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Rapid Method for Determination of Kanamycin and Dibekacin in Serum by Use of High-Pressure Liquid Chromatography

HIROAKI KUBO,[1]* YOSHIE KOBAYASHI,[1] AND TAKASHI NISHIKAWA[2]

School of Pharmaceutical Sciences, Kitasato University, 5-9-1, Shirokane, Minato-ku, Tokyo 108,[1] and School of Medicine, Kitasato University, 1-5-1, Kitasato, Sagamihara-shi, Kanagawa 228,[2] Japan

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A rapid, simple, and accurate method for the determination of kanamycin and dibekacin in serum by use of high-pressure liquid chromatography is described. The serum proteins were precipitated with 3.5% perchloric acid containing sodium octanesulfonate. After centrifugation, a sample of the supernatant was directly injected into the chromatograph. The determination of kanamycin and dibekacin was performed by a combination of reverse-phase, ion-pair chromatography, postcolumn derivatization with *o*-phthalaldehyde, and fluorescence detection. The correlation coefficients with fluorescence polarization immunoassay were 0.996 for kanamycin and 0.957 for dibekacin.

Kanamycin and dibekacin are aminoglycoside antibiotics used for the treatment of serious gram-negative-bacterium infections. Like other aminoglycoside antibiotics, kanamycin and dibekacin have narrow therapeutic ranges and exert nephro- and ototoxicity. Therefore, monitoring of kanamycin and dibekacin levels in serum is necessary for safe and effective therapy. The advantages and disadvantages of various methods for the determination of aminoglycoside antibiotics were reviewed by Maitra et al. (8) Various methods of high-pressure liquid chromatography were also reviewed by Nilsson-Ehle (9). Recently, sensitive methods of high-pressure liquid chromatography have been reported for the determination of aminoglycoside antibiotics (2–4). Most of these methods require either pre- or postcolumn derivatization for fluorescence detection. These methods involve time-consuming pretreatment such as solvent or column extraction of aminoglycoside antibiotics in serum.

We have previously reported the simple methods for the determination of gentamicin and tobramycin in serum by high-pressure liquid chromatography with *o*-phthalaldehyde postcolumn derivatization (5–7).

This report describes a simpler procedure for the determination of kanamycin and dibekacin in serum than the pretreatment of previous methods. The values obtained by the proposed method were compared with those obtained by fluorescence polarization immunoassay.

MATERIALS AND METHODS

Chemicals and reagents. Kanamycin sulfate and dibekacin sulfate were obtained from Meiji Seika, Tokyo, Japan; *o*-phthalaldehyde was from Nakarai Chemicals, Kyoto, Japan; Brij 35, 2-mercaptoethanol, and sodium octanesulfonate were from Wako Pure Chemicals, Osaka, Japan; and disodium 1,2-ethanedisulfonate was from Tokyo Kasei Kogyo, Tokyo, Japan. Distilled water and acetonitrile were of liquid chromatographic grade. All other chemicals were of reagent grade.

The protein precipitant reagent was prepared to contain 10 mM sodium octanesulfonate in 3.5% perchloric acid.

The mobile phase for kanamycin was prepared to contain 22 mM disodium 1,2-ethanedisulfonate and 5 mM sodium octanesulfonate in a water-acetonitrile mixture (80:20, vol/vol) adjusted with acetic acid to about pH 3.5. The mobile phase for dibekacin was prepared to contain 37 mM disodium 1,2-ethanesulfonate and 5 mM sodium octanesulfonate in a water-acetonitrile mixture (80:20, vol/vol) adjusted with acetic acid to about pH 3.5.

The *o*-phthalaldehyde reagent was prepared for the use of sodium borate buffer instead of potassium borate buffer by the method of Anhalt (1).

Apparatus and chromatographic conditions. The chromatographic equipment was constructed from commercially available components.

Chromatographic separations were performed with a Waters QA-1 Analyzer equipped with an autosampler with Waters 300-μl limited-volume inserts, an automatic injector (10-μl volume for kanamycin and 20-μl volume for dibekacin), a single-stroke pump, a Guard-PAK C_{18} precolumn (10 μm, 4- by 8-mm inside diameter), and a radial-compression separation unit which consisted of a Radial-PAK C_{18} cartridge (10 μm, 10- by 8-mm inside diameter) and a compression chamber for compressing the cartridge, all from NIHON Waters Ltd., Tokyo, Japan.

The column effluent was introduced into a Waters M 105 Reaction System equipped with a mixing tee, a reciprocating pump, a pulse-dampening device, and a reaction coil consisting of a stainless-steel tube (6- by 0.5-mm inside diameter) in a heating bath from NIHON Waters Ltd.

As a detector, a model S-FL-330 fluorometer (Soma Optics Co., Ltd., Tokyo, Japan) equipped with an L-1549-04 lamp (energy maximum at 351 nm, excitation), a 440-nm cutoff filter (emission), and a 25-μl quartz flow-cell was used. Photomultiplier voltage was 500 V, and the response range was set at 8.

The detector signal was recorded by a Waters QA-1 Data System (NIHON Waters Ltd.). The flow rate of the mobile phase was maintained at 1.5 ml/min. The *o*-phthalaldehyde reagent was pumped at a flow rate of 1.0 ml/min.

Procedure. Serum (50 μl) was added to the 300-μl limited-volume insert containing a 50-μl protein precipitant reagent and was then vortex mixed for a few seconds. After centrifugation of the insert for 2 min in a model KM-15200 centrifuge (Kubota Co., Tokyo, Japan) at 15,000 × *g*, the insert was put into the autosampler. A sample of the supernatant in the insert was injected into the column. Normal

* Corresponding author.

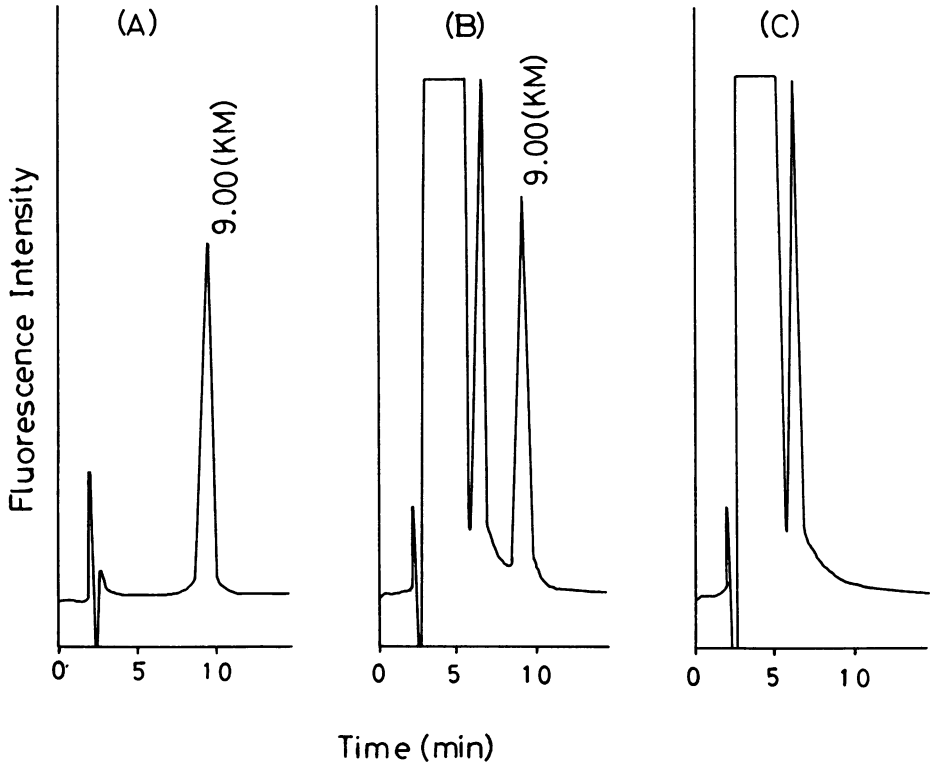

FIG. 1. Chromatograms of kanamycin standard (A), kanamycin-spiked control serum (15 μg/ml) (B), and kanamycin-free control serum (C). KM, Kanamycin.

pooled sera spiked with various known amounts of kanamycin (3 to 50 μg/ml) or dibekacin (0.5 to 10 μg/ml) were prepared and analyzed. Peak area measurements were performed to construct the calibration curve. The levels in every serum were determined in duplicate, and the results were averaged. The specimens were obtained from Kitasato University Hospital.

Fluorescence polarization immunoassay. Fluorescence polarization immunoassay was performed with commercially available kits (Abbott-TDX-Kanamycin and Abbott-TDX Dibekacin, Dainabot, Tokyo, Japan).

RESULTS

Figure 1 shows chromatograms of kanamycin standard, kanamycin-spiked control serum, and kanamycin-free control serum. The retention time of kanamycin is 9.0 min.

Figure 2 shows chromatograms of dibekacin standard, dibekacin-spiked control serum, and dibekacin-free control serum. The retention time of dibekacin is 11.0 min.

The protein precipitant reagent-treated serum produced a chromatogram that was virtually identical with the chromatogram obtained from the standard. The antibiotic-free serum showed no peaks that would interfere with the determination of antibiotics.

Linear regression analysis of the calibration curves of kanamycin (3 to 50 μg/ml) and dibekacin (0.5 to 10 μg/ml) yielded the equations $y = 0.979x - 0.006$ ($r = 0.999$) and $y = 0.998x - 0.04$ ($r = 0.999$), respectively. The peak areas (y) were related to serum concentrations (x) with high linearity.

Repeated analyses of serum pools containing kanamycin and dibekacin at three different concentrations gave the results shown in Table 1. The within-run coefficients of variation for kanamycin and dibekacin ranged from 0.8 to 2.9% and from 1.6 to 3.1%, respectively; the between-run coefficients of variation for kanamycin and dibekacin ranged from 2.0 to 5.0% and from 1.9 to 5.6%, respectively. To estimate the analytical recovery, an aqueous solution of kanamycin (15 μg/ml) with a kanamycin-added serum (15 μg/ml) and an aqueous solution of dibekacin (4 μg/ml) with a dibekacin-added serum (4 μg/ml) were analyzed, and their peak areas were compared. The recoveries of kanamycin and dibekacin, based on the average of three samples, were 99.0 and 98.5%, respectively.

The results obtained by the proposed method were compared with those obtained by a fluorescence polarization immunoassay. The regression equation, number of observations, and correlation coefficient were $y = 1.027x - 1.090$, $n = 44$, and $r = 0.996$ for kanamycin and $y = 0.998x - 0.206$, $n = 42$, and $r = 0.957$ for dibekacin (y, the proposed method; x, fluorescence polarization immunoassay).

DISCUSSION

We have previously reported precipitation of serum proteins with methanol to determine gentamicin or tobramycin in serum (5–7). Gentamicin sulfate and tobramycin sulfate are very soluble in methanol, but kanamycin sulfate and dibekacin sulfate are very poorly soluble in methanol. Therefore, precipitation of serum proteins was performed with perchloric acid containing sodium octanesulfonate.

As the precipitate of serum proteins adhered tightly to the bottom of the 300-μl limited-volume inserts after centrifugation, the supernatant was pulled into the automatic injector without disturbing the precipitate.

Reverse-phase, ion-pair chromatography was effective in eliminating the interference caused by low-molecular-weight primary amines in serum, which would give fluorescent

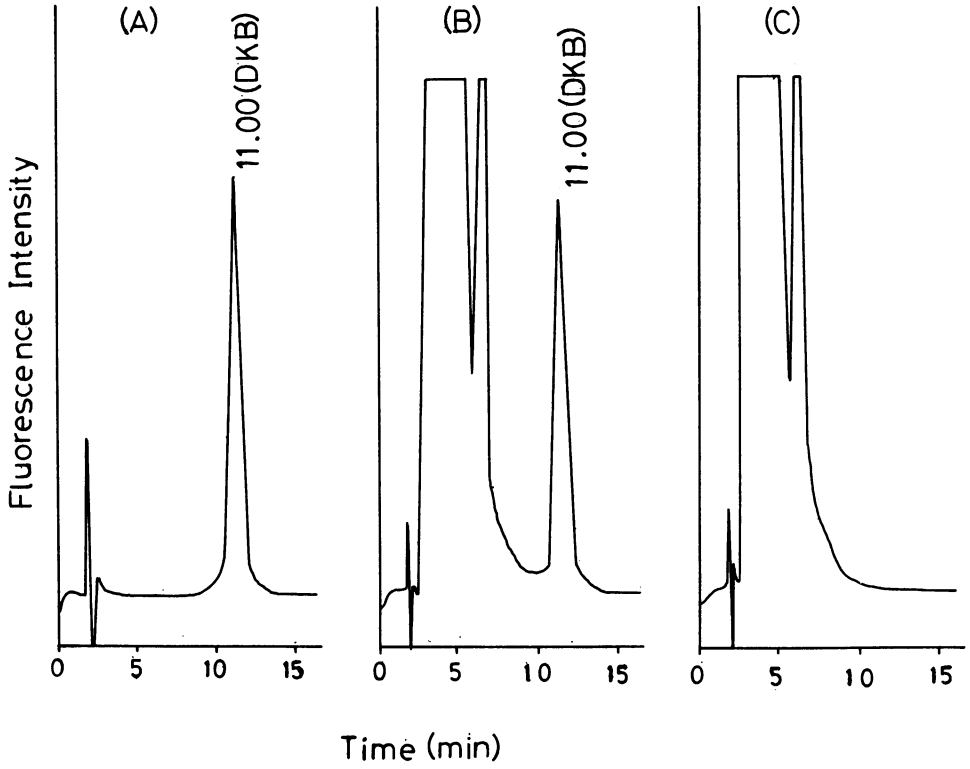

FIG. 2. Chromatograms of dibekacin standard (A), dibekacin-spiked control serum (8 μg/ml) (B), and dibekacin-free control serum (C). DKB, Dibekacin.

products with the *o*-phthalaldehyde. Kanamycin and dibekacin were separated from these amines by the use of octanesulfonate, because the ion pairs of kanamycin with four amino groups and those of dibekacin with five amino groups were held more strongly on the reverse-phase column than were the ion pairs of other amines with fewer amino groups.

Disodium 1,2-ethanedisulfonate was added to adjust the retention times of kanamycin and dibekacin.

The proposed method simplifies the sample pretreatment greatly by avoiding tedious steps such as solvent or column extraction and reduces the analysis time significantly. Total analysis time including sample pretreatment and chromatographic separation was less than 15 min.

The advantages of this method are speed, simplicity, accuracy, and good reproducibility. Therefore, the method can be used for routine monitoring of therapeutic drugs.

TABLE 1. Within-run and between-run precision

Aminoglycoside	Concn (μg/ml)	Precision (n = 8)			
		Within-run		Between-run	
		±SD (μg/ml)	CV[a] (%)	±SD (μg/ml)	CV (%)
Kanamycin	5.00	0.145	2.9	0.250	5.0
	15.00	0.225	1.5	0.300	2.0
	30.00	0.240	0.8	0.660	2.2
Dibekacin	1.00	0.031	3.1	0.056	5.6
	4.00	0.092	2.3	0.152	3.8
	8.00	0.128	1.6	0.152	1.9

[a] CV, Coefficient of variation.

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