Simple Assay for Clindamycin in the Presence of Aminoglycosides

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The presence of aminoglycoside antibiotics interferes with the determination of clindamycin levels by standard microbiological assay. A new bioassay method of quantitation of clindamycin in the presence of aminoglycosides is presented. This technique is based on the fact that incorporation of calcium chloride into assay agar blocks the antimicrobial activity of aminoglycosides against *Sarcina lutea* but allows the assay of as little as 0.31 μg of clindamycin per ml. The error of the system is less than 10%. This method is simple and adaptable to existing assay systems for clindamycin.

With the increased recognition of anaerobic bacteria as important agents of human infection, the antibiotic clindamycin has gained prominent usage because of its excellent in vitro activity and demonstrated clinical effectiveness against these organisms, particularly the genus *Bacteroides* (3). Although the levels of clindamycin achievable in serum, bile, saliva (1), synovial fluid (7), and sputum (8) have been well established, there remains a paucity of information regarding levels in other body fluids and tissues. Among these are pleural and peritoneal fluid and brain tissue. The accumulation of such data has been hampered somewhat by the frequent need to administer an aminoglycoside antibiotic to treat mixed infections involving aerobic gram-negative bacteria. Unfortunately, the presence of an aminoglycoside in a clinical specimen will invalidate the microbiological assay for content of clindamycin by standard methods that are currently available. At present, the only published method of assay of clindamycin in the presence of aminoglycosides is a rather demanding disk-plate assay method that requires the use of anaerobic technique (11).

During experiments with synthetic penicillins and aminoglycosides active against *Pseudomonas aeruginosa*, we became interested in the great variation in susceptibility to aminoglycosides that this organism displays in media containing different salt concentrations. Extension of these observations to other organisms useful in bioassay techniques revealed that low yet clinically significant concentrations of clindamycin can be measured in the presence of high levels of aminoglycosides by adding salts in appropriate concentrations to the agar-assay plate. Further refinements of this technique form the basis of this report.

MATERIALS AND METHODS

The assay organism was *S. lutea* (ATCC 9341) grown overnight in Trypticase soy broth (BBL). This organism has been previously used in many laboratories for the assay of clindamycin (1, 3, 7, 8). Large (150 mm) plastic petri dishes (Kimball, Toledo, Ohio) were layered with 20 ml of base agar (antibiotic medium 5, Difco). Melted seed agar (15 ml), also antibiotic medium 5, was cooled to 45 C and inoculated with 1.0 ml of an overnight growth of *S. lutea*, and poured immediately over the previously warmed (37 C) base agar. The pH of both base and seed agar had been adjusted to 7.0 with 1.0 N sodium hydroxide before autoclaving. The pH remained at 7.0 after sterilization. The plates were allowed to solidify on a level surface and refrigerated at 4 C until use. Plates could be stored for 1 week if refrigerated. Increasing amounts of calcium were added as the chloride salt to both base and seed agar of test plates in increments of 5.0 mM to a maximal concentration of 50 mM. When the salt is added to hot, dissolved agar, a slight opacity is created in the agar probably secondary to microprecipitation of calcium salts. This in no way interferes with subsequent measurement of zone sizes. Similarly, magnesium, as the chloride salt, was added in increments of 10 mM to provide a range of concentrations in agar from 10 to 80 mM. Sodium was added to agar as the chloride salt in increments of 50 mM through a range of 50 to 250 mM to compare the effects of monovalent versus divalent cations upon the assay.

Wells were cut in the agar with a 5-mm steel punch and the plugs were removed with a 2-mm steel punch under suction. Ten wells were placed in a circular pattern equidistant from the center of the plate. The wells were filled carefully with portions of serum containing antibiotics using a capillary tube (75 mm by 0.5 to 0.9 mm, Fisher Scientific, Pittsburgh, Pa.). Each assay was run in quadruplicate. Clindamycin

(kindly supplied by the Upjohn Co., Kalamazoo, Mich.), tobramycin (kindly supplied by Eli Lilly and Co., Indianapolis), and dessicated kanamycin and gentamicin (U.S.P. Reference Standards, Bethesda, Md.) were weighed and added to pooled human sera to make up reference standards. The respective sera were diluted serially in quantitative fashion to provide working standards of clindamycin at concentrations of 5, 2.5, 1.25, 0.6, and 0.31 µg/ml. Final working standards of gentamicin contained 20, 10 or 5 µg/ml. The final working standard of kanamycin was 100 µg/ml, and 20 µg/ml for tobramycin. Prior testing of the serum used for antibiotic dilution revealed no inhibitory effect upon the test organism. Total antibacterial activity was measured as zones of inhibition around the wells after 18 h of overnight growth at 37 C by means of a Fisher-Lilly Zone reader (Fisher Scientific, Pittsburgh, Pa.). Standard curves were plotted by the linear regression method of least squares using the logarithm of concentration and zone diameter. Statistical methods used the two-tailed t test (13).

RESULTS

We first defined the effect of gentamicin on the zone sizes of clindamycin standards. When the assay was performed in plain agar without the addition of a salt, the zones of inhibition for clindamycin concentrations of 0.31 and 0.62 µg/ml were enlarged significantly ($P < 0.001$) in presence of either 5, 10, or 20 µg of gentamicin per ml (Table 1). Furthermore, the inhibitory zone surrounding wells containing 1.25 µg of clindamycin per ml also was enlarged significantly ($P < 0.005$) in the presence of either 10 or 20 µg of gentamicin per ml.

In Fig. 1 is shown the effect of calcium chloride on the size of inhibitory zones surrounding wells containing gentamicin in concentrations of 10 and 20 µg/ml when this salt is incorporated into the agar in increasing amounts. At a concentration of 25 mM of calcium in the agar, the antimicrobial activity contained in 10 µg of gentamicin per ml was completely inhibited. Likewise at a calcium concentration of 40 mM, the activity in 20 µg of gentamicin per ml also was totally inhibited.

Progressive inhibition of gentamicin activity also was achieved by increasing concentrations of magnesium chloride through a concentration range of 10 to 40 mM. In most experiments, however, *S. lutea* proved difficult to grow in a concentration of magnesium cation greater than 40 mM. Moreover, this concentration of magnesium did not completely abolish the antimicrobial activity in 20 µg of gentamicin per ml. Sodium ions inhibited gentamicin activity but concentrations as high as 250 mM failed to ablate the antimicrobial activity in 20 µg of gentamicin per ml. Furthermore, zones of inhibition around wells containing clindamycin in any concentration were clearly less defined; inhibitory zones in agar containing sodium appeared to have "fuzzy" edges as compared with those in agar containing calcium.

The utility of incorporating calcium ions into agar for the routine bioassay of clindamycin in the presence of aminoglycosides is illustrated in Fig. 2. On a typical plate containing 75 mM/liter of calcium in the agar, the zones of inhibition are easily read around wells containing 0.31 to 5.0 µg of clindamycin per ml. By contrast, there are no zones of inhibition surrounding separate wells containing 20 µg of gentamicin per ml, 20 µg of tobramycin per ml, and 100 µg of kanamycin per ml, respectively. Thus, it is clear that clindamycin activity alone can be easily measured by this technique and that a reliable standard curve of concentration can be constructed.

Separate studies were performed to determine the effect of calcium, if any, upon clindamycin activity. In agar containing calcium, zone sizes surrounding clindamycin wells were slightly larger than in plain agar. For example, the mean zone diameter at a clindamycin concentration of 0.31 µg/ml was 10.6 mm ± 0.8 in the salt agar versus 7.2 mm ± 0.2 in plain agar. Likewise, at a clindamycin concentration of 1.25 µg/ml, the mean diameter was 18.8 mm ± 0.3 versus 15.3 mm ± 0.5 in plain agar. At 5 µg/ml, the relative values were 27.4 mm ± 0.3 versus

TABLE 1. *Augmentation of clindamycin zone size by gentamicin*

Clindamycin (µg/ml)	Gentamicin (µg/ml)			
	0	5	10	20
0.31	8.3 ± 0.4[a]	15.1 ± 0.4[b]	16.6 ± 0.4[b]	18.9 ± 0.2[b]
0.62	12.8 ± 0.4	15.7 ± 0.5[b]	16.8 ± 0.2[b]	18.7 ± 0.3[b]
1.25	16.1 ± 0.4	16.4 ± 0.4	17.5 ± 0.6[b]	18.5 ± 0.4[b]
2.5	19.8 ± 0.3	19.9 ± 0.6	18.9 ± 0.2	19.7 ± 0.5
5.0	22.7 ± 0.5	22.5 ± 0.6	22.6 ± 0.8	22.5 ± 0.5

[a] Zone size in mm, ± standard error.
[b] Significant change in zone size, $P < .005$.

22.9 mm ± 0.4. We also determined that the presence in serum of gentamicin or tobramycin at concentrations of 20 µg/ml or kanamycin (100 µg/ml) simultaneously with clindamycin does not interfere with activity of the latter antibiotic in calcium chloride agar; quadruplicate assays revealed zone diameters with and without aminoglycoside to be identical at each standard concentration of clindamycin. The relationship between zone diameter and concentration of clindamycin remains linear through antibiotic concentrations of 40 µg/ml. It should be emphasized, however, that zone sizes obtained with concentrations of clindamycin greater than 5 µg/ml are unwieldy. Furthermore, as shown in Table 1, false zone sizes in the presence of gentamicin are only encountered when concentrations of clindamycin are below 2.5 µg/ml. And finally, measurements of four inhibitory diameter zones per assay revealed an error of 10% for each clindamycin standard concentration in the salt agar; this error is acceptable for the regulation of antibiotic dosage in patients (10).
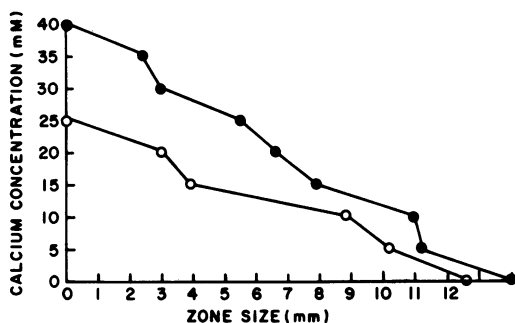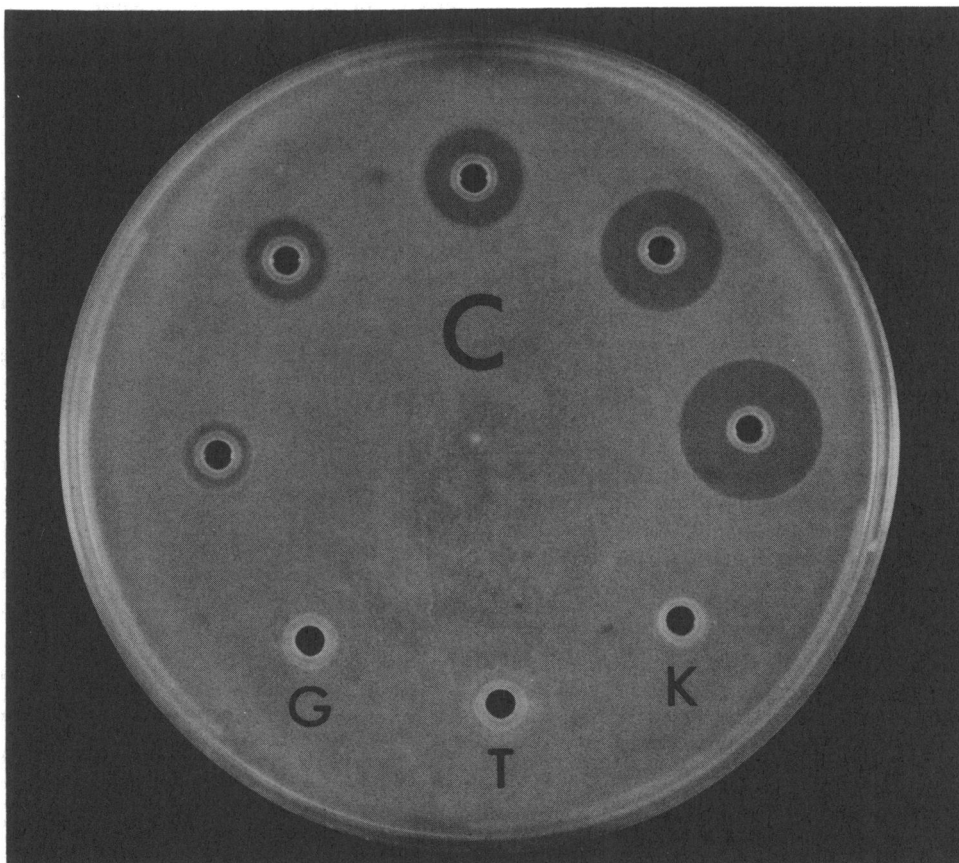
FIG. 1. *Effect of calcium (as chloride salt) on the inhibitory zone size of gentamicin. Zone size is measured as the diameter of inhibition minus well diameter. Concentration of gentamicin (µg/ml): 20 (●), and 10 (○).*

FIG. 2. *Assay plate containing a concentration of 75 mM per liter of calcium in the agar. Zones of inhibition are present surrounding wells containing clindamycin (C) at concentrations ranging from 0.31 to 5.0 µg/ml. No zones of inhibition surrounded wells containing 20 µg of gentamicin (G) per ml, 20 µg of tobramycin (T) per ml or 100 µg of kanamycin (K) per ml. Assay organism: S. lutea (ATCC 9341).*

DISCUSSION

The problem of bioassay for one antibiotic in the presence of another can usually be overcome by using an assay organism that is susceptible to one antibiotic but not the other. Unfortunately, this solution has not been applicable to the assay of clindamycin in the presence of aminoglycosides; to date all aerobic organisms used for the assay of clindamycin also have been susceptible to therapeutic concentrations of the aminoglycosides. The method described herein, whereby aminoglycoside activity is blocked by simple cationic manipulation of the assay agar, provides a simple methodology that avoids the need for a more cumbersome assay using anaerobic techniques.

The mechanism whereby salts interfere with the antibiotic activity of aminoglycosides is not yet fully understood although this phenomenon has been repeatedly documented since the work of Donovick et al. (2). These authors studied the effects of 17 different salts on the action of streptomycin against *Klebsiella pneumoniae* grown in broth that contained molar concentrations of the respective salts ranging from 4 mM through 67 mM. In comparing the effects of different salts at equimolar concentrations, the authors demonstrated that increasing salt concentrations progressively interfered with streptomycin activity as measured by change in the minimal inhibitory concentration (MIC). Salts of $MgCl_2$ and $CaCl_2$ markedly suppressed the action of streptomycin, whereas NaCl had little effect. Anions also differed in their suppressive effect with sulfate demonstrating the largest degree of inhibition.

More recently, Sakurai et al. (12) demonstrated that disks saturated with calcium gluconate facilitated the growth around their periphery of many organisms, both gram positive and gram negative, on a plate containing a concentration of kanamycin that would ordinarily inhibit growth. The intensity of this effect was not uniform among the various organisms tested. Whereas no inhibition of kanamycin activity could be demonstrated around disks containing 0.1% calcium gluconate using any of the bacteria tested, most organisms were able to grow around disks containing 5% calcium gluconate. For example, the latter disk permitted growth of *S. lutea* on agar containing 2 μg of kanamycin per ml. Rubenis et al. (9) have shown that the addition of 4% NaCl (684 mM sodium) to broth raised the MIC of gentamicin 32- to 500-fold against both gram-positive and gram-negative organisms. Likewise, the addition of salts to agar inhibits the antimicrobial

activity of gentamicin in a diffusion assay system using *Bacillus subtilis* (6).

The inhibitory effect of salts probably is not explained satisfactorily by resulting changes in osmolarity of the medium. Zimelis and Jackson (14) have shown that media made hyperosmotic by addition of 20% sucrose, yet kept low in calcium content, increased the MIC of gentamicin only twofold against several gram-negative organisms. Neither does ionic strength appear to be a major determinant of the salt inhibitory effect since Gilbert et al. (4) found no appreciable change in the MIC of gentamicin against *P. aeruginosa* over a wide range of ionic strengths. In similar fashion, changes in ionic strength do not appear to explain satisfactorily the salt effect on streptomycin activity (2).

A number of studies have suggested that the divalent cations of magnesium and calcium are unusually effective in blocking the antibiotic activity of gentamicin and tobramycin against *P. aeruginosa* (4, 5, 14). Indeed, the blocking activity of these cations in systems utilizing *Pseudomonas* is so active at low concentrations that the effect has been considered "specific" for this organism (14). Presumably the blocking activity is due to interaction of cations with *Pseudomonas* at a locus exterior to the cell membrane that results in resistance.

As reviewed above, calcium salts clearly are inhibitory to the antibiotic activity of aminoglycosides against a variety of organisms. Although this activity is most exquisite against *P. aeruginosa*, aminoglycoside activity can be blocked by calcium at a concentration that still allows luxuriant growth of many organisms, including *S. lutea* that is useful in the assay of clindamycin. Advantage has been taken of this fact to develop a new method of assay for clindamycin that is readily adaptable to simple bioassay methods already in current use.

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