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Note

Separation of gentamicin complex by ion-exchange column chromatography

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Gentamicin is a complex mixture of closely related antimicrobial substances produced by *Micromonospora purpurea*. The composition of material available for clinical use is defined in relation to three major components, gentamicin C1, C2 and C1A. Chromatographic examination has however shown the presence of at least nine components[1,2], four of which are biologically active, the additional components representing only a small proportion of the total gentamicin.

At present quantitation of the three major components is attempted either by biological assay[3] or direct densitometry[4], after separation by either paper or thin-layer chromatography. A disadvantage of these methods is the necessity for standard preparations of the individual components against which the materials being examined can be compared after separation.

A method using a direct measurement of the separated components simplifies the analysis and a chromatographic column separation in which the eluate is monitored for optical rotation has been developed and is described here.

APPARATUS AND REAGENTS

Gentamicin samples: pure components C1, C1A and C2 supplied by Dr. M. D. Yudis of Schering Corporation, Bloomfield, N.J., U.S.A.: batches of gentamicin sulphate which have been available in Great Britain.

Ion-exchange material: cellulose phosphate P-11, 7.4 mequiv./g (Whatman, Maidstone, Great Britain).

Chromatographic column: 0.9 × 15 cm (Pharmacia).

Apparatus: LKB 10200 Perspex pump: LKB 8121 gradient mixer: Thorn NPL automatic polarimeter, Type 143: Smiths Servoscribe potentiometric recorder RE 51120.

EXPERIMENTAL

The column was prepared from a suspension of cellulose phosphate in 2.0 *M* sodium chloride solution and the eluate was monitored using a flow-through microcell (pathlength 20 mm, volume 100 μl) in the polarimeter. The sensitivity of the recorder was such that an optical rotation of $\pm 0.125°$ produced full scale deflection.

The micro-cell which was jacketed was operated at approximately 16° to prevent the formation of air bubbles in the cell.

Samples of gentamicin complex (80 mg) or of the individual gentamicin components (C1, C2 and C1A) (20 mg) were dissolved in 0.5 ml of 2.0 *M* sodium chloride and applied to the top of the column. The gradient mixer, containing 60 ml each of 2.0 and 2.2 *M* sodium chloride, was connected to the column and the gradient of increasing molarity pumped through at 10 ml/h. The column eluate was continuously monitored for optical rotation. For one experiment the eluate was collected in 1-ml fractions and tested for microbiological activity and ninhydrin reactivity. Microbiological activity was determined against *Bacillus subtilis* NCTC 8236 on medium A of the British Pharmacopoeia, pH 7.0 (ref. 5). Ninhydrin reactivity was determined by measuring the extinction at 570 nm of an aliquot of each fraction after reaction with ninhydrin reagent as described in the British Pharmacopoeia[6].

Peak areas were measured with a planimeter and expressed as percentages of the total area under the recording.

RESULTS AND DISCUSSION

Column chromatography of samples of the major gentamicin components showed that both gentamicins C1 and C1A were homogenous in this system but that gentamicin C2 consisted of two components (Fig. 1). Seven components with optical activity were detected in the commercial samples of gentamicin (Fig. 2). All optically active components reacted with ninhydrin and no ninhydrin positive optically inactive components were detected. Only the major components C1, C1A and the two fractions of C2 showed biological activity.

Table I shows the composition of samples examined, quantitated on the basis of peak area. Because the specific rotations of many of the components have not yet been defined, no assessment of the composition of the samples can be made on a weight basis. Where artificial mixtures have been used interpretation on a weight basis can be made from the measured optical rotation values for the samples used in preparing the mixture. The optical rotation values did not differ significantly and

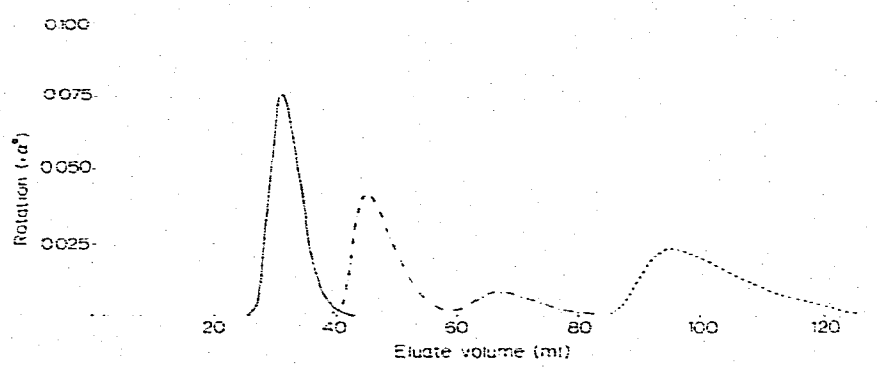

Fig. 1. The ion-exchange chromatography of gentamicins C1 (———), C2 (— · — · —) and C1A (·····), passaged separately on columns of cellulose phosphate with a salt gradient increasing from 2.0 to 2.2 *M* NaCl.

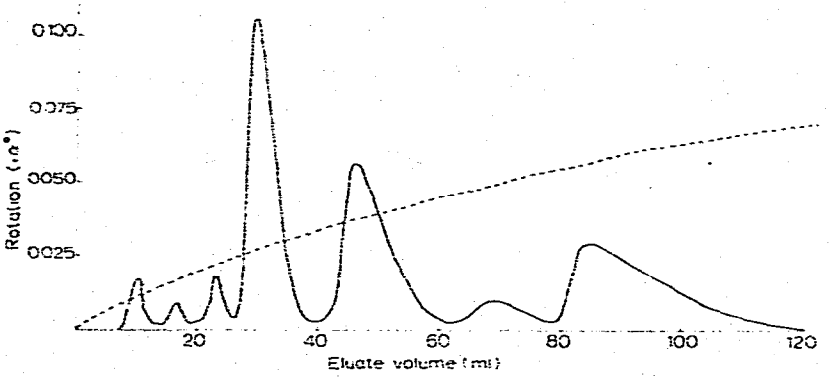

Fig. 2. The ion-exchange chromatography of gentamicin complex (sample B), passaged on a column of cellulose phosphate with a salt gradient increasing from 2.0 to 2.2 *M* NaCl (---------).

TABLE I

AREA OF EACH COMPONENT AS A PERCENTAGE OF THE TOTAL AREA RECORDED FOR THE OPTICAL ROTATION OF THE GENTAMICIN SAMPLES

I, II and III are minor components preceding the eluation of C1 (see Fig. 2).

Sample	*I*	*II*	*III*	*C1*	*C2(I)*	*C2(II)*	*C1A*
A	3.42	0.95	2.31	33.30	30.95	6.08	23.00
B	2.41	1.08	2.83	32.37	28.38	5.54	27.37
B	2.07	1.02	2.71	31.78	27.46	5.40	29.56
C	1.60	1.03	1.58	34.37	34.54*	6.15	20.75
D	2.36	1.17	2.36	31.86	29.28	6.18	26.79
E	2.00	1.32	*	33.46	28.64	6.49	28.10
F	1.48	3.41	*	29.64	33.74	6.51	25.23
F	1.79	1.28	2.92	30.71	33.85	5.66	23.80
G	3.15	2.59	1.59	38.26	33.05	7.50	13.86
G	2.15	2.17	1.12	39.41	31.63	6.58	16.95
G	1.90	2.22	1.21	37.81	31.64	7.41	17.81
H	1.12	2.29	1.08	44.49	39.95	11.07	0
I	2.13	*	2.53	18.75	13.24	3.60	59.76
J	0.17	1.11	0	15.50	8.11	2.05	73.07
J	0.87	1.78	0	14.67	8.52	2.38	71.78
K	0	0	0	28.81	55.85	15.34	0
K	0	0	0	29.26	56.00	14.73	0

* Peak not well separated and measured as part of preceding peak.

TABLE II

COMPOSITION (%, w/w) OF ARTIFICIAL MIXTURES OF GENTAMICIN SULPHATE

Sample	*C1*	*C2*	*C1A*
H	51.90	48.10	0
I	19.80	20.90	59.30
J	15.00	10.00	75.00
K	30.00	70.00	0

therefore the results of percentage area are directly comparable and in good agreement with the known composition (Table II). Repeated examination of the same samples shows the reproducibility of the procedure.

The minor components detected may well be the same extra components as reported by Wagman *et al.*[7]. However the presence of a second component in gentamicin C2 has not been previously reported.

The method described here produced a good separation of the components of gentamicin complex and, being an aqueous system, it was easier to use than the column method using a two-phase solvent system previously described[7].

The method can be used to compare the composition of samples of gentamicin. When reliable values are available for the specific rotation of each component a quantitative estimate can be made of the composition of gentamicin.

NOTE ADDED IN PROOF

Details of the flow-through micro-cell are described by De Rossi[8].

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