Double-Antibody Fluorescence Immunoassay of Tobramycin

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This heterogeneous assay for tobramycin involves fluorescein-labeled tobramycin, which competes with native unlabeled tobramycin for anti-tobramycin binding sites. Bound and free labeled antigen are separated by precipitation with a second antibody. Fluorescence intensity of the resuspended precipitate is inversely proportional to native tobramycin concentration. Background interference was consistently about 10% of the total fluorescence precipitated. Assay sensitivity was sufficient to detect nanogram quantities of tobramycin per assay tube. Correlation coefficients (r) were 0.96 and 0.98 for comparisons of this assay with a microbiological assay and a radioimmunoassay, respectively. Mean analytical recovery was 101% and the CV was <10% throughout the therapeutic range.

Additional Keyphrases: drug assay • monitoring therapy • aminoglycoside antibiotics

Tobramycin is an aminoglycoside antibiotic used to treat Gram-negative bacillary infections. Proper treatment requires recognition that peak serum concentrations of <5 mg/L are generally ineffective, and concentrations exceeding 10–12 mg/L are associated with otoxic and nephrotoxic side effects (1, 2). This low therapeutic index indicates a need for monitoring serum concentrations of tobramycin so that maximum efficacy and minimum toxicity can be achieved.

Tobramycin has been assayed in serum samples by microbiological (3), radioenzymic (4), radioimmunoassay (5), and enzyme immunoassay (6) techniques. Although radioimmunoassays offer definite advantages over other techniques, it is desirable to avoid using isotopic labels because of their limited shelf life, regulations on shipping and handling radioactive materials, and the need for expensive counting equipment. Fluorescence immunoassays have been developed for several clinically important analytes (7), obviating the use of radioisotopes.

We describe a fluorescence immunoassay based on a double-antibody separation of bound and free fluorescein-labeled tobramycin. This method involves simple techniques and only minimum capital equipment, and is generally applicable to many clinically important analytes.

Materials and Methods

Reagents

Glycine buffer (0.01 mol/L). Dissolve glycine (0.938 g) and NaCl (0.731 g) in 900 mL of doubly distilled water. Adjust the pH to 2.0 with 10 mol/L HCl and dilute to 1 L with doubly distilled water.

Phosphate buffer (0.1 mol/L). Dissolve 2.5 g of bovine serum albumin (Cohn Fraction V; Eastman Kodak Co., Rochester, NY 14650) in a solution of 80 mL of KH2PO4 (18.15 g/L) and 196 mL of Na2HPO4 (19.09 g/L); add 1 g of sodium benzoate to this mixture and adjust the pH to 7.5 with NaOH.

Fluorescein-labeled tobramycin. We prepared fluorescein thio carbamyl-tobramycin by the procedure used by Watson et al. for the preparation of labeled gentamicin (8), but substituted tobramycin free base (Eli Lilly and Co., Indianapolis, IN 46288) for gentamicin. The separation step of this procedure was modified: the chromatographic elution rate was 1.2 mL/h, and 2-mL fractions were collected over a period of seven days. We pooled fractions containing the tobramycin conjugate (53–70), concentrating them by evaporation under reduced pressure to 1.1 mL to provide a stock solution of label containing 4.94 mg of immunoreactive tobramycin per liter, as assayed by radioimmunoassay. The stock label solution was stored at 4 °C until use, when it was diluted 150-fold with pH 2.0 glycine buffer to yield a working label solution containing 33 μg of tobramycin per liter. Working solutions were freshly made daily, to minimize changes in concentration from adsorption of the aminoglycoside onto glass.

Anti-sera. Bovine serum albumin was conjugated to tobramycin and injected into New Zealand white rabbits according to the procedure of Broughton and Strong (9). Anti-tobramycin sera were obtained 10–14 days after injection and frozen until used. Goat anti-rabbit immunoglobulin was supplied by the University of California at Los Angeles Medical Center.

Tobramycin standards. Dissolve 0.1 g of tobramycin free base in 100 mL of distilled water. Dilute this solution 62.5-fold with tobramycin-free normal human control sera (Hyland Diagnostics Div., Travenol Lab Inc., Costa Mesa, CA 92628) to prepare a 16 mg/L working standard. Then dilute this standard serially to obtain working standards ranging from 0.5 to 16.0 mg of tobramycin per liter. Store aliquots frozen until use.

Procedures

Separation procedure. Dilute all samples, standards, and controls 20-fold with pH 2.0 glycine buffer. Pipet 0.1 mL of diluted sera plus 0.1 mL of working label solution into separate polystyrene reaction tubes. Add anti-tobramycin serum (0.1 mL of serum dilution 100-fold in phosphate buffer) to each tube and incubate all tubes at 37 °C. After 30 min, add 0.1 mL of working goat anti-rabbit immunoglobulin solution (diluted sevenfold in phosphate buffer) and 0.1 mL of normal rabbit-serum (diluted 200-fold in phosphate buffer) to each tube. After incubating the tubes for 2 h, add 0.1 mL of a twofold dilution of saturated Na2SO4 and 0.4 mL of pH 7.5 phosphate buffer to each tube. Centrifuge all tubes at 3000 rpm and 4 °C for 30 min. Aspirate the supernate, being careful not to disturb the precipitate at the bottom of the tubes. Add 0.2 mL of 0.1 mol/L NaOH to redissolve the precipitate, then vortex-mix vigorously. After a 10-min incubation at room temperature, add 0.4 mL of phosphate buffer to each tube and transfer all redissolved precipitates to separate 6 × 50 mm glass tubes. Measure the fluorescence of these mixtures at 470-nm excitation and 520-nm emission.

Fluorometry. We used an Aminco-Bowman spectrophotofluorimeter (Model SPF-125; American Instrument Co.,

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Silver Spring, MD 20910) equipped with a xenon arc lamp source. The instrument was slightly modified by adding a flow-cell holder drilled out to 6.1 mm (i.d.) to receive the 6 × 50 mm glass tubes. Monochromator slit widths were 2 mm for both excitation and emission.

Calculations. Fluorescence intensity readings were processed with a Model CP-5000 Clinical Processor (Syva Co., Palo Alto, CA 94304). Standard concentration values were stored in the instrument’s memory and the corresponding relative intensity values were entered manually. Data were plotted logit-log and unknown concentrations were derived from a best-fit curve of standard values.

Microbiological assay. The microbiological assay of tobramycin involved a plate diffusion technique used by the Infectious Disease Department, Shands Teaching Hospital, University of Florida, Gainesville, FL.

Radioimmunoassay. Radioimmunoassay of tobramycin was done courtesy of Upjohn Laboratory Procedures, using a commercial kit supplied by Monitor Science Corp., Newport Beach, CA 92660.

Results

Fluorescein-Labeled Tobramycin

The working label dilution (33 μg of tobramycin per liter), provided a consistently stable fluorescence signal and produced a labeled-to-unlabeled antigen ratio that gave effective competition reactions for tobramycin. The ratio was similar to that used by Watson et al. (8) for gentamicin assays. This dilution provided adequate displacement for original tobramycin concentrations of 0.5 to 16.0 mg/L while maintaining good sensitivity at the low end of the standard curve. Fluorescence intensity measurements for the 0.5 mg/L standard were consistently about 12% below that of the zero binding tube. Therefore, the least detectable amount of tobramycin is less than 2.5 ng per assay tube.

The labeled conjugate was characterized by thin-layer chromatography on silica plates with ammonium hydroxide/methanol/chloroform (1/2/1 by vol) as the mobile phase. No detectable contamination from unreacted fluorescein isothiocyanate or tobramycin was found in the stock label solution by this method. Additionally, virtually 100% of total label fluorescence could be precipitated in zero binding tubes by the double antibody, which further indicates the purity of the label.

Antiserum Dilution Curve

We assayed blank tubes with eight different antiserum concentrations before selecting the appropriate dilution of tobramycin antiserum. The antiserum dilution curve (Figure 1) demonstrates that increasing fluorescence is precipitated as the antiserum becomes more concentrated and a decrease as antibody excess is reached. We chose to use 100-fold diluted because this consistently provided 50% binding of label at zero tobramycin concentration, a value we considered desirable in maintaining good displacement in the antigen–antibody competition reaction. The antiserum dilution curve remained relatively unchanged after three and one-half months, indicating good stability of both label and antiserum. However, the exact dilution of both label and antiserum may vary with each preparation and must be determined when the lot is changed.

Other Analytical Variables

Standard curve. A standard curve showing decreasing fluorescence precipitated as unlabeled tobramycin concentration increases is shown in Figure 2. The curve demonstrated acceptable linearity on a logit-log plot, with a correlation coefficient (r) of 0.991. We did not extend the upper end of the curve beyond 16.0 mg/L original tobramycin concentration because of the decreased precision at higher concentrations. Analytical data collected by comparing sample fluorescence intensity values to best-fit logit-log plots of standard curves showed no significant change over three and one-half months, again indicating good shelf life of reagents used.

Specificity. Various antibiotics were added to tobramycin-free normal human control serum to give 5000, 500, 50, and 5 mg/L concentrations and were assayed by the double-antibody fluorescence immunoassay procedure. Kanamycin, which differs from tobramycin by one substituent group, showed the only significant cross reactivity (68%). Gentamicin and cephalothin cross reacted slightly, but not more than 0.1%. Other antibiotics found to show no cross reactivity were neomycin, vancomycin, carbenicillin, clindamycin, methicillin, ampicillin, chloramphenicol, cephradine, penicillin V, and eszafolin.

Precision. To test the precision of the double-antibody method, we assayed three control sera 24 times in one assay and in 10 consecutive assays (Table 1). The precision was optimally adjusted for control values within the therapeutic range.

Accuracy. Accuracy was evaluated by analytical recovery experiments in which tobramycin was added to tobramycin-
free human sera and assayed by the described method. Figure 3 shows a plot of tobramycin added vs tobramycin found for 24 samples at 10, 5, and 2.5 g/L concentrations. The line of best fit is very similar to the line of equivalence, indicating good accuracy at the concentrations studied. The mean percentage analytical recovery for the three concentrations was 101%.

**Patients’ Samples**

Serum samples from patients receiving tobramycin therapy were assayed by the present assay, a microbiological assay, and a radioimmunoassay. For 44 samples analyzed by the radioimmunoassay (y) and the fluorescence immunoassay (x), the linear regression equation was \( y = 1.03x - 0.06 \) (\( r = 0.98; S_x = 0.42 \)). An additional 22 samples had values of <1.0 mg/L by both methods; these results were not included in the correlation plot.

Patients’ samples (24 selected) with tobramycin exceeding 1.0 mg/L were assayed by both the fluorescence immunoassay (x) and the bioassay (y). The correlation plot of the two assays yielded a linear regression equation of \( y = 1.22x - 0.41 \) (\( r = 0.96; S_x = 0.64 \)). The precision of the particular bioassay used is such that only one significant figure is validly reported; therefore, the lower correlation coefficient of \( r = 0.96 \) was expected, considering the relatively imprecise bioassay results.

Fluorescence background from all assay components, excluding the label, was found to be about 10% of the value for the zero binding sample, with little variation (±2%). Most of this background, however, was attributable to light scattering rather than endogenous fluorescence.

To assess the fluorescence contributions of individual sample components, we compared sample blank mixtures containing no label with their corresponding assay mixtures. In various samples that were noticeably hemolyzed or turbid, the variation for the individual sample was not more than 1.0% of the fluorescence signal from assay mixtures. We considered this small contribution negligible, and made no blank subtractions.

**Discussion**

These studies indicate that the double-antibody fluorescence immunoassay is applicable to the quantitation of tobramycin in human serum. Problems usually associated with homogeneous fluorescence immunoassays have been overcome, for the most part, by the separation step. Fluorescence background contribution due to individual sample components was negligible. In addition, the background contribution attributable to intrinsic sample fluorescence and light scatter remained relatively constant and should not cause problems with routine analysis. Scatter interferences could be considerably decreased if square sample cells were used. It is important to use disposable glassware throughout the assay, to avoid carryover due to adsorption of the label onto glass and plastic. Use of the glycine buffer minimizes adsorption of the aminoglycoside and label onto glass.

A source of error associated with homogeneous procedures is intrinsic light absorbance of the serum samples. We avoid this problem by including the separation step; the good correlation with the radioimmunoassay method, also free of such interferences, demonstrates this advantage. The double-antibody method also avoids solid-phase fluorescence measurements, which generally require modified instrumentation.

This method is less sensitive than radioimmunoassay techniques for tobramycin (5). However, the detection limit for our method is more than adequate for clinical usefulness and could be decreased by adjusting assay conditions so that more dilute samples could be assayed. The precision and accuracy of this assay compare well with those of the corresponding radioimmunoassay (5), enzyme immunoassay (6), radioenzymic (4), and bioassay (3) methods. Correlation is good between our assay and widely used radioimmunoassay and bioassay procedures. The specificity of the method with the particular antiserum used was excellent for all drugs tested except kanamycin, which would not be expected to be present in patients’ samples.

With close attention to reagent preparation, technical manipulations, and fluorometry, the described fluorescence immunoassay combines several advantages of previous methods and eliminates many of their disadvantages. Fluorescein-labeled tobramycin can be easily and economically prepared, and avoids restrictions associated with isotopic labels. All other reagents are inexpensive, can be easily obtained, and have excellent shelf life. No elaborate detection equipment is necessary for this method, and any modern conventional fluorometer should be adequate for routine assay purposes. The 4-h analysis time, is somewhat long as compared with some other methods, but for much of this period no hands-on attention is required, and the assay could be shortened by combining reagents. The logit-log standard curve is linear over the range of interest and we encountered no significant specific or nonspecific interferences. In theory, the
method is generally applicable to many analytes now assayed by radioimmunoassay and is limited only by the type and quality of fluorometer used.

References