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Rapid, Simple Enzyme Immunoassay for Gentamicin

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An enzyme immunoassay for gentamicin is described. The assay is accurate and specific and needs only simple equipment.

Gentamicin is a widely used antibiotic, but the difference between therapeutic and toxic serum levels is relatively small. It is, therefore, necessary to measure its concentration in serum. We report a simple, rapid, and accurate assay for this drug which needs no radioactive material or special equipment.

The assay is a homogenous enzyme immunoassay; that is, all reactions take place in solution and no phase separation step is involved, as is needed in the enzyme-linked immunosorbent assay and radioimmunoassay. The immunological reactions involved are therefore rapid, and no centrifugation step is involved. The principle is similar to that developed for measuring certain other drugs with a reaction rate analyzer (E.M.I.T.; Syva Corp., Palo Alto, Calif.) (7). The technique described here differs in requiring only a simple spectrophotometer.

Apart from antiserum specific for gentamicin, the only special reagent needed for this assay is a conjugate of enzyme covalently linked to gentamicin. When antigentamicin antibody is added to this conjugate, the binding of antibody to the enzyme via the gentamicin ligands causes a large reduction in enzyme activity. The mechanism of this inhibition has been discussed by Rowley et al. (6). However, if unlinked gentamicin (as in a test or standard) is also present, some antibody will bind this so that less will be available for binding and inhibiting the enzyme. Therefore, the more gentamicin present, the more active will be the enzyme. The enzyme used is glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*; it is easily assayed by following reduced nicotinamide adenine dinucleotide production spectrophotometrically. No interfering enzyme activity is present in body fluids.

MATERIALS AND METHODS

Antigentamicin serum was produced in rabbits by injecting a conjugate of gentamicin and thyroglobulin made by using the carbodiimide procedure (3). To 40 mg of gentamicin sulfate (Roussel Laboratories Ltd., Wembley, Middlesex, United Kingdom) in 0.5 ml of water, pH 5, was added 20 mg of porcine thyroglobulin (Sigma Chemical Co., St. Louis, Mo.). When this had dissolved, 100 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Sigma) freshly dissolved in 0.5 ml of water was added dropwise with stirring and incubated for 1.5 h at room temperature. The mixture was then dialyzed for 2 days at 4°C against 1 liter of 0.01 M potassium phosphate–0.15 M sodium chloride, pH 7.2 (phosphate-buffered saline), with four buffer changes.

Rabbits were immunized with two intramuscular injections of a total of 1 mg of antigen in an emulsion of 0.25 ml of Freund complete adjuvant plus 0.25 ml of phosphate-buffered saline. Similar booster injections of 0.1 mg of antigen followed at 2-month intervals; the rabbits were bled after the first booster injection, and the serum was stored at −20°C.

Gentamicin-enzyme conjugate was also prepared, using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride. Gentamicin sulfate, 100 mg, was dissolved in 0.4 ml of water, and the pH was adjusted to 6 with a 10 M sodium hydroxide solution. To this was added glucose-6-phosphate dehydrogenase from *L. mesenteroides* (1,000 nicotinamide adenine dinucleotide phosphate units of lyophilized powder, about 4 mg, type XXII; Sigma). The mixture was cooled to 0°C, and a freshly made solution of 200 mg of carbodiimide per ml was added to give a final concentration of 10 mg/ml. The mixture was incubated for 3 h at 0°C then dialyzed for 2 days at 4°C against 1 liter of 0.05 M tris(hydroxymethyl)aminomethane–0.15 M sodium chloride, pH 7.8, with six buffer changes. Inclusion of tritiated gentamicin (Radiochemical Centre, Amersham, United Kingdom) showed that between 5 and 15 molecules of gentamicin bound each enzyme molecule. An enzyme activity loss of about 50% was commonly observed during this conjugation process. To preserve the dialyzed gentamicin-enzyme conjugate, sodium azide and bovine serum albumin were added to 1 and 10 mg/ml, respectively, and it was kept at 4°C.

To perform the enzyme immunoassay, four reagents were needed: (i) gentamicin samples, (ii) substrate-antibody reagent, (iii) enzyme reagent, and (iv) borate solution.

Gentamicin sample was undiluted serum or standards in the range 1.25 to 20 mg/liter. Substrate-antibody reagent was 6.25 mg of β-nicotinamide adenine dinucleotide (grade AA1; Sigma) per ml and 6.25 mg of D-glucose-6-phosphate monosodium salt (Sigma) per ml in 0.1 M sodium acetate, pH 5.4, to which antigentamicin serum was added. Enzyme reagent contained gentamicin-enzyme conjugate diluted in 0.3

M tris(hydroxymethyl)aminomethane (pH 7.8)–75% (vol/vol) newborn calf serum (Gibco, Paisley, United Kingdom). Disodium tetraborate (0.1 M; BDH Chemicals Ltd., Poole, United Kingdom) was used to stop the enzyme reaction.

To perform the assay, 20 μl of gentamicin sample was added to each plastic (not glass) tube followed by 100 μl of substrate-antibody reagent. The tubes were warmed to 37°C in a water bath, and 100-μl amounts of enzyme reagent were added at timed intervals. After exactly 10 min, the reaction was stopped by adding 2.5 ml of 0.1 M borate.

The amount of antigentamicin serum present in the substrate-antibody reagent was adjusted to give maximum absorbance difference over the range of gentamicin concentrations used. For standards in the range 1.25 to 20 mg/liter, this was done by assaying in the normal way water (*a*), 5 mg of gentamicin per liter (*b*), and 1 g of gentamicin per liter (*c*) using substrate solutions to which various amounts of antigentamicin serum were added. If the correct amount of antigentamicin serum is present, then the absorbance given by the midrange standard *b* will be half the sum of the absorbances given by *a* and *c*. With excess antigentamicin serum, the absorbance of *b* will be less than half the sum of *a* and *c*, and conversely with insufficient antigentamicin serum. The substrate-antibody reagent used in the assay contained 3.5% pooled antiserum.

The amount of gentamicin-enzyme conjugate in the enzyme reagent was that required to give a final absorbance of 0.7 above the blank with the 20-mg/liter standard.

Serum blanks were prepared similarly, but with enzyme reagent omitted. For each gentamicin solution, two tests and one blank were prepared, and the differences between corresponding test and blank absorbances were used for constructing the standard line and for interpolating the unknowns.

Standard solutions were prepared in water, since only one blank needed then to be prepared for the five standards that were usually used. The absorbance values, which were stable for several hours at room temperature, were measured at 340 nm with a Unicam SP500 spectrophotometer.

Bioassays were performed with an agar well plate diffusion technique with Oxoid Isosensitest agar and *Klebsiella* NCTC 10896 as the indicator strain.

RESULTS

A standard line is shown in Fig. 1. Absorbance on a linear scale was plotted against gentamicin concentration on a logarithmic scale, when a slightly sigmoid curve was obtained. The performance of the assay was investigated by assaying with either the bioassay or the immunoassay 38 sera from patients receiving therapy with gentamicin and other drugs. The results are shown in Fig. 2, and a correlation coefficient of 0.95 was obtained.

A gentamicin recovery experiment was performed in which 42 gentamicin-free sera were each mixed with aqueous gentamicin (100 mg/liter) to give a final concentration of 5 mg/liter and assayed with the immunoassay. The mean percent error was −0.76% and the standard deviation of the percent error was 8.59%. The minimum level of gentamicin measurable to this degree of accuracy was about 0.1 mg/liter.

The specificity of the assay was tested by assaying a gentamicin-containing serum to which a high concentration of another antibiotic was added. No interference was noted from a wide range of antibiotics, including kanamycin, streptomycin, amikacin, tobramycin, neomycin, spectinomycin, cefazolin, carbenicillin, benzylpenicillin, clindamycin, novobiocin, vancomycin, and metronidazole at 1 g/liter and fusidic acid, trimethorpim, sulfamethoxazole, nalidixic acid, tetracycline, erythromycin, and chloramphenicol at 100 mg/liter.

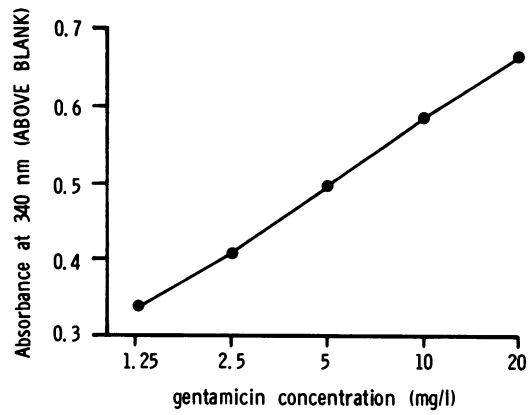

FIG. 1. *Gentamicin standard line.*

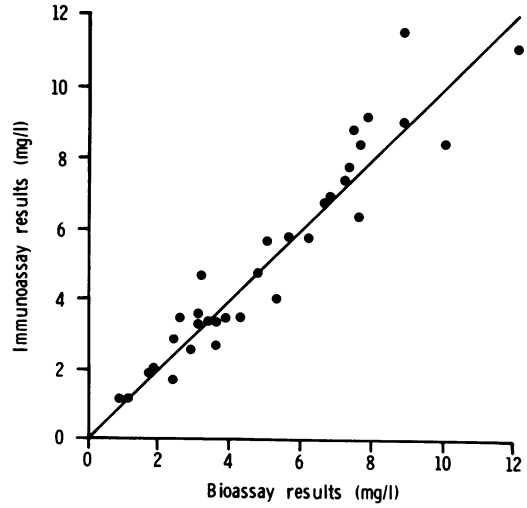

FIG. 2. *Assay of gentamicin-containing patient sera, using the bioassay and the enzyme immunoassay.*

The substrate-antibody reagent was stable for at least 2 months at −20°C and for at least 2 weeks at 4°C. Both the gentamicin-enzyme conjugate and the enzyme reagent were stable for at least 6 months at −20°C, and they were also stable at 4°C, provided that an antimicrobial agent such as sodium azide was present.

DISCUSSION

We have described a simple and accurate gentamicin assay which needs less than 0.1 ml of serum and gives results in under 1 h. The only special equipment needed is accurate pipettes for dispensing 20 and 100 μl, a clock with a second hand, a 37°C water bath, and a spectrophotometer capable of measuring absorbance at 340 nm. No sample dilution is involved, as is the case with most immunoassays for gentamicin, such as the radioimmunoassay (4) and fluoroimmunoassay (8, 9). Although this does mean that larger amounts of antiserum are needed, one rabbit can produce in 1 year enough antiserum for 100,000 tubes.

There are a number of methods for assaying aminoglycosides in serum, some of which have already been mentioned. Radioimmunoassays are expensive to perform and require expensive counting equipment, and the reagents have limited shelf lives. A new immunoassay, the homogenous reactant-labeled fluorescent immunoassay, has recently been described (2) which appears to overcome some of these disadvantages. The acetyltransferase method (1) is very satisfactory but again requires a scintillation counter. The most commonly used method, the bioassay, in many hands has poor accuracy (5) and usually takes 18 h to perform. We feel that with respect to accuracy, speed, and simplicity, the method described in this report has much to commend it.

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