Liquid Chromatographic Determination of Gentamicin in Serum with Spectrophotometric Detection

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Abstract

A simple, accurate, and specific liquid chromatographic procedure is described for the determination of gentamicin in 50 μL of serum. Gentamicin and sisomicin (IS) were converted into their trinitrophenyl derivatives by reaction with a water soluble derivatizing agent at 70°C for 30 minutes. The derivatives were extracted from the crude mixture with chloroform, which was separated, evaporated, and the residue reconstituted in acetonitrile. Gentamicin was separated into three major isomers (C_1, C_2, and C_{1a}) on a reversed-phase octyl column by eluting with a mobile phase. The eluted compounds were detected at 340 nm and quantified from their peak heights or peak areas. Chromatography was complete in $<$11.0 minutes. The lower limit of detection for gentamicin was $<$0.5 mg/L, analytical recoveries varied from 96.6% to 99.3%, linearity extended to 15.0 mg/L, and day-to-day precision was between 2.2% to 2.9%.

Introduction

Gentamicin, 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); thus, it is essential to monitor serum concentrations during therapy.

Gentamicin serum levels have been determined by microbiological, radioenzymatic, and immunoassays (1,2). The microbiological assays are inexpensive and simple, but suffer from several deficiencies including slow turnaround times due to a long incubation period, limited specificity due to 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 post column derivatization for fluorescence detection. Recently, a liquid chromatographic method for the analysis of gentamicin with ultraviolet detection was reported (9). The assay required precolumn derivatization of gentamicin with 1-fluoro-2,4 dinitrobenzene and subsequent analysis of the derivatized product on a reversed-phase column. Unfortunately, this assay required a long analysis time (45 min), incompletely derivatized the gentamicin isomers, and was not able to separate the C_1 and C_2 derivatives. Liquid chromatographs equipped with a variable-wavelength spectrophotometric detector are routinely used in many clinical laboratories for therapeutic drug monitoring, and use of this equipment may be advantageous to the use of more expensive and complicated pre- and post-column fluorescence apparatus.

The method presented is well suited for the routine assay of gentamicin with commonly available LC equipment. The method is simple, precise, sensitive, specific, and results correlate well with existing immunoassay methods.

Materials and Methods

Instrumentation

A Series 2 or Series 3 LC equipped with a Model LC-100 column oven, a Model LC-75 variable wavelength detector, and a Sigma 10 data system (Perkin-Elmer) were used. The 15-cm × 4.6-mm, reversed-phase, Ultrasphere-octyl column (Altex Scientific) was mounted in the oven. The sample was injected into a Model 7105 valve (Rheodyne) mounted on the chromatograph. Polypropylene tubes, 1.5-mL capacity, and an Eppendorf Model 5412 centrifuge were from Brinkmann Instruments.

Reagents

All chemicals used were of reagent grade. Acetonitrile, methanol, and chloroform, all distilled in glass, were obtained from Burdick and Jackson.

The mobile phase consisted of acetonitrile:phosphate buffer (66:34 v/v), where 660 mL of acetonitrile was mixed with 340 mL of 20 mmol/L phosphate buffer (2.68 g KH_2PO_4/L water), and adjusted to pH 3.0 with phosphoric acid. The *Tris* buffer was made by dissolving 24.2 g of Trizma base (Sigma) in 100 mL distilled water. This solution was made to 2 mol/L and pH 10.3, and was stable for at least one year at 4°C.

The 2,4,6,trinitrobenzene-1-sulfonic acid, 250 g/L, was made by dissolving 2.5 g trinitrobenzenesulfonic acid (TNBS; Sigma) in 10 mL of acetonitrile. This solution is stable for one month at 4°C. Gentamicin C_1, C_{1a}, and C_2 isomers, and sisomicin for the internal standard (IS) were obtained from Schering Corp.

The gentamicin stock solution was prepared by dissolving 25 mg equivalent of gentamicin activity in 100 mL water. The stock solution is stable for at least six months at 4°C. The working gentamicin serum standards (2.5 and 10 mg/L) were prepared by diluting 100 and 400 μL of stock standard with 9.9 and 9.6 mL of drug-free serum, respectively. Serum standards are stable for at least one month at 4°C. The stock IS was prepared by dissolving 25 mg sisomicin in 100 mL acetonitrile. This solution is stable at 4°C for at least six months. The working IS was prepared by diluting 2 mL stock IS with 98 mL acetonitrile. The solution is stable at 4°C for one month.

Procedure

To 50 μL of serum, standard, and control in a 1.5 mL polypropylene tube, 20 μL of 2 mol/L *Tris* buffer and 100 μL working IS (sisomicin, 5 mg/L) was added. The mixture was vortex-mixed and centrifuged for one minute in an Eppendorf centrifuge at 15,000 g. The supernate was decanted into a second set of appropriately labeled polypropylene tubes, then 30 μL TNBS solution was added. The tubes were capped, vortexed, and placed in a 70°C heating block for 30 minutes. Each sample was transferred into a labeled 12-mL glass centrifuge tube, 2 mL of chloroform added, and shaken for 3 minutes. The samples were centrifuged at 2,000 rpm (210 × g) for 4 minutes, the top layer aspirated, and the chloroform layer evaporated, in an Evapo-mix at 50°C. The sample was reconstituted in 100 μL acetonitrile, and 50 μL of sample was injected into the chromatograph and eluted with mobile phase at a flow rate of 2.0 mL/min. The column was maintained at 50°C and the column effluent was monitored at 340 nm.

Calculations

Serum standards were carried through the procedure and used to prepare a standard curve based on relative peak height or peak area ratios of gentamicin C_1, C_{1a}, and C_2 isomers. The concentration of gentamicin isomers in the specimen was calculated by direct comparison with the data obtained from the standard curve of the serum standards. The total concentration of gentamicin was calculated by adding the concentration of each isomer.

Results and Discussion

Procedural Details

The optimum conditions were derived by varying the reagent concentration, reaction temperature, reaction time, pH, and the composition of buffers. A large excess of the derivatizing agent, TNBS (18,000 to 20,000 molar ratio) was necessary to yield a single derivative quantitatively for each gentamicin isomer in ◄30 minutes. TNBS reacts with primary amino groups of amino acids and peptides 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). TNBS was dissolved in acetonitrile to expedite the derivatization, deproteinize the serum, and solubilize the non-polar gentamicin derivatives. At ambient temperature, a reaction time of 19 to 20 hours was necessary for complete derivatization. At temperatures lower than 70°C and with reaction times of ◄30 minutes, multiple derivatives of the gentamicin isomers formed due to incomplete reaction. At 70°C, the derivatization was complete in ◄30 minutes. At temperatures higher than 70°C, decomposition of gentamicin derivatives resulted. Therefore, 70°C and a 30-minute reaction time were selected as the optimum conditions for derivatization. The pH of the reaction mixture was critical for complete derivatization. The optimum pH for this reaction was found to be between 9.5 and 10.0. In highly alkaline solutions, the reaction was accompanied by appreciable decomposition of TNBS and of the gentamicin derivatives.

Several different buffers were tried for the derivatization reaction. Carbonate, phosphate, and borate buffers at pH 9.5 to 10 were unsuitable because of their low buffering capacity in this pH range, insolubility in organic solvents, or in the case of borate, its complexation with gentamicin hydroxyl groups. The high molarity of the *Tris* buffer used (2 mol/L) was necessary to maintain the reaction pH above 9.5 in the presence of the large excess of strong acids (TNBS and picric acid) used.

When the crude derivatized mixture (50 μL) was injected into a reversed-phase octyl column, a large solvent front peak was observed due 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:phosphate buffer (66:34 v/v) at a flow rate of 2.0 mL/min; total chromatographic time was about 10 minutes. The solvent front was considerably reduced by extracting the gentamicin derivatives with chloroform. Sisomicin, a structurally related aminoglycoside, was used as an IS to compensate for any losses due to the derivatization and extraction process. Chloroform extraction also prolonged the useful life of the analytical column.

Various chromatographic conditions (composition and pH of the mobile phase, column temperature, and detection wavelength) were investigated to achieve optimum conditions. Variation in the mobile phase included ratios of acetonitrile:tetrahydrofuran:phosphate buffer, namely 55:2:43, 60:2:38, and acetonitrile:phosphate buffer 66:34 (v/v). The elution order of gentamicin isomers and sisomicin were unaffected, but the retention time and resolution were affected by varying the organic solvent concentration of the mobile phase. The optimum resolution between various gentamicin isomers and sisomicin was obtained with a 66:34 mixture of acetonitrile:phosphate buffer.

The pH of the phosphate buffer was adjusted to 3.0 to minimize the peak tailing observed at higher pH. An above-ambient temperature (50°C) was used to avoid variations in retention time and to reduce the solvent viscosity for optimum column efficiency.

The λ_{max} of the trinitrophenyl derivatives of gentamicin and sisomicin was 340 nm, a wavelength at which interference from exogenous and endogenous serum constituents is minimized. The limit of detection of the assay was 0.5 mg/L when 50 μL of serum was used.

Analytical Variables

Repeated analysis of serum specimens containing gentamicin at two concentrations gave the results shown in Table I. The within-day CV ranged from 1.3% to 3.5%; the day-to-day CV ranged from 2.2% to 2.9%. More than 20 different drug free

Table I. Precision of Assays for Gentamicin in Serum at Two Concentrations

	Within-day (n = 10)			Day-to-day (n = 15)		
Concentration	Mean (mg/L)	SD	CV (%)	Mean (mg/L)	SD	CV (%)
A	1.51	0.020	1.3	1.50	0.033	2.2
B	5.89	0.206	3.5	6.00	0.174	2.9

SD = standard deviation
CV = coefficient of variation

Table II. Analytical Recovery of Gentamicin from Serum*

Gentamicin Added (Concn. mg/L)	Gentamicin Recovered (Concn. mg/L)	Recovery (%)
1.52	1.51	99.3
3.05	2.97	97.4
6.10	5.89	96.6
15.25	15.10	99.3

*n = 5

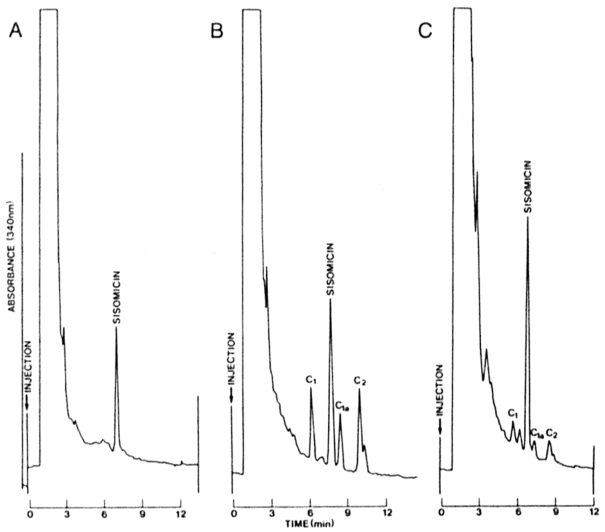

Figure 1. Sample chromatograms of A) drug-free serum; B) serum containing 8.5 µg/mL of gentamicin, showing isomers C_1, C_{1a}, and C_2; C) serum containing 1.6 µg/mL of gentamicin, showing isomers C_1, C_{1a}, and C_2.

sera and plasma samples were processed to ascertain background peak interference at the elution times corresponding to the gentamicin isomers and sisomicin. The background calculated from these samples was <0.1 mg/L, and did not interfere with the analyses.

Gentamicin was added to drug-free serum in amounts equivalent to 1.8 to 15.5 mg/L. A constant amount of IS was added to each sample, which was processed as described above. Concentration and peak height ratios were linearily related over this range. Analytical recoveries for gentamicin from low therapeutic to toxic concentrations ranged from 96.6% to 99.3% (Table II).

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

Comparison with Immunoassays

To assess the accuracy of the method, results for 90 sera from patients receiving gentamicin were compared with results obtained with established commercially available radioimmunoassay (RIA) (Gentamicin ^{125}I RIA kit, New England Nuclear) and EMIT methods. The regression data comparing this LC method with the RIA method were: n = 57, r = 0.995, y-intercept = 0.025 mg/L, and slope = 0.959. The regression data comparing the LC method to EMIT were: n = 33, r = 0.981, slope = 0.967, and y-intercept = 0.074 mg/L. These correlation studies indicate that the LC method is accurate and specific for measuring gentamicin in serum. Sample chromatograms are illustrated in Figure 1.

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