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MICRO-DETERMINATION OF TOBRAMYCIN 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 tobramycin in serum is described using pre-column derivatisation with 1-fluoro-2,4-dinitrobenzene and subsequent chromatographic analysis on a reversed-phase column with ultraviolet detection. Gentamicin is used as the internal standard. The sensitivity is 0.5 mg/l with 50-μl samples. Precision, expressed as the coefficient of variation, is 3% or better in the concentration range 0.5—16 mg/l. The absolute recovery of tobramycin is 41%.

The analyses of serum samples obtained in an in vivo experiment correlated well with the results from a microbiological assay. The influence of variation of derivatisation conditions and the implications for the reliability of the internal standardisation were studied. The 2,4-dinitrophenyl tobramycin derivative was synthesized and its structure was proved to be the fully derivatized tobramycin. Side-products of the derivatisation reaction were isolated.

INTRODUCTION

A high-performance liquid chromatographic (HPLC) method for the determination of gentamicin and sisomicin in serum was previously reported by us [1, 2]. The assay is based on pre-column derivatisation of the aminoglycoside with 1-fluoro-2,4-dinitrobenzene (FDNB) and subsequent analysis of the derivatised products on a reversed-phase type column with ultraviolet (UV) detection. In the present paper we describe the determination of tobramycin in serum, using gentamicin as the internal standard.

For "perfect" internal standardisation, tobramycin and gentamicin should react in a completely similar way with the derivatisation reagent. The reliability of internal standardisation was tested under varying derivatisation conditions. In order to establish its structure, the dinitrophenyl tobramycin derivative was

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synthesized on a preparative scale. With the help of this purified derivative, the absolute recovery of tobramycin was measured.

FDNB reacts with primary and secondary amino groups [3]. The tris(hydroxymethyl)aminomethane (Tris) added to the reaction mixture also contains a primary amino group, so a reaction between FDNB and Tris can be expected to take place. Furthermore, FDNB hydrolyses in alkaline solutions to 1-hydroxy-2,4-dinitrobenzene [3]. To establish the presence of these products, and of possible other side-products of the derivatisation reaction, the composition of the reaction mixture was investigated.

Other chromatographic determinations of tobramycin in serum have been reported [4—11]. The method described in the present paper has the advantages of small sample volume (50 µl), simple sample preparation, and the convenience offered by HPLC with UV detection.

EXPERIMENTAL

Instrumentation

The chromatographic equipment was described previously [1]. UV absorption measurements, UV spectra and infrared (IR) spectra were obtained with a Pye-Unicam SP-500 Series 2 single-beam spectrophotometer, a Shimadzu UV-200 double-beam recording spectrophotometer, and a Jasco IRA-1 grating infrared spectrophotometer, respectively. Mass spectra were recorded on an AEI MS 902 electron-impact mass spectrometer. Element analyses were carried out by the Element Analytical Section of the Institute for Organic Chemistry TNO, Utrecht, The Netherlands, under the supervision of W.J. Buis. The microbiological assays were carried out by the National Institute of Public Health (RIV), Bilthoven, The Netherlands, using the agar-well diffusion technique previously described [2].

Materials

Demineralized water was used. Tris(hydroxymethyl)aminomethane (Tris), 1-fluoro-2,4-dinitrobenzene (FDNB), acetic acid, methanol, acetone and ethyl acetate were p.a. grade from Merck (Darmstadt, G.F.R.). Acetonitrile, "zur Synthese", silica-gel 60, 70—230 mesh, and pre-coated silica-gel 60 F_{254} plates were also from Merck. Chloroform, 4 *M* sodium hydroxide, 25% ammonia and 10% hydrochloric acid were of Dutch Pharmacopoeia quality (Ph. Ned. VI and VIII, respectively) all from Brocacef (Maarssen, The Netherlands).

The preparations for injections — Obracin® (Eli Lilly, St.-Cloud, France) and Garamycin® (Essex, Heist-op-den-Berg, Belgium) — contained the equivalents of 40 g/l tobramycin and 40 g/l gentamicin, respectively. Tobramycin, lot OEJ 90, stated potency 975 µg/mg, was obtained by courtesy of Eli Lilly Nederland (Utrecht, The Netherlands). All antibiotic concentrations were calculated relative to potency [12, 18]. When the weight of the chemical substance tobramycin is meant it is indicated by the notation: (by weight).

Pooled human serum from ambulatory 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.

HPLC conditions

The mobile phase was prepared by mixing 340 ml of water (filtered through a 0.2-μm filter) with 660 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.). Ultraviolet detection was made at 365 nm. Chromatography was performed at room temperature.

Procedures

Procedure A: serum sample preparation. Dispense into a centrifuge tube 50 μl of the serum sample, add 50 μl of a solution containing 20 g/l Tris in water, also containing the equivalent of 20 mg/l gentamicin (the internal standard), and vortex. Add 200 μl of acetonitrile and vortex. Centrifuge 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 heat-seal the ampoule. Place in a water-bath at 80°C for 45 min. Break the seal and inject 150 μl into the chromatograph.

Procedure B: in vivo experiment and bioassay comparison study. A healthy 81-kg volunteer received 80 mg of tobramycin by intramuscular injection. Blood samples were collected at regular time intervals, and the serum was separated and stored at −18°C. Tobramycin standards were prepared in pooled human serum. Standards and serum samples were analysed in duplicate in one run according to procedure A. The same serum samples and standards were also analysed in duplicate by the microbiological assay.

Procedure C: synthesis of the 2,4-dinitrophenyl tobramycin derivative. Obracin® (2 ml) was mixed with 4 ml of acetonitrile containing 180 mg of FDNB and 225 μl of 4 *M* sodium hydroxide. The mixture was transferred to an 8-ml vessel, closed with a septum, and placed in a water-bath at 80°C. At regular time intervals the mixture was tested for alkalinity; if necessary, more sodium hydroxide solution was added by means of a syringe. During the derivatisation reaction an oily, reddish precipitate developed, especially upon the addition of sodium hydroxide. The reaction was considered to be complete when addition of more sodium hydroxide solution did not give rise to the formation of more precipitate. The reaction mixture was neutralised by the addition of hydrochloric acid and diluted with acetonitrile. After filtration, the acetonitrile solution was evaporated under reduced pressure and the yellow residue was reconstituted in the smallest possible volume of a chloroform—acetonitrile mixture (11.5:8.5). A glass column (100 cm × 3.5 cm I.D.) was filled with a slurry, prepared by mixing about 400 ml of silica gel, activated at 100°C for 12 h, and about 400 ml of a chloroform—acetonitrile mixture (11.5:8.5). After drainage of the column, the yellow solution was put on top of the column. After washing the column with 50 ml of chloroform, the column was eluted with a chloroform—acetonitrile mixture (11.5:8.5) and 20-ml fractions were collected. The fractions were screened by thin-layer chromatography on silica-gel plates (activated at 100°C) with chloroform—acetonitrile (11.5:8.5) as the mobile phase. The appropriate fractions (160—260 ml of the eluate) were combined and the solvent was evaporated under reduced pressure. The residue was recrystallized from a water—acetone mixture and dried overnight at 105°C under reduced pressure and over diphosphorus pentoxide, since the substance showed hygroscopic properties.

Procedure D: recovery measurements. Tobramycin lot OEJ90 was investigated for tobramycin content and moisture content by non-aqueous titration and Karl Fischer titration, respectively, according to the U.S. Pharmacopeia [12]. The purity of tobramycin lot OEJ90 was investigated by thin-layer chromatography as described by Pauncz and Harsányi [13], with the detection of the spots carried out according to the method of Wilson et al. [14].

The absolute recovery of tobramycin was estimated by spiking blank serum with tobramycin in lot OEJ90 to obtain serum spiked at a concentration of 4 mg (by weight)/l. Also, a solution of the purified 2,4-dinitrophenyl tobramycin derivative in the mobile phase was prepared. Serum sample preparation and chromatography were performed in quadruplicate according to procedure A (no internal standard). The solution of the purified derivative was injected in quadruplicate.

The yield of the derivatisation reaction was estimated by preparing from blank serum a quantity of supernatant in a way analogous to procedure A (no internal standard). A quantity of this supernatant was spiked with tobramycin lot OEJ90 to obtain a concentration of 0.67 mg (by weight)/l, corresponding to a serum concentration of 4 mg (by weight)/l. The spiked supernatant was derivatised and chromatographed, in quadruplicate, according to procedure A. A solution of the purified tobramycin derivative was also injected in quadruplicate.

As a check on the results of the two former experiments, the recovery of tobramycin from the deproteinisation step was estimated by spiking two identical quantities of blank serum samples with the same amount of tobramycin, one before and the other after the addition of acetonitrile. The added amount of tobramycin corresponded to a serum concentration of 4 mg/l.

RESULTS AND DISCUSSION

For the determination of gentamicin and sisomicin, with tobramycin as the internal standard, a mobile phase composed of 700 ml/l acetonitrile was used. No separation of the tobramycin derivative from an interfering peak in the blank was then obtained [2]. However, with a mobile phase composed of 660 ml/l acetonitrile, separation of the tobramycin derivative from the interfering peak was obtained. Gentamicin was added as the internal standard; the peak of the gentamicin C_{1a} derivative was used for the calculation of the peak height ratios. Representative chromatograms are shown in Fig. 1.

Precision, linearity and sensitivity

Serum samples with eight different concentrations of tobramycin, ranging from 0.25 to 16 mg/l, were analysed in one run. The results are summarised in Table I. In the range 0.5—16 mg/l a linear calibration line was obtained; throughout this range the coefficient of variation was 3% or less. At lower concentrations deviations from linearity were observed. The equation for the linear least-squares regression line in the range 0.5—16 mg/l is: PHR = 0.037 (± 0.050) + 0.398 × tobramycin concentration, $r = 0.998$ (39 samples), in which PHR is the peak height ratio (tobramycin:gentamicin C_{1a}), and the number between brackets is the 95% confidence interval of the intercept. The intercept has a

95% confidence interval which includes the origin, so the calibration line passes through the origin. In the therapeutic use of tobramycin, peak serum concentrations of 5—8 mg/l and trough concentrations of 1—2 mg/l are encountered [15]. So, the linear part of the calibration line includes the therapeutic range.

TABLE I

PEAK HEIGHT RATIOS (PHR) OF DERIVATISED TOBRAMYCIN TO INTERNAL STANDARD (GENTAMICIN C_{1a}) AND COEFFICIENTS OF VARIATION (C.V.) OBTAINED WITH SERUM SAMPLES (50 µl) CONTAINING 0.25—16 mg/l TOBRAMYCIN

Concentration (mg/l)	n*	PHR	PHR/ concentration	C.V. (%)
0.25	6	0.166	0.66	4
0.50	6	0.252	0.50	2
1	6	0.420	0.42	2
2	6	0.873	0.44	3
4	6	1.67	0.42	2
8	6	3.17	0.40	2
12	6	4.68	0.39	2
16	3	6.65	0.42	1

*n = number of determinations.

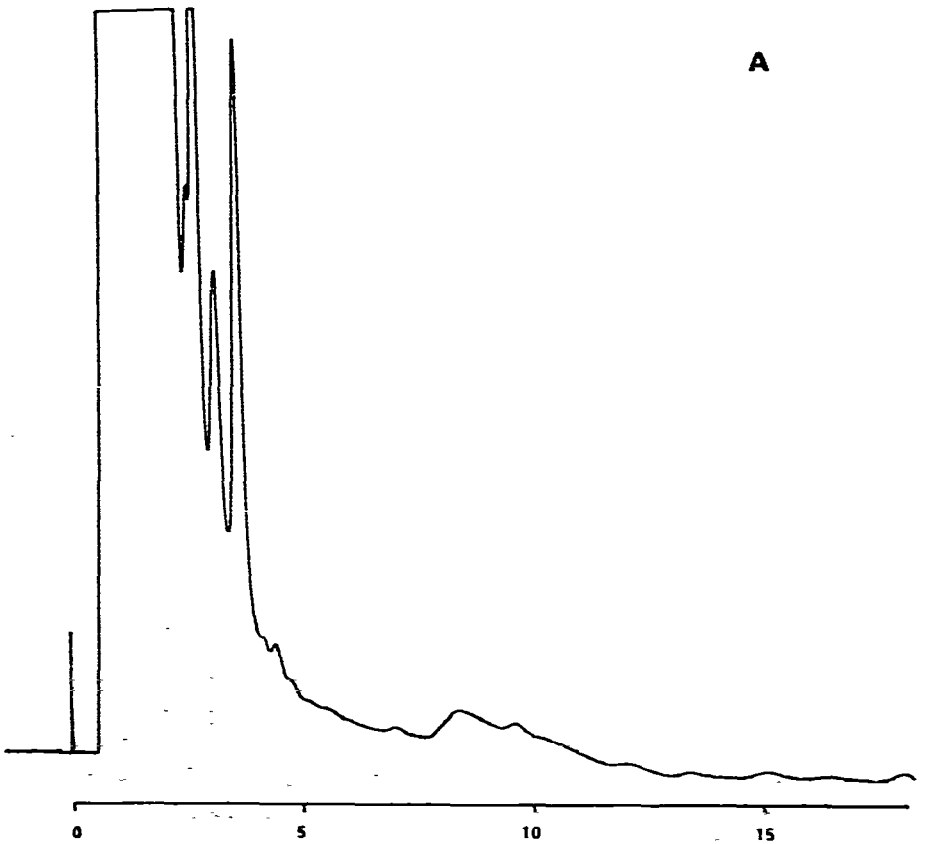

Fig. 1.

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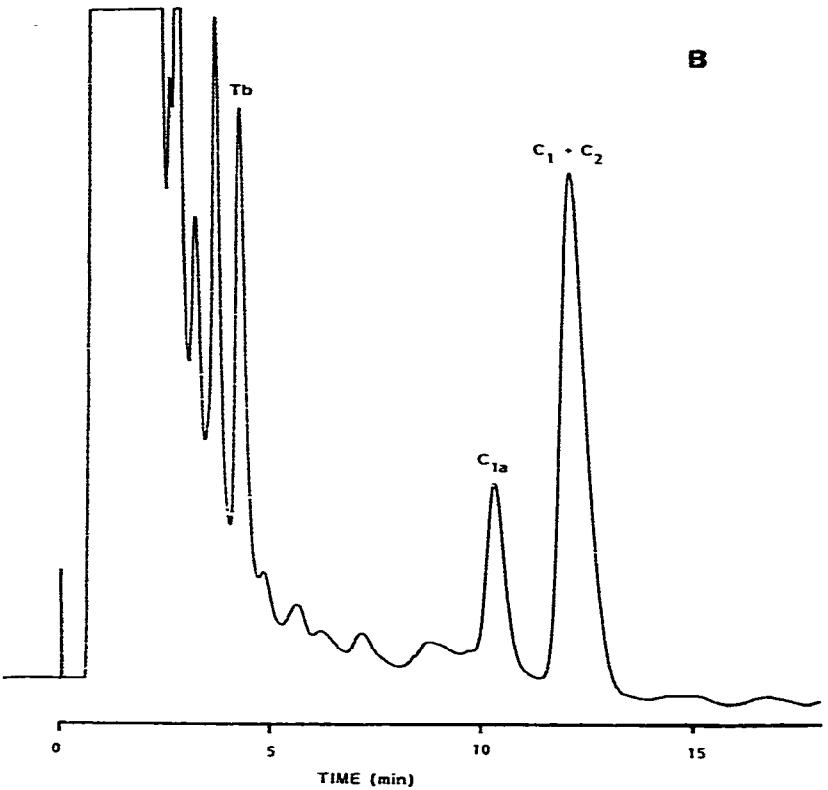

Fig. 1. HPLC of serum samples. The detector setting was 0.02 a.u.f.s. Chromatogram A was obtained from 50 μl of blank serum. No internal standard was added. Chromatogram B was obtained from 50 μl of blank serum, spiked at a concentration of 4 mg/l tobramycin, with gentamicin as the internal standard. Tb = tobramycin derivative; C_{1a} = gentamicin C_{1a} derivative; $C_1 + C_2$ = gentamicin C_1 and gentamicin C_2 derivatives (not separated).

In vivo experiment and bioassay comparison study

The results are summarized in Fig. 2. The correlation between the values found by the two methods is good. The correlation line shows no significant intercept, and the slope differs not significantly from one, so both methods can be regarded as free of systematic errors.

Characterisation of the derivative of tobramycin

(1) A solution of the recrystallised yellow substance in the mobile phase was chromatographed as described under Experimental (HPLC conditions). Only one peak was obtained with the same retention time as the tobramycin derivative after derivatisation of tobramycin-containing serum samples.

(2) The purity of the derivative was also investigated by thin-layer chromatography on silica gel, activated at 100°C with mobile phase chloroform—acetonitrile (11.5:8.5). Only one yellow spot with $R_F = 0.48$ was observed. The obtained chromatogram was sprayed with ninhydrin, according to the description of Wilson et al. [14]. The yellow spot did not change colour, indicating that all amino groups were derivatised.

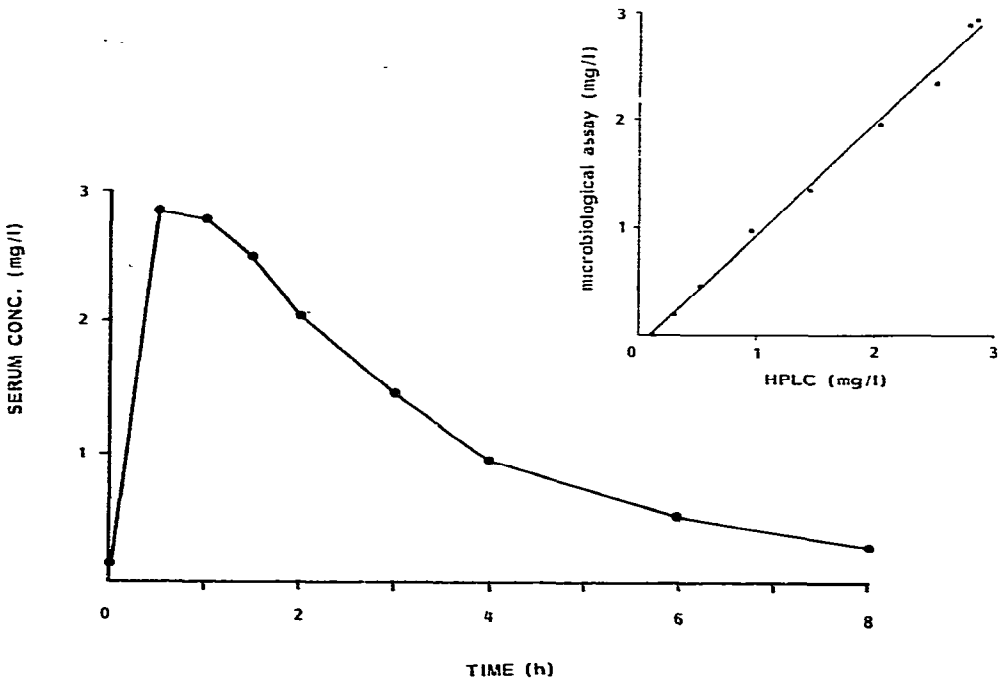

Fig. 2. Serum concentration of tobramycin as a function of time obtained in one volunteer following intramuscular injection of 1 mg/kg body weight. Each value is the mean of duplicate HPLC determinations. Inset: linear least-squares regression analysis of the tobramycin concentrations in the sera from the in vivo experiment, determined by HPLC and microbiological assay. Equation: $Y = -0.11\ (\pm\ 0.13) + 1.04\ (\pm\ 0.07)\ X$, $r = 0.997$ (9 data pairs), where Y = result of the HPLC assay (mean of duplicate determinations), and X = result of the microbiological assay (mean of duplicate determinations). Numbers in parentheses are 95% confidence intervals.

(3) The results of the element analyses were: C 43.58%, H 3.63%, N 15.62%, O 36.08%, found; C 44.42%, H 3.65%, N 16.19%, O 35.75%, calculated for $C_{48}H_{47}N_{15}O_{29}$ = tobramycin-(2,4-dinitrophenyl)$_5$.

(4) A solution of the substance in methanol showed the UV spectrum of the N-2,4-dinitrophenyl group with maxima at 260 nm and 350 nm. The specific absorption, $A_1^{1\%}{}_{cm}$, measured at 350 nm, was found to be 674. Assuming the derivative to be tobramycin-(2,4-dinitrophenyl)$_5$, the molar absorptivity, ϵ, was calculated from the observed specific absorption and the assumed molecular

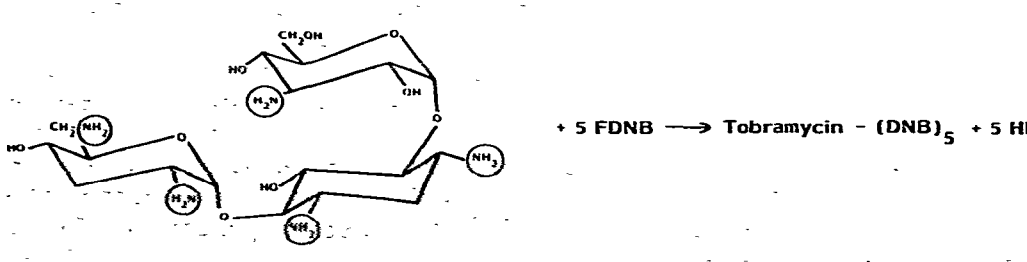

Fig. 3. Derivatisation of tobramycin with 1-fluoro-2,4-dinitrobenzene (FDNB).

weight. ϵ was found to be 8.74×10^4 l/mol · cm. The value of ϵ for the N-2,4-dinitrophenyl group is 1.7×10^4 to 1.8×10^4 l/mol · cm [16], thus 4.9—5.1 dinitrophenyl groups are present in the derivative, which is in good accordance with the expected number of 5. Other reports [1, 17] are also in support of a structure consisting of the five-fold derivatised tobramycin (see Fig. 3).

Recovery

By non-aqueous titration, the tobramycin content of lot OEJ 90 was found to be 86.7% (by weight); the moisture content was found to be 10.3%. From these data, the potency, calculated on the anhydrous basis, is 964 μg/mg [12]; this is in accordance with the stated potency (975 μg/mg). No impurities related to tobramycin could be detected by thin-layer chromatography. It was concluded that lot OEJ90 contained 867 μg (by weight)/mg of the chemical substance tobramycin. The absolute recovery of tobramycin was measured at 4 mg/l and found to be 41% (S.D. = 1%, n = 4). The recovery of tobramycin, added after the protein precipitation was 53% (S.D. = 1%, n = 4). From these data, the recovery of tobramycin from the deproteinisation can be calculated to be 77% (S.D. = 2%). By direct measurement, this recovery from the protein precipitation was found to be 75% (S.D. = 1%, n = 4), which is in accordance with the calculated value.

The recovery of tobramycin in the derivatisation reaction is rather low. Whether this is due to less-favourable derivatisation conditions, or to the difficulty of derivatising all five amino groups of the tobramycin molecule is not yet clear. Up to now, however, we have not been successful in finding other derivatisation conditions which give higher yields (see below).

The low over-all absolute recovery does not affect the reliability of the determination as this recovery is sufficiently reproducible. Until now, no results on the absolute recovery have been published in reports dealing with the chromatographic determination of aminoglycoside antibiotics in serum.

Internal standardisation

We investigated the extent to which the derivatisation conditions could be varied without affecting the peak height ratios of derivatised tobramycin with respect to derivatised gentamicin C_{1a}, and with respect to gentamicin $C_1 + C_2$. In general, we found that under derivatisation conditions far less favourable than those used in the determination, i.e. lower Tris and FDNB concentrations, lower reaction temperature and shorter reaction time, the peak heights are lower for all derivatives, but that the derivatisation of the gentamicin components is more affected than the derivatisation of tobramycin. It is concluded that tobramycin is more easily derivatised than the gentamicin components. Despite internal standardisation, it is advisable, therefore, to do the complete sample preparation for standards and unknown samples together in one run. However, the proposed internal standardisation was found to be effective in compensating for the increase in peak height observed after the storage of the derivatised samples [1]. After storage for 16 h at room temperature we found that peak height ratios were increased by less than 10%.

Side-products of the derivatisation reaction

A solution of 100 mg of FDNB and 30 mg of Tris in 3 ml of a water—acetonitrile mixture (1:2) was heated at 80°C for 45 min. A chromatogram of the derivatisation mixture is shown in Fig. 4. The four substances giving rise to the peaks I, II, III and IV were isolated. Peak I was identified as 1-hydroxy-2,4-dinitrobenzene. The isolated substance and the reference substance have identical UV spectra — which show a pH shift — and identical chromatographic properties in various HPLC systems. 1-Hydroxy-2,4-dinitrobenzene is known to be formed by hydrolysis of FDNB in alkaline solution [3].

Peak II was identified as 2-[N-(2,4-dinitrobenzene)amino]-2-hydroxymethyl-1,3-propanediol, the product of the reaction between Tris and FDNB. Its structure was confirmed by mass spectrometry; the UV spectra and the IR spectrum were in accordance with this structure.

Peak III was identified as the excess FDNB in the reaction mixture.

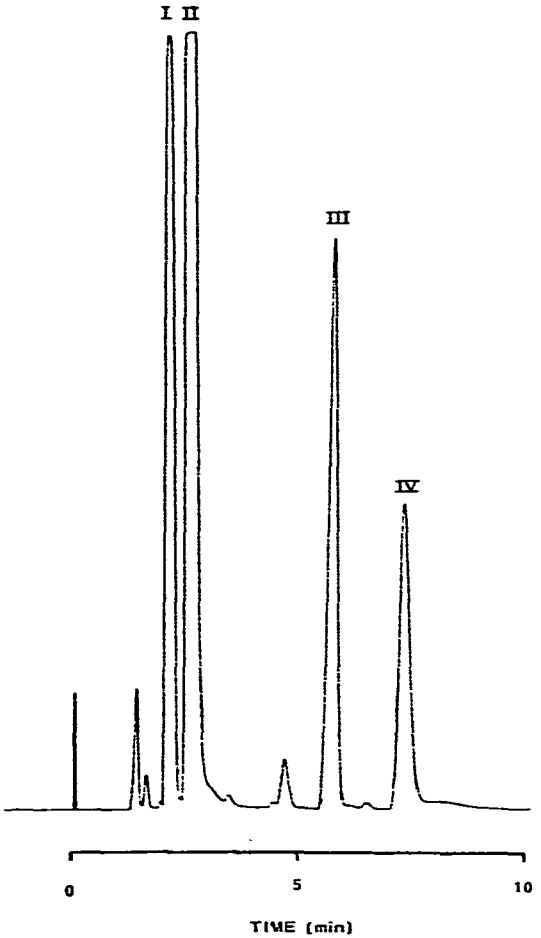

Fig. 4. HPLC of the derivatisation mixture. The detector setting was 2.0 a.u.f.s. Experimental details: mobile phase, 500 ml/l acetonitrile and 500 ml/l water; flow-rate, 1.5 ml/min; injection volume, 7 μl. Other conditions as described in Fig. 1. For identity of the peaks see Results (side-products of the derivatisation reaction).

Substance IV gave the UV spectrum of the N-2,4-dinitrophenyl group, but its structure could not be elucidated with certainty.

As Tris was expected to react with FDNB, we made a search for other substances or buffers that could be useful to alkalinise serum prior to deproteinisation. Tested were solutions in water of 10 g/l sodium carbonate, sodium acetate, triethanolamine and ammonia; 1.5 g/l triethylamine and N,N-diisopropylethylamine; 0.1 *N* sodium hydroxide and 0.1 *N* tetrabutylammonium hydroxide in a propan-2-ol—methanol mixture. Aqueous solutions of 10 g/l dibasic sodium phosphate, sodium bicarbonate and borax were incompatible with an acetonitrile—water mixture (2:1). We were unable, however, to find a suitable substitute for Tris. The best results were obtained with N,N-diisopropylethylamine which, being a tertiary amine, is not attacked by FDNB. After optimising the derivatisation parameters for this amine, we found essentially the same absolute recovery for tobramycin as with the Tris procedure. However, the recovery for gentamicin with N,N-diisopropylethylamine was far less than with the Tris procedure.

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