Amikacin Assay in Serum by High-Performance Liquid Chromatography

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A high-performance liquid chromatographic method for the quantitative determination of amikacin in serum is described. The antibiotic was separated from serum by adsorption on silica gel. The adsorbed drug was then derivatized with o-phthalaldehyde and eluted with ethanol. The derivatized amikacin was separated by reverse-phase, high-performance liquid chromatography and quantitated by fluorometry. A linear response for serum samples containing amikacin ranging from 1.0 to 15 µg/ml was obtained. Other antibiotics including various aminoglycosides did not interfere with the amikacin assay. Comparison with a standard microbiological assay gave a correlation coefficient of 0.99. This chemical assay is sensitive and specific under the conditions tested and can be performed rapidly.

Amikacin is an aminoglycoside antibiotic currently used for treatment of aerobic gram-negative bacillary infections. The pharmacokinetic characteristics of amikacin are similar to those of kanamycin (4), and, like other aminoglycoside antibiotics, its major adverse effects are alteration of renal function and ototoxicity (12). To minimize these toxicities and to assure therapeutic serum concentrations, frequent and careful monitoring of serum levels of amikacin is essential. Currently available methods for quantitating amikacin in serum are microbiological assay (2), radioenzymatic assay (13), and radioimmunoassay (6).

Previously the application of high-performance liquid chromatography (HPLC) for quantitating gentamicin (1, 7, 9), tobramycin (S. K. Maitra, T. T. Yoshikawa, J. L. Hansen, M. C. Schotz, and L. B. Guze, Am. J. Clin. Pathol., in press), netilmicin (10), and a variety of other antimicrobial agents (T. T. Yoshikawa, S. K. Maitra, M. C. Schotz, and L. B. Guze, in G. L. Hawk, ed., Biological/Biomedical Applications of Liquid Chromatography, in press) has been reported. In this report, we describe the application of HPLC for the separation and detection of amikacin.

MATERIALS AND METHODS

Absolute methanol, water, silicic acid (60–200 mesh), silane-treated glass wool, tripotassium ethylendiaminetetraacetate (EDTA), 2-mercaptoethanol, and o-phthalaldehyde (Fluoropa) are the same as described previously (7). Acetonitrile (liquid chromographic grade) came from Burdick & Jackson Laboratories, Inc., Muskegon, Mich.; amikacin, USP reference standard, was kindly donated by Bristol Laboratories, Syracuse, N.Y.

Chromatographic eluent. The solvent used for the mobile phase was a mixture of methanol–water-acetonitrile (65:30:5, by volume) containing 2 g of tripotassium EDTA per liter (designated “methanol-EDTA”). The methanol-EDTA was passed through a membrane filter (Millipore Corp.), 0.6-µm pore size (7), and desalted under reduced pressure.

Chromatographic equipment. Separation was performed with a liquid chromatograph (model no. ALC/GLC 200, Waters Associates, Milford, Mass.). This instrument was equipped with a model 6000 solvent delivery system, a model U6K Universal injector, and a MicroBondapak C18 column (30 cm by 4 mm), all obtained from Waters Associates. The eluant was monitored by a fluorometer (model no. J4-7461, American Instrument Co., Silver Spring, Md.) equipped with an 18-µl flow cell, a 7-50 primary filter (Corning Glassware, Corning, N.Y.), which has 70% maximum transmission at 350 nm (50% minimum transmission from 330 to 375 nm), secondary filters 38A and 2E (Kodak Wratten, Eastman Kodak, Rochester, N.Y.), having 70% transmission from 420 to 490 nm and 90% transmission from 415 to 700 nm, respectively, and a photomultiplier tube 931B (Radio Corporation of America, Harrison, N.J.). Fluorescence was quantitated by measuring peak areas recorded on an integrating recorder (model no. HP380A, Hewlett-Packard, Avondale, Pa.).

o-Phthalaldehyde (derivatizing) reagent. Absolute ethanol was diluted to 95% by volume with deionized water. Two hundred milliliters of 95% ethanol was adjusted to pH 10 with 50 µl of potassium hydroxide solution (450 g/liter). o-Phthalaldehyde (200 mg) was dissolved in 40 ml of this 95% ethanol,
and 0.4 ml of undiluted 2-mercaptoethanol (11) was added. When the o-phthalaldehyde reagent was stored under nitrogen at 4°C after each use, over a period of at least 5 days, no significant changes were observed in the recovery of amikacin. New reagent was prepared each week.

In vitro and in vivo serum samples. Aqueous solutions of amikacin were prepared. Serum standards were prepared by taking 0.1-ml samples of aqueous solutions of amikacin and adding them to 0.9-ml samples of dog or human sera. The serum standards were incubated at ambient temperature for at least 20 min. Blanks were prepared by substituting water for the drug. No differences in chromatographic separation and quantitation were noted when comparisons were made between human and dog sera containing amikacin. A healthy 70-kg man received 350 mg of amikacin mixed in 200 ml of dextrose solution (50 g/liter) and infused intravenously during 30 min. Blood samples were obtained immediately after, and for intervals up to 12 h after, completion of the infusion. Three dogs, approximately 30 kg each, also received 7.5 mg of amikacin per kg of body weight in a dextrose solution infused intravenously during a 5-min period. Blood samples were obtained immediately after, and for as long as 4 h after, completion of infusion.

Chemical assay, extraction, and derivatization. The procedure used was similar to that employed for assay for tobramycin (Maitra et al., in press). A silica gel column was prepared by plugging a disposable Pasteur pipette with silane-treated glass wool and adding silica gel powder to a height of 4.0 cm. The column was washed with 2.0 ml of 66 mM potassium phosphate buffer, pH 9. One milliliter of phosphate buffer was added to a 1.0-ml serum sample containing amikacin and applied to the silica gel column. The serum sample tube was rinsed twice, each time with 1.0 ml of water. The aqueous washings were then added to the column and eluted with the aid of pressure from a rubber bulb. The total eluate of 4.0 ml was discarded. One milliliter of o-phthalaldehyde reagent was applied to the silica gel column, immediately followed by 2.0 ml of 95% ethanol (pH 10). The eluate (3.0 ml) containing the derivatized amikacin was mixed by Vortexing without delay and immediately heated to 50°C for 5 min. The mixture was stirred, placed in an ice bath, and, within 3 to 4 min, passed through a Millipore filter (pore size, 0.6 μm). The filtrate was stored on ice, in the dark, until ready for injection into the chromatograph.

Separation, detection, and quantitation. A 25-μl sample of the filtered, derivatized amikacin was injected into the chromatograph. Amikacin was eluted with methanol-EDTA as the mobile phase at a flow rate of 2.0 ml/min and detected by determining relative fluorescence. The filters used in the fluorometer covered the extinction and emission wavelengths described for derivatives of o-phthalaldehyde (8). Quantitation was based on integration of peak areas with an integrating recorder previously calibrated with known concentrations of amikacin. Peak areas were also measured manually (peak height × width at one-half peak height). The attenuation of the recorder was 128, with a photomultiplier sensitivity of 10. The chart

![Figure 1: Area of the peak at a retention time of 7.5 min as a function of amikacin concentration. Symbols: (O) aqueous standard; (△) serum standard. See text for details of assay.](http://aac.asm.org/)

**Fig. 1. Area of the peak at a retention time of 7.5 min as a function of amikacin concentration. Symbols:** (O) aqueous standard; (△) serum standard. See text for details of assay.
speed was 0.5 cm/min, with a maximum slope sensitivity of 0.3 mV/min.

**Microbiological assay.** The present HPLC method was compared with a microbiological assay (7). Amikacin standards were prepared in both pooled dog and human sera.

**RESULTS**

**Standard curve.** Aqueous and serum standard curves were constructed at varying concentrations of amikacin, which was dissolved in 0.1 ml of water and then mixed with 0.9 ml of either water or serum and 1.0 ml of phosphate buffer. These samples were passed through a silica gel column and processed as described in Materials and Methods. Figure 1 shows both the aqueous and serum standard curves. It appears that the serum standard curve yielded 80 to 85% of the aqueous standard values at each concentration ranging from 1.0 to 15 μg/ml (Fig. 1). This discrepancy has not been further investigated. In all our calculations, corrections were made by using the aqueous standard values.

When serum samples containing differing amikacin concentrations were quantitated by using the serum standard curve, the results were the same whether human or dog serum was used. The lowest amikacin concentration that could be accurately measured in serum was 1.0 μg/ml.

**Separation and detection.** Figure 2 shows a typical chromatogram obtained from an eluate from serum containing amikacin. The retention time of amikacin was 7.5 min. Extracts of serum containing no amikacin produced no interfering peaks. Two small peaks eluting between 4 and 7 min were consistently present in the serum samples containing amikacin, and they may be degradation products resulting from heating the drug or contaminants closely related to semisynthetic amikacins (3). Identification of these peaks has not been pursued. It was, however, necessary to heat the derivatized amikacin for 5 min at 50°C and then immediately place it on ice in the dark until it was injected into the chromatograph (within 3 h). In an unheated sample, when injected within 5 min after elution from the silica gel column, two major peaks were seen with retention times of 3.5 and 7.5 min (not shown). In the unheated sample injected 60 min after elution, the 3.5-min peak disappeared significantly whereas the 7.5 min peak had increased in size. Moreover, unheated samples stored for longer than 60 min showed a gradual decrease of fluorescence intensity of the 7.5-min peak (not shown). Heating the derivatized amikacin mixture essentially eliminated the fluorescence peak eluted at 3.5 min and stabilized the fluorescence peak at 7.5 min. Fluorescence loss was minimized to less than 5% during a 3-h period by placing the sample on ice in the dark.

**Precision.** Precision for the HPLC assay of serum samples containing amikacin was calculated at concentrations of 2.5 and 10 μg/ml. At each concentration, six samples were extracted separately, derivatized, and chromatographed in duplicate on the same day. The relative standard deviations for amikacin at 2.5 and 10 μg/ml were 5 and 6%, respectively. Day-to-day variations in the recovery from serum samples containing 2.5 and 10 μg of amikacin per ml were within 5% when tested over a period of 2 weeks.

**Correlation of HPLC assay with microbiological assay.** Sera from three dogs receiving amikacin intravenously were assayed in duplicate by microbiological and HPLC assays. Figure 3 illustrates the rapid disappearance of amikacin from the serum of a single dog, as determined by both methods. More than 50% of...
the drug was removed from the serum within 30 min in all three dogs. The insert in Fig. 3 shows the correlation between the two assays. The correlation coefficient \( r \) was calculated to be 0.99 for serum samples from all three dogs.

Serum samples from a healthy man to whom amikacin was administered intravenously were also assayed in duplicate by both the microbiological and HPLC methods. Figure 4 shows the rapid decrease in amikacin concentrations from serum as determined by these assays. The correlation between the two methods was again good, with \( r \) calculated to be 0.99 (not shown). Table 1 shows concentrations of amikacin in sera, as determined by both the HPLC and microbiological assays, from five patients from whom blood samples were obtained at random times. The amikacin values obtained were quite similar when determined by either method. This suggests the feasibility of the HPLC assay for routine clinical monitoring.

**Specificity.** No interfering peaks were found in pooled dog serum extracts or in sera from three individual dogs and five individual, healthy human volunteers. It was also observed that mixing other antibiotics with 10 \( \mu \)g of amikacin per ml of serum caused no interference with the HPLC assay (Table 2).

**DISCUSSION**

The method described here is similar to our previously reported HPLC assay for gentamicin (7) and recently developed tobramycin assay (Maitra et al., in press). Like the other aminoglycoside antibiotics, amikacin binding to protein is negligible (4). Therefore, the drug does
not require extraction from serum by protein-precipitating agents. Because most aminoglycoside antibiotics adsorb to silica gel, amikacin was removed from serum by passing it through a silica gel column. Derivatization of the drug was necessary because a sample of amikacin (1 mg/ml) had no significant adsorption between 240 and 360 nm. Amikacin was derivatized directly on the column and eluted from the silica gel with ethanol. Like tobramycin (Maitra et al., in press), derivatized amikacin was also unstable and consisted of two major components. Heating the derivatized sample stabilizes the 7.5-min peak and causes the 3.5-min fluorescence peak to decrease. The mechanism may involve a chemical shift. Rapid cooling of the heated sample prevented decrease in the antibiotic fluorescence intensity of the 7.5-min peak over a period of 3 h.

In all of our HPLC assays of aminoglycoside antibiotics (i.e., gentamicin [7], tobramycin [Maitra et al., in press], and amikacin), we have separated these drugs by reverse-phase chromatography, preceded by precolumn derivatization. Precolumn derivatization is used as a pre-clean-up step and is likely to result in the elimination of interfering materials (5).

The HPLC assay can be performed in 60 min compared with 24 to 48 h for the usual microbiological assay methods. A rapid amikacin assay is vital for clinicians who must make decisions regarding appropriate dosage of this antibiotic for critically ill patients or for those who have renal dysfunction. This HPLC assay has also been shown to be precise and highly reproducible. When precision was calculated from serum samples containing amikacin at concentrations of 2.5 and 10 µg/ml, the relative standard deviations were 5 and 6%, respectively. Furthermore, when these samples were processed in the identical fashion on different days for 2 weeks, highly reproducible results were obtained with a variation of only 5%. Finally, the present assay was specific for amikacin. Antibiotics other than aminoglycosides (Table 2) were not detected with this procedure and did not alter the recovery of amikacin. Other aminoglycosides, i.e., gentamicin, tobramycin, sisomicin, and netilmicin, lose greater than 50% of their fluorescence with the present methodology and also chromatograp with retention times different from those of amikacin (Table 2). Recovery of amikacin was not affected by the addition of other aminoglycoside antibiotics to serum samples.

We have tested our HPLC assay on sera from three dogs and six human subjects. In all cases, our results indicate that the HPLC assay correlates well with the microbiological assay. The variation between the two assays, when tested in five individual patients (Table 1), was never over 10%.

The HPLC assay can measure amikacin in serum at concentrations as low as 1.0 µg/ml. This sensitivity is more than adequate for routine clinical purposes. It would appear, then,
TABLE 2. Interference and recovery studies with different antibiotics in combination with amikacin in serum

| Antibiotic added to amikacin* | Amt added (μg/ml) | Retention time of antibiotic* (min) | Recovery of amikacin (%) |
|-----------------------------|------------------|-----------------------------------|-------------------------|
| None                        |                  | Amikacin                          | Other antibiotics       |
| Tobraamycin                 | 10               | 7.50                              | 100                     |
| Gentamicin                  | 10               | 7.55                              | 17.2                    |
| Sisomicin                   | 10               | 7.50                              | 22.9, 36.5, 54.5        |
| Netilmicin                  | 10               | 7.56                              | 47.0                    |
| Carbenicillin\(^{7}\)       | 500              | 7.55                              | 98                      |
| Ampicillin                  | 100              | 7.56                              | 97                      |
| Methicillin\(^{7}\)         | 100              | 7.60                              | 103                     |
| Penicillin G\(^{7}\)        | 100              | 7.58                              | 96                      |
| Chloramphenicol\(^{7}\)     | 50               | 7.58                              | 98                      |
| Cephalothin\(^{7}\)         | 50               | 7.58                              | 97                      |
| Clindamycin\(^{7}\)         | 25               | 7.56                              | 98                      |

* 10 μg/ml.

\(^{7}\) The retention and resolution of aminoglycosides depend on the age of the MicroBondapak column.

\(^{7}\) Retention times of gentamicin components C\(_1\), C\(_2\), and C\(_3\).

\(^{7}\) The present method probably destroys most of the netilmicin derivative. Unheated netilmicin derivative elutes at 55.8 min. All other aminoglycosides also lose over 50% of their fluorescence intensity with this procedure.

* These antibiotics do not yield fluorescent derivatives with this procedure.

that this amikacin assay employing HPLC is feasible for routine clinical use.

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