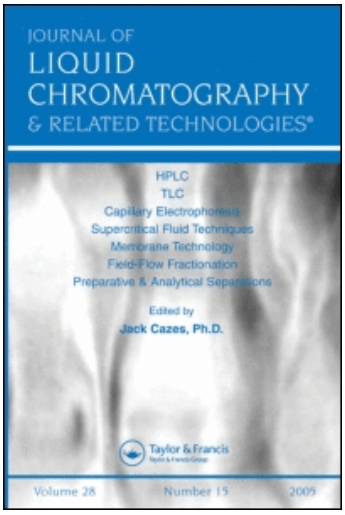

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Micro-Scale Method for Determination of Tobramycin in Serum Using High-Performance Liquid Chromatography

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To cite this Article Kubo, Hiroaki , Kinoshita, Toshio , Kobayashi, Yoshie and Tokunaga, Ken(1984) 'Micro-Scale Method for Determination of Tobramycin in Serum Using High-Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 7: 11, 2219 — 2228
To link to this Article: DOI: 10.1080/01483918408068871
URL: http://dx.doi.org/10.1080/01483918408068871

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(11), 2219-2228 (1984)

MICRO-SCALE METHOD FOR DETERMINATION OF TOBRAMYCIN IN SERUM USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A rapid, simple, accurate, and micro-scale method for the determination of tobramycin, sisomicin and netilmicin in serum using high-performance liquid chromatography has been developed. The method is sensitive to 0.3 µg/ml using only 20 µl of serum. The serum is deproteinized with methanol containing an internal standard: sisomicin for the tobramycin, netilmicin for the sisomicin, and sisomicin for the netilmicin. After centrifugation, a counter-ion reagent is added to the supernatant, then an aliquot of the solution is injected into the chromatograph. Tobramycin, sisomicin and netilmicin are separated by reversed-phase, ion-pair chromatography and detected by fluorescence using continuous-flow, post-column derivatization with o-phthalaldehyde. For the tobramycin, within-run and day-to-day variation was below 2.5%. Correlation of this method with microbiological assay and homogeneous enzyme immunoassay was good.

INTRODUCTION

Tobramycin is an aminoglycoside antibiotic used for the treatment of serious gram-negative infections. As with other aminoglycoside antibiotics, tobramycin has a narrow therapeutic range and exerts nephro- and oto-toxicity. Therefore, monitoring

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of tobramycin levels in serum is necessary for safe and effective therapy. The advantages and disadvantages of various methods for the determination of tobramycin were reviewed by Maitra et al (1) and Nilsson-Ehle (2). Some of these methods use fluorescence detection with pre- or post-column derivatization of tobramycin. Other methods employ ultraviolet absorption detection with pre-column derivatization of tobramycin. All these methods require time-consuming pretreatment such as solvent or column extraction of tobramycin in serum and require a large volume(50 µl-250 µl) of serum. We have previously reported a micro determination of gentamicin in serum by high-performance liquid chromatography (3). This report describes a modified procedure, using an internal standard, as well as the applicatin of the method to the determination of tobramycin in serum. The values determined by this method were compared with those by a microbiological assay and those by a homogeneous enzyme immunoassay. This method is also applicable for the analysis of sisomicin and netilmicin.

MATERIALS AND METHODS

Chemicals and Reagents

Tobramycin sulfate (manifested potency, 964 µg/mg) was obtained from Shionogi Co. (Osaka, JAPAN). Sisomicin sulfate (646 µg/mg) and netilmicin sulfate (628 µg/mg) were supplied by Essex Nippon K.K. (Shiga, JAPAN). o-Phthalaldehyde was purchased from Nakarai chemical Ltd.(Kyoto, JAPAN); sodium octanesulfonate and disodium 1,2-ethanedisulfonate were from Kanto Chemical Co.(Tokyo, JAPAN). De-ionized and distilled water was used. Methanol used was of liquid chromatographic grade. All other chemicals were of reagent grade.

Counter-ion reagent was prepared to contain 0.2 M disodium 1,2-ethanedisulfonate and 0.01 M sodium octanesulfonate in water, adjusted to pH 2.5 with acetic acid.

Mobile phase was prepared to contain 0.1 M disodium 1,2-ethanedisulfonate and 0.005 M sodium octanesulfonate in a water-methanol mixture (64:36, v/v), adjusted to pH 3.5 with acetic acid.

o-Phthalaldehyde reagent was prepared according to the method of Anhalt and Brown (4).

Methanol solutions of internal standard were prepared by dissolving 0.5 mg of sisomicin sulfate or netilmicin sulfate in 5 µl of 70% perchloric acid and making up to 200 ml with methanol.

Apparatus and Chromatographic Conditions

The chromatographic system consisted of a Solvent Delivery System 6000A, a Universal Injector U6K and a Radial-Pak C_{18} column (10 cm x 8 mm I.D., particle size 10 µm) with radial compression module (RCM-100), all from Waters Assoc.(Milford, Mass, U.S.A.), was used. The flow-rate of mobile phase was maintained at 2.0 ml/min. The o-phthalaldehyde reagent was delivered with a Model 3000 pump (Waters Assoc.) at a flow rate of 0.8 ml/min to the column effluent via a mixing T-piece. A reaction coil consisting of a stainless steel tube (5 m x 0.25 mm I.D.) was placed between the mixing T-piece and a detector. As a detector, a Model S-FL-330 fluorometer(Soma Optics Co., Ltd. Tokyo, JAPAN) equipped with a FL4BLB lamp (energy maximun at 365 nm, excitation), a 440 nm cutoff filter (emission) and a 70 µl quartz flow-cell was used. Chromatography was performed at room temperature.

Procedure

Twenty µl serum in a 1.5-ml tapered polypropylene centrifuge tube was vortex-mixed with 100 µl of the methanol solution of internal standard for a few seconds. The tube was then centrifuged at 7,800g in a Model H-25FL centrifuge (Kokusan Co., Tokyo, JAPAN) for 1 min. Then 160 µl of the counter-ion reagent were directly added to the tube, which was vortex-mixed again for a few seconds and centrifuged at 7800g for 1 min. A 200 µl aliquot of this mixture was injected into the chromatograph.

Standard sera spiked with various known amounts of tobramycin (potency, 1.85 - 14.83 μg/ml), sisomicin(potency, 1.66 - 13.33 μg/ml) or netilmicin(potency, 1.63 -13.06 μg/ml) were prepared and treated in an identical fashion to that described above. The peak height ratios between tobramycin and sisomicin as internal standard, sisomicin and netilmicin as internal standard, or netilmicin and sisomicin as internal standard were calculated to construct a calibration curve. Tobramycin in patient sera was determined in duplicate and the results averaged.

Homogeneous enzyme Immunoassay

Homogeneous enzyme immunoassay was performed by using commercially available kits (Emit®-amd, Syva Co., Palo Alto, CA., U.S.A.).

Microbiological Assay

Microbiological assay was performed by a paper disc-diffusion technique, using antibiotic medium No. 5 (Difco Labaratories, Detroit, MI., U.S.A.) and Bacillus subtilis ATCC 6633 as a test organism.

RESULTS

The chromatogram obtained from a standard mixture of tobramycin, sisomicin and netilmicin dissolved in mobile phase is shown in Fig. 1. The retention times for tobramycin, sisomicin and netilmicin are 9.0, 12.5 and 17.0 min, respectively. The antibiotics are well-resolved chromatographically. Figure 2 shows chromatograms obtained from the antibiotics-spiked serum without and with a counter-ion. The chromatogram obtained from the supernatant of methanol-treated serum gave different results from that obtained from the standard mixture of antibiotics. The solution of methanol-treated serum containing the counter-ion reagent resulted in a chromatogram that was virtually identical with the chromatogram obtained from the standard mixture. Figure

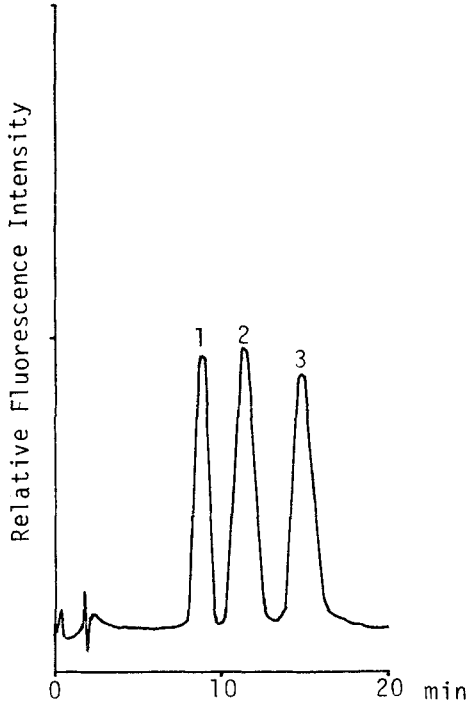

Figure 1. Chromatogram of standard mixture.
1.tobramycin, 2.sisomicin, 3.netilmicin.

3 shows typical chromatograms obtained from tobramycin-free serum that was treated with the methanol and from patient serum containing tobramycin that was treated with the methanol solution of internal standard. The tobramycin-free serum showed no peaks that would interfere with the determination of tobramycin. Linear regression analysis of the calibration curve of tobramycin(1.85 -14.83 μg/ml) yielded the equation, Y=0.101X - 0.057(r=1.000). The peak height ratios(Y) were related to serum tobramycin concentration (X) with high linearity. Those of the calibration curve of sisomicin(1.66- 13.33 μg/ml) and netilmicin(1.63-13.06 μg/ml) were Y=0.136X + 0.002(r=0.999), and Y=0.100X + 0.003(r=0.993), respectively. The limit of detection for these antibiotics is 0.3

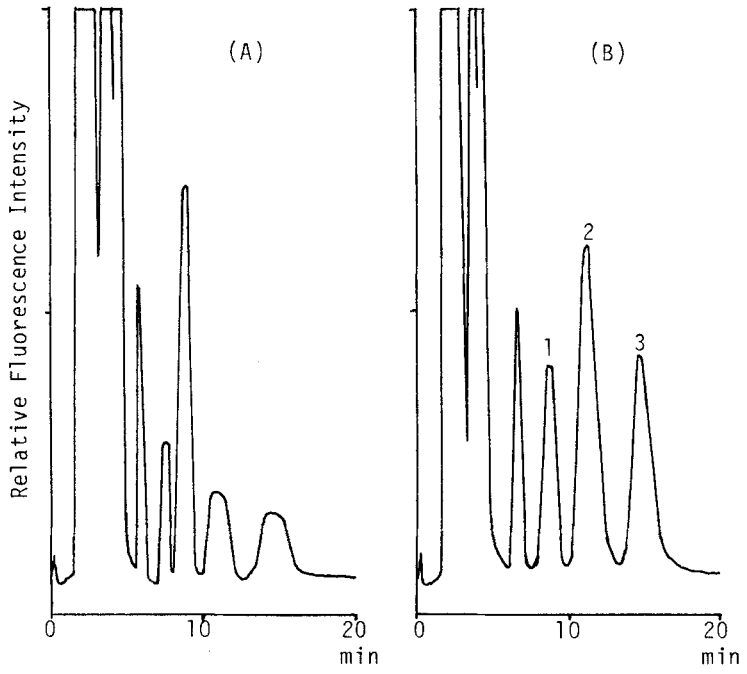

Figure 2. Chromatograms of antibiotics-spiked serum (A):without and (B):with a counter-ion.
1.tobramycin, 2.sisomicin, 3.netilmicin.

μg/ml. This sensitivity is high enough for routine clinical purposes. In order to estimate the analytical recovery, an aqueous solution of tobramycin (5.5 μg/ml) and a tobramycin-added serum (5.5 μg/ml) were analyzed and their peak height ratios compared. The recovery was excellent (97.5-102.5%).

Within-run and day-to-day precision were determined on two serum pools containing tobramycin 3.5 μg/ml and 10.3 μg/ml. As Table 1 shows, the coefficient of within-run variation was less than 2.2% and that of day-to-day variation was less than 2.5%.

The results obtained by the proposed method on patient sera were compared with those by a microbiological assay and those by a homogeneous enzyme immunoassay (Fig. 4). The correlation coefficients were 0.956 and 0.988, respectively. The correlation between the three methods is good.

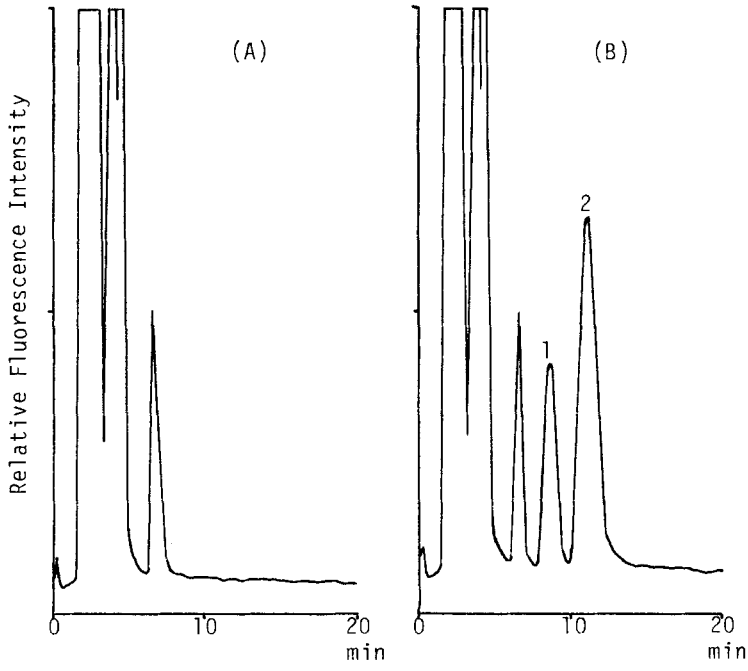

Figure 3. Chromatograms of (A):tobramycin-free serum and (B): tobramycin-containing patient serum.
1.tobramycin, 2.sisomicin(internal standard).

TABLE 1

Coefficient of Variation(%) for Analyses of Tobramycin in Serum

Concentration(μg/ml)	Within-run(n=10) (%)	Day-to-day(n=10) (%)
3.5	2.2	2.5
10.3	2.0	2.3

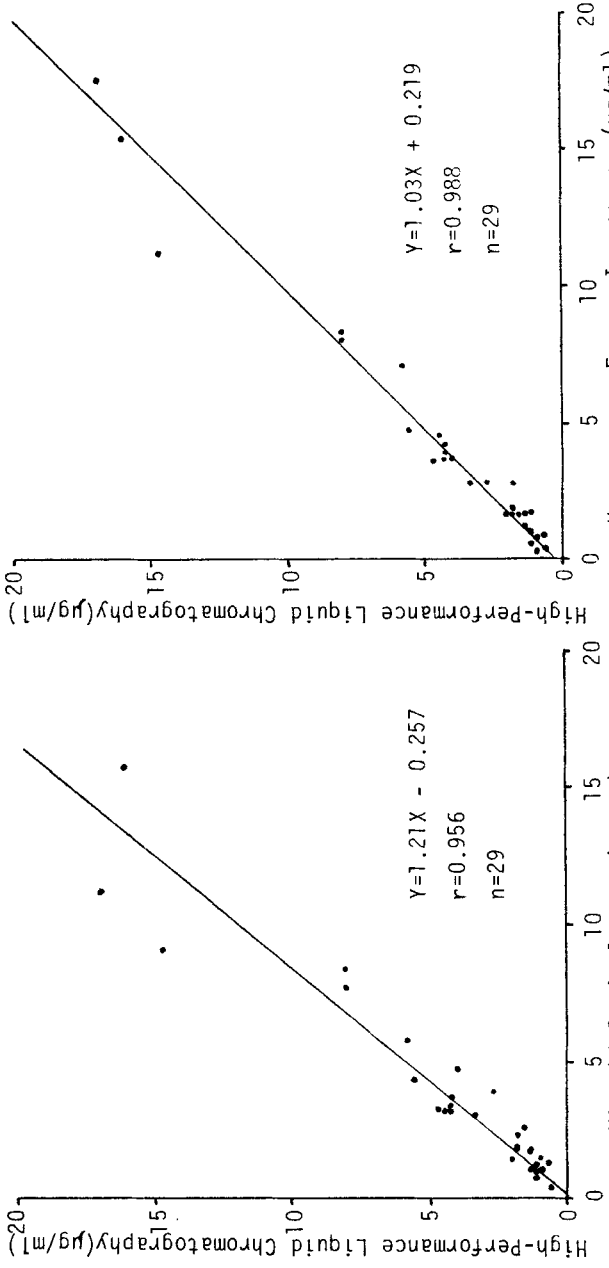

Figure 4. Comparisons with the microbiological assay and with the homogeneous enzyme immunoassay.

DISCUSSION

As we reported previously, precipitation of serum proteins with methanol was essentially complete (3,5). The methanol content in the supernatant of methanol-treated serum is higher than that in the mobile phase solvent and a tobramycin in the supernatant could not form an ion-pair with a counter-ion in the mobile phase during separation on the column. Therefore, it is necessary that the supernatant be diluted with the counter-ion reagent prior to injection into the chromatograph.

Sisomicin sulfate and netilmicin sulfate are very poorly soluble in methanol, but sisomicin perchlorate and netilmicin perchlorate are very soluble in methanol. Therefore, the methanol solutions of internal standard were prepared by dissolving sisomicin sulfate and netilmicin sulfate with perchloric acid and diluting with methanol.

Reversed-phase, ion-pair chromatography was effective in eliminating the interference caused by serum components such as amines, amino acids and small peptides which would form fluorescent products with the o-phthalaldehyde. Tobramycin, sisomicin and netilmicin were separated from these serum components by the use of sodium octanesulfonate. Because tobramycin, sisomicin and netilmicin have five amino groups, their ion-pairs were held more strongly on the reversed-phase column than the ion-pairs of other serum components which have fewer amino groups. By the addition of disodiumn 1,2-ethanedisulfonate, the resolution of tobramycin , sisomicin and netilmicin which have subtle molecular differences could be increased. The retention times for tobramycin, sisomicin, netilmicin and these components could also be shortened.

The method described here simplifies the sample pretreatment greatly by avoiding tedious steps such as solvent or column extraction, and reduces the analysis time significantly. The method is sensitive and accurate. Each analysis requires only 20 µl of serum, and the result can be obtained in less than 15 min for the tobramycin.

This method can be used for pharmacokinetic studies and for routine therapeutic monitoring of pediatric patients.

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