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MICRO DETERMINATION OF GENTAMICIN IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION

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SUMMARY

A procedure for the high-performance liquid chromatographic determination of gentamicin in serum is described using pre-column derivatisation and UV detection. The serum proteins are precipitated with acetonitrile and the gentamicin components in the supernatant are derivatized with 1-fluoro-2,4-dinitrobenzene. The reaction products are chromatographed on a microparticulate C_{18} reversed-phase column and detected at 365 nm. Sample volumes of 50 μl are sufficient for the determination of gentamicin concentrations in, and well below, the therapeutic range.

INTRODUCTION

Gentamicin is a valuable antibiotic in the treatment of patients with gram-negative infections. Monitoring the serum concentration of gentamicin is considered to be helpful in achieving optimal therapeutic levels and in avoiding toxic reactions [1, 2]. Gentamicin serum levels are determined most often by microbiological methods or by radioimmunoassay, but modern chromatographic methods can offer an alternative. A gas—liquid chromatographic method for the assay of aminoglycoside antibiotics in serum has been reported [3, 4]. The method requires a precipitation step and an evaporation step, followed by a two-stage derivatisation of first the hydroxyl groups and then the amino groups.

High-performance liquid chromatographic (HPLC) methods for measuring aminoglycoside antibiotic levels in serum have also been described recently [5—10]. In all these methods fluorescence detection of the derivatized aminoglycosides was used. High-performance liquid chromatographs with UV detectors are rapidly becoming standard equipment in hospital laboratories. An

HPLC method based on UV absorption detection rather than on fluorescence detection should therefore be useful for monitoring the serum levels of these compounds.

In the present investigation a simple HPLC method, based on a method reported by Bangert and Grossman [11], was developed for gentamicin, requiring only 50 μl of serum. The method uses pre-column derivatisation, separation of the components of the reaction mixture on a reversed-phase column and UV detection of the chromatographed products at 365 nm.

MATERIALS AND METHODS

Apparatus and chromatographic conditions

The chromatographic system consisted of a Solvent Delivery System 6000A, a Universal Injector U6K with a 2-ml injection loop (or an Autosampler WISP 710), a UV Absorbance Detector 440 equipped with a 365-nm filter and a μBondapak C_{18} column (30 cm × 3.9 mm I.D., particle size 10 μm) all from Waters Assoc. (Milford, Mass., U.S.A.). Retention times and peak areas were measured with the chromatographic Data System SP4000 of Spectra-Physics (Santa Clara, Calif., U.S.A.)

The mobile phase consisted of 1 g/l tris(hydroxymethyl)aminomethane in water (300 ml/l), adjusted with hydrochloric acid to pH 7, and acetonitrile (700 ml/l). This mixture was passed through a 0.2-μm filter and de-aerated ultrasonically. The flow-rate was maintained at 1.5 ml/min. Chromatography was performed at room temperature.

Reagents and glassware

Demineralised water was used throughout. Hydrochloric acid, 2 mol/l in water, was prepared by diluting 12 mol/l hydrochloric acid (p.a., Merck, Darmstadt, G.F.R.). 1-Fluoro-2,4-dinitrobenzene (FDNB) and tris(hydroxymethyl)aminomethane (Tris) were both p.a. grade from Merck; acetonitrile was "zur Synthese" from Merck.

Garamycin®, an injection of gentamicin sulphate in water containing the equivalent of 40 g of gentamicin per litre, was from Essex (Heist-op-den-Berg, Belgium). The gentamicin components C_1, C_{1a}, and C_2 as their sulphate salts were by courtesy of Schering (Bloomsfield, N.J., U.S.A.); the exact purity and water content of these components were not known. All antibiotic concentrations were calculated relative to potency unless otherwise indicated.

Human serum obtained from ambulant patients was frozen and stored at −18° within three days of collection. Once defrozen it was used the same day.

Centrifuge tubes were of 7 ml capacity with glass stoppers and the conical end drawn to a fine point. Ampoules had a capacity of 1 ml.

Solutions

All solutions and the acetonitrile were passed through a 0.2-μm filter before use.

Solution 1: gentamicin sulphate in water, containing the equivalent of 4 mg of gentamicin per litre.

Solution 2: Tris 20 g/l in water.
Solution 3: FDNB 250 g/l in acetonitrile.
Solution 4: FDNB 170 g/l in acetonitrile.

Procedures

Procedure A: derivatisation of aqueous gentamicin solutions. Dispense into an ampoule 50 µl of solution 1, 50 µl of solution 2, 200 µl of acetonitrile and 20 µl of solution 3. Close the ampoule and place in a water-bath at 80° for 45 min. Inject 175 µl into the chromatograph.

Procedure B: derivatisation of serum samples. Dispense 50 µl of serum into a centrifuge tube, add 50 µl of solution 2 and vortex for 15 sec. Add 200 µl of acetonitrile and vortex for 15 sec, then centrifuge at 2500 *g* for 5 min. Transfer 200 µl of the supernatant into an ampoule, add 20 µl of solution 4. Close the ampoule and place in a water-bath at 80° for 45 min. Inject 175 µl into the chromatograph.

Procedure C: in vivo experiment. A healthy 80-kg volunteer received 1 mg of gentamicin per kg by intramuscular injection. Blood samples were collected at regular time intervals by venipuncture. Serum was separated from the collected blood and stored at −18°. Gentamicin standards were prepared in pooled serum with a gentamicin injection from the same lot that was administered to the subject. Serum samples (in duplicate) and standards were analyzed in one run according to procedure B.

Aqueous dilutions of this injection were also analysed following procedure A. Peak heights of the C_{1a} and of $C_1 + C_2$ derivatives were measured, and the ratio (peak height $C_1 + C_2$ derivatives)/(peak height C_{1a} derivative) was calculated for all serum samples and for the gentamicin injection.

RESULTS

Gentamicin consists of three major components, C_1, C_{1a}, and C_2, which are present in roughly equal amounts. By running the purified gentamicin components through procedure A, the elution order of the gentamicin derivatives was determined. The C_1 derivative eluted a few seconds before the C_2 derivative; however, with the gentamicin mixture, the C_1 and C_2 derivatives appeared as one single peak in the chromatogram. The C_{1a} derivative was well separated from the others, with a retention time of 1.5 min less. The purified gentamicin components yielded the same peak area per mol (by weight) within the experimental error. Fig. 1 shows chromatograms obtained from blank serum, and from serum spiked with gentamicin.

The optimal derivatisation conditions were determined by investigating the effect of pH, FDNB concentration and of reaction time and temperature on the derivatisation of aqueous gentamicin solutions. The results are depicted in Fig. 2–4.

Precision and linearity

Serum samples with five different concentrations of gentamicin, ranging from 1 to 16 mg/l were analyzed in one run using procedure B. The results are summarized in Table I.

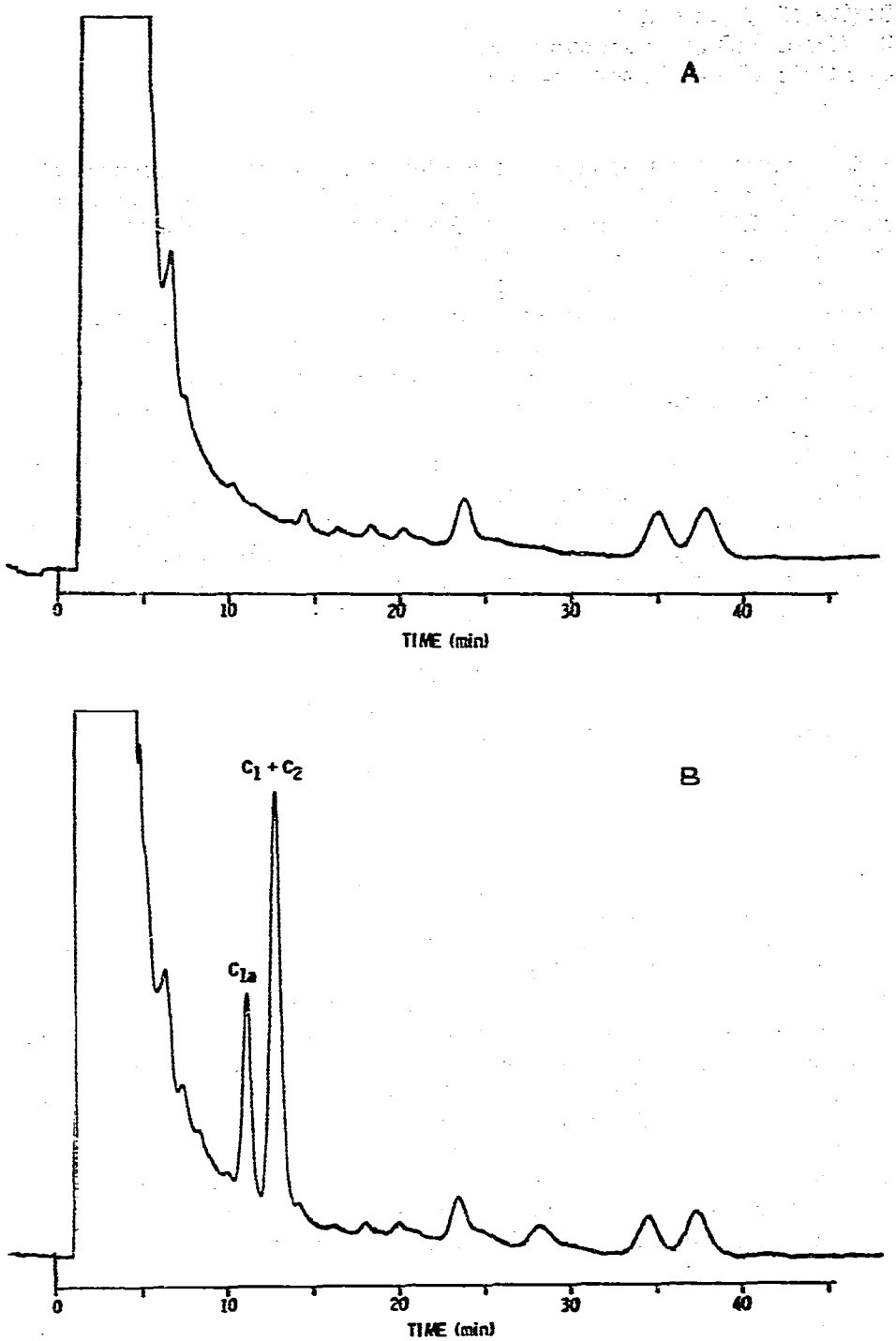

Fig. 1. HPLC of serum samples. Chromatogram A was obtained from 50 μl of blank serum, chromatogram B from 50 μl of blank serum, spiked with gentamicin to a concentration of 4 mg/l. Detector setting was 0.01 a.u.f.s.

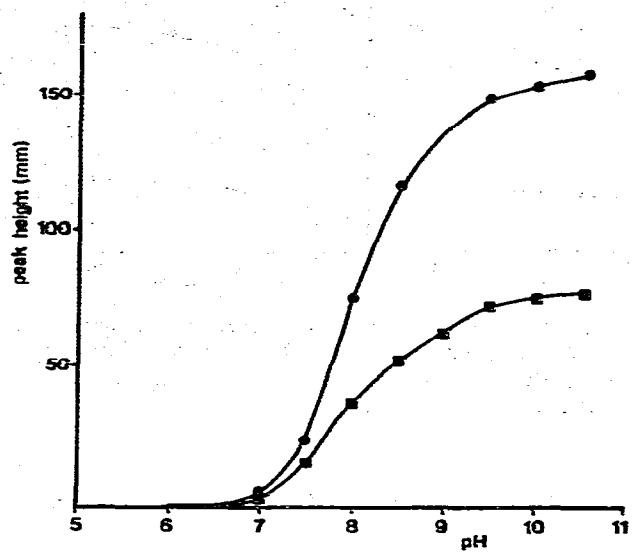

Fig. 2. Effect of pH of the Tris solution on the peak heights of the derivatized gentamicin components C_1 and C_2 (•); and C_{1a} (▪). Experimental details: procedure A, modified by adding different quantities of hydrochloric acid to solution 2. Each point represents the mean value of three or four determinations. Peak heights are based on a detector setting of 0.01 a.u.f.s.

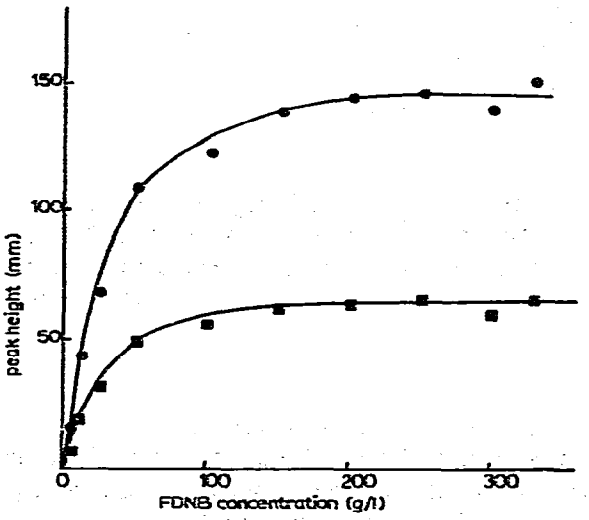

Fig. 3. Effect on FDNB concentration of solution 3 on the peak heights of the derivatized gentamicin components. Symbols as in Fig. 2. Experimental details: procedure A, modified by varying the FDNB concentration of solution 3. Each point represents the mean value of three or four determinations.

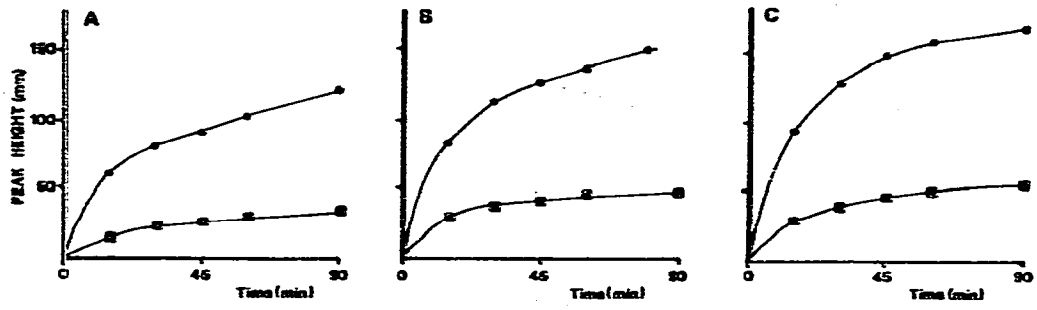

Fig. 4. Effect of the reaction time on the peak heights of the derivatised gentamicin components, at reaction temperatures of 65° (A), 80° (B) and 100° (C). Symbols as in Fig. 2. Experimental details procedure A, modified by varying reaction time and temperature. Each point represents the mean value of three determinations.

TABLE I

PEAK HEIGHTS AND COEFFICIENTS OF VARIATION OF THE DERIVATIZED GENTAMICIN COMPONENTS

Serum samples of 50 µl were used.

Gentamicin concentration (mg/l)	n	Component C_{1a}			Components C_1 and C_2		
		PH* (mm)	PH/concentration	C.V. (%)	PH (mm)	PH/concentration	C.V. (%)
1	6	19	19	4.8	40	40	3.7
2	6	32	16	0.9	74	37	3.0
4	5	68	17	2.7	147	37	1.7
8	6	152	19	5.9	312	39	5.4
16	6	311	19	5.5	627	39	4.9

*Peak heights (PH) are based on a detector setting of 0.01 a.u.f.s.

Sensitivity

Samples of pooled serum containing gentamicin at 0.33 mg/l were analyzed according to procedure B. The value of the ratio of the mean peak height of the derivatisation products and the gentamicin concentration was found to be 17 (C.V. = 12%; $n = 6$) for the C_{1a} component, and 35 (C.V. = 4.3%; $n = 6$) for the $C_1 + C_2$ components.

Specificity

Serum samples from a patient subjected to a combined therapy of gentamicin and ampicillin, and serum samples from a patient treated with gentamicin, trimethoprim and sulphamethoxazole, were analyzed by procedure B. No interfering peaks could be discerned in the chromatograms.

Recovery

Recovery was estimated by spiking serum samples with gentamicin at a concentration of 4 mg/l, before and after the acetonitrile treatment, respectively. Both sera were analyzed six times in one run, according to procedure B. Recoveries were found to be 83% (S.D. = 4%; $n = 6$) for the C_{1a} component, and 84% (S.D. = 2%; $n = 6$) for the $C_1 + C_2$ components.

Comparison of sera from different persons

Twenty-seven sera, obtained from different persons, and pooled serum, were spiked with gentamicin, each to the same concentration (6 mg/l). Each of the individual sera, in duplicate, and the pooled serum (six determinations), were analyzed in one run according to procedure B.

The average peak heights of the C_{1a} derivative peak, obtained from the individual sera and from the pooled serum were 101.4 mm (S.D. = 3.2 mm; n = 27) and 100.0 mm (S.D. = 2.2 mm; n = 6), respectively. For the peak of the C_1 + C_2 derivatives an average peak height of 223.6 mm (S.D. = 5.4 mm; n = 27) was obtained with the individual sera, while pooled serum determinations yielded a value of 220.4 mm (S.D. = 5.0 mm; n = 6). (Peak heights were based on a detector setting of 0.01 a.u.f.s.)

In vivo experiment (Procedure C)

The ratio of the combined peak heights of the C_1 and C_2 components to the peak height of the C_{1a} component was found to be the same in the serum samples and in the administered injection, and this ratio remained constant in the serum samples collected at different times after the injection. This ratio was found to be 2.27 (S.D. = 0.09; n = 21) for the serum samples, and 2.29 (S.D. = 0.13; n = 6) for the injection.

Gentamicin serum concentrations, calculated by measuring the C_{1a} derivative peak, did not differ significantly from the serum concentration found by measuring the peak of the C_1 + C_2 derivatives. The average of four values obtained for the gentamicin concentration (one C_{1a}-based and one C_1 + C_2-based, in duplicate) was taken as the final value.

The serum concentration—time curve of gentamicin in the volunteer is shown in Fig. 5. A half-life of 2.4 h was calculated from the data obtained.

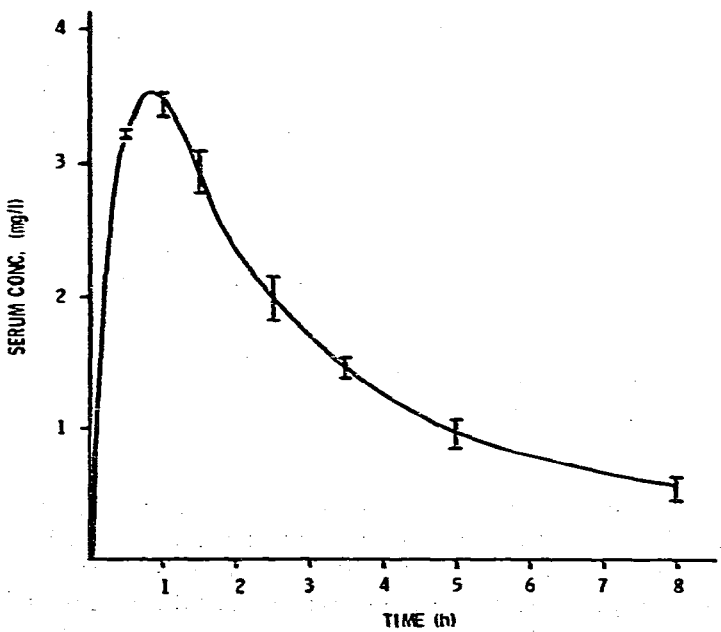

Fig. 5. Serum concentration of gentamicin as a function of time obtained in a volunteer following an intramuscular injection of 1 mg per kg body weight. Each value is the mean (± S.D.) of four values from two determinations (see Results).

DISCUSSION

Deproteinisation

Deproteinisation of gentamicin-containing serum samples, by precipitation with acetonitrile after alkalinisation, has been reported previously [6, 10]. An organic buffer system was chosen for making the samples alkaline, because the usual inorganic buffer compounds tend to precipitate in the acetonitrile—water mixture.

Derivatisation

The peak heights of the derivatized products increased with higher pH values of the added buffer solution. Above pH 9.5 no significant increase in the peak heights was observed (Fig. 2). A mixture of equal volumes of human serum and of Tris solution 2 resulted in a solution of pH 9.6. The peaks of the derivatized gentamicin components increased with higher reagent concentrations, up to an FDNB concentration of about 200 g/l (Fig. 3). FDNB at 250 g/l in acetonitrile was chosen as the derivatisation reagent for procedure A, and at 170 g/l in acetonitrile for procedure B, resulting in the same FDNB concentration in the derivatisation mixture for both procedures. Time—temperature curves were constructed with reaction temperatures of 65°, 80°, and 100°, covering reaction times of up to 90 min (Fig. 4). In none of the resulting plots was a constant peak height achieved, indicating that under the experimental conditions derivatisation was not complete.

As the experimental error was somewhat larger when derivatisation took place at 100° compared with at 80°, the latter temperature was chosen as the derivatisation temperature for the assay. A reaction time of 45 min was selected, in order to keep the time needed for the total assay within acceptable limits. Gentamicin solutions, which were derivatized for 45 min at 80°, showed an increase in peak heights of 14 ± 2% for both product peaks after storage of the reaction mixtures for 24 h at room temperature. When the solutions were injected within 4—5 h of derivatisation, the increase in peak height remained within experimental error.

Stoichiometry of the derivatisation reaction

After derivatisation of equal (molar) amounts of the three purified gentamicin components, the resulting peak areas did not differ significantly. These results are in agreement with a reaction in which every amino group, primary as well as secondary, reacts with one FDNB molecule. Evidence that all of the available amino groups of aminoglycosides can be derivatized with FDNB was also presented by Tsuji et al. [12].

Chromatography

Methanol—water mixtures and acetonitrile—water mixtures were investigated as possible mobile phases. The best results were obtained with the acetonitrile—water mixture described above. The mobile phase was buffered at pH 7 to prevent column damage due to the repeated injection of alkaline samples. Acetonitrile "zur Synthese" was found to be fully satisfactory since detection was performed at 365 nm.

The time required for a complete chromatogram was 45 min, due to the appearance of four small peaks after the gentamicin derivative peaks (Fig. 1). Three of these peaks were observed in the chromatogram from blank serum. One peak, with a retention time of about 28 min, also appeared in chromatograms resulting from aqueous gentamicin solutions, but not in the reagent blank chromatogram; this peak can therefore probably be ascribed to a minor component of the gentamicin complex.

To reduce the analysis time samples were injected at 20-min intervals; this gave a partial overlap of the chromatograms but did not affect the gentamicin peaks. Use of an autosampler made this procedure feasible. Alternatively one might use a flow-rate of 3.0 ml/min; this does not seriously affect the resolution of the peaks, but twice the amount of mobile phase is needed.

Calibration curve and analysis of serum samples

As is apparent from the data in Table I, the calibration curve proved to be a straight line through the origin, over the studied concentration range, for both the C_{1a} and the $C_1 + C_2$ components. No significant differences were observed between the results obtained with pooled serum and with 27 sera from different persons. The method can therefore be applied to the analysis of different serum samples, despite the fact that the derivatisation of the gentamicin components is not complete.

Gentamicin injections from different batches contain different amounts of the C_1, C_{1a}, and C_2 components. With the method described above, gentamicin C_1 and gentamicin C_2 are not resolved. The potencies of the three components may not be the same [13]. It is therefore advisable to construct calibration curves using the same batch of gentamicin that was administered to the patient(s).

From the in vivo experiment it can be concluded that the pharmacokinetic parameters of the three components do not differ significantly. Similar results have been reported previously [14]. This allows computation of the gentamicin concentration in the serum of patients from either one of the two gentamicin peaks, by peak height or by peak area measurements. If the corresponding gentamicin injection solution is not available for calibration, the relatively small differences in potency between the three components might be ignored, and the sum of the peak areas of the two gentamicin peaks can be used for the construction of the calibration curve and for the calculation of the gentamicin level in serum samples from the patient(s), as has been proposed by Anhalt et al. [5].

The serum concentration—time curve, and the gentamicin half-life, obtained by the in vivo experiment, are in good agreement with the data obtained by other investigators using a microbiological assay [15].

Advantages of the proposed method

The method as described above is simple and suitable for automation. Fluorescence detection after derivatisation with *o*-phthalaldehyde [5, 6, 8, 9] or with dansyl chloride [7, 10] offers, in principle, greater sensitivity than UV detection; however, the sensitivity obtained with the proposed method is quite sufficient for monitoring gentamicin levels in the therapeutic range, in serum

samples no larger than 50 μl. The lowest concentration of gentamicin that can be determined in 50-μl serum samples with acceptable precision was found to be about 0.3 mg/l of serum.

ACKNOWLEDGEMENTS

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