THE SEPARATION AND DIFFERENTIATION OF THE GENTAMICIN COMPLEX

HUBERT MAEHR* AND CARL P. SCHAFFNER
Institute of Microbiology, Rutgers, The State University, New Brunswick, N.J. (U.S.A.)
(Received May 12th, 1967)

INTRODUCTION

Our successful separations of the neomycin and catenulin antibiotic complexes by ion-exchange resin chromatography[4] suggested the technique's possible efficacy in resolution of the new, broad-spectrum antibiotic complex, gentamicin, produced by several *Micromonospora* species[2,8,7], and classified as one of the 2-deoxystreptamine antibiotics[5]. After elution of the antibiotic acivity from weak-acid ion exchange resins, followed by neutralization of the effluent, gentamicins C_1 and C_2 were precipitated by sodium dodecylbenzenesulfonate solution. An ion-exchange resin process involving Amberlite IRA 400 in the hydroxide form converted the gentamicin dodecylbenzenesulfonate to the free base form. Gentamicins C_1 and C_2 were separated as their N-acetyl derivatives by partition chromatography; the antibiotic activity from N-acetyl gentamicins regenerated upon alkaline hydrolysis[6].

We here describe ion-exchange resin chromatography as key in preparative separation of the entire antibiotic complex; in addition to low cost, high capacity, desalting and decolorizing the antibiotic, the procedure offers easy solute recovery with the possibility of conductometric effluent analysis; future papers will describe preparative methods leading to individual gentamicins after ion-exchange resin chromatography.

APPARATUS AND REAGENTS

Antibiotic sample. A gift of gentamicin complex in the free base form with gentamicins C_1, C_2, and D partly removed, was a generous donation of the Schering Corporation, Bloomfield, N.J.

Ion-exchange resin. The strong-base ion-exchange resin, Dowex 1-X2, 100–200 mesh, was used without further purification or sizing.

Chromatographic column. A glass tube to accommodate a resin bed 75 × 670 mm with space for backwashing the column was fitted with a fritted-glass bottom and stopcock.

Conductivity bridge. The Industrial Instruments Model RC-16B conductivity bridge, operating at a frequency of 1000 c.p.s., was modified with a voltage divider in the output circuit to provide for recorder readout. A glass flow-through cell, with

* Present address: Hoffman-La Roche, Inc., Chemical Research Department, Nutley, N.J. 07110.

platinum electrodes, having a cell constant of 0.2 cm^{-1}, was used without special temperature control.

Recorder. The Leeds and Northrup Spidomax H recorded the 0–10 mV range.

Thin-layer chromatography. Chromatography was performed on non-activated layers of Brinkmann Silica Gel G, 0.75 mm thickness, prepared with a Desaga thin-layer spreader (Brinkmann Instruments Inc., Westbury, New York).

Ninhydrin spray. 125 mg ninhydrin were dissolved in 25 ml 1-butanol and 25 ml acetone.

Solvent systems. The following solvent systems were used: (I) chloroform–methanol–28 % ammonium hydroxide–water (1:4:2:1, v/v); (II) chloroform–methanol–28 % ammonium hydroxide (2:1:1, v/v); (III) chloroform–methanol–28 % ammonium hydroxide–water (10:5:3:2, v/v), lower phase, *cf.* ref. 1; (IV) 1-butanol–pyridine–water (3:2:2, v/v).

EXPERIMENTAL

Ion-exchange resin chromatography

The resin was converted to the hydroxide form with 10 resin-bed volumes of 8 % carbonate-free sodium hydroxide solution. All washings and elutions were with distilled, carbonic acid-free water; displacing the sodium hydroxide solution from the resin by back-washing swelled the resin bed approximately 20 %. In each separation on this column, 30–50 g of the free base gentamicin complex in 50 % aqueous solution charged the column, with a column flow rate not exceeding 0.7 cm per min; manual fraction collection, according to the continuously recorded conductivity of the column effluent, afforded individual fractions for evaporation under reduced pressure and subsequent freeze drying.

Thin-layer chromatography

Plates to be sprayed with ninhydrin solution received 0.1–0.2 mg portions of the antibiotic fractions; developed plates, dried at 100°, sprayed with pyridine, were reheated to aid removal of ammonia traces. Bioautographic detection of the spots, employing *Staphylococcus aureus* as test organism, utilized antibiotic concentrations of 0.01 mg; the pyridine and heat treatments of the plates were omitted.

Paper chromatography

N-acetylated gentamicin fractions were prepared and chromatographed in the manner prescribed for the N-acetylation and chromatography of neomycins and catenulins[4].

RESULTS AND DISCUSSION

Typically, 35 g of gentamicin complex were dissolved in 30 ml of water and washed into the resin bed, with measurement of the effluent volume beginning at the point of complete percolation of the charge into the resin bed, and with conductivity of the effluent thereafter continuously recorded. The elution graph appears as Fig. 1. Of the ten fractions collected, the first appears as a sharp peak at 1510 ml in the conductivity diagram; its corresponding solute exhibited high ash content with only traces of biological activity.

All following fractions displayed high activity against the test organism, Fig. 2 being a photograph of a thin-layer chromatogram of these same fractions. Fraction aliquots were developed with solvent system II and the spots located with ninhydrin. A parallel experiment located the spots by bioautography, the correlation affording qualitative agreement between ninhydrin color and zones of inhibition. The effluent volumes of the anion-exchange resin column (Fig. 1) designated the chromatographed

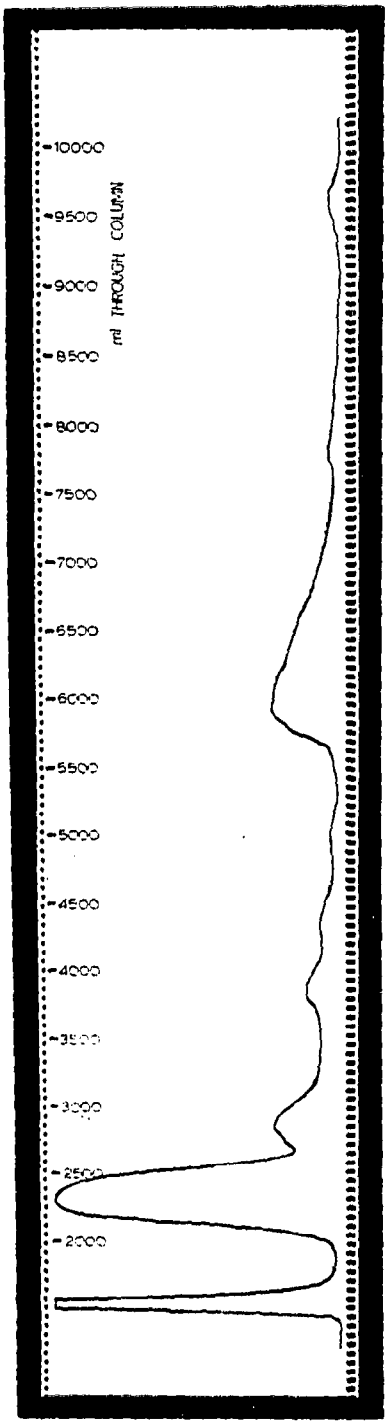

Fig. 1. Elution graph of gentamicin complex.

fractions in Figs. 2 and 4, the second peak in the elution graph (1950–2670 ml), representing the mixture of the three major antibiotics, gentamicins C_1, C_2, and D, being nearly quantitatively separated from the remaining constituents of the complex. Fig. 2 indicates the overlapping of C_1 and C_2 as well as the slower travel of gentamicin D.

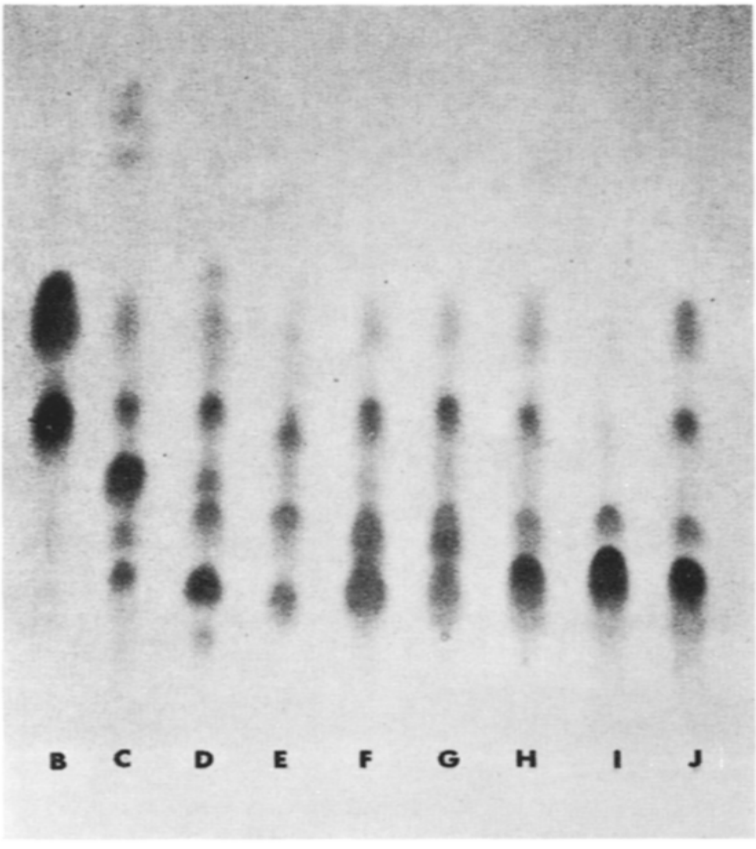

Fig. 2. Thin-layer chromatography of gentamicins. (B) 1950–2670 ml; (C) 2670–3155 ml; (D) 3155–3485 ml; (E) 3485–4175 ml; (F) 4175–4770 ml; (G) 4770–5110 ml; (H) 5110–5550 ml; (I) 5550–9340 ml; (J) 9340–10120 ml.

Complete separation of components C_1, C_2, and D was obtained by solvent system III; the usual technique being insufficient, proper separation demanded a threefold development with drying of the plates at room temperature between developments, although similar results could be obtained in a large chromatographic jar for a minimum developing time of 24 h. The relative migration distances are reproducible; R_F values, of course, are not constant under such conditions.

Fig. 3 shows a two-dimensional thin-layer chromatogram of the entire gentamicin complex prior to ion-exchange resin chromatography, with antibiotics C_1, C_2, and D quantitatively separated and appearing in approximately equal concentrations. Remaining components of the complex do not travel far from the point of sample application; many cannot be detected at all due to low concentration.

The following six fractions, within the 2670–5550 ml effluent interval, more or less overlap and are complex mixtures with the major spot of the third (2670–3155 ml, *cf.* Figs. 2 and 4), and sixth fraction (4175–4770 ml, *cf.* Fig. 2), as well as the three

minor components of the third fraction with the highest R_F values (*cf.* Fig. 2), inactive against the test organism at the dose level employed.

Bioautographic location of a thin-layer chromatographic plate, developed with solvent system II, indicates sixteen different, active substances, all being ninhydrin positive as deduced from a parallel experiment. Table I summarizes the R_F values of these antibiotics and indicates the column chromatographic fractions with the relatively highest concentrations of these antibiotics.

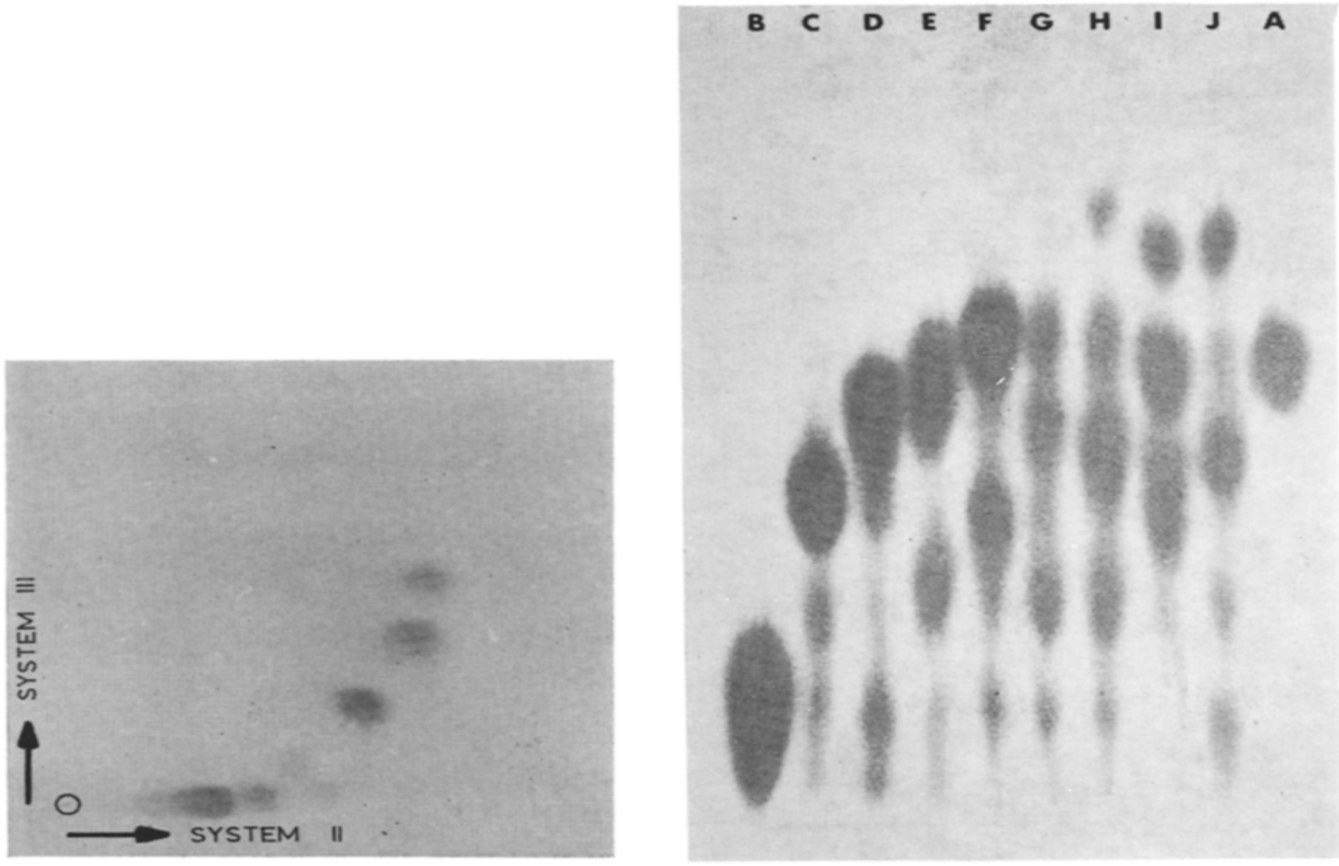

Fig. 3. Two-dimensional thin-layer chromatography of gentamicin complex.

Fig. 4. Paper chromatography of N-acetylated gentamicin fractions. (B) 1950–2670 ml; (C) 2670–3155 ml; (D) 3155–3485 ml; (E) 3485–4175 ml; (F) 4175–4770 ml; (G) 4770–5110 ml; (H) 5110–5550 ml; (I) 5550–9340 ml; (J) 9340–10120 ml; (A) pure N-acetylgentamicin A.

TABLE I

SUMMARY OF COLUMN AND THIN-LAYER CHROMATOGRAPHIC CHARACTERISTICS OF GENTAMICIN ANTIBIOTICS

	Column effluent volumes (ml)								
	1950–2670	*2670–3155*	*3155–3485*	*3485–4175*	*4175–4770*	*4770–5110*	*5110–5550*	*5550–9340*	*9340–10120*
$R_F \times 100$	42 54 58	18 24	9 17 26 64	13 39	22	—	26 45	18	59

Fig. 4 illustrates descending paper chromatography of the N-acetylated gentamicin fractions, revealing a number of additional components; their direct classification as gentamicin antibiotics is impossible due to biological inactivity of N-acetyl derivatives.

The last major fraction, 5550–9340 ml, contains gentamicin A as the major constituent[3]. N-Acetylgentamicin A exhibits a R_F value of 0.30; Fig. 4 displays the position of pure N-acetylgentamicin A and shows three concomitant constituents of the gentamicin A fraction not revealed by TLC. Evidently, paper chromatography of the N-acetyl derivatives supplements very favorable the thin-layer chromatographic separations.

Important, related antibiotics are compared with the major gentamicins by TLC with solvent system I; the solvent front migrated 16.5 cm, the results are summarized in Table II.

TABLE II

THIN-LAYER CHROMATOGRAPHIC COMPARISON OF VARIOUS ANTIBIOTICS

Antibiotic	$R_F \times 100$
Streptomycin	0
Dihydrostreptomycin	0
Bluensomycin	11
Streptolin complex	11, 15, 20, 25
Neomycin B	26
Neomycin C	29
Paromomycin	34
Kanamycin A	38
Kanamycin C	51
Kanamycin B	56
Gentamicin A	60
Gentamicin D	69
Gentamicin C_1	71
Gentamicin C_2	76

ACKNOWLEDGEMENT

This work was supported in part by funds made available through Public Health Service Grant No. AI-06182.

SUMMARY

Column chromatography on a strongly basic ion-exchange resin, with water as eluent, fractionated the gentamicin antibiotic complex; each fraction, representing different mixtures of antibiotics, further separated upon thin-layer chromatography. Sixteen antibiotics were clearly differentiated. Paper chromatography of the N-acetyl derivatives supplemented thin-layer chromatography, detecting many additional constituents of the complex. A number of important, basic, water-soluble antibiotics were chromatographically compared with the major gentamicin antibiotics.

REFERENCES

1 T. IKEKAWA, F. IWAMI, E. AKITA AND H. UMEZAWA, *J. Antibiotics (Tokyo)*, 16A (1963) 56.
2 G. M. LUEDEMANN AND B. C. BRODSKY, in *Antimicrobial Agents and Chemotherapy—1963*, American Society for Microbiology, Washington, 1963, p. 116.
3 H. MAEHR, *Ph. D. Thesis*, Rutgers University, New Brunswick, 1964.
4 H. MAEHR AND C. P. SCHAFFNER, *Anal. Chem.*, 36 (1964) 104.
5 J. P. ROSSELET, J. MARQUEZ, E. MESEK, A. MURAWSKI, A. HAMDAN, C. JOYNER, R. SCHMIDT, D. MIGLIORE AND H. L. HERZOG, in *Antimicrobial Agents and Chemotherapy—1963*, American Society for Microbiology, Washington, 1963, p. 14.
6 G. H. WAGMAN AND M. J. WEINSTEIN, *J. Med. Chem.*, 7 (1964) 800.
7 M. J. WEINSTEIN, G. M. LUEDEMANN, E. M. ODEN AND G. H. WAGMAN, in *Antimicrobial Agents and Chemotherapy—1963*, American Society for Microbiology, Washington, 1963, p. 1.
8 M. J. WEINSTEIN, G. M. LUEDEMANN, E. M. ODEN, G. H. WAGMAN, J. P. ROSSELET, J. A. MARQUEZ, C. T. CONIGLIO, W. CHARNEY, H. L. HERZOG AND J. BLACK, *J. Med. Chem.*, 6 (1963) 463.