Serum Gentamicin Assay by High-Performance Liquid Chromatography


We describe a high-performance liquid chromatographic method for the quantitative determination of gentamicin in serum. The antibiotic was separated from serum by passage through a silicic acid column, derivatized with o-phthalaldehyde, and eluted with ethanol. The derivatized gentamicin was then separated into all three of its major components by reversed-phase chromatography and quantified by fluorometry. Concentrations in serum as low as 0.5 mg of gentamicin per liter could be accurately determined. A standard curve showed a linear response for serum containing gentamicin at concentrations ranging from 0 to 20 mg/liter. Tobramycin, amikacin, ampicillin, penicillin G, methicillin, carbenicillin, chloramphenicol, clindamycin, and cephalothin did not interfere with the gentamicin assay. Comparison with an accepted microbiological assay yielded a correlation coefficient of 0.99. This chemical assay is rapid (less than 30 min), sensitive, accurate, specific, and appears to be applicable to other aminoglycosides.

Additional Keyphrases: measurement of aminoglycosides in serum · fluorometry · silicic acid column · o-phthalaldehyde reagent · monitoring therapy · antibiotic assay

Gentamicin is an effective aminoglycoside antibiotic for treatment of serious Gram-negative bacillar infections. Like other aminoglycoside chemotherapeutic agents, gentamicin has a narrow therapeutic range. Consequently, monitoring its concentration in serum has been urged for maintaining safe yet therapeutic concentrations (1, 2). Microbiological (3, 4), enzymatic (5, 6), hemagglutination inhibition (7), and radioimmunoassays (8, 9) have been developed for gentamicin in serum and other biological fluids.

Recently, high-performance liquid chromatography has been successfully applied to the quantitative determination of several antimicrobial agents in serum and other biological fluids (10-16). This report describes such a method, with which the three major components of gentamicin—C1, C1a, and C2—in serum can be measured. The technique involves a simple extraction procedure, derivatization, separation by reversed-phase chromatography, and measurement of the fluorescent products. The method is rapid, accurate, sensitive, and specific for gentamicin.

Materials and Methods

Absolute methanol was glass distilled. Water was de-ionized and glass distilled daily. Tripotassium ethylenediaminetetraacetate (EDTA) was purchased from Eastman Organic Chemicals, Rochester, N. Y. Silicic acid (60-200 mesh), purchased from J.T. Baker Chemical Co., Phillipsburg, N. J. 08865, was washed with water, ethanol and methanol, and air dried. Silane-treated glass wool was from Applied Science Laboratories, Inc., State College, Pa. 16801. o-Phthalaldehyde (Fluoropa) was obtained from Durrum Chemical Corp., Palo Alto, Calif. 94303, and 2-mercaptoethanol from Sigma Chemical Co., St. Louis, Mo. 63178. Gentamicin sulfate, USP Reference Standard, containing 650 μg of of anhydrous gentamicin base per milligram of powder and gentamicin components C1, C1a, and C2 were obtained by courtesy of Schering Drug Corp., Kenilworth, N. J. 07033. All other chemicals used were reagent grade and are commercially available.

Chromatographic eluent. The solvent used was a mixture of methanol/water (79/21 by vol) containing 2 g of tripotassium EDTA per liter (designated “methylol-EDTA”). The tripotassium ethylenediaminetetraacetate was added to the mobile phase because of previous experiments, which showed that the tripotassium salt gave sharper and better-resolved peaks than were obtained with the dipotassium salt or in the absence of ethylenediaminetetraacetate. The mixture was passed through a Millipore filter (pore size, 0.6 μm, “Solvinert”; Millipore Corp., Bedford, Mass. 01730) and de-aerated under reduced pressure.

Chromatographic equipment. Separation was performed with a liquid chromatograph (Model No.

---

Research Service, Veterans Administration, Wadsworth Hospital Center, Los Angeles, Calif.; Departments of Medicine, Harbor General Hospital, Torrance, Calif.; UCLA School of Medicine, Los Angeles, Calif.; and the Departments of Infectious Diseases and Clinical Chemistry, the University of Lund, Sweden.

1 Address correspondence to this author at: Division of Infectious Disease, Harbor General Hospital, 1000 West Carson St., Torrance, Calif. 90509.

Received Aug. 8, 1977; accepted Sept. 19, 1977.
was added 0.5 ml of 12% silicic acid and the column was maintained at 4°C for at least five days. After this period, the column was washed with water at a flow rate of 2.0 ml/min for 30 min, and then eluted with nitrogen at a flow rate of 2.0 ml/min. The eluate was monitored for fluorescence intensity, and the elution was performed with a photomultiplier tube. The retention times of gentamicin C1, C1a, and C2 were determined by comparing retention times with known concentrations of these compounds. Gentamicin peaks for C1, C1a, and C2 were identified by comparing retention times with chromatographed individual peaks.

The retention times of gentamicin C1, C1a, and C2 were 4.7, 7.2, and 9.5 min, respectively. Fig. 1 shows the chromatograms of extracts of dog serum containing 5 mg of gentamicin per liter, dog serum without gentamicin, and human serum without gentamicin. The chromatograms were obtained using the MicroBondapak C18, 30 cm x 4 mm i.d.; mobile phase: methanol-EDTA, 78/21 by vol; flow rate: 2.0 ml/min; sample size: 100 μl; detection: Fluorescence intensity measurements, at excitation maximum 360 nm and emission maximum 430 nm.

Chemical assay, extraction, and derivatization. A silicic acid column was prepared by plugging a disposable Pasteur pipet with silanized glass wool and then adding dry silicic acid to a height of 1.0 cm (about 150 mg). The column was treated with 1.0 ml of water. A 0.5-ml serum sample containing gentamicin was diluted with water to a volume of 2.0 ml, vortex-mixed, and applied to the silicic acid column. The serum sample tube was rinsed with 1.0 ml of water, which was then added to the column, and eluted under the help of pressure from a rubber bulb. The total eluate (3.0 ml) was discarded and within 5 min 0.5 ml of the o-phthalaldehyde reagent was applied to the silicic acid column. The derivatized gentamicin was then eluted from the column by adding 1.5 ml of ethanol. The ethanol eluent was passed through a Millipore filter (pore size, 0.6 μm) and stored in the dark until injected.

Separation, detection, and quantitation. An aliquot (100 μl) of the filtered sample was injected into the chromatograph. Gentamicin was eluted with methanol-EDTA as the mobile phase at a flow rate of 2.0 ml/min and detected by determining relative fluorescence (excitation maximum 360 nm and emission maximum 430 nm). Quantitation was based on integration of peak areas with an integrating recorder previously calibrated with known concentrations of gentamicin. Gentamicin peaks for C1, C1a, and C2 were identified by comparing retention times of each gentamicin component when chromatographed individually.

Microbiological assay. The microbiological assay with which the present method was compared was performed in duplicate by a standard agar well diffusion technique using antibiotic media No. 11 (Difco Laboratories, Detroit, Mich. 48232). Gentamicin standards were prepared in pooled sera. The test organism was Bacillus subtilis (ATCC 6633). The seeded agar was incubated at 37°C and the diameter of the zone of growth inhibition was measured 16–18 h later. Zone size was plotted on semilogarithmic graph paper vs. known concentrations of gentamicin.

Results

All three major components of gentamicin, C1, C1a, and C2, were well separated from each other and had retention times of 4.7, 7.2, and 9.5 min, respectively (Figure 1). Extracts of serum blanks produced no interfering peaks. At least one minor peak did elute prior to the C1 peak but was absent in extracts of serum not containing gentamicin. This peak could represent minor
components of the gentamicin complex previously described (18).

Analytical recovery studies were conducted after constructing an absolute standard curve, where 0.1 ml of drug was mixed with 0.5 ml of o-phthalaldehyde reagent and 1.4 ml of ethanol. Serum samples containing gentamicin were processed as described and the values were then compared. We found that with serum concentrations of gentamicin exceeding 2 mg/liter, recovery was between 95 to 105%, and with concentrations near 1.0 mg of gentamicin per liter of serum the recovery was consistently 80% based on the absolute standard curve. A standard curve determined from serum samples was linear over a range of concentrations from 0 to 20 mg/liter (Figure 2).

Precision for the chemical assay was calculated for serum samples of gentamicin at concentrations of 1.0 and 10.0 mg/liter. Six samples at each concentration were extracted separately, derivatized, and chromatographed on the same day. The coefficients of variation (relative standard deviations) for gentamicin C₁, C₁ₐ, and C₂ at 1.0 mg/liter were 5.6, 7.5, and 5.1%, respectively; at 10.0 mg/liter, the values were 4.2, 4.4, and 3.9%, respectively. Day-to-day variations in recovery from serum samples containing 1.0 and 10.0 mg/liter were within 6%. Preliminary studies with standard solutions and serum-based samples showed that tobramycin and amikacin did not interfere with the gentamicin assay procedure, nor did standard solutions of penicillin, methicillin, carbenicillin, ampicillin, cephalothin, clindamycin, and chloramphenicol.

Sera from a healthy volunteer to whom gentamicin was administered intravenously were assayed in duplicate by the microbiological as well as the chemical method. Figure 3 illustrates the rapid decrease in gentamicin concentrations from serum as determined by both assays. The correlation coefficient between results by the two methods was 0.99 (Figure 3, insert). Values obtained by the chemical assay are expressed as the sum of all three components of gentamicin. The values obtained by the microbial assay were higher than those by the chemical method. A possible explanation for the higher microbiological values may be the presence of other minor components of gentamicin (18) that have antimicrobial activity but are not measured by the chemical assay.

Discussion

This method does not require extraction from serum by protein-precipitating agents but takes advantage of gentamicin's property of partitioning or adsorbing to silicic acid. The gentamicin is derivatized directly on the silicic acid column and the derivatized compounds are then eluted from silicic acid with ethanol. While the present manuscript was in preparation, Anhalt (17) described a serum gentamicin assay by high-performance liquid-chromatography [Ed. note: see also the paper by Peng et al. in the October 1977 issue of this journal]. However, no data on correlation with a microbiological (or other) assay nor possible interference by other commonly used drugs were presented. Anhalt's method is based on extraction of gentamicin from serum with CM-Sephadex, separation by reversed-phase ion-pair high-performance chromatography, followed by post-column derivatization with o-phthalaldehyde.
In contrast, in the present method precolumn derivatization is used, a technique that is more likely to eliminate interfering materials and in addition does not require any additional equipment for postcolumn derivatization (20).

Our assay for gentamicin is sensitive enough for routine clinical use. Its major advantages over microbiological assays are rapidity (assay can be performed in less than 30 min), accuracy, reproducibility, and specificity. Preliminary studies suggest that it is highly specific for gentamicin. Other antimicrobial agents, including aminoglycosides such as tobramycin and amikacin, do not interfere with this assay. This potential interference by other aminoglycosides is one major disadvantage of radioenzymatic assays for gentamicin (21). Although radioimmunoassays for gentamicin have greater sensitivity (nanogram amounts) (9), this offers no real clinical advantage. A major advantage of the present technique over microbial, enzymatic, and radioimmunoassay methods is that all three major components of gentamicin can be quantitated with a single assay. This may have relevance for future pharmaco-kinetic studies in terms of relative toxicity of individual components. Finally, this procedure appears potentially applicable for quantifying the other aminoglycosides used in clinical practice: kanamycin, tobramycin, and amikacin.

We gratefully acknowledge a travel grant to Dr. Nilsson-Ehle from the Council for International Exchange of Scholars, Washington, D. C. Several of us are belatedly indebted to Dr. J. O'Connor for his help in initiating these studies on high-performance liquid chromatography. We thank Ms. Sharon Shibata for performing the microbiological assay and Ms. Adrienne Bigcas for preparation of this manuscript. This study was supported in part by funds from the Veterans Administration. The in vivo portion of this study was performed on one of the investigators, who gave informed consent.

References