# Chemical Determination of Component Ratio and Potency of Gentamicin Complex

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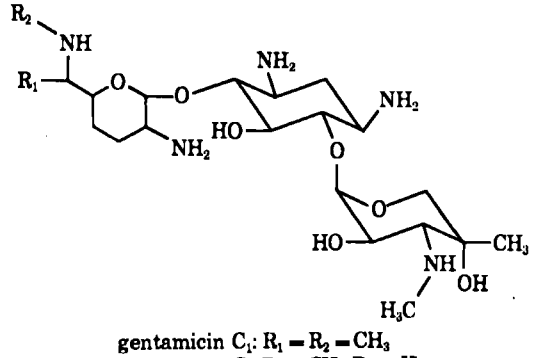
**Abstract** □ A rapid chemical method for estimating the total gentamicin potency as well as for quantitating the individual components in one step by direct densitometry after resolution on TLC is described. Because the amount of each component is read directly from the plate after separation of impurities and the values are interpolated from standards treated in an identical manner, this method is more accurate for estimating total potency than the official microbiological assay in which biologically active impurities may interfere.

**Keyphrases** □ Gentamicin—simultaneous component variation analysis and potency determination, TLC–direct densitometry □ Microbiological potency, gentamicin—simultaneous determination with component variation analysis, TLC–direct densitometry □ TLC–direct densitometry—simultaneous component variation analysis and potency determination, gentamicin □ Direct densitometry and TLC—simultaneous component variation analysis and potency determination, gentamicin □ Antibiotics—method for simultaneous component variation analysis and potency determination

Gentamicin is a broad spectrum oligosaccharide antibiotic produced by *Micromonospora purpurea*. It has been shown by paper chromatography (1) to consist of three active components, $C_1$, $C_2$, and $C_{1a}$ (Structure I). These have also been separated by column (1) and ion-exchange (2) chromatography. The drawbacks of the column method are the comparative difficulty of column packing and equilibration, the large eluant volume, and the high adsorbent to antibiotic ratio required for resolution. This method was recently improved on a preparative scale by Cooper *et al.* (3).

The present USP (4) and BP (5) requirements for this antibiotic consist of a microbiological estimation of potency. In addition, the USP has set limits on the allowable percentage of the individual components in the gentamicin complex and prescribes a differential paper chromatographic bioassay for their control. Wagman *et al.* (6) also published a chemical method for quantitating the various components by direct densitometry after paper chromatography. This method, however, involves an 18-hr. development of the paper chromatogram.



gentamicin $C_1$: $R_1 = R_2 = CH_3$
$C_2$: $R_1 = CH_3$, $R_2 = H$
$C_{1a}$: $R_1 = R_2 = H$

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This paper reports a rapid chemical method for estimating the total gentamicin potency as well as for quantitating the individual components, all in one step by direct densitometry after resolution on TLC. This method, which has already been applied successfully to the analysis of the tetracycline (7) and erythromycin (8) antibiotics, is much faster than the official microbiological procedures.

## EXPERIMENTAL

**Standard Solutions**—Gentamicin sulfate working standard (potency 586 mcg./mg.) was assayed in two independent laboratories by the differential microbiological assay and was found to contain, on a weight basis, 164 mcg. $C_1$-base, 217 mcg. $C_2$-base, and 204 mcg. $C_{1a}$-base/mg. gentamicin sulfate. It was dissolved in distilled water to a concentration of 15.00 mg. gentamicin sulfate/ml., giving a standard solution containing 2.46 mg. $C_1$-base, 3.26 mg. $C_2$-base, and 3.06 mg. $C_{1a}$-base/ml.

**Assay Solutions**—Accurately weighed samples of gentamicin sulfate were dissolved in water to a concentration of about 30 mg. gentamicin sulfate/ml. In the case of the injection preparation, a volume (1 μl.) equivalent to about 30 mcg. gentamicin sulfate was applied directly to the TLC plate.

**TLC**—Precoated analytical silica gel 60[1] plates (20 × 20 cm., 0.25-mm. thickness) were heated for 1 hr. at 135° prior to use. The solvent system was composed of the lower layer (150 ml.) of a mixture of methanol–chloroform–28% ammonium hydroxide

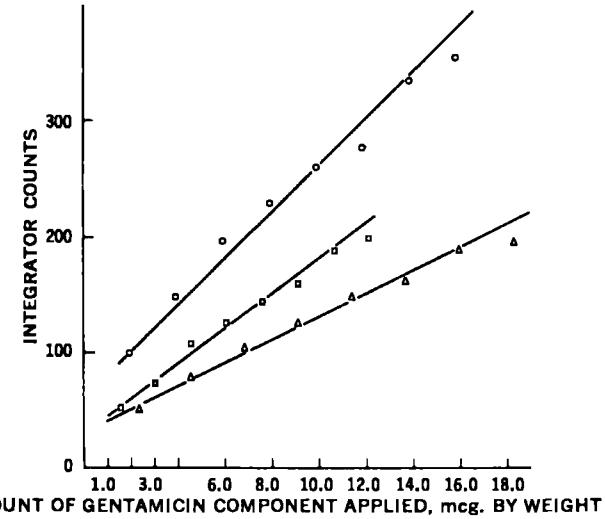


**Figure 1**—*Representative calibration curves for the gentamicin components in the gentamicin sulfate standard. Key:* □, *gentamicin* $C_1$; △, *gentamicin* $C_2$; *and* ○, *gentamicin* $C_{1a}$.

[1] E.M. Reagents, E.M. Laboratories Inc., Elmsford, NY 10523

Table I—Determination of Components in Bulk Samples by the Densitometric Method

| Sample | Percent by Weight of Components $C_1$ | $C_2$ | $C_{1a}$ |
|---|---|---|---|
| Bulk A | 25.2 | 44.5 | 30.2 |
| Bulk B | 34.8 | 40.1 | 25.1 |
| Bulk C | 25.3 | 44.9 | 29.8 |
| Bulk D | 27.7 | 41.6 | 30.7 |
| Bulk D[a] | 29.1 | 40.5 | 30.5 |
| Injection E | 35.1 | 44.6 | 20.3 |

[a] Results obtained by differential bioassay method.

(1:1:1). This was passed through 100 g. of anhydrous sodium sulfate before being placed in a chromatographic chamber lined with Whatman 3-mm. filter paper. The chamber was saturated with solvent for 1 hr. prior to use. Samples were applied to the plates using 1-μl. micropipets[2]. One- and 3-μl. aliquots of the reference solution and a 1-μl. aliquot of the sample to be assayed, with a concentration lying within those of the two reference amounts, were applied across the plate 2 cm. from the lower edge as alternate spots, giving a total of eight measurements, four for the standard and four for the unknown.

The plate was developed to a height of 15 cm. (80 min.), removed from the tank, dried at 135° for 20 min., and allowed to cool. Uniform spraying with 10 ml. of a freshly prepared solution of ninhydrin (1.0 g.) in a mixture of 95% ethanol (50 ml.) and glacial acetic acid (10 ml.), followed by heating for 15 min. at 135°, revealed the gentamicins as magenta spots on a white background. These were analyzed the same day by direct densitometry.

**Densitometry**—Measurements were performed on a double-beam chromatogram analyzer[3] with the following settings: monochromator, 500 nm.; spectral resolution, 10 nm.; double-beam mode; motor drive speed, 2.03 cm. (0.8 in.)/min.; and recorder chart speed, 2.54 cm. (1.0 in.)/min. Spots were scanned in the direction of solvent flow and the areas were quantitated by means of the digital integrator.

A plot of peak area (integrator counts) *versus* amount of each component (micrograms) yielded linear calibration curves for amounts between 2 and 12 mcg. of each component per spot (Fig. 1). In practice, duplicate spots of two reference concentrations, within the values of which the unknown concentration will be located, are applied to the plate; curves are prepared for each individual plate by plotting the integrals of the observed peaks for the four reference spots *versus* the amount (micrograms) of each component present in the reference solution. The values of each component in the four unknown samples are found by interpolation from the respective curve (Fig. 2). The results from each of the three components are added and expressed as a percentage by weight of the total. The individual values of each component (in micrograms) are then converted into micrograms of gentamicin base activity

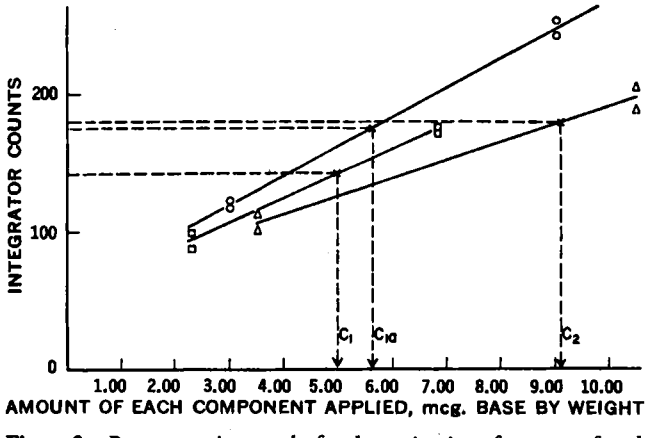


**Figure 2**—*Representative graphs for determination of amount of each component in a gentamicin sample. Values of components are found by interpolation from the respective plot. Key: □, gentamicin $C_1$; △, gentamicin $C_2$; and ○, gentamicin $C_{1a}$.*

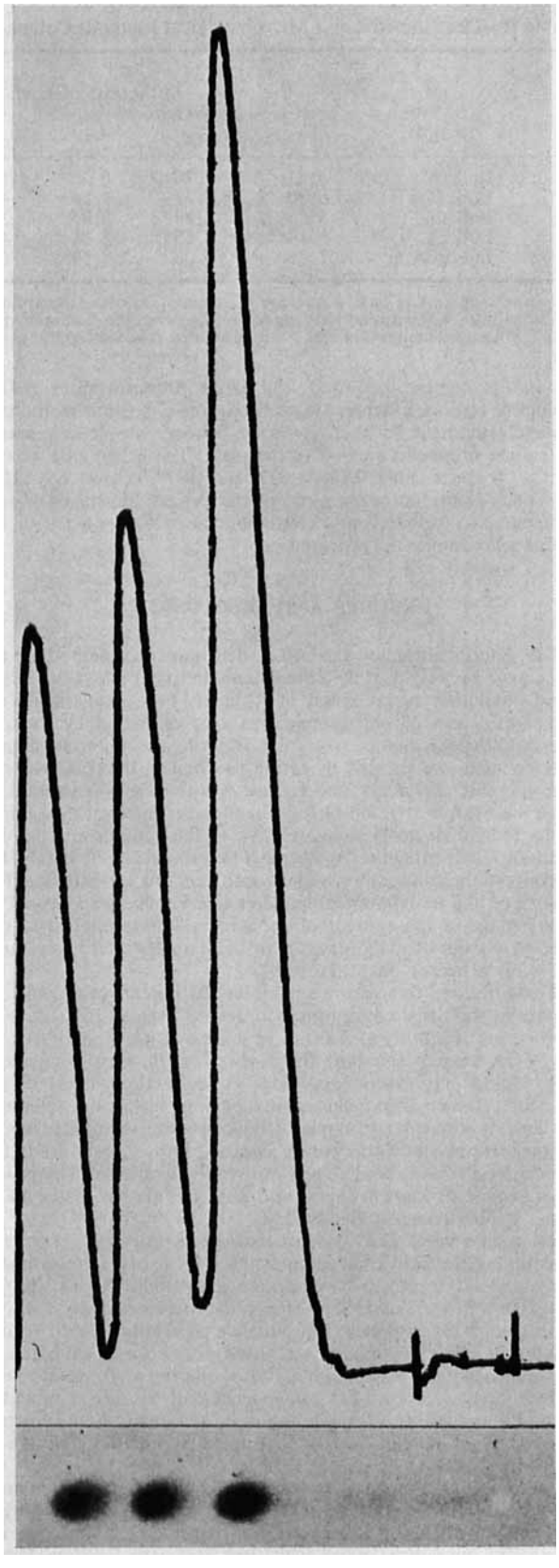

**Figure 3**—*TLC separation and densitometer scan of gentamicin complex. Starting at origin on the right, the spots are gentamicin $C_{1a}$, $C_2$, and $C_1$, respectively.*

[2] Drummond Scientific Co.
[3] Vis-UV, Farrand Optical Co., Mount Vernon, NY 10550

Table II—Densitometric and Microbiological Methods Comparison

| Sample | Amount[a] of Each Component — By Weight $C_1$ | By Weight $C_2$ | By Weight $C_{1a}$ | By Activity $C_1$ | By Activity $C_2$ | By Activity $C_{1a}$ | Densitometric Assay[b] — Potency[c] | Coefficient of Variation | Microbiological Assay Potency[c] |
|---|---|---|---|---|---|---|---|---|---|
| Bulk A | 5.21 | 9.20 | 6.25 | 4.10 | 9.42 | 6.12 | 655 | 4.4 | 641[d] |
| Bulk B | 6.30 | 7.27 | 4.54 | 4.95 | 7.44 | 4.44 | 561 | 9.8 | 561 |
| Bulk C | 5.05 | 8.98 | 5.95 | 3.97 | 9.19 | 5.81 | 632 | 2.8 | 641 |
| Bulk D | 5.10 | 7.65 | 5.65 | 4.00 | 7.82 | 5.52 | 578 | 5.7 | 577 |
| Injection E | 7.95 | 10.08 | 4.59 | 6.25 | 10.31 | 4.48 | 1052[e] | 6.3 | 1000[f] |

[a] In micrograms of base. [b] Average of a minimum of six determinations for each value reported. [c] In micrograms of base per milligram of gentamicin sulfate. [d] Determinations made by Bio-Research Laboratories, Pointe Claire, Quebec, Canada, with required pharmacopeial precision of ±5%. [e] Assayed contents of vial. [f] Amount present based on label claim.

by multiplying by the Food and Drug Administration (FDA) biological response factors (4), totaled, and expressed as the percentage gentamicin base activity in the 30-mcg. sample of gentamicin sulfate originally applied to the plate. These response factors for $C_1$, $C_2$, and $C_{1a}$ are 0.786, 1.023, and 0.977, respectively. Thus from each plate, four estimations of the percent by weight of each component as well as four determinations of the potency of the gentamicin complex are provided.

## RESULTS AND DISCUSSION

The determination by the direct densitometric method of the percentage by weight of the components in four bulk samples and one formulation is presented in Table I. For comparison, the weight variation of one sample was also estimated by the differential bioassay method (4). Table II shows the amounts of each component by weight and by activity as well as the total potency assayed by densitometry and by the microbiological method. By using the FDA correction factors for the microbiological response of the individual gentamicins relative to the gentamicin complex standard (assayed against the same FDA standard), it is possible to convert the amount by weight into amount by activity. The activity of the individual components when totaled and taken as a percentage of the amount of gentamicin sulfate applied to the plate gave values for the potency of the complex that agree quite well with the microbiological results.

It was decided to use amount by weight instead of amount by activity in the densitometry method and to convert the values to micrograms of activity at the end. In this way the eventual use of a new FDA master standard (of perhaps a different component ratio) would only involve applying new correction factors in the last step. Rather than using pure components as the reference standard, a commercial sample of bulk gentamicin sulfate, whose composition and potency were accurately known, was used; the pure materials are expensive and not readily available. Any gentamicin sample of known weight and potency relative to the FDA master standard may be employed.

Although several TLC systems have been proposed (1) for the resolution of the individual gentamicins, the use of the lower phase of methanol–chloroform–concentrated ammonium hydroxide (1:1:1) gave the best separation. This system was originally used by Cooper *et al.* (3); however, reproducible separations could be obtained only when the solvent was dried prior to use. Commercial precoated silica gel plates, although not offering any improvement in resolution, were used for convenience and because they had a more uniform surface for quantitation by reflectance densitometry. An example of the TLC separation and densitometric quantitation is shown in Fig. 3.

The use of ninhydrin for detection of the spots after chromatography also required improvement. Kantor and Selzer (9) reported that ninhydrin was unsatisfactory for densitometry since the intensities of the colors varied with time and with fraction. However, the inclusion of acetic acid ensures an optimum pH of about 5, as required for the reaction (10), and gives bright-red spots on a white background, which were shown by densitometry to be stable for up to 6 hr. (repetitive scanning gave a variation of less than 1%). Other potential problems due to different responses of the gentamicin components to the spray and unevenness of spraying are easily overcome when standards and unknowns are compared under identical conditions on the same plate.

This TLC–direct densitometric assay technique offers several improvements over the methods presently available for the gentamicin component variation analysis and potency determination. In the first place both tests are combined in one experiment. Second, it accomplishes these tests in a much faster time and with the same precision as the microbiological assay. It is well known that antibiotics of the deoxystreptamine group may be mixtures, and FDA has set limits on allowable percentages. Maehr and Schaffner (2) showed this also to be true for the gentamicin complex. Because the amount of each component is read directly from the plate after separation of impurities and the values interpolated from standards are treated in an identical manner, the densitometric method is more accurate for estimating total potency than the official microbiological assay in which biologically active impurities may interfere.

## REFERENCES


(1) G. H. Wagman, J. A. Marquez, and M. J. Weinstein, *J. Chromatogr.*, **34**, 210(1968).

(2) H. Maehr and C. P. Schaffner, *ibid.*, **30**, 572(1967).

(3) D. J. Cooper, M. D. Yudis, H. M. Mariglians, and T. Traubel, *J. Chem. Soc., C*, **1971**, 2878.

(4) "The United States Pharmacopeia," 18th rev., Mack Publishing Co., Easton, Pa., 1970.

(5) "The British Pharmacopoeia," Pharmaceutical Press, London, England, 1968, Addendum 1971.

(6) G. H. Wagman, J. V. Bailey, and M. M. Miller, *J. Pharm. Sci.*, **57**, 1319(1968).

(7) C. Radecka and W. L. Wilson, *J. Chromatogr.*, **57**, 297(1971).

(8) C. Radecka, W. L. Wilson, and D. W. Hughes, *J. Pharm. Sci.*, **61**, 430(1972).

(9) N. Kantor and G. Selzer, *ibid.*, **57**, 2170(1968).

(10) E. Stahl, "Thin-Layer Chromatography: A Laboratory Handbook," 2nd ed. (in translation), Springer-Verlag New York, New York, N. Y., 1969, p. 747.


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