without first-pass metabolism. The nasal route may be of practical value for the administration of this hormone.

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Colorimetric Determination of Gentamicin, Kanamycin, Tobramycin, and Amikacin Aminoglycosides with 2,4-Dinitrofluorobenzene

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Abstract □ The reaction of 2,4-dinitrofluorobenzene (Sanger's reagent) is used to form colored products with aminoglycoside antibiotics. Stopping the progress of the reaction with acid after a fixed time allows aqueous solubility to be maintained while discharging any color due to excess reactant. The choice of an appropriate analytical wavelength results in adherence to Beer's law. Although this colorimetric method is not expected to be stability-indicating, it is convenient and should be useful in content uniformity determinations for pharmaceutical dosage forms (*e.g.*, ointments).

Keyphrases □ Colorimetry—aminoglycosides, 2,4-dinitrofluorobenzene □ Aminoglycosides—2,4-dinitrofluorobenzene, colorimetric determination

Most assays for monitoring aminoglycoside antibiotics which are mentioned in the Federal Register (1) are plate-cup bioassays. A number of chemical assays (2) have been developed for use with pharmaceutical dosage forms and content determinations. These assays are useful, in some cases, as rapid control procedures featuring high precision, but do not actually measure bioactivity; most are amine reagents (3). We found in our preliminary work that certain pharmaceutical formulation excipients do not react with 2,4-dinitrofluorobenzene (Sanger's reagent) (4–6); therefore, this reagent could be useful when determining aminoglycosides. Sanger's reagent was originally used to detect terminal amino groups in insulin, and was utilized recently (7) for postcolumn HPLC-derivatization and detection of neomycin sulfate.

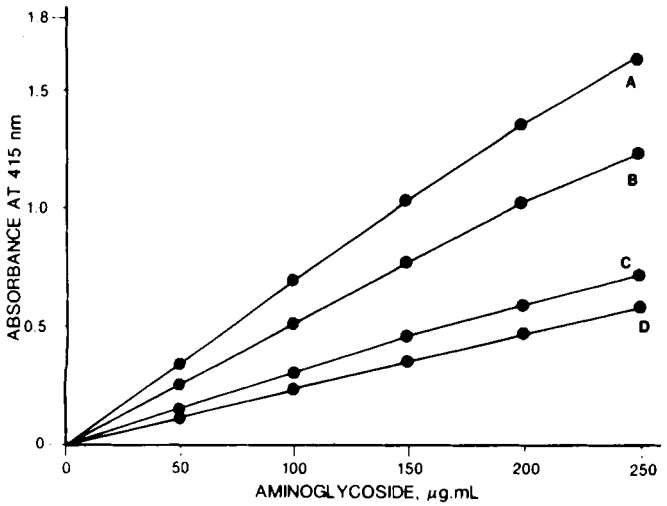

Figure 1—*Absorbance of aminoglycosides at 415 nm with 20-min reaction time. Key: (A) amikacin, (B) tobramycin, (C) kanamycin, (D) gentamicin.*

This paper reports the direct one-phase determination of gentamicin sulfate, kanamycin sulfate, tobramycin, and amikacin. The reaction was allowed to proceed under ambient conditions and then it was stopped, after an appropriate time, by acidification. The analytical wavelength chosen was not the absorption maximum, but one which permitted measurement in the aqueous-alcoholic medium without problem precipitation and with adherence to Beer's law over the range of 0–1000 μg/mL (Fig. 1). A typical reaction profile of gentamicin sulfate is outlined in Fig. 2.

EXPERIMENTAL SECTION[1]

Colorimetric Measurement—Exactly 5.0 mL of an aqueous solution containing ~500 μg of the aminoglycoside being tested was transferred to a 10-mL glass-stoppered flask or tube. Similarly, a series of flasks or tubes containing

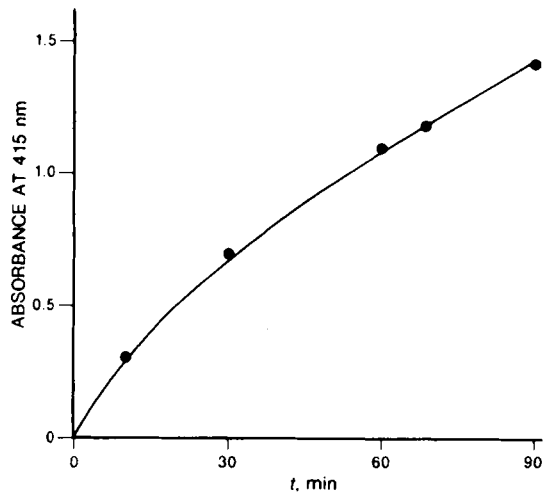

Figure 2—*Gentamicin sulfate reaction profile.*

[1] Gentamicin sulfate (lot GMC-6M-6024, potency 581 μg/mg) was obtained from the Schering Corp., Kenilworth, N.J. Kanamycin sulfate (lot 76F-140, potency 780 mg/mL) and amikacin (lot 74F-1805, potency 934 mg/mL) were both obtained from Bristol Labs., Syracuse, N.Y. The tobramycin (lot OCU55, potency 960 μg/mg) was made available through the courtesy of Eli Lilly and Co., Indianapolis, Ind. Sanger's reagent, 2,4-dinitrofluorobenzene (lot 101547, purity 98%), was obtained from the Aldrich Chemical Co., Milwaukee, Wis. Measurements were made on a Cary Model 15 UV-visible spectrophotometer.

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Table I—Stability of Developed Color after Acidification

	Absorbance Values	
	Initial	$T + 17$ h at Room Temperature
Blank	0.066	0.069
Gentamicin sulfate	0.321	0.318
Kanamycin sulfate	0.419	0.419
Amikacin	0.747	0.747
Tobramycin	0.586	0.586

5-mL aliquots of standard solutions containing 250, 500, 750, 1000, 1250 μg per 5.00 mL of the aminoglycoside were prepared. Using a definite sequence and a convenient but reproducible time interval between reagent additions, the following operations were performed on each sample and standard solution.

One milliliter of a 2% aqueous $NaHCO_3$ solution was added to each tube. The tubes were stoppered and mixed. Exactly 2.0 mL of a freshly prepared ethanolic (95%) solution of 2,4-dinitrofluorobenzene[2] (5, 6) containing 0.25 mL/100 mL was added to each tube. The contents of the tube were mixed thoroughly. After 20 min for tobramycin, amikacin, and kanamycin sulfate, or 30 min for gentamicin sulfate, the solution was neutralized by the addition of 0.5 mL 1 M HCl. After tapping the cell gently to remove any CO_2 bubbles, the absorbance was determined at 415 nm in the spectrophotometer using 1 cm cells and distilled water as a reference liquid.

RESULTS AND DISCUSSION

Inasmuch as adherence to Beer's law was obtained, the concentration of each aminoglycoside was determined by direct ratio to the diluted reference standard. Since the yellow color, due to excess reagent, is discharged by the acid solutions, no reagent blank was required when assaying colorless solutions. The *RSD* of each of the aminoglycosides tested was: gentamicin sulfate ±1.4%, kanamycin sulfate ±0.86%, tobramycin ±1.6%, and amikacin ±1.1%. The color that developed after acidification was found to be stable for at least 17 h (Table I). Use of filter photometers or other instruments with wide spectral band-pass may possibly result in deviations from Beer's law at 415 nm.

A probe experiment was also conducted to check the stability of the response of aqueous solutions of these antibiotics to the assay. Dilutions of the individual antibiotics in unbuffered aqueous solutions were prepared at levels of ~10 mg/100 mL. The solutions were sampled for their color response and then rechecked after 35 d under ambient conditions (~21.5°C in laboratory light) (Table II).

Compatibility of the Method with Various Pharmaceutical Excipients used for Topical Administration of the Antibiotics—Five grams of an ointment base[3] was mixed for 5 min with 20 mL of water and filtered[4]. The clear filtrate was used as a gentamicin sulfate diluent instead of water in the assay method and compared to a standard in distilled water. The average of three consecutive comparisons of these gentamicin solutions showed water extract absorbance values only 100.5% of those obtained with distilled water diluent solutions which demonstrates the lack of significant interference from the excipients.

Phosphate buffers are often used in connection with the bioassays of aminoglycosides such as gentamicin sulfate, however, they were found to interfere with the color development reaction. Those studied were 0.1–1.0 M buffers adjusted to pH 7.0. An average of eight measurements of four buffer strengths at pH 7 produced only 31.2% of the color development of a sample in distilled water.

Potential Automated Modifications—The requirement for precise timing during the initial reaction step and the need for caution to prevent exposure of Sanger's reagent to the skin indicates that, if automated, this method might reasonably be expected to have wide application.

Table II—Color Responses of Amino Functions after 35 d at 21.5°C in Unbuffered Aqueous Solutions

	Initial mg/mL	Final mg/mL	% Initial
Gentamicin sulfate	10.1	10.1	100
Kanamycin sulfate	9.74	10.5	107
Amikacin	10.3	10.2	99.0
Tobramycin	10.7	11.1	104

[2] Exercise appropriate caution; handle in hood with protective gloves and clothing.

[3] Ointment consisted of: 0.267 g of Wool Alcohols B.P., 0.8015 g of Amerchol C, 1.87 g of Multivax W445 White Wax, and 2.06 g of isopropyl myristate.

[4] Selas Flotronics Ag filter (0.2 μg apertures).

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