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CHROMATOGRAPHIC SEPARATION OF THE COMPONENTS OF THE GENTAMICIN COMPLEX

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SUMMARY

By means of paper and thin-layer chromatography using the lower phase of a solvent mixture consisting of chloroform–methanol–17% ammonium hydroxide (2:1:1, v/v), the gentamicin antibiotic complex is shown to consist of three components, named C_1, C_{1a} and C_2. Methods are given for preparative separation of these antibiotics by use of cellulose and Chromosorb W chromatographic column procedures utilizing the solvent system described for paper and TLC.

INTRODUCTION

WEINSTEIN and co-workers[1] and ROSSELET and co-workers[2] had reported that the antibiotic gentamicin consisted of two closely related isomeric pseudo-oligosaccharides referred to as gentamicin C_1 and gentamicin C_2 which had essentially identical polarities. The two components were separable only after acetylation and partition chromatography on cellulose powder using a heptanol–pyridine–water system in a ratio of 6:4:3 and subsequent deacetylation of the separated N-acetyl derivatives by reflux for 70 h in 1.2 *N* sodium hydroxide.

Fig. 1 illustrates the chromatographic pattern of gentamicin N-acetyl C_1 and gentamicin N-acetyl C_2 on Whatman No. 1 paper developed in the heptanol–pyridine–water system. The compounds were detected by the starch-potassium iodide method

Fig. 1. Chromatogram of N-acetyl gentamicin C_1 and N-acetyl gentamicin C_2 on Whatman No. 1 paper using heptanol–pyridine–water (6:4:3). Compounds visualized by starch–potassium iodide spray.

of PAN AND DUTCHER[3], and the spot closest to the origin is C_1 and the faster moving one is C_2.

WEINSTEIN and co-workers[4] recently reported that the gentamicin complex produced by submerged fermentations of *Micromonospora purpurea* NRRL 2953 consists of three antibiotic components which have been designated as gentamicin C_1, gentamicin C_2, and gentamicin C_{1a} respectively. The data presented shows the biological properties of the individual components to be essentially the same.

This paper describes techniques for the separation of the three gentamicin components and it will also demonstrate that gentamicin C_1 referred to in the original paper was actually composed of a mixture of two antibiotics which are now designated as C_1 and C_{1a} and that C_2 was homogeneous.

EXPERIMENTAL AND RESULTS

Thin-layer chromatography

Separation of these antibiotic moieties has been accomplished with thin layer or paper chromatography using the lower phase of a solvent system composed of chloroform–methanol–17 % ammonium hydroxide in a ratio of 2:1:1. This system is a modification of the method reported by IKEKAWA *et al.*[5] in 1963.

Thin-layer chromatography of the gentamicin complex was carried out on plates coated with Silica Gel G. Plates were developed for 16–18 h.

Detection of spots was accomplished by two methods: (1) by the use of reagents; and (2) by a microbiological procedure. The first method was carried out by the usual technique of spraying the plates with 0.25 % ninhydrin in pyridine–acetone and consequent heating at 105° for several minutes. The zones appear as purple or blue spots against a white background. Also the starch–potassium iodide reagent spray can be used, with which the zones show up as dark blue spots against a white background.

Microbiological detection of the antibiotic zones was carried out by laying the plate face-down on a sheet of Whatman No. 1 paper in contact with agar seeded with *Staphylococcus aureus* ATCC 6538P. After 10 min the paper and plate were removed and after a suitable incubation period the zones of inhibition, representing the gentamicin components, were observed.

Paper chromatography

The method of separation utilizing the thin-layer plates is extremely slow compared to most thin-layer techniques. Increased speed of separation of the components was realized by use of paper chromatography utilizing the chloroform–methanol–ammonia system. Upper phase was placed in the bottom of the chromatographic jars several hours prior to use, and lower phase was used to develop the papergrams which were run descending on Whatman No. 1 paper in insulated chambers, for 5 h at 25°. The solvent was allowed to drip off the papers during development.

Detection of zones was accomplished by the previously described ninhydrin technique or by the bioautographic method. In the latter, the paper was placed directly on the agar, prepared as previously described, for a 10-min period prior to incubation.

The complex separated into three zones as seen by the microbiological method

illustrated in Fig. 2. These zones corresponded to those observed using the thin-layer chromatographic technique except that the paper chromatographic method was more sensitive and rapid. The zone of inhibition nearest to the front is identified as C_1, the zone following designated as C_2 and that closest to the origin, C_{1a}.

Fig. 3 shows a similar pattern with ninhydrin sprayed chromatograms. The R_F values of the ninhydrin spots are identical with those of the corresponding zones of inhibition.

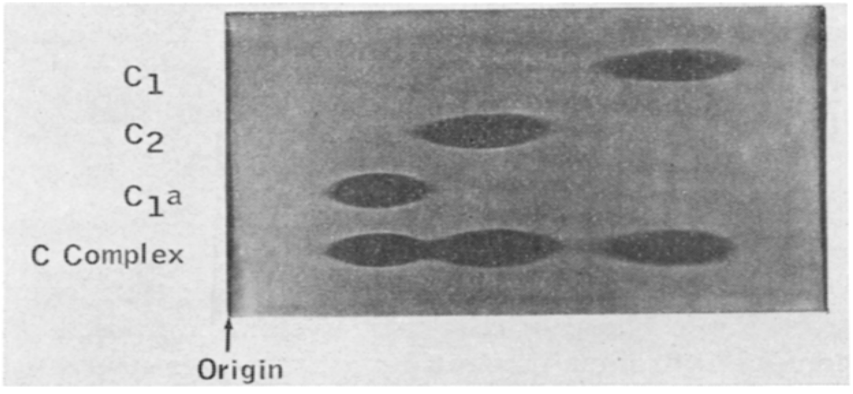

Fig. 2. Bioautograph of gentamicins. Chromatogram run on Whatman No. 1 paper using lower phase of chloroform–methanol–17% ammonia (2:1:1) and plated against *S. aureus* ATCC 6538P.

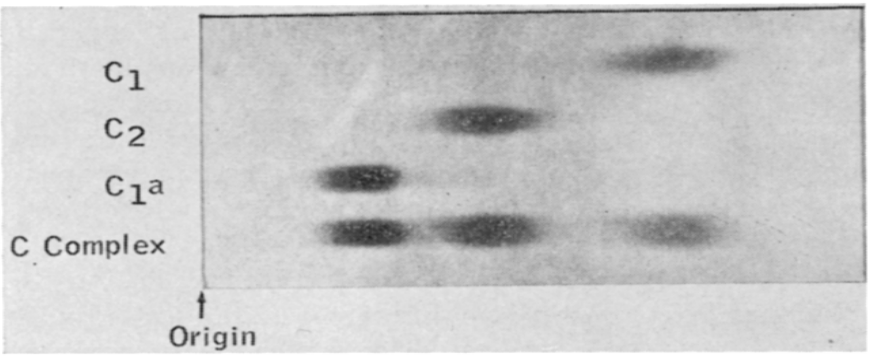

Fig. 3. Ninhydrin sprayed chromatogram of gentamicins run on Whatman No. 1 paper using lower phase of chloroform–methanol–17% ammonia (2:1:1).

Cellulose column chromatography

As a result of the success of the paper chromatographic separations of the gentamicin complex, it appeared feasible to attempt preparative column chromatography of the mixture using cellulose powder and the predescribed solvents.

Whatman No. 1 cellulose powder was packed in small segments in a column having an inner diameter of 2.4 cm, to a height of 30 cm. The upper phase of the solvent system comprised of chloroform–methanol–17% ammonia (2:1:1, v/v) was run through the column until a yellow band of impurities emerged and the column was allowed to drain.

A 200 mg quantity of gentamicin sulfate (equivalent to 129 mg of free base) was mixed with 2 g of cellulose powder, packed atop the cellulose in the column and wet with a small quantity of upper phase. Lower phase was allowed to run through the column at the rate of 2 ml per min; 16 ml fractions were collected every 8 min.

Aliquots of each fraction were spotted on filter paper and tested with ninhydrin

reagent to determine the presence or absence of antibiotic. All of the antibiotic (positive test) was located in fractions 12 through 49. Paper chromatography of each of the fractions determined that the three component peaks were located as seen in Table I. Like fractions, converted to their free bases on the column, were pooled and concentrated to dryness. Weights of peak fractions (Table I) indicated a 94 % recovery of starting material.

TABLE I

CHROMATOGRAPHIC SEPARATION OF THE GENTAMICIN COMPLEX USING A CELLULOSE COLUMN

Starting material: 200 mg of gentamicin complex sulfate (129 mg base equivalent). Total yield: 121.5 mg (94%).

Component	*Fraction No.*	*Weight (mg) (base)*
C_1	12–19	58.2
C_2	23–33	50.8
C_{1a}	38–49	12.5

Paper chromatography of the bases and bioautography of the papergrams against *Staphylococcus aureus*, showed that the C_1 and C_2 components were free of impurities and that C_{1a} contained approximately 5 % of C_2. Rechromatography of the C_{1a} fraction using the described column resulted in isolation of this component free of the C_2 component.

Chromosorb chromatography

A major problem in separation of quantities of the gentamicin complex into its three components is scale-up of existing procedures. Major disadvantages of the above methods are that thin layer or paper chromatography can be used only for detection or preparation of minute quantities of material and cellulose powder chromatography is time-consuming because of the care that must be taken in packing the column.

An improved technique has been devised utilizing the solvent system previously described and Chromosorb W® an inert adsorbent. Although the mechanics of the separation are essentially similar to those described, this procedure is more easily carried out, does not require hand packing, can be scaled up readily and, because it uses a low ratio of adsorbent to gentamicin complex, can be used to separate large quantities of material.

Chromosorb W (60–100 mesh) manufactured by Johns-Manville, New York, N.Y. was slurried with the upper phase of chloroform–methanol–17 % ammonia (2:1:1,v/v) and filtered using suction on a Buchner funnel until excess solvent was removed. The Chromosorb was packed into a column having an I.D. of 3 cm to a height of 50 cm, in 5 cm segments (approximately 150 g as dry Chromosorb). An alternate method which has been used is to pour the slurry into the column and remove excess solvent by suction from the bottom of the column. One liter of lower phase was run through the column to wash the Chromosorb.

A 3 g portion of gentamicin base was dissolved in 10 ml of methanol, adsorbed to the smallest possible quantity of Chromosorb and dried under vacuum using a Rinco film evaporator. This mixture was packed atop the column and wet with a

small quantity of lower phase. The column was eluted with lower phase at the rate of 1 ml/min, collecting five fractions/h for a total of 231 fractions. Fractions were tested as described under cellulose column chromatography and the three major component peaks were located as shown in Table II, pooled, decolorized on IRA401S (OH^-) resin and concentrated to dryness. Paper chromatography of the bases and bioautography of the papergrams indicated that all components were free of microbiologically active impurities. Weights and bioassay data for the dried bases are presented in Table II.

TABLE II

CHROMATOGRAPHIC SEPARATION OF THE GENTAMICIN COMPLEX USING A CHROMOSORB W COLUMN
Starting material: 3 g gentamicin complex base.

Component	*Fraction Nos.*	*Purified fractions (weight, g)*	*Antibiotic activity* (μg/mg)*
C_1	35–60	1.01	833
C_2	95–140	0.74	1050
C_{1a}	165–231	0.31	1050

* Average of four assays against complex standard.

DISCUSSION

Thin-layer and paper chromatography were used to show that the gentamicin complex is made up of three components and not two as was previously reported. These components can now be separated by column chromatography on cellulose or Chromosorb using the chloroform–methanol–ammonia system.

Acetylation of each of the three gentamicin components and chromatography of the derivatives indicated that the N-acetyl gentamicins C_1 and C_{1a} moved as a single spot and differed from the R_F of the N-acetyl C_2 zone as illustrated in Fig. 4. This is in direct agreement with the original assumption that gentamicin was a mixture of only two components and would explain why three acetylated zones did not appear. The chromatographic behavior of the gentamicin components as evidenced by the

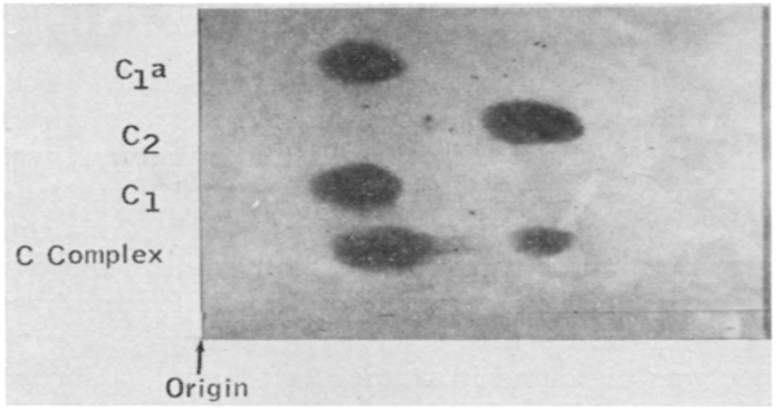

Fig. 4. Chromatogram of N-acetyl gentamicins showing identity of C_1 and C_{1a}. Whatman No. 1 paper; heptanol–pyridine–water (6:4:3). Spots visualized by starch–potassium iodide spray.

data presented in this paper, appears to confirm earlier views indicating very close structural relationship between these compounds.

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