Comparative Assessment of In Vitro Inactivation of Gentamicin in the Presence of Carbenicillin by Three Different Gentamicin Assay Methods

STEVEN C. EBERT, JAMES H. JORGENSEN, DAVID J. DRUTZ, and WILLIAM A. CLEMENTI

Departments of Pharmacology, Pathology, and Medicine, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284

Received 9 April 1984/Accepted 9 July 1984

Inactivation of gentamicin (G) is known to occur secondarily to the formation of complexes with certain beta-lactam antibiotics. However, aminoglycosides in the presence of aminoglycoside-beta-lactam complexes may not be recognized uniformly by all assay methods. We tested this hypothesis by using mixtures of G plus carbenicillin (C), with and without the addition of penicillinase, in pooled sera under several in vitro conditions: at 25 and 35°C and at low and high C concentrations. Samples were assayed for G with the EMIT and TDx methods. A microbiological assay was performed with a strain of Klebsiella pneumoniae resistant to C. In the presence of C (500 μg/ml) at 35°C, the initial G concentration of 5 μg/ml decreased markedly after 48 h as assessed by all three assay methods. However, significantly greater degradation was noted when samples were measured by microbiological assay and TDx than by EMIT. Differences between assays were less marked when mixtures were studied at a lower temperature and with a lower G to C ratio (5 μg of G plus 100 μg of C per ml). The addition of penicillinase to the antibiotic mixture prevented the degradation of G over time when measured by all three assay systems. We concluded (i) that EMIT measures higher serum concentrations of G than do TDx or microbiological assays when complexes of G and C are present and (ii) that the addition of penicillinase to serum samples containing C and G would be effective in preventing G degradation during prolonged (>24 h) periods between the time of sampling and assay.

Apparent inactivation of gentamicin via formation of complexes with carbenicillin or other beta-lactam antibiotics is known to occur both in vitro and in vivo (8, 12, 13). Inactivation in vivo has been described primarily in patients with severe renal impairment (2, 13, 14, 17). The assay methods used to quantify the extent of gentamicin inactivation have included radioimmunoassay (3, 6, 12, 14), radioenzymatic assay (5, 10, 11, 16), and high-pressure liquid chromatography (11). All of these assay methods have been able to demonstrate inactivation that is comparable in extent to that seen with microbiological assays (BA's), which measure only biologically active gentamicin in vitro (10, 12, 14, 16). However, comparisons of the EMIT method (enzyme-linked immunoassay) (Syva Co., Palo Alto, Calif.), or the TDx method (Abbott Laboratories, North Chicago, Ill.), a fluoropolarimetric assay, each of which is a common clinical assay, with microbiological methods for characterizing this inactivation have yet to be described.

We questioned whether the EMIT or TDx assay system for gentamicin would be able to distinguish active gentamicin as measured by BA in the presence of gentamicin-carbenicillin complexes. Our suspicions were based on serum gentamicin concentration values measured by EMIT which were consistently higher than values measured by BA in patients who had received concomitant carbenicillin therapy. The following case is an example of such an occurrence: a 64-year-old female with a long history of diabetes mellitus was admitted to our institution for treatment of a case of right lower extremity osteomyelitis. The patient had a substantial degree of renal dysfunction (serum creatinine, 5.5 mg/dl; blood urea nitrogen, 96 mg/dl). The osteomyelitis progressed despite antibiotic therapy, and a below-the-knee amputation was performed. Subsequently, the patient became febrile and developed an infection at the site of amputation, which, when cultured, revealed the presence of Pseudomonas aeruginosa. Therapy with carbenicillin (2 g intravenously [i.v.] every 6 h) and gentamicin (60 mg i.v. every 12 h) began. After 4 days of combined therapy, a sample of serum was obtained for assay immediately after a dose of carbenicillin was injected and 6 h after the last dose of gentamicin was injected. The sample was assayed for gentamicin ca. 12 h later. The measured gentamicin concentration was 3.9 μg/ml by the EMIT method but less than 1.0 μg/ml by BA. Based on these and similar clinical observations, we undertook a study which was designed to compare gentamicin degradation during in vitro incubation with carbenicillin, using the EMIT, TDx, and gentamicin BA methods.

MATERIALS AND METHODS

Study design. Gentamicin (Garamycin, Schering Laboratories, Bloomfield, N.J.) and carbenicillin (Geocillin, J. B. Roerig, Div. Pfizer Inc., New York, N.Y.) in amorphous powder forms were diluted in sterile water to prepare stock solutions of 1 and 25 mg/ml, respectively. These solutions were then added to pooled human sera to yield mixtures containing selected concentrations of each antibiotic.

The experiment was conducted in two phases. In the first phase, an attempt was made to maximize the rate of gentamicin inactivation by carbenicillin. Therefore, gentamicin (final concentration, 5 μg/ml) was mixed with high concentrations of carbenicillin (500 μg/ml) and was incubated in pooled human sera at 25 and 35°C.
The second phase was designed to more closely simulate the in vitro conditions which would occur clinically when a serum sample is obtained and processed for assay. Gentamicin (5 μg/ml) was combined with both high (500 μg/ml) and low (100 μg/ml) concentrations of carbenicillin. Another mixture was prepared in which 1 ml of penicillinase (107 Kersey units per ml; BBL Microbiology Systems, Cockeysville, Md.) was combined with 5 ml of serum containing 5 μg of gentamicin and 500 μg of carbenicillin per ml. These three mixtures were kept at room temperature.

With the exception of those containing penicillinase, all mixtures were composed of at least 95% serum. The mixtures remained intact for 48 h. Samples from each mixture were taken in a volume necessary to perform the three assays (0.5 ml). Each sample was assayed immediately after sampling at 0, 6, 24, and 48 h. Gentamicin was measured in duplicate by the EMIT assay system, the TDx system, and the BA. For comparisons between EMIT and microbiological methods, each experimental condition was repeated on three occasions over a 6-month period. For comparisons with the TDx method, experiments were performed on a single occasion.

Finally, to assess the effect of penicillinase alone on gentamicin stability, 1 ml of penicillinase was combined with 5 ml of serum containing 5 μg of gentamicin per ml and incubated at 35°C.

**Assay comparison.** Data were acquired to assess the analytical performance of each method in measuring gentamicin from freshly prepared mixtures. Serum mixtures containing carbenicillin (500 μg/ml) and gentamicin (in concentrations ranging from 1.0 to 10 μg/ml) were prepared as above and assayed immediately by all three methods.

**BA.** The BA used was similar to that described by Lund et al., utilizing a strain of Klebsiella pneumoniae (ATCC 27799) which was resistant to carbenicillin (7). Samples containing 0.25 ml of an overnight tryptic soy broth culture (ca. 106 organisms per ml) were added to tubes containing 25 ml of molten antibiotic medium 11 (Difco Laboratories, Detroit, Mich.) at 50°C. The contents of these tubes were then poured into petri dishes (diameter, 15 cm). After the medium had cooled to room temperature, 10 5-mm wells were cut in the agar equidistant from the plate edge. Samples (20 μl) of serum containing 1, 5, and 10 μg of gentamicin per ml were added to two wells each (gentamicin standards). Samples (20 μl) of an unknown were added to each of the remaining four wells in each plate. In each case, samples of the same composition were placed in wells on opposing edges of the plate to minimize any effect of nonuniformity of the agar thickness. The plates were incubated for 4 h at 35°C. Standard curves were prepared, and unknown concentrations were determined by plotting the logarthm of gentamicin concentration versus the mean inhibition zone diameter measured by calipers to the nearest 0.1 mm.

**Statistical analysis.** Gentamicin measured by all three assays was expressed as a percentage of the initial gentamicin concentration (5 μg/ml). Measured gentamicin concentrations at time zero for a carbenicillin to gentamicin ratio of 500:5 at 35°C were 5.15, 5.01, and 5.12 μg/ml for EMIT, BA, and TDx, respectively. Similar values were obtained for other experimental conditions. Each gentamicin measurement was expressed as the mean fraction of gentamicin remaining ± one standard deviation. Correlation between assays was determined by linear least-squares regression analysis. Differences between values measured during incubation by the three assay methods and between mixtures were tested by Student-Newman-Keuls and one-way analysis of variance. The alpha value for significance was set at 0.05.

**RESULTS**

**Assay comparison.** Each assay method demonstrated a significant linear relationship throughout the measured gentamicin range of 1 to 10 μg/ml (Table 1). Neither the slopes nor the intercepts of the regression lines for each method differed significantly between each assay comparison. The regression equations for each assay comparison were as follows: EMIT versus TDx, TDx = 1.03(EMIT) + 0.24; EMIT versus BA, EMIT = 1.04(BA) - 0.47; BA versus TDx, BA = 0.96(TDx) + 0.34.

The regression equations for each assay method compared with spiked gentamicin concentrations were as follows: EMIT = 0.91x + 0.454; TDx = 0.93x + 0.26; BA = 0.95x + 0.06.

**Effect of carbenicillin concentration on gentamicin stability.** Concentrations of gentamicin declined to a greater extent when combined with 500 than with 100 μg of carbenicillin per ml at room temperature (Table 2). At 48 h, EMIT measured 61.8 ± 3.1% and 98.3 ± 2.4% of the initial concentrations of gentamicin when combined with 500 and 100 μg of carbenicillin per ml, respectively (P < 0.001). After 48 h of incubation at 25°C, EMIT measured a greater fraction of gentamicin remaining than did either TDx or BA; TDx measured 32.9 ± 0.7% and 84.5 ± 2.0% gentamicin remaining at 25°C and at carbenicillin concentrations of 500 and 100 μg/ml. The values obtained from BA were not significantly different from those obtained with TDx. Similar observations occurred at the higher carbenicillin concentration after 24 h of incubation. With the exception of the 6-h values at the carbenicillin concentration of 100 μg/ml under 25°C, EMIT consistently measured a significantly greater amount of gentamicin than either the TDx or BA.

**Effect of temperature.** A comparison of mixtures of gentamicin (5 μg/ml) plus carbenicillin (500 μg/ml) incubated at 35 and 25°C showed an increased rate of gentamicin degradation at the higher temperature (Table 2). For EMIT and TDx assays, the differences due to temperature did not achieve statistical significance until after 24 h, whereas with the BA a significant difference was noted as early as 6 h after mixing. After 48 h at 35°C in the presence of carbenicillin (500 μg/ml), gentamicin declined to 14.9 ± 3.1% of initial concentrations when measured by BA and 17.4 ± 1.6% by TDx. However, a significantly greater fraction of gentamicin (52.5 ± 7.0%) was measured by the EMIT method under these conditions. Significant differences were also noted between the EMIT method and the other two assays in the fraction of

**TABLE 1. Comparison of three assay methods with freshly prepared standards of gentamicin containing 500 μg of carbenicillin in sera**

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<th>Standard gentamicin concn (μg/ml)</th>
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* Linear progression analysis comparing BA, EMIT (E), and TDx significant correlations: BA versus E, r² = 0.96 (P < 0.001); BA versus TDx, r² = 0.97 (P < 0.001); E versus TDx, r² = 0.99 (P < 0.001).
gentamicin remaining at 6 and 24 h of incubation. At 6 h under these conditions (35°C), the BA measured significantly lower gentamicin concentrations than did either EMIT or TDx. Since the BA requires an additional 4 h of incubation, it is possible that continued inactivation of gentamicin occurred during this time. As the inactivation period became larger, the fraction of gentamicin inactivated during BA incubation contributed less to the total amount of gentamicin inactivated. Good agreement occurred between TDx and BA at 24 and 48 h of incubation. Consistent with the observations at 35°C, EMIT measured a greater fraction of gentamicin remaining at both 24 and 48 h at 25°C than did either TDx or BA. Again the TDx and microbiological methods were in good agreement at 24- and 48-h time periods.

Effect of penicillinase. Penicillinase prevented the inactivation of gentamicin by carbenicillin (500 μg/ml) as measured by all three assays (Table 3). For example, addition of penicillinase to gentamicin-carbenicillin mixtures resulted in the presence of 105.0 ± 2.7%, 109.5 ± 12.1%, and 99.2 ± 3.1% of initial gentamicin concentrations after 48 h at 25°C as measured by TDx, EMIT, and BA, respectively.

DISCUSSION

The results of this study suggest that the EMIT system measures higher gentamicin values than the TDx and BA when gentamicin-carbenicillin complexes are present in sufficient concentrations. Since the assays all correlated well in the presence of carbenicillin but before inactivation, the differences in the measured gentamicin concentrations in the presence of carbenicillin as measured by EMIT versus TDx and BA cannot be explained by differences in precision or sensitivity among the three different assay methods.

Mixtures which showed the greatest extent of inactivation (i.e., 5 μg of gentamicin plus 500 μg of carbenicillin per ml at 35°C for 48 h) demonstrated the largest discrepancies between EMIT and the other two assays. Conversely, in mixtures with little inactivation or in early (i.e., 6-h) specimens, little or no discrepancy was observed. For example, a mixture of 5 μg of gentamicin and 500 μg of carbenicillin per ml at room temperature for 48 h yielded 61.8 ± 3.1% of initial concentrations by EMIT compared with 29.2 ± 3.2% by BA (P < 0.001) and 32.9 ± 0.7% by TDx (versus EMIT, P < 0.001). Under conditions in which minimal gentamicin inactivation occurred (i.e., 5 μg of gentamicin plus 100 μg of carbenicillin per ml at 25°C for 6 h), broad agreement between each of the three assays was observed.

The mechanism most consistent with our findings is that of a decline in gentamicin concentrations when gentamicin is combined with carbenicillin, with a concurrent increase in gentamicin-carbenicillin complexes. The ability of the EMIT system to differentiate these complexes from parent gentamicin appears to be less specific. Since the TDx assay was in good agreement with the BA, this method appears to be more specific in measuring biologically active gentamicin than the EMIT assay. In only two experimental conditions were the TDx values significantly greater than the values obtained from BA (Table 2). These observations are compatible with the longer incubation time required by BA (4 h). An alternative explanation is provided by the regression analysis of each assay method compared with spiked concentrations. The intercept values of 0.45 (EMIT), 0.26 (TDx), and 0.06 (BA) represent ca. 90, 50, and 2.0%, respectively, of the initial gentamicin concentration (5 μg/ml) used in the incubation experiments. Although these values did not differ significantly from zero, a slight overestimate of measured value would have occurred for EMIT and TDx.
The results of this study showing a dependency of the rate and extent of gentamicin inactivation upon carbencillin concentration, length of incubation, and temperature are similar to those reported by others (6, 10). Pickering and Gearhart (10) showed that the extent of gentamicin inactivation during incubation in human sera with carbencillin (600 μg/ml) at 24 h was 41 ± 19% of the initial concentration. Further inactivation occurred at 72 h resulting in only 17 ± 4.3% of initial gentamicin remaining. In that study, gentamicin was measured by using a radioenzymatic method. Although the experimental conditions were slightly different than those in the present study, the fraction of gentamicin remaining at 24 h was similar to our values when measured by BA (35.6 ± 7.2%) and TDX (38.1 ± 0.3%) but not by EMIT (62.3 ± 7.0%).

Preliminary data of interest demonstrating that the EMIT method incompletely detects the aminoglycoside-beta-lactam interaction under in vitro conditions when compared with the BA have been recently reported (M. Pfaller, G. Granich, and P. Murray, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C314, p. 364).

Since inactivation of gentamicin by carbencillin via amide formation results in minimal structural changes in the gentamicin molecule (4), it is possible that the antibody used in the EMIT gentamicin assay displays some affinity for the inactivated species, resulting in elevated gentamicin values. The product information booklet included with the gentamicin assay kit does not address possible cross-reactivity of gentamicin-beta-lactam complexes with the assay antibody (Product Information, Gentamicin EMIT Assay, Syva Company, Palo Alto, Calif.). Interference of structurally related compounds has been shown to occur for other EMIT assays; Aldwin and Kabakoff (1) demonstrated affinity of the EMIT phenytoin assay for the p-hydroxylated metabolite of phenytoin. Pape (9) described cross-reactivity of the EMIT lidocaine assay with the anesthetic meperidine.

From a clinical standpoint, the majority of serum samples from patients receiving both gentamicin and carbencillin therapy contain 100 μg of carbencillin or less per ml and would be assayed for gentamicin within 6 to 8 h after sampling. For these samples, either the TDX or EMIT gentamicin assay would be adequate, since minimal in vitro gentamicin inactivation was shown to occur during this time interval and at low concentrations of carbencillin.

In contrast, in samples from patients with higher serum carbencillin concentrations (i.e., those with poor renal function, receiving full doses of carbencillin), the rate of in vitro inactivation of gentamicin is greater; however, differences in measured gentamicin concentration between EMIT, TDX, and BA are still minimal after 6 h at room temperature. The extent of inactivation (11 to 18%) by these three assays may be important when precise dose adjustments are warranted. However, if these samples were to remain at room temperature for longer periods of time (24 to 48 h), a substantial amount of inactivation would occur, and important differences would exist between EMIT and the other two methods. Therefore it would appear prudent to add penicillinase to these samples. Based on previous work, storage of samples at −70°C also appears to prevent gentamicin inactivation, and this would be an alternative procedure.

The clinical assay of choice of samples left for prolonged (24 h or more) periods of time or of those from patients with severely impaired renal function, in which in vivo inactivation may occur, is uncertain. The EMIT assay will minimize effects of in vitro inactivation because of its nonspecificity; however, it may overestimate biologically active gentamicin concentrations in patients with in vivo production of gentamicin-carbencillin complexes. The issue of whether these complexes should be measured (i.e., whether they are toxic or are hydrolyzed in vivo to yield active gentamicin) has yet to be resolved.

The results of this study suggest (i) that it is possible to demonstrate that the EMIT gentamicin assay measures higher gentamicin concentration values than do TDX or BA when gentamicin-carbencillin complexes are present, (ii) that either EMIT or TDX may be used to measure gentamicin concentrations in the majority of patients receiving carbencillin if the assay is performed promptly after sampling, (iii) that serum samples containing gentamicin and carbencillin which are not to be assayed promptly (i.e., within 24 h) will show less inactivation by EMIT than by the other two assays but should have penicillinase added to them to prevent excessive in vitro inactivation, and (iv) that the therapeutic and toxic potential of gentamicin-carbencillin complexes formed in vivo in patients with severe renal dysfunction should be investigated before their clinical significance can be assessed.

LITERATURE CITED


