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## Note

# Pharmacognostical studies of *Tabernaemontana* species

## XX*. Ion-pair droplet counter-current chromatography of indole alkaloids from suspension cultures

R. VAN DER HEIJDEN*, A. HERMANS-LOKKERBOL, R. VERPOORTE and A. BAERHEIM SVENDSEN

*Center for Bio-Pharmaceutical Sciences, Division of Pharmacognosy, Leiden University, P.O. Box 9502, 2300 RA Leiden (The Netherlands)*

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The genus *Tabernaemontana* (Apocynaceae) consists of about 120 species, distributed over the (sub)tropical parts of the world. Over 250 different indole alkaloids have been isolated from *Tabernaemontana* species, many of them of pharmacological interest[2,3]. In order to study the biosynthesis of these alkaloids and to obtain a reliable source of these compounds, we initiated callus and suspension cultures of *Tabernaemontana* species[4]. A callus culture of *T. elegans* was found to produce some dimeric indole alkaloids[5]. Pawełka and Stöckigt[6] isolated six monomeric indole alkaloids from a suspension culture of *T. divaricata*.

Recently we developed an ion-pair droplet counter-current chromatographic (DCCC) separation of alkaloids[7]. This system consists of McIlvaine buffer (pH 4) containing 1 *M* sodium perchlorate–methanol–chloroform (3:5:5), using the aqueous phase as the mobile phase (ascending mode). By using a gradient with a decreasing concentration of the counter ion, the retention of the alkaloids studied could be reduced. In this paper, we report on the application of such a system to alkaloid extracts, isolated from cell cultures of *Tabernaemontana* species. The use of thiocyanate as a counter ion is also reported.

The compounds studied are listed in Table I.

## EXPERIMENTAL

### *DCCC*

The separations were carried out on a Model A DCC chromatograph (Tokyo Rikakikai, Tokyo, Japan) with 300 capillaries (400 × 2 mm I.D.) in series. The eluate was collected in fractions of 200 drops (*ca.* 5 ml) by means of a fraction collector (LKB 7000 Ultrorac). The system used was chloroform–methanol–McIlvaine buffer (5:5:3) in the ascending mode.

* For Part 19, see ref. 1.

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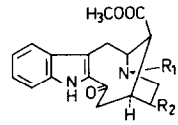
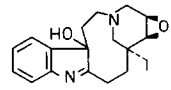
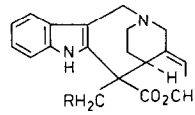
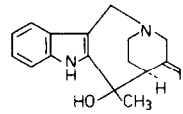
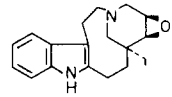
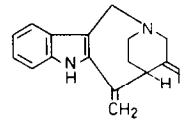
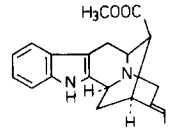
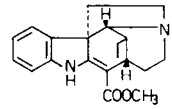
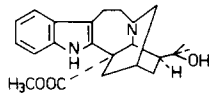
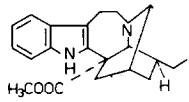
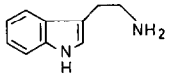
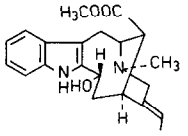
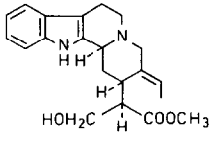
TABLE I

COMPOUNDS STUDIED

| No. | Name | Structure | R | $R_1$ | $R_2$ |
|---|---|---|---|---|---|
| 1 | Tabernaemontanine |  | | $CH_3$ | $\beta$-$C_2H_5$ |
| 2 | Perivine | | | H | =CH–$CH_3$ |
| 3 | Vobasine | | | $CH_3$ | =CH–$CH_3$ |
| 4 | Voaphylline hydroxyindolenine |  | | | |
| 5 | Vallesamine |  | OH | | |
| 6 | 16-Hydroxy-16,22-dihydroappariceine |  | | | |
| 7 | Voaphylline |  | | | |
| 8 | Apparicine |  | | | |
| 9 | Pericyclivine |  | | | |
| 10 | Tubotaiwine |  | | | |
| 11 | 19-S-Heyneanine |  | | | |
| 12 | Coronaridine |  | | | |

(*Continued on p. 412*)

TABLE I (*continued*)

| *No.* | *Name* | *Structure* | *R* | $R_1$ | $R_2$ |
|---|---|---|---|---|---|
| 13 | Tryptamine |  | | | |
| 14 | Vobasinol |  | | | |
| 15 | Isositsirikine |  | | | |
| 16 | O-Acetylvallesamine | See compound 5 | $CH_3COO$ | | |

The McIlvaine buffer (0.025 *M* citrate and 0.05 *M* phosphate) was adjusted to pH 4.2 with phosphoric acid. Sodium perchlorate and potassium thiocyanate were added to the buffer in decreasing concentrations in order to obtain a stepwise gradient. All mobile phases were saturated with stationary phase. The gradients used are indicated in Figs. 1–4. The fractions obtained by DCCC were compared by thin-layer chromatography (TLC).

*TLC*

The TLC eluent was chloroform–methanol (9:1) on silica gel F254 fertigplatte (Merck) in saturated chambers[8]. All solvents used were distilled before use or were of analytical-reagent grade. The detection reagent was 0.2 *M* iron(III) chloride in 35% perchloric acid. The plates were subsequently heated with a hot-air blower.

*Identification of the alkaloids*

For the identification of the alkaloids from the suspension culture of *T. divaricata*, see ref. 1. The identification of the alkaloids from the suspension cultures of *T. elegans* and *T. orientalis* are under investigation.

*Suspension cultures*

The suspension cultures were initiated and subcultured as described previously[1,4]. A 1.5-kg amount of biomass from the *T. divaricata* suspension was extracted with 96% ethanol, dried over anhydrous sodium sulphate and concentrated under reduced pressure. About 1 g of crude extract was injected.

Biomass from *T. orientalis* and *T. elegans* were extracted with 96% ethanol, concentrated under reduced pressure, extracted at pH 4 (phosphoric acid) with diethyl ether and extracted at pH 9 (dilute sodium hydroxide) with chloroform. The

chloroform layers were dried over anhydrous sodium sulphate and evaporated to dryness. The yield for *T. elegans* was 0.004% of crude alkaloid on a fresh-weight basis. Before injection, the extracts were dissolved in a mixture of mobile and stationary phases.

## RESULTS

### *DCCC of an extract of a T. divaricata suspension culture with perchlorate as counter ion*

For the separation of the alkaloids produced by a suspension culture of *T. divaricata* a two-step perchlorate gradient was used (Fig. 1). After screening the fractions with TLC, approximately twenty alkaloids could be detected. Some of them were pure: voaphylline hydroxyindolenine (4) (fraction No. 140), apparicine (8) (fraction No. 240) and coronaridine (12) (fraction No. 310). The main products were voaphylline (7), voaphylline hydroxyindolenine (4), apparicine (8) and tubotaiwine (10). No alkaloids could be removed from the stationary phase; it contained mainly triterpenes. TLC provided a second, uncorrelated, separation technique by which the fractions from DCCC could be further purified.

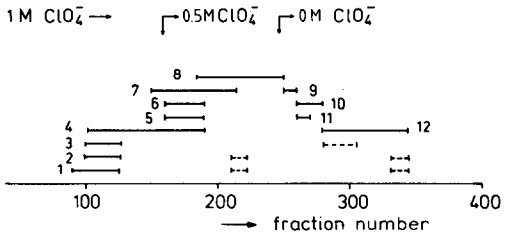



Fig. 1. Ion-pair DCCC of an extract of a *T. divaricata* suspension culture with perchlorate as counter ion. 1 = Tabernaemontanine; 2 = perivine; 3 = vobasine; 4 = voaphylline hydroxyindolenine; 5 = vallesamine; 6 = 16-hydroxy-16,22-dihydroapparacine; 7 = voaphylline; 8 = apparicine; 9 = pericyclivine; 10 = tubotaiwine; 11 = 19-S-heyneanine; 12 = coronaridine. Broken lines, unidentified (minor) components.

### *DCCC of an extract of a T. divaricata suspension culture with thiocyanate as counter ion*

When the same extract was chromatographed with a two-step thiocyanate gradient (Fig. 2), it resulted in the coelution of tabernaemontanine (1), vallesamine (5), 16-hydroxy-16,22-dihydroapparicine (6) and voaphylline (7). Apparicine (8) and tubotaiwine (10) were eluted over a number of fractions, but they were obtained pure. Coronaridine was still not eluted after 500 fractions, but it could be recovered from the stationary phase.

### *DCCC of an extract of a T. elegans suspension culture with perchlorate as counter ion*

To improve the separation, a gradient consisting of four (smaller) steps was used. With TLC 23 alkaloids were detected in the fractions and eight of them were

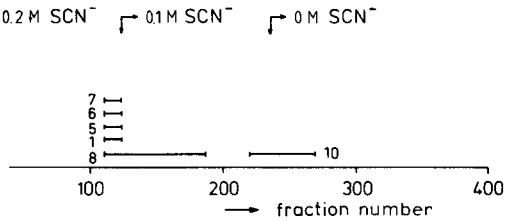



Fig. 2. Ion-pair DCCC of an extract of a *T. divaricata* suspension culture with thiocyanate as counter ion. Alkaloid identification as in Fig. 1.

identified (Fig. 3). This suspension culture produced a large amount of tryptamine, which is not retained in the DCCC system. Using this gradient also a separation of apparicine (8) and tubotaiwine (10) was obtained (compare Fig. 1). Both were eluted in a relative small number of fractions. Most fractions contained two or more compounds that could be separated by TLC.

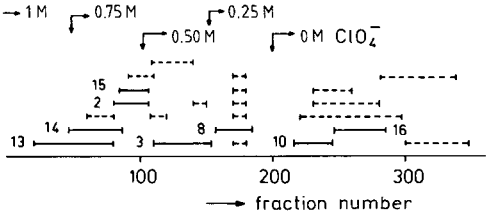



Fig. 3. Ion-pair DCCC of an extract of a *T. elegans* suspension culture with perchlorate as counter ion. 13 = Tryptamine; 14 = vobasinol; 15 = isositsirikine; 16 = O-acetylvallesamine; other alkaloids as in Fig. 1.

*DCCC of an extract of a T. orientalis suspension culture with perchlorate as counter ion*

To improve the separation of the rapidly eluting compounds, a separation was performed in which a high concentration of perchlorate (1 *M*) was maintained up to fraction 250. Although the retention was enhanced by this type of gradient, the main compounds voaphylline (7) and apparicine (8) could not be separated by DCCC (Fig. 4). Identification of other fractions, about twenty different alkaloids, is still under investigation.

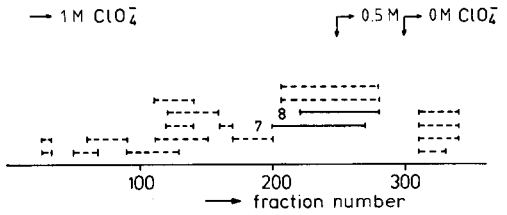



Fig. 4. Ion-pair DCCC of an extract of a *T. orientalis* suspension culture with perchlorate as counter ion. Alkaloid identification as in Fig. 1.

## DISCUSSION

For crude alkaloid extracts, ion-pair DCCC proved to be extremely useful. The complex mixtures of alkaloids (*ca.* twenty compounds) were separated in series of fractions, several of which contained only one alkaloid. Fractions containing mixtures of alkaloids could be easily separated by using an uncorrelated separation method, *e.g.*, preparative TLC, using silica gel as the stationary phase.

Comparing the two counter ions used, perchlorate gave better results than thiocyanate, the separation being more selective and the bands of alkaloids narrower. Depending on the alkaloids to be separated, the perchlorate gradient can be adjusted. Higher levels of the counter ion increase the retention time. Smaller steps in reducing the counter-ion concentration can also be used to improve the resolution.

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