Measurement of Gentamicin by Radioimmunoassay

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ABSTRACT

A sensitive, specific and precise procedure for the measurement of serum gentamicin by radioimmunoassay is presented. The method is rapid, convenient, and highly reliable for this very important measurement. Studies designed to evaluate the validity and reproducibility of the assay are presented and discussed.

Introduction

Gentamicin is an aminoglycoside antibiotic which has been demonstrated to be effective in the treatment of infection by aerobic Gram-negative bacteria. However, one of the potential dangers associated with the use of gentamicin in clinical practice is the narrow range which exists between therapeutic and toxic concentrations of the antibiotic. Serum gentamicin concentrations between 4 and 12 mg per liter are generally considered to be optimum. Serum drug concentrations which exceed 12 mg per liter may lead to ototoxicity and nephrotoxicity, while concentrations below 4 mg per liter may be inadequate for treatment. As with many drugs, the concentration of serum gentamicin is very difficult to predict without a reliable quantitative method in patients with impaired renal function. As a result of the potential toxicity associated with the administration of gentamicin, the routine monitoring of serial serum gentamicin concentrations is of great clinical significance.

The microbial assay was one of the earlier methods developed for the determination of serum gentamicin concentrations. This assay requires a long incubation time and is potentially less accurate owing to interferences by other antibiotics present in the sample. Over the past six years, other more accurate and precise methods have been published which include the enzymatic assay, radioimmunoassay, fluorescence polarization immunoassay, fluorescence immunoassay, high pressure liquid chromatography, and enzyme immunoassay. All of these newer methods have demonstrated the acceptable accuracy and precision required for the serial monitoring of patients receiving gentamicin.

Here are reported the data collected during the validation of a pre-precipitated double antibody radioimmunoassay (RIA) method for serum gentamicin, which uses...
125I-gentamicin as the tracer. The assay is a modification of the pre-precipitated double antibody radioimmunoassay originally described by Hales and Randle. The method is simple, fast, sensitive and precise.

**Principle**

The RIANEN® gentamicin radioimmunoassay is a pre-precipitated, double antibody radioimmunoassay procedure. The primary antiserum (sheep), generated against a gentamicin-bovine serum albumin conjugate, is pre-reacted with anti-sheep (goat) gamma globulin to form a pre-precipitated primary and secondary antibody complex. A sample of patient serum or gentamicin standard and a known amount of 125I-labelled gentamicin derivative are placed in a test tube to which a known quantity of the pre-precipitated antibody complex is added to initiate the reaction. During the specified incubation period, the 125I-labelled gentamicin derivative and the unlabelled gentamicin present in the patient serum or standards compete for the available antibody binding sites on the pre-precipitated antibody complex. After incubation, the 125I-labelled gentamicin derivative and the unlabelled gentamicin not reacting with the anti-gentamicin serum are separated from the antibody-bound gentamicin by centrifugation. As the concentration of the unlabelled gentamicin in standard or patient specimens increases, the amount of labelled gentamicin bound to the pre-precipitated complex decreases, and vice versa. If the concentrations of the labelled gentamicin and antibody complex are constant, by using several known concentrations of unlabelled gentamicin, a standard curve can be generated which can be used for quantifying unknown specimens.

**Materials and Methods**

Gentamicin Radioimmunoassay Kit "RIANEN" which was purchased contains all of the reagents necessary for performing the assay.

**GENTAMICIN ANTISERUM COMPLEX**

Gentamicin antiserum, produced in sheep against gentamicin-bovine serum albumin conjugate, is pre-precipitated with goat anti-sheep gamma globulin in 10 mM phosphate buffer, pH 7.4.

**Radioiodinated Gentamicin**

The iodinated derivative of gentamicin has a structure similar to that described previously. The tracer is prepared in 10 mM phosphate buffer, pH 7.4. The specific activity is approximately 100 c per g.

**Blank Antiserum Complex**

Normal sheep serum pre-reacted with an antiserum to sheep gamma globulin. This blank antiserum complex is used to determine the non-specific binding.

**Gentamicin Standards**

The standards are prepared in gentamicin-free serum at concentrations of 1.0, 2.0, 4.0, 8.0 and 16.0 mg per liter.

**Gentamicin, Tobramycin, and Amikacin**

Gifts were received of gentamicin sulfate (Garamycin®), Tobramycin sulfate (Nebcin®) standard solution (1000 mg per liter), and Amikacin sulfate (Amikin®).

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* Purchased from New England Nuclear, North Billerica, MA 01862.
Radioimmunoassay Procedure

Aliquots (50 µl) of the individual gentamicin standards (0, 1.0, 2.0, 4.0, 8.0, and 16.0 mg per liter), controls, and unknowns were diluted with 5.0 ml of deionized water. Duplicate disposable polypropylene tubes (12 x 75 mm) were marked for each standard, control and unknown. Fifty µl of each 1:101 dilution were pipetted into appropriately marked tubes followed by the sequential additions of 500 µl of 125I-gentamicin and 500 µl of the pre-precipitated anti-gentamicin serum. The tubes were vortex-mixed for three to five seconds between additions. Duplicate non-specific binding tubes were prepared by adding 50 µl of deionized water, 500 µl of 125I-gentamicin, and 500 µl of the blank antiserum complex to duplicate tubes. The total counts were determined by counting duplicate 500 µl aliquots of the 125I-gentamicin. All of the tubes were vortex-mixed gently for three to five seconds and incubated for 10 minutes at room temperature. Following this incubation period, all of the tubes (with the exception of the total count tubes) were centrifuged at 1600 × g for 10 minutes at 4°, and the supernates were discarded. The tubes were blotted on absorbent paper to remove residual radioactivity from the lips of the tubes, and the radioactive counts in the individual precipitates were determined by counting in an automatic gamma scintillation spectrometer for two minutes.

Calculations

The duplicate counts for the total counts, standards, and patient specimens were averaged. The standards, patient specimens, and controls were calculated as a percentage of the zero standard in order to determine the percent binding of the antiserum for each of the other standards, patient specimens, and controls. The calculations were performed as follows:

\[
\text{percent } B = \left( \frac{\text{Average counts of standard or specimen-blank}}{\text{Average counts of zero standard-blank}} \right) \times 100
\]

The standards were subjected to linear regression analysis of the logit transformation of the percent bound versus the log10 of the gentamicin concentration. From this analysis, the slope and the y-intercept of the standard line were obtained. Using the formula for a straight line (where y = logit of the percent bound and x = log10 of the gentamicin concentration), the concentration of gentamicin in each patient specimen and control was computed.

Results

Sensitivity and Specificity of the Anti-Gentamicin Serum

In figure 1 is illustrated a typical semi-logarithmic standard curve for the gentamicin radioimmunoassay. The assay demonstrated a detection limit, defined as the smallest quantity of gentamicin which could routinely be distinguished from the zero standard, of approximately 900 pg. This detection limit corresponds to a serum concentration of approximately 1.0 mg per liter. The binding of the 125I-gentamicin derivative to the anti-gentamicin serum in the absence of unlabelled gentamicin averaged 55 ± 5 percent of the total radioactivity. The non-specific binding (125I-gentamicin, zero standard, and blank antibody complex) averaged 5.0 ± 0.5 percent of the total radioactivity. Although the standard curve shown in figure 1 indicates a high standard of 16.0 mg per liter, all patient and control specimens ≥ 14.0 mg per liter were routinely diluted with an equal vol-

\[\text{Searle Model 1285, Searle Analytic, Inc., Des Plaines, IL 60016.}\]
The volume of gentamicin-free serum and reassayed. The final value is corrected for dilution.

Evaluations of the cross-reactivities of tobramycin and amikacin in the gentamicin assay are depicted in figure 2. Tobramycin at a concentration as high as 1000 mg per liter did not result in a demonstrable displacement of the 125I-labeled gentamicin derivative from the antibody complex. Similarly, amikacin at a concentration of approximately 2000 mg per liter did not affect the accuracy of the gentamicin assay. Furthermore, the following antibiotics were assayed individually with the radioimmunoassay procedure for gentamicin at a concentration of 1280 mg per liter and were found not to cross-react with the anti-gentamicin serum: streptomycin, clindamycin, penicillin, erythromycin, lincomycin, tetracycline, kanamycin, natidixic acid, chloramphenicol, vancomycin, polymyxin B, dicloxacillin, methicillin, and cephalothin.

The specificity of the anti-gentamicin serum was further evaluated by determining the parallelism of the radioimmunoassay. In figure 3 are illustrated the data obtained following the analysis of serial dilutions of three specimens collected from patients known to have received gentamicin as part of their therapy. The excellent proportionality observed (figure 3) between the amount of gentamicin quantified and the quantity of specimen analyzed for all three specimens documents the immunochemical similarity between the gentamicin measured in serum and the gentamicin used as the reference standard.
ANALYTICAL RECOVERY

The accuracy of the gentamicin assay was determined by adding known quantities of crystalline gentamicin (5.0, 10.0, and 20.0 mg per liter) to sera collected from patients receiving gentamicin as part of their therapy. The data presented in table I indicate that the analytical recovery of gentamicin from the sera of these patients averaged 102 percent.

INTRA-ASSAY AND INTER-ASSAY VARIATION

The intra-assay variation of the method was determined by assaying three different quality control pools on the same day (n = 20). The inter-assay variation was determined by assaying the same control pools over a period of 50 consecutive assays. The data accumulated from both studies are summarized in table II. The intra-assay variation was generally < 10 percent at a gentamicin concentration of approximately 3.2 mg per liter and < 5 percent for values between 10 and 16 mg per liter. Similar precision data were observed for the inter-assay variations.

Discussion

The radioimmunoassay technique described is an excellent method for the determination of gentamicin by the clinical chemistry laboratory. The rapidity, sensitivity and specificity characteristics of the method render it useful both as an aid to the clinician in arriving at accurate management decisions and as a tool for investigators studying the pharmacodynamics of gentamicin.

Sources of Error

Serum or EDTA plasma collected by standard procedures may be used in this assay. If plasma samples are used, they should be fresh, not previously frozen. Serum samples may be stored between 2°C and 8°C for assay within 48 hours or frozen for longer periods of time. Specimens must be free of particulate matter, such as red cells, fibrin strands, or insoluble proteinates, because they may interfere with the method. The gentamicin standards, control sera and all patient specimens must be diluted in polystyrene or polypropylene tubes to minimize adsorption to container walls. Dilution of sera containing gentamicin in glass containers results in substantial adsorption of the antibiotic to the surface of the container.

In order to obtain an assay value which represents the steady-state physiological level, peak gentamicin levels should be obtained within one-half hour at the end of the infusion for intravenously administered gentamicin or one hour after intramuscular injection. Trough levels

<table>
<thead>
<tr>
<th>Specimen</th>
<th>A (mg/liter)</th>
<th>B (mg/liter)</th>
<th>C (mg/liter)</th>
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<tbody>
<tr>
<td>1</td>
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<td>5.0</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>7.1</td>
<td>20.0</td>
<td>24.4</td>
</tr>
</tbody>
</table>

*Recovery (percent) = \( \frac{C - A}{B} \times 100 \).

Intra-Assay Variation

<table>
<thead>
<tr>
<th>Control</th>
<th>Number 1</th>
<th>Number 2</th>
<th>Number 3</th>
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<tbody>
<tr>
<td>Mean (mg/liter)</td>
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<td>15.6</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.3</td>
<td>0.5</td>
<td>0.7</td>
</tr>
<tr>
<td>C.V. (percent)</td>
<td>9.4</td>
<td>4.7</td>
<td>4.5</td>
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</table>

Inter-Assay Variation

<table>
<thead>
<tr>
<th>Control</th>
<th>Number 1</th>
<th>Number 2</th>
<th>Number 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (mg/liter)</td>
<td>2.9</td>
<td>11.5</td>
<td>17.9</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.3</td>
<td>0.6</td>
<td>1.0</td>
</tr>
<tr>
<td>C.V. (percent)</td>
<td>9.4</td>
<td>5.0</td>
<td>5.8</td>
</tr>
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</table>
should be obtained just prior to the subsequent dose.

Resumé of Clinical Investigations

Peak gentamicin levels of 4 to 8 μg per ml are generally considered acceptable for treating serious Gram-negative bacillary infections. \(^13\) Trough values of 2 μg per ml or greater\(^7\) and peak values of 12 μg per ml are generally considered acceptable for treating serious Gram-negative bacillary infections. \(^13\) Trough values of 2 μg per ml or greater\(^7\) and peak values of 12 μg per ml or greater\(^11,20,21\) have been associated with toxicity. Peak and trough gentamicin levels should be collected during the first day of treatment and every three to four days thereafter in patients with stable renal function. Elderly patients and those with pre-existing renal impairment should be monitored prior to and after every dose to avoid toxicity.

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References