Evaluation of Serum Gentamicin Assay Procedures for a Clinical Microbiology Laboratory

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Four methods for the measurement of serum gentamicin concentration were evaluated with respect to cost-effectiveness, accuracy, and precision. Gentamicin concentration was determined in 112 clinical samples by the *Staphylococcus epidermidis* agar diffusion bioassay procedure in routine service in our laboratory at the time this study was initiated. Appropriate portions of these clinical samples were frozen and later thawed for remeasurement of gentamicin by bioassay or for measurement of gentamicin in one of three other systems. These included the Enzymatic Radiochemical Assay, the Diagnostic Products Corporation Radioimmunoassay and the New England Nuclear Corporation Radioimmunoassay. In addition, gentamicin dissolved in horse serum at 2, 4, 6, 8, 10, and 12 μg/ml was aliquoted, frozen, and later thawed for assay in each of the above systems. The data were analyzed for evidence of constant and proportional bias as well as for accuracy and precision.

Gentamicin is an aminoglycoside antibiotic used frequently in the treatment of gram-negative bacillary infections. It has a low therapeutic index and is potentially ototoxic and nephrotoxic. Serial monitoring of serum levels is an important factor in adjusting dosage of this antibiotic, especially if compromised renal function is present (3, 4, 7). Our laboratory has used for several years a modification of the agar diffusion bioassay procedure (1, 6) to monitor serum gentamicin levels. This procedure is time-consuming, labor-intensive, and suffers from a lack of specificity (e.g., interference by other antimicrobial agents). More specific and rapid assays have been available for several years and include the enzymatic acetylator assay and the radioimmunoassay. Recently the enzymatic assay became commercially available as the Enzymatic Radiochemical Assay (ERA). The radioimmunoassay (RIA) is now available from a number of commercial sources. Previous comparative studies (5) indicate that each method has advantages and disadvantages which should be evaluated by individual laboratories to determine the method best suited to their needs. The purpose of the present study was to evaluate the ERA and two commercially available RIAs with a view to selecting the most appropriate method for measuring serum gentamicin levels of patients at our hospital.

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MATERIALS AND METHODS

Samples containing gentamicin. Two types of samples were used in this study. The first type consisted of 112 serum samples taken from patients receiving gentamicin. The concentration of gentamicin in each sample was determined initially upon receipt by the microbiology service by using the bioassay procedure described below. The remaining serum was stored frozen at 0°C in tightly capped plastic tubes. Between 2 and 12 months later, the samples were thawed, and appropriate portions were refrozen at 0°C in tightly capped plastic tubes. Between 1 and 5 months after the portions were refrozen, they were thawed, and gentamicin concentration was remeasured in the bioassay or in the ERA, Diagnostic Products Corporation Radioimmunoassay (DP-RIA), or New England Nuclear Corporation-RIA (NEN-RIA) as described below. The second type of sample assayed was made by dissolving gentamicin sulfate (Schering Corp., Kenilworth, N.J.; potency, 537 μg/mg) in sterile, filtered (0.45 μm Nalgene filter; Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.) horse serum (HS; Microbiological Associates, Walkersville, Md.). Gentamicin was dissolved in HS to a concentration of 24 μg/ml. Appropriate dilutions of this solution were made by using HS to obtain solutions containing gentamicin at concentrations of 2, 4, 6, 8, 10, and 12 μg/ml, respectively. Amounts of these solutions were frozen (0°C) in tightly capped plastic tubes and thawed between 1 and 5 months later for measurement of gentamicin concentration in each of the four assay systems.

With minor modifications, the assay performed in our laboratory was similar to that (1, 6) formerly used by the Mayo Clinic, Rochester, Minn. Neomycin assay agar (30.5 g; BBL Microbiology Systems, Cockeysville,
Md.) was dissolved in 1 liter of distilled water. Portions of 195 ml were added to flasks, and the pH was adjusted to the range of 7.3 to 7.5 by the addition of 1 N HCl. Flasks were stored refrigerated at 4°C for up to 2 weeks. After melting the agar in a flask and cooling it to 45 to 50°C, 8 ml of a very dense suspension of *Staphylococcus epidermidis* (ATCC 27626) was added, and the contents of the flask were mixed by swirling. Approximately 8 ml of this seeded agar was poured as a layer into a standard petri dish (100 by 15 min) and, after cooling, the dish was wrapped in plastic and stored refrigerated for up to 1 week. Plates were brought to room temperature before use. Gentamicin standards for the bioassay were prepared from gentamicin sulfate (Schering Corp.) by dissolving crystalline drug in sterile distilled water to a concentration of 240 μg/ml. Portions of this solution were stored frozen (0°C) for several months. Before each bioassay, a portion was thawed and diluted with HS to obtain a solution of 12 μg/ml. Serial dilutions of this were then made to obtain standards in HS at 4, 3, 2, and 1 μg/ml. Sample preparation for the bioassay was as follows. Three polypropylene tubes (12 by 75 mm; Falcon Plastics, Oxnard, Calif.) were labeled as u (undiluted), 1:2 (1:2 dilution, vol/vol), or 1:4 (1:4 dilution, vol/vol). Serum (four hundred μl) containing gentamicin was added to the tube labeled u. A 20-μl portion of a solution containing β-lactamase was added, and the contents of the tube were mixed. (β-Lactamase was obtained from Whatman Biochemicals Ltd., Maidstone, Kent, England, as a lyophilisate containing a minimum of 50 U of β-lactamase II and 500 U of β-lactamase I per vial; the lyophilisate was reconstituted in 3 ml of sterile distilled water and stored refrigerated for up to 2 weeks.) To the tube labeled 1:2 was added 200 μl of the solution from tube u and 200 μl of HS. After mixing, 200 μl from tube 1:2 was added to tube 1:4 and mixed with 200 μl of HS. Duplicate absorbent paper disks (Schleicher and Schuell, Inc., Keene, N.H.; 740E, 6.35-mm diameter) were saturated with 20 μl of the appropriate solution containing gentamicin. The disks were placed on duplicate plates containing *S. epidermidis* seeded agar prepared as described above. Plates were covered and incubated at 35°C for a minimum of 12 h. The size of the zone of inhibition of growth was read to the nearest 0.1 mm by Mitutoyo Dial Calipers (Laboratory Supplies Co., Inc., Hicksville, N.Y.) from duplicate plates. Duplicate values were averaged and rounded to the nearest 0.1 mm. Standard curves plotting zone diameter size in millimeters (usually between 10 and 14 mm) on the y-axis and concentration of the standard in micrograms (between 1 and 4) per milliliter on the x-axis were constructed on semilogarithmic paper. Average zone sizes for samples were used to determine micrograms per milliliter from the x-axis. Only dilutions of samples giving zone sizes on the linear portion of the standard curve were used. Appropriate multiplication was performed to correct for dilution of samples with HS and with β-lactamase solution. For a given sample, values obtained from u, 1:2, or 1:4 tubes were averaged as appropriate to obtain the concentration of gentamicin.

**ERA.** The ERA was obtained as 100-tube kits from PL Biochemicals, Inc., Milwaukee, Wis. The basis of the method (2) is the acetylation of gentamicin with [*]C]acetel coenzyme A by gentamicin acetyltransferase. The [*]C]acetelgentaminic is isolated on phosphocelulose filter disks. A summary of the details of the procedure is included for clarity. Portions of frozen clinical samples or HS containing gentamicin at 0, 2, 10, and 20 μg/ml were prepared from the kit. A working reagent for each assay was prepared from the kit being used and contained 10 volumes of buffer solution, 1 volume of [*]C]acetel coenzyme A, and 1 volume of gentamicin acetyltransferase. Sixty μl of this working solution was pipetted into each polypropylene tube (12 by 75 mm). All samples were assayed in duplicate. At timed intervals of 30 s, 10 μl of sample was added to the appropriate assay tube, the contents were mixed, and the tube was placed in a 37°C water bath. Exactly 10 min later in timed sequence, 50 μl of each reaction mixture was pipetted onto a numbered phosphocelulose paper disk previously mounted on a stainless steel pin stuck in a polystyrene mounting board. After allowing at least 1 min for the reaction product to adsorb to the paper, all disks and pins were removed from the mounting board and dropped into a beaker containing a minimum of 10 ml of distilled water for each sample disk to be washed. The contents of the beaker were swirled gently for 2 min, the distilled water was decanted, and the beaker was refilled with the same volume of distilled water. The contents of the beaker were swirled and allowed to stand at least 2 min. A third wash was performed in the same manner. The pin and paper disks were transferred to an absorbent paper towel. Pins were removed, and disks were arranged in numerical order. Each disk was blotted gently and placed in an appropriately numbered scintillation vial. To each vial was added 0.5 ml of ammonium hydroxide (1.4 mol/liter), thoroughly wetting the paper disk. After allowing at least 1 min to elapse, 12 ml of scintillation cocktail (Packard Insta-Gel; Packard Instrument Co., Downers Grove, Ill.) was added to each vial, the contents were swirled, and the vials were counted for 1 min. Standard curves were prepared by plotting counts per minute × 10^3 on the y-axis versus micrograms per milliliter of standard on the x-axis. Average counts per minute for duplicate experimental samples were used to determine the number of micrograms per milliliter.

**DP-RIA.** Kits in the 100- and 500-tube size were obtained from the Diagnostic Products Corporation, Los Angeles, California. Portions of clinical samples or HS containing gentamicin were thawed. Gentamicin standards for construction of the standard curve were prepared from each kit (0, 1, 2, 4, 8, and 16 μg/ml). All gentamicin solutions were diluted 1:201 (vol/vol) in tris(hydroxymethyl)aminomethane buffer before assay, and all samples were assayed in duplicate. To each polypropylene test tube (12 by 75 mm) was added 100 μl of either diluted standard, clinical sample, or HS-gentamicin sample. [125I]Gentamicin (100 μl) was then added to each reaction tube, and the contents were mixed thoroughly. The tubes for determination of total counts (T tubes) were set aside and not processed further. To all remaining tubes, except nonspecific binding (NSB) tubes, was added 100 μl of reconstituted gentamicin antiserum (primary antibody). To
all tubes was added 100 µl of reconstituted goat anti-rabbit gamma globulin (secondary antibody). All tubes were blended in a Vortex mixer followed by incubation at room temperature (21 to 24°C) for 5 min. To each tube was added 2.0 ml of cold 6% (wt/vol) polyethylene glycol-normal saline solution. All tubes were centrifuged (Sorvall RC-3 refrigerated centrifuge) at 2,030 × g for 20 min at 4°C. The supernatant solution was decanted from all tubes, and the rim was blotted. All tubes were counted (Micromedics 588 Gamraa Counter) for 1 min. Duplicate values were averaged. Correction for nonspecific binding was made by subtracting the average counts in the NSB tubes from all other average values. The standard curve was constructed by plotting percent of maximum binding on the y-axis versus the natural logarithm of concentration of gentamicin standard on the x-axis. Averaged values of percent maximum binding for clinical samples and HS-gentamicin samples were used to determine gentamicin concentration in micrograms per milliliter.

NEN-RIA. Kits in the 100- and 500-tube size were obtained from the New England Nuclear Corp., Boston, Mass. The methodology for this double antibody RIA is similar to that described above. Important differences are as follows. This kit contains two quality control samples labeled I and II containing gentamicin at nominal concentrations of 3 and 12 µg/ml, respectively. All gentamicin solutions are diluted 1:101 (vol/vol) in distilled water before assay. Diluted sample (50 µl) is added to each polypropylene test tube (12 by 75 mm). [125I]gentamicin tracer solution (500 µl) is added to each tube. Total count tubes are set aside and not processed further. The antiserum complex in this kit contains both primary and secondary antibodies pre-mixed in polyethylene glycol solution. After allowing the antiserum complex solution to come to room temperature, 500 µl is added to each tube except nonspecific binding tubes. To the latter tubes is added 500 µl of blank antiserum complex. All tubes are blended in a Vortex mixer and then incubated at room temperature for 10 min. After centrifuging at 1,000 × g for 10 min at 4°C, the supernatant liquid is decanted, and the rim of each tube is blotted. All tubes are counted for 1 min in a gamma counter. Construction of the standard curve and determination of results is performed in a manner similar to that described above.

Duration of the study. The duration of this study was 7 months. During month 1, 8% of the ERA experiments were performed. During month 2, the percentages of assays performed were: ERA, 42%; bioassay, 33%; DP-RIA, 15%; and NEN-RIA, 0%. In month 3, the percentages were: ERA, 33%; bioassay, 33%; DP-RIA, 15%; and NEN-RIA, 0%. In month 4, the percentages were: ERA, 17%; bioassay, 26%; DP-RIA, 0%; and NEN-RIA, 0%. In month 5, 8% of the bioassay experiments were performed. In month 6, 35% of the DP-RIA and 50% of the NEN-RIA experiments were performed. In month 7, the percentages were the same as for month 6. No consistent significant changes in the mean values for the HS gentamicin samples were noted over this 7-month period with any of the assay methods.

RESULTS

Figure 1 shows a standard curve for the bioassay based on 12 separate experiments. The x-axis shows the gentamicin concentration in micrograms per milliliter, whereas the y-axis shows the size in millimeters of the diameter of the zone of growth inhibition. The data are expressed as the mean plus or minus one standard deviation. Figure 2 displays the standard curve for the ERA based on 12 experiments. The x-axis depicts gentamicin concentration (micrograms per milliliter) and the y-axis shows counts per minute × 10⁵ of reaction product. The data are graphed as the mean plus or minus one standard deviation. The curve appears to be linear from 2 to 20 µg/ml. The standard curve
for the DP-RIA is shown in Fig. 3; on the x-axis is the natural logarithm of the gentamicin concentration, whereas the y-axis shows the percentage of maximum binding of $[^{125}I]$gentamicin. The mean plus or minus the standard deviation from 20 experiments is shown. Figure 4 shows the standard curve for the NEN-RIA with the natural logarithm of the gentamicin concentration on the x-axis and the percent maximum binding of $[^{125}I]$gentamicin on the y-axis. The mean plus or minus one standard deviation of 20 experiments is shown.

In Fig. 5 are the data comparing the repeated measurement of gentamicin in HS; the x-axis shows the calculated concentration (micrograms per milliliter), i.e., the concentration resulting from dilution of gentamicin powder in HS, whereas the y-axis shows the mean of 12 experiments in which gentamicin concentration was determined by each of four methods. A method which measured gentamicin in an ideal fashion would give a line in Fig. 5 with slope of 1.0 and y-intercept of 0. The NEN-RIA appears to overestimate the gentamicin concentration, whereas the bioassay appears to underestimate the concentration. Two of the methods (ERA and DP-RIA) gave curves which were very nearly superimposed on the theoretical line of identity.

Table 1 presents the results of linear regression analysis of the data in Fig. 5. For all methods it can be seen that the degree of correlation between the observed means and the calculated values was very high, with the correlation coefficient ($r$) approaching a value of 1.0. A measure of methodologic (constant) bias is provided by testing the hypothesis that the y-intercept of the regression line for a given method is significantly different from 0 (i.e., is the value of $b$ in the regression equation significantly different from 0?). The results are presented in the column labeled $P$, and the hypothesis that the NEN-RIA has a y-intercept significantly different from 0 cannot be rejected. It should be noted that the control material included with the NEN-RIA kit gave values (mean of 12 experiments plus or minus one standard deviation) of 2.9 ± 0.7 and 11.9 ± 1.3 µg/ml for control materials I and II.
respectively. The nominal values of these materials were reported as 3.0 and 12.0 μg/ml. Thus, the method appears to have been in control by the criteria of the quality control material included with the kit.

Since the 95% confidence interval excluded the value 1.0 for the slope in the bioassay, the bioassay appears to exhibit proportional bias.

Table 2 presents precision data for each method. The results are expressed as coefficients of variation at each concentration for which gentamicin was measured. There was no outstanding difference in the range of coefficient of variation values for the different assay systems.

Table 3 shows the observed means from Fig. 5 expressed as percentages of the calculated values. Five of six of the recovery values for the bioassay were less than 100%. Most of the values for the ERA and DP-RIA were approximately 100%. All of the values for the NEN-RIA were greater than 100%.

Table 4 shows the correlation coefficients for the regression lines obtained by plotting measured gentamicin concentration in each method against each of the other methods for 112 clinical samples. Inspection of Table 4 reveals that the correlation coefficients were smaller when the original bioassay was considered the reference method. There are several considerations which may account for this observation. First, all of the repeat bioassays were performed by one individual (C.H.L.) over a relatively short period of time (1 to 5 months), whereas the original bioassays were performed by a number of individuals over a relatively longer time period (approximately a 12-month period). Second, it is possible that a chemical change related to storage of the frozen portions occurred during the time of the study so that the repeat bioassay, ERA, DP-RIA, and NEN-RIA might have been performed on samples that had changed since the original bioassay. Because the repeat bioassay was performed under sample conditions similar to the other methods, it is appropriate to use the repeat bioassay as the reference method with which to compare the results of the other methods for the clinical samples.

The data in Fig. 6 show the results of a linear regression analysis in which the results for clinical samples measured in the ERA (y-axis) are compared to the results in the repeat bioassay (x-axis). The results for clinical samples mea-
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Table 6 shows the cost of determining a single serum gentamicin concentration as a function of the method used and as a function of the size kit from which materials are obtained, as well as the cost of obtaining a single gentamicin level when it is run in a batch of six levels. The data are given in terms of method used as well as size of kit used.

DISCUSSION

Consideration of the experiments performed with HS containing gentamicin suggests that the NEN-RIA was unsatisfactory because of the apparent constant bias. Similarly, the bioassay appeared to have a proportional bias. The portion of the study utilizing clinical samples did not indicate the presence of significant constant
bias in the ERA, DP-RIA, or NEN-RIA, when compared to the bioassay reference method. However, each of these methods shows proportional bias, compared to the repeat bioassay. The data on recovery argued against two methods: the bioassay appeared to underestimate, whereas the NEN-RIA appeared to overestimate, gentamicin concentration to some extent. Two of the methods, the ERA and DP-RIA, appeared very similar with respect to accuracy (the percent recoveries were very close to 100%). Cost analysis of these methods argued in favor of one of the commercial methods. Another consideration may be the existing equipment in a laboratory, such as scintillation or gamma counters.

LITERATURE CITED