

Colorimetric Method for the Determination of Vinblastine, an Alkaloid from *Vinca rosea* I

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A reproducible colorimetric method is described for the determination of vinblastine, an antileukemic alkaloid from *Vinca rosea* Linn.

VINBLASTINE, an alkaloid from the ornamental shrub *Vinca rosea* Linn. was first reported as vincalucoblastine¹ by Noble, Beer, and Cutts (1). On the basis of analytical and physical data the empirical formula of $C_{46}H_{58}N_4O_9$ (2) has been suggested for vinblastine. Spectral studies of this and parent alkaloids, catharanthine, and vindoline indicated that vinblastine represented a new type of dimeric alkaloid containing indole and indoline moieties (3). Vinblastine has been shown to contain the following functional groups: 2-COOCH₃, OCOCH₃, OCH₃ (aromatic), OH (free), OH (hydrogen bonded), NH (indole), and NCH₃. The appropriate derivatives of vinblastine as well as functional group analysis corroborate the presence of these groups (4).

Since some 20 different alkaloids have been obtained from this plant (5), a specific method for the detection and determination of vinblastine and its salts were highly desirable. The alkaloid produces color reactions with several reagents. If about 1 mg. of the alkaloid is heated with 50% hydrochloric acid containing 0.2% vanillin, a pink color is produced. With concentrated sulfuric acid containing traces of formaldehyde, a light wine color results. Concentrated sulfuric acid gives no color at room temperature; nitric acid gives a yellow color. A faint pink color is obtained with the alkaloid when heated with 40–60% sulfuric acid. Ten per cent sulfuric acid containing 1% of cerium sulfate gives a dark orange color which rapidly changes to yellow. A dark blue color is obtained by dissolving vinblastine in a 1% solution of ferric ammonium sulfate in 75% sulfuric acid. If vinblastine is dissolved in formic acid and several drops of 5% sodium hypochlorite solution are added, an orange color develops which fades to yellow.

After experimentation with many reagents, it was found that addition of vinblastine to an appropriate mixture of acetic anhydride, acetyl chloride, pyridine, and sulfuric acid followed by heating, produced a deep rose color. This color development is specific for vinblastine and, up to now, has not been observed with any other alkaloid reported from *V. rosea*. The specificity of this reaction has been

corroborated qualitatively by paper chromatography of the alkaloids from *V. rosea* (6).

This paper describes the details for the colorimetric determination of vinblastine.

EXPERIMENTAL

Moisture Determination.—Vinblastine sulfate contains moisture which can be removed by vacuum drying at about 50° without damage to the alkaloid. The anhydrous material, however, is very hygroscopic and picks up about 2% moisture in 2 minutes when allowed to stand under room conditions. Equilibrium is reached in about 20 minutes, resulting in a moisture content of about 10%. The most convenient method of handling the compound is to allow it to reach equilibrium, at which time separate samples are weighed for the color assay and for the moisture determination. The moisture is determined by vacuum drying at 50° for 16 hours.

Apparatus and Reagents.—A Beckman model DU spectrophotometer with 1-cm. glass cells was used in this laboratory, but any suitable spectrophotometer may be used.

A constant temperature oil bath was used to insure the exclusion of condensation water during the heating period. The oil bath used was designed to hold eight 10-ml. volumetric flasks so that a standard and three samples, in duplicate, could be run simultaneously.

Care must be taken to insure the exclusion of water from the reagents and glassware.

Acetyl chloride, analytical reagent grade.

Acetic anhydride, analytical reagent grade. This reagent must contain a minimum of 97% $(CH_3CO)_2O$, and must pass the U.S.P. test for reducing substances. At least 24 hours before use, 0.25 ml. of acetyl chloride is added to each 500 ml. of acetic anhydride. The activated acetic anhydride can be stored for at least 3 months. The metallic lining of the acetic anhydride bottle cap should be replaced with an inert plastic material. A corroded metallic liner may indicate an undesirable reaction which reduces the color formation.

Pyridine, analytical reagent grade. Water content must be less than 0.1%.

Sulfuric acid, concentrated, analytical reagent grade.

Preparation of the Color Reagent.—The color reagent must be freshly prepared immediately prior to use as indicated in the procedure.

By means of a graduate, 35 ml. of acetic anhydride is transferred to a 100-ml. glass-stoppered Erlenmeyer flask and cooled under running water. While swirling and cooling, 1 ml. of concentrated sulfuric acid is added. With continued cooling and swirling, 35 ml. of pyridine is added in about three equal portions.

Procedure.—About 3 mg. of standard vinblastine sulfate, and about 3 mg. of the sample preparation are weighed accurately to the nearest 0.01 mg. Each is transferred quantitatively to separate 10-ml. volumetric flasks. From this point on, the assay should be performed without delay. The

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¹ In compliance with the A.M.A. Council on New Drugs, its name has been changed to vinblastine. The sulfate salt of this alkaloid under the name of Velban (vinblastine sulfate, Lilly) has recently been made available for the treatment of Hodgkin's disease and choriocarcinoma.

standard and sample are dissolved and diluted to the mark with the color reagent and well mixed. Two 2-ml. aliquots of the standard solution are transferred to separate 10-ml. volumetric flasks labeled "standard." Two 2-ml. aliquots of the sample solution are transferred to separate 10-ml. volumetric flasks labeled "sample." To each of the four flasks 3 ml. of the color reagent is added by pipet. The flasks are placed simultaneously in an oil bath which has been preheated to $80 \pm 1^\circ$. After about 2 minutes of heating, the flasks are stoppered loosely with glass stoppers. The flasks are heated a total of exactly 20 minutes at $80 \pm 1^\circ$, then removed from the bath and cooled to room temperature under running water. Each flask is diluted to the mark with color reagent and well mixed. The absorbance of each is measured on a suitable spectrophotometer in 1-ml. cells at 574 and 538 $m\mu$, using distilled water as a reference.

The ratio A_{574}/A_{538} is determined. This value must be within the range of 1.20 to 1.25 for relatively pure vinblastine. Any value outside this range indicates the color has been incorrectly developed. In the case of crude alkaloid mixtures containing less than 30% vinblastine, the ratio may be slightly less than 1.20.

Calculations.—

$$\frac{\text{Abs.}_{574} \text{ sample} \times \text{mg. standard}}{\text{Abs.}_{574} \text{ standard} \times \text{mg. sample}} \times 100 =$$

% vinblastine sulfate

$$\frac{\% \text{ vinblastine sulfate}}{1.000 - \frac{\% \text{ loss of wt. on drying}}{100}} =$$

% vinblastine sulfate, anhydrous

DISCUSSION

Standard Curve.—A plot of the absorbance *versus* concentration was found to be linear and to pass through the origin, obeying Beer's law from 5–70 mcg. per ml. (Fig. 1). The reproducibility of the method is entirely satisfactory and will be discussed in a later paper.

Stability of the Color.—The color is stable for about 20 minutes after which time it begins to increase slowly at 574 $m\mu$, and more rapidly at 538 $m\mu$ (Fig. 2). This differential rate of increase of the color at the two wavelengths was responsible for the choice of 574 $m\mu$ as the wavelength for quantitative measurements.

Influence of Sulfuric Acid.—One milliliter of sulfuric acid seems to be the optimum amount to add to the 70 ml. of 1:1 acetic anhydride:pyridine mixture. The addition of more or less sulfuric acid reduces the color intensity and conformity to Beer's law is reduced to a much narrower range of concentrations.

Influence of Acetyl Chloride.—Acetyl chloride has an important influence on the color development. Without acetyl chloride, the color develops a pronounced yellow tint and the absorption peak at 574 $m\mu$ is shifted to lower wavelengths. With a few lots of acetic anhydride it was not necessary to add acetyl chloride. In these cases it is supposed that trace amounts of acetyl chloride were present due to the method of synthesis of the anhydride.

Characterization of the Color Produced.—It is possible to isolate a deep rose oil from the reaction

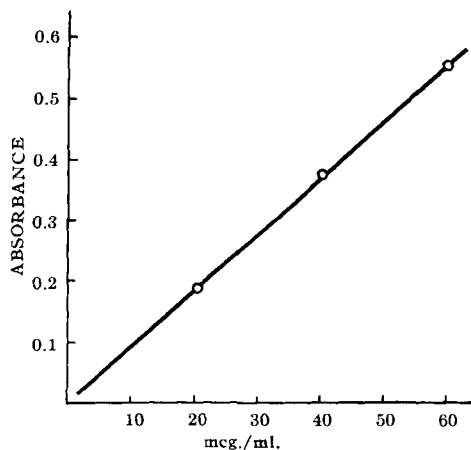


Fig. 1.—Calibration curve of vinblastine sulfate.

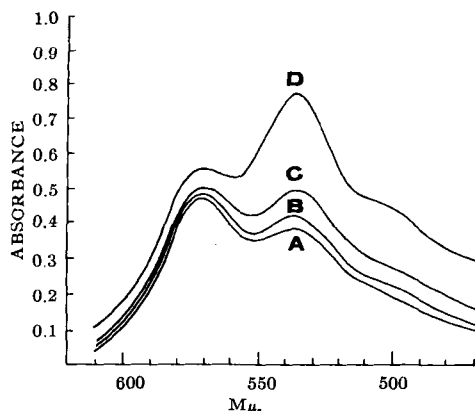


Fig. 2.—Cary recording spectrophotometric curves of the color. A, Immediately after diluting to volume; B, 1 hour after diluting to volume; C, 2 hours after diluting to volume; D, 4 hours after diluting to volume.

mixture after the color has been developed. The excess reagent was removed from the mixture by several washings and decantations with large amounts of ether. The oily residue rapidly changed to a bright brown color. When color reagent was added to the residue, the original rose color was reconstituted; however, the ratio A_{574}/A_{538} was slightly diminished.

The chemistry of the color formation is not clear at the present time. There is a possibility that the color is due to a halochromic effect of the reaction mixture. Clarification on this point may result from further elucidation of the structure of this alkaloid, presently being conducted in these laboratories.

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