Journal of Chromatography, 291 (1984) 165–174
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,577

REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC CHARACTERISTICS OF INDOLE ALKALOIDS FROM CELL SUSPENSION CULTURES OF *CATHARANTHUS ROSEUS*

JEAN-PIERRE RENAUDIN
Laboratoire de Physiologie Cellulaire Végétale CNRS-INRA, CNRS, BP No. 1, 91190 Gif-sur-Yvette (France)
(First received October 13th, 1983; revised manuscript received January 5th, 1984)

SUMMARY

The reversed-phase high-performance liquid chromatographic characteristics of eight indole alkaloids of *Catharanthus roseus* have been investigated, with reference to the eluting power, pH and the ionic strength of the eluent. The relative elution volumes of the alkaloids can be changed by altering one of these parameters.

Crude alkaloid extracts of cells are fractionated on a silica column prior to analysis. The principal alkaloids, ajmalicine and serpentine, are reversed from the silica in different fractions. On chromatography of these fractions with isocratic eluents the two alkaloids elute within 20 min. Their peak heights are linearly correlated with the amount injected and thus can be used for their quantitation.

INTRODUCTION

Cell suspension cultures of the periwinkle *Catharanthus roseus* (Apocynaceae) biosynthesize indole alkaloids, among which ajmalicine and serpentine predominate[1–4]. For their determination, thin-layer chromatography (TLC) associated with ceric ammonium sulphate (CAS) spray reagent[5] and UV detection is specific and fairly sensitive (detection limit less than 50 ng), but of low accuracy. High-performance liquid chromatography (HPLC) on a reversed-phase column is commonly used for alkaloid analysis. It is a rapid, sensitive and accurate method[6]. Several *Catharanthus* alkaloids can be eluted on reversed-phase columns[4,7–9], and gradient systems have already been applied to plant[8] or cell[9] extracts. But no study has been made of the influence of slight modifications of the mobile phase composition on the retention behaviour of these alkaloids. This paper describes the retention behaviour of several *Catharanthus* alkaloids with simple isocratic eluents made of methanol and buffered aqueous solution. The elution order of some alkaloids can change when the eluent is modified (water content, pH and ionic strength); this provides a convenient choice of mobile phases for various separations.

However, HPLC analysis of crude alkaloid extracts of *Catharanthus* cells takes a long time[4,8,9] because of the many alkaloids in these extracts. Peak identification

0021-9673/84/$03.00 © 1984 Elsevier Science Publishers B.V.

is also difficult. Fractionation of crude extracts before HPLC analysis overcomes these problems. A procedure using a silica column for the rapid separation of alkaloids into three fractions is presented here. Ajmalicine and serpentine levels in cells are also reported.

EXPERIMENTAL

HPLC

Chromatographic separations were carried out on a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph equipped with a Model U6K injector, Model 6000 A pump and Model M440 absorption detector set at an excitation wavelength of 254 nm. The reversed-phase column was μBondapak C_{18}, 10 μm (30 cm × 3.9 mm I.D., Waters Assoc.). A guard column with 37–50 μm Bondapak C_{18}/Corasil phase protected the column against contamination from cell extracts.

Chemicals

Ajmalicine and serpentine tartrate were from Fluka (Buchs, Switzerland), tryptamine hydrochloride from Sigma (St. Louis, MO, U.S.A.), and vinblastine sulphate from Eli Lilly (Saint-Cloud, France). Other alkaloid standards, 19-epiajmalicine, tetrahydroalstonine, catharanthine hydrochloride and vindoline, were given by Dr. H. P. Husson of the Institut de Chimie des Substances Naturelles, C.N.R.S., Gif-sur-Yvette, France. Among these alkaloids, only vinblastine has not yet been found in cell cultures of *Catharanthus*.

The HPLC solvent was a mixture of methanol (analytical grade, Prolabo, France) and 5 m*M* diammonium hydrogen phosphate (pH 7.3) aqueous solution. Prior to use, the mobile phase was filtered at 0.45 μm and degassed in a sonic bath. The pH of the aqueous phase was checked with a Heito PSD11 pH meter.

Ethanol used for cell extraction was distilled in our laboratory. Diethyl ether and chloroform (stabilized with ethanol 0.4–0.8% w/w) and diethylamine were analytical grade from Prolabo.

Extraction of plant cells

The culture and growth characteristics of the cell line C20 of *C. roseus* have already been reported[10]. For alkaloid extraction, cells are separated from their liquid culture medium by vacuum filtration on a sintered glass filter No. 2. Cells of 1–10 g fresh weight were extracted three times for 15 min with 30 ml of ethanol at 60°C. After filtration, the ethanolic extract was evaporated at 60°C under reduced pressure in a rotary evaporator and partitioned between 5% sulphuric acid and diethyl ether (1:1). The organic phase was washed with one volume of 5% sulphuric acid. The acid solution was adjusted to pH 10 with 10 *M* sodium hydroxide and extracted three times with chloroform (1:1). The organic phase was evaporated to dryness to obtain the crude alkaloid extract.

This extract was then solubilized in 1 ml of chloroform and fractionated on a prepacked silica column (50 μm neutral silica, 1 ml internal volume: Sep-Pak silica, Waters Assoc.). It was successively eluted with 14 ml of chloroform (Fraction 1), 10 ml of chloroform–methanol (9:1) (Fraction 2) and 15 ml of chloroform–methanol–diethylamine (75:20:5) (Fraction 3). The flow-rate was between 10 and 20 ml min^{-1}.

The fractions were evaporated to dryness and recovered in 0.5–2.0 ml of methanol for HPLC measurements.

TLC

This was performed on precoated silica plates (Kieselgel F254, 0.25 mm, Merck, F.R.G.) with chloroform–methanol (98:2) or acetone–toluene–methanol–27% ammonia (9:8:2:1) as eluents. After a CAS spray[5], plates were examined at 254 nm. For semi-quantitative assessments, the same amounts as those used for HPLC were analysed and their spot intensities were compared.

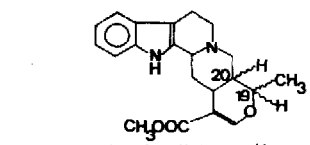
Serpentine reduction

The reduction of serpentine to ajmalicine provided a check for the identity of the serpentine peak. Sodium borohydride (*ca.* 200 mg) was added to 0.5 ml of Fraction 3 and diluted with 2 ml of methanol. The solution was then kept in the dark for a day, an excess of water was added and the solution was extracted with chloroform. The chloroform extract was dehydrated over anhydrous sodium sulphate and evaporated to dryness.

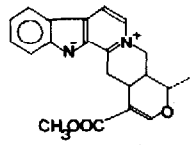
RESULTS

Elution characteristics of indole alkaloids

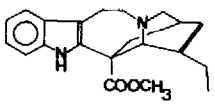
All the alkaloids listed in Fig. 1 can be retained and eluted on the reversed-phase column using an isocratic eluent methanol and 5 m*M* diammonium hydrogen



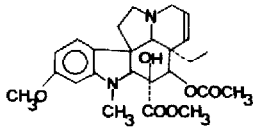
19-Hβ,20-Hβ ajmalicine $pK_a = 6.3$
19-Hβ,20-Hα tetrahydroalstonine $pK_a = 5.8$
19-Hα,20-Hβ 19-epiajmalicine



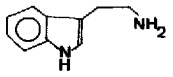
serpentine $pK_a = 10.8$



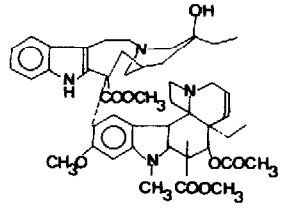
catharanthine $pK_a = 6.8$



vindoline $pK_a = 5.5$



tryptamine $pK_a = 10.2$



vinblastine $pK_a = 5.4$ and 7.4

Fig. 1. Chemical structures and acidity constants of some indole alkaloids of *C. roseus*. The pK_a values are from ref. 1.

TABLE I

PARAMETERS OF k' AS A FUNCTION OF THE METHANOL CONTENT OF THE MOBILE PHASE

Standard alkaloid solutions were analysed with a mobile phase containing methanol and 5 mM diammonium hydrogen phosphate pH 7.3 in water. The methanol percent (P) of the mobile phase was tested at n different values from 55 to 85%. Other chromatographic conditions are given in Fig. 3. The capacity factor k' of each alkaloid was measured. It is indicated for the two extreme values of P. The equation for ln k' as a function of P is given, with its correlation coefficient r.

Alkaloid	n	k'		$\ln k' = aP + b$		
		$P = 55\%$	$P = 85\%$	a	b	r
Ajmalicine and 19-epiajmalicine	13	11.9	0.67	−9.60	7.86	−0.994
Tetrahydroalstonine	11	20.2	0.92	−10.3	8.67	−0.997
Catharanthine	13	13.0	0.90	−8.90	7.46	−0.996
Vinblastine	6	31.8	0.68	−12.8	10.5	−0.994
Vindoline	13	6.7	0.47	−8.90	6.80	−0.992
Tryptamine	13	2.7	2.7	−0.07	1.04	−0.073

phosphate. This salt was preferred to carbonate in order to avoid pH modifications during solvent degassing. The pH of the aqueous solution had to be greater than 7.0 in order to retain strong bases and less than than 7.5 not to destroy the bonded phase. The pH used was 7.3 ± 0.1.

The capacity factor, k', of most alkaloids increased exponentially when the methanol percentage was decreased between 85 and 55% (Table I and Fig. 2). This is typical for reversed-phase systems[6]. However, the two compounds with higher pK_a, serpentine and tryptamine, did not follow this rule (Fig. 2). For serpentine, the exponential increase only occurred below 70% methanol. The elution volume of tryp-

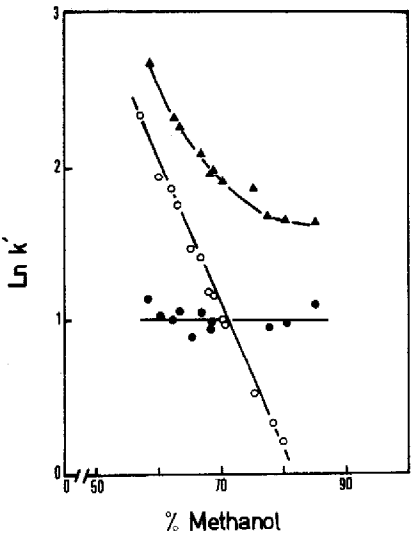


Fig. 2. Variation of ln k' with the methanol percentage P of the mobile phase for ajmalicine, serpentine and tryptamine. Conditions are given in Table I. ○ = Ajmalicine; ▲ = serpentine; ● = tryptamine.

tamine appeared to be independent of the eluting strength. When the methanol content of the mobile phase was higher than 90%, the capacity factors of all the alkaloids increased again.

Fig. 3 shows chromatograms of reference samples for different mobile phases, between 62 and 90% methanol. A complete separation of all eight alkaloids simultaneously was not accomplished. The elution volumes were always less than 50 ml in these conditions. When the methanol content was changed from 90 to 62%, the elution volume of ajmalicine altered from 4.6 to 25.0 ml and that of serpentine from 13.0 to 28.0 ml. Ajmalicine could not be separated from 19-epiajmalicine but was

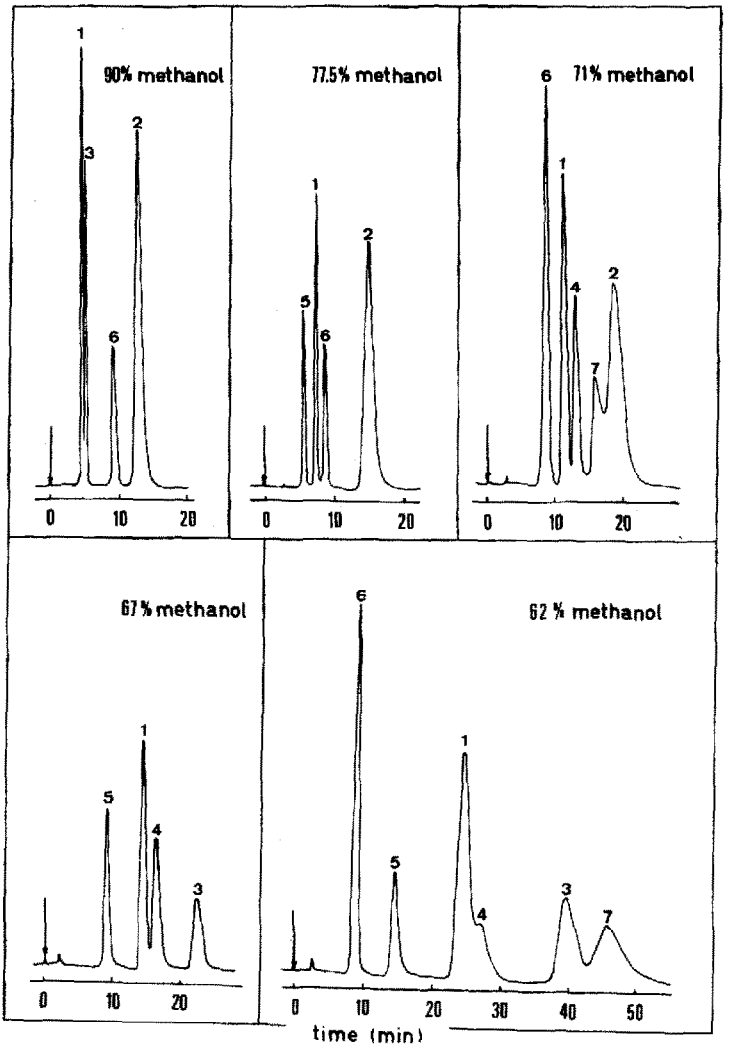


Fig. 3. Chromatograms of authentic standards. Chromatographic conditions: column, μBondapak C_{18} with a guard column; detection, 254 nm; eluent, methanol–5 m*M* diammonium hydrogen phosphate pH 7.3 in water. The methanol percentage is indicated for each chromatogram. Flow-rate, 1 ml min^{-1}. Peaks: 1 = ajmalicine; 2 = serpentine; 3 = tetrahydroalstonine; 4 = catharanthine; 5 = vindoline; 6 = tryptamine; 7 = vinblastine.

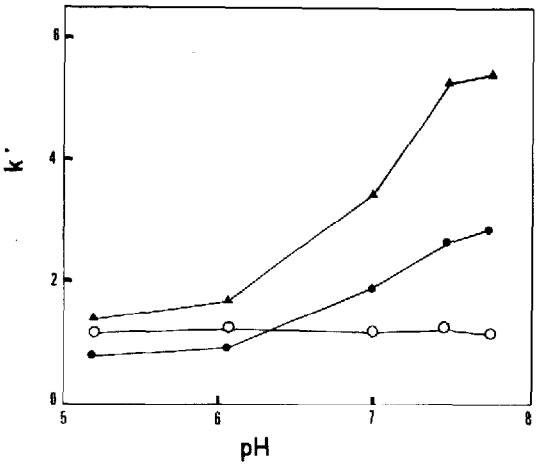


Fig. 4. Variation of k' with the pH of the aqueous mobile phase. The eluent was methanol–5 mM diammonium hydrogen phosphate. The pH of the aqueous solution of the mobile phase was adjusted with 1 N hydrochloric acid before mixing with alcohol. The flow-rate was 2 ml min^{-1}. Other conditions are given in Fig. 3. ○ = Ajmalicine; ▲ = serpentine; ● = tryptamine.

resolved from tetrahydroalstonine, another isomer that was always more retained. The elution order of the alkaloids changed with the methanol content of the mobile phase and was not simply the reverse of that of a normal-phase separation system[5]. As in normal-phase chromatography, serpentine was strongly retained, with tryptamine, even at a high methanol level (90%). This factor could be exploited for their separation from other alkaloids. These two alkaloids also had broader peaks with more tailing than the others.

The plate number of the column with a guard column for ajmalicine and its isomers, and for catharanthine and vindoline, was *ca.* 1600 when the eluent contained 67% methanol. It was *ca.* 1000 for vinblastine, which is a dimeric alkaloid. This difference is in accordance with a detailed study of reversed-phase HPLC of monomeric and dimeric *Catharanthus* alkaloids[8]. With an eluent containing 77.5% methanol, the plate number was only 900 for tryptamine and 700 for serpentine. The use of a guard column induced some peak broadening.

TABLE II

VARIATION OF k' WITH THE IONIC STRENGTH OF THE AQUEOUS PHASE

Mobile phase		*k'*			
Methanol (%)	*$(NH_4)_2HPO_4$ in the aqueous phase (mM)*	*Ajmalicine*	*Catharanthine*	*Tryptamine*	*Serpentine*
80	5	1.2	–	3.7	6.7
	10	1.2	–	2.7	5.2
66.5	5	3.9	4.7	2.7	7.5
	20	3.8	4.5	1.7	5.0

These results indicate that tryptamine and serpentine are retained on the stationary phase by forces different from those acting on the other alkaloids. This was confirmed by a study of the influence of the pH and ionic strength of the aqueous eluent. Contrary to any prediction based on pK_a values, the capacity factor of ajmalicine was scarcely affected by pH modification between 5.2 and 7.7 (Fig. 4); similar results were obtained with catharanthine and vinblastine. Theoretical calculations would predict larger variations[6]. A good resolution of ajmalicine and catharanthine only occurred at pH values greater than 7.0.

In contrast, changes in the capacity factor were much more pronounced for serpentine and tryptamine. Decreases of 2.7 and 2.2 times, respectively, were observed when the pH was changed from 7.7 to 5.2 (Fig. 4). Likewise, when the ionic strength was increased from 5 to 20 m*M* diammonium hydrogen phosphate only k' values of serpentine and tryptamine decreased, with no change for ajmalicine (Table II). Finally, although the elution of alkaloids on other identical columns was qualitatively the same as described above, the absolute retention volume may differ between columns. With an old column, for example, alkaloids with low pK_a values were retained less (the elution volume of ajmalicine being 1–5 ml lower) but tryptamine and serpentine were retained more (their elution volumes being 2.5 and 3.5. ml higher, respectively). Degradation of the hydrophobic phase and an increase of free silanol groups may explain this phenomenon. Tryptamine and serpentine are mainly present

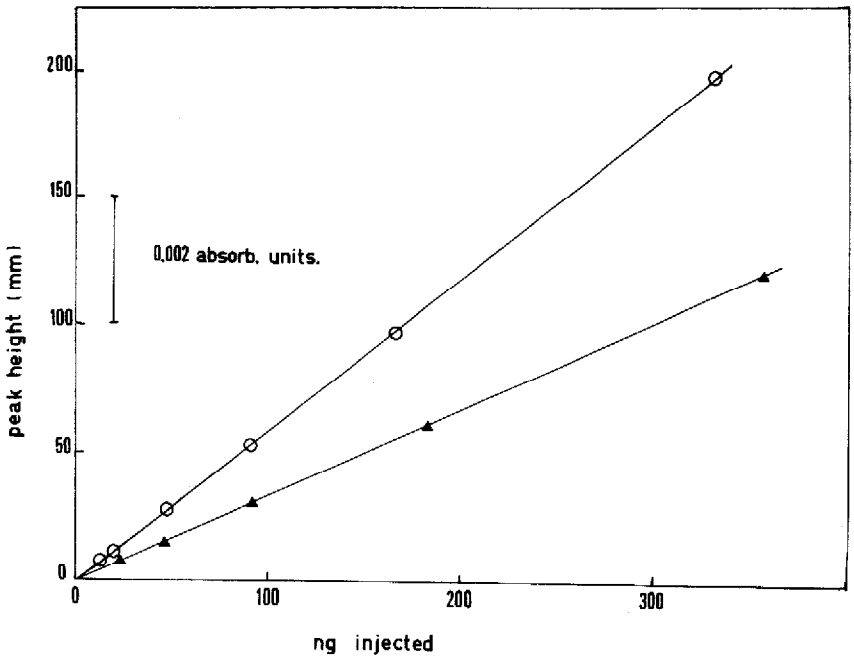


Fig. 5. Relationship between peak height and amount injected for ajmalicine and serpentine. Chromatographic conditions are given in Fig. 3. Standard solutions of alkaloids were eluted with methanol–5 m*M* diammonium hydrogen phosphate pH 7.3 in the following respective percentages: 67:33 for ajmalicine (○) and 77.5:22.5 for serpentine (▲). Two separate injections were made from independent solutions for each point, the mean deviation being less than 4%. For ajmalicine the slope is 1.72 ng mm^{-1} and for serpentine 3.04 ng mm^{-1}.

in their ionized form in the column, and can adsorb on the silanol groups, either residual or liberated by C_{18} phase degradation. Other alkaloids are mainly present in their un-ionized form and are retained on the hydrophobic phase.

Ajmalicine and serpentine can be quantified by peak height measurements (Fig. 5). A linear correlation exists between peak height and the amount injected if the methanol percentage is between 62 and 85%. The detection limit of the method, corresponding to a 1-cm high peak at 0.005 A.U.F.S. is 9 ng for ajmalicine and 15 ng for serpentine, with the experimental conditions of Fig. 5.

Application to cell extracts

The cell line C20 of *C. roseus* synthesizes mainly tryptamine, ajmalicine, serpentine and catharanthine[10] (Renaudin, unpublished results), in addition to many other unidentified alkaloids. Analysis of crude alkaloid extracts with the above HPLC

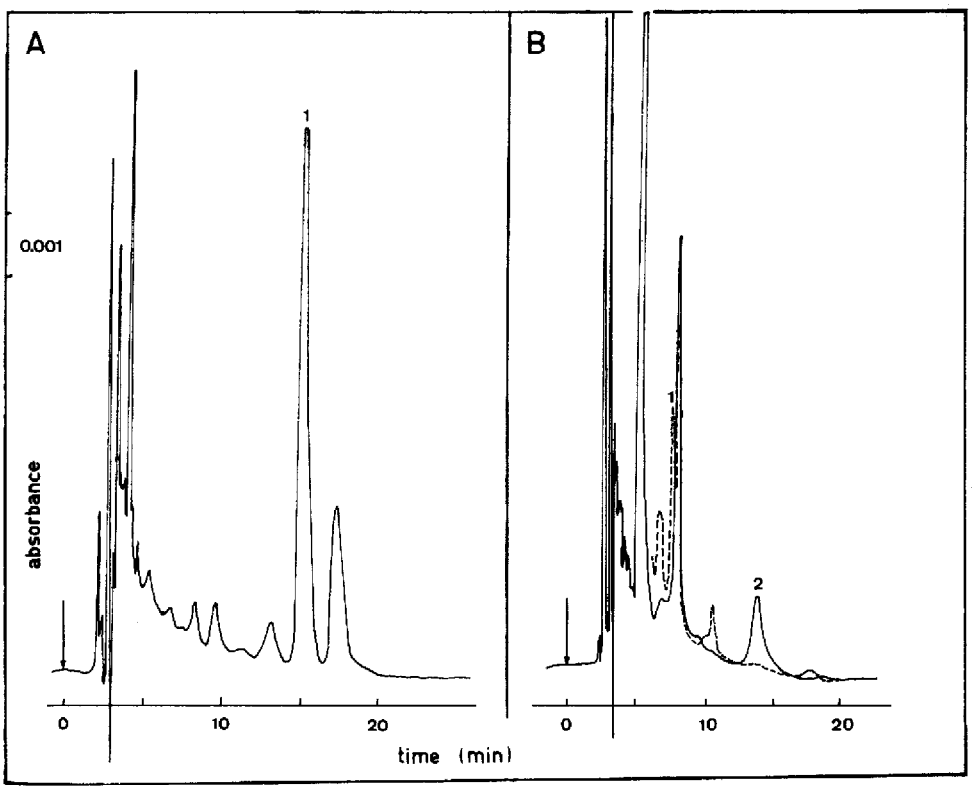


Fig. 6. Chromatograms of cell extracts. Cells of 3.2 g fresh weight were extracted and the alkaloids fractionated on Sep-Pak silica as indicated in Experimental. Fractions 1 and 3 were evaporated to dryness, then taken up in 1 ml of methanol of which 15 μl was injected. Chromatographic conditions as given in Fig. 3. Peaks: 1 = ajmalicine; 2 = serpentine. (A) Fraction 1 containing ajmalicine was analysed with the eluent methanol–5 m*M* diammonium hydrogen phosphate pH 7.3 (67:33). From the peak height, the ajmalicine concentration in cells was estimated at 4.4 μg g^{-1} fresh weight for this batch. (B) Fraction 3 containing serpentine was analysed with the eluent methanol–5 m*M* diammonium hydrogen phosphate pH 7.3 (77.5:22.5). From the peak height, the serpentine concentration in cells was estimated at 1.5 μg g^{-1} fresh weight for this batch. The dashed line is the chromatogram of the fraction after reduction by sodium borohydride and formation of ajmalicine.

system is complicated by their low solubility in methanol, rendering difficult the dissolution in small volumes. Moreover, some peaks are not well separated. Prior fractionation of alkaloids was therefore accomplished according to their polarity on a silica column ("Experimental"). Fraction 1 was shown by HPLC and TLC of standards and cell extracts to contain all the ajmalicine and its two isomers, and 80–90% of the total catharanthine and vindoline. Vinblastine was completely recovered in Fraction 2. Tryptamine and serpentine were totally in Fraction 3. These fractions were soluble in small volumes of methanol. They were conveniently analysed with the isocratic HPLC eluents discussed above.

From cell extracts, Fraction 1, containing ajmalicine was analysed with the eluent methanol–5 m*M* diammonium hydrogen phosphate pH 7.3 (67:33). The elution volume of ajmalicine was 15.5 ml (Fig. 6A). Except for some rapidly eluting substances, ajmalicine appeared as the major product of Fraction 1. The ajmalicine peak was identified by co-chromatography with authentic standard and by semi-quantitative TLC of Fraction 1, associated with specific CAS detection. The peak following the ajmalicine peak was shown to be identical with that of catharanthine.

Fraction 3 of cell extracts, which contains tryptamine and serpentine, was analysed with the eluent methanol–5 m*M* diammonium hydrogen phosphate pH 7.3 (77.5:22.5). The elution volume of serpentine was 14.4 ml (Fig. 6B). It was the last product of the fraction to elute. The serpentine peak was identified by co-chromatography with authentic standard and semi-quantitative TLC of Fraction 3. Moreover, reduction of Fraction 3 alkaloids with sodium borohydride, which is known to reduce serpentine to ajmalicine, led to complete disappearance of the serpentine peak and to formation of a peak co-migrating with ajmalicine (confirmed by TLC).

Ajmalicine and serpentine may be quantified with the calibration curves of Fig. 5. Preliminary assays were made with cells from suspensions at 5 days and gave average values of 3.9 $\mu g\ g^{-1}$ fresh weight for ajmalicine and 1.2 $\mu g\ g^{-1}$ fresh weight for serpentine (0.0060 and 0.0019% dry weight, respectively). Reproducibility of peak height measurement was satisfactory (standard error less than 4% with 15-μl injection volumes). The main variation was in levels from one batch to another. Chromatograms of cell extracts were reproducible.

Characterization of other alkaloids from cells and their liquid culture medium is now in progress and confirms the need for a preliminary purification on silica.

ACKNOWLEDGEMENTS

The author is indebted to Dr. H. P. Husson from the Institut de Chimie des Substances Naturelles, C.N.R.S., Gif-sur-Yvette, France, for the generous gift of alkaloid standards, to Mrs B. Cervoni for typing the manuscript and to Dr. S. Brown for correcting the English text.

REFERENCES

1 G. H. Svoboda and D. A. Blake, in W. I. Taylor and N. R. Farnsworth (Editors), *The Catharanthus Alkaloids*, Marcel Dekker, New York, 1975, p. 45.
2 W. G. W. Kurz, K. B. Chatson, F. Constabel, J. P. Kutney, L. S. L. Choi, P. Kolodziejczyk, S. K. Sleigh, K. L. Stuart and B. R. Worth, *Phytochemistry*, 19 (1980) 2583.
3 J. Stockigt and H. J. Soll, *Planta Med.*, 40 (1980) 22.

4 W. G. W. Kurz, K. B. Chatson, F. Constabel, J. P. Kutney, L. S. L. Choi, P. Kolodziejczyk, S. K. Sleigh, K. L. Stuart and B. R. Worth, *Helv. Chim. Acta*, 63 (1980) 1891.
5 N. R. Farnsworth, R. N. Blomster, D. Damratoski, W. A. Meer and L. V. Cammarato, *Lloydia*, 27 (1964) 302.
6 P. R. Brown and A. M. Krstulovic, *Anal. Biochem.*, 99 (1979) 1.
7 S. Görog, B. Herenyi and K. Jovánovics, *J. Chromatogr.*, 139 (1977) 203.
8 M. Verzele, L. De Taeye, J. Van Dyck, G. De Decker and C. De Pauw, *J. Chromatogr.*, 214 (1981) 95.
9 W. Kohl, B. Witte and G. Höfle, *Planta Med.*, 47 (1983) 177.
10 D. Courtois and J. Guern, *Plant Sci. Lett.*, 17 (1980) 473.