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DETERMINATION OF SISOMICIN, NETILMICIN, ASTROMICIN AND MICRONOMICIN IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for monitoring serum levels of the aminoglycoside antibiotics sisomicin, netilmicin, astromicin, and micronomicin, using an amino acid analysis system is described. The procedure involves sample preparation with a CM-Sephadex column, and quantitation using internal standards chosen from each of the other aminoglycosides.

The aminoglycosides were separated by reversed-phase ion-pair chromatography on Zorbax C_8 and ODS columns, and detected by the post-column derivatization technique.

The calibration curves of serum concentration for sisomicin, netilmicin, astromicin, and micronomicin were linear over the ranges 0.32—22.8 μg/ml, 0.17—11.6 μg/ml, 0.1—6.3 μg/ml, and 1.0—30 μg/ml, respectively. The coefficients of variation were 2.5, 2.8, 3.1 and 1.9%, at the serum concentrations of 1.3, 1.45, 1 58 and 2.5 μg/ml, respectively (n = 6).

Determination by the internal standard method using another aminoglycoside gives accurate and reproducible results. This method is applicable also to other aminoglycoside antibiotics.

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INTRODUCTION

Aminoglycoside antibiotics are used for the treatment of serious infections caused by gram-negative bacilli. Because of their ototoxicity and nephrotoxicity, careful monitoring of blood levels is required in order to obtain rational therapy.

Various methods have been developed for the determination of aminoglycoside antibiotics in serum, including microbiological assay, radioenzymic assay, homogeneous enzyme immunoassay, and high-performance liquid chromatography (HPLC).

Determination using microbiological methods is time-consuming and their quantitative variation, which is inherent to microbes, makes its application to drug monitoring difficult. Recently, many authors have reported the determination of aminoglycosides in serum using HPLC because of its accuracy and precision in quantitation [1—5]. Since aminoglycosides have several primary amino groups, they are easily derivatized, and these same authors have described ultraviolet detection of 1-fluoro-2,4-dinitrobenzene derivatives (pre-label) [1, 2] and fluorescence detection of *o*-phthalaldehyde derivatives (post-label) [3—5].

We have already reported the determination of tobramycin in serum by HPLC adopting *o*-phthalaldehyde post-label derivatization [6], this report describes the application of this method to the analysis of sisomicin, netilmicin, astromicin, and micronomicin in serum. Since there is so far no kit commercially available in Japan for the enzymic immunoassay of these antibiotics, this method is valuable from a therapeutic viewpoint.

EXPERIMENTAL

Apparatus

The Shimadzu liquid chromatographic amino acid analysis system consisted of an LC-3A pump equipped with a column oven CTO-2A and a sample injector SIL-1A, a reagent pump PRR-2A, and a fluorescence detector FLD-1

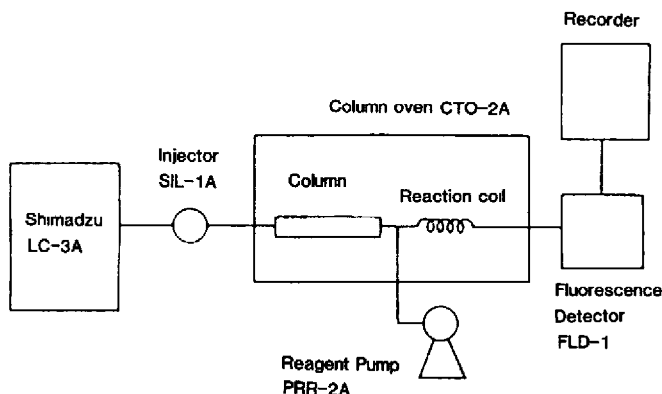


Fig. 1. Flow diagram of the analytical system. Flow-rates: LC-3A: 0.8 ml/min (Zorbax C_8), 1.5 ml/min (Zorbax ODS); PRR-2A (reagent pump)· 0.5 ml/min. Column oven temperature CTO-2A: 55°C

with a fluorescence lamp (300—450 nm, maximum 360 nm) and an emission filter EM-4. Fig. 1 shows the flow diagram of the chromatographic system.

The columns used were Zorbax C_8 (5 μm, 15 cm × 4.6 mm I.D.) for the analysis of sisomicin (SISO), netilmicin (NTL), and astromicin (ASTM), and Zorbax ODS (5 μm, 15 cm × 4.6 mm I.D.) for micronomicin (MCR). The reaction coil, made of stainless steel (70 cm × 0.5 mm I.D.), was stored in the column oven and maintained at 55°C to promote the reaction.

Reagents

Sisomicin sulphate and netilmicin sulphate were obtained from Essex Nippon K.K. (Osaka, Japan); astromicin and micronomicin were obtained from Kyowa Hakko Kogyo (Tokyo, Japan). Concentrated aqueous stock solutions of these aminoglycoside antibiotics were stored refrigerated at 4°C and were diluted before use. All antibiotics concentrations were calculated by their potency. *o*-Phthalaldehyde was obtained from Merck (Darmstadt, F.R.G.); 30% solution of Brij-35 was from Technochemical (Tokyo, Japan). Sodium *p*-toluenesulphonate and 2-mercaptoethanol were from Wako (Osaka, Japan). All other chemicals were of analytical grade.

Mobile phase and reaction reagent

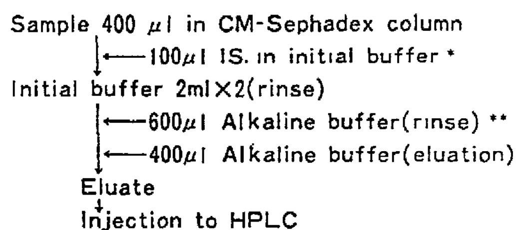
The mobile phase contained 25 m*M* sodium *p*-toluenesulphonate as a counter-ion for reversed-phase ion-pair chromatography and 20 m*M* sodium dihydrogen phosphate dihydrate; sodium perchlorate anhydrous was added to the mobile phase to adjust the retention time of each aminoglycoside. The pH of the mobile phase was adjusted to about 2.0 with perchloric acid.

The stock reagent was prepared by dissolving 9.4 g of boric acid and 4.8 g of sodium hydroxide in 400 ml of distilled water. Just before use, the reaction reagent was prepared by the addition of 6 ml of an ethanolic solution of 400 mg of *o*-phthalaldehyde, 1 ml of 2-mercaptoethanol, and 2 ml of a 30% solution of Brij-35. The reaction reagent was then diluted to 500 ml, and the pH was adjusted to about 10.5 with sodium hydroxide.

Sample preparation

Amino acids in serum interfere with the determination of aminoglycosides because *o*-phthalaldehyde reacts with primary amines and gives fluorescent products. So it is necessary to remove amino acids as well as proteins prior to injection. We adopted and modified the preparation procedure of Anhalt [3].

Fig. 2 shows the procedure to separate aminoglycosides from interfering compounds in serum by a short column with a bed volume of 1 ml of CM-Sephadex (C-25). This column was washed with the initial buffer (0.4 *M* sodium acetate) and the serum sample was applied to the column. The internal standard chosen from other aminoglycosides was dissolved in the initial buffer, and 100 μl of the solution were added to the column. The column was washed with 2 ml of initial buffer twice in succession. The eluting buffer was changed to 10 m*M* sodium hydroxide in the initial buffer (alkaline buffer), and 600 μl of this buffer were added to the column. After the column had been drained, 400 μl of alkaline buffer were added and the eluate collected was injected into the HPLC system.



* 0.4 M Sodium acetate.

** 10 mM Sodium hydroxide in initial buffer.

Fig. 2. Sample preparation with a CM-Sephadex column.

RESULTS AND DISCUSSION

Fig. 3 shows chromatograms of sisomicin-spiked serum and the corresponding blank serum. Tobramycin was used as the internal standard at a concentration of 9.4 μg/ml. Chromatogram A is the blank serum containing the internal standard, and B is the sisomicin spiked at a concentration of 22.8 μg/ml. The relation between the peak height ratio (SISO/TOB) and the sisomicin concentration in the serum was linear over the range 0.32—22.8 μg/ml. Linear regression analysis of this calibration yielded the equation $Y = 0.034X + 2.23 \cdot 10^{-3}$ ($r = 0.9997$).

The coefficient of variation (C.V.) of the peak height ratio was 2.5%, which was determined by analyzing six individually prepared serum samples containing 1.3 μg/ml sisomicin and 9.4 μg/ml tobramycin (Table I).

Fig. 4 shows chromatograms of netilmicin-spiked serum and its blank. Astromicin was used as the internal standard at a concentration of 6.3 μg/ml. Chromatogram A is the blank and B is the netilmicin-spiked serum (11.6 μg/ml). The calibration curve was linear over the range 0.17—11.6 μg/ml. Linear regression analysis of this calibration curve resulted in the equation $Y = 0.101X - 8.5 \cdot 10^{-3}$ ($r = 0.9998$). The C.V. for netilmicin was 2.8% at a concentration of 1.5 μg/ml ($n = 6$) (Table I).

In order to analyze astromicin, netilmicin was used as the internal standard

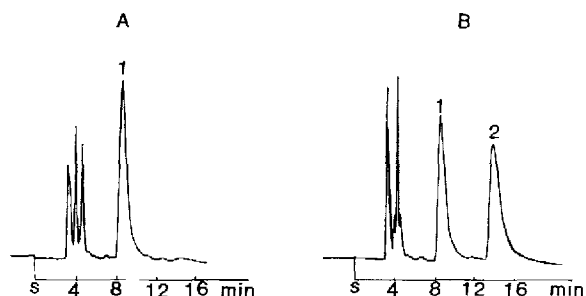


Fig. 3. Chromatograms of serum spiked with sisomicin and blank serum. Peaks: 1 = tobramycin (IS), 2 = sisomicin. (A) Blank serum: tobramycin (IS) 9.4 μg/ml. (B) Spiked serum: sisomicin 22.8 μg/ml. Mobile phase: 25 m*M* sodium *p*-toluenesulphonate, 20 m*M* sodium dihydrogen phosphate dihydrate, 0.3 *M* sodium perchlorate (anhydrous), pH 2.0 with perchloric acid. Flow-rate 0.8 ml/min; sensitivity ×64; injected volume 10 μl.

TABLE I

COEFFICIENTS OF VARIATION FOR THE DETERMINATION OF AMINOGLYCOSIDES IN SERUM

	C.V. (%)	Serum concentration (μg/ml)
Sisomicin	2.5	1.3
Netilmicin	2.8	1.45
Astromicin	3.1	1.58
Micronomicin	1.9	2.5

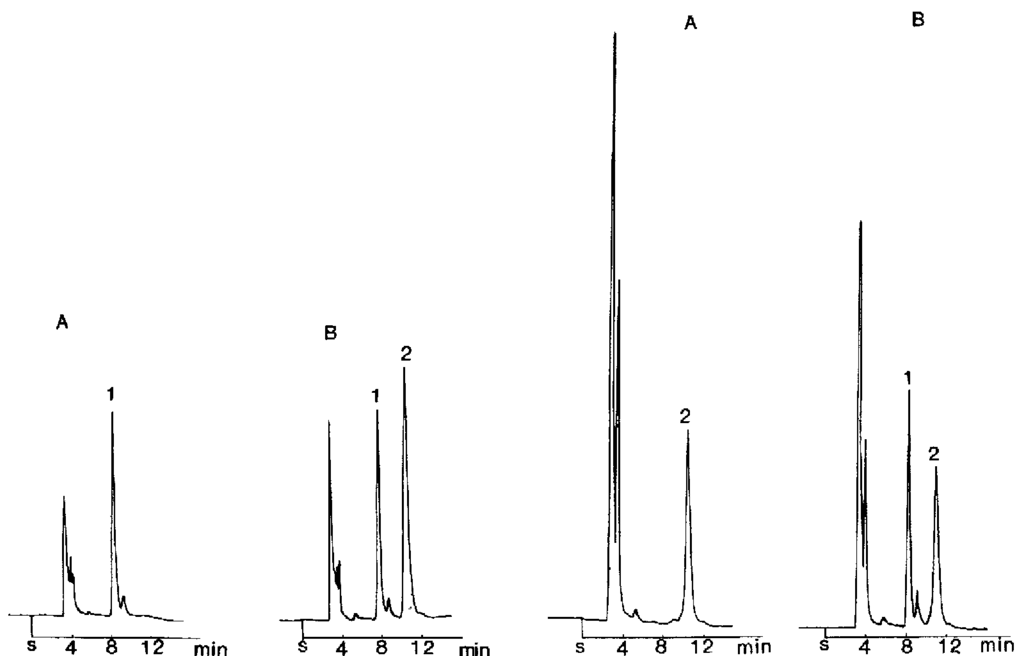


Fig. 4. Chromatograms of serum spiked with netilmicin and blank serum. Peaks: 1 = astromicin (IS), 2 = netilmicin. (A) Blank serum: astromicin (IS) 6.3 μg/ml. (B) Spiked serum: netilmicin 11.6 μg/ml. Mobile phase: 0.6 *M* sodium perchlorate (anhydrous); other conditions are the same as in Fig. 3. Injected volume 20 μl. These analytical conditions were used for the experiments with netilmicin and astromicin.

Fig. 5. Chromatograms of serum spiked with astromicin and blank serum. Peaks: 1 = astromicin, 2 = netilmicin (IS). (A) Blank serum: netilmicin (IS) 5.8 μg/ml. (B) Spiked serum: astromicin 6.3 μg/ml.

at a serum concentration of 5.8 μg/ml. Typical chromatograms of serum containing astromicin and blank serum are shown in Fig. 5. Chromatogram A is the blank serum and B is the astromicin-spiked serum (6.3 μg/ml). The calibration curve is linear from 0.1 to 6.3 μg/ml. The equation obtained from linear regression analysis is $Y = 0.244X - 7.75 \cdot 10^{-3}$ ($r = 0.9997$). The C.V. for astromicin was 3.1% at a concentration of 1.6 μg/ml ($n = 6$) (Table I).

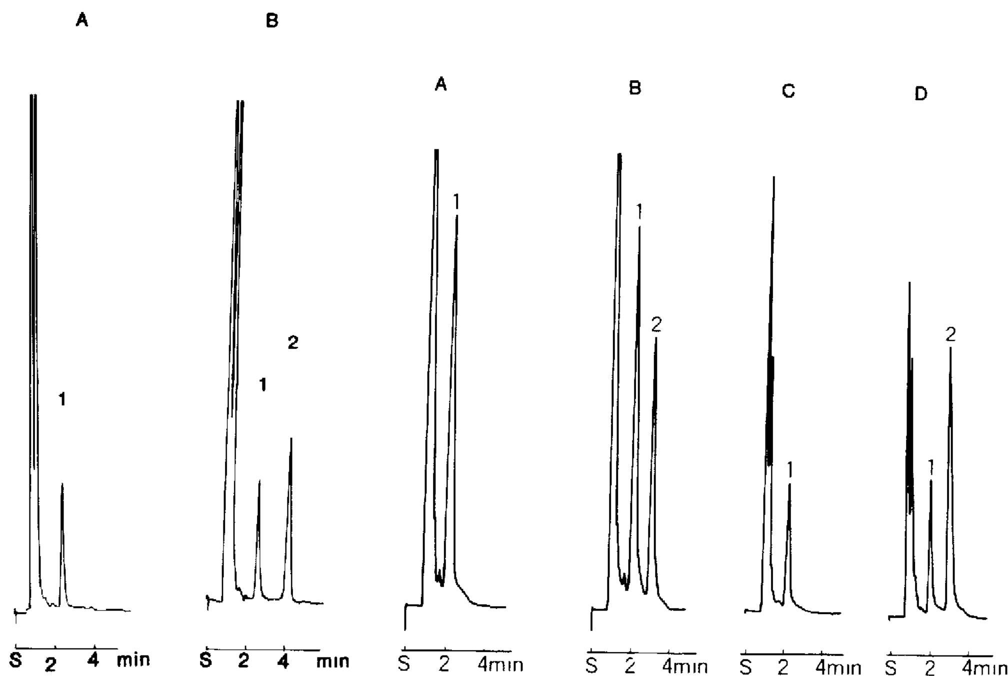


Fig. 6. Chromatograms of serum spiked with micronomicin and blank serum. Peaks: 1 = sisomicin (IS), 2 = micronomicin. (A) Blank serum: sisomicin (IS) 40 μg/ml. (B) Spiked serum: micronomicin 7.5 μg/ml. Mobile phase: 1.0 *M* sodium perchlorate (anhydrous); other conditions are the same as in Fig. 3; injected volume 50 μl.

Fig. 7. Chromatograms of serum and urine after the administration of micronomicin to a patient. Peaks: 1 = sisomicin (IS), 2 = micronomicin. (A) Blank urine: sisomicin (IS) 40 μg/ml. (B) Sample urine. (C) Blank serum: sisomicin (IS) 40 μg/ml. (D) Sample serum. Injected volumes: 150 μl (A, B) and 50 μl (C, D). Other analytical conditions are the same as in Fig. 6.

Experiments concerning sisomicin to astromicin were carried out using the Zorbax C_8 column; analytical conditions are described in the legends to the relevant figures.

The column was changed to Zorbax ODS for the analysis of micronomicin. Sisomicin was chosen as the internal standard from the other aminoglycosides, and it was spiked in serum at a concentration of 40 μg/ml. Fig. 6 shows the chromatograms of micronomicin-spiked serum (B, 7.5 μg/ml) and the blank (A). Fig. 7 shows chromatograms of serum and urine after the administration of micronomicin to a patient. Chromatograms A and C are blank urine and serum, B and D are sample urine and serum, respectively. The linearity of the calibration curve was from 1.0 to 30 μg/ml. The C.V. was 1.9% at a concentration of 2.5 μg/ml (n = 6) (Table I). Linear regression analysis of this curve resulted in the equation $Y = 0.082X - 11.7 \cdot 10^{-3}$ ($r = 0.9998$).

Linearity of the calibration curves which cover the clinically observable ranges of each aminoglycoside, and the low C.V. values indicate that the determination of aminoglycosides in serum by this method is accurate and reproducible. By using internal standards chosen from other aminoglycosides, manipulation errors during sample preparation, or injection errors, are avoided

and accurate determination of aminoglycoside antibiotics in serum is attained even when many samples are treated at the same time.

Sample preparation was examined by various methods such as organic solvent deproteinization, the ion-exchange resin method, and short-column extraction with CM-Sephadex (C-25). Short-column extraction with CM-Sephadex based on Anhalt's method [3] resulted in the highest recovery.

In Anhalt's method, 0.2 *M* sodium sulphate was used as the initial buffer, but its application to the analysis of netilmicin resulted in two split peaks in our experiments. This phenomenon was avoided by changing the initial buffer to 0.4 *M* sodium acetate, but the exact mechanism is unknown (see Fig. 2).

During the experiments for micronomicin, 400 μl of alkaline buffer were added to elute aminoglycoside from the short column. Another 400 μl of alkaline buffer were then added to the short column and the 800 μl of eluate collected were used as sample. This method gave a recovery rate (almost 100%) higher than that obtained by washing the short column once to elute (around 90% recovery: data not shown). But we carried out experiments following the procedure in Fig. 2, in order to avoid sample dilution and complicated procedures. Deproteinization and extraction were performed simultaneously in a short column, so the procedure was easy and fast. Besides that, because of the separation selectivity of reversed-phase ion-pair chromatography, other aminoglycoside antibiotics hardly interfere with the determination.Total analysis time including sample preparation and chromatographic separation was less than 30 min.

In conclusion, the advantages of this method are speed, accuracy, and good reproducibility. Therefore, this method is applicable for therapeutic drug monitoring.

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