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Note

Liquid chromatographic determination of amikacin in serum with spectrophotometric detection

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Amikacin, an aminoglycoside antibiotic, is widely used against aerobic gram-negative bacillary infections, and, like other aminoglycoside antibiotics, has the potential for nephrotoxicity and ototoxicity if not carefully monitored [1, 2]. To minimize these toxicities and to assure therapeutic serum concentrations, frequent and careful monitoring of serum amikacin levels is essential.

Methods for measuring serum amikacin include microbiological [3], radioenzymatic [4], and immunoassays [5]. The microbiological assays are inexpensive and simple, but may suffer from several deficiencies such as slow turnaround time, 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 [6, 7].

Various liquid chromatographic (LC) procedures have been reported for the measurement of amikacin [8—10], most require either pre-column or post-column derivatization for fluorescence detection [8, 10]. 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 spectrometric 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 post-column fluorescence equipment.

We describe a method that is well suited for the routine assay of amikacin

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with commonly available LC equipment. The method is simple, precise, sensitive, selective and results correlate well with existing radioimmunoassay methods.

MATERIALS AND METHOD

Instrumentation

The chromatographic system consisted of a Series 2 or Series 3 liquid chromatograph, a Model LC-100 column oven, a Model LC-75 variable-wavelength detector, a Sigma 10 data system (all from Perkin-Elmer, Norwalk, CT, U.S.A.), and a reversed-phase 25 cm × 4.6 mm column packed with 5-μm reversed-phase octyl packing material (Ultrasphere Octyl, Altex Scientific, Berkeley, CA, U.S.A.) mounted in the oven. The sample was injected into a Model 7105 valve (Rheodyne, Cotati, CA, U.S.A.). The mobile phase consisted of acetonitrile—phosphate buffer (52:48). The flow-rate was maintained at 2.0 ml/min at 50° C, and the column effluent was monitored at 340 nm. Polypropylene tubes, 1.5-ml capacity, and an eppendorf Model 5412 centrifuge were from Brinkmann Instruments (Westbury, NY, U.S.A.). Vac Elut^{TM} vacuum chamber and Bond-Elut^{TM} C_{18} extraction columns, were purchased from Analytichem International (Harbor City, CA, U.S.A.).

Reagents

All chemicals used were of reagent grade. Acetonitrile and methanol, all distilled-in-glass, were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Mobile phase was a solution of 520 ml of acetonitrile in 480 ml of 20 mmol/l phosphate buffer, pH 3.0. Phosphate buffer, 20 mmol/l, pH 3.0, was prepared by dissolving 2.68 g of potassium dihydrogen phosphate in 1 l of distilled water. The solution was titrated to pH 3.0 with phosphoric acid. Tris buffer, 2 mol/l, pH 10.3, was prepared by dissolving 24.2 g of Trizma base (Sigma, St. Louis, MO, U.S.A) 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, was prepared by dissolving 2.5 g of trinitrobenzene sulfonic acid (TNBS, Sigma) in 10 ml of acetonitrile. This solution is stable for one month at 4°C. Wash buffer, 10 mmol/l potassium hydrogen phosphate, was prepared by dissolving 1.82 g of potassium hydrogen phosphate in 1 l of distilled water. The pH was adjusted to 8.6 with phosphoric acid. This solution is stable for at least six months at ambient temperature.

Drug standards. Amikacin and kanamycin sulfate were obtained from Bristol Labs. (Syracuse, NY, U.S.A.). The amikacin stock standard, 25 mg in 100 ml of water, is stable at 4°C for at least six months. The stock internal standard, 25 mg of kanamycin 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 ten-fold with acetonitrile, is stable for one month at 4°C. The working amikacin serum standards (5, 10, and 25 mg/l) were prepared by diluting 200, 400, and 1000 μl of stock standard with 9.8, 9.6 and 9.0 ml of drug-free serum. The serum standards are stable for at least one month at 4°C.

Procedure

Pipette 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 (kanamycin, 16.0 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 C_{18} 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 250 μl of derivatized sample. Reconnect 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 column and connect the vacuum. Pipette 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.

RESULTS AND DISCUSSION

Optimum conditions for derivatization

The optimum conditions for derivatization were derived by varying reagent concentration, reaction temperature, reaction time, pH, and the composition of buffers. A large excess of derivatizing agent, TNBS (18,000—20,000 molar ratio) was necessary to yield a single amikacin derivative quantitatively in less than 30 min. TNBS reacts 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 derivative has a high molar absorptivity at 340 nm [11]. The TNBS solution was dissolved in acetonitrile to expedite the derivatization, deproteinize the serum, and solubilize the nonpolar amikacin derivative. At temperatures lower than 70°C and reaction time shorter than 30 min, multiple derivatives of amikacin formed, due to incomplete reaction. At 70°C, the derivatization was complete in less than 30 min. 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 amikacin molecule. The optimum pH for this reaction is between 9.5 and 10.0.

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 amikacin hydroxyl groups with borate buffer. The high molarity of this Tris buffer (2 mol/l) was necessary to maintain the reaction pH above 9.5 in the presence of a large excess of strong acids (TNBS, and picric acid formed during the reaction) used.

Extraction of amikacin derivative

When 50 μl of the crude derivatized sample was injected onto a reversed-

phase octyl column, a large void volume peak was observed, due to the presence of a large excess of polar constituents (TNBS and picric acid) in the crude mixture. The amikacin sample was eluted from the reversed-phase octyl column with acetonitrile—phosphate buffer (52:48) at a flow-rate of 2.0 ml/min at 50°C, and the total analysis time was about 15 min. The solvent front was significantly reduced by extracting the amikacin and kanamycin, internal standard, derivatives from the crude mixture using a Bond-Elut C_{18} reversed-phase column. The solid-phase extraction procedure simplified the process and eliminated the large solvent front. A batch of ten samples could be extracted in less than 5 min. This extraction procedure increased the useful life of the analytical column.

Chromatography

A number of chromatographic parameters were investigated to optimize the separation in the shortest time. The composition of the mobile phase, the pH of the mobile phase, column temperature, and detection wavelength were varied to achieve optimum chromatographic conditions.

Mobile-phase variation included various ratios of acetonitrile—tetrahydrofuran—phosphate buffer, namely 45:5:50, 48:4:48, 50:4:46, and acetonitrile—phosphate buffer (52:48). The greatest resolution and sharpest peaks between amikacin and kanamycin were obtained with a mobile phase of acetonitrile—phosphate buffer (52:48). To reduce tailing, the pH of the phosphate buffer was adjusted to 3.0. At a higher pH substantial peak tailing was observed. We elected to use above ambient temperature (50°C) to avoid variations in the retention time and to reduce the solvent viscosity for optimum column efficiency.

Detection wavelength. The λ_{max} value of the trinitrophenyl derivatives of amikacin and kanamycin 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 <0.5 mg/l, when 50 μl of serum was used.

Analytical variables

Precision. Repeated analysis of serum specimens containing amikacin at two different concentrations gave the results shown in Table I. The within-day coefficients of variation (C.V.) ranged from 3.5 to 6.9%; the day-to-day C.V. ranged from 2.8 to 3.1%.

TABLE I

PRECISION OF ASSAYS FOR AMIKACIN IN SERUM

	Range ± S.D. (mg/l)	C.V. (%)
Within-day (n = 10)	9.0 ± 0.542	6.0
	24.0 ± 0.846	3.5
Day-to-day (n = 15)	9.49 ± 0.298	3.1
	23.63 ± 0.668	2.8

Background. Several drug-free sera and plasma samples were processed as described above to ascertain the level of background peak interference at the elution times corresponding to the amikacin and kanamycin. The background calculated from these samples was < 0.1 mg/l, and did not interfere with the analysis.

Analytical recovery and linearity. Amikacin was added to drug-free serum in amounts equivalent to 2.5—50.0 mg/l. A constant amount of internal standard (kanamycin) was added to each sample and processed as described above. Concentration and peak height ratios were linearly related over this range. Analytical recoveries for amikacin from low therapeutic to toxic concentrations ranged from 92.8 to 98.4% (Table II).

TABLE II

ANALYTICAL RECOVERY OF AMIKACIN FROM SERUM (n = 5)

Added (mg/l)	Recovered (mg/l)	Recovery (%)
2.5	2.5	98.4
5.0	4.7	94.0
10.0	9.6	96.1
25.0	23.6	94.4
50.0	46.4	92.8

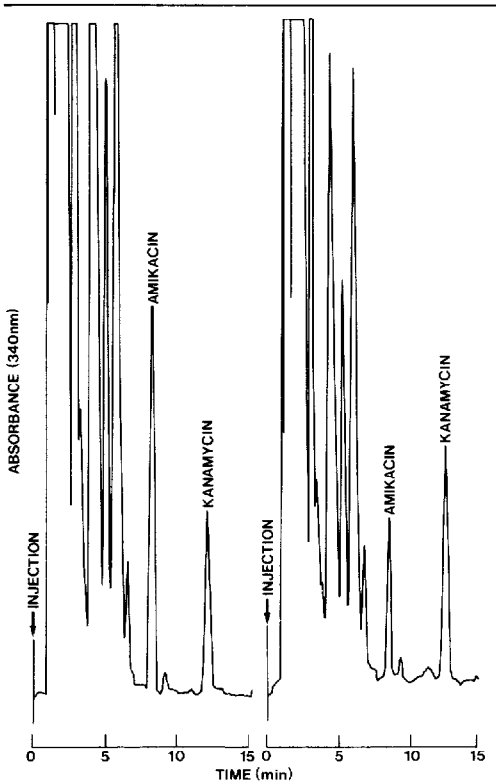

Fig. 1. Chromatograms of patient serum containing left: 26.2 μg/ml of amikacin; and right: 8.6 μg/ml of amikacin.

Interference. Potential interference with the analysis of amikacin was evaluated by chromatographing pure drug solutions and/or serum standards individually, noting retention time for each. Drugs tested but not detected under these conditions were: acetaminophen, acetazolamide, amobarbital, ampicillin, amitriptyline, caffeine, cafamandole, cefoxime, cefoxitin, cephalothin, clindamycin, chloramphenicol, chlordiazepoxide, diazepam, erythromycin, ethosuximide, nitrofurantoin, penicillin G, pentobarbital, phenobarbital, phenytoin, primidone, procainamide, N-acetylprocainamide, quinidine, salicylate, secobarbital, tetracycline, theophylline, and vancomycin. Other aminoglycoside antibiotics (gentamicin and tobramycin) did not interfere with the analysis.

Comparison with radioimmunoassay

To assess the accuracy of the method, we compared our results for 25 sera from patients receiving amikacin with results obtained with established commercially available radioimmunoassay (Amikacin ^{125}I kit, American Diagnostic, Newport Beach, CA, U.S.A.). The regression data comparing our LC method with radioimmunoassay method were: $n = 25$, $r = 0.999$, y-intercept = 0.398 mg/l, and slope = 1.047. The correlation study indicates that the LC method is accurate and selective for the determination of amikacin in serum. Sample chromatograms are illustrated in Fig. 1.

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