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Extraction and *in situ* densitometric determination of alkaloids from *Catharanthus roseus* by means of overpressured layer chromatography on amino-bonded silica layers I. Optimization and validation of the separation system

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Abstract

A simple, rapid and efficient method was developed for the separation and spectrodensitometric determination of bis-indole alkaloids, minor components of *Catharanthus roseus*. Separation was performed on amino-bonded silica gel layers using one-dimensional overpressured layer chromatographic development. Optimization of the eluent was performed by the PRISMA model followed by a factorial experimental design using the geometric mean of the normalized resolution as a response function. Peak purity test and validation data verified that the method was sufficient for separation of these closely related alkaloids.

1. Introduction

Vinblastine (VLB) and vincristine (VCR) are the most important bis-indole alkaloids of *Catharanthus roseus*, having high anti-neoplastic activity, but their determination is very difficult as they are only minor components of the plant. To determine these alkaloids in a plant extract, several closely related compounds must be separated from each other in a highly complex matrix. The industrial isolation of these important alkaloid components requires reliable and rapid analytical methods.

HPLC is often used for the purity testing of bulk drug substances of *Catharanthus* alkaloids (VLB, VCR) [1] and for measuring them in biological fluids for pharmacokinetic studies [2]. VLB and VCR of plant origin have been determined by HPLC either from cell cultures of *Catharanthus roseus* or from the plant extract itself after purification by solid-phase [3,4] or ion-pair extraction [5].

Quantitative TLC methods described for *Catharanthus roseus* cannot separate VLB and related compounds in the drug extract in a single development. These methods involve two-dimensional developments (with 2–4 runs) and elution–spectrophotometric [6,7] or densitometric [7] determination of these components.

HPLC methods used for these tasks require thorough sample pretreatment and highly purified solvents. Multi-dimensional TLC methods are laborious, time consuming and need large amounts of sorbents and solvents.

Our aim was to develop a rapid and simple liquid chromatographic method for the determination of bis-indole alkaloids, with special emphasis on VLB, from extracts of *Catharanthus*

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roseus drug. It seemed that overpressured layer chromatography (OPLC) using spectrodensitometry was suitable for this task owing to its simplicity, flexibility and modest sample pretreatment demands. This technique has been successfully applied in our laboratory for different kinds of separations on various sorbents [8–12] utilizing its higher efficiency compared with conventional TLC [13].

In this work, amino-bonded silica gel HPTLC layers were used, utilizing the different selectivity of the sorbent compared with silica gel [12]. An amino-bonded (NH_2) sorbent offers the possibility of the one-dimensional OPLC separation of *Catharanthus* alkaloids of interest.

In this first part, the optimization and validation of the separation system are presented. The second part will deal with the optimization of the extraction of VLB from the plant material utilizing the one-dimensional OPLC separation system described here.

2. Experimental

All solvents and chemicals were of analytical-reagent grade. Authenthic alkaloid standards were prepared at the Chemical Works of Gedeon Richter (Budapest, Hungary).

Amino-bonded silica gel was obtained from Merck (Darmstadt, Germany) (HPTLC precoated plate NH_2, Cat. No. 15647) or prepared by amino modification of silica gel sheets (HPTLC Aluminium sheets silica gel 60 F_{254}, Merck, Cat. No. 5548) according to Mincsovics [14] (some other sorbents from Merck were used only in preliminary experiments). For preparing dual-phase sorbents, only a section of the HPTLC silica sheet was modified by the reagent. Four edges of the sorbent layers were sealed with Impres emulsion (Laberté, Budapest, Hungary) for OPLC development [15].

Chromatograms were developed in normal unsaturated chambers (Desaga, Heidelberg, Germany) and in a Chrompres-10 overpressured layer chromatograph (Laberté, Budapest, Hungary) at room temperature. The OPLC conditions were as follows: membrane pressure, 1 MPa; linear velocity of eluent, 0.8 cm/min; and running distance, 16 cm.

The chromatograms were evaluated by spectrodensitometry at 298 nm with a computer-controlled TLC Scanner II (CAMAG, Muttenz, Switzerland) using CATS software (version 3.04).

3. Results and discussion

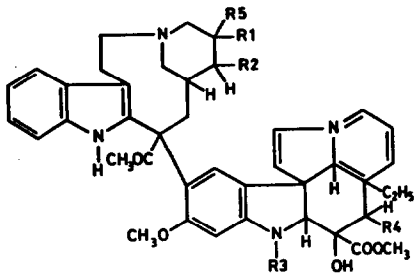
3.1. Preliminary experiments

A model mixture of authentic alkaloid standards (Table 1) and a plant extract were chromatographed on different sorbents (bare silica and octyl-, octadecyl-, cyanopropyl- and amino-bonded silica gel) in a normal unsaturated chamber (N_{us}) for the selection of a suitable stationary phase. Comparing the different sorbents tested, the resolution and spot shape of the alkaloids were the best on amino-bonded silica gel. Simple eluents gave promising separations of the standard mixture on these plates.

3.2. Optimization of eluent for OPLC developments by the PRISMA model

On the basis of preliminary experiments, hexane–dichloromethane (a), hexane–acetone (b) and hexane–2-propanol (c) mixtures were used as mobile phases in the initial OPLC systems for optimization by the PRISMA model [16]. The solvent strength of eluents used for preliminary experiments in N_{us} chambers had to be decreased for OPLC development. Relatively good separations are illustrated in Fig. 1. These densitograms show, nevertheless, that the separation of alkaloids is not yet satisfactory either for the standard mixture or for the plant extract. To improve the selectivity of separation, different combinations of the initial eluents were tested as marked in Fig. 2A by the circles. At the selectivity points 1–1–8, 8–1–1 and 3–4–3 in the triangle diagram, spots migrated to the β- or γ-front, owing to the solvent-demixing effect. Hence chromatograms obtained with these eluent combinations could not be evaluated. The chromatogram obtained with a 1:8:1 mixture of

Table 1
Structure of the alkaloids investigated



No.	Name	R1	R2	R3	R4	R5
I	Deacetylvinblastine	Et	H	Me	OH	OH
II	Vincristine	Et	H	CHO	$OCOCH_3$	OH
III	N-Demethylvinblastine	Et	H	H	$OCOCH_3$	OH
IV	Vinblastine	Et	H	Me	$OCOCH_3$	OH
V	Deacetoxyvinblastine	Et	H	Me	H	OH
VI	Leurosine	–O–		Me	$OCOCH_3$	Et

eluents a, b and c (point 1–8–1) was promising, and further experiments were therefore carried out in the neighbourhood of point 1–8–1 in the triangle as marked in Fig. 2B with circles.

Fig. 3 shows the densitogram of the best separation achieved by PRISMA at the selectivity point 2–7–1. It can be seen that the separation of the model mixture is good but with the drug extract the peak of VLB is disturbed by two slightly overlapping peaks (marked with arrows). This separation, therefore, is not suitable for the quantitative determination of the VLB content of *Catharanthus* drug extracts.

3.3. Optimization of eluent with factorial experimental design

As the OPLC PRISMA optimization system resulted in good resolution only for the compounds in the standard mixture, the separation of the plant extract components was further improved by a factorial experimental design method [17]. The 'normalized resolution' of peak pairs introduced by Gazdag *et al.* [18] for HPLC was used for characterizing the separations. The peak resolution (R_s) can also be divided to two parts as follows:

$$R_s = 1/4\sqrt{N}(\alpha - 1)/\alpha \cdot k_2'/(k_2' + 1) = 1/4\sqrt{N}D \quad (1)$$

From Eq. 1, D can be expressed as a function of capacity factors:

$$D = (k_2' - k_1')/(k_2' + 1) \quad (2)$$

Using the equation

$$R_F = 1/(1 + k') \quad (3)$$

the normalized peak resolution can be expressed with R_F values:

$$D = (R_{F_2} - R_{F_1})/R_{F_2} \quad (4)$$

where $R_{F_2} > R_{F_1}$, *i.e.*, in the case of planar liquid chromatography (*e.g.*, TLC, OPLC) the normalized resolution (D) can be regarded as the relative difference in R_F values. The use of the D value is advantageous because it can easily be determined and characterizes the separation better than the ΔR_F value. The geometric mean of the normalized resolution values was used as the response function (Y) [19] in the experimental design for characterizing the separations of different chromatograms:

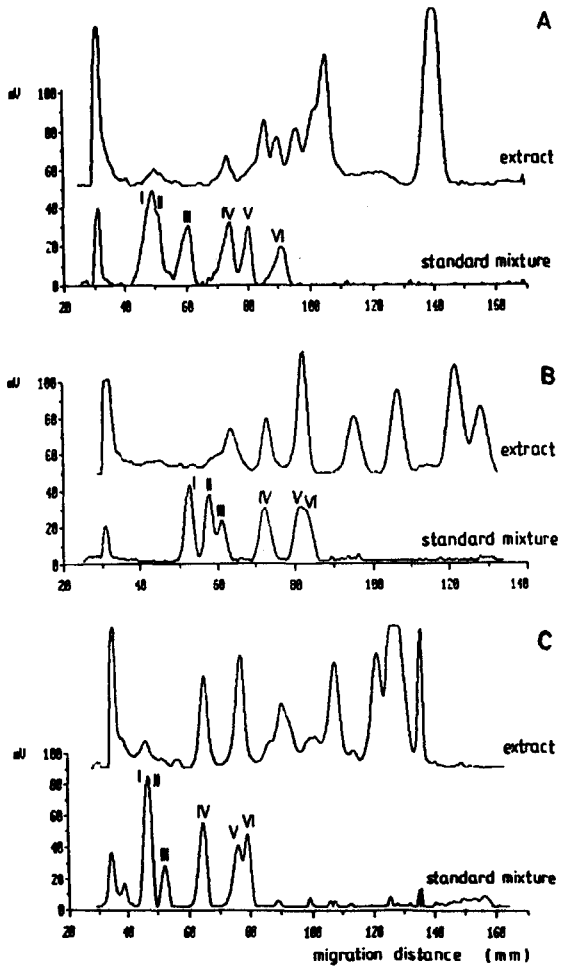


Fig. 1. Separation of alkaloids in a model mixture and a drug extract by the initial OPLC systems (a, b and c) used for optimization by the PRISMA model. Sorbent: amino-bonded silica (laboratory-modified). Eluent: (A) hexane–dichloromethane (30:70, v/v); (B) hexane–acetone (70:30, v/v); (C) hexane–2-propanol (85:15, v/v). Compound numbers as in Table 1.

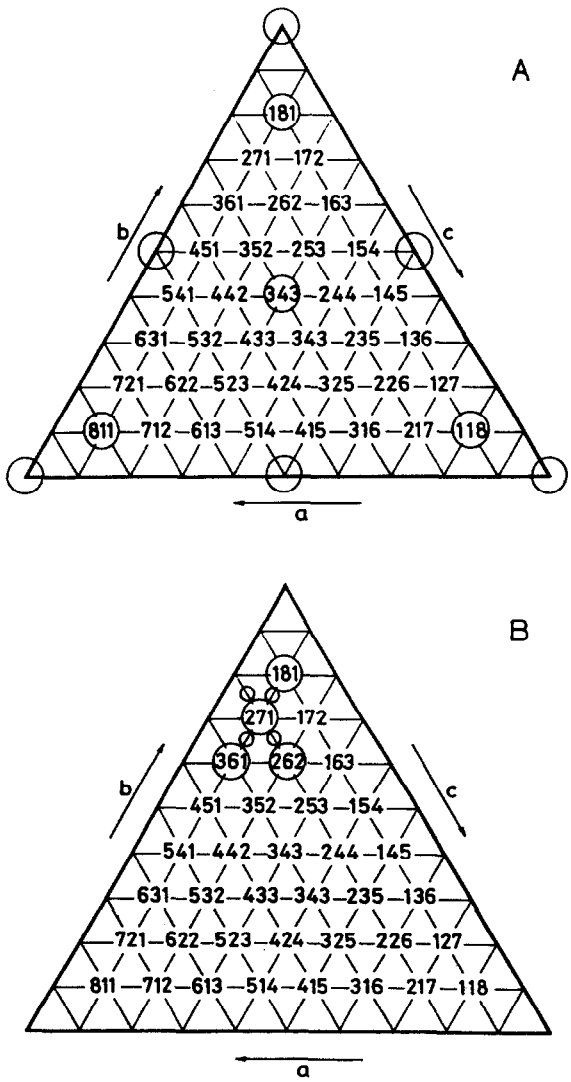


Fig. 2. Triangle diagrams of eluent compositions tried in PRISMA optimization. Vertices of the triangle correspond to the initial eluents (a, b and c). (A) Experiments in the first step; (B) experiments in the second step.

$$Y = \sqrt[n]{\prod_{i=1}^{n} D_i} \qquad (5)$$

which should be maximized in order to improve the separation. By using this function the disturbances caused by unknown adjacent peaks of VLB in drug extracts can also be taken into account.

The experimental design matrix applied and its results, *i.e.*, the values of the response function (Y) and the fitted model, are given in Table 2(A). The signs and values of the coefficients in the fitted model show that the separation of compounds can be improved by increasing the volume fraction of dichloromethane and/or decreasing that of 2-propanol. After evaluating the effects of different factors, based on the fitted model, further chromatograms were developed

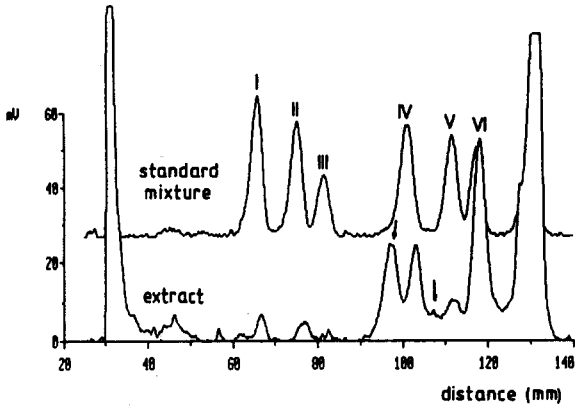


Fig. 3. Separation of compounds resulting from PRISMA optimization. The arrows show the unidentified peaks adjacent to VLB in the plant extract. Eluent: hexane–dichloromethane–acetone–2-propanol (64.5:14:21:1.5, v/v). Sorbent and other conditions as in Fig. 1. Compound numbers as in Table 1.

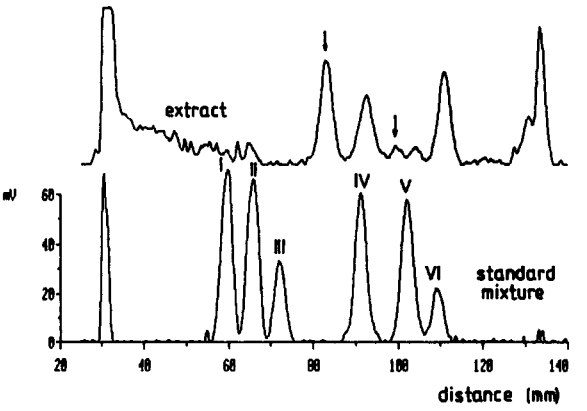


Fig. 4. Separation of compounds using the optimum eluent composition achieved by factorial experimental design. Eluent: hexane–dichloromethane–acetone–2-propanol (65:13:21:0.9, v/v). Other conditions as in Fig. 3. Compound numbers as in Table 1.

[Table 2(B)]. The best separation is illustrated in Fig. 4. It is clearly demonstrated that a baseline separation of components has been achieved both for the standard mixture and the *Catharanthus* extract (peaks adjacent to VLB in the drug extract are marked with arrows).

3.4. Check of peak purity

As VLB was separated from drug extract in a single run by one-dimensional OPLC development, the examination of the selectivity of separation is of great importance. *In situ* spectral comparison for the peak purity test was insuffi-

Table 2
Factorial experimental design matrix for eluent optimization and the response function (Y)

Serial No.	x_1	x_2	x_3	Hexane	CH_2Cl_2	Acetone	2-Propanol	$Y=\sqrt[n]{\prod_{i=1}^{n} D_i}$
(A) Initial experiments								
1	+	+	+	63	11	24	2	0.0717
2	−	+	+	67	7	24	2	0.0000
3	+	−	+	69	11	18	2	0.0708
4	−	−	+	73	7	18	2	0.0773
5	+	+	−	64	11	24	1	0.0801
6	−	+	−	67	7	24	1	0.0886
7	+	−	−	70	11	18	1	0.1106
8	−	−	−	74	7	18	1	0.0000
Fitted model: $Y = 0.0624 + 0.0181x_1 + 0.0001x_2 - 0.0096x_3$								
(B) Further experiments based on the fitted model								
9				66	11.7	21	1.1	0.123
10				65	12.6	21	1.0	0.128
11				65	13.5	21	0.9	0.125
12				64	14.4	21	0.8	0.094

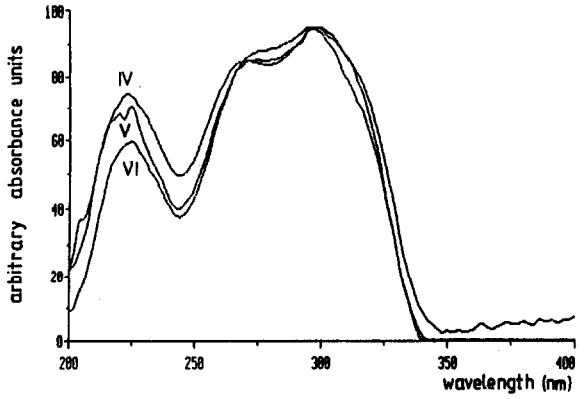


Fig. 5. UV reflectance spectra of VLB (IV), deacetoxy-VLB (V) and leurosine (VI). Chromatographic conditions as in Fig. 4.

cient because the UV reflectance spectra of the alkaloids examined are almost identical (Fig. 5).

A peak homogeneity test of the separated spots was therefore performed by two-(perpendicular)-dimensional development of the chromatograms on amino-bonded (Fig. 6A) and on laboratory-made dual-phase layers (Fig. 6B). *Catharanthus* extract containing *ca.* 3 μg of VLB was applied on the chromatoplates. The optimized eluent had been used for the first development by OPLC on amino-bonded silica. Development in the second direction was carried out either on the NH_2 phase with hexane–2-propanol (8:2, v/v) as eluent by OPLC (Fig. 6A), or on silica with ethyl acetate–benzene–ethanol–ammonia (100:5:18:1.6, v/v) as eluent [7] in a saturated normal chamber (Fig. 6B). Fig. 6 shows that no additional spot was observed in densitograms of the track of VLB in the second direction. Hence the spot of VLB is pure, *i.e.*, a single OPLC run is suitable for the determination of VLB.

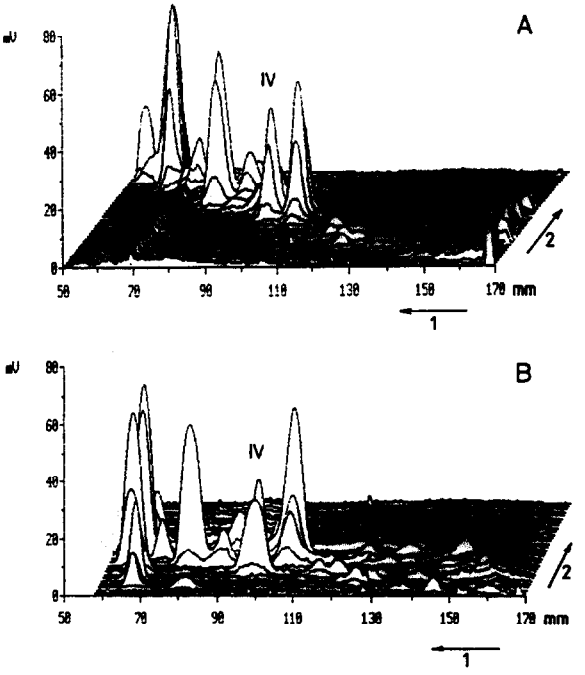


Fig. 6. Two-dimensional chromatograms of drug extract for peak purity test. Chromatographic conditions are described in the text. The arrows show the directions of developments.

3.5. Validation of the method

To apply this method for quality control purposes, it is necessary to prove its effectiveness by a validation process.

Stability tests were performed as described elsewhere [20,21]. The components of the drug extracts are stable during chromatographic development when using the optimized eluent. The drug extract was stable in methanolic solution for 3 h and also for 30 min standing on the sorbent layer before chromatography.

The calibration graph based on the peak area of VLB (y) is linear within the range 100–1000 ng of spots applied (x), as expressed by the fitted linear regression function $y = 1.51x + 41.57$. No trend was observed in the plot of residuals. Linearity of the fitted function was also verified by the F-test.

The limit of detection (LOD) for VLB was determined as described elsewhere [20] and was found to be about 50 ng.

The repeatibility of the chromatographic measurements was determined from five parallel applications of the same drug extract to the same chromatoplate: x(VLB content) = 246.8 mg/kg; S.D. = 7.1 mg/kg; R.S.D. = 2.9%.

In the ruggedness test, the effects of slight changes in the following parameters on the result of VLB determinations were studied [22]: the quality of the sorbent layer (ready-made or

laboratory-modified), volume fractions of eluent composition and OPLC equipment. Measurements were carried out with a factorial experimental design. It was found that the equipment and small changes in eluent composition have no influence on the results of measurements, but the quality of the sorbent has a moderate influence on the determination of VLB content.

4. Conclusions

Important alkaloids of *Catharanthus roseus* could be separated by a single one-dimensional OPLC development. By means of this method, the time of analysis and the sorbent and solvent consumption can be dramatically decreased compared with earlier two-dimensional methods. Vinblastine and related compounds are highly polar basic substances, therefore the amino-bonded HPTLC sorbent proved to be useful for their separation. OPLC development gave the possibility of utilizing the high resolving power of HPTLC plates over a long development distance. As the OPLC PRISMA optimization system resulted in good resolution only for the compounds in the standard mixture, the separation of the plant extract components was further improved by a factorial experimental design method. Separation of the spots in the chromatograms could be well characterized by the normalized resolution. The geometric mean of the normalized resolution values of peak pairs was successfully applied as a response function for the optimization process.

Peak purity tests were based on two-dimensional developments because there was no difference in the UV spectra of the alkaloids separated. Validation data showed that the method is suitable for routine measurements.

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6. References

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