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Note

Determination of gentamicin components C_{1a}, C_2 and C_1 in plasma and urine by high-performance liquid chromatography

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Aminoglycoside antibiotics are used extensively in medicine but have serious drawbacks with respect to ototoxicity and nephrotoxicity, particularly when used in patients with renal failure. Consequently, drug doses need to be carefully adjusted in these patients. The mechanisms for this toxicity are unknown, and no single factor has been shown to be clearly responsible. Frequent and accurate monitoring of drug concentrations in biological fluids is necessary to enable proper dose adjustments in order to achieve optimum therapeutic levels compatible with safety.

Gentamicin is a widely used aminoglycoside antibiotic, having a broad spectrum of activity against both gram-positive and gram-negative bacteria. It is a complex of three major components designated C_{1a}, C_2 and C_1 [1, 2] possessing similar antibacterial activities [3]. In a commercial preparation such as Garamycin® these components are present in roughly equal proportions [4].

Information available on gentamicin is mainly data for the complex as a whole, as the kinetic disposition of the individual fractions has been incompletely elucidated [5—7]. A variety of methods are available for the quantitative estimation of gentamicin in plasma [8]. Recently, sensitive high-performance liquid chromatographic (HPLC) procedures have also been reported. These methods involve derivatization with either *o*-phthalaldehyde [9—11], Dns chloride [12, 13] benzene sulfonyl chloride [14], 1-fluoro-2,4-dinitrobenzene [15] or fluorescamine [16], and quantitation by fluorescent or UV detection. Only three of the reported procedures claim to resolve the individual

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components of gentamicin with sample clarification being achieved either by passing samples through CM-Sephadex resin [10], silicic acid column [9] or by solvent extraction after precipitation of plasma proteins [11].

Laboratories are still in need of a rapid method for the analysis of gentamicin components, requiring small volumes of plasma or urine samples. We report here a simple, rapid and sensitive micromethod for the quantitative estimation of the individual gentamicin components in 100 μl of plasma or serum. Furthermore, an assay method for gentamicin components in urine is also presented, which employs the same HPLC system as plasma, but with a preliminary extraction procedure using Sep-Pak C_{18} cartridges. The latter procedure involves a rapid method which eliminates solvent extraction and still produces clean chromatograms.

METHODS

Chemicals and reagents

Gentamicin (USP reference standard) and the purified gentamicin components C_{1a}, C_2 and C_1 were kindly provided by Dr. J. Chiz (Schering Canada, Pointe Claire, Canada), the labelled potencies being 586, 788, 641 and 620 μg/ml of powder, respectively. *o*-Phthalicdicarboxaldehyde (OPA) was obtained from Aldrich (Milwaukee, WI, U.S.A.) and 2-mercaptoethanol from Eastman-Kodak (Rochester, NY, U.S.A.). Water was deionized and distilled; organic solvents were of chromatographic quality and all other chemicals were of reagent grade.

Chromatographic system

Separation was performed with a liquid chromatograph (Model ALC/GPC 204; Waters Assoc., Milford, MA, U.S.A.) equipped with a Waters M6000A solvent delivery system and a Waters UK6 universal injector. The column (25 cm × 30 mm I.D.) consisted of reversed-phase LiChrosorb RP-8 5 μm packing (E. Merck, Darmstadt, G.F.R.) supplied by Brownlee Labs. (Santa Clara, CA, U.S.A.) and a guard column dry-packed with Co:Pell ODS (Whatman, Clifton, NJ, U.S.A.) was used. The eluent was monitored with a Schoeffel Model FS 970 fluorometer (Schoeffel Instruments, Westwood, NJ, U.S.A.). Excitation wavelength was set at 340 nm with a Kv 418 emission cut-off filter. The mobile phase consisted of 70% acetonitrile in tris(hydroxymethyl)aminomethane (1 g/l adjusted to pH 3.0 with 1 *M* hydrochloric acid). This solution was passed through a 0.45-μm filter and degassed under vacuum; the flow-rate was maintained at 1.5 ml/min and chromatography was performed at room temperature. Extractions of urine samples were performed using Sep-Pak C_{18} cartridges (Waters Assoc.).

Derivatizing reagent

A 100-mg amount of OPA was dissolved in 1 ml methanol to which was added 0.2 ml mercaptoethanol and then made up to 20 ml with 0.4 *M* potassium borate buffer at pH 10.4. The solution was prepared every week and in an air-tight amber bottle at 4° C.

Extraction procedure and standard curve preparation

For calibration, an aqueous solution containing equal amounts of C_{1a}, C_2 and C_1 base was added to plasma and urine blank samples to provide concentrations in the range 0.25—25 μg/ml.

Plasma

To each sample of plasma or serum (100 μl in duplicate) in a 1.5-ml polypropylene micro test tube (Eppendorf) was added Tris buffer (20 g/l; 100 μl) and acetonitrile (600 μl). The solutions were mixed by vortexing (10 sec) and the precipitate sedimented by centrifugation (2000 *g*, 2 min). The proteins precipitated as a pellet at the bottom of the micro test tube and the supernatant was poured into another 1.5-ml micro test tube containing chloroform (600 μl). Following mixing and centrifugation, the top aqueous layer (100 μl) was transferred into a 500-μl micro test tube to which was added OPA solution (100 μl). The solutions were then vortexed and ethyl acetate (200 μl) was added. After mixing and centrifuging, 10—30 μl of the top ethyl acetate layer containing the derivatised gentamicin components, were injected onto the liquid chromatograph.

Urine

Sep-Pak C_{18} cartridges were conditioned by flushing with methanol (5 ml) followed by distilled water (5 ml). Urine (1 ml) was then passed through the cartridge and this was washed with distilled water (10 ml), methanol—distilled water (1:1) (10 ml) and methanol (10 ml). Flow-rate for the sample and eluting solvents through the cartridge was maintained at between 5—10 ml/min. The gentamicin components were eluted by passing 2 ml of 10% ammonia solution (28—30% NH_3, v/v) in methanol, discarding the first 0.5 ml and collecting the final 1.5 ml into micro test tubes (2 ml capacity). After evaporating off the methanol using a stream of nitrogen, the residue was reconstituted with distilled water (100 μl), derivatized with OPA solution (100 μl) and the derivatives extracted into ethyl acetate (200 μl), 10 μl of which were injected onto the column.

Radioimmunoassay

This present HPLC method was compared with a standard radioimmunoassay technique using the Beckman RIAphase Gentamicin Kit (Beckman Instruments, Fullterton, CA, U.S.A.). This liquid phase kit is based on the double-antibody test system using [^{125}I]gentamicin and specially designed for the quantitative radioimmunoassay of gentamicin.

RESULTS

Linearity and sensitivity

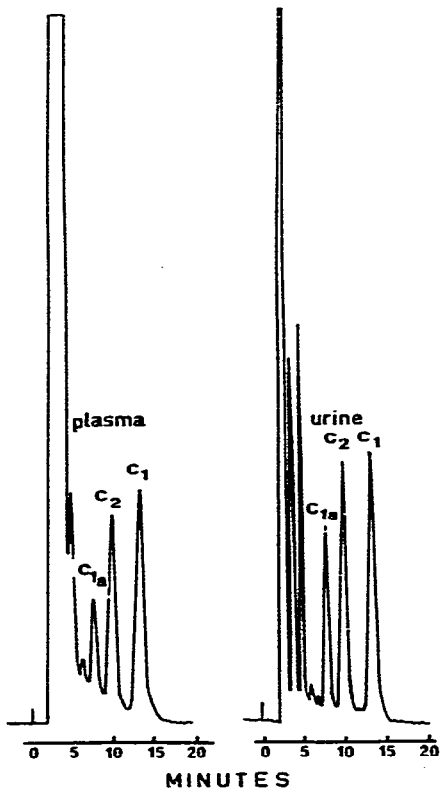
Fig. 1 shows typical chromatograms of human plasma and urine from a volunteer who received a single infusion dose of gentamicin (1.7 mg/kg). The three components of gentamicin, C_{1a}, C_2 and C_1 eluted as distinct peaks with retention times of 8, 10 and 14 min, respectively. There were no interfering peaks when compared to chromatograms of blank plasma and urine samples

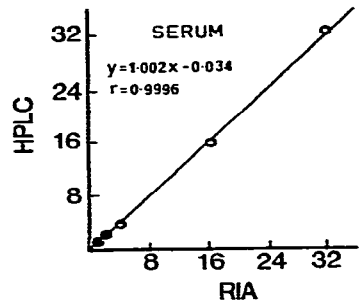
taken prior to administration of gentamicin, and analyzed by the procedures described.

Standard calibration curves for the three gentamicin components in both plasma (range 0.5—10 mg/l) and urine (range 0.5—5 mg/l) were linear (r = 0.99). The lower limit of sensitivity in both plasma and urine was 0.5 mg/l. The upper limit in plasma was as high as 40 mg/l but urine samples above 10 mg/l did not give reasonable duplicates (with 10%). This problem was resolved by either diluting urine samples above this concentration or by reducing sample volume in the Sep-Pak extraction.

Recovery, precision and specificity

Both plasma and urine recovery when compared to corresponding aqueous standards was consistent and greater than 85% for all the three components of gentamicin. The mean intra-assay coefficient of variation for all three fractions at 0.5 mg/l was 10.1% (range 6.0—14.4%) and 2.4% (range 2.2—2.5%) at 10 mg/l. OPA solutions were used for five consecutive days and new calibrations were constructed every week using freshly prepared OPA solutions; the inter-assay coefficient of variation never exceeded 8%.





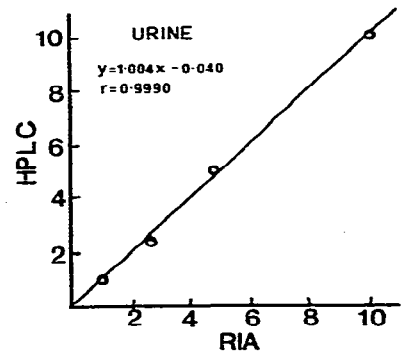


Fig. 1. Chromatograms of human plasma and urine from a volunteer who received gentamicin (1.7 mg/kg) by single infusion (20 min). The blood sample was taken 30 min after infusion and the urine sample was a 36—48-h collection.

Fig. 2. Linear regression analysis of total gentamicin concentration in human serum and urine as determined by the radioimmunological and HPLC procedures.

Our HPLC procedures for plasma and urine were compared with a standard radioimmunoassay method for total gentamicin. Fig. 2 illustrates the conformity between the two procedures. Values for total gentamicin using the HPLC method are expressed as the sum of the three components of gentamicin.

Comparison with standard aqueous solutions of drugs and other antibiotics which are frequently used concomitantly with gentamicin showed that kanamycin, amikacin, tobramycin, clindamycin, chloramphenicol, cloxacillin, cefazoline, penicillin G and netilmicin did not interfere with the assay. Netilmicin upon derivatization with OPA showed a single peak with a retention time of 17 min. Since it is a dehydrogenated analogue of gentamicin C_{1a} and due to its close chemical similarity with other gentamicin components, it serves as a useful internal standard.

Effect of pH

In order to optimize chromatographic conditions, the mobile phase (70% acetonitrile in Tris 1 g/l) was adjusted with 1 *M* hydrochloric acid to various pH values. A standard aqueous solution containing equal amounts (5 mg/l) of the three components of gentamicin was derivatised with OPA and extracted into ethyl acetate as described in Methods. After equilibration of the various mobile phases, 10 μl of the ethyl acetate layer were injected onto the chromatograph. Fig. 3 illustrates the results obtained. Clearly the mobility of gentamicin C_1 was more affected by pH than either C_{1a} or C_2. For example, at pH 7.0 the retention time of gentamicin C_1 was around 40 min. Hence the mobile phase was maintained at pH 3.0 for optimal resolution.

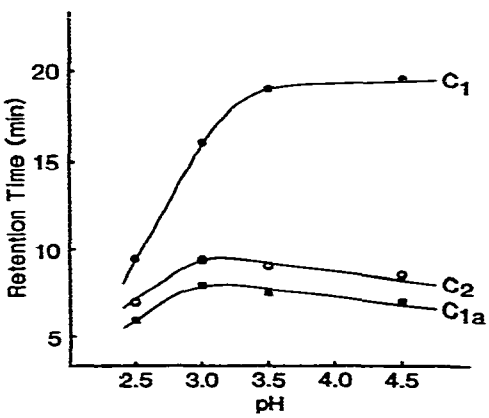


Fig. 3. Effect of pH on the mobility of gentamicin components. The mobile phase consisted of 70% acetonitrile in Tris (1 g/l) which was adjusted to the required pH with 1 *M* hydrochloric acid. Flow-rate was maintained at 1.5 ml/min.

DISCUSSION

We have described an HPLC method for the quantitative separation and analysis of gentamicin C_{1a}, C_2 and C_1 in either plasma or urine. OPA was chosen as the derivatising reagent since the reaction occurs rapidly at room temperature in an aqueous media which can be buffered appropriately for optimising reaction conditions.

There have been many HPLC methods for determination of gentamicin reported in the literature [9—16] utilizing various derivatising reagents and also differing in chromatographic conditions. The procedure reported here is simple, can be performed rapidly using relatively small sample volumes. Furthermore, it allows the simultaneous monitoring of the three major fractions of gentamicin in both urine and plasma. The time taken for elution of a complete chromatogram is less than 15 min and hence many samples can be analyzed within a day. The plasma method has an added advantage in that the assay can be conducted on a microscale level, thus requiring only small volumes of blood samples for detailed pharmacokinetic analysis in infant or adult patients.

The use of netilmicin as an internal standard in both assays minimizes variations in detector response or any chromatographic changes. It also compensates for injection and sample preparation errors.

Extraction using the Sep-Pak C_{18} cartridges has several advantages over solvent precipitation and extraction procedures. It provides chromatographically cleaner extracts and removes any material which would otherwise adsorb irreversibly to the column matrix thus prolonging the lifetime of the analytical column. Sensitivity can also be improved simply by increasing sample size.

The Sep-Pak extraction development was arbitrary and the best eluting solvent was found to be 10% ammonia solution in methanol. This combination was used previously [2] for the elution of tritiated gentamicin from urine after adsorption on an Amberlite XAD-2 resin column. By comparing subsequent extractions after 0.5-ml elutions, the first 1.5 ml after the void volume was found to elute most of the gentamicin applied to Sep-Pak cartridges. The accuracy and reproducibility was found to be within acceptable limits. However, it was necessary to evaporate this fraction to dryness in order to remove any ammonia which might react with OPA. Sep-Pak cartridges were used at least twice without loss in performance, despite the manufacturer's recommendations.

The assay is presently being used to study the kinetic disposition of the individual fractions of gentamicin in urine and plasma after single and multiple dosing in humans.

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