Radioimmunoassay and Radioenzymatic Assay of a New Aminoglycoside Antibiotic, Netilmicin

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A radioimmunoassay and a radioenzymatic assay for netilmicin, a new aminoglycoside, were developed in our laboratories to assist in the study of the pharmacology of the drug and to establish values for use in its monitoring. The assays are sensitive, precise, and rapid, giving results that correlate \( r = 0.90 \) with each other and with those of a microbiological assay in which Klebsiella pneumoniae is used as the test organism. Preliminary pharmacological studies show the drug to have a biological half-life of 135 min, which is comparable to that for other aminoglycosides.

Netilmicin, a new semi-synthetic derivative of sisomicin currently in phase 3 trials, is of considerable interest because of its broad spectrum of activity against Gram-negative bacilli and some Gram-positive cocci (1, 2). In vitro, netilmicin has a spectrum of activity similar to that of gentamicin, but is more active than are other aminoglycosides against isolates of Escherichia coli, Klebsiella spp., Enterobacter spp., and Serratia spp. (2–4). In addition, preliminary studies suggest that it is significantly less ototoxic and nephro-toxic than gentamicin in experimental animals (4, 5). We developed a radioimmunoassay for netilmicin and validated it against both a microbiological assay and a radioenzymatic assay, which is also reported. The assay was used in preliminary pharmacological studies of patients receiving the drug.

Materials and Methods

Hapten/Protein Conjugation

We conjugated netilmicin to bovine serum albumin, the procedure for conjugation and purification being as follows. Dissolve 100 mg of bovine serum albumin in 1 ml of water, adjust the pH to 6.0, and mix with 5 ml of a solution of netilmicin, 40 g/liter (Schering Corp., Bloomfield, N.J. 07003). Dissolve 800 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in 1 ml of water and add the solution slowly to the netilmicin mixture, with constant stirring. Stir the mixture for 1 h at room temperature and incubate at 40 °C for three days. After incubation, dialyze the conjugate for 16 h vs. 2 liters of the following solution: 10 mmol of sodium dihydrogen phosphate, 150 mmol of NaCl, and 1 g of sodium azide per liter, and chromatograph on a 0.9 × 25 cm column containing Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J. 08854).

Immunization

Immunize a convenient number of New Zealand albino rabbits by monthly intramuscular injections of the conjugate emulsified in complete Freund’s adjuvant as described by Hurn and Landon (6). A specimen of blood obtained from the ear vein after the third monthly injection had a final titer of 20,000.

Iodination

The iodination procedure of Bolton and Hunter (7) as modified for the aminoglycosides (8) was used to produce \(^{125}\)I-labeled netilmicin. Dissolve 200 mg of the acylating agent 3-(4-hydroxyphenyl)propionic acid-N-hydroxysuccinimide ester (“Tagit”; Calbiochem, San Diego, Calif. 92112) in 10 μl of benzene and evaporate under nitrogen. Iodinate the residue by the Chloramine T technique of Hunter and Greenwood (9). After the iodination, extract the mixture twice with 0.4 ml of benzene and rapidly evaporate the benzene extracts in a stream of nitrogen. Next add 50 μl of a solution of netilmicin 55 mg/liter in borate buffer (0.1 mol/liter, pH 9.5) to the evaporated extracts and allow the reaction mixture to stand overnight at room temperature. Stop the reaction by adding 0.2 ml of a 15 g/liter solution of glycine in the borate buffer). Purify the iodinated netilmicin by passage through a 0.9 × 30 cm column of Sephadex with the phosphate-buffered saline solution as eluent.

Radioimmunoassay Procedure

The netilmicin was radioimmunoassayed as follows. Into each assay tube place 500 μl of phosphate-buffered saline, 100 μl of standard or unknown diluted to be within the range of the standard curve, 100 μl of \(^{125}\)I-labeled netilmicin diluted to 40,000 cpm/100 μl, and 100 μl of antisera diluted 40,000 fold in a gelatin solution (1 g/liter, in phosphate-buffered saline). After thorough mixing incubate the tubes for 30 min at 37 °C and immediately add 100 μl of normal rabbit or goat sera diluted threefold in phosphate-buffered saline. Add 500 μl of a 250 g/liter solution of Polystyrene Glycerol 6000 (Carbowax; Fisher Scientific Co., Fair Lawn, N.J. 07410), vortex mix, and allow the tubes to stand for 30 min at 4 °C, then centrifuge (1500 × g, 10 min, 4 °C). Decant the supernates and measure the radioactivity of the precipitate in an automated gamma counter to determine the bound antigen. Run each standard or unknown in duplicate and include quality-control tubes in each assay.

Enzyme Preparation

The bacterium we used as a source of enzyme was a mutant of E. coli W777/JR66. Prepare an 18-h culture of this bacte-
rium in trypsin-cellose broth at 37 °C. Dilute the culture 250-fold in the broth, and incubate at 37 °C with agitation until the absorbance at 490 nm is 0.2–0.3. Centrifuge the culture (15 min, 12 000 × g, 4 °C) discard the supernate, and resuspend the pellet in 150 ml of tris(hydroxymethyl)methyamine buffer (10 mmol/liter, pH 7.4 at 25 °C, plus 30 mmol of NaCl per liter). Repeat this step and then, using the following modification of the BenVeniste and Davis (10) technique, rupture the bacterial cells. Weight the pellet and suspend it in 80 ml of buffer tris(hydroxymethyl)methyamine (3 mmol/liter, pH 7.3, plus 200 g of sucrose per liter), centrifuge (12 000 × g, 10 min, 4 °C) and discard the supernatant fluid. Remove the excess sucrose from the sides of the tube with cotton-tipped applicators. Resuspend the pellet in 32 ml of 0.5 mmol/liter MgCl₂ kept at 4 °C. Gently stir the mixture until an even suspension is obtained, then centrifuge (26 000 × g, 20 min, 4 °C). The supernatant fluid is an osmotic lysate that serves as a source of the enzyme.

Enzyme Assay

The enzyme gentamicin adenyl transferase catalyzes the transfer of the adenyl moiety from ATP to certain amino-glycosides. The adenylated amino-glycosides are positively charged and bind to negatively charged phosphocellulose paper. Any enzymes that act on ATP present in the samples or the human serum standard matrix are destroyed by heating at 56 °C for 5 min before the assay is done. The samples are assayed as follows: Add 30 μl of standard or unknown to 20 μl of a mixture of, per liter, 0.4 mol of tris(hydroxymethyl)methyamine and 64 mmol of magnesium chloride (pH 8.6) 5 μl, of dihithreitol (24 mmol/liter); 20 μl of [14C]ATP (1.2 mmol/liter, spec. act. 35 Ci/mol); and 50 μl of enzyme extract containing a minimum of 60 μg of protein. Incubate the mixture at 37 °C for 30 min, then remove 50 μl and pipet this onto a 2-cm square piece of phosphocellulose paper (Whatman no. p-81). Wash the paper twice in tris(hydroxymethyl)methyamine buffer (5 mmol/liter, pH 7.4 at 0 °C) for 5 min, dry at 37 °C, and count in a toluene-based scintillant in a liquid scintillation counter. Run each standard and control in duplicate and include quality-control samples in each assay.

Microbiological Assay

For this assay we used an agar-well technique and a carbencillin-resistant isolate of K. pneumoniae as the test organism. Produce an 18-h culture in trypsin-cellose soy broth and resuspend it in isotonic saline so that the suspension has an absorbance of 0.1 at 625 nm. Mix an 0.8-ml aliquot of the suspension with 500 ml of antibiotic medium No. 2 (Difco), which has been adjusted to a pH of 8.0. Fill Petri dishes with 14-ml portions and cut wells (0.75 mm in diameter and 0.4 mm in depth) into the agar. Fill the wells with 50 μl of the specimens, and incubate the plates at 37 °C for 18 h. Measure the zones of inhibition and compare them to standard curves. Analyze the controls, tests, and standards in triplicate.

Specimen Collections

Blood specimens were collected from patients receiving the drug (informed consent), allowed to clot, centrifuged, and the sera stored at -70 °C in plastic tubes until assayed. The pharmacokinetic studies were performed on timed samples from patients who had received 50 mg of netilmicin per square meter of body surface, by 30-min intravenous infusion.

Results

The standard curve for the radioimmunoassay was linear (logit-log plot). The sensitivity of the assay, defined as the least amount of netilmicin that can be significantly (P < 0.05) distinguished from no drug, was 200 pg per tube. Within-run precision was estimated by measuring 20 replicates at low therapeutic range (2 mg/liter), mid-therapeutic range (4 mg/liter), and high therapeutic range (8 mg/liter); the respective coefficients of variation were 4.4, 3.0, and 7.6%. Between-assay precision for 15 assays at each of these three concentrations was 16.0, 9.0, and 8.0%, respectively. Analytical recovery of netilmicin added to patients' serum samples was 90% (CV, 7.55%).

We measured the cross reactivity of the netilmicin radioimmunoassay with other antibiotics by determining the amount of drug necessary to produce 50% inhibition of antibody binding to 125I-labeled netilmicin. The efficiency of this inhibition was related to the amount of unlabeled netilmicin and is expressed as percentage cross reactivity. The following antibiotics cross reacted less than 1%: carbencillin (Beecham-Massengill), cephamandole (Parke-Davis), neomycin (Upjohn), kanamycin, streptomycin, tobramycin (Lilly), and cephaloridine (Schering). The closely related aminoglycoside gentamicin (Schering) cross reacted less than 3%.

The affinity of the netilmicin antisera for 125I labeled netilmicin was measured by two different techniques, Scatchard plot (11) and an equilibrium technique (12). The results obtained by these techniques were 2.0 × 10 and 2.4 × 10 liters/mole, respectively. The specific activity of the 125I-labeled netilmicin was about 44 Ci/g, with an incorporation of 5.8 × 10⁻³ atoms of 125I per molecule of antibiotic. The standard curve of the radioenzymatic assay was linear when the celluose bound counts were related to the linear concentration of the netilmicin with a sensitivity of 200 ng/tube. Table 1 shows the results of statistical analysis of data on 21 specimens, each analyzed by all three methods. The correlation between the radioenzymatic assay and the radioimmunoassay was good, the correlation between the microbiological assay and the other two less good.

Previous reports have shown that both gentamicin and tobramycin are biologically and immunologically inactivated by incubation with carbencillin in vitro (13, 14) and in certain instances this may be of clinical significance (15). Therefore we designed experiments to determine whether netilmicin measured by radioimmunoassay would decrease in immuno-reactivity after incubation with carbencillin. Netilmicin (2 mg/liter) was incubated at 37 °C with carbencillin (50, 100, and 200 mg/liter) in normal donors' sera. Samples were removed and frozen after incubation for 0, 24, 48, and 72 h. The results of this incubation showed that the relative immuno-reactivity of netilmicin decreased in a similar manner to tobramycin (10). Pharmacokinetic analysis of the elimination of the drug from four patients receiving it by 1-h intravenous infusion showed that the drug is distributed as a two-compartment model, with a β elimination half-life of 135 min. These results are comparable to the data published for the other aminoglycoside.

Discussion

The potential ototoxicity and nephrotoxicity of aminoglycoside antibiotics make it desirable to monitor the concentrations of these drugs in serum. The mode of administration of these drugs varies from intramuscular injections (16) and short intravenous infusions (17) to continuous intravenous infusions (18), each requiring a differing approach to monitoring but all having in common the need for a sensitive, precise, and rapid assay. We previously have described radioimmunoassays for gentamicin (8), sisomicin (19), and tobramycin (10). In addition, we compared three different
The assay systems for tobramycin (10) and suggested that the final choice of method depends on the personnel and equipment available.

Netilmicin is a new aminoglycoside that is yet to be thoroughly studied in man. With the availability of the radiolmmunoassay system, pharmacokinetic studies in man can be undertaken. The radioimmunoassay described in this paper is precise, sensitive, and specific. It compares favorably with microbiological and radioenzymatic assays. The use of polyethylene glycol instead of charcoal (20) as a separating agent eliminates the critical time dependency (21) and it is easier to pipet. The bound phase is counted thus improving precision. Additionally, the test-tube trays provided for the Model 1285 Automatic Gamma Counter (Searle Analytical) can be used to assist mechanization.

The radioenzymatic procedure was first described for assaying gentamicin (22) and subsequently the R-mediated enzyme gentamicin adeny1 transferase was partly purified and characterized by Smith and Smith (23). The radioenzymatic assay can be used to assay most aminoglycoside antibiotics. The aminoglycoside amikacin is not adenylated by this enzyme but is acetylated by another R-mediated enzyme, kanamycin acetyl transferase. It can be assayed by using [3H]-acetyl coenzyme A as a co-substrate (24). The radioenzymatic assays are advantageous in that the same reagents can be used to assay for most aminoglycosides. They are precise but require expertise and the use of liquid-scintillation counting equipment, which is not available in all clinical laboratories.

The microbiological assay is inexpensive but requires long incubation times and is subject to interference by other antimicrobial agents. An additional problem with microbiological assays is the difficulty of interpreting results, especially when variations in support media or double zonation are encountered; this phenomenon makes precise reading of the inhibition zone very difficult. The high y-intercept and the relatively poor correlation between the microbiological assay and both the radioenzymatic and the radioimmunoassay make it the least desirable method of monitoring patients receiving netilmicin.

Having validated the radioimmunoassay procedure, we studied four patients with normal renal function who were receiving netilmicin by short intravenous infusion to determine the β elimination half-life of the drug. The half-life of 135 min is similar to those reported for the other aminoglycosides (25). Schentag (26) reported long terminal half-life of gentamicin with specimens taken up to 300 h after therapy. In our laboratories, we have noted long terminal half-lives for tobramycin and are presently evaluating the terminal half-life of netilmicin.

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**Table 1. Comparison of Results by the Three Methods**

<table>
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<tr>
<th>Method</th>
<th>y-axis</th>
<th>x-axis</th>
<th>Coef. of correl. (r)</th>
<th>Regression anal.</th>
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<td>Radioenzymatic</td>
<td>Radioimmunoassay</td>
<td>0.90</td>
<td>y = 1.04x + 0.01</td>
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<tr>
<td>Microbiological</td>
<td>Radioimmunoassay</td>
<td>0.90</td>
<td>y = 1.4x + 1.5</td>
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<tr>
<td>Microbiological</td>
<td>Radioenzymatic</td>
<td>0.82</td>
<td>y = 1.18x - 0.66</td>
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**References**