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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF INDOLE ALKALOIDS IN A SUSPENSION CULTURE OF *TABERNAEMONTANA DIVARICATA**

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SUMMARY

A method is reported for the determination of alkaloids in cell cultures of *Tabernaemontana* species. The alkaloids were isolated by means of C_8 pre-concentration columns. After recovery they were analysed on a Phenyl type of column using phosphate buffer (pH 3.9)–acetonitrile–2-methoxyethanol (80:15:5) as the mobile phase. Detection was effected at 275 and 313 nm and peak-height ratios were used for identification.

INTRODUCTION

A number of terpenoid–indole alkaloids are used in therapy and are usually isolated from plants belonging to the families Apocynaceae, Loganiaceae and Rubiaceae. Many of these plants are rare tropical species, which limits the possibilities of using them as a source of drugs. Therefore, in recent years research has focused on the production of the indole alkaloids by means of cell and tissue culture methods. The genus *Tabernaemontana* (Apocynaceae) is an example, and several interesting activities have been reported for alkaloids isolated from plants of this genus[1]; however, most plants are difficult to obtain in large amounts. Therefore, we started cell cultures of several *Tabernaemontana* species. Several of them have been studied for their alkaloid contents[2–4], and like Pawelka and Stöckigt[5], we found a number of alkaloids in the cultures. For further optimization of the culture conditions for alkaloid production a method for the determination of the alkaloids was needed. In this paper we report a selective extraction method and a high-performance liquid chromatographic (HPLC) system suitable for the determination of the indole alkaloids present in cell cultures of *Tabernaemontana*.

* Dedicated to Prof. Dr. Finn Sandberg on the occasion of his retirement.

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EXPERIMENTAL

Chemicals

Yohimbine · HCl (98% pure) and vincamine were obtained from C. Roth (Karlsruhe, F.R.G.). Dihydroquinine (free base) was a kind gift from ACF Chemiefarma (Maarssen, The Netherlands). Other alkaloids were isolated from plants or cell cultures of *Tabernaemontana* species. Their identities were confirmed by co-chromatography and from mass and NMR spectral data.

All solvents used were either of analytical-reagent grade or chemically pure; in the latter instance they were distilled prior to use. All reagents were of analytical-reagent grade.

Pre-concentration columns (RP-18, RP-8 and Kieselguhr) were obtained from J. T. Baker (Deventer, The Netherlands).

Biological material

Cell suspension cultures of *T. divaricata*, initiated as described previously[2], were grown on an MS medium[8] containing 2,4-D (1 mg/l), zeatin (1 mg/l) and sucrose (30 g/l) in the light (150 lux) at 28 ± 1°C on gyrotary shakers at 140 rpm (New Brunswick, G 10-21; Edison, NJ, U.S.A.). They were routinely subcultured every seventh day by adding 20 ml of a 7-day-old culture to 50 ml of fresh medium.

Extraction

Lyophilized plant material was extracted with methanol by grinding three times for 5 min using an Ultra Turrax. In the residual plant material no alkaloids could be detected (data not shown). The extracts were concentrated under reduced pressure and dissolved in a mixture of 1 ml of methanol, 1 ml of internal standard solution (50 μg/ml dihydroquinine) and 8 ml of 0.05 *M* sodium phosphate buffer solution (pH 7.0). After filtration (glass-fibre No. 8; Schleicher & Schüll, Dassel, F.R.G.) the filtrate was applied to an RP-8 pre-concentration column, previously prepared by washing with 3 ml of methanol and 3 ml of 0.05 *M* sodium phosphate buffer solution (pH 7.0). After application, the column was washed with 1.2 ml of 0.05 *M* sodium phosphate solution (pH 9.5)–isopropanol (70:30) and 1 ml of 0.05 *M* sodium phosphate solution (pH 9.5)–isopropanol (95:5). The alkaloids were eluted with 1.5 ml of 0.05 *M* sodium phosphate buffer solution (pH 2.0)–isopropanol (60:40). The eluate was evaporated to dryness, the residue was dissolved in 0.5 ml of HPLC mobile phase and 25–100 μl of this solution were injected in the HPLC system. A suspension culture medium was adjusted to pH 7.0 by adding 2 ml of 0.50 *M* sodium phosphate buffer solution (pH 7.0). After adding 1 ml of internal standard solution the medium was filtered (glass-fibre No. 8) and applied to an RP-8 pre-concentration column. The washing and elution steps were the same as described above. The whole extraction procedure is summarized in Fig. 1.

HPLC

The apparatus consisted of an LKB 2150 pump (LKB, Bromma, Sweden) combined with a WISP 710B automated injection system (Waters Assoc., Milford, MA, U.S.A.), two LKB 2158 Uvicord fixed-wavelength detectors operating at 275 and 313 nm, a Shimadzu Chromatopac C-R3A computing integrator (Shimadzu,

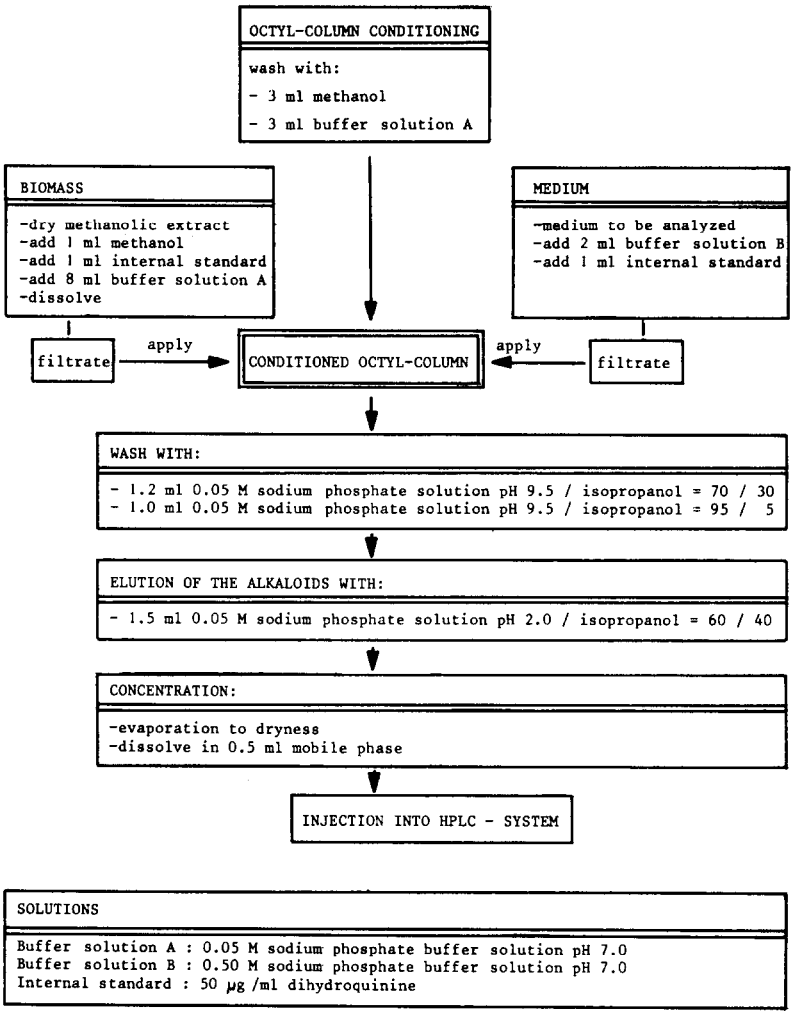


Fig. 1. Summary of the extraction method.

Kyoto, Japan) and a two-channel recorder (Kipp and Sons, Delft, The Netherlands). The column was a Waters Assoc. µBondapak-Phenyl (300 × 3.9 mm I.D.) and the eluent was 0.05 *M* sodium dihydrogen phosphate solution (6.8 g/l $NaH_2PO_4 \cdot H_2O$, pH adjusted to 3.9 with phosphoric acid)–acetonitrile–2-methoxyethanol (80:15:5) at a flow-rate of 2 ml/min[6,7]. Peak areas were used for calculations of the injected amounts of alkaloids.

Recovery

In order to test the recovery of yohimbine, vincamine and dihydroquinine over a range of concentrations, increasing amounts of a solution containing known amounts of these alkaloids were concentrated on the extraction column and were

extracted according to the method summarized in Fig. 1. All extractions were performed in duplicate.

Reproducibility

In order to test the reproducibility of the recovery of *Tabernaemontana* alkaloids, nine 1-ml samples of a methanolic extract of a suspension culture of *T. divaricata* were worked up according to Fig. 1. All extractions were performed using the same RP-8 pre-concentration column, which was regenerated by successive washings with 5 ml of methanol, 5 ml of ethyl acetate, 5 ml of methanol and 3 ml of 0.05 *M* sodium phosphate buffer solution (pH 7.0) after each extraction. All extracts were evaporated to dryness and dissolved in 0.5 ml of mobile phase, and 100 μl were injected in duplicate. The peak areas of coronaridine, apparicine, tubotaiwine, voaphylline and dihydroquinine on the chromatograms were determined. For dihydroquinine the absolute recovery was determined and for the other alkaloids the peak area after the first extraction was taken to represent 100% recovery.

RESULTS AND DISCUSSION

Extraction of the alkaloids

For the extraction of drugs from biological matrices, pre-concentration on extraction columns is widely used nowadays. They have the advantage over the classical solvent solvent extraction methods that compounds present in small amounts in large volumes can be selectively isolated in a short time without the need for large amounts of organic solvents. Such methods have also been reported for the determination of alkaloids. For the determination of *Catharanthus roseus* alkaloids (which are closely related to the *Tabernaemontana* alkaloids) in cell cultures, Kohl *et al.*[9] found Extralut normal-phase and cation-exchange columns to be suitable for the isolation of the alkaloids. They were compared with a classical solvent partitioning system. Renaudin[10] and Morris[11] used reversed-phase (C_{18}) type columns for the purification of the crude extracts.

To study the performance of the various types of pre-concentration columns, three commercially available alkaloids were selected that could serve as model compounds for the *Tabernaemontana* alkaloids: yohimbine, which has been found in *T. affinis*[12], vincamine, which was isolated from *T. rigida*[13], and dihydroquinine, which could be used as an internal standard in the HPLC system (see below).

The method described by Kohl *et al.*[9] using a normal-phase Extralut column gave good results for yohimbine, but vincamine was strongly retained on the stationary phase, and even 10 ml of chloroform did not elute the alkaloid completely from the column. Using a crude extract from a cell culture it was found that phenolic and terpenoid compounds were not completely separated from the alkaloids with this method. Using a C_{18} type of column a similar problem was encountered. Vincamine was strongly retained[14], a problem previously also encountered with cinchonamine[15]. By using a C_8 type of column this problem could be overcome. Yohimbine, vincamine and dihydroquinine could be recovered with satisfactory yields over a wide range of concentrations (Fig. 2). The phenolic and terpenoid compounds could be eluted from the column with sodium phosphate solution (pH 9.5) containing 5% or 30% of isopropanol, respectively. The alkaloids were recovered with sodium

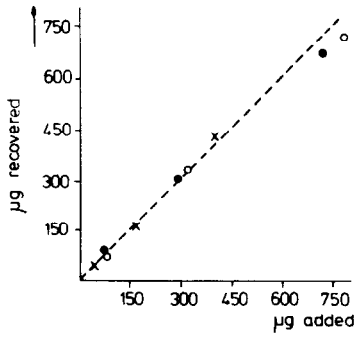
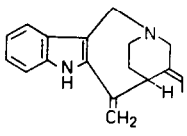


Fig. 2. Recovery of (●) yohimbine, (×) vincamine and (○) dihydroquinine using the extraction method summarized in Fig. 1. ----, 100% recovery, mean values, $n = 2$.

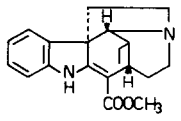
phosphate buffer (pH 2) containing 40% of isopropanol. The method is summarized in Fig. 1.

The method was tested for its reproducibility in the analysis of cell material of a suspension culture of *T. divaricata*, which produces indole alkaloids including apparicine, tubotaiwine, voaphylline and coronaridine[4]. The same material was analysed nine times using the same pre-concentration column, which was regenerated after each extraction. The results are presented in Table I. The internal standard dihydroquinine had an average recovery of 88%. The recovery of coronaridine, apparicine and tubotaiwine appeared to have a good reproducibility and correlated with the recovery of the internal standard. Voaphylline, which had the lowest k' value of the alkaloids studied in the HPLC system, was also only weakly retained on the pre-concentration column, resulting in somewhat disparate recovery data. Vobasine, another alkaloid with a low k' value, showed an excellent recovery in this method (95%), however.

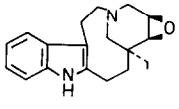
The pre-concentration columns could therefore well be regenerated after use. After nine extractions the column still showed the same characteristics.



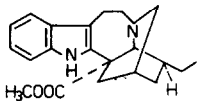
Apparicine



Tubotaiwine



Voaphylline



Coronaridine

TABLE I

REPRODUCIBILITY OF THE ANALYSIS ACCORDING TO FIG. 1

Nine 1-ml portions of a methanolic extract of a suspension culture of *T. divaricata* were extracted on the same pre-concentration column, which was regenerated after each extraction

Extraction No.	*Dihydroquinine recovery** (%)	*Coronaridine recovery**** (%)	*Tubotaiwine recovery**** (%)	*Appariceine recovery**** (%)	*Voaphylline recovery**** (%)
1	77.1	100	100	100	100
2	89.6	119	116	109	94
3	83.7	104	105	102	103
4	90.0	111	117	113	104
5	87.8	115	109	108	104
6	88.3	119	113	116	119
7	95.7	127	121	119	101
8	92.3	117	111	115	105
9	82.7	117	118	124	113
Mean**	87.5 ± 5.6	114 ± 8	112 ± 7	112 ± 8	105 ± 7

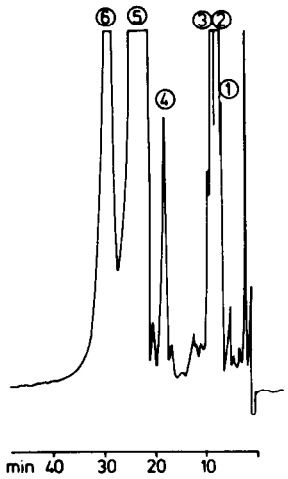
* Absolute recovery: mean value for two injections of the same extract (maximal difference between two injections was 3.7%).

** ± Standard deviation.

*** Relative recovery: the first extraction was taken to represent 100%.

HPLC

A number of systems have been reported for the HPLC determination of *Catharanthus* alkaloids[16]. An HPLC system was reported by Perera *et al.*[17] for the determination of the alkaloids of *T. dichotoma*, but it did not give satisfactory results



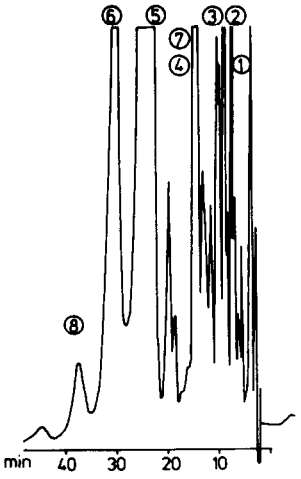
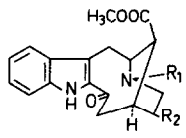


Fig. 3. Chromatogram of an extract of a *T. divaricata* suspension culture. Detection at 313 nm. peaks: 1 = perivine; 2 = tabernaemontanine; 3 = vobasine; 4 = dihydroquinine (internal standard); 5 = apparicine; 6 = tubotaiwine.

Fig. 4. Chromatogram of an extract of a *T. divaricata* suspension culture. Detection at 275 nm. Peaks: 1–6 as in Fig. 3; 7 = voaphylline; 8 = coronaridine.



	R_1	R_2
Perivine :	H	$=CH-CH_3$
Vobasine :	CH_3	$=CH-CH_3$
Dregamine :	CH_3	α-C_2H_5
Tabernaemontanine:	CH_3	β-C_2H_5

for cell culture extracts. Also, the systems reported for the determination of *C. roseus* alkaloids in cell cultures were not satisfactory for the *Tabernaemontana* alkaloids[9,10,18]. A system used for the determination of *Cinchona* alkaloids[6,7], however, proved useful for the *Tabernaemontana* alkaloids. By reducing the pH below that in the system employed for the *Cinchona* alkaloids, the separation of the major alkaloids in *T. divaricata* cell cultures, apparicine, tubotaiwine, voaphylline and coronaridine, was achieved. In addition to these major alkaloids, at least ten minor alkaloids can be observed (Figs. 3 and 4). Yohimbine, vincamine and dihydroquinine were tested as internal standards. The last compound, which so far has not been isolated from *Tabernaemontana*, proved to be the most suitable. Its retention behaviour is much more strongly influenced by changes in pH than are the *Tabernaemontana* alkaloids. Depending on the peaks present in a chromatogram, pH changes can be used to resolve the peak of the internal standard from those of the alkaloids from the cell extracts.

UV detection was chosen. Although fluorescence detection can be used, the wavelengths of excitation and emission for the different alkaloids vary too much to allow the determination of all the major components. The wavelength of UV detection chosen was 275 nm as most of the alkaloids have a maximum here. A second UV detector working at 313 nm was coupled in series in order to identify alkaloids with an acylindole, vinylindole or α-methyleneindolenine chromophore. Peak-height ratios were used for identification.

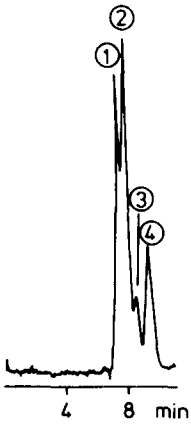


Fig. 5. Separation of the closely related corynanthean alkaloids. Peaks: 1 = perivine; 2 = tabernaemontanine; 3 = vobasine; 4 = dregamine.

TABLE II

k' VALUES OF SOME INDOLE ALKALOIDS AND RELATED COMPOUNDS AND THEIR 275:313 nm ABSORBANCE RATIO USING THE HPLC SYSTEM 0.05 *M* SODIUM DIHYDROGEN PHOSPHATE BUFFER (pH 3.9)–ACETONITRILE–2-METHOXYETHANOL (80:15:5)

The k' value of the internal standard (dihydroquinine) is 11.72.

Alkaloid/compound	k'	*Absorbance ratio (275:313 nm)*
Tryptophan	0.55	–*
Sarpagine	1.22	3.9
Tryptamine	1.39	–
Norharmane	2.12	1.2
Tetrahydroharmane	2.70	1.8
Harmane	2.71	1.7
Perivine	3.99	0.08
Stemmadenine	4.29	–
Tabernaemontanine	4.34	0.08
Vobasine	4.81	0.08
Dregamine	5.39	0.08
Vallesamine	5.62	–
Yohimbine	6.00	–
Methuenine	6.30	0.08
Voaphylline	7.28	–
Vincamine	8.37	–
12-Methoxyvoaphylline	9.34	–
19-*epi*-Voacristine	10.03	2.7
19-*R*-Heyneanine	11.14	–
O-Acetylvallesamine	12.10	–
Pericine	13.48	0.6
Apparicine	15.55	0.6
Ajmalicine	16.50	–
Tubotaiwine	16.93	0.5
Catharanthine	17.03	–
Coronaridine	20.03	–
Serpentine	30.38	0.5

* –, No absorption at 313 nm.

The HPLC system not only separates alkaloids with very different structures, but also its selectivity is well demonstrated by the separation of the closely related alkaloids perivine, vobasine, dregamine and tabernaemontanine (Fig. 5). In Table II the retention data for a series of alkaloids are summarized, including the peak-height ratio at 275 and 313 nm.

Using this HPLC method in combination with the extraction and purification method, the concentrations by the major alkaloids determined in a *T. divaricata* cell suspension culture were 2 mg/l coronaridine, 7 mg/l tubotaiwine, 7 mg/l apparicine and 24 mg/l voaphylline.

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