Liquid-Chromatographic Determination of Tobramycin in Serum with Spectrophotometric Detection

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We describe a simple, precise, accurate, and specific liquid-chromatographic procedure for determination of tobramycin in 50 μL of serum. Tobramycin and the internal standard (sisomicin) are quantitatively converted into their trinitrophenyl derivatives by reaction with a water-soluble derivatizing agent (2,4,6-trinitrobenzenesulfonic acid) at 70 °C for 30 min. The derivatives are extracted from the crude reaction mixture by using a reversed-phase Bond-Elut C18 column, and separated on a reversed-phase octyl column with a mobile phase consisting of an acetonitrile/phosphate buffer (70/30 by vol) at a flow rate of 3.0 mL/min. The eluted compounds are detected at 340 nm, and quantified from their peak areas. Chromatography is complete in <4.5 min at the optimum column temperature of 50 °C. The lower limit of detection for tobramycin is <0.2 mg/L. Analytical recoveries for tobramycin varied from 94 to 99%, linearity extended to 25 mg/L, and day-to-day precision (CV) was between 4.6 and 5.1%. Numerous drugs and antibiotics tested do not interfere. Results correlate well (r > 0.95) with those by radioimmunoassay and EMIT®.

Tobramycin, an aminoglycoside antibiotic, is widely used against Gram-negative bacterial infections. It has a narrow therapeutic range, and can cause severe nephro- and ototoxicity at serum concentrations exceeding 12 mg/L (1), which makes it essential to monitor its concentration in serum during therapy.

Methods for measuring serum tobramycin include microbiological, radioenzymatic, and immunoassays (1, 2). The microbiological assays are inexpensive and simple, but may suffer from several deficiencies such as slow turnaround time because of a long incubation, limited specificity because of interferences by other antimicrobial agents, and variable precision. The enzymatic and immunoassays can be more specific and accurate, but they also depend on the purity of the enzyme and the specificity of the antibodies. Interferences and cross reactions have been reported for both techniques (3, 4).

Of the liquid-chromatographic (LC) procedures reported for the measurement of aminoglycoside antibiotics (5–9), most require either precolumn or postcolumn derivatization for fluorescence detection (5–8). Recently, a liquid-chromatographic method of analysis for tobramycin, with ultraviolet detection, was reported by Barends et al. (9). The assay is based on precolumn derivatization of the tobramycin with 1-fluoro-2,4-dinitrobenzene and subsequent analysis of the derivatized product on a reversed-phase column. Unfortunately this assay was limited by a long analysis time (15 min), poor analytical recovery of tobramycin (41%), and formation of several undesired products from the derivatizing agent. These methods may not be suitable for routine clinical laboratory application because of the complicated procedures, the need for specialized equipment, and the length of the procedure. Liquid chromatographs equipped with a variable-wavelength spectrophotometric detector are routinely used in many clinical laboratories for therapeutic drug monitoring, and have advantages over methods involving the more expensive and complicated pre- and postcolumn fluorescence apparatus.

We present a method that is well suited for the routine assay of tobramycin with commonly available liquid-chromatography equipment. The method is simple, precise, sensitive, specific, and results correlate well with those by existing immunoassay methods.

Materials and Methods

Instrumentation

We used a Series 3 or Series 2 liquid chromatograph equipped with a Model LC-100 column oven, a Model LC-75 variable wavelength detector, and a Sigma 10 data system (all from Perkin-Elmer Corp., Norwalk, CT 06856). The 25 cm × 4.6 mm reversed-phase column (Ultrasphere-octyl; Altex Scientific Inc., Berkeley, CA 94710) was mounted in the oven. The sample was injected into a Model 7105 valve (Rheodyne, Cotati, CA 94928) mounted on the chromatograph. Polypropylene tubes, 1.5-mL capacity, and an Eppendorf Model 5412 centrifuge were from Brinkmann Instruments, Inc., Westbury, NY 11590.

Reagents

All chemicals used were of reagent grade. Acetonitrile, tetrahydrofuran, and methanol, all distilled in glass, were obtained from Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442.

Mobile phase: Mix 700 mL of acetonitrile and 300 mL of 50 mmol/L phosphate buffer (6.8 of KH2PO4 per liter of water). Adjust the pH to 3.5 with phosphoric acid.

Tris buffer, 2 mol/L, pH 10.3: Dissolve 24.2 g of Trizma base (Sigma Chemical Co., St. Louis, MO 63178) in 100 mL of distilled water. This solution is stable for at least a year at 4 °C.

2,4,6-Trinitrobenzene-1-sulfonic acid, 250 g/L: Dissolve 2.5 g of trinitrobenzenesulfonic acid (TNBS, Sigma) in 10 mL of acetonitrile. This solution is stable for one month at 4 °C.

Stock wash buffer, 1 mol/L K2HPO4: Dissolve 87 g of K2HPO4 in 500 mL of distilled water. This solution is stable for a year at 4 °C.

Working wash solution, methanol/phosphate buffer (0.1 mol/L K2HPO4, pH 8.5), 50/50 by vol: Transfer 10 mL of stock wash buffer into a 250-mL graduated cylinder, add 90 mL of distilled water, followed by 100 mL of methanol. Adjust the pH to 8.5 with phosphoric acid. This solution is stable for a year at ambient temperature.

Vac Elut® vacuum chamber and Bond-Elut® C18 extraction columns, were purchased from Analytichem International, Inc., Harbor City, CA 90710.

Comparison methods: The Tobramycin Gamma Coat 125I radioimmunoassay kit was obtained from Clinical Assays, Cambridge, MA 02139. The EMIT kit for tobramycin was obtained from Syva Co., Palo Alto, CA 94303.
Drug standards. We obtained tobramycin from Eli Lilly and Co., Indianapolis, IN 46285, and sisomicin from Schering Corp., Bloomfield, NJ 07003. The tobramycin stock standard, 25 mg in 100 mL of water, is stable at 4 °C for at least one year. The stock internal standard, 25 mg of sisomicin in 100 mL of acetonitrile, is stable at 4 °C for at least six months. The working internal standard, prepared by diluting the stock internal standard 25-fold with acetonitrile, is stable for one month at 4 °C. Prepare the working tobramycin serum standards (5 and 10 mg/L) by diluting 100 and 200 μL of stock standard with 9.9 and 9.8 mL of drug-free serum, respectively; the serum standards are stable for at least one month at 4 °C.

Procedure

Pipet 50 μL of serum standards, controls, or unknown into 1.5-mL polypropylene tubes. Add 25 μL of 2 mol/L Tris buffer and 100 μL of working internal standard (sisomicin, 10 mg/L) to each tube. Vortex-mix and centrifuge all tubes for 1 min in an Eppendorf centrifuge at 15 000 × g. Decant the supernate into a second set of appropriately labeled polypropylene tubes, then add 30 μL of TNBS solution. Cap, vortex-mix, and place the tubes in a 70 °C heating block for 30 min. For each sample, place a Bond-Elut C₁₈ extraction column on the top of the Vac-Elut chamber, and connect the vacuum to the chamber. Pass two column volumes of methanol and two column volumes of water through each column. Disconnect the vacuum, and fill each column with 700 μL of working wash solution, followed by approximately 200 μL of derivatized sample. Re-connect the vacuum to the chamber, and pass three column volumes of working wash solution through each column. Disconnect the vacuum, and place a rack of labeled 10 × 75 mm glass tubes in the Vac-Elut chamber, corresponding to each Bond-Elut column. Pipet 300 μL of acetonitrile onto each column and connect the vacuum. After collecting the eluate in the tubes, remove the rack from the vacuum chamber, shake the tubes to mix the eluate, and inject 50 μL of each eluate onto the liquid chromatograph. Elute the column with acetonitrile/phosphate buffer (70/30 by vol) at the rate of 3.0 mL/min at 50 °C, and monitor the column effluent at 340 nm.

Results and Discussion

Procedural Details

Optimum conditions for derivatization. We arrived at the optimum conditions by varying reagent concentration, reaction temperature, reaction time, pH, and the composition of buffers. A large excess of the derivatizing agent, TNBS (18 000–20 000 molar ratio) was necessary to yield a single tobramycin derivative quantitatively in <30 min. TNBS reacted with primary amino groups of amino acid and peptide in the aqueous solution at pH 8 and at room temperature without any undesirable side reactions. The resulting trinitrophenyl derivatives had a high molar absorptivity at 340 nm (10). The TNBS solution was dissolved in acetonitrile to expedite the derivatization, deproteinize the serum, and solubilize the nonpolar tobramycin derivative. At ambient temperature a reaction time of 19–20 h was necessary for complete derivatization. At temperatures lower than 70 °C and reaction time <30 min, multiple derivatives of tobramycin formed, owing to incomplete reaction. At 70 °C, the derivatization was complete in <30 min. If the temperature was raised above 80 °C, there was substantial decomposition of the tobramycin derivative. Therefore, we selected 70 °C and 30-min reaction time as the optimum conditions for derivatization.

The pH of the reaction mixture was critical for complete derivatization. Below pH 9, derivatization was incomplete and slow because of the basic nature of the tobramycin molecule. The optimum pH for this reaction is between 9.5 and 10.0. In strongly alkaline solutions the reaction was accompanied by appreciable decomposition of TNBS and of the tobramycin derivative. Several different buffers were tried for the derivatization reaction. Carbonate, phosphate, and borate buffers at pH 9.5–10 were unsuitable because of their low buffering capacity in this pH range, insolubility in organic solvents, or complexation of tobramycin hydroxyl groups with borate buffer. The high molarity of Tris buffer (2 mol/L) was necessary to maintain the reaction pH above 9.5 in the presence of a large excess of the strong acids (TNBS and picric acid) used.

Extraction of tobramycin derivative. When the crude derivatized mixture (50 μL) was injected onto a reversed-phase octyl column, a large void-volume peak was observed, owing to the presence of a large excess of polar constituents (TNBS and picric acid) in the crude mixture. In this instance, the reversed-phase octyl column was eluted at 50 °C with a mobile phase containing acetonitrile/tetrahydrofuran/phosphate buffer 50 mmol/L (60/3/37 by vol) at a flow rate of 2.0 mL/min; total chromatography time was about 10 min. The solvent front was considerably reduced by extracting the tobramycin derivative with ethyl acetate. We used sisomicin, a structurally related aminoglycoside, as an internal standard to compensate for any losses in derivatization and extraction process. We extracted the tobramycin and sisomicin derivatives from the crude mixture with Bond-Elut C₁₈ reversed-phase columns. The solid-phase extraction procedure simplified the process, and eliminated the large solvent front. A batch of 10 samples could be extracted in <5 min. The chromatographic time was <4.6 min at a flow rate of 3.0 mL/min (Figure 1). This extraction method also prolonged the useful life of the analytical column. We have used the same analytical columns to analyze more than 1500 serum samples.

Chromatography. We evaluated various chromatographic conditions (the composition of the mobile phase, the pH of the mobile phase, the column temperature, and the detection wavelength) by injecting 50 μL of the crude reaction
mixture containing approximately 2 μg of the tobramycin derivative.

Mobile-phase variation included various ratios of acetonitrile/phosphate buffer, namely 50/50, 60/40, 65/35, 75/25 (by vol), and acetonitrile/tetrahydrofuran/phosphate buffer (60/37/3 by vol). The elution order of tobramycin and sisomicin were unaffected, but the retention time and resolution were affected by increasing the acetonitrile concentration. Some resolution of tobramycin, sisomicin, and gentamicin C1a isomer was lost when the proportion of acetonitrile exceeded 75%. Adding tetrahydrofuran to the mobile phase reduced the tailing of the tobramycin peak associated with the injection of the crude reaction mixture; however, the solid-phase extraction method eliminated the solvent front, thus eliminating the need for tetrahydrofuran in the mobile phase. By this extraction method the chromatography was rapid (<4.5 min) with virtually no solvent front.

The pH of the phosphate buffer was adjusted to 3.5 to minimize the peak tailing observed at higher pH. We elected to use above-ambient temperature (50°C) to avoid variations in the retention time that would be related to variations in the ambient temperature and to reduce the solvent viscosity for optimum column efficiency.

Detection wavelength. The λmax of the trinitrophenyl derivatives of tobramycin and sisomicin is 340 nm, a wavelength at which interference from exogenous and endogenous serum constituents was also minimized.

Sensitivity. The limit of detection of the assay was <0.2 mg/L, when 50 μL of serum was used. The signal-to-noise ratio was >5 at 0.1 mg of tobramycin per liter.

Analytical Variables

Precision. Repeated analysis of serum specimens containing tobramycin at two concentrations gave the results shown in Table 1. The within-day CV ranged from 4.0 to 4.9%; the day-to-day CV ranged from 4.6 to 5.1%.

Background. We processed more than 20 different drug-free sera and plasma samples to ascertain background peak interference at the elution times corresponding to tobramycin and internal standard. The background calculated from these samples was <0.1 mg/L, and it did not interfere with the analyses.

Analytical recovery and linearity. Tobramycin was added to drug-free serum in amounts equivalent to 1–25 mg/L. A constant amount of internal standard was added to each sample, which was then processed as described above. Concentration and peak area ratios were linearly related over this range. Analytical recoveries for tobramycin from low therapeutic to toxic concentrations ranged from 94 to 98.6% (Table 2).

Interference. We evaluated potential interference with the analysis of tobramycin by chromatographing pure drug solutions and (or) serum standards (concentrations >100 mg/L) individually, noting retention times for each. Drugs tested but not detected under these conditions were: acetaminophen, acetazolamide, amobarbital, ampicillin, amitriptyline, caffeine, cefamandole, cefoxime, cefoxitin, cephalothin, clindamycin, chloramphenicol, chlordiazepoxide, di-azepam, erythromycin, ethosuximide, glutethimide, imipramine, methaqualone, moxalactam, nafcillin, nitrofurantoin, penicillin G, pentobarbital, phenobarbital, phenytoin, primidone, procainamide, N-acetylprocainamide, quinidine, salicylate, secobarbital, tetracycline, theophylline, and vancomycin. Other aminoglycoside antibiotics (gentamicin, amikacin, and kanamycin) did not interfere with the analysis. Amikacin and kanamycin eluted in the solvent front (<1 min); gentamicin isomers were completely resolved from tobramycin.

Comparison with Immunoassays

To assess the accuracy of the method, we compared our results for 100 sera from patients receiving tobramycin with results obtained with established commercially available radioimmunoassay and EMIT methods. 

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<th>Added Concn, mg/L</th>
<th>Recovered Mean, mg/L</th>
<th>Recovery, %</th>
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<tr>
<td>2.5</td>
<td>2.47</td>
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<td>5.0</td>
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<td>25.0</td>
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Table 1. Precision of Assays for Tobramycin in

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<th>Within-day (n = 10)</th>
<th>Day-to-day (n = 15)</th>
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<tr>
<td>Mean, mg/L</td>
<td>Mean, mg/L</td>
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<tr>
<td>(and SD) CV, %</td>
<td>(and SD) CV, %</td>
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<tr>
<td>4.7 (0.189)</td>
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<td>9.3 (0.45)</td>
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References