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Note

Improved microdetermination of gentamicin and sisomicin in serum by high-performance liquid chromatography with ultraviolet detection

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A high-performance liquid chromatographic (HPLC) method for the determination of gentamicin in serum was previously reported [1]. In the present paper we describe a modified procedure, using an internal standard, as well as the application of the method to the determination of sisomicin in serum. To our knowledge no other chromatographic determination of sisomicin in serum has been reported. The HPLC determination of sisomicin is compared with a microbiological assay. An in vivo experiment in which the sisomicin concentration is determined following intramuscular administration of the drug is reported.

MATERIALS AND METHODS

Chemicals and reagents
The water used was demineralized. 1-Fluoro-2,4-dinitrobenzene (FDNB), tris(hydroxymethyl)aminomethane (Tris) and acetic acid were p.a. grade from Merck (Darmstadt, G.F.R.). Acetonitrile, "zur Synthese", was also from Merck.
The preparations for injections — Garamycin® (Essex, Heist-op-den-Berg, Belgium), Obracin® (Eli Lilly, St.-Cloud, France) and Extramycin® (Bayer, Leverkusen, G.F.R.) — contained the equivalents of 40 g/l gentamicin, 40 g/l tobramycin and 50 g/l sisomicin, respectively. The gentamicin components C_1, C_{1a} and C_2 were obtained as their sulphate salts (by courtesy of Schering, Bloomsfield, NJ, U.S.A.). Sisomicin sulphate, lot Pt 489 851, standard substance was by courtesy of Bayer Nederland (Mijdrecht, The Netherlands). All antibiotic concentrations were calculated relative to potency.

Pooled human serum from ambulant patients was frozen and stored at —18°C within three days of collection.

Stoppered polypropylene centrifuge tubes of 1.5 ml capacity, and ampoules of 0.5 ml capacity were also used.

Chromatographic conditions

The chromatographic instrumentation was described previously [1].

The mobile phase was prepared by mixing 300 ml of water (filtered through a 0.2-μm filter) with 700 ml of acetonitrile (filtered through a 0.2-μm filter) and 1 ml of acetic acid, and deaerated ultrasonically. The flow-rate was 3.0 ml/min. A μBondapak C_{18} column (30 cm × 3.9 mm I.D., particle size 10 μm) was used (Waters Assoc., Milford, MA, U.S.A.). Chromatography was performed at room temperature. Ultraviolet detection was made at 365 nm. The detector signal was recorded at two different attenuation settings.

Procedures

Procedure A: derivatization of aminoglycosides. Dispense into a centrifuge tube 50 μl of serum or aqueous aminoglycoside solution; add 50 μl of a solution containing 20 g/l Tris in water, also containing the equivalent of 160 mg/l tobramycin (the internal standard), and vortex. Add 200 μl of acetonitrile and vortex. Centrifuge, in the case of serum samples, at 2500 g for 5 min. Transfer 200 μl of the supernatant into an ampoule, add 20 μl of FDNB in acetonitrile (170 g/l) and close the ampoule. Place in a water-bath at 80°C for 45 min. Inject 150 μl into the chromatograph.

Procedure B: in vivo experiment and bioassay comparison study. A healthy 70-kg volunteer received 75 mg of sisomicin by intramuscular injection. Blood samples were collected at regular time intervals, and the serum was separated and stored at —18°C. Sisomicin standards in the range 0—4 mg/l were prepared by adding appropriate quantities of aqueous solutions of sisomicin sulphate standard substance to pooled serum. Standards and serum samples were analyzed in duplicate in one run according to procedure A.

The serum samples, obtained from the in vivo experiment, were also analyzed in duplicate by a microbiological assay, against the same sisomicin standard substance. This bioassay was carried out by the National Institute of Public Health (RIV), Department of Chemotherapy, Utrecht, The Netherlands, using an agar well diffusion technique. The antibiotic medium was DST (Oxoid, Basingstoke, Great Britain). The test organism was *Staphylococcus aureus* Alkmaar, a strain resistant to penicillin, cefalothin, sulfonamide antibiotics, chloramphenicol and streptomycin but susceptible to aminoglycoside antibiotics. The seeded agar was incubated overnight at 37°C and the diameter of the zones of growth inhibition was measured. Zone sizes were plotted against the log values of the concentrations of the standards to obtain a straight line.

RESULTS AND DISCUSSION

The aminoglycoside antibiotics sisomicin and tobramycin and the three main components of the gentamicin complex (gentamicin C_1, C_{1a} and C_2) are closely related (see Table I). These aminoglycosides have the same number of amino

TABLE I

RETENTION OF 2,4-DINITROPHENYL DERIVATIVES OF AMINOGLYCOSIDES RELATIVE TO GENTAMICIN C_{1a}, AND FUNCTIONAL GROUPS

	$RRT_{C_{1a}}$*	Expected No. of derivatized amino groups	No. of hydroxyl groups	No. of methyl groups
Tobramycin	0.47	5	5	0
Sisomicin	0.97	5	3	2
Gentamicin C_{1a}	1	5	3	2
Gentamicin C_2	1.15	5	3	3
Gentamicin C_1	1.15	5	3	4

*Retention time relative to gentamycin C_{1a}.

groups, and there is evidence that all five amino groups of these antibiotics are derivatized by FDNB [1]. Tobramycin has two more hydroxyl groups than the other substances; consequently the tobramycin—dinitrophenyl derivative is the most polar of these derivatives, and has the shortest retention time in this reversed-phase system. The influence of the number of methyl groups on the retention behaviour is much less than the influence of the number of hydroxyl groups. The gentamicin C_2 derivative contains one more methyl group (C—C bond) than the gentamicin C_{1a} derivative. This gives rise to a slightly longer retention time for the gentamicin C_2 derivative due to the less polar nature of the more methylated derivative. In the gentamicin C_1 derivative, one further methyl group is present (N—C bond), but no further increase in retention time was observed.

Sisomicin is a dehydro analog of gentamicin C_{1a}. The retention behaviour of the derivatives of these two compounds is very similar.

With the chromatographic system used, the tobramycin derivative is not completely resolved from peaks near the solvent front. At a high concentration of tobramycin (160 mg/l) the contribution of the blank to the peak height of the tobramycin derivative was found to be only 4% (see Fig. 1). By monitoring the effluent of the column at two different attenuation settings, tobramycin at this high concentration could be used as internal standard in the determination of gentamicin and sisomicin in serum.

Recovery

Recovery of sisomicin from the deproteinization step was estimated by spiking blank serum samples with the same amount of aminoglycoside before and after the addition of acetonitrile; the same procedure was followed for determining the recovery of tobramycin.

The recovery of sisomicin, measured at 4 mg/l, was 84% (S.D. = 6%; $n = 6$). The recoveries of gentamicin C_{1a} and gentamicin $C_1 + C_2$, measured under the same conditions, were determined previously and found to be 83% and 84% respectively [1]. The recovery of tobramycin, measured at 160 mg/l, was 64% (S.D. = 2%; $n = 6$).
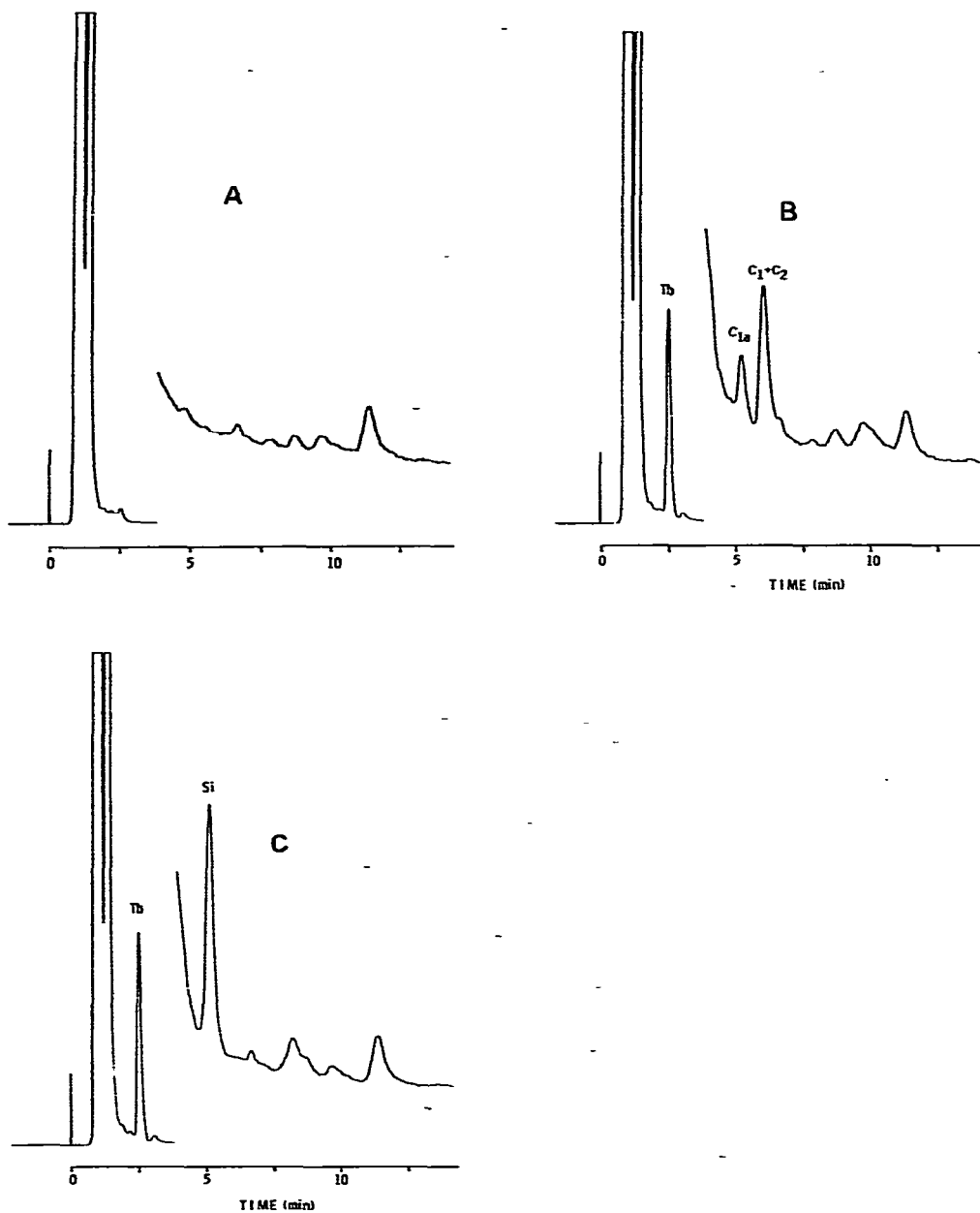
Fig. 1. HPLC of serum samples. Detector settings: 0—3.8 min, 1.0 a.u.f.s.; 3.8—14 min, 0.01 a.u.f.s. Chromatogram A was obtained from 50 μl of blank serum. No internal standard was added. Chromatogram B was obtained from 50 μl of blank serum, spiked to a concentration of 4 mg/l gentamicin with tobramycin as internal standard added. Chromatogram C was obtained from 50 μl of blank serum, spiked to a concentration of 4 mg/l sisomicin with tobramycin as internal standard added. Tb = tobramycin derivative; Si = sisomicin derivative; C_{1a} = gentamicin C_{1a} derivative; C_1+C_2 = gentamicin C_1 and gentamicin C_2 derivatives (not separated).

Other authors also report low recoveries if serum containing a high concentration of tobramycin is deproteinized with acetonitrile [2]. However, as tobramycin is always added at the same concentration, this lower recovery still permits the use of 160 mg/l tobramycin as the internal standard, because the recovery was found to be sufficiently reproducible.

Precision, linearity and sensitivity

Sera were spiked with different quantities of gentamicin, or sisomicin, to obtain concentrations ranging from 0.5 to 16 mg/l, and were analyzed in one run according to procedure A. The results are summarized in Tables II, III and IV. As is apparent from the data provided, the calibration curves are straight lines in the concentration range studied. The 95 per cent confidence interval of the intercept of the calibration line for sisomicin includes zero, so this calibration line passes through the origin. The calibration lines for gentamicin C_{1a} and gentamicin $C_1 + C_2$ have intercepts that do not pass through the origin at this level of confidence. However, the intercepts are small and can be neglected for all practical purposes. The sensitivity of the method is sufficient to allow the determination of sisomicin and gentamicin at concentrations in, and well below, the therapeutic range using 50-μl serum samples.

TABLE II

PEAK HEIGHT RATIOS (PHR) OF DERIVATIZED GENTAMICIN COMPONENTS TO INTERNAL STANDARD (TOBRAMYCIN) AND COEFFICIENTS OF VARIATION (C.V.) OBTAINED WITH SERUM SAMPLES (50 μl) CONTAINING 0.5—16 mg/l GENTAMICIN

Analyses at each concentration were performed in six-fold.

Gentamicin C_{1a}				Gentamicin C_1 and C_2		
Concentration (mg/l)	PHR*	PHR/con-centration	C.V. (%)	PHR*	PHR/con-centration	C.V. (%)
0.5	0.055	0.111	10	0.110	0.220	2
1	0.095	0.095	4	0.214	0.214	3
2	0.192	0.096	2	0.417	0.209	3
4	0.384	0.096	1	0.850	0.213	2
8	0.745	0.093	1	1.666	0.208	1
16	1.462	0.091	3	3.268	0.204	2

*Peak heights of the tobramycin and gentamicin derivatives based on detector settings of 1.0 a.u.f.s. and 0.01 a.u.f.s., respectively.

In vivo experiment and bioassay comparison study

For the HPLC assay, the working calibration curve obtained from the standard sera gave an intercept with a 95 per cent confidence interval that included the origin, so the working calibration curve was recalculated forcing this line through the origin [3]. The concentrations in the serum samples, obtained in the in vivo experiment, were calculated from this calibration curve. The serum concentration—time curve for sisomicin, obtained by the HPLC assay, is shown in Fig. 2. The comparison of the values obtained from the

TABLE III

PEAK HEIGHT RATIOS (PHR) OF DERIVATIZED SISOMICIN TO INTERNAL STANDARD (TOBRAMYCIN) AND COEFFICIENTS OF VARIATION (C.V.) OBTAINED WITH SERUM SAMPLES (50 μl) CONTAINING 0.5—16 mg/l SISOMICIN

Analyses at each concentration were performed in six-fold.

Concentration (mg/l)	PHR*	PHR/concentration	C.V. (%)
0.5	0.162	0.324	9
1	0.291	0.291	4
2	0.539	0.270	4
4	1.052	0.263	3
8	2.109	0.264	4
12	3.283	0.272	5
16	4.255	0.266	2

*Peak heights of the tobramycin and sisomicin derivatives based on detector settings of 1.0 a.u.f.s. and 0.01 a.u.f.s., respectively.

TABLE IV

STANDARD CURVES FOR AMINOGLYCOSIDES IN SERUM

Aminoglycoside	Range (mg/l)	n*	Equation**		
			Slope	Intercept ± 95 per cent confidence interval	r
Gentamicin C_{1a}	0.5—16	36	0.091	0.012 ± 0.008	0.9993
Gentamicin C_1+C_2	0.5—16	36	0.204	0.017 ± 0.014	0.9996
Sisomicin	0.5—16	42	0.267	0.011 ± 0.037	0.9986

*Number of determinations.
**Estimated by linear least-squares regression analysis.

determinations by the HPLC method and the microbiological method is also shown in Fig. 2. The correlation found is comparable, or better, than those reported in other studies in which chromatographic assays for aminoglycoside antibiotics in human serum are compared to a microbiological assay [4—9].

An elimination half-life of 1.8 h for sisomicin was estimated from the serum concentration—time curve. Doenicke et al. [10], summarizing the results of several investigators, found a half-life of 2 h for sisomicin in healthy subjects.

Advantages of the proposed method

The determination of gentamicin in serum previously reported [1] requires six transfers of accurately measured volumes of liquids. By introducing an internal standard only two accurately measured liquid transfers are necessary. However, as most of these transfers are performed using repeating dispensers and an autosampler injection device, this advantage is comparatively small. In our opinion, the main advantage of internal standardisation is here that a check
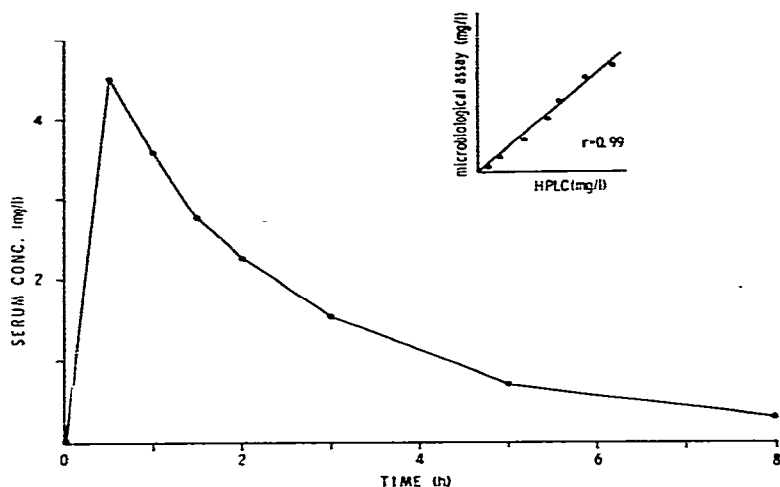
Fig. 2. Serum concentration of sisomicin as a function of time obtained in a volunteer following an intramuscular injection of 1.07 mg/kg body weight. Each value is the mean of two HPLC determinations. Inset: least linear-squares regression analysis of the sisomicin concentrations in the sera from the in vivo experiment, determined by HPLC and microbiological assay. Equation: $Y = -0.04 (\pm 0.24) + 0.91 (\pm 0.10) X$ (8 data pairs), where X = result of the HPLC assay (mean of duplicate determinations) and Y = result of the microbiological assay (mean of duplicate determinations); number between brackets: 95 per cent confidence interval.

of the peak heights of the internal standard (which should remain constant in all the chromatograms) provides a means of spotting irregularities due to incorrect sample handling, or fluctuations in the derivatization conditions and/or chromatographic conditions, thereby improving the reliability of the results.

Sisomicin, as compared to gentamicin, is more easily determined by chromatographic methods. Gentamicin consists of three main components, and the ratios between these components may vary, making calibrations in chromatographic procedures more cumbersome [1, 4]. On the other hand, sisomicin consists of one major component, and calibrations can be made against a reliable standard substance.

ACKNOWLEDGEMENTS

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