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Note

High-performance liquid chromatographic assay of the major components of gentamicin in serum

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Several methods are available for monitoring gentamicin in biological fluids in the clinical setting. Traditionally microbiological assay has been the most common assay technique used but this method is slow, often inaccurate and subject to interference by other antibiotics [1]. Recently a number of new methods have been published that are faster, more specific and more accurate. These methods include radioimmunoassay, fluorescence immunoassay, enzyme immunoassay and the adenylation methods [2,3].

These assays do not include a chromatography step prior to analysis and hence the individual gentamicin components (C_1, C_{1a} and C_2) are quantified as a combined amount. For determination of the individual components, combined thin-layer chromatography–microbiology [4] and high-performance liquid chromatographic (HPLC) assays [5–8] are available. However, some of these methods are limited by the requirement for post-column derivatisation [5] or unsuitable mobile phases [6]. The present communication details a simple precolumn derivatisation HPLC assay for the analysis of the individual gentamicin components in plasma.

EXPERIMENTAL

Gentamicin sulphate containing 563 μg gentamicin base per milligram of powder with a component ratio of C_1 25.4%, C_{1a} 31.7%, C_2 42.9%, was supplied by Essex Labs. (Sydney, Australia). All solvents were specially purified to HPLC grade. Water was deionised and glass-distilled daily. All other chemicals were

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reagent grade. The *o*-phthalaldehyde reagent was prepared according to the method of Maitra et al. [6].

Chromatographic equipment

Separation was performed using a Waters M6000A solvent delivery system fitted with a U6K injector. A C_{18} μBondapak column (particle size 10 μm; 300 mm × 3.9 mm I.D.; Waters Assoc., Milford, MA, U.S.A.) was used in combination with a guard column (μBondapak C_{18}/Porasil B; Waters Assoc.; 23 mm × 3.9 mm I.D.). Detection of fluorescent products in the eluent was performed using a Schoeffel Model FS9701C fluorometer (Schoeffel Instrument, Westwood, NJ, U.S.A.) with fluorescence excitation at 260 nm and emission detection at 418 nm. Detector signal was recorded on a dual-channel Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.). The mobile phase was 1% triethylamine (TEA) solution (adjusted to pH 6.2 ± 0.1 with phosphoric acid)–methanol (79:21) mixture. The flow-rate was 2 ml/min. Injections were made with a 25-μl Hamilton syringe.

Sample preparation

Preparation of derivatised gentamicin from serum samples was performed by the method of Maitra et al. [6] with some modification. In this method, a disposable Pasteur pipette was plugged with silanized glass wool and about 150 mg of dry silicic acid were added to form a silicic acid column of 1.0 cm height. The column was treated with 1.0 ml of water. Serum (0.5 ml) was diluted to 2.0 ml with water, vortexed, and applied to the silicic acid column. The serum sample tube was rinsed with 1.0 ml water which was then applied to the column and eluted with the help of pressure from a rubber bulb. The eluate was discarded. Immediately *o*-phthalaldehyde reagent (0.5 ml) was applied to the column and allowed to stand for 30 s. The reagent was eluted with the aid of pressure from the rubber bulb and the eluate was discarded. The derivatised gentamicin was then eluted from the column by adding 1.5 ml of methanol. The eluent was vortexed, centrifuged and stored in the dark until injected. A 20-μl aliquot of the sample was injected onto the column.

Preparation of standard curves

Plasma standards were prepared by spiking drug-free plasma with known amounts of a freshly prepared aqueous solution of gentamicin to produce concentrations of 1–20 mg/l total gentamicin (representing 0.14–2.86 mg/l C_1, 0.18–3.57 mg/l C_{1a} and 0.24–4.83 mg/l C_2).

The standards were then assayed in the described manner. Standard curves were prepared by plotting the peak heights of the components versus concentration.

RESULTS

Chromatograms of blank serum and serum from a subject following intravenous (i.v.) administration of gentamicin are shown in Fig. 1. The three major

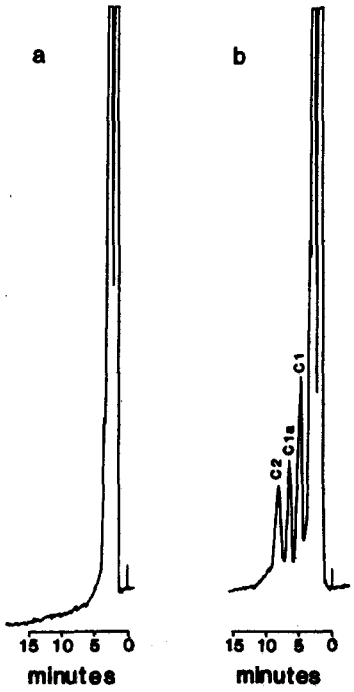

Fig. 1. Chromatograms of (a) blank serum and (b) serum from the same subject 80 min after i.v. administration of 1 mg/kg lean body mass dose of gentamicin complex. The gentamicin concentrations are estimated to be: C_1, 0.38 mg/l; C_{1a}, 0.57 mg/l; C_2, 0.45 mg/l.

gentamicin components were well separated from each other and eluted in the order of C_1, C_{1a}, C_2 and 4.8, 6.8 and 8.6 min, respectively.

Peak heights and serum concentrations were linearly related for each of the three components over the concentration range used for the standard curves. Their equations were: C_1: $y=311.40x+12.84$ ($r=0.991$); C_{1a}: $y=131.6x+1.5$ ($r=0.995$); C_2: $y=124.7x+4.8$ ($r=0.996$) where y is the peak height of the gentamicin component, x is the concentration of the component and r is the correlation coefficient. The detection limits of this assay (defined at a signal-to-noise ratio of 3:1) were C_1, 20 μg/l; C_{1a}, 80 μg/l; C_2 80 μg/l.

The intra- and inter-day reproducibility of the assay are shown in Table I.

DISCUSSION

At the time of this study, the only HPLC methods capable of separating the major gentamicin components were those of Anhalt [5], Maitra et al. [6] and Back et al. [9]. The method of Anhalt [5] used post-column derivatisation which, although reliable, has some disadvantages including the need for additional pumping equipment and a mixing chamber (not available in many laboratories), a high consumption of reagent and an increase in baseline noise due to the pumping of derivatising agent through the detector [8]. Pre-column derivatisation is simpler, requiring no additional machinery and is likely to eliminate more inter-

TABLE I

INTRA-DAY AND INTER-DAY VARIATION OF GENTAMICIN IN SERUM

Concentration (mg/l)		Coefficient of variation (%)		
		C_1	C_{1a}	C_2
2	Intra-day ($n=5$)	6.76	11.66	7.02
	Inter-day ($n=6$)	8.87	19.76	14.82
10	Intra-day ($n=5$)	6.05	5.13	6.36
	Inter-day ($n=10$)	6.49	7.22	10.35

fering material [6]. The methods of Maitra et al. [6] and Back et al. [9] use pre-column derivatisation and are identical except in the manner of sample extraction. In the method of Maitra et al. [6] the derivatising agent, *o*-phthalaldehyde, is included in the eluent subsequently injected onto the column. However, during the development of the present assay, the inclusion of this reagent led to a decrease in sample peak height throughout the day. As the *o*-phthalaldehyde reagent is alkaline (pH 10.4), its repeated injection may have destroyed the silica skeleton of the stationary phase of the pre-column and column. It was found that by discarding the derivatising eluate, then eluting the derivatised gentamicin with methanol that this problem was overcome. To assess that this method

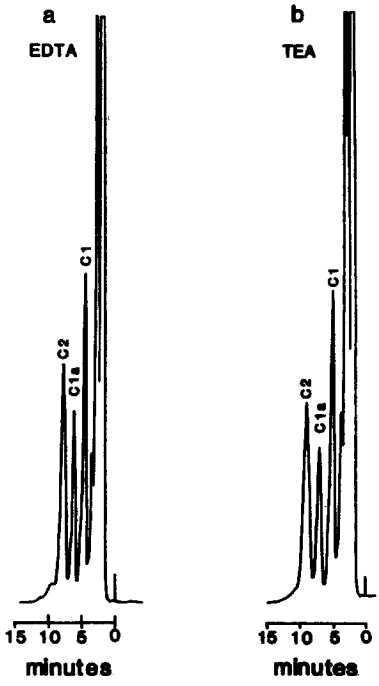

Fig. 2. Chromatograms of serum spiked with C_1 (1.43 mg/l), C_{1a} (1.785 mg/l) and C_2 (2.415 mg/l) and assayed (a) using the mobile phase of Maitra et al. [6] containing EDTA and (b) the mobile phase of the present method containing triethylamine (TEA).

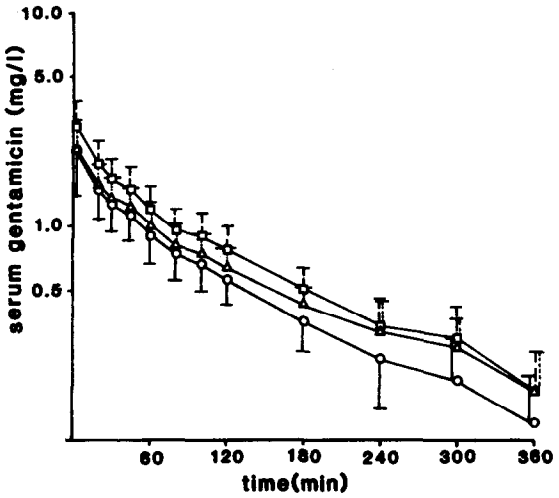

Fig. 3. Mean (±S.D.) serum gentamicin C_1 (○), C_{1a} (□) and C_2 (△) concentrations found in twelve healthy adult volunteers following i.v. administration of a 1 mg/kg (lean body mass) dose of gentamicin complex.

still provided satisfactory recovery, comparison was made of samples containing 0.1 ml of drug solution, 0.5 ml *o*-phthalaldehyde reagent and 0.9 ml methanol (total volume 1.5 ml), which were vortexed, centrifuged and then injected onto the column, with samples prepared as described (1.5 ml methanol eluent). This comparison showed that the peak heights were higher when the reagent was not included. In the automated method of Essers [8], the problem was overcome by pre-saturating the derivatising solution with silica. Maitra et al. [6] used excitation and emission wavelengths of 340 nm and 418 nm, respectively. In this study it was found that these wavelengths resulted in an unacceptably low peak height/noise ratio. Excitation at 260 nm and emission at 418 nm provided negligible background noise and good sensitivity, giving optimal peak height/noise ratios.

In the development of this assay, the EDTA–methanol mobile phase of Maitra et al. [6] was used initially and this provided separation of the components with retention times (C_1, 5.6 min; C_{1a}, 7.2 min; C_2, 9.6 min) similar to those of the published method (C_1, 4.7 min; C_{1a}, 7.2 min; C_2, 9.5 min). However, continued use of this mobile phase over a few days led to loss of peak resolution. The pH of the mobile phase (8.75) was higher than the top of the optimum pH range (2–7) for bonded-phase silica-based column packings [10]. At this pH the silica matrix is likely to dissolve and release of bonded groups from the surface may occur. These factors may account for the loss of resolution experienced in this study. A change from EDTA to 1% TEA adjusted to pH 6.2±0.1 with phosphoric acid gave resolution similar to that initially experienced using the EDTA–methanol mobile phase [R_s (EDTA) C_1–C_{1a}=1.06, C_{1a}–C_2=0.90; R_s (TEA) C_1–C_{1a}=1.16, C_{1a}–C_2=1.00) (Fig. 2). This mobile phase provided stable chromatograms over several weeks with the same column and injection of hundreds of samples.

This assay has been applied to the study of the pharmacokinetics of the gen-

tamicin components in healthy adults. Fig. 3 shows the mean plasma gentamicin component concentrations versus time profile obtained following i.v. administration of a 1 mg (total gentamicin) per kg dose to twelve healthy young adults.

This paper presents a simple, rapid assay for the determination of the three major components of gentamicin (C_1, C_{1a} and C_2) with sufficient sensitivity for use in clinical and pharmacokinetic studies.

REFERENCES

1 D.S. Reeves and M.J. Bywater, J. Antimicrob. Chemother., 1 (1975) 103.
2 R.M. Ratcliffe, C. Mirelli, E. Moran, D. O'Leary and R. White, Antimicrob. Agents Chemother., 19 (1981) 508.
3 G.R. Matzke, C. Gwizdala, J. Wery, D. Ferry and R. Starnes, Ther. Drug Monit., 4 (1982) 195.
4 W.L. Wilson, G. Richard and D.W. Hughes, J. Pharm. Sci., 62 (1973) 282.
5 J.P. Anhalt, Antimicrob. Agents Chemother., 11 (1977) 651.
6 S.K. Maitra, T.T. Yoshikawa, J. Hansen, I. Nilsson-Ehle, W.J. Palin, M.C. Schotz and L.B. Guze, Clin. Chem., 23 (1977) 2275.
7 J. Marples and M.D.G. Oates, J. Antimicrob. Chemother., 10 (1982) 311.
8 L. Essers, J. Chromatogr., 305 (1984) 345.
9 S.-E. Back, I. Nilsson-Ehle and P. Nilsson-Ehle, Clin. Chem., 25 (1979) 1222.
10 R.W. Giese, Clin. Chem., 29 (1983) 1331.