ether produced 0.4 Gm. (57%) of crystals melting at 148–150°. Carrara and co-workers (11) reported the melting point of phenylserine hydrochloride to be 157°.

α - Hydroximino - β - (p - methoxyphenyl)-propionic Acid.—A 5.3 Gm. (0.024 mole) quantity of *p*-methoxybenzylmalonic acid was dissolved in 50 ml. of ether, and 4.85 Gm. (0.047 mole) of butyl nitrite was added. The mixture was placed in an ice bath and stirred while hydrogen chloride was passed into the beaker until none of the gas was absorbed. Stirring and cooling were continued for 20 minutes, and the reddish reaction mixture was warmed on a hot water bath until the ether had evaporated. A residue of slightly colored solid remained; this was dissolved in warm alcohol and reprecipitated by the addition of cold water to yield 4.95 Gm. (96%) of colorless crystals of α-hydroximino-β-(*p*-methoxyphenyl)-propionic acid. The recrystallized product melted at 156°. Hamlin and Hartung (12) reported a melting point of 156–157° for this compound. Subsequent reactions gave yields of 90–95%.

O-Methyltyrosine.—To a solution of 4 Gm. (0.019 mole) of the α-hydroximino-β-(*p*-methoxyphenyl)-propionic acid in 100 ml. ethyl alcohol was added 3 Gm. of a 10% palladium catalyst. To the mixture was then added 10 ml. of 36% hydrochloric acid; the mixture was shaken with hydrogen at a pressure of 10 Atm. The theoretical quantity of hydrogen was absorbed in 3.5 hours, and the mixture was filtered to remove the catalyst. The solvent was removed under reduced pressure on a hot water bath, and the residue was dissolved in 15 ml. of warm distilled water and titrated to neutral with 0.1 *N* sodium hydroxide using methyl red indicator. Cooling of the neutral solution precipitated 3.5 Gm. (93%) of *O*-methyltyrosine. The melting point, 295–296° with decomposition, agreed with the value reported (13, 14).

DISCUSSION

Phenylglyoxylohydroxamyl cyanide (II) was prepared by nitrosating phenacylchloride and reacting the resulting isonitroso compound with potassium cyanide. Both keto and cyano groups are known to promote nitrosation of adjacent aliphatic carbon atoms. Indeed, Pasquale (15) obtained good yields of II by nitrosating phenacyl cyanide. We were not, however, able to prepare *p*-hydroxyphenylglyoxylohydroxamyl cyanide (IV*a*) by nitrosating *p*-hydroxyphenacylcyanide, nor are reports available indicating that 3,4-dihydroxyphenylglyoxylohydroxamylcyanide (IV*b*) can be prepared by nitrosating dihydroxyphenacylcyanide. This would indicate that substituents adjacent to carbonyl groupings drastically affect nitrosation reactions.

The potential of these compounds as enzyme inhibitors or alternate precursors of norepinephrine were not investigated; however, a group of the National Institutes of Health (16, 17) reported recently on the activity of a number of related compounds as substrates and inhibitors of dopamine-β-oxidase.

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Assay Methods for Some *Vinca rosea* Alkaloids II

By IVAN M. JAKOVLJEVIC, L. DAVID SEAY, and REGINALD W. SHAFFER

Colorimetric and fluorometric methods are presented for quantitative determination of some *Vinca* alkaloids. The identification is accomplished with a new color reagent—ceric ammonium sulfate in phosphoric acid—and two thin-layer chromatographic systems.

THE ALKALOIDS from *Vinca rosea* Linn. represent a new approach in the chemotherapeutic treatment of a variety of human neoplasms (6).

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In Part I a colorimetric method which was very specific for vincaleukoblastine[1] (vinblastine) was described (5). Since that time, only leurocristine[1] (vincristine)—another dimeric indole-indoline compound isolated later (8, 10)—has been shown to give the same reaction as vincaleukoblastine. By the vincaleukoblastine method (5), leurocristine exhibits the same color curve as

[1] The A.M.A. Council on Drugs has approved vinblastine and vincristine as generic names for these alkaloids. Vincaleukoblastine is marketed as Velban (vinblastine sulfate) by Eli Lilly and Co., Indianapolis, Ind.

TABLE I.—COLOR REACTIONS OF SOME *Vinca rosea* LINN. ALKALOIDS

Name	1% Ceric Ammonium Sulfate in 85% H_3PO_4, RT	1% Ferric Ammonium Sulfate in 85% H_3PO_4 After 10 Min. in Water Bath	1% Ferric Ammonium Sulfate in 75% H_2SO_4, RT
Vincaleukoblastine	Purple	Violet	Blue
Leurocristine	Blue violet	Pink	Blue → grey-blue
Leurosine	Pink	Pale yellow	Yellow → green
Leurosidine	Yellow → copper	Violet (strong)	Dark blue
Vindoline	Purple (strong)	Blue-grey	Purple (strong)
Catharanthine	Indigo → green	Yellow	Blue-violet
Virosine	Sky blue → yellow	Colorless	Pink → green
Perivine	Green → brown	Pale yellow	Umber
Reserpine	Sky blue → yellow	Lemon yellow	Blue → green
Vincarodine	Blue → green	Colorless	Blue → green → umber
Neoleurosidine	Violet → blue-violet	Magenta	Green → blue
Tetrahydroserpentine	Yellow → umber	Colorless	Violet → blue → green
Isoleurosine	Copper	Yellow → orange	Green → blue
(Indole)	Dark brown	Yellow	Yellow → green

vincaleukoblastine. However, at the maximum absorption peak, leurocristine has an extinction coefficient 25% lower than that of vincaleukoblastine.

There are still 35 alkaloids which have been isolated from *Vinca rosea* Linn. (*Catharanthus roseus* G. Don) in the Organic Chemical Development and Research Laboratories, Eli Lilly and Co. (11, 12); it is the purpose of this paper to give quantitative means for determining some of them.

It is very interesting that at room temperature a 1% solution of ceric ammonium sulfate in 85% phosphoric acid is an excellent reagent for distinguishing between most of these alkaloids.

A 1% solution of ferric ammonium sulfate in 85% phosphoric acid gives quite different colors from that of the ceric salt, but requires heating in a water bath.

Ferric ammonium sulfate, as a 1% solution in 75% sulfuric acid, offers at room temperature a third series of different colors—a great help in the identification of these alkaloids.

Table I gives the color reactions produced by these three reagents. Approximately 200–300 mcg. of the alkaloid dissolved in about 1 ml. of the corresponding reagent will give colors as shown in Table I.

EXPERIMENTAL

Leurosine

Analytical data for leurosine and vincaleukoblastine (3) show them as isomeric compounds: $C_{46}H_{56}N_4O_9$ having the dimeric indole-indoline group with a demonstrable oncolytic activity. The ultraviolet spectrum of leurosine has a maximum at 214 mμ in ethanol. Electrometric titration shows pKa′ at 7.5 in water (7).

The sulfate salt, m.p. 238–242° dec., has been used for the quantitative determination described below.

Materials.—Ferric ammonium sulfate, crystals, A.R.; phosphoric acid 85%, A.R.; and acetone, A.R., all from Mallinckrodt Chemical Works are utilized.

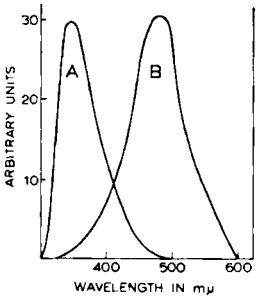

Fig. 1.—Excitation (A) and emission (B) spectra of leurosine sulfate fluorophor.

Color Reagent.—Five grams of ferric ammonium sulfate is dissolved in about 400 ml. of phosphoric acid in a 500-ml. volumetric flask by heating in a boiling water bath. The solution is cooled to room temperature and made up to volume with phosphoric acid. To prepare a reagent which is stable for several months, the larger crystals found in the reagent bottle should be pulverized and used.

Apparatus.—An Aminco-Bowman spectrophotometer (American Instrument Co., Inc., Silver Springs, Md.), equipped with 1-cm. square silica cells was used for the determination of excitation and emission spectra. A Turner model 110 fluorometer (G. K. Turner Associates) equipped with a set of matched borosilicate glass test tubes was employed for all fluorometric measurements. A filter 7–60 (transmitting light between 350 and 400 mμ) was employed as a primary filter, and a filter 2A (transmitting at 415 mμ and above) was employed as secondary filter.

Procedure.—One milligram of the standard leurosine sulfate and 1 mg. of the sample preparation are weighed to the nearest 0.01 mg. and transferred into separate 100-ml. volumetric flasks. The standard and the sample are dissolved and diluted to the mark with acetone. Two 1-ml. aliquots of the standard solution and two 1-ml. aliquots of the sample solution, equivalent to 10 mcg. of leurosine sulfate each, are transferred to separate 10-ml. volumetric flasks. The solvent is evaporated with a mild jet of air. To each of the four flasks, 5 ml. of the color reagent is added by a graduated cylinder. A reagent blank is prepared by adding 1 ml. of acetone and 5 ml. of the color reagent to a 10-ml. volumetric flask. The flasks

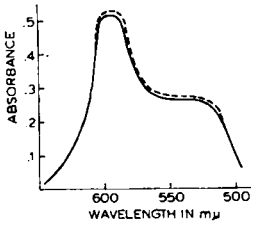

Fig. 2.—Cary recording spectrophotometric curve of the leurosidine chromophor. Key: ——, initially; - - - -, after 24 hours.

are placed simultaneously in an oil bath of 70 ± 3° and, after heating for 2 minutes, they are stoppered with glass stoppers. The heating is continued for a total of 40 minutes. The contents are cooled to room temperature and diluted to the mark with phosphoric acid.

In the example given, the concentration of the final solution is 1 mcg. of leurosine sulfate per milliliter, but as low as 0.02 mcg. can be easily estimated. The calibration curve passes through the origin and obeys Beer's law within this concentration range. It was noted also that the fluorophor formed is extremely stable for up to 8 hours after being diluted with phosphoric acid.

Figure 1 gives excitation and emission spectra of the fluorophor.

Leurosidine

Leurosidine is another dimeric indole-indoline compound having an ultraviolet spectrum essentially identical to that of leurosine. In chloroform as a solvent, even the infrared spectrum is similar to that of leurosine (10), m.p. 208–211° dec. pKa′ 5.5 and 8.8 in 33% dimethylformamide. $[\alpha]_D^{26} = +55.8°$ in chloroform (10, 11).

When leurosidine is treated with ceric ammonium sulfate color reagent, it exhibits a fluorescence which is approximately one-half that of leurosine; however, leurosidine exhibits a reddish color also which is not observed with leurosine. The same reddish color is given by vincaleukoblastine and leurocristine if treated with the same reagent. The fluorescence of vincaleukoblastine is about 30% of that of leurosine, and leurocristine has practically no fluorescence.

Materials.—Ferric ammonium sulfate, crystals, A.R.; phosphoric acid 85%, A.R., both from Mallinckrodt Chemical Works; and sulfuric acid concentrate, reagent, E. I. du Pont de Nemours & Company, Inc., are employed.

Color Reagent.—Five grams of ferric ammonium sulfate is dissolved in about 400 ml. of phosphoric acid in a 500-ml. volumetric flask by heating in a boiling water bath. The solution is cooled to room temperature, 10 ml. of sulfuric acid is added, and the volume made up to 500 ml. with phosphoric acid. As mentioned above, only the large crystals should be pulverized and used.

Apparatus.—A Beckman model DU spectrophotometer with 1-cm. glass cells is used.

Procedure.—Three milligrams each of the standard leurosidine sulfate and the sample is weighed to the nearest 0.01 mg., transferred to separate 10-ml. volumetric flasks, dissolved in water, and diluted up to volume. Two 1-ml. aliquots of both standard and sample solution are transferred into individual 10-ml. volumetric flasks; 2 ml. of water is added to each of the flasks. The solution is made to volume with the color reagent, and the flasks placed for 40 minutes in a water or oil bath at 100 ± 3°. After the heating period, the flasks are cooled to room temperature and, if necessary, the volume is corrected to 10 ml. using the color reagent. A reagent blank is prepared by transferring 3 ml. of water to a 10-ml. volumetric flask and diluting to volume with the color reagent. The reagent blank is heated simultaneously with the standard and the sample. The dark blue chromophor exhibits a peak at 605 mμ, where the calculations are made, and a well defined shoulder at 570 mμ. After 24 hours, both the peak and the shoulder will be found at the same place, except that their absorbances are slightly higher (Fig. 2).

The calibration curve made with 5–40 mcg./ml. of leurosidine sulfate in the final solution passes through the origin and obeys Beer's law.

Vindoline

Vindoline is considered the major alkaloid in the leaves of *Vinca rosea* Linn. On the basis of analytical and physical data, the empirical formula $C_{25}H_{32}N_2O_6$ has been found (4, 11). Vindoline is combined with the indole moiety in several dimeric *Vinca* alkaloids, m.p. 154–155°. pKa′ 5.5 in 66% dimethylformamide. $[\alpha]_D^{26} = -18°$ (chloroform). Ultraviolet absorption curve shows peaks at 212, 250, and 304 mμ in ethanol (2).

Vindoline dissolves in concentrated sulfuric acid and hydrochloric acid without color; in concentrated nitric acid or nitric acid diluted no more than 1:3, it dissolves giving a dark ruby red.

The strong red developed with ferric ammonium sulfate described below has been found specific for vindoline and until now has not been observed with any other alkaloid from *Vinca rosea* Linn., as seen in Table I.

Materials.—Ferric ammonium sulfate, crystals, A.R., Mallinckrodt Chemical Works; sulfuric acid concentrate, reagent, E. I. du Pont de Nemours & Company, Inc.; and acetic acid glacial, A.R., Mallinckrodt Chemical Works are utilized.

Color Reagent.—One gram of ferric ammonium sulfate is dissolved in 25 ml. of water and filtered if necessary. While swirling and cooling in ice water, 75 ml. of concentrated sulfuric acid is added in small increments. The reagent is stable for 2 days, after which time it becomes turbid and should be discarded.

Apparatus.—A Beckman model DU spectrophotometer with 1-cm. glass cells is employed.

Procedure.—One milligram each of the standard vindoline and the sample are weighed to the nearest 0.01 mg., transferred to separate 50-ml. volumetric flasks, and dissolved in and made up to volume with glacial acetic acid. Two 2-ml. aliquots of both standard and sample solutions, each equivalent to 40 mcg., are transferred into individual 10-ml. volumetric flasks. To each flask is pipeted 3 ml. of glacial acetic acid; the solution is made to volume with the color reagent. As soon as the reagent is added, a red (lavender) is observed. Although heat develops by mixing acetic acid with the color reagent, a contraction of the volume can be observed. The mixture is homogenized by inverting the flasks several times and is allowed to cool to room temperature. The final volume is made 15 minutes after the color reagent is added.

The reagent blank consists of 5 ml. glacial acetic acid and 5 ml. of the color reagent treated at room temperature as standard and sample.

The absorbances are measured at 538 mμ where the only peak appears. The color is stable for at least 4 hours, after which time the mixture becomes a little opalescent. The calibration curve is linear, passes the origin, and obeys Beer's law in concentrations from 1–10 mcg./ml.

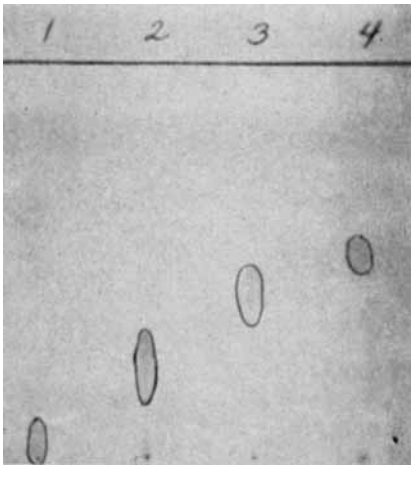

Fig. 3.—Solvent: 5% EtOH in acetonitrile; plate: 0.5*N* LiOH/alumina; sample: 50 mcg. Key: *1*, neo-leurosidine; *2*, leurosidine; *3*, vincathicine; *4*, leurocristine.

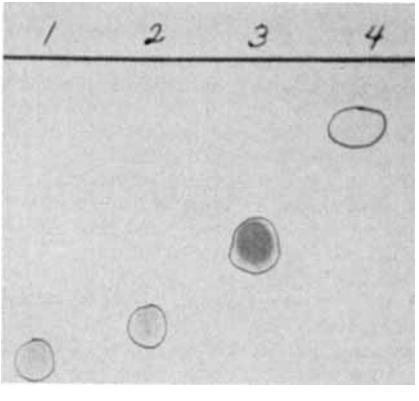

Fig. 4.—Solvent: 30% acetonitrile in benzene; plate: 0.5*N* LiOH/alumina; sample: 50 mcg. Key: *1*, leurosine; *2*, vincaleucoblastine; *3*, vindoline; *4*, catharathine.

THIN-LAYER CHROMATOGRAPHY

The chromatographic investigation of the *Vinca* alkaloids, as with most alkaloids, has been done mainly by employing paper chromatography (9). This technique has much to offer for identification purposes, but it suffers somewhat with respect to reproducibility and rigid control of conditions. There are also some limitations on the type of detection techniques employed. Thin-layer chromatography offers a better approach to the identification of most *Vinca* alkaloids (1, 10); the two systems discussed below are felt to have special advantages over paper chromatography as a means of identification. The methods are less time consuming, more reproducible, and more specific as a result of the use of a new color reagent.

The work discussed here was first performed by spotting the free alkaloids on silica gel plates prepared in the usual manner. Ethyl acetate, absolute ethanol, or a mixture of the two gave satisfactory results, except for tailing of several of the alkaloids.

Alumina plates (aluminum oxide Fluka for thin-layer chromatography) prepared in the usual manner eliminated some of the tailing, but not all separations were complete. Finally, the use of alumina plates prepared with 0.5 *N* lithium hydroxide worked well with a solvent containing 5% absolute ethanol in acetonitrile (v/v), except for some tailing of leurosine. The tailing of leurosine was eliminated with a 30% acetonitrile in benzene solution (v/v). The last system separates leurosine and vincaleukoblastine quite well, while other alkaloids travel too slowly. By making use of both systems, all the alkaloids mentioned below can be separated and identified readily (see Figs. 3 and 4).

All experiments were performed employing large chromatographic chambers (4 × 11 × 12 in.) which contained blotting paper wicks covering both narrow sides of the chamber and one of the large sides for a distance of approximately 9 in. from the bottom. The sulfate salts of the alkaloids studied thus far behave the same as their corresponding free alkaloids in a given system. The samples were prepared as 1% solutions in chloroform and have been stable with respect to chromatography for at least 24 hours if the solutions are stored in a refrigerator. Normally, 200 mcg. of the sample is spotted which permits the detection of as low as 1 to 3% of any other alkaloid which may be present.

The detection of the alkaloids may be accomplished by either of two methods. Most of them may be seen under a low wavelength ultraviolet light when the plate is viewed against a white background. All of the alkaloids react well with a

Table II.—Some Characteristics of Several *Vinca* Alkaloids

Name	Ultraviolet Fluorescence	Color	*Rf* Values 5% EtOH in Acetonitrile	*Rf* Values 30% Acetonitrile in Benzene
Neo-leurosidine	Purple	Brown → lavender	0.04	...
Leurosidine	Purple	Lavender	0.23	...
Vincathicine	Bluish-white	Pink → yellow	0.41	...
Leurocristine	Quenches	Lavender → pink	0.51	...
Leurosine	Blue	Bluish lavender	...	0.27
Vincaleukoblastine	Purple	Lavender	...	0.36
Vindoline	Quenches	Violet → pink	...	0.56
Catharanthine	Quenches	Yellow	...	0.85

mixture of 1% ceric ammonium sulfate in 85% phosphoric acid. If the color reagent is diluted with an equal volume of water, it is more conveniently sprayed; however, more than one spraying may be required with the diluted reagent to obtain sharp and stable colored zones. Table II shows the colors obtained, the ultraviolet characteristics, and the R_f values of the alkaloids in the two systems desiccatot discussed above.

Some precautions have been necessary to obtain the best results. The plates should be oven-dried at 105° for at least 30 minutes before using them. The dried plates may be stored for weeks in a suitable desiccator without noticeable effects on their performance. Color development is best if the plates are heated to 100° immediately after they are removed from the chamber and are kept warm for at least 5 minutes before spraying them. As mentioned above, the diluted color reagent is the best for the detection purposes in spite of the necessity of repeated sprayings. The colors are quite stable overnight if the plate is kept warm (90–100°) and no moisture is allowed to come into contact with the plate. The colors fade considerably if the plate is allowed to cool and absorb moisture from the air. The color development will be poor if the spraying is delayed more than 30 minutes after removing the plate from the solvent system, even though the plate is kept warm.

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Technical Articles

New Rheometer for Determining Rheological and Viscoelastic Properties of Fluids

By DUNCAN E. McVEAN* and JERE E. GOYAN‡

A rising cylinder rheometer is described which can be used to study the flow properties of fluids in the low shear regions similar to those caused by a falling particle. The rheometer may also be used to study viscoelastic properties of fluids. The rheometer was used to obtain flow curves in the low shear region of several pharmaceutical suspending agents. Bingham yield values were obtained by extrapolating methods from these flow curves. Dynamic viscosities of these materials were obtained by analysis of their relaxation behavior. The dynamic viscosities are compared with the plastic viscosities of the materials in the low shear range.

THE COMMONLY used suspension vehicles exhibit either pseudoplastic or plastic flow properties (1). Flow curves for these fluids over a wide range of shear rates may be obtained from viscometers currently in use (2).

However, the measurement of shearing forces at the very low shear rates produced by a falling particle is difficult to obtain with available viscometers. An example of the magnitude of desirable shear may be obtained as follows. Consider a 100-μ particle with a density of 2.00 falling in a Newtonian fluid having a density of 1.00 and a viscosity of 10 poise. The maximum shear rate (at the equator of the sphere) will be 0.16 reciprocal seconds. It was therefore decided to build a viscometer which could produce viscometric data at these low shear rates. An additional advantage to such an instrument would be the more precise determination of yield value obtainable because of the shorter extrapolation to zero shear rate.

The development of a new viscometer seemed desirable because of the difficulties that would be

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