TABLE I—RECOVERIES WITH THE PAPER CHROMATOGRAPHIC METHOD

Batch	mg. Glyceryl Guaiacolate/Tablet—Actual	Found	% Recovery
A	195.0	195.2	100.1
		191.0	98.0
B	195.0	193.6	99.3
		194.0	99.5
C	195.0	195.0	100.0
		193.2	99.1

four more times using fresh portions of chloroform, but using the same 0.4 *N* hydrochloric acid solution. The combined chloroform extracts are diluted to yield a concentration of 1 mg./ml. of glyceryl guaiacolate. One hundred microliters of this extract is placed on paper chromatographic strips[1] (20 in. long) impregnated with the lower phase of the following solvent system: benzene–ethanol–water (2:1:1). Blank strips and strips with a standard solution of the same concentration (1 mg./ml.) are prepared similarly. The strips are allowed to equilibrate for 2 hr. in a tank saturated with the above-mentioned solvent system, and developed with the upper phase of the solvent system (developing time approximately 3 hr.). Glyceryl guaiacolate and caffeine spots are located with a UV lamp (R_f of glyceryl guaiacolate is 0.52, R_f of caffeine, 0.70).

Glyceryl guaiacolate spots from two strips are combined and eluted with 10 ml. ethanol. The absorbance of these solutions is read at 275 mμ with a spectrophotometer and corrected for the paper blank. (The absorbance readings of the sample without correction are generally over 13 times the magnitude of the paper blank reading. Paper blanks and placebos yield absorbance readings of approximately 0.020 units.) The concentration of glyceryl guaiacolate is calculated against the standard, similarly corrected for the paper blank.

[1] Whatman No. 1.

The results for three different batches of glyceryl guaiacolate tablets, with a known concentration of glyceryl guaiacolate, are given in Table I.

CONCLUSIONS

An average recovery of 99.3% (S.D. = ±1.05) may be achieved by the paper chromatographic method of assay described for glyceryl guaiacolate tablets. This method is simple, reliable, and specific. The specificity is achieved by the separation of the desired component by the paper chromatography followed by its determination at the appropriate wavelength in the ultraviolet region. The concentrations of glyceryl guaiacolate estimated were in the order of 195 mg. However, the design of this method permits estimations down to approximately 0.5 mg. of glyceryl guaiacolate or as high as might be desired. Furthermore, this method permits the quantitative determination of the other ingredients present in the formulation, such as acetylsalicylic acid, chlorpheniramine maleate, and caffeine, by the conventional methods.

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Keyphrases

Glyceryl guaiacolate tablets
Caffeine-aspirin-chlorpheniramine-glyceryl guaiacolate formulation
Paper chromatography–separation
UV spectrophotometry–analysis

Colorimetric Assay and Improved Method for Identification of Vancomycin Hydrochloride

By JAMES R. FOOKS, IAIN J. MCGILVERAY, and ROBERT D. STRICKLAND*

Procedures are described for the qualitative and quantitative determination of vancomycin hydrochloride by means of spectrophotometry and thin-layer chromatography. The Folin-Ciocalteau reaction which forms the basis of both procedures is also amenable to the estimation of vancomycin factor A, a commonly occurring impurity.

VANCOMYCIN, an antibiotic obtained from *Streptomyces orientalis* (1), is retained for use against Gram-positive cocci resistant to the more common antibiotics and for treatment of patients allergic to penicillins and cephalosporins (2). The structure of this drug has yet to be fully established, but chemical studies (3–5) have demonstrated the presence of carboxyl, amino, and phenolic groups. Glucose and amino acid fragments have also been identified after partial hydrolysis. Vancomycin as obtained from *S.*

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* Present address: Biochemistry Department, Ottawa Civic Hospital, Ottawa, Ontario, Canada.

orientalis may contain an unidentified fraction, factor A, which has similar chemical properties (5).

The USP XVII (6) refers to the Federal Register (7) for identity and assay procedures. Tests described in B.P. 1963 (8) and the Federal Register for identity and potency of vancomycin depend on microbiological assay using *Bacillus subtilis*. The official paper chromatographic methods (7, 8) for identifying vancomycin and estimating vancomycin factor A also employ this microorganism.

Betina included vancomycin in a systematic study of antibiotics by paper chromatography (9). Lightbown and de Rossi (10) described the separation of four biologically active compounds from vancomycin international reference standard by electrophoresis in agar gel. No reports could be found in the literature of a substitute for the time-consuming paper chromatographic and bioautographic procedures used in the characterization of vancomycin hydrochloride.

The object of this paper is to supplement and improve the present standards by providing chemical methods for the identification and assay of the antibiotic and a more rapid and sensitive means of separating it from closely related substances.

EXPERIMENTAL

Vancomycin hydrochloride injectable and vancomycin factor A used in this study were kindly supplied by the Eli Lilly & Co., Indianapolis, Ind. The "fresh" sample was dated 1966 and the "aged" had an expiration date in 1960. Vancomycin hydrochloride USP reference standard was obtained from USP Reference Standards, New York, N. Y.

Colorimetric Assay—In the Folin-Ciocalteau method the reagents were sodium carbonate 5% aqueous and Folin-Ciocalteau phenol reagent.[1]

A solution of vancomycin USP reference standard (1 ml.) representing 10 mcg./ml. was treated with sodium carbonate (1 ml.) reagent followed by diluted 1:3 Folin-Ciocalteau reagent (0.5 ml.). The solution was then made up to a volume of 5 ml. The development of color in this solution was followed by absorbance measurements at 725 mμ every 15 min. for 2 hr. A reagent blank was prepared by replacing the antibiotic with distilled water.

A calibration curve was obtained with standard amounts of vancomycin hydrochloride USP reference standard. The Folin-Ciocalteau and sodium carbonate reagents were added as described above and the absorbance of each solution at 725 mμ was measured after 1 hr.

Two commercial samples of vancomycin hydrochloride were assayed using this standard curve.

Microbiological Assays—The assay procedure for vancomycin hydrochloride described in the Federal Register was used (7).

Tests for Identity and Purity Using Thin-Layer Chromatography (TLC)—*Chromatographic Plates*—(*a*) Glass plates (20 × 20 cm.) were coated to a thickness of 250 μ with a slurry of Kieselguhr G (Merck) (40 Gm.) in distilled water (80 ml.) according to the method of Stahl (11) and left to dry overnight. They were used within 2 days without preactivation. (*b*) Glass plates were coated as above with Silica Gel G (Merck), but activated at 105° for 10 min. before use.

Solvent Systems—(*a*) Benzene–*n*-butanol–water (20:20:100) aqueous phase. (*b*) Methanol–water (1:99).

Detection Agent—(*a*) Sodium carbonate 20% aqueous and (*b*) Folin-Ciocalteau phenol reagent diluted 1:2 with distilled water.

Samples of an aqueous solution of vancomycin hydrochloride and factor A each representing 10 mcg. were applied to the same point on a Kieselguhr-coated plate by means of a Hamilton microsyringe. After drying in a stream of warm air, the plates were inserted in a previously equilibrated, filter paper-lined tank saturated with solvent system (*a*) and chromatographed to a height of 15 cm. (approximate time taken 50 min.). The residual solvent was removed immediately in a stream of warm air and the spots located by spraying with 20% sodium carbonate solution followed by Folin-Ciocalteau reagent. Gray-blue areas were observed for both vancomycin hydrochloride and factor A.

The sequence was repeated for 5 mcg. of vancomycin and factor A on Silica Gel G with solvent system (*b*). Approximate R_f values in each system were calculated from 10 plates. The limit of detection was determined by spotting dilutions of vancomycin hydrochloride USP reference standard and factor A separately in amounts ranging from 10 to 0.01 mcg. on five plates. Both compounds were visible at concentrations below 0.1 mcg.

The approximate R_f values of a number of drugs which also react with Folin-Ciocalteau reagent were compared by TLC in the above systems.

Quantitative TLC by Densitometry—The instrument used to scan the plates was a Photovolt densitometer (model 520M) equipped with a TLC stage, attached to a Varicord recorder (model 42B), a search unit C, and an Integraph integrator. A blue filter (max. 420 mμ) and a slit 0.1 mm. wide and 6 mm. long were used. The distance between plate and search unit was maintained at 1 mm. with a response setting of 10.

Thin-layer chromatograms of 2–10 mcg. quantities of vancomycin hydrochloride were developed with solvent system (*a*) on Kieselguhr. The plates were dried without delay in a stream of warm air and then sprayed immediately with 20% sodium carbonate solution. After drying thoroughly, they were sprayed with freshly prepared Folin-Ciocalteau reagent (1:3) and dried for 15 min. in a stream of warm air.

Densitometric scanning began as soon as the sprayed plate was dry and each spot was measured separately. The densitometer was vectored to the highest reading at the center of the spot, the plate clamped firmly in position, and the densitometer readjusted to the lowest background reading. Subsequently automatic scanning was effected in the direction of solvent flow. The operation was performed in a darkened room to prevent interference from stray light.

[1] Folin-Ciocalteau phenol reagent manufactured by Hartman-Ledden Co., Philadelphia, Pa.

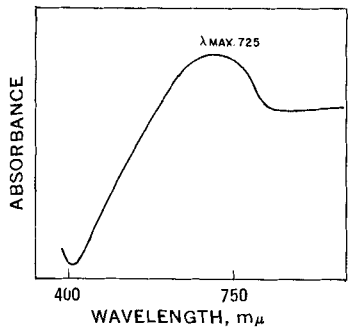

Fig. 1—Reaction of vancomycin with Folin-Ciocalteau reagent. Absorption curve of the product.

TABLE I—TIME OF COLOR DEVELOPMENT *vs.* ABSORBANCE FOR VANCOMYCIN WITH FOLIN-CIOCALTEAU REAGENT

Time, min.	Absorbance
0	0.075
15	0.116
30	0.148
45	0.162
60	0.166
75	0.173
90	0.178
105	0.180
120	0.186

The integrated areas were evaluated to give a standard curve. The process was repeated and some mixtures of vancomycin and factor A were estimated from the curve.

RESULTS AND DISCUSSION

The presence of phenolic groups in vancomycin has been demonstrated (1, 5). The phenolic moieties have been found to give the typical Folin-Ciocalteau reaction resulting in a blue-gray color (absorption maximum at 725 mμ) (Fig. 1). Color intensity was found to increase rapidly up to 1 hr. and, though a slight increase occurred after this time (Table I), the readings taken were sufficiently reproducible for assay purposes. The pH of the solutions produced by the reaction conditions averaged 9.6. When the pH was increased using more concentrated sodium carbonate solutions (*i.e.*, 20%), the color intensity of the product was lower and carbon dioxide was evolved which interfered in the measurement of color. Under the conditions used, Beer-Lambert's law was obeyed over the range 0.4–100 mcg./ml. Experimental results showing a comparison of the three vancomycin samples are shown in Table II which also lists the microbiological results. Both sets of results compare favorably.

Higgins *et al.* (3) observed that vancomycin, after hydrolysis in acid or alkaline solution, reacted with ninhydrin to give a positive test for amino groups. In our efforts to obtain a quantitative response with this reaction, various conditions were investigated for acid hydrolysis of vancomycin and for color development with the ninhydrin reagent. Results using the method of Yemm and Cocking (12) devised for amino acids were found to be inconsistent, and this reaction cannot be recommended as useful for the determination of vancomycin.

TABLE II—DETERMINATION OF SAMPLES OF VANCOMYCIN—COMPARISON OF COLORIMETRIC AND MICROBIOLOGICAL RESULTS

Sample	Absorbance	Concn. Found, mcg./ml.	Concn. Expected, mcg./ml.	%	% Microbiological
USP (reference)	0.151	10	10.0	100	100
Aged	0.129	8.50	10.0	85	85
Fresh	0.156	10.25	10.0	102.5	102

TABLE III—R_f VALUES OF SEVERAL ANTIBIOTIC DRUGS IN TWO SOLVENTS[a]

	System (*a*)	System (*b*)
Vancomycin	0.98	0.54
Factor A	0.87	0.24
Bacitracin	...	0.10
Chlortetracycline HCl	0.13	0.18
Gramicidin	...	0
Griseofulvin	...	0.4[b]
Sodium methicillin	0.98	0.80
Novobiocin	0.98[b]	0 to 0.4[b]
Oxytetracycline hydrochloride	0.87[b]	0.15
Polymyxin B sulfate	...	...
Tyrocidine hydrochloride	0	0
Tyrothricin	0	0

[a] Spray reagent: Folin-Ciocalteau. [b] Streaking; no reaction.

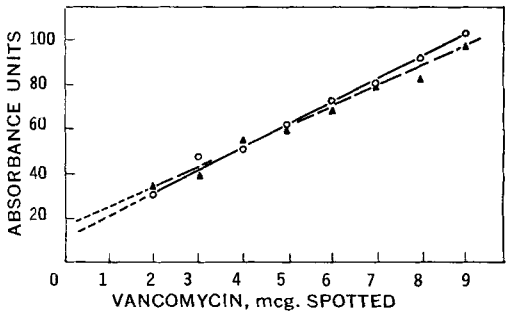

Fig. 2—Standard curves from TLC densitometry. Key: ○, USP vancomycin; ▲, USP vancomycin with 1 mcg. factor A.

The systems described for thin-layer chromatography were chosen after an empirical study of many solvent-adsorbent variations. Solvent system (*a*) was satisfactory for the separation of vancomycin from factor A up to the 20-mcg. level. Adverse tailing effects were observed with greater concentrations. With solvent system (*b*) on silica gel, effects of overloading were apparent with even less material and the system, though very rapid, is recommended only as an auxiliary for separation of vancomycin from other drugs as seen in Table III. From this table it is evident that system (*a*) does not differentiate methicillin and novobiocin from vancomycin. In solvent system (*b*), however, they

exhibit different R_f values. Table III shows the average R_f value of vancomycin and factor A in the two solvent systems.

Vancomycin and factor A could be determined semiquantitatively by densitometry over a range from 2 to 10 mcg. Reliable scanning was difficult with greater amounts because of streaking, especially where mixtures of the two components were involved. Figure 2 shows the linear curve of vancomycin (USP reference standard) concentration *versus* average spot density calculated from five plates and the effect of factor A on this curve. Factor A has little or no effect on the lower range (3 to 6 mcg.) but appears to inhibit color development at higher levels.

The vancomycin factor A used in this work was impure, as it contained approximately 20% vancomycin as estimated by TLC. The R_f values quoted as "factor A" are for the major, slower running spot. In making the densitometer readings for vancomycin in mixtures it was necessary to begin the scan from the lowest background reading between the spots for vancomycin and factor A.

The Folin-Ciocalteau assay is obviously not specific for vancomycin hydrochloride and should not be used in the absence of other criteria of identity. One such procedure is thin-layer chromatography which also detects the presence of interfering materials including factor A. Where nonphenolic impurities occur, the Folin-Ciocalteau method is recommended as the most convenient assay. Where impurities such as factor A are encountered, the semiquantitative assay of vancomycin by TLC with Folin-Ciocalteau spray followed by densitometry provides an alternative.

These data show that the chemical methods may supplement the current microbiological procedures for the qualitative and quantitative determination for this drug.

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Keyphrases

Vancomycin HCl
Colorimetry—analysis
Folin–Ciocalteau reaction
Microbiological analysis
TLC–densitometric scanning
Factor A–vancomycin—TLC separation

Technical Articles

Design and Evaluation of a Miniature Air-Suspension Coating Apparatus

By HAL N. WOLKOFF, GEORGE PINCHUK, and PAUL H. SHAPIRO

Experiments were conducted, using a miniature air-suspension coating apparatus designed and built in these laboratories, to determine the feasibility of uniformly coating gram quantities of various sized and shaped particles. The coating chamber used has an inner diameter of 1.22 in. and an overall length of 4.875 in. A schematic drawing of the apparatus is presented and the function of each component as well as its critical dimensions are discussed. The coating capabilities of the apparatus were explored using nonpareil seeds, tablets of several sizes and shapes, and hard gelatin capsules. Data are presented to compare the relative coating efficiency of the miniature unit with the widely used 6-in. Wurster apparatus. The results indicate that minor differences do exist in terms of coating efficiency but that the two units are comparable with respect to the uniformity of coating which can be achieved.

AIR-SUSPENSION coating techniques have generated considerable interest since Wurster's disclosures in 1953 and 1957 (1, 2). The process has been widely used on an experimental basis and, to a lesser extent, in commercial production. It has as its major advantages simplicity of operation, versatility, rapidity, and uniformity of the final product. As opposed to conventional pan coating techniques, this process provides for rigorous control over the many factors responsible for batch-to-batch variability.

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