Studies on *Catharanthus* alkaloids

III. Separation of vincaleukoblastine, leurocristine, leurosine and leurosidine by thin-layer chromatography

A report by BEER in 1955[1] that extracts from the Madagascan periwinkle, *Catharanthus roseus* (L) G. Don (*Vinca rosea* L.) elicit leukopenic activity, and a subsequent report on the isolation of the leukopenic and antineoplastic alkaloid vincaleukoblastine (VLB) from this plant by NOBLE, BEER AND CUTTS[2] have led to numerous subsequent reports from other laboratories on the separation of alkaloids from this plant. To date, a total of 59 alkaloids[2–30] have been reported isolated from *C. roseus*, four of which exert marked antineoplastic activity. Leurocristine, leurosine, and leurosidine, isolated by SVOBODA[22,23], in addition to VLB by NOBLE *et al.*[2], are reported to be active against a wide variety of neoplasms in experimental animals[2,31,32]. Further, leurocristine (vincristine) and vincaleukoblastine (vinblastine) are now available for routine use in the treatment of certain human neoplasms. Leurocristine (Oncovin®, Lilly) is employed for the treatment of acute lymphocytic and monocytic leukemias in children[31,33,34], whereas vincaleukoblastine (Velban®, Lilly) has been shown to be of value in treating Hodgkin's disease[31,35] and choriocarcinoma[31,36].

Certain studies now in progress in our laboratories require a method for identification and estimation of each of the four active, dimeric, antineoplastic alkaloids present in *C. roseus*. In particular, we are concerned with the development of a rapid, reproducible, and effective procedure for use in screening commercial samples of *C. roseus* for the presence of VLB, leurocristine, leurosine and leurosidine.

A report by JAKOVLJEVIC *et al.*[37] has shown that alumina plates prepared with 0.5 *N* lithium hydroxide, in conjunction with a 5 % absolute ethanol in acetonitrile (v/v) eluent, will effectively separate leurosidine (R_F 0.23) and leurocristine (R_F 0.51) by thin-layer chromatography. However, the same adsorbent matrix, together with a 30 % acetonitrile in benzene (v/v) eluent was required to separate leurosine (R_F 0.27) and VLB (R_F 0.36). These investigators did not demonstrate a method for separating all four active dimeric alkaloids from a single mixture.

Similarly, in a report by CONE *et al.*[38] in which adsorbent layers of silica prepared with water, silica prepared with 0.5 *N* KOH, or alumina were used, in conjunction with various combinations of seven different eluent systems, separation of VLB, leurocristine, leurosine and leurosidine from a mixture by means of thin-layer chromatography was not possible. No other reports concerned with this problem have been found in the literature.

At this time we wish to present a method found useful for separating and identifying the four closely related dimeric alkaloids (VLB, leurocristine, leurosine and leurosidine) by conventional Silica Gel G thin-layer chromatography, together with the useful alkaloid detecting ceric ammonium sulfate chromogenic spray reagent developed by JAKOVLJEVIC *et al.*[37], and first reported by CONE *et al.*[38] as a useful aid in differentiating certain of the *Catharanthus*-derived alkaloids.

Experimental

Preparation of plates. Silica Gel G plates were prepared in the usual manner with

a spreading applicator designed to produce a 250 μ matrix*. The prepared plates were activated by heating at 95–105° for 30 min in a circulating-air oven followed by cooling to room temperature in a desiccator over calcium sulfate. All plates were utilized within 72 h of activation, a precaution found necessary to insure reproducibility.

Sample preparation. The alkaloid samples, as free bases, were dissolved in benzene (2 mg/1 ml). Leurocristine, VLB, leurosine and leurosidine decompose as free bases under ordinary storage conditions and they are usually only available as the relatively stable sulfates. Each alkaloid (as sulfate) was accurately weighed, dissolved in a minimum volume of distilled water, made alkaline with NH_4OH, and extracted several times with benzene. The benzene extracts from each alkaloid were combined, dried over anhydrous sodium sulfate, filtered, and taken to dryness *in vacuo* using a flash evaporator set at 35°. Sufficient benzene was then added to each sample giving a concentration of 2 mg of alkaloid for each 1 ml of solvent (2 μg/1 μl).

The solutions of VLB and leurocristine free base (in benzene) were found to be stable for several weeks if kept frozen. However, leurosine and leurosidine as free base in benzene were found to decompose within two or three days, under the same storage conditions, to a point where interpretation of the major alkaloid component following chromatography of each sample was impossible.

Although 20 μg (10 μl of prepared sample) of each alkaloid could be readily detected with the ceric ammonium sulfate (CAS) reagent following chromatographic elution, 20 μl of each sample (40 μg of alkaloid) was found to be desirable because of the production of a more pronounced and stable chromogenic effect.

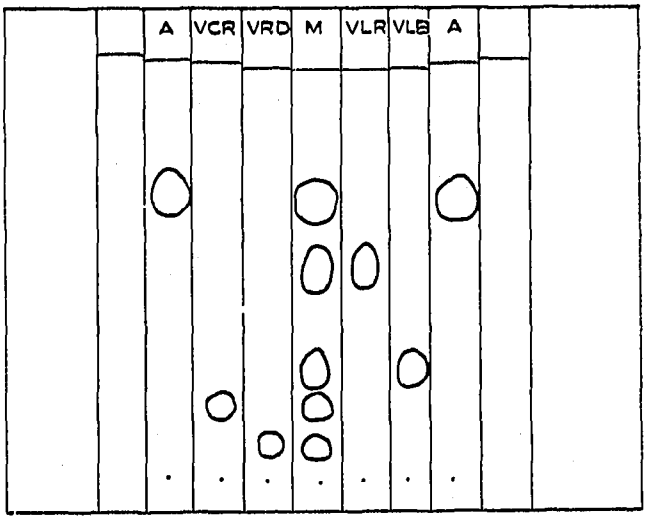
Uni-dimensional chromatography. To insure against sample swerving effects, the matrix on each plate was scored with a dissecting needle at parallel 10 mm intervals, in the same direction that the adsorbent matrix was originally spread on the plates, to give 20 separate channels. The two end channels were not utilized. Points for application of the samples were marked with a needle 15 mm from the bottom edge of the plate and centered within each channel. This general procedure has been previously described by McLAUGHLIN *et al.*[39] and appears to be beneficial in maintaining reproducibility.

Following removal of the plates from the desiccator and scoring the matrix as described, 20 μl of each alkaloid sample was applied by means of a micropipet to the point previously marked 15 mm from the bottom edge of the plate. A stream of air during application of the samples facilitated rapid evaporation of the solvent. Ajmalicine (10 μl = 20 μg) is also applied to the same spot as a reference alkaloid.

Each plate was then placed into an equilibrated (30 min) glass developing chamber ($8^1/_2$ in. × 4 in. × $8^1/_2$ in.), lined with eluent-moistened Whatman No. 1 filter paper and containing 100 ml of a chloroform–methanol (95:5) eluent. Freshly prepared eluent was used for each plate. Elution was allowed to continue until a solvent front of 100 mm (measured from the point of sample application) was attained. The plates, following a drying period in air of several minutes, were then sprayed lightly with the CAS reagent. The chromogenic reaction for each alkaloid was recorded and the separated alkaloid spots were outlined and the respective R_F values were calculated.

A typical separation of VLB, leurocristine, leurosine and leurosidine under

* The Desaga apparatus (Brinkmann Instruments, Inc., New York) was used to prepare 200 × 200 mm glass plates of the adsorbent matrix.



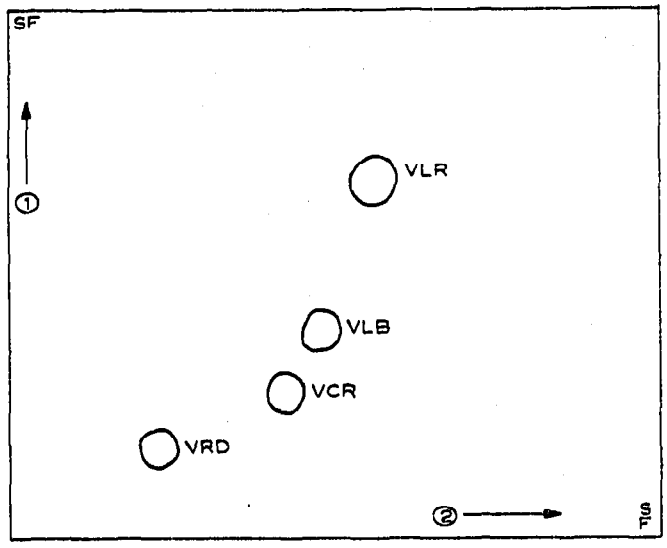


Fig. 1. A typical thin-layer chromatogram of vincaleukoblastine (VLB), leurocristine (VCR), leurosine (VLR), leurosidine (VRD) and ajmalicine (A), singly and in mixture (M). Matrix: Silica Gel G. Eluent: chloroform–methanol (95:5). Ascending development to a solvent front of 100 mm. Average development time 10–12 min.

Fig. 2. A typical two-dimensional thin-layer chromatogram of vincaleukoblastine (VLB), leurocristine (VCR), leurosine (VLR) and leurosidine (VRD). Matrix: Silica Gel G. Eluent in first direction: chloroform–methanol (95:5) to a solvent front (SF) of 120 mm. Average time of development 23–31 min. Eluent in second direction: methanol to a solvent front of 120 mm. Average development time 25–30 min.

these conditions is presented in Fig. 1. The mean R_F values for the alkaloids, together with their chromogenic behavior following application of the CAS reagent are presented in Table I.

Two-dimensional chromatography. Silica Gel G plates, prepared as previously described, were used for this procedure. Application of the alkaloid samples was made at the lower left corner of each plate at a point 15 mm from the bottom and 15 mm from the edge, in such a manner that the first direction of eluent flow would be the same as described for the uni-dimensional procedure.

TABLE I

THIN-LAYER CHROMATOGRAPHIC SEPARATION OF VINCALEUKOBLASTINE, LEUROCRISTINE, LEUROSINE AND LEUROSIDINE*

Alkaloid	*R_F value***	*Chromogenesis following CAS reagent*		
		Immediate	*After 15 min*	*After 1 h*
Leurosidine	0.06 ± 0.01	Orange-brown	Fades to tan	Tan
Leurocristine	0.16 ± 0.03	Blue	Light blue	Light blue
Vincaleukoblastine	0.24 ± 0.02	Orange-brown	Lavender	Light lavender
Leurosine	0.45 ± 0.03	Orange-brown	Yellow	Yellow
Ajmalicine (reference)	0.64 ± 0.03	Yellow-green	Yellow	Yellow

* On Silica Gel G plates (250 μ) using a chloroform–methanol (95:5) eluent. Development time was 10–12 min for 100 mm solvent front.

** Data were derived only from plates on which the reference alkaloid R_F value fell within a range of 0.60–0.68 and the solvent front (100 mm) was attained within 9–13 min.

Elution of each plate in an equilibrated (30 min) paper-lined chamber ($8^1/_2 \times 4 \times 8^1/_2$) with freshly prepared chloroform–methanol (95:5) was continued until a solvent front of 120 mm was attained. Each plate was then removed, air-dried, rotated 90°, and introduced into a second equilibrated chamber having the same dimensions and containing 100 ml of fresh methanol. Elution was continued until a 120 mm solvent front was again attained, following which the plate was removed, air-dried, and lightly sprayed with the CAS reagent (Fig. 2).

Results and discussion

The conditions described herein effectively and rapidly resolve a mixture of vincaleukoblastine, leurocristine, leurosine and leurosidine. It should be pointed out that fresh solvents must be used for each plate chromatographed since we have observed that plates chromatographed in previously used eluent will not lead to a separation of the two slower moving alkaloids leurosidine and leurocristine. Leurosidine, under these conditions, frequently remains at the point of sample application and leurocristine moves only slightly.

The alkaloid samples should be applied to the plates as quickly as possible after removal from the storage desiccator and elution should follow immediately. Plates prepared for more than 72 h, even when stored in a desiccator, were found to produce unreliable separations of the four alkaloids. As reported by JAKOVLJEVIC *et al.*[37], we have also observed a necessity for spraying the chromatograms with CAS reagent within 30–60 min following elution in order to insure reproducible chromogenic reactions.

With reference to the uni-dimensional thin-layer chromatographic procedure described, we have observed a high degree of R_F value reproducibility by considering as invalid all data derived from plates on which the reference alkaloid (ajmalicine) R_F value was determined to be outside the range of 0.60–0.68. Further, if the time required to attain a 100 mm solvent front was less than 9 or more than 13 min, R_F data derived from such plates were observed to be unreliable. No significant difference was noted in the R_F value of the alkaloids chromatographed singly or in mixture.

A measure of reliability concerning the relative position of the four alkaloids on chromatograms, following the two-dimensional procedure, was found to be in the time required to attain a 120 mm solvent front with each eluent. Considerable variation has been observed only on those plates in which a solvent front (120 mm) time falling outside the range of 23–31 min for chloroform–methanol (95:5) and 24–31 min for methanol, was noted.

In attempting to locate any one of the four dimeric antineoplastic alkaloids in crude mixtures, uni-dimensional thin-layer chromatography is of no value. However, it is possible to prepare a mixture of alkaloids from *C. roseus* by selective extraction that will contain the alkaloids vincaleukoblastine, leurocristine, leurosine, leurosidine, as well as others, but with a number of interfering alkaloids removed. Two-dimensional thin-layer chromatography of such a mixture, in the manner reported herein, separates the four active alkaloids from others which react to produce similar chromogenic reactions with the CAS reagent. It should be pointed out that leurosine, leurosidine, leurosivine, isoleurosine, neoleurosidine, VLB and desacetyl VLB all react similarly with the CAS reagent. Similarly, leurocristine and neoleurocristine produce identical colors with this reagent. Full details for a method that will separate

and allow identification of all 59 *Catharanthus roseus* alkaloids will be reported at a later date.

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