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Chromatographic separation of some minor components of the gentamicin complex

Paper, thin-layer, ChromAR®, and preparative separations of the major gentamicin components (C_1, C_{1a}, C_2) have been described by WAGMAN and co-workers[1,2] using a solvent mixture consisting of chloroform–methanol–17 % ammonium hydroxide. It had been previously reported by WEINSTEIN *et al.*[3], that two minor components, referred to as gentamicins A and B were co-produced with the gentamicin complex. Gentamicin A has been isolated and characterized by MAEHR[4]. It has now been demonstrated that the minor components consist of at least four related antibiotics identified as gentamicins A, B, B_1 and X.

This publication describes techniques for separation of the minor constituents produced in the gentamicin fermentation by *Micromonospora purpurea* NRRL 2953. This work was made possible by the availability of large quantities of the complex mixture since these components, representing only a small proportion of the total content, are not readily seen after chromatography of crude gentamicin.

Experimental and results

Separation of these antibiotic moieties has been accomplished with paper or ChromAR® chromatography in various solvent systems as follows, utilizing complex mixtures of the antibiotic components isolated as described later.

System No. 1. A solvent mixture consisting of chloroform–methanol–17 % ammonium hydroxide (2:1:1) was used to separate the components of the antibiotic complex. The quantity spotted on the papers for gentamicins A, B, X, and B_1 was 5 µg. The upper phase was placed in the bottom of the chromatographic jars several hours prior to use and lower phase was used to develop the paper chromatograms which were run downwards on Whatman No. 1 paper in insulated chambers, for 16 h at 25°. The solvent was allowed to drip off the paper during development.

Microbiological detection of the antibiotic zones was carried out by placing the paper directly on an agar plate seeded with *Staphylococcus aureus* ATCC 6538P.

TABLE I

CHROMATOGRAPHY OF MINOR GENTAMICIN COMPONENTS

	System 1 (*mm from origin*)	*System 2* R_F B_1[a]	*System 3* R_F
Gentamicin A_1[b]	0.0	0.36	0.10
Gentamicin A	0.0	0.41	0.16
Gentamicin B	9.0	0.52	0.22
Gentamicin X	13.5	0.55	0.28
Unknown component	27.0	0.72	0.32
Gentamicin B_1	27.0	1.00	0.40

[a] $R_F\ B_1 = \frac{\text{distance component from origin}}{\text{distance component } B_1 \text{ from origin}}$.

[b] Present in component A as a trace.

After 10 min the paper was removed and after a suitable incubation period the zones of inhibition, representing the gentamicin components, were observed. Very little movement of the minor components was seen in this system as described in Table I.

System No. 2. A solvent system was prepared consisting of 2-butanone-*tert.*-butanol-methanol-28% ammonium hydroxide (16:3:1:6). This is a modification of a solvent mixture described by MAJUMDAR AND MAJUMDAR[5]. The chromatographic jars were equilibrated for several hours prior to use. The antibiotic complex was spotted on Whatman No. 1 paper and run downwards for 16 h. The solvent was allowed to drip off the paper during development. Microbiological detection of the antibiotic components was carried out as described above.

R_F values relative to gentamicin B_1, the most mobile component, are described in Table I.

System No. 3. The lower phase of a chloroform-methanol-28% ammonium hydroxide (1:1:1) system was used to separate the minor components on ChromAR® sheet 500 (silicic acid-glass fiber sheet). The chromatograms were developed in equilibrated insulated chambers, ascending, for 50 min. Microbiological detection was carried out as described above. R_F values are detailed in Table I.

Column chromatography

At the end of the gentamicin fermentation, the antibiotics produced are extracted from the broth by adsorbing the total antibiotic complex onto a cation-exchange resin type such as Amberlite® IRC-50 in the ammonium cycle, and the crude antibiotic mixture eluted with dilute ammonium hydroxide. The eluate is decolorized by passage through an anion-exchange column containing Amberlite® IRA-401S resin in the hydroxyl form. After decolorization, the eluate is concentrated *in vacuo* to a solution containing from about 30% to 60% solids.

This solution contains all of the antibiotics produced in the fermentation. Removal of the gentamicin C components is accomplished by adsorbing the crude concentrate on a column of Dowex® 1 X2 and selectively eluting the gentamicin C components as a group with deionized water. The co-produced minor components are eluted from the Dowex 1 X2 column subsequent to the gentamicin C components and the aqueous eluate concentrated *in vacuo* to a suitable volume and lyophilized.

A 2-kg portion of fermentation product from which most of the gentamicin C had been removed was dissolved in 4.5 l of hot methanol. The solution was clarified by filtration, cooled to room temperature with exposure to air and seeded with gentamicin A. Gentamicin A crystallized as its colorless carbonate salt which was filtered, washed sparingly with methanol and dried at 80° *in vacuo* affording 120 g of the product.

The mother liquor and washings were combined and concentrated *in vacuo*. Based on paper chromatography this material contained residual A and C components together with B, B_1, and X. This material was chromatographed as follows: Two chromatographic columns 10 cm I.D. × 150 cm long were connected in series and filled with silica gel (Baker) which had previously been conditioned by contact with the lower phase of a mixture of chloroform-methanol-28% ammonium hydroxide (1:1:1). A solution of 750 g of the residue was prepared in a minimum volume (approx. 2 l) of the lower layer of the same solvent mixture. The antibiotic solution was pumped onto the first column with a metering pump attached to a pressure valve set at

5 p.s.i. The column was eluted by pumping solvent to the top at a flow rate of about 58 ml/min and collecting fractions of approximately 2000 ml each. The effluent was monitored by thin-layer and paper chromatography as described earlier. Appropriate fractions were combined and evaporated to dryness. Residual gentamicin C was eluted first, followed by an unknown component, B_1, X and B. When elution of gentamicin B was almost complete the solvent was changed to a mixture comprising equal volumes of methanol and conc. ammonium hydroxide to elute gentamicin A. The pooled fractions containing either gentamicin B or gentamicin B_1 were dried, dissolved in ethanol and concentrated to a residue. Repetition of the foregoing step resulted in crystalline suspensions of the ethanol solvates of gentamicin B and gentamicin B_1.

TABLE II

COLUMN CHROMATOGRAPHIC SEPARATION OF MINOR GENTAMICIN COMPONENTS

Fraction No.	*Component*	*Weight*[a] *(g)*
11–12	Fore-run	4.8
13–21	Gentamicin C	110
22–26	Unknown component + Gentamicin B_1	196.8
27–29	Gentamicin B_1	42.0
30–34	Gentamicins B_1 + X	67.8
35	Gentamicin X	12.3
36–40	Gentamicins X + B	74.9
41–50	Gentamicin B	85.8
51–80	Gentamicins B + A	165.0

[a] Not corrected for moisture.

A summary of the fractionation and yields of the gentamicin components are shown in Table II. Detailed chemical and biological studies on some of these antibiotics will be published elsewhere.

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