of SA at essentially one dose level, and consistency at one level is no test of a model. Since it is known that SA elimination kinetics do change with dose, more information is needed before a model can be proposed which will adequately describe the observed data at all dose levels of this drug.

APPENDIX

The mass equations describing the scheme in Fig. 3 are:

$$-\dot{m}_1 = k_{12}\cdot m_1 + k_{13}\cdot m_1 - k_{21}\cdot m_2 \quad \text{(Eq. 1}a\text{)}$$

$$\dot{m}_2 = k_{12}\cdot m_1 - k_{21}\cdot m_2 \quad \text{(Eq. 2}a\text{)}$$

$$\dot{m}_3 = k_{13}\cdot m_1 - k_{34}\cdot m_3 - k_{35}\cdot m_3 + k_{43}\cdot m_4 \quad \text{(Eq. 3}a\text{)}$$

$$-\dot{m}_4 = -k_{34}\cdot m_3 + k_{43}\cdot m_4 \quad \text{(Eq. 4}a\text{)}$$

$$-\dot{m}_5 = -k_{35}\cdot m_3 \quad \text{(Eq. 5}a\text{)}$$

$$\text{Dose} = m_1 + m_2 + m_3 + m_4 + m_5 \quad \text{(Eq. 6}a\text{)}$$

where m_x is the amount of durg in compartment x. The analog computer program for this scheme is shown in Fig. 9.

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Keyphrases

Pharmacokinetics—aspirin, salicylic acid
Aspirin, *N*-methylglucamine salt—IV administration
Salicylic acid, *N*-methylglucamine salt—IV administration
Two-compartmental open system—pharmacokinetic model
UV spectrophotometry—analysis
Fluorometry—analysis
GLC—analysis

Differential Ninhydrin Chromatographic Assay for the Gentamicin Complex

By GERALD H. WAGMAN, JANET V. BAILEY, and MORTON M. MILLER

A differential ninhydrin chromatographic assay has been devised to quantitatively determine the proportions of the three gentamicin components in gentamicin preparations. The technique consists of separation of the antibiotics on paper using the lower phase of a solvent system composed of chloroform, methanol, and 17 percent ammonium hydroxide (2:1:1 v/v). After development, strips containing the antibiotic are treated with ninhydrin reagent, developed, and color intensities read on an integrating scanner from which component proportions can be determined. Results are in excellent agreement with the microbiological method.

Recently, Weinstein *et al.* (1) reported that preparations of the gentamicin complex consist of three antibiotic components which have been designated C_1, $C_{1}a$, and C_2 and that there are no significant differences in their biological properties. It became desirable therefore to devise methods for detection, isolation, and assay of these components. Thin-layer, paper, and column chromatographic separation techniques have been described by Wagman *et al.* (2) using a solvent system composed of chloroform–

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methanol–17% ammonium hydroxide (2:1:1 v/v).

A differential paper chromatographic microbiological assay has been reported (3) which utilizes this solvent system and is based on bioanalytical methods for determinations of penicillin mixtures. The method consists of chromatographing samples containing gentamicin together with a series of standards on paper strips, bioautographing the strips against *Staphylococcus aureus* on standardized assay plates, and measuring the diameters of the zones of inhibition of the separated components. Standard curves are constructed, and the concentration of each of the components in a sample of unknown composition is determined from an appropriate curve.

A chemical method has now been devised to carry out the differential analysis of the gentamicin components and is described here. While this new technique uses the chromatographic solvent system as reported in the bioanalytical assay, it does not require biological procedures. After chromatographic separation the papers are treated with ninhydrin, heated at 105° to develop the color, and run through an integrating scanner. The quantities of the individual components are determined by the peak intensities of the chromatogram zones. Although the quantity of material required in this method is somewhat greater than in the microbiological assay procedure, the technique is simple and reproducible.

EXPERIMENTAL

Apparatus and Materials

(*a*) Standard rectangular chromatographic tanks with troughs 30.5 × 30.5 × 61 cm. high (12 × 12 × 24 in.); insulate the outsides completely with paper.

(*b*) Schleicher and Schuell No. 589 blue ribbon chromatographic paper cut to size 20 × 58 cm. (along grain).

(*c*) Integrating scanner—instrument used for the determinations to be described was the model RB Analytrol, manufactured by Beckman Instruments, Inc., Fullerton, Calif.

(*d*) Reagents—the purest available reagent grade chemicals were used. The 17% ammonium hydroxide was made by mixing 600 ml. concentrated NH_4OH with 400 ml. water. Gentamicin C_1, C_{1a}, and C_2 standards were obtained as the sulfates and converted to their respective free bases by use of small columns of IRA 401S resin (Rohm and Haas) in the hydroxyl form and the solutions lyophilized. All standards were dried at 60° under vacuum for 3 hr. prior to use. Ninhydrin reagent (triketo hydrindene hydrate) was prepared fresh daily as a 0.25% solution by dissolving in a 1:1 mixture of pyridine–acetone.

Methods

Chromatographic Solvent and Conditions—The solvent is prepared by mixing together chloroform, methanol, and 17% NH_4OH in a ratio of 2:1:1 (v/v) in a separator and allowing the layers to separate. The lower layer is placed in the bottom of the chromatographic jar, and the upper layer is placed in a beaker with a wick at the bottom of the jar. The jar is equilibrated for several hours prior to use. The lower layer is used for development of the chromatograms. Optimum temperature for development is in a range of from 20 to 25°, and it is important that the temperature at which the chromatograms are run be maintained at a constant level.

Preparation of Standards and Samples—Gentamicin standards consisting of individual solutions of the C_1, C_{1a}, and C_2 components, either all as the sulfates or all as free bases, are dissolved in water to give concentrations of exactly 50 mg./ml. Equal volumes of each component standard are mixed together to give a solution containing the three components in a 1:1:1 ratio. Three replicate standard solutions are prepared in this manner. Samples in which the ratios of the gentamicin components are to be determined are dissolved in water to give a concentration of about 50 mg./ml.

Preparation and Development of Chromatograms—A line is drawn approximately 7 cm. from the end of each strip and 5-μl. quantities of each standard mixture are pipeted twice on triplicate spots equidistant from each other. This results in three chromatographic sheets containing a total of nine standard mixtures at a level of 500 mcg./spot.

The samples to be assayed are spotted in exactly the same manner as the standards, three spots for each unknown, using 10 μl./spot (two increments of 5 μl.).

The papers are placed in the chromatographic jars and solvent added to the troughs immediately. No equilibration is necessary. The papers are developed, descending, for 18 hr., and air dried for 15 min. to remove solvent. The sheets are pulled through a trough of ninhydrin reagent, dried in a fume hood, and heated for 5 min. at 105° to develop the color. The gentamicin C_1 zone is found nearest to the front, followed by C_2, and the zone closest to the origin is designated as C_{1a}. A chromatogram showing the relative positions of the components is illustrated in Fig. 1.

Scanning Chromatograms and Computations—The chromatograms are cut into strips 3.2 cm. (1¼ in.) in width and reference lines are marked on both ends of the papergrams beyond the last zone and before the origin. The strips are put through the integrating scanner using a slit width of 1.5 mm., and the zone intensities are automatically plotted and integrated. The chromatogram is lined up with

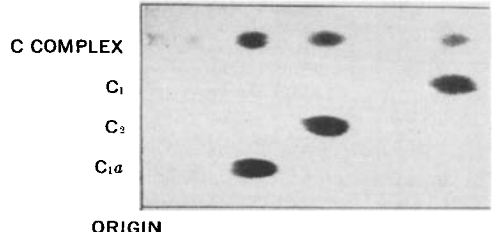

Fig. 1—Ninhydrin-treated chromatogram illustrating separation of gentamicin components using chloroform–methanol–17% ammonia (2:1:1 v/v) solvent system.

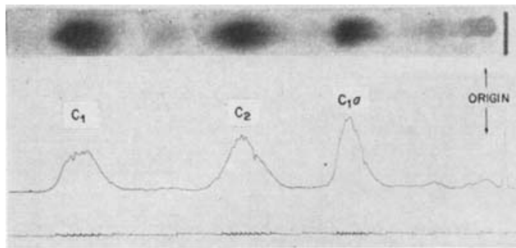

Fig. 2—Integrated scan of ninhydrin reaction following paper chromatography. Instrument used was Beckman Analytrol model RB, slit width = 1.5 mm. Upper curve is densitometer scan; lower, sawtooth line is integrator trace.

its scan using the reference marks, and the total scan area and area under each peak is determined for each chromatographic strip. A typical chromatogram of a gentamicin mixture and its integrated scan is illustrated in Fig. 2. Ratios of ninhydrin intensity of standards in a mixture containing equal weights of components can then be calculated as will be described. Results for each group of nine reference standards are averaged to obtain a final set of values.

Comparison of ninhydrin reactivity for each of the gentamicin base component standards showed that the intensity of color varied with $C_{1}a > C_2 > C_1$. This was true also for the sulfates, but the ratios were somewhat different. For the free bases, if $C_{1}a$ is assigned a ninhydrin peak value of 1.00, then an equal quantity of C_2 results in a less intense color reaction and a value of 0.58 (58% of the intensity of color for an equal weight of C_2 compared to $C_{1}a$). For the C_1 component, the value is 0.52. Therefore, in order to calculate the correct proportions of components in an unknown mixture, it is necessary to multiply the C_1 and C_2 peak integrations by the appropriate factor (the reciprocal of the color intensity) to compensate for the differences in intensity between those components and $C_{1}a$. Similarly, for the sulfates, this compensation must also be made. The peak intensities and correction factors (reciprocals) for the bases and sulfates are summarized in Table I.

Samples of unknown composition are run in a manner similar to that for the standard mixtures. After integration of component peaks, the peak areas are multiplied by the appropriate factors for each compound and the corrected intensities for each zone are added together. The percentage of each component can be calculated by dividing the number of integrations for each chromatogram zone by the total number for the complex. The three replicate sets of chromatogram data are averaged to obtain final results.

TABLE I—COMPARISON OF INTENSITY OF NINHYDRIN REACTIONS ON CHROMATOGRAMS FOR INDIVIDUAL COMPONENTS OF THE GENTAMICIN COMPLEX

Derivative	Component	Relative Intensity of Ninhydrin Spot	Factor, Reciprocal of Intensity
Base	$C_{1}a$	1.000	1.00
	C_2	0.578	1.73
	C_1	0.518	1.93
Sulfate	$C_{1}a$	1.000	1.00
	C_2	0.485	2.06
	C_1	0.397	2.52

RESULTS AND DISCUSSION

Assay responses using the ninhydrin differential assay were compared to results obtained by the previously described microbiological method on six lots of gentamicin sulfate. Using the antibacterial potencies of C_1, $C_{1}a$, and C_2 gentamicins (assayed against the same working standard) it was possible to convert the percentages by weight to percentages based on activity.

Table II indicates the average assay results obtained using the ninhydrin assay both by weight and activity compared to the results obtained for the same samples in the microbiological assay. Assay variation as determined by a series of completely randomized analyses of variance indicates the residual error to be in excellent agreement with the biological procedure.

It has been found that a significantly high correlation ($p < 0.01$) exists between the biological assay and the ninhydrin assay, both by weight and activity. The product-moment correlation coefficient indicates the degree of association between the two assay procedures where -1.0 indicates perfect negative correlation and $+1.0$ indicates perfect positive correlation. These data are summarized in Table III and are a reflection of the data given in Table II, where each observation is a mean of three values.

TABLE III—CORRELATION OF NINHYDRIN DIFFERENTIAL ASSAY WITH MICROBIOLOGICAL DIFFERENTIAL ASSAY FOR GENTAMICIN SULFATE BATCHES

Component	Correlation Coefficient: Weight	Activity
C_2	0.87	0.94
$C_{1}a$	0.89	0.88
C_1	0.87	0.94

TABLE II—AVERAGE ASSAY VALUE OF GENTAMICIN SULFATE BATCHES BY LOT AND METHOD

	Ninhydrin Assay								
	By Weight, %			By Activity, %			Microbiological Assay, %		
Lot No.	C_1	C_2	$C_{1}a$	C_1	C_2	$C_{1}a$	C_1	C_2	$C_{1}a$
GMC-4J-5I	46.9	36.2	16.9	41.2	40.8	18.0	42.2	39.2	18.7
60-1197-65I	46.3	33.1	20.6	40.6	37.4	22.0	41.2	34.1	24.9
60-1197-64I	35.6	32.2	32.2	30.6	35.6	33.8	36.5	32.9	30.6
GMC-4J-1-4C	32.4	42.6	24.9	27.5	46.6	25.9	29.1	40.5	30.5
60-1197-61I	44.8	33.2	21.9	36.0	39.4	24.6	38.7	37.8	23.5
60-1197-60	40.2	41.8	18.0	34.7	46.4	19.0	36.8	43.5	19.7

TABLE IV—AVERAGE ASSAY VALUE OF GENTAMICIN BATCHES CONVERTED TO THEIR FREE BASES BY LOT AND METHOD

	Ninhydrin Assay						Microbiological Assay		
	By Weight, %			By Activity, %					
Lot No.	C_1	C_2	C_{1a}	C_1	C_2	C_{1a}	C_1	C_2	C_{1a}
GMC-4J-5I	42.5	37.2	20.3	36.9	40.8	22.3	42.2	39.2	18.7
60-1197-65I	43.1	34.0	23.0	37.7	37.2	25.1	41.2	34.1	24.9
60-1197-64I	35.8	35.3	28.8	30.7	38.2	31.1	36.5	32.9	30.6
GMC-4J-1-4C	31.0	41.0	28.0	26.3	43.9	29.9	29.1	40.5	30.5
60-1197-61I	38.8	37.1	24.0	33.5	40.4	26.1	38.7	37.8	23.5
60-1197-60	37.5	42.1	20.3	32.3	45.7	22.0	36.8	43.5	19.7

TABLE V—CORRELATION OF NINHYDRIN DIFFERENTIAL ASSAY WITH MICROBIOLOGICAL ASSAY FOR GENTAMICIN BATCHES CONVERTED TO THEIR FREE BASES

	Correlation Coefficient	
Component	Weight	Activity
C_1	0.98	0.97
C_{1a}	0.98	0.98
C_2	0.93	0.96

As a second step, the six samples under consideration were converted to their free bases by means of an ion-exchange resin. The identical assay was performed using factors for the ninhydrin intensities of the compounds determined on known mixtures of the free bases of all of the components (Table I). The average response for these samples by weight and activity are presented in Table IV and are compared to the results obtained in the microbiological differential assay.

The correlations between the two assays using the free base mixtures measured by weight and activity are shown in Table V. All of the correlations are significant ($p < 0.01$).

The differential ninhydrin assay which has been described for the quantitative determination of the components of the gentamicin complex is highly reproducible and easier to perform than is the microbiological method. A significant correlation exists between the results obtained in the two procedures, and this new assay should therefore be very useful in determining proportions of the three gentamicin components in mixtures of the complex.

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Keyphrases

Gentamicin complex—differential analysis
Paper chromatography—separation, analysis
Ninhydrin—color reagent
Integrating scanner—color intensity

Dissolution Kinetics of Drugs in Human Gastric Juice—the Role of Surface Tension

By PER FINHOLT and SISSEL SOLVANG

The kinetics of *in vitro* dissolution of phenacetin and phenobarbital in human gastric juice have been determined and compared to those in hydrochloric acid containing various amounts of polysorbate 80. The surface tension of the dissolution medium is shown to have an appreciable effect on the dissolution kinetics of the drugs studied.

DURING THE LAST YEARS, a number of rate studies on the dissolution of drugs have been conducted. An excellent review of these is given by Wurster and Taylor (1). Various dissolution media have been used, but, surprisingly enough, not that solvent, in which the dissolution process takes place *in vivo*—that is, human gastric juice.

The purpose of this investigation was to study the rate of dissolution of drugs in human gastric juice. In addition, a closer examination of the

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