Journal of Chromatography, 348 (1985) 229–240
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 032

QUANTITATIVE DETERMINATION AND SEPARATION OF ANALOGUES OF AMINOGLYCOSIDE ANTIBIOTICS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received May 29th, 1985; revised manuscript received July 12th, 1985)

SUMMARY

Commercial bulk products and pharmaceutical drug formulations of aminoglycoside antibiotics obtained by fermentation (kanamycin, gentamicin, sisomicin and tobramycin) or by synthesis (amikacin) were analysed with high-performance liquid chromatography on a C_8 reversed-phase column. The method is based on a pre-column derivatization of the aminoglycosides with a 2,4,6-trinitrobenzenesulphonic acid reagent and UV detection (350 nm). The quantitative determination was carried out *vs.* an external standard; both peak heights and areas were used. A gentamicin mixture was separated into five or four components, depending on the column used. Amikacin was separated from its possible regioisomers and kanamycin A was easily separated from its minor components B and C.

INTRODUCTION

Aminoglycoside antibiotics are therapeutically important for the treatment of severe infections caused by Gram-negative bacilli[1]. However, as their major drawbacks are ototoxicity and nephrotoxicity[2], it is necessary to monitor accurately their concentrations in serum and plasma. Moreover, bulk drugs and pharmaceutical dosage forms described in official pharmacopoeias require potency and purity tests for certification, such as the Code of Federal Regulations[3].

Several methods have been published for their assay in both biological fluids and in final drugs based on high-performance liquid chromatography (HPLC) with appropriate derivatization, as almost all members of this class have no visible or UV absorption. In most procedures a fluorescence reagent was used with pre-column[4–17] or post-column[18–21] derivatization, with fluorimetric or UV detection, while only

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0021-9673/85/$03.30

Getek *et al.*[22] separated the components of gentamicin, without derivatization, using an electrochemical detector.

Our approach was based on a pre-column derivatization of the aminoglycoside by reaction with 2,4,6-trinitrobenzenesulphonic acid[23], which gave rise to a chromophore with a peak maximum in the UV region, eluted on a C_8 reversed-phase column and detected at 350 nm. The method was applied with positive results to the separation and determination of the main components of a gentamicin mixture and of amikacin from its regioisomers, which were obtained as by-products during its synthesis. Kanamycin, sisomicin and tobramycin were analysed in the same way.

EXPERIMENTAL

Materials and reagents

The purified Gentamicin components C_1, C_{1a}, C_2 and C_{2a}[16] were prepared by one of the authors (G. M.). Gentamicin sulphates, laboratory reference standard, were obtained from the United States Pharmacopeia (labelled potency 663 μg/mg) and from the WHO (labelled potency 641 I.U./mg).

Gentamicin sulphate injectable dosage forms were purchased from pharmacies (Gentalyn, Essex, Italy and Gentamen, Pierrel, Italy). Gentamicin sulphate bulk drugs were of Pierrel, Bulgarian and Chinese origin. Tobramycin sulphate (Nebicina, Lilly, Italy) and Sisomicin sulphate (Sisomin, Essex) as injectable forms were also purchased from pharmacies. Amikacin base, laboratory reference standard, was provided by the United States Pharmacopeia (labelled potency 914 μg/mg). Samples of final dosage forms were obtained internally (Pierami) or purchased from pharmacies (BB-K8, Bristol, Italy). Bulk drugs were Pierrel products. Regioisomers[24] BB-K6, BB-K11 and BB-K29 were prepared in the synthesis department.

Kanamycin A sulphate, together with kanamycin B (kanendomicin) was provided by Meiji (Japan), while the laboratory reference standard was provided by the United States Pharmacopeia (labelled potency 779 μg/mg).

2,4,6-Trinitrobenzensulphonic acid, pyridine, potassium hydroxide, potassium dihydrogen phosphate, acetic acid and carbon tetrachloride were purchased from Carlo Erba (Milan, Italy). All reagents were of analytical-reagent grade. LiChrosorb 100 (5 μm), trimethylchlorosilane, hexamethyldisilazane, trioctylchlorosilane, acetonitrile and methanol were purchased by E. Merck (Darmstadt, F.R.G.); the last two reagents were of HPLC grade.

HPLC equipment

A Millipore–Waters instrument was used, consisting of a 6000 A pump, a WISP 710 B automatic sampler, a Model 730 printer–plotter and a Model 480 spectrophotometric detector (350 nm).

Commercial 5-μm RP-8 columns (25 × 0.46 cm I.D.) were used to separate routinely the various aminoglycoside antibiotics. Columns (25 × 0.46 cm I.D.) were also prepared and packed in our laboratories and were used mainly for the separation of the five components of gentamicin.

Stationary phase preparation

LiChrosorb 100 (5 μm) (10 g) was treated in toluene (150 ml) with trioctyl-

chlorosilane (10 ml) and hexamethyldisilazane (1 ml) at boiling temperature for 10 h. After concentration to half its volume under vacuum and filtration of the treated phase, the solid was again suspended in toluene (70 ml) with addition of trimethylchlorosilane (10 ml) and hexamethyldisilazane (1 ml). After boiling for 10 h, the mixture was concentrated to half its volume under vacuum, filtered and the solid washed thoroughly with toluene and acetonitrile. Finally, the bonded phase was washed with several portions of distilled water at 50°C with vigorous shaking until it became hydrophobic. The phase was filtered, washed with acetonitrile and carbon tetrachloride and dried under vacuum. With 2.5 g of this stationary phase it is possible to fill a 25 × 0.46 cm I.D. column.

Chromatographic conditions

The oven temperature was maintained at 45°C with an eluent flow-rate of 1.5 ml/min. The mobile phase consisted of 0.02 *M* potassium phosphate buffer adjusted at pH 7.5 with 40% (w/v) potassium hydroxide solution, filtered through a 0.2-μm filter and degassed ultrasonically, followed by addition of the necessary amounts of acetonitrile and methanol (Table I). In amikacin analysis and in the separation of the gentamicin mixture into four components only the buffer concentration was 0.015 *M*.

TABLE I

COMPOSITION OF MOBILE PHASES

Component	*Amikacin*	*Gentamicin**	*Kanamycin*	*Sisomicin*	*Tobramycin*
Phosphate buffer (ml)	400	400	400	400	400
Acetonitrile (ml)	320	493	460	506	460
Methanol (ml)	106	120	150	160	150

* This ratio gives rise to the separation of five components of the mixture; a slight change [0.015 *M* phosphate buffer–acetonitrile–methanol (400:506:149)] is able to separate only the major four components.

The antibiotic concentration before derivatization was in the range 0.01–0.15% (w/v) and the volume injected was 25 μl for all aminoglycosides except gentamicin (35 μl).

Derivatization procedure

To a vial a 0.5% (w/v) aqueous solution of 2,4,6-trinitrobenzenesulphonic acid (1 ml), the aqueous solution of the aminoglycoside (100 μl) and pyridine (1.5 ml or 1.25 ml for gentamicin and sisomicin) were added, in this sequence. The vials were tightly sealed and heated at 70°C for 15 min together with the reference standard vials. After cooling under tap water, acetic acid (1 ml) (alternatively acetonitrile could be used) was added and a suitable volume was injected into the chromatograph.

RESULTS AND DISCUSSION

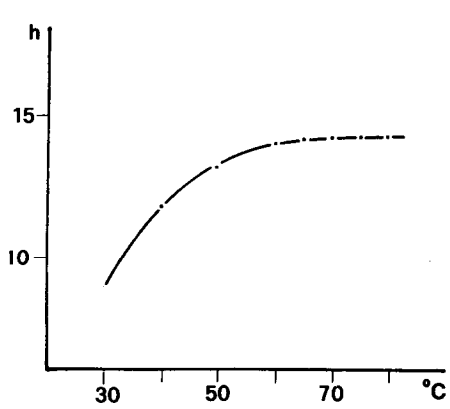
The proposed procedure is based on the pre-column derivatization of the

aminoglycoside antibiotics with 2,4,6-trinitrobenzenesulphonic acid, a reagent used originally by Satake *et al.*[23] for the spectrophotometric determination of primary amines, amino acids and peptides with the modification that was later introduced by Snyder and Sobocinski[25]. The resulting 2,4,6-trinitrophenylamine gave absorption spectra with a first main maximum at 350 nm and a second at about 420 nm. The reaction was formerly[26] used for the determination of kanamycin, gentamicin and tobramycin in samples free from interfering amino compounds. We preferred to use the pre-column derivatization as it speeds up the whole procedure. The best conditions for derivatization were optimized for each aminoglycoside; Figs. 1 and 2 show the relationship between the development of the amikacin chromophore and temperature and time, respectively. After this screening, we found that 15 min at 70°C were sufficient for all the aminoglycosides. The stability of the chromophore varies from compound to compound (Fig. 3); the addition of acetic acid ensures the stabilization of this value for at least 10 h; gentamicin shows only a slight decrease (less than 5% in 10 h). The quantitative determination was carried out *versus* external reference standards; peak heights rather than peak areas were used for calculation. The data in Table II confirm the validity of the method.

As gentamicin[27] is one of the oldest aminoglycosides used in human therapy and as it is a mixture of at least five components[16], various HPLC methods for its determination have been developed.

As many of the HPLC methods for the determination of gentamicin in biological fluids[4–7,9–12,14,18,20,21] were used, no particular care was taken with regard to the separation of its components; only a few methods[8,13,15,16,19,22] refer to bulk drugs and pharmaceutical dosage forms. Most of them are based on a very similar derivatization procedure with *o*-phthalaldehyde and 2-mercaptoethanol[13,19] or mercaptoacetic acid[8,15,16] and ion-pair chromatography on a reversed-phase[8,15,16,19] with fluorescence[13,19] or UV[8,15,16] detection.

Only Freeman *et al.*'s method[8], which was later developed by Claes *et al.*[16], gives a chromatogram with four well resolved peaks corresponding to C_1, C_{1a}, C_{2a} and C_2 components, according to the elution time. This method is now officially rec-



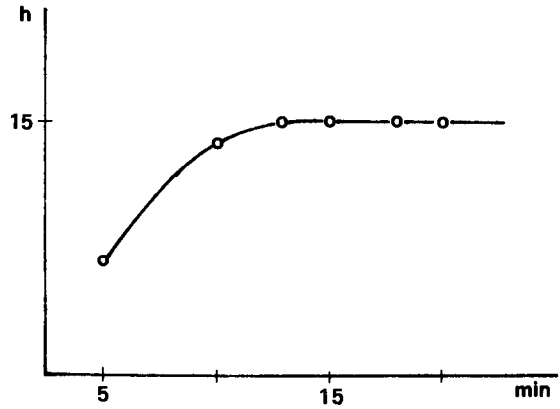


Fig. 1. Peak height of amikacin *vs.* temperature with a time of derivatization of 15 min.

Fig. 2. Peak height of amikacin *vs.* time of derivatization at 70°C.

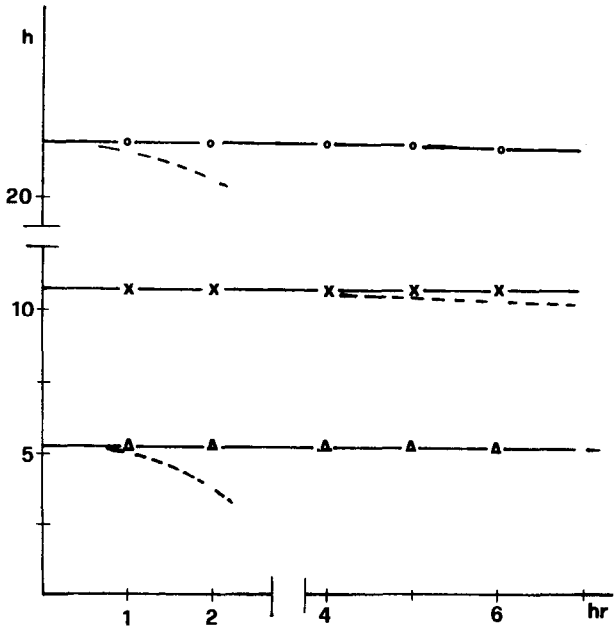


Fig. 3. Peak heights of some aminoglycosides *vs.* time after addition of acetic acid: (○) gentamicin C_1; (×) kanamycin; (△) sisomicin; or without the addition (dotted line).

ommended in the British Pharmacopoeia (BP)[28] for the determination of the component ratio, the sum of C_{2a} and C_2 being considered to be equivalent to the C_2 component of FDA[29]. As the area response factors are different for the four components, as indicated in Claes *et al.*'s paper[16], the method of calculation described in the BP gives results that are not representative of the actual ratio of gentamicin components.

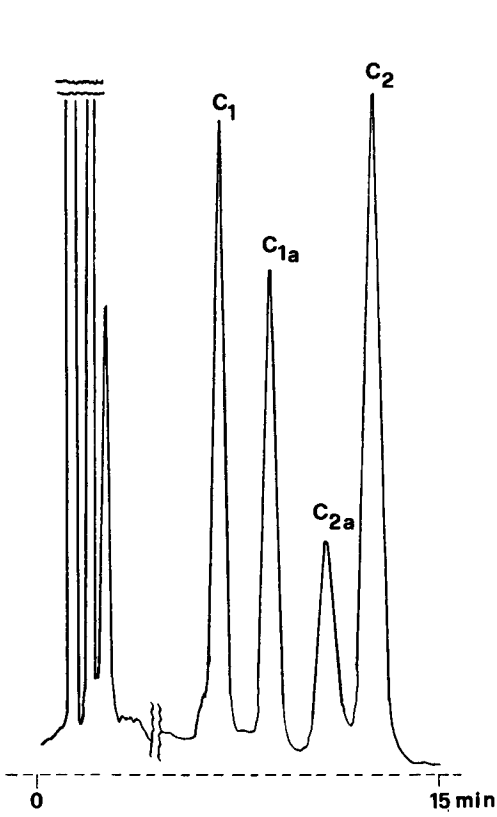
Figs. 4 and 5 show the chromatograms that were obtained in the separation of the four main components of gentamicin with the same order of elution as Freeman *et al.*'s method[8], and of a fifth component, different from C_{2b}[16], being present in small amounts but in all the samples that were analysed. The separation of this fifth component, so far not identified, is made possible by the use of a reversed-phase that

TABLE II

VALIDATION OF HPLC ANALYSIS OF AMINOGLYCOSIDE SULPHATE ANTIBIOTICS

Compound	*Average**	*Standard deviation*	*Coefficient of variation (%)*	*Lower and upper reliability limit (±5%)*
Kanamycin A	79.3	0.6	0.75	78.8–79.7
Sisomicin	61.2	0.44	0.72	60.8–61.5
Tobramycin	80.9	0.2	0.23	80.8–81.1
Amikacin	69.3	0.7	1.0	68.8–69.8

* Calculated as free base from ten different analyses.



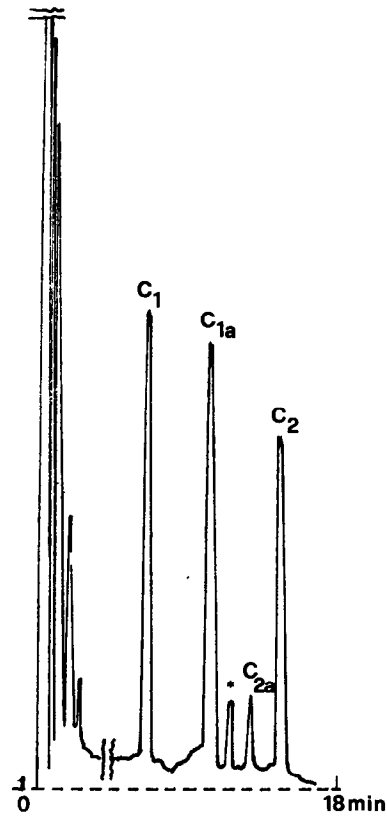


Fig. 4. Chromatogram of gentamicin (batch 080/13) with separation into the four main components.

Fig. 5. Chromatogram of Gentamicin (batch 080/13). The fifth component marked with an asterisk is unidentified.

was prepared (as described under Experimental) in our laboratories and from a slight change in the mobile phase (Table I). This method is routinely applied not only to gentamicin bulk drugs and injectable preparations but also in the control of fermentation and in the screening of highly producing strains.

Fig. 6 shows the calibration graph of the four main components of gentamicin obtained when the above described analysis was used; it can be seen that the response factor is different for each analogue, so that the exact quantitative determination of the component ratio, as required officially[28,29], can be obtained only with respect to an external reference standard. Table III reports data on the validation of gentamicin components analysis.

For gentamicin, the method here described may represent an alternative to that described in the BP; both offer the advantage of being rapid and precise and can be easily applied. A further need to measure accurately the gentamicin components derives from the fact that each analogue may show a variable degree of toxicity[2,30].

Amikacin[31] was obtained by acylation of the amino group in position 1 of kanamycin A with L-(−)-4-amino-2-hydroxybutyric acid after protection and ester-

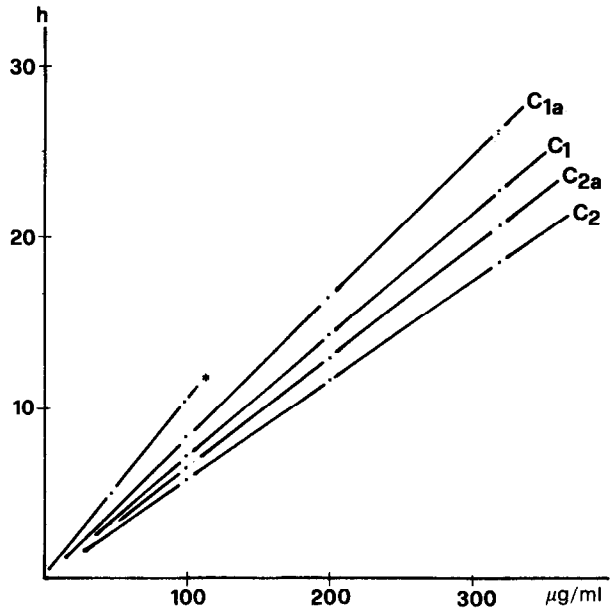


Fig. 6. Calibration graphs for gentamicin components. The line marked with an asterisk refers to the unidentified component.

TABLE III

VALIDATION OF HPLC ANALYSIS OF GENTAMICIN SULPHATE

Sum of five components taken as 100%*.

Run No.	C_1 *(%)*	C_{1a} *(%)*	C_{2a} *(%)*	C_2 *(%)*	*5th Component (%)***
1	29.6	22.1	10.2	37.9	1.7
2	28.9	22.1	10.6	37.5	1.7
3	28.9	22.2	9.8	37.3	1.7
4	28.8	22.3	10.7	36.0	1.7
5	28.7	22.0	10.4	36.3	1.5
6	29.3	21.8	10.4	37.3	1.7
7	29.6	21.6	11.1	37.3	1.6
8	29.2	22.0	10.8	36.6	1.8
9	28.9	22.6	10.3	36.5	1.7
10	29.1	28.1	10.3	37.5	1.6
Average	29.10	22.07	10.44	37.01	1.67
Standard deviation	0.3	0.26	0.36	0.6	0.1
Coefficient of variation (%)	1.1	1.2	3.45	1.65	5.4

* Calculated from HPLC area on batch 080/13 (area of each component peak expressed as a percentage of the sum of the five peak areas). For comparison, see Table IV of ref. 16. The same figures calculated *vs.* standards are C_1 31.5%, C_{1a} 26.1%, C_{2a} 12.4%, C_2 27.2% and fifth component 1.7%.

** Unidentified; C_{2b}[16] has a retention time lower than that of C_1.

ification. As the molecule of kanamycin has four primary amino groups, it is possible during the synthesis to obtain three more isomers[24] of amikacin that differ only in the position of the acylation of the side-chain. Depending on the synthesis used[31–33], a different pattern and concentration of isomers may be present in the final solution, before the purification, which is usually obtained by an ion-exchange column.

Fig. 7 shows the chromatogram of an artificial mixture of all the by-products and kanamycin, which are potential impurities in the final drug and which are completely resolved from the peak of amikacin. Depending on the amount injected, it is possible to determine each isomer at levels down to 0.2%. Fig. 8 shows the slight differences in the calibration graphs of the four isomers; therefore, quantitative determination can be achieved only by using the proper standards. Table IV compares the official microbiological method of analysis specified by the FDA[34] for certification and the assay based on the HPLC procedure (faster).

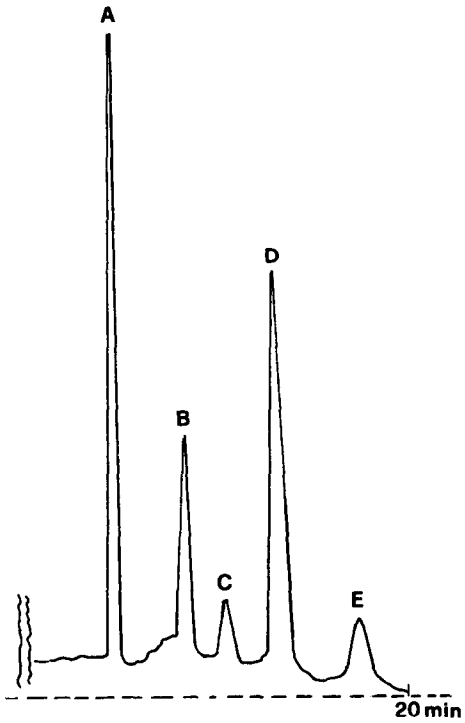


Fig. 7. Chromatogram of an artificial mixture of (A) amikacin, (B) BB-K29, (C) BB-K6, (D) kanamycin and (E) BB-K11.

Our original method[35] for the determination of amikacin in bulk and the Pierrel product was later[36] applied, with minor changes, comparing three dosage forms available commercially in Italy; the method was used only to determine the separation of amikacin from BB-K29. Other workers[17] described pre-column derivatization with *o*-phthalaldehyde and the use of a fluorescence detector for the separation of the main regioisomers of amikacin. The separation of BB-K29 from amikacin gives poor results and makes the determination of the former at low levels difficult.

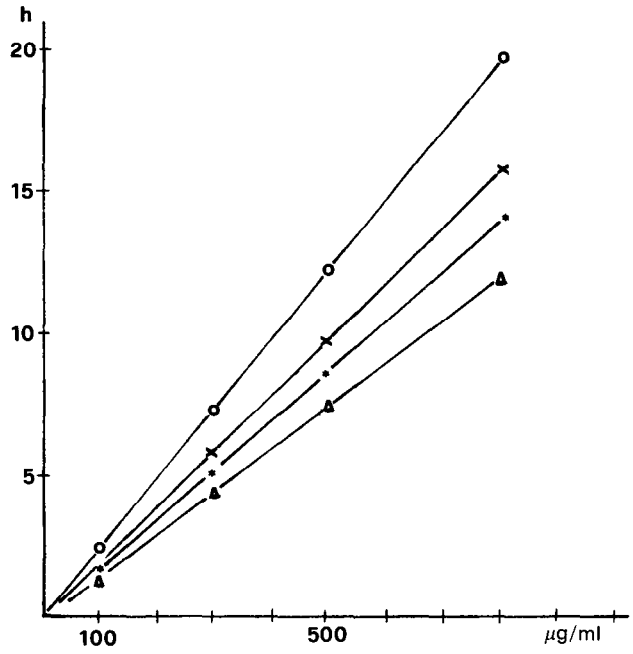


Fig. 8. Peak height of (○) amikacin, (×) BB-K29, (*) BB-K6 and (△) BB-K11 *vs.* concentration.

TABLE IV

COMPARISON OF HPLC ANALYSIS AND OFFICIAL MICROBIOLOGICAL DETERMINATION OF AMIKACIN SULPHATE (AS FREE BASE)

Sample No.	*Amikacin sulphate (%)**	
	HPLC	*Microbiological method*
1	69.8	67.0
2	67.8	67.0
3	69.5	67.0
4	65.0	66.5
5	69.7	67.5
6	67.5	67.5
7	69.0	69.0
8	69.3	67.5
9	69.6	69.0
10	69.3	69.0
Average	68.65	67.7

* Mean of three determinations.

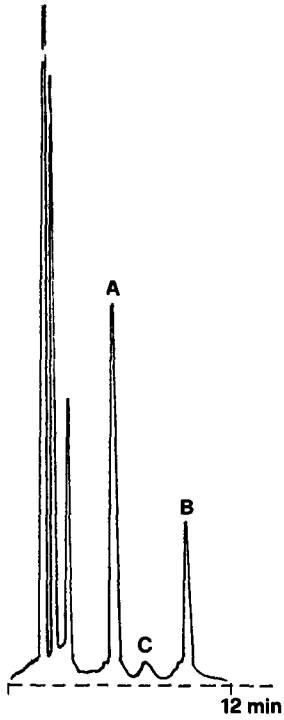
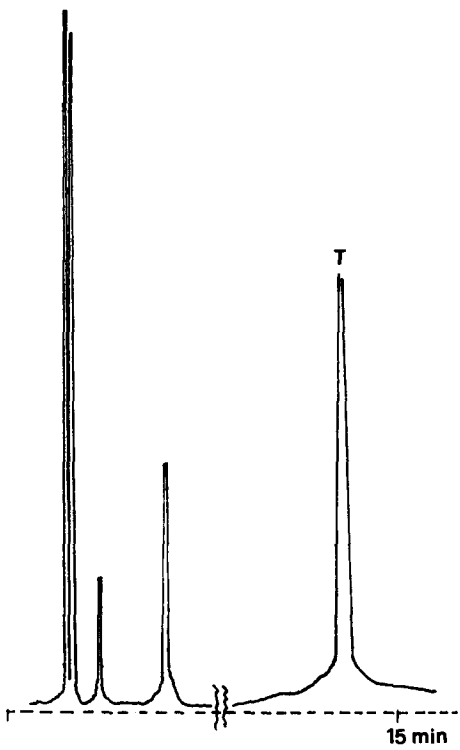


Fig. 9. Chromatogram of kanamycin derivatives A and B. C is probably kanamycin C.



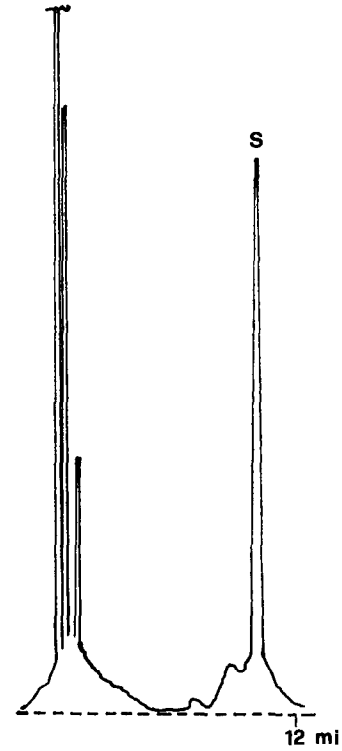


Fig. 10. Chromatogram of tobramycin sulphate injectable form.

Fig. 11. Chromatogram of sisomicin sulphate injectable form.

The same method that is used for gentamicin and amikacin can also be used for the separation and determination of kanamycin components (A and B), the latter being the normal major by-product that is present in the bulk. The official FDA monograph[37] sets a limit on the amount of kanamycin B in kanamycin sulphate, which is determined by a time-consuming and tedious microbiological assay. In less than 10 min it is possible to have an accurate assay of the amount of kanamycin B in the mixture (Fig. 9) using the described HPLC procedure.

Figs. 10 and 11 show the HPLC traces obtained when analysing drug dosage forms of tobramycin and sisomicin, respectively. Detection of the latter compound is possible at 215 nm, owing to the double bond in the sisosamine moiety, with no prior derivatization and with only minor modification of the mobile phase (the ratio between phosphate buffer and acetonitrile being 7:3).

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