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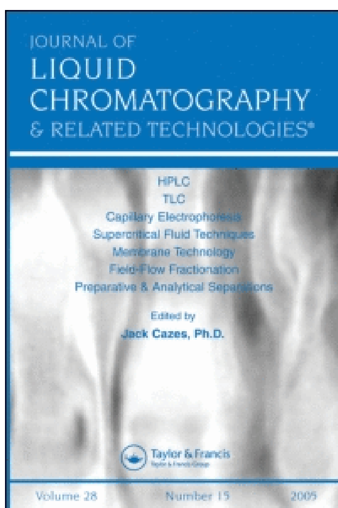

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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To cite this Article Cabanes, A. , Cajal, Y. , Haro, I. , Anton, J. M. Garcia , Reig, F. and Arboix, M.(1991) 'Gentamicin Determination in Biological Fluids by HPLC, Using Tobramycin as Internal Standard', Journal of Liquid Chromatography & Related Technologies, 14: 10, 1989 — 2010

To link to this Article: DOI: 10.1080/01483919108049669

URL: http://dx.doi.org/10.1080/01483919108049669

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JOURNAL OF LIQUID CHROMATOGRAPHY, 14(10), 1989-2010 (1991)

GENTAMICIN DETERMINATION IN BIOLOGICAL FLUIDS BY HPLC, USING TOBRAMYCIN AS INTERNAL STANDARD

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ABSTRACT

An improved procedure to quantify gentamicin in biological fluids is presented. The antibiotic is isolated from plasma and urine by using a silica column, and measured by reversed-phase chromatography. Pre-column derivatization with o-phthalaldehyde to form fluorescent products for detection is used. The method can accurately measure 0.3 mg of gentamicin per liter, and standard curves showed a linear response in plasma and urine at concentrations ranging from 0 to 10 mg/liter. The different processes described in the literature for aminoglycoside isolation are discussed and evaluated with reference to the present results. Moreover, the

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use of several antibiotics as internal standards is described. This procedure is useful to quantify plasma and urine concentrations of gentamicin administered free or encapsulated in liposomes.

INTRODUCTION

Aminoglycoside antibiotics are valuable in treatment of serious Gram-negative bacillary infections (1). However, their use is limited due to potential renal and otovestibular toxicity. While renal damage is reversible and well controlled during clinical treatment, ototoxic side effects can cause permanent loss of inner ear function (2).

Monitoring of blood levels has been recommended to assure effective therapy and avoidance of toxicity with this drug, particularly when used in patients with renal failure.

Several methods based on high-performance liquid chromatography have been developed to quantify gentamicin in biological fluids. These methods involve isolation of gentamicin from serum or urine, chemical derivatization to enhance ultraviolet absorbance or to produce fluorescent compounds, and resolution of the three major components.

Deproteinization of biological samples been described by precipitation with acetonitrile (3,4) or

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trichloroacetic acid, ion exchange gel chromatography (5,6), methanol treatment (7) or column chromatography.

Due to the lack of absortion in the ultraviolet range it is necessary to derivatize gentamicin molecules for detection by HPLC. The reagents most frecuently used are 1-fluoro-2,4-dinitrobenzene (8), o-phthalaldehyde (3,9), fluorescamine (10) and dansyl chloride (3).

To monitor fluorescence there are two options, pre-column or post-column derivatization. Pre-column derivatization yield samples containing fewer interfering substances.

Moreover, the use of an internal standard would be of great help minimizing errors due to extraction and derivatization processes as well as variations in detector response.

In our hands none of these steps are devoid of practical problems being the reproducibility and the choice of a suitable internal standard the most importants.

We report here a simple, rapid and sensitive method for the quantitative estimation of the individual components of gentamicin, using tobramycin as internal standard. This method involves a simple

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extraction procedure, pre-column derivatization with OPA, separation by reversed-phase chromatography, and measurement of the fluorescent products. This procedure is valid also to quantify gentamicin encapsulated in liposomes.

MATERIALS AND METHODS

Reagents

Gentamicin sulphate (Danish Powder Tabletting Factory Ltd. ApS., the labelled potency being 638,4 µg/mg of drug powder) was gently supplied by Infavet. Tobramycin was from Sigma (potency 940 µg/mg); o-phthalaldehyde and sodium heptanesulphonate were obtained from Scharlau.

Methanol, acetonitrile, 2-mercaptoethanol, ethanol and Silica Gel 60 (particle size 0.040-0.063 mm) were from Merck.

Water was deionized and distilled. All other chemicals were of reagent grade and are comercially available.

Instrumentation

Analyses were carried out in a Merck-Hitachi HPLC system (L-6200 intelligent pump) equipped with a

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chromatographic integrator Merck-Hitachi D-2000. Samples were injected using a Rheodyne 7125 injection valve with a 25 µl-loop.

The eluent was monitored with a Perkin Elmer LS-1 spectrofluorimeter; chromatographic separation was performed on columms (125 mm x 4 mm) packed with Lichrosorb RP 18, particle size 5 µ (Merck).

The mobile phase consisted of 80% methanol and 20% acetic acid (10% aqueous), containing 4.5 g of sodium heptanosulphonate per liter for the assay of aqueous samples; and 75% methanol, 25% acetic acid (10% aqueous) for serum and urine samples.

The eluents were filtered (0.22 µm, Millipore filter) and degassed under vacuum; the flow rate was mantained at 1 ml/min and chromatography was performed at room temperature. Excitation wavenlength was set at 340 nm and KV 418 nm filter was used for the emission.

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o-phthalaldehyde reagent (OPA)

Borate buffer was prepared from boric acid (0.4 M) and adjusted to pH 10.5 with 40 % aqueous sodium hydroxide.

50 mg of o-phthalaldehyde were dissolved in 1 ml of methanol and 40 µl of 2-mercaptoethanol were added

to this solution. The mixture was gently shaken till decoloration, and 9 ml of borate buffer (pH 10.5) were added.

This solution was considered useful only when recently prepared (maximum two days) and was stored at 4°C in amber vials.

Internal Standards

A previous comparative study was carried out by adding a known amount of several antibiotics to the plasma or urine samples before any manipulation. The drugs assayed as potential internal standards were: tobramycin, kanamycin and colistin.

The retention time and the shape of the peaks after extraction and derivatization moved us to select tobramycin sulphate as the most suitable molecule for the quantitative analysis of gentamicin. The concentration of the internal standard was adjusted to give a peak having about the same area as a standard containing 3 mg of gentamicin per liter.

It was necessary to modify slightly the chromatographic conditions to obtain a good elution profile. Finally the mobile phase for the serum and urine samples determination was 75% methanol and 25% acetic acid (flow 1 ml/min.)

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Serum treatment and derivatization

Gentamicin was separated from interfering compounds in serum by passage through a silica column. A disposable polypropylene column or a Pasteur pippete were used. The column was filled with 150 mg of dry silica gel 60 and treated with 1.0 ml of water. To 0.5 ml serum, 0.1 ml of tobramycin solution (5 mg/l) used as internal standard were added and diluted to a final volume of 2 ml with distilled water. The solution was mixed by vortexing and introduced into the mini-column; the eluted liquid was discarded. 0.5 ml of OPA solution were added and the derivatized gentamicin and tobramycin were eluted with 1.5 ml of ethanol; this solution was directly injected into the chromatograph.

Urine extraction and derivatization procedures

The urine under test was diluted with distilled water to ten times its volume, and 0.5 ml of diluted sample were analyzed as described for serum determination.

In vitro and in vivo serum samples

Solutions of each antibiotic were prepared in rabbit serum, in rabbit urine and in water (Milli Q).

Four male (3 Kg) healthy New Zealand White rabbits were given 3 mg of gentamicin sulphate/ Kg of body weight via an ear vein, or the equivalent amount of gentamicin entrapped in liposomes (PC/Chol 1:1). Previously a baseline (0 time) blood sample was taken. Additional blood samples were collected from the marginal vein of the opposite ear at 5, 10, 15, 30, 45 minutes and 1, 1.5, 2, 3, 4, 5 and 6 hours after injection. Samples were centrifuged within 2 hours after collection and serum stored at -20°C until assay.

For the urine collection every rabbit was catheterized; the bladder was emptied and urine was obtained every 1 or 2 hours after administration of gentamicin.

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RESULTS

HPLC separation

Fig.1 and Fig. 2 show typical chromatograms for gentamicin from rabbit plasma and rabbit urine, respectively. Although gentamicin has three components, only two of them, namely C1a and C2 with retention times 9 min and 14 min respectively, can be clearly quantified. The retention time of internal standard was 5 min.

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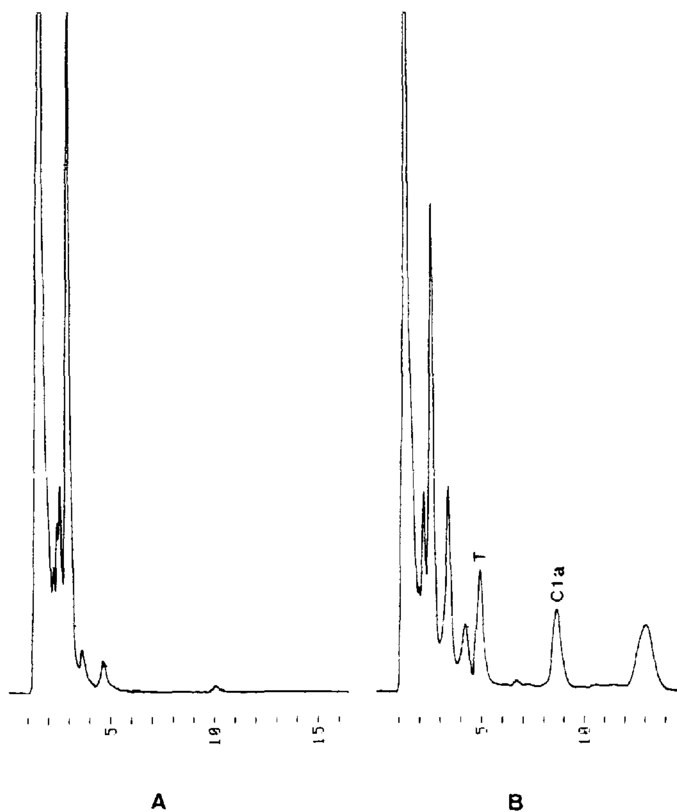

Figure 1.- Chromatograms of: (A) rabbit plasma blank; (B) gentamicin from rabbit plasma containing 5 mg/l.

Rabbit plasma and urine blanks usually showed no interfering peaks.

Many of the peaks that eluted earlier in the chromatograms could not be identified because of detector overloading, but they were probably the OPA excess and the C1 component of gentamicin.

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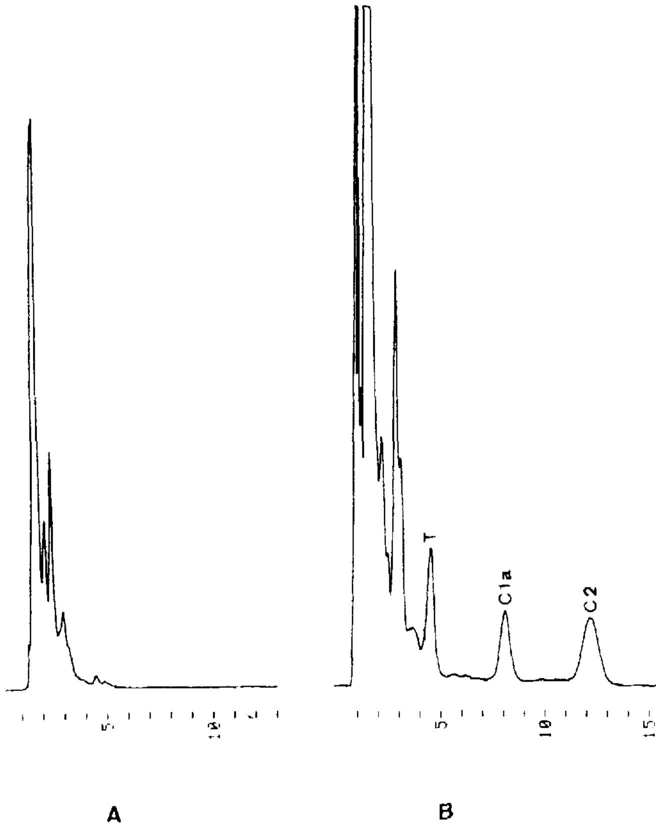

Figure 2.- Chromatograms of: (A) rabbit urine blank; (B) gentamicin from rabbit urine containing 5 mg/l.

Analytical recovery and linearity

The recovery of gentamicin components and tobramycin in plasma and urine samples ranged between 91 to 106%.

Standard curves were prepared for each component of gentamicin from the averages of duplicate analyses

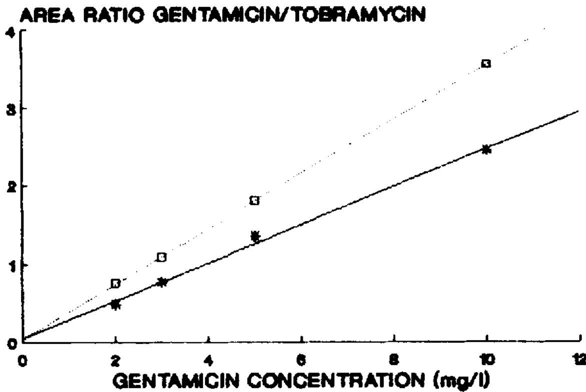

Figure 3.- Linear regression analysis of gentamicin concentration in serum samples and peak areas for the components C1a and C2.
Linear regression equations:
C1a (*) $y = 0.0540 + 0.241\,x$; $r = 0.9970$
C2 (□) $y = 0.0331 + 0.353\,x$; $r = 0.9999$

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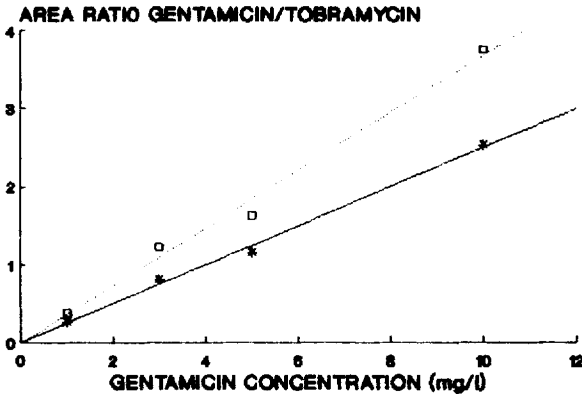

Figure 4.- Linear regression analysis of gentamicin concentration in urine samples and peak areas for the components C1a and C2.
Linear regression equations:
C1a (*) $y = 0.0006 + 0.250\,x$; $r = 0.9977$
C2 (□) $y = -0.0101 + 0.369\,x$; $r = 0.9944$

TABLE 1
Comparison of standard curves for gentamicin in rabbit plasma.

CURVE No	CURVE REGRESSION PARAMETERS Intercept	slope	r
		(C1a component)	
1	0.0288	0.249	0.9957
2	0.0411	0.236	0.9990
3	0.0382	0.235	0.9942
4	0.0707	0.237	0.9960
5	0.0665	0.248	0.9943
		(C2 component)	
1	0.0095	0.349	0.9992
2	0.0381	0.347	0.9995
3	0.0249	0.357	0.9996
4	0.0698	0.353	0.9988
5	0.0875	0.357	0.9980

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of standards prepared in serum and urine (Fig. 3 and Fig. 4).

The lower limit of sensitivity both in plasma and urine was 0.3 mg/l.

Table 1 and Table 2 show a comparison of standards curves for gentamicin sulphate in rabbit plasma and rabbit urine respectvely. Coefficients of correlation (r) indicate that gentamicin concentrations in plasma give linear standard curves in the range from 0 to 10 mg/l.

TABLE 2
Comparison of standard curves for gentamicin in rabbit urine

CURVE No	CURVE REGRESSION PARAMETRES Intercept	slope	r
		(C1a component)	
1	0.0343	0.206	0.9864
2	-0.0139	0.242	0.9906
3	-0.0293	0.274	0.9979
4	-0.0031	0.241	0.9904
5	0.0460	0.238	0.9994
		(C2 component)	
1	-0.0550	0.355	0.9900
2	-0.0437	0.368	0.9887
3	0.0660	0.375	0.9975
4	0.0380	0.362	0.9916
5	0.0066	0.374	0.9977

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Standard curves in urine were linear in the same range.

Precision studies

Table 3 shows the results of the within-day study conducted at four different concentrations; the within-run precision was estimated by analyzing five samples of each concentration on the same day. Day-to-day precision studies were estimated by analyzing three samples of each concentration on five

TABLE 3
Precision studies within day

PLASMA SAMPLES

	target value (mg/l)	mean (mg/l)	sd	cv (%)
C1a	2	2.076	0.013	0.64
	3	3.012	0.145	4.8
	5	5.106	0.275	5.3
	10	10.07	0.301	4.9
C2	2	2.105	0.067	3.17
	3	3.013	0.145	4.8
	5	4.662	0.312	6.6
	10	10.35	0.171	1.6

URINE SAMPLES

	target value (mg/l)	mean (mg/l)	sd	cv (%)
C1a	1	1.092	0.041	3.7
	3	3.181	0.174	5.4
	5	4.943	0.193	3.9
	10	10.034	0.503	5.0
C2	1	0.934	0.054	5.7
	3	3.290	0.096	2.9
	5	5.112	0.017	0.3
	10	10.323	0.310	3.0

• five sample of each concentration were analyzed on the same day.

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TABLE 4
Precision studies between day

PLASMA SAMPLES

	target value (mg/l)	mean (mg/l)	sd	cv(%)
C1a	2	2.176	0.108	4.95
	3	3.064	0.107	3.4
	5	5.10	0.174	3.4
	10	10.21	0.510	4.99
C2	2	2.269	0.089	3.93
	3	3.116	0.155	4.96
	5	4.80	0.238	4.93
	10	10.15	0.424	4.17

URINE SAMPLES

	target value (mg/l)	mean (mg/l)	sd	cv(%)
C1a	1	1.223	0.034	2.7
	3	3.349	0.120	3.5
	5	5.165	0.077	1.5
	10	10.28	0.235	2.2
C2	1	1.190	0.061	5.1
	3	3.391	0.071	2.1
	5	4.956	0.224	4.5
	10	10.21	0.077	0.7

• three samples of each concentration were analyzed on five different days.

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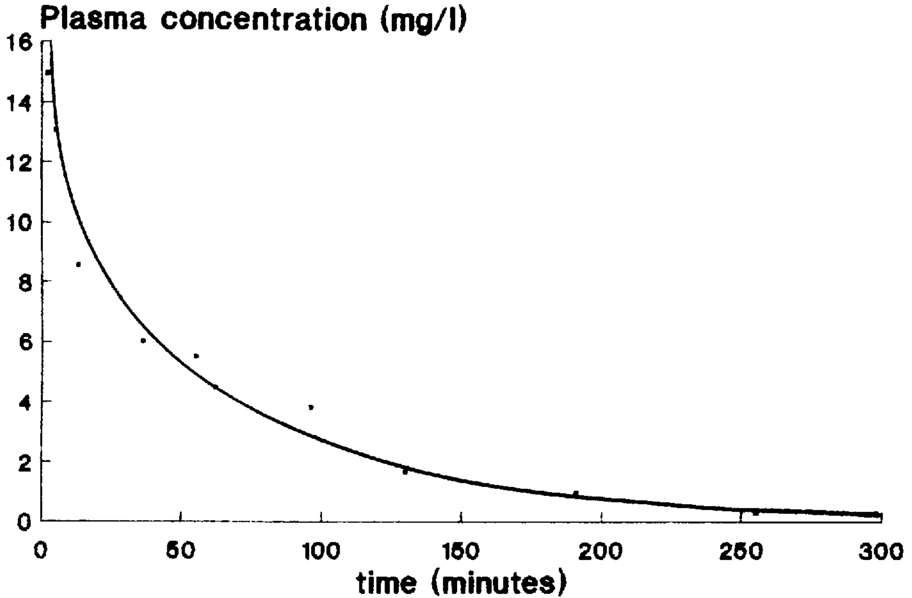

Figure 5.- Plasma concentration of gentamicin as a function of time following an IV injection of 3 mg per kg of body weight.

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differents days (Table 4). In both cases the reproducibility was good.

In vivo experiments

Gentamicin concentration data after an IV administration of 3 mg of gentamicin sulphate/Kg of body weight, were found to be best fitted by a bicompartimental model (fig. 5), with r^2 =0.9973. Half life, as determined from the terminal phase, was

86 minutes. Calculation of total body clearance provided a mean of 8.6 ml/min/kg of body weight and the apparent volume of distribution, calculated from the area under the curve for each animal was 0.506 l/kg.

DISCUSSION

We have described an HPLC procedure for the determination of gentamicin in plasma and urine.

Concerning the extraction procedure several approaches were assayed. Due to the high solubility of gentamicin in water it is not possible to isolate this molecule by extraction with organic solvents and a previous process of deproteinization of biological samples was necessary. The method based on precipitation by trichloroacetic acid treatment, gave no reproducible results because the drug partially precipitated with proteins and gentamicin was not totally recovered in any experiment. The use of other water miscible organic solvents such as methanol, ethanol, acetonitrile or acetone promoted the precipitation of proteins as a fine particulate suspension and the supernatant, after centrifugation, was not completely clear.

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Another extraction method assayed was ion exchange chromatography. The biological fluid containing the drug was placed directly into a C-25 Sephadex column and biological contaminants and gentamicin were eluted succesively by using buffers of different pH. This procedure was not completely satisfactory.

On the contrary, the separation of gentamicin from serum and urine by passage through a silica columm and derivatization directly in the columm improved the accuracy and the recovery, and therefore this was the method of choice.

It was also judged mandatory the use of an internal standard to minimize assay variation, although on the majority of aminoglycoside assays documented in the literature, internal standards have not been reported. Only two methods described by Anhalt (3) and D'Souza (1) use internal standards to quantify gentamicin concentrations. In this papers netilmicin and a synthetic derivative (1-N-Acetylgentamicin C1) were used as internal standards.

In order to decide the most suitable internal standard a previous comparative study was carried out by adding a known amount of several antibiotics

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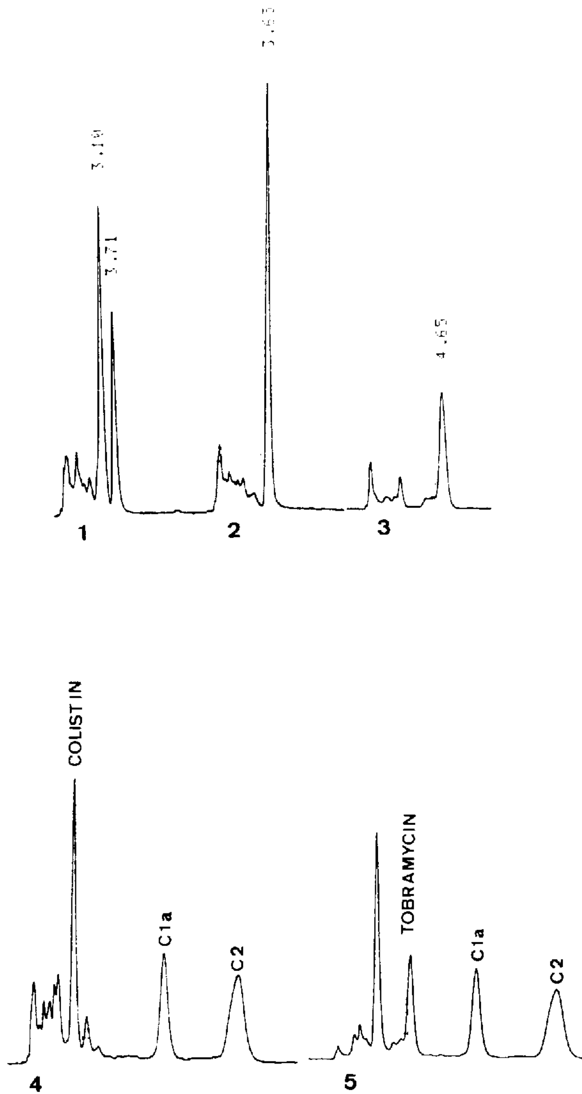

Figure 6.- Chromatographic profiles corresponding to the different antibiotics assayed as internal standard.
(1) Kanamycin A and B (1 mg/l), (2) Colistin (3 mg/l), (3) Tobramycin (0.5 mg/l), (4) Colistin and C1a and C2 gentamicin components, (5) Tobramycin and C1a and C2 gentamicin components.

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to the plasma or urine samples before any manipulation. Fig 6 shows the chromatographic profiles of aqueous samples containing colistin, kanamycin and tobramycin. This last antibiotic was the molecule giving less interferences, and was used throughout the analytical work.

As far as the derivatization reagent was concerned, o-phthalaldehyde was chosen since the reaction occurs rapidly at room temperature in aqueous media and proved to be completed in 1 to 3 minutes. Methods employing pre-column derivatization techniques require knowledge of the structural stability of the formed derivatives. This stability depends on the reducing agent employed in the reaction. It can be seen that the derivatives formed with mercaptoethanol decay significantly within 15 minutes of formation (7). Derivatives with short half-lives may require individual sample preparation with precise timing prior to injection.

This method can be used directly to assay other aminoglycosides. However, slight modifications may be necessary to optimise quantification of these compounds.

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