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Note

Micro determination of gentamicin in serum by high-performance liquid chromatography

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Gentamicin is a widely used aminoglycoside antibiotic for treatment of serious infections with gram-negative bacilli. It has, however, a narrow therapeutic range and exerts adverse side-effects of nephrotoxicity and ototoxicity. Therefore, monitoring of gentamicin levels in serum is necessary for the best therapeutic effects.

Recently, several methods using high-performance liquid chromatography (HPLC) have been developed for determination of the three major components of gentamicin in serum or plasma [1—11]. Some of these methods have used fluorescence detection with pre-column or post-column derivatization of gentamicin [1—8]. Others have employed ultraviolet absorption detection with pre-column derivatization of gentamicin [8—10]. All these methods require time-consuming sample pretreatment and a large volume of sample for the determination of gentamicin in serum or plasma.

This report describes a simple micro-scale method for the analysis of gentamicin in serum.

EXPERIMENTAL

Reagents

Gentamicin sulfate (manifested potency, 552 μg/mg) was obtained from Shionogi (Osaka, Japan), *o*-phthalaldehyde was from Nakarai Chemicals

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(Kyoto, Japan), Brij-35 from Wako Pure Chemicals (Osaka, Japan), sodium octanesulfonate from Kanto Chemicals (Tokyo, Japan) and disodium 1,2-ethanedisulfonate from Tokyo Kasei Kogyo (Tokyo, Japan). De-ionized distilled water was used throughout this investigation. Acetonitrile and methanol were 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 about 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—acetonitrile mixture (85:15, v/v) adjusted to about pH 3.5 with acetic acid.

o-Phthalaldehyde (OPA) reagent was prepared according to the method of Anhalt and Brown [5].

Apparatus and chromatographic conditions

Fig. 1 shows the flow diagram of the chromatographic system. A Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.) was used to deliver the mobile phase (1.5 ml/min) and a Model U6K universal injector (Waters Assoc.) was used for injection of samples. A μBondapak C_{18} column (particle size 10 μm, 30 cm × 3.9 mm I.D., Waters Assoc.) was used for the analyses. The OPA reagent was delivered at a flow-rate of 0.8 ml/min to the column effluent via a mixing T-piece with a Model 3000 pump (Waters Assoc.). A reaction coil consisting of a Teflon tube (5 m × 0.25 mm I.D.) was placed in a 50°C water-bath between the mixing T-piece and a detector. A Model S-FL-330 fluorometer (Soma Optics, Tokyo, Japan) equipped with a FL4BLB lamp (energy maximum at 365 nm, excitation), a 440-nm cut-off filter (emission) and a 70-μl quartz flow-cell was used to detect the fluorescent product. Detector signals were recorded with a Model VP6621A recorder (Matsushita Communication Industrial, Osaka, Japan).

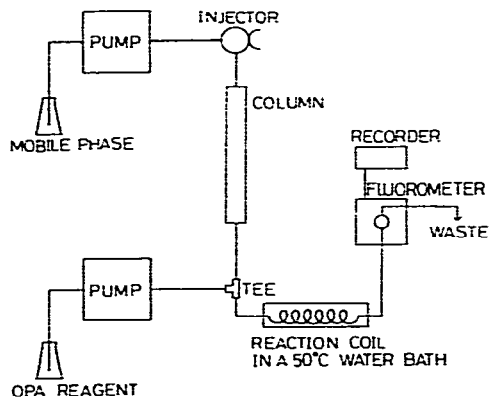

Fig. 1. Flow diagram of the chromatographic system used for separation and fluorescence detection.

Procedure

A 20-μl serum sample in a 1.5-ml tapered polypropylene centrifuge tube was vortex-mixed with 100 μl of methanol for a few seconds, then centrifuged at about 7800 *g* in a Model H-25FI centrifuge (Kokusan, Tokyo, Japan) for 1 min. Then 200 μl of the counter-ion reagent were directly added to the supernatant, which was vortex-mixed again for a few seconds and centrifuged at about 7800 *g* for 1 min. A 240-μl aliquot of the supernatant containing the counter-ion reagent was injected into the chromatograph.

Standard serum samples supplemented with various concentrations of gentamicin (potency 2.7—16.5 μg/ml) were prepared for chromatographic analysis. Peak area measurements were used to construct the standard curve.

Every sample was analysed in duplicate. The sum of the areas of gentamicin components was measured and averaged.

RESULTS

It was observed frequently that gentamicin-containing sera after deproteinization gave inconsistent chromatograms. To avoid this problem, a counter-ion reagent was added to the protein-free supernatant prior to injection of samples into the chromatograph. It was found that the chromatograms obtained after this treatment were virtually identical with those obtained with standard gentamicin dissolved in mobile phase.

Typical chromatograms of gentamicin components from a patient's serum and of serum blanks with and without antibiotic supplementation are shown in Fig. 2. Individual components were identified by their retention times. The serum blanks so far examined showed no interfering peaks. A small peak appearing between gentamicin C_2 and C_1 was observed with standard gentamicin samples as well as with extracts of patient's sera. The peak presumably corresponds to a minor component of a gentamicin C complex.

Linear regression analysis of the standard curve from 2.7 to 16.5 μg/ml yielded the following equation: $y = 0.792x - 0.126$ ($r = 0.999$). A good linear relationship was obtained between peak areas and gentamicin concentrations in

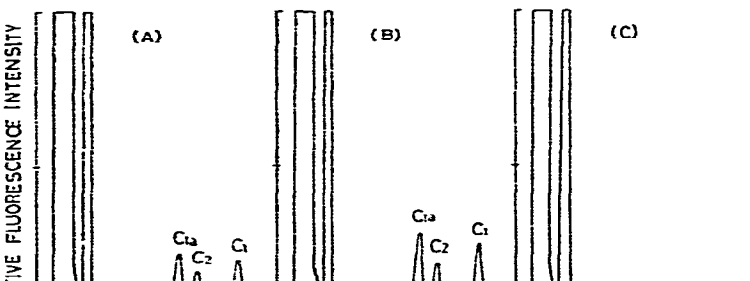

Fig. 2. Chromatograms of gentamicin components: (A) a patient serum; (B) a serum blank supplemented with 10.0 μg of gentamicin per ml; (C) a serum blank. C_{1a} = gentamicin C_{1a}, C_2 = gentamicin C_2, C_1 = gentamicin C_1.

serum in the range studied, even though no internal standard was used in the method. The limit of sensitivity of this method is about 0.5 μg/ml.

Analytical recovery of gentamicin added to serum was determined by comparing the peak areas from a serum sample containing 5.5 μg of gentamicin per ml with the results obtained from an aqueous standard of the same concentration. The recovery was between 97 and 103%.

The precision of this method is summarized in Table I. Within-run precision was estimated by analyzing ten aliquots of each of two serum pools containing gentamicin in concentrations of 5.5 and 11.0 μg/ml, respectively. Day-to-day precision was estimated by analyzing an aliquot of each pool for ten days. The within-run precision was less than 2.5% and day-to-day precision was less than 3.2%.

The results obtained by the proposed method were compared with those obtained using a conventional microbiological method (Fig. 3). The correlation coefficient was 0.934.

TABLE I

PRECISION STUDIES

Gentamicin concentration in serum (μg/ml)	Coefficient of variation (%)	
	Within-run (n = 10)	Day-to-day (n = 10)
5.5	2.5	3.2
11.0	2.0	2.3

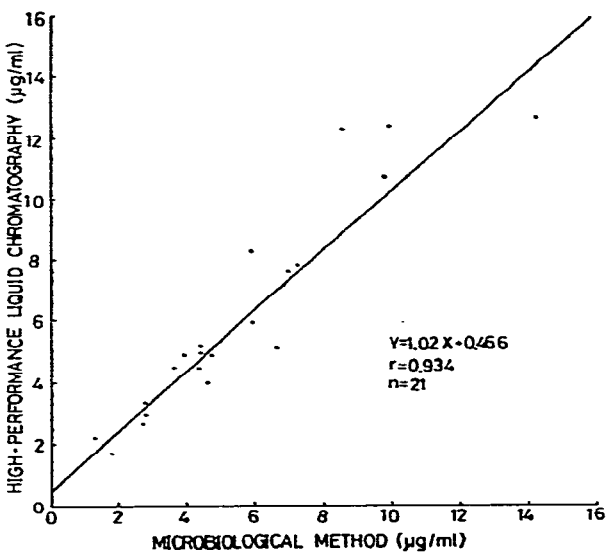

Fig. 3. Comparison with the microbiological method.

DISCUSSION

As reported previously [12], at a serum/methanol ratio of 1:5 (v/v), deproteinization was essentially complete. The counter-ion reagent could be

added directly to the supernatant without disturbing the precipitate of serum proteins denatured with methanol which adhered tightly to the bottom of the tapered polypropylene centrifuge tubes after centrifugation. No serum proteins were detected by the Lowry method in the supernatant containing the counter-ion reagent.

The method described above simplifies sample pretreatment greatly by avoiding tedious steps such as solvent extraction [1, 4, 6—11] and column chromatography [2, 3, 5] and reduces the analysis time significantly.

Ion-pair chromatography was effective in eliminating the interference caused by serum components such as amino acids, small peptides and amines, which form fluorescent products with the OPA reagent during gentamicin determination. In the presence of sodium octanesulfonate the retention time of gentamicin with five amino groups was longer than of those with fewer amino groups. The addition of disodium 1,2-ethanedisulfonate increased the resolution of the three major components of gentamicin with subtle molecular differences. The attempts to use sodium sulfate [7], potassium methanesulfonate or sodium methylsulfate instead of disodium 1,2-ethanedisulfonate resulted in the appearance of an interfering peak and poor resolution of the gentamicin components.

This procedure for the determination of gentamicin in serum appears to be potentially applicable to the determination of other aminoglycosides used in clinical treatment such as kanamycin, tobramycin and amikacin. Serum volumes of as little as 20 μl provide a further advantage for monitoring pediatric patients.

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