of cephalosporinase was done by simply choosing a gram-negative isolate with a low susceptibility to cephalothin. Although an *E. cloacae* was used here, other groups, such as *Pseudomonas* species or *B. cereus* (6), may prove equally satisfactory.

β-Lactamases produced by gram-negative bacteria are generally observed to be intracellular or cell-bound in contrast to those produced by gram-positive bacteria (5). However, exposure of a gram-negative culture to subinhibitory concentrations of cephalosporin releases at least some of the cell-bound enzymes into the broth (5). The use of this technique averted the requirement for sonic treatment or other mechanical lysing of the cells.

All activity due to a cephalosporin antibiotic must be removed from the specimen before attempting to assay the remaining antibiotic. Therefore, the potency of the crude enzyme preparation must be assayed to insure the destruction of the expected cephalosporin levels. A potency assay was used to predict the maximal serum levels of cephalosporin that could be destroyed by this method.

Enzyme titers achieved by the above method proved sufficient to destroy peak concentrations of cephalosporins found in the serum of patients with normal renal function, i.e., about 50 µg/ml or less (3, 4). Concentrations of certain cephalosporins in excess of 100 µg/ml, as in azotemic patients (3), require special consideration. In such cases, the specimen should be appropriately diluted to bring the cephalosporin concentration into the range for enzymatic destruction. Care must be taken not to dilute the serum specimen beyond the detectable limit of the antibiotic being monitored. For instance, if the critical serum level of the monitored antibiotic were 10 µg/ml and the lower detectable limit of the assay of that antibiotic were 0.6 µg/ml, a high dilution of the serum specimen may dilute a critical level (greater than 10 µg/ml, in this case) of the antibiotic to a level not detectable by the assay. A specimen containing 20 µg of the monitored antibiotic per ml, if diluted 1:50, would fall below the lower limit of the assay, rendering a potentially dangerous level of the antibiotic undetected.

Determination of the cephalosporin concentration in a mixture by subtracting the individually determined aminoglycosidic or polymyxin activity from a "total activity" of the mixture is most likely an invalid procedure. Another assay method might be utilized to quantitate the
cephalosporin activity in the mixture. By buffering the assay medium at a pH value outside the optimal range for the non-β-lactam antibiotic, the effect of this antibiotic on the cephalosporin assay may be minimized. However, the validity of this procedure would have to be proven before clinical application could be made.

Assay values for aminoglycosidic antibiotics may be determined within 8 hr from collection of the serum specimen. Growth characteristics of the assay organism, *B. bronchiseptica*, require incubation of the polymyxin or colistin assay plates for at least 16 hr, bringing the minimal time between collection of the specimen and reading the assay to about 18 hr.

This method provides a rapid clinical laboratory assay for determining serum concentrations of potentially toxic antibiotics administered in combination with cephalosporins or penicillins.

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LITERATURE CITED