Homogeneous Reactant-Labeled Fluorescent Immunoassay for Therapeutic Drugs Exemplified by Gentamicin Determination in Human Serum

John F. Burd, Raphael C. Wong, John E. Feeney, Robert J. Carrico, and Robert C. Boguslaski

We applied a homogeneous reactant-labeled fluorescent immunoassay to the measurement of therapeutic drug concentrations in human serum, exemplified here by gentamicin. A derivative of umbelliferyl-β-galactoside was coupled covalently to the drug and this conjugate was found to be nonfluorescent under assay conditions. The drug/dye conjugate was a substrate for bacterial β-galactosidase and yielded a fluorescent product. When the drug/dye conjugate was bound to anti-gentamicin antibody it was inactive as an enzymatic substrate. This inactivation was relieved by the presence of gentamicin in competitive binding reactions. Hence, the rate of production of fluorescence was proportional to the gentamicin concentration. The fluorescent assay yielded values which compared favorably to a radioimmunoassay for gentamicin in clinical serum samples (r = 0.94, standard error of estimate = 0.66 mg/liter). The fluorescent assay requires only 1 μl of serum and offers several advantages over existing techniques: sensitivity, specificity, simplicity, and the obviation of radioisotopes.

Additional Keyphrases: drug assay • antibiotics • monitoring therapy • immunochemistry • fluorometry • competitive protein-binding assay

Gentamicin is a broad spectrum aminoglycoside antibiotic currently of great value in the treatment of severe infections of Gram-negative bacteria. Optimum therapeutic concentrations of gentamicin in human serum lie between 4 and 12 mg/liter (9 to 27 μmol/liter). Below this range the drug is ineffective; higher concentrations may lead to ototoxicity and nephrotoxicity (1). Within recent years several methods have evolved for measuring the concentration of gentamicin in serum and other biological specimens. These methods include microbiological assay (2), enzymatic assay (3), RIA1 (4), and a fluorescence polarization immunoassay (5). We wish to report a new fluorescent immunoassay for measuring gentamicin in human serum. This work extends our previous studies on reactant-labeled immunoassays (6–8).

The reaction principles on which this fluorescent immunoassay is based are presented schematically in Figure 1. In the method a drug/fluorescent dye conjugate is used as a substrate for an enzyme. The drug/dye conjugate must be nonfluorescent under assay conditions and must react with the enzyme to yield fluorescent products (Figure 1, I). When the drug/dye conjugate interacts with an antibody produced against the drug (Figure 1, II), a complex forms that prevents interaction of the bound conjugate with the enzyme. In competitive binding reactions (Figure 1, III), drug present in the serum sample competes with the drug/dye conjugate for binding sites on the antibody. The amount of drug/dye conjugate available for reaction with the enzyme is therefore controlled by the concentration of free drug in the serum sample. Hence, the rate of increase in fluorescent intensity is proportional to the drug concentration in the sample. Because the drug/dye conjugate bound to the antibody is not active as an enzymatic substrate, no separation steps are required.

To perform this fluorescent immunoassay for gentamicin, one adds 1 μl of the serum standard or unknown to a reagent composed of a buffered solution of antiserum and bacterial β-galactosidase (β-D-galactoside galactohydrolase; EC 3.2.1.23), mixes, adds the drug/dye conjugate, and monitors the rate of production of fluorescence.

Results of assays of clinical samples were compared to results obtained by an RIA method. Also, the specificity, sensitivity, accuracy and precision of the fluorescent assay were evaluated.

Materials and Methods

Apparatus

Fluorescence was measured with an Amino-Bowman spectrophotofluorometer (American Instrument Co., Silver Springs, Md. 02910) equipped with temperature
control for the sample compartment. Excitation and emission wavelengths were 400 and 453 nm, respectively. Reaction rates were monitored with a Model 7100 B strip-chart recorder (Hewlett-Packard, Rolling Meadows, Ill. 60008). Polystyrene disposable cuvettes (Evergreen Scientific, Los Angeles, Calif. 90058) were used for routine measurements. Reaction rates are presented as the change in arbitrary fluorescence units per minute. All fluorescence measurements were performed at 25 °C.

Absorbance was measured with a Model 2000 spectrophotometer (Gilford Instrument Labs, Inc., Oberlin, Ohio 44074) or a Model 16 spectrophotometer (Cary Instruments, Monrovia, Calif. 91016).

Optical rotations were measured with a Model 241 polarimeter (Perkin-Elmer Corp., Norwalk, Conn. 06856). Infrared spectra were recorded with a Perkin-Elmer 237 spectrophotometer. NMR spectra were measured at 60 MHz with a Model T-60 spectrometer (Varian Instruments, Palo Alto, Calif. 94303) and chemical shifts are reported in parts per million down field from an external tetramethylsilane standard.

Reagents

Enzyme. Escherichia coli-derived β-galactosidase was used (Grade IV; Sigma Chemical Co., St. Louis, Mo. 63178). One IUB unit (U) hydrolyzes 1.0 µmol of o-nitrophenyl-β-D-galactoside per minute at pH 7.2 at 37 °C. The enzyme preparation used in the present studies had a specific activity of 745 U per milligram of protein. Enzyme concentrations are reported as nanograms of protein per assay.

Buffer. Bicine buffer (Nutritional Biochemicals Corp., Cleveland, Ohio 44128) was used (50 mmol/liter, pH 8.2 at 25 °C).

Drugs. Gentamicin, sisomicin, netilmicin, the purified gentamicin components C1, C2, and C1A (Schering Corp., Bloomfield, N. J. 07064), kanamycin, and amikacin (Bristol Laboratories, Syracuse, N. Y. 13201), and tobramycin (Eli Lilly & Co., Indianapolis, Ind. 46206) were provided by the manufacturers. Other drugs tested (Table 3) were obtained by prescription.

Drug/Fluorescent Dye Conjugate

The synthetic scheme for preparing the drug/dye conjugate, β-galactosyl-umbelliferone-sisomicin (Figure 2, III), is presented in Figure 2. Gentamicin is...
a mixture of three components (9); sisomicin was used to prepare the drug/dye conjugate. It is a single compound, which differs from gentamicin C1A only by the presence of one carbon–carbon double bond (10). Thus, sisomicin is immunochemically similar to the gentamicins (11).

We prepared the 3-carboethoxy-7-hydroxycoumarin (Figure 2, I) using a Knoevenagel condensation of 2,4-dihydroxybenzaldehyde (Aldrich Chemical Co., Milwaukee, Wis. 53233) with diethylmalonate in acetic acid, benzene, and piperidine (12). The potassium salt of β-[7-(3-carboxycoumarinoyl)]-D-galactoside (Figure 2, II) was prepared by the reaction of 3-carboethoxy-7-hydroxycoumarin and 2,3,4,6-tetraacetyl-α-D-galactosyl bromide (Sigma Chemical Co.) as described by Leaback for the preparation of methylumbelliferonyl-β-D-galactoside (13). The potassium salt of this compound was purified by chromatography on silica gel-60 (E. Merck, St. Louis, Mo. 63116) with a gradient of n-butanol/methanol/water (4/2/1 by vol) and methanol/water (1/6). After recrystallization from acetone–water, the corrected melting point of the product was 258–263 °C (decomp.). Analysis: calculated for C16H15O10K: C 47.28%, H 3.73%, K 9.62%; found: C 47.30%, H 3.74%, K 9.34%. Optical rotation [α]D20 = –77.40 (ig. H2O). NMR (2H2O, δ 8.2 (s, 1H), 7.6 (m, 1H), 7.0 (m, 2H), 5.1 (s, 1H), and 4.0 (m, 6H). Infrared analysis (KBr) indicated a carbon–oxygen double bond and a carbon–carbon double bond (1705 and 1620 cm⁻¹).

β-Galactosyl-umbelliferone-sisomicin (Figure 2, III) was prepared by mixing 50 mg (117 µmol) of the potassium salt of β-[7-(3-carboxycoumarinoyl)]-D-galactoside with 171 mg of sisomicin sulfate (223 µmol of sisomicin free base) in 2 ml of water. The pH was adjusted to 3.8 by dropwise addition of 1 molar HCl. The solution was cooled in an ice bath and 30 mg (150 µmol) of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Pierce Chemical Co., Rockford, Ill. 61105) was added. After 2 h the mixture was chromatographed at 25 °C on a 2.5 × 50 cm column of CM-Sephadex C-25 (Pharmacia Laboratories, Inc., Piscataway, N. J. 08854), 5.8-ml fractions were collected, and their absorbance was monitored at 345 nm. The column was washed with 200 ml of 50 mmol/liter ammonium formate to elute unreacted β-galactosyl-umbelliferone-sisomicin. A linear gradient, formed with 400 ml of 50 mmol/liter and 400 ml of 1.8 mol/liter ammonium formate, was applied to the column. A peak of material absorbing at 345 nm eluted at approximately 1.4 mol/liter ammonium formate. After the gradient, the column was washed with 600 ml of 1.8 mol/liter ammonium formate. Three 345-nm absorbing peaks were eluted in this wash. Unreacted sisomicin was eluted well separated from the last 345-nm absorbing peak.

The carbodiimide-activated reaction (Figure 2) presumably leads to the formation of amide bonds between the carboxylic acid of β-[7-(3-carboxycoumarinoyl)]-galactoside and the primary amino groups of sisomicin (Figure 2). The major peak of β-galactosyl-umbelliferone-sisomicin (the last 345-nm absorbing peak) was used in the present studies. Ammonium formate was removed by lyophilization. Because the absorptivity of this compound is currently unknown, the relative concentration is presented in terms of A345 units.¹

Antiserum

Antibodies against gentamicin were produced as described (4).

Gentamicin Serum Samples

Sera from patients receiving gentamicin were provided by Dr. K. Cario (South Bend Medical Foundation, South Bend, Ind. 46601). These samples were used to compare the fluorescent immunoassay with a RIA for gentamicin.

RIA for Gentamicin

In comparison studies, gentamicin was determined with a commercial RIA kit (Monitor Science Corp., Newport Beach, Calif. 92663).

Procedures

Fluorescent immunoassay for gentamicin in human serum. A reagent, prepared in 50 mmol/liter Bicin buffer (pH 8.2), contained β-galactosidase (25 ng/ml) and anti-gentamicin antiserum (an amount sufficient to decrease the reaction rate to 20 to 30% of the rate observed in the absence of antibody). To 2 ml of this reagent was added 1 µl of serum standard or unknown. After mixing, 5 µl of an aqueous solution of β-galactosyl-umbelliferone-sisomicin (0.125 A345 units/ml) was added and the rate of increase in fluorescence was monitored for 2 to 3 min. All solutions were kept at 25 °C, except the β-galactosyl-umbelliferone-sisomicin, which was kept in an ice bath.

Results

The absorbance spectrum of the drug/dye conjugate, β-galactosyl-umbelliferone-sisomicin, showed an absorbance maximum at 345 nm. When the conjugate was hydrolyzed with bacterial β-galactosidase to remove the galactose moiety, the absorbance at 345 nm decreased and a new maximum appeared at 402 nm. The absorbance of the enzyme-treated conjugate was 1.46 times that of the untreated conjugate.

Analysis of the fluorescence spectrum of the conjugate revealed a similar shift in the maximum wavelength. Before enzyme treatment, the conjugate exhibited excitation and emission maxima at 350 and 394 nm, respectively. After hydrolysis with β-galactosidase, a 15-fold increase in fluorescence was observed, with new excitation and emission maxima of 409 and 445 nm. Hence, under the conditions of the fluorescent assay (excitation and emission wavelengths of 400 and 453) ¹ One A345 unit is the quantity of material contained in 1 ml of a solution that has an absorbance of 1.0 at 345 nm when measured with a 1-cm light path.
the unreacted conjugate contributed negligible fluorescence.

The reaction of β-galactosyl-umbelliferone-sisomicin with β-galactosidase was examined. In 2-ml reaction volumes containing 0.0006 $A_{345}$ units of the drug/dye conjugate, we observed a linear response between reaction rate and β-galactosidase concentration between 5 and 80 ng of enzyme per reaction.

Likewise, we observed a linear relation between reaction rate and conjugate concentration between 0.00015 and 0.096 $A_{345}$ units per reaction when 50 ng of enzyme was reacted with β-galactosyl-umbelliferonesisomicin. All reaction rates were linear for at least 3 min. Based upon this information, 0.0006 $A_{345}$ units of conjugate with 50 ng of β-galactosidase in 2-ml volumes was used in the following experiments.

The effect of antiserum to gentamicin on the ability of the drug/dye conjugate to function as a substrate for β-galactosidase was examined. Various amounts of antiserum were added to 2.0 ml of buffered β-galactosidase. The drug/dye conjugate was added and the reaction rate determined. As the amount of antiserum increased, the reaction rate decreased (Figure 3). Addition of normal rabbit serum did not affect the reaction rate (Figure 3). Apparently the reaction between the antibody and the conjugate was complete in the time required for mixing the reagents, because incubation of the conjugate with the antibody before adding enzyme did not alter the results. Based upon this experiment, an amount of antiserum sufficient to inhibit the reaction rate by 70 to 80% was chosen for the competitive binding reactions.

For the standard curve, gentamicin standards were prepared from 0 to 14 μg/ml (mg/liter) in normal human serum and assayed as described in Materials and Methods. The strip-chart recording of the rates for the reactions containing 1 μl of the 0, 6, and 12 μg of gentamicin standards per milliliter are shown in Figure 4. Figure 5 shows the standard curve of the reaction rate vs. gentamicin concentration in serum standards. No difference was observed for standards prepared in
buffer vs. standards prepared in serum. Varying the time of incubation of the standards with the antibody/enzyme reagent from 0.25 to 60 min before adding the drug/dye conjugate did not alter the standard curve. Hence, the assay can be performed as rapidly as the reagents can be mixed. As shown in Figure 5, the standard curve approximates a straight line between 0 and 10 μg/ml. The sensitivity of the assay (zero level plus 2 SD) was 0.8 ng of gentamicin.

To establish the reliability of the fluorescent immunoassay in clinical situations, we determined gentamicin by the fluorescent assay in 66 serum samples from patients receiving therapy. We compared the results with those obtained by RIA. As shown in Figure 6, results by the two methods agreed very well (correlation coefficient = 0.94). The regression line determined by the method of least squares was y = 1.07x — 0.19, with the standard error of estimate equal to 0.66 μg/ml. Previous work has established the correlation of results by RIA with those by the microbial assay (14).

To establish the precision of the fluorescent assay, we examined standards of 2, 6, and 10 μg of gentamicin per milliliter 20 times each in one day. In addition, these standards were analyzed in triplicate on 10 different days. The results are presented in Table 1. The statistical analysis was performed on both the reaction rates and the concentration as determined from standard curves. The observed precision is similar to that observed for RIA (14).

In recovery experiments, 2, 4, and 6 μg of gentamicin per milliliter was added to each of six clinical serum samples with low gentamicin concentrations (1.7 to 3.0 μg of gentamicin per milliliter). The percentage analytical recovery for the different gentamicin concentrations in the six samples was 88.5, 103, 106, 93.3, 87.7, and 109%. In another experiment, 50, 35, 15, and 5 μl of 50-fold dilutions of five clinical serum samples with high gentamicin concentrations were examined. The results (Table 2) illustrate that the observed values, when multiplied by the dilution factor, are equivalent. These

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**Table 1. Precision of Fluorescent Immunoassay**

<table>
<thead>
<tr>
<th>Serum standard, μg/ml</th>
<th>2 μg/ml</th>
<th>Mean</th>
<th>SD</th>
<th>CV %</th>
<th>6 μg/ml</th>
<th>Mean</th>
<th>SD</th>
<th>CV %</th>
<th>10 μg/ml</th>
<th>Mean</th>
<th>SD</th>
<th>CV %</th>
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<tbody>
<tr>
<td><strong>Rate</strong></td>
<td>5.9-6.5</td>
<td>7.0</td>
<td>0.5</td>
<td>7.1</td>
<td>10.2-11.6</td>
<td>10.7</td>
<td>0.5</td>
<td>3.0</td>
<td>13.7-15.2</td>
<td>14.2</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Intra-assay variation</strong></td>
<td>0.5-3.4</td>
<td>1.7</td>
<td>0.6</td>
<td>35.3</td>
<td>5.3-6.9</td>
<td>6.0</td>
<td>0.3</td>
<td>5.0</td>
<td>6.0</td>
<td>2.8</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>6.6-10.0</td>
<td>1.2-2.5</td>
<td>12.6</td>
<td>16.7</td>
<td>10.3</td>
<td>8.3</td>
<td>9.3</td>
<td>6.9</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Inter-assay variation</strong></td>
<td>10.6-15.4</td>
<td>2.8</td>
<td></td>
<td></td>
<td>4.4-7.2</td>
<td>5.0</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Range</strong></td>
<td>13.6-19.3</td>
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<td>0.5</td>
<td>15.1</td>
<td>8.6-11.7</td>
<td>6.0</td>
<td>0.7</td>
<td>1.5</td>
<td></td>
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<td></td>
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<tr>
<td><strong>Mean</strong></td>
<td>16.1</td>
<td>1.5</td>
<td>0.7</td>
<td>1.5</td>
<td>10.1</td>
<td>0.8</td>
<td>1.5</td>
<td>0.7</td>
<td></td>
<td></td>
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<tr>
<td><strong>SD</strong></td>
<td>19.3</td>
<td>2.8</td>
<td>5.1</td>
<td>2.8</td>
<td>6.9</td>
<td>9.3</td>
<td>9.3</td>
<td>6.9</td>
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Table 2. Effect of Sample Dilution on Fluorescent Assay Results

<table>
<thead>
<tr>
<th>Sample</th>
<th>50 µl</th>
<th>35 µl</th>
<th>15 µl</th>
<th>5 µl</th>
<th>Mean ±SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.5</td>
<td>11.3</td>
<td>12.2</td>
<td>12.5</td>
<td>11.9±0.6</td>
<td>5.0</td>
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<tr>
<td>2</td>
<td>—</td>
<td>20.0</td>
<td>18.7</td>
<td>20.5</td>
<td>19.7±0.9</td>
<td>4.6</td>
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<tr>
<td>3</td>
<td>8.85</td>
<td>8.0</td>
<td>9.5</td>
<td>8.5</td>
<td>8.7±0.6</td>
<td>6.9</td>
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<tr>
<td>4</td>
<td>8.65</td>
<td>8.0</td>
<td>8.83</td>
<td>5.0</td>
<td>7.6±1.8</td>
<td>23.7</td>
</tr>
<tr>
<td>5</td>
<td>10.1</td>
<td>10.7</td>
<td>12.3</td>
<td>8.0</td>
<td>10.3±1.8</td>
<td>17.5</td>
</tr>
</tbody>
</table>

* Corrected values are observed values times the dilution factor.

experiments show that there are no factors in serum that adversely affect the fluorescent assay.

The activity of β-galactosidase in human serum has been reported to be extremely low (13). In addition, the β-galactosidase in human serum is very unstable and has a low pH for maximum enzymatic activity (pH 4.1). To test for β-galactosidase in clinical serum samples, 10 sera were chosen at random and 5 µl of each (five times the amount required for the fluorescent assay) was incubated with 0.0006 A345 units of β-galactosyl-umbelliferone-sisomicin in 2 ml buffer. After observing the fluorescence for 5 min, we added 50 ng of bacterial β-galactosidase and monitored the fluorescence again. The bacterial β-galactosidase was added to check for factors that might enhance or inhibit its enzymatic activity and thereby interfere with assay results. None of the serum samples exhibited enzyme activity. Furthermore, all rates observed after adding the bacterial enzyme were identical (rate = 15.0±1.6).

The specificity of the immunoassay was studied. Seventeen drug preparations were examined for cross reactivity in the fluorescent immunoassay. From 1 to 10 000 ng of each drug was added to 2.0 ml of reagent containing antibody and bacterial β-galactosidase. β-Galactosyl-umbelliferone-sisomicin was then added and the reaction rate was determined. The amounts of the various drugs required to obtain 50% of the maximum reaction rate is shown in Table 3. Only those drugs with structures closely related to the gentamicins showed cross reactivity. These results agree with previous studies of drug cross reactivities with a gentamicin RIA (4,11). Because the specificity of both assay techniques resides in the antiserum, this agreement was expected.

Discussion

These studies indicate that the reactant-labeled fluorescent immunoassay is readily applicable to the determination of gentamicin in human serum. The requirements for the fluorescent immunoassay outlined previously (6) are satisfied by the system described herein. Namely, the drug/dye conjugate is nonfluorescent under assay conditions and it reacts with β-galactosidase to produce a fluorescent product. The enzymatic reaction is inhibited by antibody and the inhibition is reversed by the presence of gentamicin. As described previously (6), the inhibition of the enzymatic reaction can also be used as an assay for specific binding proteins.

The use of a β-galactoside linkage offers several advantages over the ester-linked system described previously (6), especially for determining the concentration of compounds in human serum. Human serum contains high esterase activity, which would interfere with an assay based on esterase activity; β-galactosidase activity in human serum is negligible. Also, β-galactoside linkages are more stable than ester bonds under the assay conditions, with the result that there is negligible background hydrolysis in the absence of enzyme. Furthermore, the antibody-induced hydrolysis observed with the ester-linked system (6) was not observed with the β-galactosyl linkage. Our experience has also shown that bacterial β-galactosidase is a very stable enzyme, even in a very dilute solution.

The fluorescent immunoassay combines several of the advantages of previous methods for gentamicin determination and eliminates many of their disadvantages. The fluorescent assay: (a) is homogeneous (no separation steps are required to separate antibody-bound and free drug/dye conjugate); (b) is rapid and requires minimal manipulation of a small sample (1 µl required); (c) uses stable, nonisotopic reagents; (d) possesses sensitivity, specificity, precision, and accuracy similar to that of RIA; (e) yields a linear standard curve over the range of interest; (f) is amenable to total automation; and (g) may be applicable to a variety of biologically important molecules.
We thank Lenora Coyle for excellent technical assistance, and the following individuals for generously providing materials: Mr. Gerald Wagman (Schering Corp.), for the purified gentamicin components, C1, C1A, and C2; Dr. Jerald C. Nelson (Loma Linda University), for a sample of antiserum to gentamicin; and Dr. Karen Carifo (South Bend Medical Foundation), for the clinical serum samples.

References