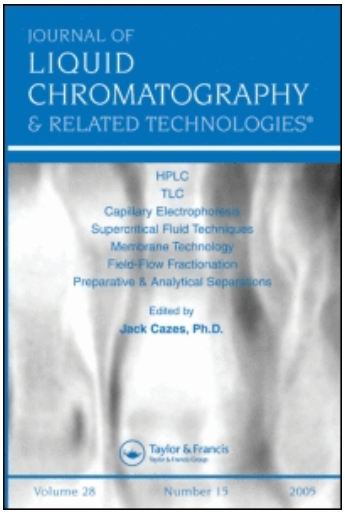

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DETERMINATION OF AMIKACIN ISOMERS BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

A high-pressure liquid chromatographic (HPLC) method for quantitative monitoring of amikacin isomers is described. Four isomers, BB-K8, BB-K29, BB-K6 and BB-K11 were applied to a silica gel column. While adsorbed, the isomers were derivatized with o-phthalaldehyde and the derivatized products eluted with ethanol. A decrease in the fluorescence of the derivatized products with time was observed. Heating at 50°C for 5 min produced products with stable fluorescence for at least three hours. Using the fluorescent properties of the amikacin derivative for detection, the four isomers of amikacin were separated by reverse phase (HPLC). A linear relationship from 1 to 10 μg/mL was obtained for all four isomers.

INTRODUCTION

Amikacins are semisynthetic aminoglycoside antibiotics (1), and one of their isomers, BB-K8, is currently being used for treatment of aerobic gram-negative bacillary infections. These semisynthetic amikacin products were produced to cope with the increas-

ing problem of R-factor mediated resistance to aminoglycoside antibiotics (2,3). Knowledge of this mechanism of resistance to aminoglycosides led to specific structural modification of kanamycin A and the production of various amikacin derivatives. Amikacin BB-K8 and its three isomers (Figure 1) have been synthesized by reacting kanamycin A with carbobenzoxy-N-hydroxy succinimide and L(-)-γ-amino-α-hydroxybutyric acid (4). These isomers are characterized by their melting points and relative rf values on thin layer chromatography (1,4). On HPLC, it is clear that these semi-

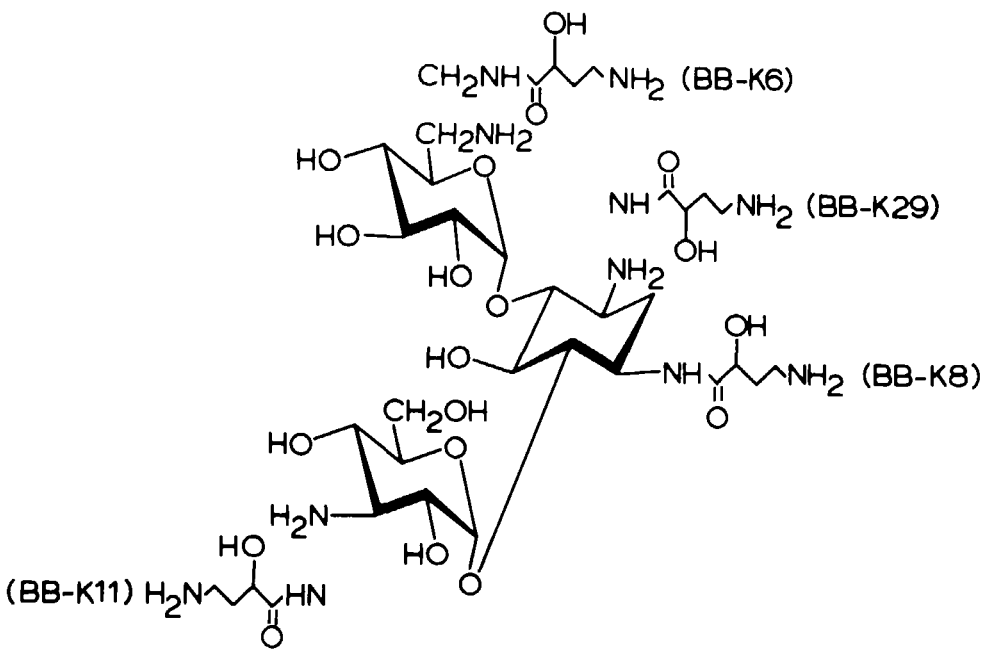

Figure 1. Structures of amikacin isomers. The L(-)-γ-amino-α-hydroxybutyric acid at position indicated gives the isomer shown in parenthesis.

synthetic isomers contain by-products of the synthetic reaction. For example, during the synthesis of one of the isomers, BB-K11, BB-K29 and BB-K6 would also be produced (4).

In order to perform pharmacological and toxicity studies of these semisynthetic drugs, pure isomers and a specific method for quantitation are necessary. We have described in this report a method for monitoring and quantitating the four isomers of amikacin, i.e., BB-K8, BB-K29, BB-K6 and BB-K11.

EXPERIMENTAL

Reagents:

Silicic acid (60-200 mesh), o-phthalaldehyde, tripotassium ethylenediaminetetraacetate (EDTA), silane-treated glass wool, methanol, ethanol, distilled water and 2-mercaptoethanol were purchased or prepared as described previously (5). Amikacn isomers BB-K6, BB-K8, BB-K11 and BB-29 were kindly donated by Bristol Laboratories.

Equipment:

High-pressure liquid chromatography separations were performed on Waters Associates Model No. ALC/GLC 200 equipped with a Altex reverse pack C_{18} column (25 cm x 4 mm). The mobile phase was a mixture of methanol/water (70:30 v/v) containing 2 g of tripotassium EDTA per liter (5). Prior to use, the mobile phase was passed through Solvinert 0.6 μm millipore filter and de-aerated under reduced pressure. The eluent was monitored with a fluorometer (Model No. J4-7461; American Instrument Co.) equipped with an 18 μl flow cell, a 7-60 primary filter (Corning Glassware, 70% maximum

transmission at 350 nm, 50% minimum transmission from 330-375 nm) and secondary filters 38A and 2E (Kodak, wratten; Eastman Kodak, 70% transmission from 420-480 nm and 95% transmission from 415-700 nm, respectively). A 931B photomultiplier tube from Radio Corporation of America was used. (Fluorescent intensity was measured on an integrator recorder (Model No. HP3380A, Hewlett-Packard.)

PROCEDURES

Derivatizing Reagent:

Fifty microliters of 40% potassium hydroxide were combined with 195 mL absolute ethanol and 5 mL of water. This corresponded to pH 10. o-phthalaldehyde (200 mg) was dissolved in 40 mL of the above, pH 10. ethanol and mixed with 0.4 mL of undiluted 2-mercaptoethanol (6). This reagent was stored under nitrogen at 4°C after each use for a period of no longer than five days.

Chemical Assay:

This procedure was similar to the amikacin and tobramycin assays previously described (6,7). A 4.0 cm x 0.5 cm silica gel column (6) was washed with 2 mL of 66 mM potassium phosphate buffer, pH 9. A one milliliter sample containing an amikacin isomer was diluted to 2 mL with phosphate buffer and was applied to the silica gel column. The sample tube was rinsed twice, with 1 mL of water and also applied to the column. A total of 4 mL of the eluate was discarded. One milliliter of the o-phthalaldehyde reagent was applied to the column and immediately followed by 2 mL of 95% ethanol, pH 10. The derivatized isomer was recovered in the 3 mL elute. This mixture was vortexed and immediately heated at

50°C for 5 min, vortexed again and placed immediately into an ice bath. The solution was passed through a Millipore filter (0.6 μm, pore size) and the resulting filtrate was kept in the dark on ice until analyzed.

A 25 μL sample of the above amikacin derivative was injected into the column and eluted with mobile phase at a flow rate of 2. mL/min. Relative fluorescence was quantitated by integration of peak areas. Peak areas were some times measured manually (peak height x width at ½ peak height). The attenuation of the recorder was 128 with a photomultiplier sensitivity of 10. The chart speed was 0.5 cm/min with a maximum slope sensitivity of 0.3 mv/min.

In vitro serum samples:

Standard aqueous solution of amikacins were prepared. Serum samples containing 0.1 mL aliquots of aqueous solution and 0.9 mL aliquots of dog or huma sera were incubated at ambient temperature for at least 20 min.

RESULTS AND DISCUSSION

Separation and Detection:

Figure 2 shows chromatograms of all four isomers. Isomers dissolved in water or sera (human or dog) showed no difference in retention times. The retention times of all four isomers were different (Figure 2). The recovery of all isomers from serum compared to that of the aqueous sample was 80-90%. This discrepancy has not been further investigated.

Figure 3 shows aqueous standard curves for amikacin and its three isomers. All isomers showed a linear relationship at concen-

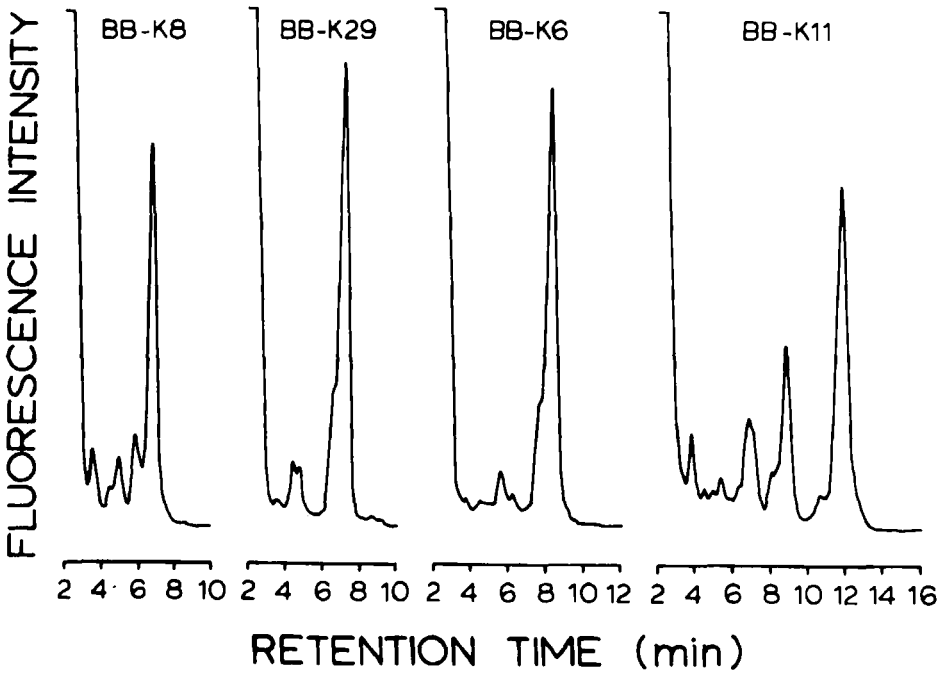

Figure 2. Typical HPLC chromatograms of the amikacin and its isomers. Serum samples containing 10 μg/mL each of BB-K8, BB-K29 and BB-K6 and 20 μg/mL of BB-K11 were used. The retention times were the following: BB-K8, 6.6 min; BB-K29, 7.1 min; BB-K6, 8.3 min; and BB-K11, 11.7 min. The two peaks eluting prior to BB-K11 corresponds to the retention times of BB-K29 and BB-K6.

trations between 0 and 10 μg/mL. The slope of the curve for BB-K11 was markedly decreased compared to other isomers. A lowest concentration of 1 μg/mL for all four isomers of amikacin could be accurately measured.

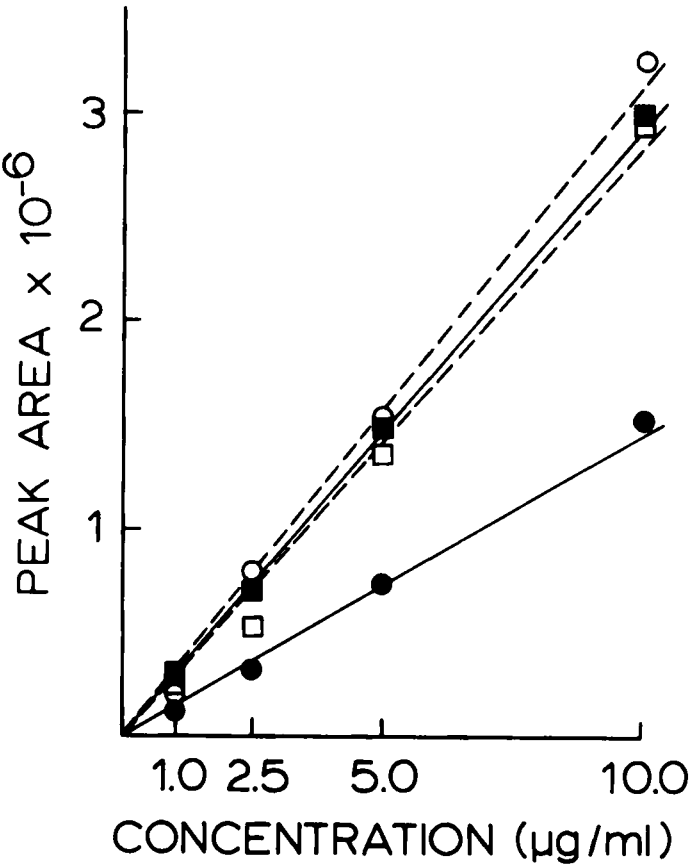

Figure 3. Areas of the peaks at retention times corresponding to BB-K8, BB-K29, BB-K6 and BB-K11 as a function of isomer concentration. Standards were prepared in water and processed through silica gel column as described. 0,BB-K6; ◘, BB-K8; ■ ,BB-K29; and ●, BB-K11.

Fluorescence studies of unheated samples:

We have previously shown that other aminoglycosides, amikacin (6) and tobramycin (7), when mixed with o-phthalaldehyde produce

two fluorescent products which can be separated by chromatography. It was observed that on storage of the derivatized products, the fluorescence of the earlier eluting peak decreased, whereas the fluorescence of the later eluting peak increased. This same phenomenon was observed with all isomers of amikacin as shown in Figures 4 and 5. As shown in Figure 4, the fluorescence of the earlier peak for BB-K29 is completely gone within 70 min. Whereas the fluorescence of the earlier peak for BB-K6 takes approximately twice as long (Figure 5). In the cases of BB-K8 (Figure 4) and BB-11 (Figure 5), the fluorescence of the earlier peaks did not disappear within 180 min. In all cases the fluorescence of the later peaks increased with time. This increase in fluorescence of the late peaks was essentially reciprocal to the disappearance of fluorescence of the earlier peaks.

Actual chromatographic profiles for all the isomers after 3 and 180 min are also shown in Figures 4 and 5 inserts. The same reciprocal relationship for the early and late peaks described above is easily discernable. The chemical basis for the decreased fluorescence of the early peaks and increased fluorescence of the late peaks is not understood. It is also apparent that the time required for each isomer to yield an equal amount of fluorescence for the two peaks is different. Perhaps this is an indication of the participation of L(-)-γ-amino butyric acid in the derivative formation by o-phthalaldehyde.

We have previously shown that the fluorescence of the late peak can be stabilized for 180 min by heating tobramycin and amika-

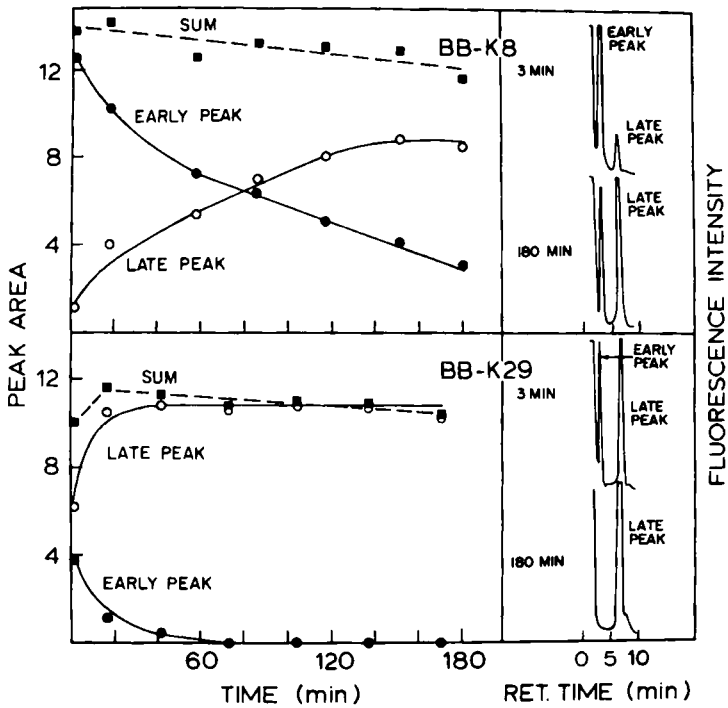

Figure 4. Fluorescence of unheated sample with time. To 10 μg/mL of each aqueous isomer 1 mL o-phthalaldehyde reagent and 1 mL ethanol (pH 10) were added and injected with time. See details in the text. ●, fluorescence of earlier eluting peak; 0, fluorescence of late eluting peak; and ■, total fluorescence of both peaks. Inserts represents HPLC profile of isomers injected at time 3 min and 180 min. The retention times of earlier eluting peaks for BB-K8 and BB-K29 were 3.43 and 3.22 min respectively. The late eluting peaks were 6.66 and 7.10 min respectively.

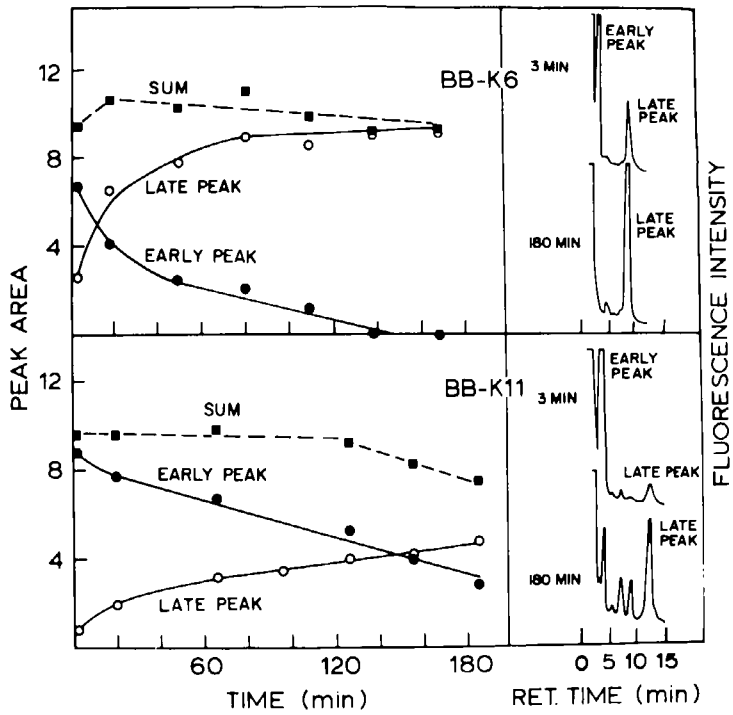

Figure 5. Fluorescence of unheated sample with time. To 10 μg/mL of BB-11 1 mL o-phthalaldehyde reagent and 1 mL ethanol (pH 10) were added and injected with time. ●, fluorescence of earlier eluting peak; 0, fluorescence of late eluting peak; and ■ , total fluorescence of both peaks. Inserts represents HPLC profile of isomers injected at time 3 min and 180 min. The retention times of earlier eluting peaks for BB-K6 and BB-K11 were 3.0 and 3.70 respectively. Similarly the late eluting peaks were 8.35 and 11.8 min respectively.

cin BB-K8 derivatives (6,7). Figures 4 and 5 show that total fluorescence of the two peaks slightly decreases with time for all isomers. Hence heat treatment was utilized to yield a stabilized late eluting peak (Figure 2). Thus the fluorescence loss of the stabilized late peak for all isomers were minimized to less than 5% during a three-hour period.

Fluorescent Studies in Mixtures:

Figure 6 shows that the retention times of isomers are not effected when 10 μg each of BB-K6, BB-K8 and BB-K11 are reacted with o-phthalaldehyde and chromatographed. An aqueous blank did not produce interfering peaks. Small peaks produced in serum blank at 6.54 min may cause some interference when BB-K8 is measured in serum. However, addition of 5% acetonitrile to the mobile phase removed this interference (6). All possible combinations of three and two isomers were studied and no interference with retention times as well as recoveries were observed (not shown). These results may suggest that intermolecular derivative formation with o-phthalaldehyde is unlikely. The relative standard deviation and day-to-day variation for all isomers were within 5% when tested over a period of two weeks.

CONCLUSION

We have found that aminoglycoside antibiotics, gentamicin, tobramycin, and amikacins, because of their chemical nature and their polarity adsorb to silicic acid. Hence these compounds can be purified from serum or other mixtures by silicic acid chromato-

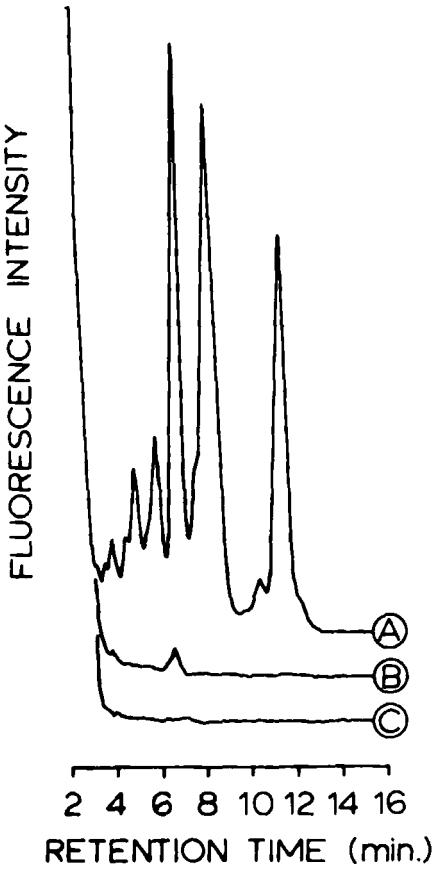

Figure 6. HPLC profile of 3 amikacin isomers.

A. Combination of aqueous solution BB-K8 (10 μg), BB-K6 (10 μg) and BB-K11 (20 μg) were processed through silica gel column as described. A combination in serum gave similar profile and quantitative recovery. The retention times were BB-K8, 6.6 min; BB-K6, 7.06 min; and BB-K11 11.7 min respectively.

B. Water blank

C. Serum blank

graphy since the derivatization is accomplished directly on the column and the derivatives formed are more nonpolar, these can be eluted with suitable organic solvents. This precolumn derivatization step has resulted in clear separations and flat baselines. We have also found that gentamicin, tobramycin, netilmicin, sisomicin and many other penicillin derivatives do not interfere with amikacin assay with the present methodology (6).

This method can accurately measure 1 μg/mL of amikacin and its three isomers and can be utilized for purity estimation of commercial samples. It is evident that samples of BB-K11 have contaminants which elute at the retention times of BB-K29 and BB-K6. During the synthesis of BB-K11 with carbobenzoxy-N-hydroxysuccinimide and Kanamycin A, BB-K29 and BB-K6 may be by-products. Separation and monitoring of these mixtures may be difficult and time-consuming to assay by conventional chromatographic techniques (2,3,4). This HPLC method may be desirable for this latter purpose and could also be utilized in pharmacological studies of these isomers and other semisynthetic amikacin derivatives.

ACKNOWLEDGMENT

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