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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINATION OF GENTAMICIN IN BIOLOGICAL FLUIDS

S.E. WALKER*,* and P.E. COATES

The Faculty of Pharmacy, University of Toronto, Toronto, Ontario (Canada)

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SUMMARY

A selective and sensitive method for the determination of gentamicin in plasma and urine by high-performance liquid chromatography has been developed. Following deproteinization, the gentamicin is reacted with fluorescamine to produce a fluorescent derivative. This reaction mixture is directly chromatographed on a cation-exchange column using as mobile phase acetonitrile—phosphoric acid (7:3). The gentamicin components elute as a single peak. Using 0.1 ml of plasma, quantitation of gentamicin concentrations as low as 1 mg/l are possible. Possible interference from other aminoglycosides and antibiotics is discussed.

INTRODUCTION

A variety of techniques are available for the quantitative determination of gentamicin and other aminoglycosides in biological fluids. Until recently sensitive high-performance liquid chromatographic (HPLC) methods were not available since gentamicin does not possess an absorbing chromophore and, therefore, does not lend itself to UV detection. This necessitates derivatization of gentamicin to allow detection with the required sensitivity. Aminoglycosides have been derivatized with *o*-phthalaldehyde (OPA) [1—3], dansyl chloride [4] and 1-fluoro-2,4-dinitrobenzene (FDNB) [5], all of which are suitable for HPLC analysis using either fluorescent or UV detection. Those assays using OPA utilize a normal column chromatographic technique for sample preparation. Thereafter, HPLC results in multiple peaks which correspond to the various components [1—3]. Peng et al. [4] report derivatization of gentamicin with dansyl chloride. Their derivatization procedure re-

*Present address: Department of Pharmacy, Sunnybrook Medical Centre, 2075 Bayview Avenue, Toronto, Ontario M4N 3M5, Canada.

quires heat and is time-consuming. Also, in our laboratory, duplication of their reaction and chromatographic conditions resulted in variable chromatograms with more than their reported two peaks. The derivatization procedure using FDNB is the most time-consuming, requiring incubation of gentamicin with FDNB at 80°C for 45 min [5].

Quantitation of the individual components may be important from a research aspect. However, in the clinical situation quantitation of the individual components or combinations thereof, is of little value.

Fluorescamine is a third derivatizing agent which produces fluorescent products [6—8]. Fluorescamine has advantages over the other two fluorescent derivatizing agents, in that (1) the nucleophilic reaction with an amine proceeds at a much faster rate, being complete within seconds [6] and (2) excess fluorescamine is hydrolyzed to non-fluorescent products [6].

We report the development of an HPLC assay which utilizes fluorescamine as the derivatizing agent. On chromatography the derivatized gentamicin yields only a single product, which we feel is an advantage. The assay has been used to analyse serum and urine samples obtained following a single intravenous dose of gentamicin to rabbits.

MATERIALS AND METHODS

Reagents

The components of gentamicin sulphate and the mixture (referred to as gentamicin) were donated by Schering (Bloomfield, NJ, U.S.A.). The labelled potencies are 620, 641, 788 and 551 μg/mg of powder for C_1 C_{1a} C_2 and gentamicin respectively. Fluorescamine [4′-phenylspiro(furan-2(3H),1′-phthalal)-3,3′-dione] was obtained from Sigma (St. Louis, MO, U.S.A.). All organic solvents were glass distilled (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). Other chemicals were reagent grade.

Chromatographic system

The chromatographic system consisted of a solvent delivery pump (Altex Model 100A), connected to a sample loop injector (Altex Model 905-42) fitted with a 175-μl sample loop. A chromatographic fluorescence detector (Schoeffel FS 970) monitored the effluent. The excitation wavelength was set at 275 nm and a KV 418 emission cut-off filter was used to select the fluorescence for detection. The mobile phase consisted of acetonitrile—phosphoric acid (5 g/l) (7:3). This was pumped through a cation-exchange column (Partisil SCX; 250 × 3.6 mm, particle size 10 μm) at 2 ml/min. The chromatograms were recorded on a 10-mV potentiometric chart recorder (Hewlett-Parkard Model 7101B-06-07) at a chart speed of 0.2 in./min.

Analytical variables affecting the gentamicin—fluorescamine reaction in water

Initially, the reaction between gentamicin, dissolved in water and fluorescamine, dissolved in acetone, was investigated with respect to the following variables: (1) pH of the reaction mixture; (2) buffer strength of the reaction mixture; (3) amount of fluorescamine required. Optimum conditions were evaluated on the basis of peak height using the aforementioned chromatographic conditions.

Sample preparation

Plasma. In a clean dry 15-ml test tube, 100 µl of a plasma sample and 800 µl of 0.05 mol/l potassium phosphate (pH 4.5) were mixed with 50 µl of 1.0 mol/l sodium hydroxide. After briefly vortexing, 2.5 ml of acetonitrile are added. The tube was again vortexed and then centrifuged at RFC = 2500 *g* for 2 min. After centrifugation the supernatant was decanted into a second tube containing 2.0 ml of methylene chloride. This tube was vortexed for 30 sec and then centrifuged for 2 min. Following centrifugation, 500 µl of the upper aqueous layer were pipetted into a third tube with 40 µl of 0.1 mol/l hydrochloric acid. The tube was then vortexed and during mixing, 200 µl of acetone containing 240 µg of fluorescamine were added. Fifty microlitres of the resultant clear yellow solution were chromatographed. Standards of 1, 2, 5, 10, 15, 30 and 40 mg/l were prepared and treated in an identical fashion to that described above.

Gentamicin in human plasma can be quantitated by the above method. However, prior extraction with methylene chloride is not necessary.

Urine. Into a clean dry 15-ml test tube, 50 µl of a urine sample were mixed with 500 µl of 0.05 mol/l phosphate buffer (pH 7.35). During mixing, 200 µl of acetone containing 240 µg of fluorescamine were added and 50 µl of the resulting clear yellow solution were injected into the liquid chromatographic system.

Rabbit study

Rabbits were weighed and placed in restraining cages. Angiocaths (Deseret; Sandy, UT, U.S.A.) were placed in the marginal veins of each ear. Under mesmerization, a Foley catheter (No. 8 Fr. Paediatric Foley Catheter; Acmi, Sullivan, IN, U.S.A.) was inserted into the bladder via the urethra. The dose of gentamicin (Cidomycin, 3.5 mg/kg; Roussel, Montreal, Canada) was administered via one of the ear veins, thereafter plasma and urine samples were taken at selected times over a 6-h period.

RESULTS

Reaction conditions in aqueous solution

The reaction between gentamicin (in aqueous solution) and fluorescamine (dissolved in acetone), was investigated with respect to pH, buffer strength and the amount of fluorescamine required. From these investigations, it appeared that gentamicin reacts optimally with fluorescamine between a pH of 7.2 and 8.0 when the molar strength of the phosphate buffer (pH 7.35) exceeds 0.03 *M*.

The amount of fluorescamine required to give greatest peak height and linear standard curves was determined to be about 10 *M* fold. This 10 *M* fold excess is required (to maintain linearity in a standard curve), regardless of pH, or amount of gentamicin. Once the molar ratio of fluorescamine to gentamicin falls below 10 the standard curve begins to deviate from linearity.

Using these optimum conditions, equal concentrations of each of the three major components of gentamicin, C_1 C_{1a} and C_2 were derivatized and chromatographed individually and together. When chromatographed individually, the peak heights and retention times were identical. When chromatographed together the components eluted as a single peak.

Recovery from plasma

The analysis of gentamicin utilizing the above-mentioned optimum reaction conditions and only an initial protein precipitation step, using acetone, proved to be sufficient for human plasma. However, attempts to reproduce these results with gentamicin in rabbit plasma yielded less than adequate recovery. This problem was circumvented by utilizing the extraction mentioned under Methods.

Since it was expected that amines in plasma would react with fluorescamine, the 10-fold molar ratio was re-investigated for a 100-μl sample of rabbit plasma containing 40 mg/l of gentamicin sulphate. The results, plotted in Fig. 1, demonstrate that at least an 80—100 fold molar excess is required to give maximum peak height and, therefore, linear standard curves in the concentration range from 0—40 mg/l of gentamicin sulphate.

Evaluation of protein precipitants (acetone and acetonitrile) and back-extracting solvents (diethyl ether, ethyl acetate, hexane, and methylene chloride) led to the choice of acetonitrile—methylene chloride as the best protein precipitant/back-extracting solvent combination. In addition to extracting the greatest amount of interfering substance, this combination of solvents leaves the aqueous layer (containing gentamicin) above the organic layer, making it easier to remove.

Interference from other drugs was tested by subjecting them to the procedures developed for gentamicin and chromatographing the products. Of the drugs tested (quinine, quinidine, amiloride, tobramycin, amikacin, netilmicin, ampicillin, cloxacillin, ticarcillin and cefazolin) only tobramycin and netilmicin produced a peak sufficiently close to the gentamicin peak to interfere with its quantitation. Retention times for gentamicin, tobramycin and netilmicin were 2.25, 2.42 and 2.25 min respectively. The recovery of gentamicin from a 100-μl plasma sample relative to water is about 93%.

Comparison of a series of standard curves for gentamicin in rabbit plasma,

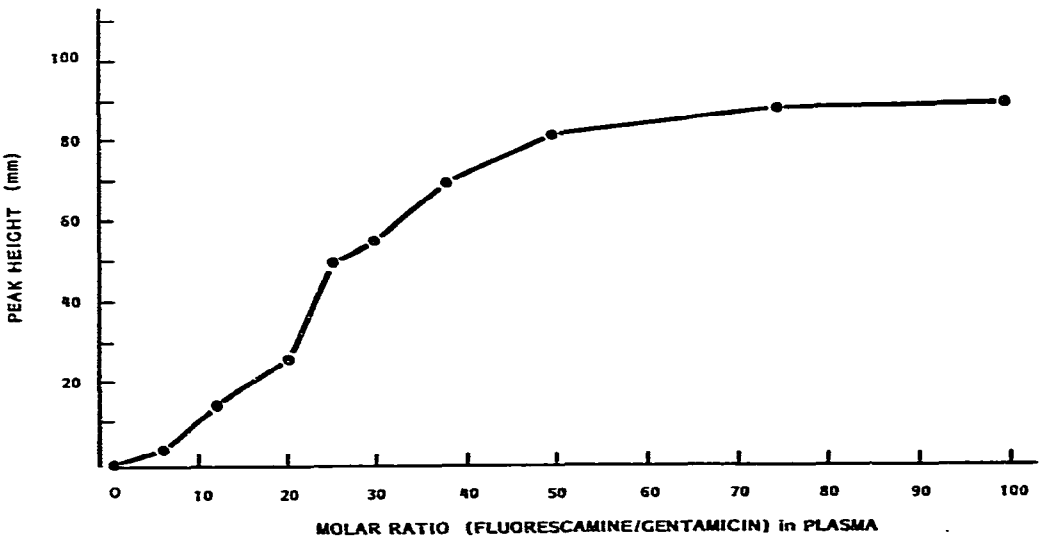

Fig. 1. Investigation into the optimum molar ratio of fluorescamine/gentamicin required with a 100-μl rabbit plasma sample.

produced on different days, demonstrates the reproducibility of the overall assay procedure (Table I). The intra-assay coefficient of variation, which never exceeded 3.5%, and the coefficients of determination (r^2) indicate that gentamicin concentrations in plasma produce linear and reproducible standard curves in the range from 0 to 40 mg/l. Replicates of unknowns, analysed on different days, demonstrated that the inter-assay coefficient of variation is less than 2%. Blank plasma showed no peaks which would interfere with the peak height quantitation of gentamicin (Fig. 2). The limit of sensitivity of this assay is about 1.0 mg/l of gentamicin sulphate. This corresponds to about 5 ng injected.

Standard curves in urine, according to the method described previously, were linear in the range from 0 to 71 mg/l. This, and the reproducibility of the overall assay technique is demonstrated by the low coefficients of variation and r^2 values observed in standard curves (Table II). Blank rabbit urine showed no peaks which would interfere with the peak height quantitation of gentamicin (Fig. 3). The limit of sensitivity of this procedure is about 1.0 mg/l.

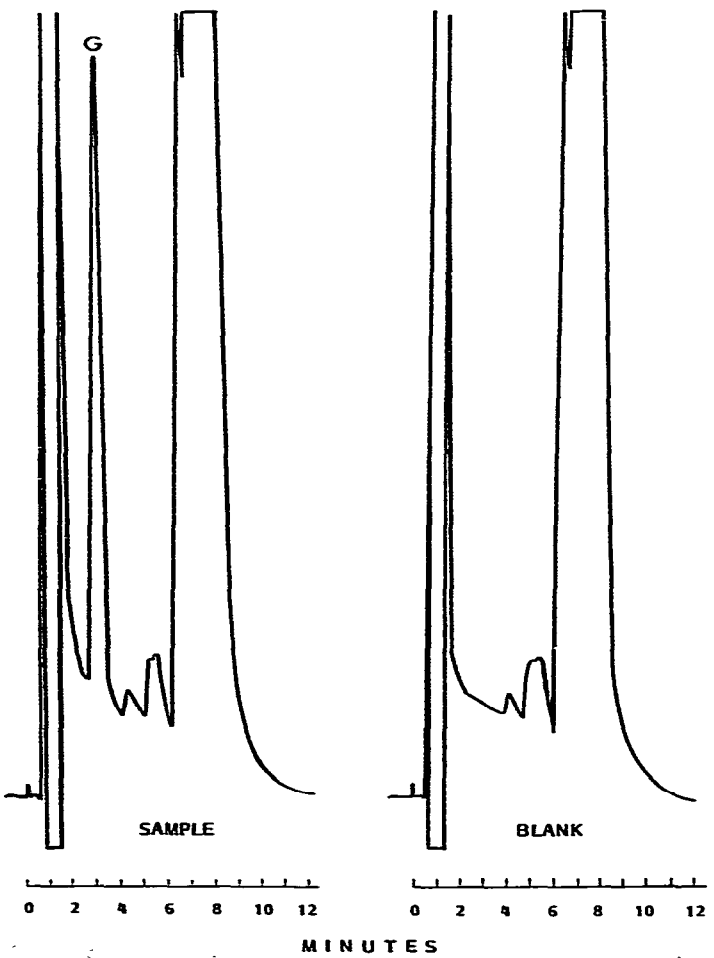

Fig. 2. Typical chromatograms of a blank rabbit plasma sample and one containing 19.1 μg/ml of gentamicin (G).

TABLE I

COMPARISON OF STANDARD CURVES FOR GENTAMICIN SULPHATE IN RABBIT PLASMA

Curve No.	Curve regression parameters			Coefficient of variation (%) at various gentamicin concentrations*		
	r^2	Slope	Intercept	Concentration (μg/ml)		
				5.93	11.68	19.10
1	0.9987	3.25	—0.21	2.56	0.80	0.65
2	0.9994	4.26	—0.79	2.47	2.89	1.09
3	0.9999	2.59	0.17	2.24	1.94	1.43
4	0.9982	2.49	0.73	0.00	3.45	2.11
5	0.9996	3.81	0.25	3.27	1.12	2.69
6	0.9977	3.17	—0.65	3.02	1.02	0.00
7	0.9996	2.86	0.24	0.00	0.86	2.80

*Coefficient of variation based on at least four replicates.

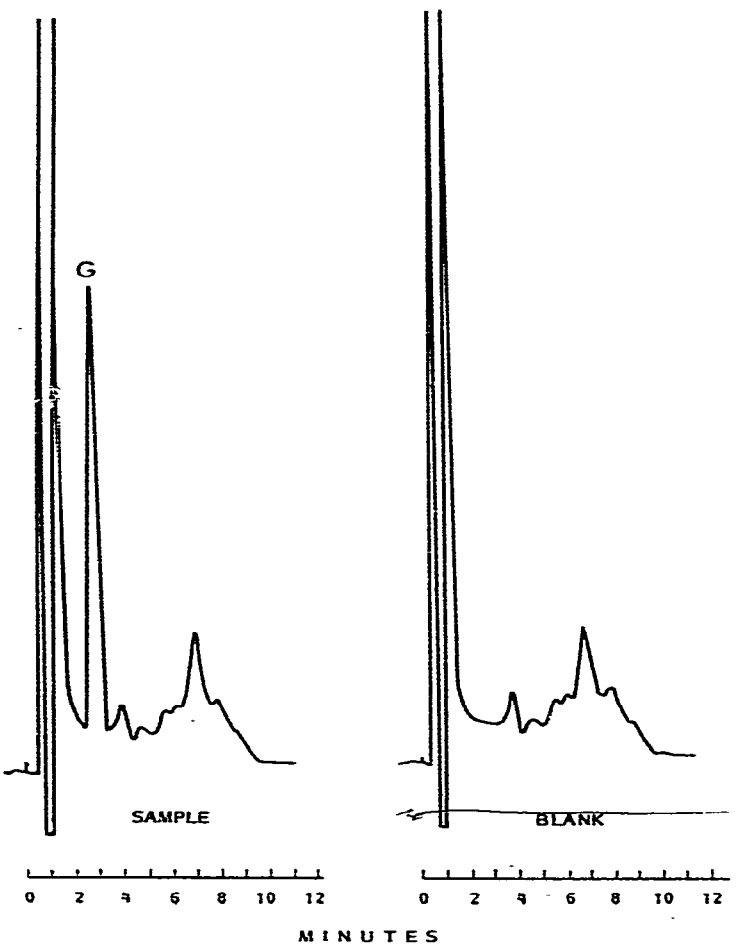

Fig. 3. Typical chromatograms of a blank rabbit urine sample and one containing 52.3 μg/ml of gentamicin (G).

This assay has been used extensively to monitor gentamicin concentrations in plasma and urine in rabbits. These rabbits were given approximately 3.5 mg/kg of gentamicin as an intravenous bolus. Gentamicin shows a biexponential decline with a mean half-life ($t_{½\beta}$) of about 54 min (21 experiments) (Fig. 4). This agrees closely with the half-life reported by Peng et al. [4] using their dansyl chloride derivatization assay.

TABLE II

COMPARISON OF STANDARD CURVES FOR GENTAMICIN SULPHATE IN RABBIT URINE

Curve No.	Curve regression parameters			Coefficient of variation (%) at various gentamicin concentrations*			
	r^2	Slope	Intercept	Concentration (μg/ml)			
				17.00	36.46	52.31	71.00
1	0.9997	2.09	−0.64	0.00	1.88	3.28	0.39
2	0.9963	2.13	1.60	0.92	0.00	0.00	0.54
3	0.9995	2.33	0.55	0.00	2.38	0.47	0.94
4	0.9989	1.90	−0.10	2.32	1.00	0.70	1.06

*Coefficient of variation based on at least four replicates.

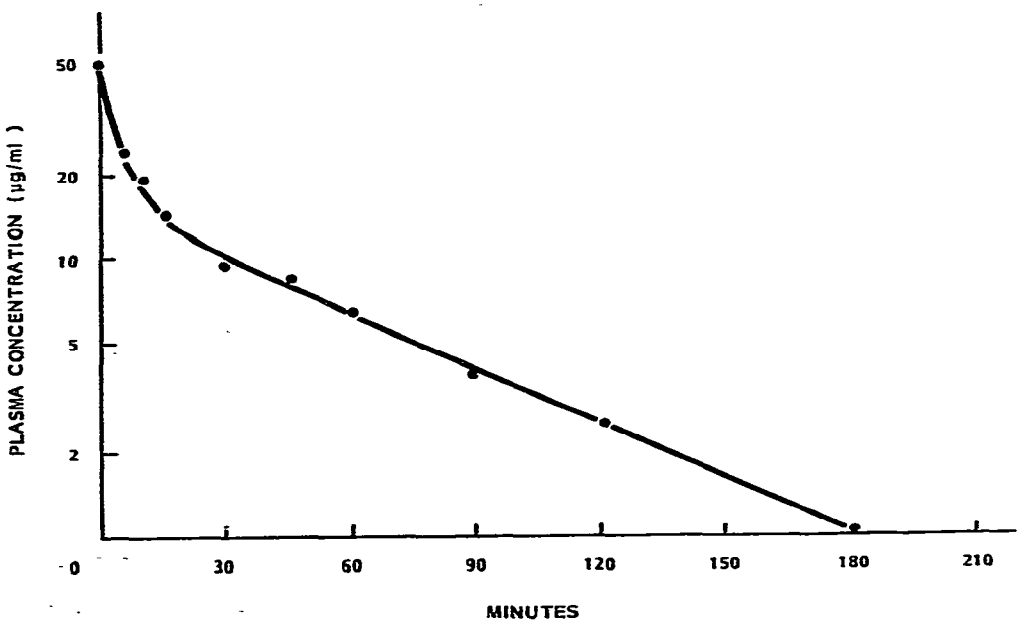

Fig. 4. Plasma concentration—time profile in a 4-kg rabbit following a 3.5 mg/kg dose of gentamicin given as a bolus over 30 sec.

DISCUSSION

We have described a HPLC procedure for the determination of gentamicin in rabbit and human plasmas, and in rabbit urine. In each case the quantitation of gentamicin was achieved after derivatization with fluorescamine.

After the establishment of optimum conditions (when working with human plasma), a protein precipitation step was required before buffer addition and derivatization. However, with rabbit plasma derivatization immediately following protein precipitation resulted in extremely low recovery of gentamicin. The reason for this effect is unknown but it is not unlike the situation reported by Peng et al. [4]. Dilution of the plasma with phosphate buffer alkalinised with sodium hydroxide circumvented this problem. It also produced a very dilute supernatant with respect to gentamicin. Back-extraction of this supernatant not only removed some of the acetonitrile, but also basic compounds (possibly amines) which could interfere with the derivatization process. Since gentamicin is very soluble in water and insoluble in organic solvents, no gentamicin was lost during this procedure.

The back-extraction procedure did not remove all of the endogenous amines from the plasma as evidenced by the large peak which elutes from the column between 6 and 8 min. This large peak remained regardless of the extracting solvent used. Consequently, the elution time for each sample was significantly increased.

The arrival at our chromatographic system was quite pragmatic. The reaction of fluorescamine with an amine produces a carboxyl group on the fluorescamine moiety. Initially, assay development was centred around this carboxyl group using an anion-exchange column and a mobile phase of pH 6—7. However, the appearance of broad, asymmetrical peaks suggested the presence of unreacted amino groups. Therefore, we selected to use a cation-exchange column with a solvent of low pH to suppress ionization of the carboxyl group and to effect separation on the basis of protonated amino groups. Thus, we developed a chromatographic system which produced sharp symmetrical peaks due to gentamicin from human and rabbit plasmas, and rabbit urine.

HPLC methods for gentamicin have the advantages of rapid turnover time, specificity and sensitivity. Furthermore, we feel that this assay has a certain advantage over other presently available HPLC methods, in that the gentamicin components elute as a single peak. This should be particularly useful in a clinical setting. Also, this method can be used directly to assay other aminoglycosides. However, slight modifications (in both reaction and HPLC conditions) may be necessary to optimise quantitation of these compounds.

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