Enzyme Immunoassay for Gentamicin

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We describe a gentamicin assay in which a peroxidase–gentamicin conjugate competes with gentamicin for binding to a gentamicin antibody adsorbed to a polystyrene solid phase. The assay can be completed in 30 min and requires 50 µl of diluted serum. The precision and accuracy are equivalent to that of a radioimmunoassay technique and the reagents are stable for several months.

Additional Keyphrases: well technique rather than tube technique · drug assay · peroxidase–gentamicin conjugate

Gentamicin is an antibiotic with potentially ototoxic and nephrotoxic side effects. Because these toxic effects are more common if the concentrations in serum exceed 10 mg/liter while therapeutic effects are decreased if they are less than 2 mg/liter (1, 2), assay of gentamicin in serum may provide information as to whether an effective therapeutic concentration of the drug has been obtained.

Gentamicin has been assayed in serum by microbiological techniques (3) and more recently by radioimmunoassay methods (4, 5) that provide a more rapid estimation. Enzyme immunoassay has been applied to assay of several drugs in serum (6, 7) and we describe here an enzyme immunoassay method for assay of gentamicin that is based on a technique reported by Saunders and Bartlett (8).

In this technique, gentamicin antibody is adsorbed to the polystyrene surface of a multi-well reaction tray and peroxidase–gentamicin conjugate is mixed with a gentamicin standard or serum in each reaction well. After this competition reaction is completed, the extent of conjugate binding is observed by adding a chromogenic substrate for the peroxidase reaction. The absorbance of the product is inversely proportional to the amount of gentamicin in the reaction well.

Materials and Methods

Reagents

Potassium phosphate solution, 0.5 mol/liter. Dissolve 116 g of K₂HPO₄·3H₂O in 1 liter of water. The solution is stable for six months at room temperature.

Bovine serum albumin, 2.5 g/liter. Add 0.8 ml of bovine serum albumin (300 g/liter, Hyland Division Travenol Laboratories, Inc.) to 100 ml of water. This solution is stable for four months at 4 °C.

Citrate buffer 1, 0.1 mol/liter, pH 4.0. Dissolve 2.3 g of citric acid monohydrate in 90 ml of water. Adjust the pH to 4.0 with the potassium phosphate solution (0.5 mol/liter) and dilute to 100 ml with water. This solution is stable for four months at 4 °C.

Citrate buffer 2, 0.1 mol/liter, pH 2.8. Dissolve 2.3 g of citric acid monohydrate in 90 ml of water. Adjust the pH to 2.8 with the potassium phosphate solution (0.5 mol/liter) and dilute to 100 ml with water. This solution is stable for four months at 4 °C.

Wash solution. Dissolve 5 ml of the surfactant Tween 80 and 9 g of NaCl in 1 liter of water. Adjust the pH to 7.6. This solution is stable for two months at room temperature.

Chromogenic reagent. Dissolve 0.20 g of 2,2'-azino-di(3-ethyl-benzthiazolino)-6'-sulfonate (Boehringer Mannheim Corp.) in 10 ml of water. This solution is stable for six months at 4 °C.

Working substrate solution. Add 0.5 ml of chromogenic reagent and 0.1 ml of hydrogen peroxide (5 g/liter) to 10 ml of citrate buffer 1. This solution is stable for 8 h at room temperature.

Reaction tray. Polystyrene, 96-well, flat-bottom microtiter trays are available from Linbro Chemical Co., New Haven, Conn. 06605.

Peroxidase–gentamicin conjugate. A conjugate of horseradish peroxidase (Type VI, Sigma Chemical Co.) and gentamicin was prepared according to the two-step procedure described by Nakane and Kawai (9), except that the final chromatographic step was omitted. The first step of this procedure produces a “peroxidase aldehyde,” which we coupled to gentamicin in a second reaction by reacting 2.5 ml of the peroxidase aldehyde solution with 0.57 mg of gentamicin according to the described protocol (9). We sterilized the final dialyzed product by filtration through an 0.45-µm filter (Millipore Corp.). The filtrate is stored in sterile glass tubes at 4 °C and is diluted 500-fold with isotonic NaCl solution (9 g/liter) before the assay.

Gentamicin antibody. Human thyroglobulin was conjugated to gentamicin and the subsequent immunization and antibody production in rabbits was performed according to Mahon and Wilson (5). The affinity constant of this antiserum is approximately 5 × 10⁸ mol⁻¹, and we dilute the antiserum 500-fold with isotonic saline before applying it to the reaction tray.

Preparation of the reaction tray. Fill the wells in the reaction tray with bovine serum albumin solution (2.5 g/liter). After 10 min, empty and wash the plate wells twice with wash solution and dry the plate. Add 50 µl of gentamicin antibody (diluted 500-fold) to each well and dry overnight at room temperature. Wash each well five times with wash solution and shake dry. The tray is now ready for use, or it may be stored at room temperature for at least one month. When only a portion of a reaction tray is used, the remainder may be covered with plastic film (Microtiter plate sealers; Cooke Laboratory Products, 900 Slaters Lane, Alexandria, Va. 22314).

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Gentamicin standards. Dissolve 100 mg of gentamicin sulfate (571 mg of gentamicin per gram of powder; Schering Corp., Kenilworth, N.J.) in 100 ml of distilled water. Dilute this solution 19-fold with a gentamicin-free serum pool to obtain a 30 mg/liter stock standard. This stock standard is serially diluted to obtain the working standards, which are stable for at least two weeks at 4 °C.

Procedure

Dilute all sera, standards, and controls 10-fold with isotonic saline. Add 50 μl of the diluted gentamicin-peroxidase conjugate and then immediately add 50 μl of each diluted specimen to duplicate antibody-coated reaction tray wells. After 10 min, wash each well five times with wash solution. Add the working substrate solution (100 μl) to each well and after 10 min add 0.1 ml of 1 mol/liter HCl to each well to inhibit the peroxidase reaction. Transfer the contents of each well into 1 ml of citrate buffer 2 and measure the absorbance of this mixture at 410 nm.

Calculations

Use the absorbance readings for the gentamicin standards to construct a graph of absorbance vs. concentration and use this graph to convert the absorbances of controls and patients to concentration.

Results

Antibody Concentration

An appropriate dilution of our gentamicin antibody was selected after assaying gentamicin standards in reaction wells coated with three different antibody concentrations (Figure 1). We converted the data in Figure 1 to a logit-log plot and found acceptable linearity in all of the standard curves, with a slope of -0.648 to -0.669 and correlation coefficients of 0.989 to 0.996. We chose an antibody dilution of 500-fold because it gave the highest correlation coefficient (0.996) while providing comparable sensitivity at concentrations lower than 1 mg/liter as well as maintaining displacement between 10 and 15 mg/liter.

Gentamicin-Peroxidase Conjugate

We obtained the appropriate dilution of the enzyme-labeled conjugate for use in our assay by diluting the conjugate 100-, 500-, and 1000-fold, and using each dilution for assaying a series of gentamicin standards (Figure 2). The data illustrate that a 500-fold conjugate dilution provides adequate displacement for gentamicin concentrations between 2 and 10 mg/liter, while maintaining good sensitivity below 2 mg/liter. By extrapolating the variation at zero gentamicin concentration (6.5%), we find the least detectable concentration to be about 0.4 mg/liter, or 2 mg of gentamicin per assay. We converted the data in Figure 2 to a logit-log plot (Figure 3) by assuming that the absorbance at zero gentamicin concentration is equivalent to 100% B0 and then calculating the percent binding values corresponding to the other gentamicin concentrations. These tabulated data yield a logit-log correlation coefficient of 0.985 for the 500-fold dilution and 0.986 for the 100- and 1000-fold dilutions. Additionally, a 500-fold dilution provides a gentamicin value at 50% displacement of 3.1 mg/liter, as compared to 11.7 and 1.8 for the 100- and 1000-fold dilutions, respectively. From these data we conclude that a 500-fold dilution of conjugate yields an optimum standard curve for our assay. However, it should be noted that the exact dilution may vary with each preparation of conjugate and must be determined after each conjugate preparation is completed.
Table 1. Effect of Serum Dilution on Recovery of Gentamicin

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<tr>
<th>Serum dilution, fold</th>
<th>Gentamicin, mg/liter</th>
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<tr>
<td></td>
<td>Expected</td>
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<tr>
<td>3</td>
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<td>6</td>
<td>11.1</td>
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<td>12</td>
<td>5.6</td>
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<td>96</td>
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Fig. 4. Binding of the gentamicin–peroxidase conjugate during the initial reaction period
After the gentamicin–peroxidase conjugate was incubated in the antibody-coated reaction well for the indicated times, each reaction well was washed and then incubated with substrate solution for 10 min.

Protein Effect

To test the effect of serum proteins on the assay accuracy, we diluted a serum supplemented with gentamicin and assayed each dilution (Table 1). Good agreement was found between the expected and assayed values, which indicates an insignificant protein effect on this assay.

Reaction Intervals

Two separate reaction intervals in this assay sequence require evaluation. In the first reaction, the gentamicin–enzyme conjugate competes with gentamicin for binding sites on the antibody and for equilibrium saturation techniques the antigen–antibody reaction should proceed to equilibrium (10). We determined the time required for this competitive reaction to reach equilibrium by adding the gentamicin–peroxidase conjugate to a series of reaction wells which had been coated with antibody as described. After the reaction times indicated in Figure 4, the wells were washed and the extent of reaction was monitored by adding the substrate solution, waiting 10 min, and measuring the absorbance of the solution in each reaction well. In this way we observe that the antigen–antibody reaction is complete within 5 min after the conjugate is added. However, in order to accommodate small differences in reagent addition times, we recommend incubating the reactants in the initial reaction for 10 min. Because the antigen and antibody equilibrate rapidly, we tested the effect of adding gentamicin standards before adding the peroxidase conjugate, a reversal of addition order specified in the recommended procedure. As expected, when the standards were added first, the final absorbance readings were 20 to 30% lower.

The second reaction interval that must be carefully regulated is the final assay step in which the bound gentamicin–peroxidase conjugate converts the chromogen to a colored product. This enzyme activity should be measured in substrate excess to assure accurate quantitation. In establishing the appropriate incubation interval for the peroxidase reaction, we incubated different dilutions of gentamicin–peroxidase conjugate in the antibody-coated reaction well without gentamicin standard for 10 min, washed the reaction tray, and added 100 μl of substrate solution. At the times indicated in Figure 5 the reaction was inhibited by adding 100 μl of 1 mol/liter HCl. When the conjugate is diluted 500-fold the enzyme reaction proceeds linearly after substrate is added, with a change of absorbance of about 0.050/min for the first 10 min of the reaction interval. After 10 min the reaction becomes nonlinear. Additionally, the peroxidase reaction rate is nonlinear after 3–4 min, when a less diluted enzyme-conjugate solution is added to the reaction well. To ensure that excess substrate is available during the reaction interval, we incubated peroxidase–gentamicin conjugate (diluted 500-fold) in the antibody-coated reaction well and then assayed the activity of the bound enzyme in the presence of increasing concentrations of substrate as described in procedure. The data in Figure 6 indicate that saturation or zero-order kinetics obtain at substrate concentrations above 1.5 mmol/liter. In our assay we specify 2.0 mmol/liter chromogen to assure zero-order kinetics during the reaction interval.

After addition of the HCl to inhibit the reaction there is no
significant change in absorbance for at least 30 min. After the reaction mixture has been diluted in Buffer 2, there is no significant absorbance change for at least 24 h.

**Precision**

We tested the precision of this assay by assaying three control sera 10 times in one assay and in 10 consecutive assays (Table 2). As expected, the precision was best for the control value in the mid-range of the standard curve.

**Accuracy**

We evaluated the accuracy of this method by comparing it with an radioimmunoassay method (11) (Figure 7).

**Discussion**

We have adapted an enzyme immunoassay technique described by Sanders and Bartlett (8) to an assay for gentamicin in serum. We use gentamicin labeled with peroxidase in a competition reaction with gentamicin standard in a fashion analogous to most radioimmunoassay procedures. The utility of this competitive approach is illustrated by its use to measure several antigens (12), including such diverse analytes as cortisol (13) and alpha-fetoprotein (14). In these particular techniques soluble enzyme–antigen conjugates and solid-phase antibodies are used. Our approach is similar except that we use polystyrene reaction trays rather than individual test tubes. The multi-reaction trays can be washed and otherwise manipulated more rapidly than test tubes and thus a significant amount of technician time is conserved.

To perform enzyme immunoassays accurately and precisely, one must control two critical reactions. The first is the competition antigen–antibody reaction, which must proceed to equilibrium (10). With our reaction conditions this equilibrium is reached at 5–10 min of reaction time, relatively short as compared to other reported methods (7, 8, 13). Although a direct comparison of methods is difficult because of the different reagents involved, we attribute our shorter reaction interval to the use of higher antibody and antigen concentrations, which promote faster reactions. After equilibrium is obtained and unbound antigen is washed from the well, the second critical reaction is performed, i.e., the enzyme reaction. The activity of the enzyme bound to the solid phase antibody via its link to gentamicin is a reflection of the gentamicin concentration during the competitive antigen–antibody reaction, and thus the enzyme activity must be measured accurately to obtain a quantitative estimate of gentamicin in the tested sample. Several factors can influence the accuracy with which the enzyme activity must be measured and, in particular, one must ensure that there is a constant conversion rate during the reaction interval, i.e., zero-order kinetics. This requires a balance between amount of enzyme–conjugate bound in the reaction well, amount of substrate added, and total time of incubation, as well as control of reaction variables such as pH and temperature. In our method, peroxidase is conjugated to gentamicin and competes with gentamicin for binding sites on the solid-phase antibody. Maximum binding, and hence maximum enzyme activity, will occur when the gentamicin concentration is zero. Therefore, to test our system for linearity of substrate conversion when the enzyme concentration is highest, we incubated the conjugate with antibody in the absence of gentamicin. In this way we have shown that we maintain a constant substrate conversion during the initial 10 min of reaction in the presence of maximum enzyme concentration. This reaction is neither linear beyond 10 min nor linear after shorter incubations with higher concentrations of enzyme–conjugate. This implies that we are using a maximum enzyme–conjugate concentration compatible with zero-order kinetics during the specified 10 min incubation. Our reaction conditions could be altered (e.g., more dilute enzyme–conjugate) to ensure linear enzyme kinetics for longer reaction periods, but one should expect that lower conjugate concentrations will result in lower absorbance readings in addition to increasing the total reaction time.

This method is less sensitive than radioimmunoassay techniques with use of $I^{125}$-labeled gentamicin (11). However, our detection limit of 0.4 mg/liter is well within that required for clinical utility and could be extended if necessary. That is, because the sera are diluted 10-fold before assay and we could demonstrate no effect of increasing the protein concentrations in the assay, greater sensitivity could be obtained by assaying sera at lower dilutions.

The precision of this assay compares well with that of a radioimmunoassay method and is highest for gentamicin concentrations in the therapeutic range. Of the several factors that can influence the precision of the assay, the reaction tray preparation is one of the most important. Precoating the polystyrene tray with albumin as described in the Reagents section above prevents unequal antibody binding and lowers the nonspecific conjugate binding. However, we occasionally observe that specific lots of reaction trays bind less (sometime as much as 1/2 less) of the usual amount of antibody to their polystyrene surface. As demonstrated in Figure 1, a twofold decrease in antibody concentration applied to the reaction well does not greatly affect the shape of the standard curve but does lower the final absorbance reading. These lower readings do not significantly alter the assay's accuracy and an increase in the antigen–antibody or the enzyme reaction intervals can be used to produce higher absorbance readings. Although absolute absorbance readings may change with reaction tray preparations, we observed reasonably small variations in control values when different reaction tray preparations were
used. For instance, the variations shown in Table 2 are derived from assays performed with three separate reaction trays, and these variations compare well with those obtained with a radioimmunoassay method in our laboratory.

A second factor that has led to some differences in antigen binding (and subsequent absorbance values) during the antigen–antibody reaction is the stability of the peroxidase-gentamicin conjugate. We have stored the conjugate in sterile aliquots at 4 °C as well as at −20 °C. We find either temperature is generally adequate for long-term storage. The absolute stability of the conjugate depends on the quality of reagent, care of reagent preparation, and the extent to which the reagent-preparation steps are followed. In this regard, we have noted that the borohydride reduction step is particularly important. When this step was omitted, the conjugate lost half of its immunological activity within three days at 4 °C. Occasionally, we observe a loss of immunological activity in a particular conjugate preparation, even when all apparent precautions have been taken. Although we have not discovered the exact reason for this sudden loss, accidental trace contamination of the conjugate preparation with H2O2 may occur, which can lead to rapid destruction of its immunologic activity.

In summary, this technique provides a rapid, precise, and accurate assay for gentamicin, which does not require use of radioisotopes. This solid-phase enzyme immunoassay technique of using a multi-well polystyrene reaction tray should be adaptable to many other assays.

References