Chromatographic Separation and Bioassay of the Gentamicin Complex

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The gentamicin complex consists of three components, C_1, C_{1a}, and C_2, which are separated by paper chromatography using the lower phase of a solvent system of chloroform, methanol, and 17% ammonia (2:1:1). Two identical chromatograms are developed. On one, the positions of these components at R_f's of approximately 0.75, 0.35, and 0.50, respectively, are located by means of ninhydrin. On the other, corresponding areas are cut out, eluted from the paper with 0.1 *M* potassium phosphate buffer, pH 8.0, and assayed microbiologically. This procedure determines the relative amounts of the three gentamicin fractions, and is superior to the original official FDA procedure that measured only two fractions.

THE ISOLATION of gentamicin, an antibiotic complex from *Micromonospora purpurea*, was reported in 1963 (1, 2). Initially, this complex was considered to be a mixture of two fractions (C_1 and C_2). The invariance quotient of these two fractions is determined by acetylating the gentamicin sample with ^{14}C-labeled acetic anhydride, separating the two acetyl derivatives by paper chromatography, and measuring the relative amounts of the two fractions by radioautography (3). Further work (4), utilizing the technique of descending paper chromatography with a solvent system composed of chloroform, methanol, and 17% ammonia, revealed that C_1 consists of two fractions, C_1 and C_{1a}, making three fractions altogether. The exact structures of the gentamicin fractions are unknown, but they are amino-sugar antibiotics resembling the neomycin-kanamycin-streptomycin groups.

In order to characterize various batches of gentamicin with a view toward assuring compositional similarity of different batches, an analytical procedure for the gentamicin complex was developed, in which paper chromatography is used to separate the fractions and microbiological assay to measure the activity of each fraction.

EXPERIMENTAL

Materials—Reagent grade solvents and chemicals. Chromatographic paper, Whatman No. 2, 14.25 × 46 cm. Rule an origin line 9 cm. from the top of the paper and mark two dots on the origin line 4 cm. from each edge of the paper.

Apparatus—A standard cylindrical chromatography jar (30.5 cm. diameter, 61 cm. high) with a tightly fitting, ground-glass contact top and equipped for descending chromatography.

Solvent System—Equilibrate 200 ml. of chloroform and 100 ml. of methanol with 100 ml. of 17% ammonia [ammonium hydroxide + water (3 + 2)] by shaking in a separator. Without allowing the phases to separate, add the entire mixture to the chromatography jar and allow 24 hr. for saturation. Prepare another equilibration mixture as above but allow the phases to separate and use the lower phase only as the chromatographic solvent.

Ninhydrin Reagent—Prepare a modified Barrollier reagent (5) as follows: To 1 g. of ninhydrin and 0.1 g. of cadmium acetate, add 3 ml. of water and 1.5 ml. of glacial acetic acid, and shake. Add 100 ml. of *n*-propanol and shake until solution is complete. Store this solution in a brown bottle under refrigeration.

Procedure—Prepare an aqueous solution containing 40 mg. of the sample/ml. Apply 5 µl. to each of the two dots on the chromatographic paper and allow to dry. In like manner, prepare another paper. Place the papers in separate troughs in the chromatographic chamber, and fill the two troughs with the chromatographic solvent. Chromatograph until the solvent front reaches the bottom of the paper (approximately 3.5 hr.). Remove the papers and dry them in a hood for 30 min. Cut each paper in half lengthwise; spray one half with ninhydrin reagent, and place it in a drying oven at 100° for 1 min.

The gentamicin fractions appear as reddish zones; the zone farthest from the origin is C_1, the zone nearest is C_{1a}, and the middle zone is C_2. Using this half as a guide, cut the other half of the paper into segments representing, respectively, the C_1, C_2, and C_{1a} fractions. Cut each segment thus obtained into small strips and put the strips into a separate 125-ml. glass-stoppered flask. To each flask add 50 ml. of 0.1 *M* potassium phosphate buffer, pH 8.0, and swirl the flasks mechanically for 30 min. Decant each solution into a test tube and allow the paper to settle. Pipet 4.0 ml. of the clear solution into a 25-ml. volumetric flask and make to volume with the same buffer. Assay these solutions by the microbiological plate assay, using *Staphylococcus epidermidis* as the test organism (6).

Computations—The percentage of each fraction is calculated as follows:

$$\% C_1 = \frac{A_1}{504} \times \frac{100}{B}$$

$$\% C_{1a} = \frac{A_{1a}}{626} \times \frac{100}{B}$$

$$\% C_2 = \frac{A_2}{656} \times \frac{100}{B}$$

where:

A_1 = concentration of the assayed C_1 solution in mcg./ml.

A_{1a} = concentration of the assayed C_{1a} solution in mcg./ml.

A_2 = concentration of the assayed C_2 solution in mcg./ml.

$$B = \frac{A_1}{504} + \frac{A_{1a}}{626} + \frac{A_2}{56}$$

504, 626, and 656 are the activities of C_1 sulfate, C_{1a} sulfate, and C_2 sulfate, respectively, compared to the gentamycin sulfate standard.

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TABLE I—COMPARISON OF KNOWN AND ASSAYED VALUES OF THE GENTAMICIN FRACTIONS

Gentamicin Fraction	% Present	% Found ± SD
C_1	37.8	37.2 ± 2.04
C_{1a}	33.3	32.7 ± 2.17
C_2	28.9	30.0 ± 2.26

RESULTS AND DISCUSSION

A synthetic mixture of the three gentamicin fractions was prepared and assayed six times. The average recoveries for the fractions ranged from 98 to 104% (Table I).

Seven production lots of gentamicin sulfate were analyzed. The results (Table II) show a pattern of relative fractional composition which is rather consistent for this type of sample.

A differential microbiological assay of the gentamicin complex has been suggested which involves the use of a bioautographic procedure (7). Microgram and submicrogram amounts of antibiotic are spotted on narrow strips of paper for chromatographing. The chromatographed papers are placed on a seeded agar plate for the determination of antibacterial activity in which the sizes of the zones of inhibition produced by the sample are compared with those of the standard. A serious problem that may occur is the production of spurious zones caused by contamination, atmospheric and otherwise. This could be minimized by meticulous handling in a clean environment.

In the procedure described in the experimental section of this paper, any such possible interference is reduced more than 300-fold because microgram amounts of antibiotic are placed on the paper, and they are eluted and diluted even more to obtain concentrations suitable for microbiological assay. Another feature of this new procedure is that there is no need for reference standards of the fractions, since the dose-response curves for the three gentamicin components have the same slopes (4). Once the positions of the three fractions have been located on the chromatogram, the relative proportions are determined by microbiological assay using a single reference standard of gentamicin sulfate rather than three individual standards.

The solvent system of chloroform–methanol–17% ammonia (2:1:1) was used by Ikekawa *et al.* (8) for the separation of various antibiotics by TLC. Gentamicin was not included, as it was unknown at that time. Moreover, the upper layer of this solvent system was used, which is unsatisfactory for the separation of the gentamicin fractions. The lower layer must be used in the procedure described here.

The chromatography tank must be completely saturated for a satisfactory and rapid separation.

TABLE II—ANALYSIS OF PRODUCTION LOTS OF GENTAMICIN SULFATE

Lot No.	% C_1 ± SD	% C_{1a} ± SD	% C_2 ± SD
1	37.0 ± 1.73	27.0 ± 2.13	36.1 ± 0.96
2	34.0 ± 2.41	34.3 ± 4.20	31.7 ± 3.66
3	39.5 ± 2.26	25.2 ± 1.66	35.3 ± 1.80
4	40.7 ± 1.56	26.4 ± 1.19	32.9 ± 1.80
5	29.7 ± 1.40	32.8 ± 3.00	37.5 ± 2.36
6	38.3 ± 3.29	20.6 ± 1.89	41.1 ± 2.38
7	40.7 ± 2.14	21.0 ± 2.21	38.4 ± 2.81

The appearance of two phases on the paper is an indication that the tank is not completely saturated. It may be possible to obtain an acceptable chromatographic separation under this condition but the time of development would be increased so that the separation and elution could not be finished in one day.

Other papers such as Whatman No. 1 and S&S 589 Blue R were not as satisfactory; the time of development was longer and the separation of the fractions not as consistently good.

An attempt to assay the three fractions colorimetrically after a ninhydrin spray by determining the absorbances on a densitometer proved unsatisfactory. The intensities of the colors varied with time and with the fraction. Also, trace amounts of ammonia in the atmosphere caused the paper to gradually turn pink, thus interfering with the base line.

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Keyphrases

Gentamicin complex—separation, bioassay
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Ninhydrin—color reagent
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