An Enzymatic Radioassay for Gentamicin

Richard V. Case¹ and Louis M. Mezel²

An enzymatic, radiochemical assay procedure for measuring serum gentamicin by use of gentamicin 3-acetyltransferase and [acetyl-¹⁴C]acetyl-Coenzyme A is described and evaluated. The enzyme stoichiometrically and quantitatively transfers a radioactive label to the analyte during a 10-min incubation at 37 °C. The labeled gentamicin is then adsorbed onto phosphocellulose paper discs, which are washed to remove unreacted [acetyl-¹⁴C]-acetyl-Coenzyme A and counted in a liquid scintillation counter for 1 min each. The assay detects gentamicin in concentrations as low as 0.2 mg/liter and gives a linear response to concentrations as high as 20 mg/liter. Sisomycin, a structural analog of gentamicin, is measured by the procedure, and tobramycin and netilmicin are slightly reactive. No other interferents were found among other aminoglycosides, other antibiotics, or substances endogenous to serum. Results by the new method are compared to those by radioimmunoassay and a microbiological method.

Additional Keyphrases: monitoring therapy • Gentamicin E-R-A kit • RIA, growth-inhibition assays compared

Gentamicin, an aminoglycoside (aminocyclitol) antibiotic, is widely used in the treatment of serious or life-threatening infections caused by such organisms as Pseudomonas aeruginosa, Proteus species, Escherichia coli, and members of the Klebsiella-Enterobacter-Serratia group. Its mechanism of action is probably analogous to that of streptomycin: interaction with the ribosome to induce inappropriate amino acid incorporation into a protein during its synthesis (1).

As are the other antibiotics of the aminoglycoside class, gentamicin is toxic in man, manifested as loss of hearing or of vestibular function (otoxicity) and impairment of renal function (nephrotoxicity). The symptoms of toxicity may be transitory or permanent. Early clinical reports of gentamicin trials implied a relationship between toxicity and maximum drug concentration (2, 3). More recently, a strong correlation has been demonstrated between maintenance of high minimum concentrations of gentamicin and the appearance of toxicity (4). The manufacturer of gentamicin recommends that "serum concentrations of aminoglycosides should be monitored when feasible, to assure adequate levels and to avoid potentially toxic levels" (5).

Several methods for measuring gentamicin concentrations have been described, but only two techniques are now widely used in the United States: radioimmunoassay (RIA) and a microbiological growth-inhibition assay.

In radioimmunoassay of gentamicin (6), the relationship between concentration of analyte and observed radioactivity can be made linear by use of a log-logit transformation, but many standards and controls typically are required. In all of the gentamicin radioimmunoassays now commercially available (251) is used, which places a practical limit of about two months on the shelf-life of the reagent. Additional drawbacks to the RIA approach include the many manipulations involved, including centrifugation, and the continual change in counting rate of the standards because of the short half-life of the isotope.

In the microbiological assay, standards and specimens containing gentamicin are pipetted onto small discs of filter paper, which are then placed onto an agar plate that has been seeded with a test bacterium or its spores. Antibiotic diffusing out of the paper disc inhibits the growth (or germination of spores) of the test organism, the area of inhibition increasing in proportion to the concentration of antibiotic. Commercial versions of this approach are also available. Its principal drawbacks are its lack of sensitivity and precision, the length of time required, and interference from other antibiotics.

We have attempted to overcome these drawbacks with the enzymatic procedure described in this report. In our procedure, a serum specimen containing gentamicin is incubated in buffered solution with [acetyl-¹⁴C]acetyl-Coenzyme A and an enzyme, gentamicin 3-acetyltransferase (EC 2.3.1.60). The enzyme transfers the labeled acetyl group to the antibiotic. After incubation, the reaction mixture is pipetted onto phosphocellulose paper, to which the labeled antibiotic binds very tightly. The excess [acetyl-¹⁴C]acetyl-Coenzyme A is washed out and the radioactivity in the paper is counted in a liquid scintillation counter. The procedure is calibrated by use of gentamicin dissolved in a matrix of normal human serum.

The technique of adsorbing the highly cationic radiolabeled aminoglycosides to phosphocellulose paper was first described by Davies et al. (7), who used the technique to assay enzymes that modify aminoglycosides. Gentamicin adenyl transferase (no EC no. assigned), a related enzyme, was used by Smith et al. (8) in the first report of a clinically useful assay of gentamicin based on this approach. The purification of gentamicin 3-acetyltransferase was reported by Williams and Northrop (9). A preliminary report of the present work has been given (10).

Methods and Materials

Gentamicin 3-acetyltransferase used in these studies was not less than 50% pure, as assessed by comparison to the specific activity reported for the purified enzyme (9).

Gentamicin used as the primary standard in these studies was obtained from USP-NF Reference Standards, Rockville,
2146

sodium sulfate, Coenzyme X contained: acetate transferase.

Acetyl-gentamicin 5 ml, Acetyl-gentamicin 5 ml, used for the determination of gentamicin A and gentamicin 3-acetyltansferase; 60 μl of this working reagent was mixed with 10 μl of sample in a small disposable test tube and incubated at 37 °C, 10 min. Then 50 μl of this reaction mixture was transferred to a phosphocellulose paper disc suspended above the polystyrene mounting board by being impaled with a pin. After a 1-min adsorption period, the discs were dropped into a beaker of distilled water and washed by soaking for 2 min and decanting. The wash was repeated twice. The paper discs were then blotted to remove excess water and transferred to liquid-scintillation counting vials. One-half milliliter of 1.4 mol/liter ammonium hydroxide was added to each vial, followed by 9 ml of the liquid scintillation fluid described by Bray (12). This and other liquid scintillation fluids were obtained from New England Nuclear Corp. Counting time was 1 min per sample.

For most of the studies reported here we used a Packard Model 3320 liquid scintillation counter. The utility of other radiation detection instruments (e.g., gas-flow counters) has not been evaluated.

In these studies the lower and upper discriminator settings of the liquid scintillation counter were 040 and 1000, respectively, and the gain was set at 8.5%. These settings, routinely used in our laboratory for 14C in solution, gave a counting efficiency of 50% to 60%. Slightly higher counting efficiencies are obtainable with other settings.

Results

Optimization of Reaction Conditions

The pH optimum for the enzymatic reaction is between pH 7 and 8 (10). Tris-HCl buffer, pH 7.5, at a final concentration of 0.07 mol/liter was used in all the studies reported here.

Figure 1 depicts the rate of the enzymatic acetylation of gentamicin at two enzyme concentrations. At the higher concentration of enzyme, the reaction was complete in 10 min, and no further change occurred during 20 additional minutes of incubation at 37 °C. At the lower enzyme concentration, the reaction was essentially complete in 10 min, but there was a slight increase in adsorbed radioactivity on longer incubation.
We chose to use the higher concentration in the present method.

In other experiments, the equilibrium of the reaction was measured spectrophotometrically. For the forward reaction, 1.0 μmol of acetyl-Coenzyme A (0.9 mg) and 1 μmol of gentamicin (0.6 mg) were combined with 0.2 U of gentamicin 3-acetyltransferase in 3.0 ml of Tris-HCl buffer, pH 7.5, and incubated at 37 °C. For the reverse reaction, 1 μmol of acetyl-gentamicin (0.6 mg), prepared as described in Methods and Materials, and 1 μmol of Coenzyme A (0.8 mg) were mixed with enzyme and buffer as above. The time-dependent formation (forward reaction) or loss (reverse reaction) of the Coenzyme A thiol function was measured by adding one volume of the reaction mixture to four volumes of a 1 mmol/liter solution of 5,5'-dithio-bis(2-nitrobenzoic acid) and determining the absorbance at 412 nm (23). In the forward reaction, a rapid increase in absorbance occurred until a 1.0 equivalent of thiol had formed about after about 20 min. In the reverse reaction, a slight decrease in the amount of thiol was independent of the presence of acetyl-gentamicin and was attributed to spontaneous auto-oxidation of Coenzyme A. No enzymatic reaction was observed in 1 h. We concluded that the equilibrium for the reaction strongly favors acetyl-gentamicin formation, and that in the presence of excess acetyl-Coenzyme A, the reaction would be essentially complete.

Williams and Northrop (9) studied the reaction kinetics of gentamicin acetyltransferase, and reported a $K_m$ for acetyl-Coenzyme A of 3.0 ± 0.2 $\times 10^{-6}$ mol/liter. Figure 2 shows the effect of different concentrations of [acetyl-$^{14}$C]acetyl-Coenzyme A on the extent of the enzyme-catalyzed reaction in the present assay protocol. At the lowest concentrations, increases in labeled acetyl-Coenzyme A concentration produced large increases in the adsorbed radioactivity, indicating that the coenzyme is at least partly limiting in the reaction. At a concentration of labeled CoA of $1.1 \times 10^{-5}$ mol/liter, further increases in coenzyme concentration gave negligible increases in adsorbed radioactivity, indicating that the coenzyme is not limiting at this concentration. Thus we chose $1.1 \times 10^{-5}$ mol of labeled acetyl-Coenzyme A per liter as the concentration to be used in all subsequent experiments and in the final protocol.

Adsorbed radioactivity as a function of gentamicin concentration is shown in Figure 3. Dose–response is linear throughout the therapeutic range and into the toxic range. Although the relation becomes nonlinear above a gentamicin concentration of 20 mg/liter, a reasonably good direct relation exists even at the highest concentration studied, 40 mg/liter.

Several commercially available scintillation cocktails were examined for their compatibility with the test system. Several performed acceptably, but the counting efficiency of some gradually changed during several hours. If uncontrolled, this situation could lead to incorrect test results. Bray's solution did not do this, and we chose to use it for the rest of the studies reported here.

Because many scintillation cocktails and modifications are in routine laboratory use, a single system is recommended. Other scintillation cocktails may be adequate, but the laboratory should verify their compatibility with the present method before use.

Performance Characteristics

**Precision.** Precision within run was evaluated by performing multiple analyses on sera containing gentamicin at a therapeutic concentration, 3.8 mg/liter, or at a toxic concentration, 16.9 mg/liter. The standard deviations and coefficients of variation found were 0.13 mg/liter (3.3%) and 0.39 mg/liter (2.3%), respectively.

Day-to-day precision of the procedure was evaluated by analyzing a single specimen in 10 runs on eight different days. The mean concentration was 9.2 mg/liter, the standard deviation 0.23 mg/liter, and the coefficient of variation 2.6%.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gentamicin added, mg/liter</th>
<th>Gentamicin measured, mg/liter</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>12.0</td>
<td>12.3</td>
<td>102.5</td>
</tr>
<tr>
<td>B</td>
<td>8.0</td>
<td>7.8</td>
<td>97.5</td>
</tr>
<tr>
<td>C</td>
<td>1.0</td>
<td>1.0</td>
<td>100</td>
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CLINICAL CHEMISTRY, Vol. 24, No. 12, 1978 2147
Yes
Yes
Yes
Yes
Yes
Yes
Yes

5.1
2.1
0.2

concn.,

Gentamicin
20.4
mg/liter

the

drugs

added

Gentamicin

assuming

expected

netilmicin

concentrations

analyzing

by

F-test

gentamicin

concentrations

by

recovery,

1

Streptomycin

Netilmicin

Kanamycin

Sisomicin

Amikacin

Table 2. Sensitivity Studies

<table>
<thead>
<tr>
<th>Nominal concn.</th>
<th>Mean (n = 6) measured concn. mg/liter</th>
<th>SD</th>
<th>Dispersion same for both concns.?</th>
<th>Value of t</th>
<th>Difference significant?</th>
</tr>
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<tbody>
<tr>
<td>0.0</td>
<td>0.07</td>
<td>0.006</td>
<td>Yes (by F test)</td>
<td>48.9</td>
<td>Yes</td>
</tr>
<tr>
<td>0.2</td>
<td>0.25</td>
<td>0.007</td>
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<td></td>
<td>Yes</td>
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<tr>
<td>0.9</td>
<td>0.90</td>
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<td>9.57</td>
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<tr>
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<td>0.035</td>
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<td>5.62</td>
<td>0.117</td>
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<td>6.0</td>
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<tr>
<td>11.0</td>
<td>11.09</td>
<td>0.281</td>
<td>Yes</td>
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<tr>
<td>12.5</td>
<td>12.64</td>
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</table>

<table>
<thead>
<tr>
<th>Table 3. Specificity of Gentamicin E•R•A™ for Aminoglycosides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. added, mg/L</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Tobramycin</td>
</tr>
<tr>
<td>Amikacin</td>
</tr>
<tr>
<td>Sisomicin</td>
</tr>
<tr>
<td>Kanamycin</td>
</tr>
<tr>
<td>Netilmicin</td>
</tr>
<tr>
<td>Streptomycin</td>
</tr>
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</table>

Accuracy. Standard-addition studies were used to assess the analytical recovery of the procedure. Sera containing gentamicin at the concentrations indicated in Table 1 were prepared and analyzed according to the assay protocol. Table 1 also shows the analytical results and the percent analytical recovery, which averaged 100%.

Sensitivity. The sensitivity of the procedure was assessed by preparing pairs of sera containing slightly different concentrations of gentamicin at each of four concentrations spanning the range of clinical interest. We analyzed each of the eight sera in sextuplicate and calculated means and standard deviations. At each of the four concentrations the F-test was applied to the paired sera to determine the similarity of dispersion. The t-test was then used to determine the confidence limits at which each of these differences in concentration could be distinguished (Table 2).

Specificity. The specificity of the test system was assessed by adding aminoglycosides other than gentamicin to sera and analyzing for gentamicin. Sera were prepared containing three concentrations of each compound (Table 3). Tobramycin and netilmicin gave a slight reaction. Sisomicin, which is 4,5-dehydrogentamicin C1a, reacted very much like gentamicin except that it was only 89% as reactive at the highest concentration tested. Amikacin, kanamycin, and streptomycin were unreactive in this test.

Interference by other antibiotics was also studied (Table 4). Sera were prepared containing the highest clinically expected concentration of each, a concentration determined by assuming the rapid equilibration into 5 liters of blood of the maximum or loading dose recommended for a 70-kg subject. The sera were assayed in the absence of added gentamicin. None of the compounds tested gave the gentamicin reaction. Gentamicin at a final concentration of 20 mg/liter was then added to each of the sera and the assay was repeated. The results indicate no significant interference from any of the drugs tested, with the possible exception of carbenicillin. In the presence of carbenicillin, the measured concentration of gentamicin, 18.8 mg/liter, was not significantly different (95% confidence limits) from the expected 20.0 mg/liter.

Gentamicin and carbenicillin are known to react to form a covalent adduct (14), so this result was investigated further. Carbenicillin (final concentration, 2.0 g/liter) was added to sera containing gentamicin (20 mg/liter) at the intervals before assay indicated in Table 5, the sera were incubated at 25°C, and then assayed for gentamicin by the present method. The result shows a time-dependent decrease in the gentamicin concentration. This is consistent with the formation of a gentamicin-carbenicillin adduct that is not a substrate for the enzymatic reaction.

When serum containing completely inactivated gentamicin was mixed with an equal volume of serum containing 20 mg of gentamicin per liter and the mixture assayed immediately, the gentamicin concentration was found to be 10.2 mg/liter, or 102% of the expected value. Therefore, under the conditions of the assay, the inactivated gentamicin did not interfere with accurate gentamicin determination.

We evaluated the possibility of interference by lipemia, icterus, or hemolysis by obtaining such sera and measuring
recovery at two different gentamicin concentrations. Cholesterol, triglyceride, bilirubin, and hemoglobin concentrations of several sera are shown in Table 6, as is the lack of effect of the abnormal concentrations of these substances on gentamicin measurements with the present procedure.

In another experiment, sera from 39 hospitalized patients who were not receiving gentamicin were analyzed for gentamicin, then gentamicin was added to each serum and the analysis was repeated. In the absence of added gentamicin, no false positive was observed. The mean analytical recovery of 20 mg of added gentamicin per liter was 20.2 mg/liter (SD, 0.85 mg/liter).

Finally, we compared the present enzymatic approach to the assay of gentamicin to a radioimmunoassay procedure. Fifty-seven serum specimens were obtained from patients who were receiving a course of gentamicin therapy. Each specimen was analyzed by an RIA procedure and by the Gentamicin E-R-A™ procedure, and regression analysis by the least squares method was performed. The results are presented in Figure 4.

We also compared the present method with the microbiological growth-inhibition assay. A physician-volunteer administered gentamicin to himself and subsequently took blood samples [in a study of the pharmacokinetics of gentamicin, other results of which will be reported elsewhere (15)]. The serum specimens were analyzed by the Serassay™ microbiological procedure and by the present method. The results are shown in Figure 5.

**Discussion**

We have described here a novel enzymatic method for measuring gentamicin in human serum.

In the present procedure, 14C-labeled acetyl-Coenzyme A was chosen over the tritium-labeled compound, to maximize counting efficiency. An enzymatic assay of gentamicin with use of tritium-labeled acetyl-Coenzyme A has been advocated (16), but the procedure involves elution of the acetyl-gentamicin from phosphocellulose in a separate step in order to obtain an acceptable counting efficiency. We considered this to be too laborious for routine clinical use. It was noted above that the counting efficiency obtained in the present method was 50–60% with instrument settings routinely used in our laboratory. This was considered acceptable in the perspective of the theoretical, instrument-imposed counting efficiency maximum of about 95% for 14C.

The reaction conditions were chosen such that the formation of the reaction product, acetyl-gentamicin, is independent of the concentrations of enzyme and acetyl-Coenzyme A, respectively. The reaction is directly proportional to gentamicin concentration, and the relationship of measured radioactivity to serum gentamicin concentration is both direct and linear over a wide range.

The precision of the present method is of the same order of magnitude as the expected cumulative pipetting error, both within-run and day-to-day.
The sensitivity of the present method was assessed at four gentamicin concentrations spanning the range of clinical interest. The following concentrations could be distinguished from one another at the 99.5% confidence levels: 0 and 0.2 mg/liter; 0.9 and 1.1 mg/liter; and 11.0 and 12.5 mg/liter. Concentrations of 5.6 and 6.0 mg/liter could be distinguished at the 99%, but not at the 99.5% confidence limits. We consider the precision and sensitivity to be completely satisfactory in the perspective of the clinical significance of these gentamicin concentration differences.

The test is highly specific for gentamicin. Sisomicin, a structural analog of gentamicin C16, is measured by the test. Two other aminoglycosides, tobramycin and netilmicin, are detected to a small extent. These drugs would not likely be given in combination with gentamicin.

The reaction of gentamicin with carbenicillin has been reported previously (14). The adduct formed is not a substrate for the enzyme used in the present method. Furthermore, the presence of the gentamicin–carbenicillin adduct in a specimen did not interfere with accurate measurement of unaltered gentamicin in that specimen. Interference due to other antibiotics, icterus, lipemia, hemolysis, and substances present in the serum of hospitalized patients selected at random have been sought. In no case was either a false positive or a false negative observed.

A good correlation (r = 0.90) was found when the present method was compared with a microbiological growth inhibition assay. In a study of hospitalized patients receiving a course of gentamicin therapy, the present method was found to correlate well (r = 0.92) with results by radioimmunoassay.

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References