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Note

Purification and separation of pyrrolizidine alkaloids from Boraginaceae on a polystyrene–divinylbenzene resin

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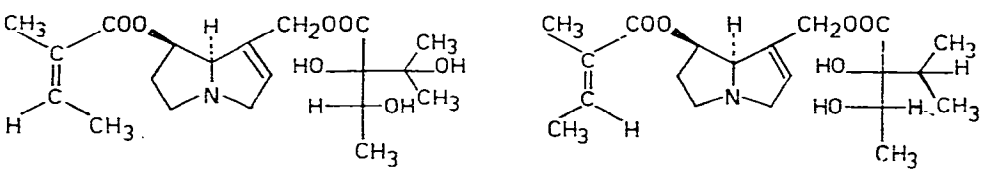
Interest in pyrrolizidine alkaloids has increased considerably in recent years owing to their antitumour and hepatotoxic properties[1–4]. Many isolation procedures have been described by a large number of investigators in the screening of various plant materials. The most commonly used technique is column chromatography with silica gel[5–9], alumina[10,11], Kieselguhr[12,13] or cellulose[14,15]. If the resolution is poor, preparative thin-layer chromatography [16–18], which has the disadvantage of being rather elaborate, or countercurrent distribution[19] could be employed.

XAD, a macroreticular polystyrene–divinylbenzene copolymer, has shown to be an effective adsorbent of organic molecules from aqueous solutions[20–23]. No ion-exchange or pore exclusion mechanisms are involved[24]. Column separations of organic compounds on XAD-2 have been much less employed than simple concentration or isolation procedures[20,25].

In this paper we describe methods for the purification and separation of mono- and diester pyrrolizidine alkaloids from Boraginaceae on XAD-2 columns using gradient elution with acidified methanol–water mixtures. The separation technique was initially developed for an improved isolation of echimidine (I) and symphytine (II) (Fig. 1), major alkaloids in Symphytum species, which could not be separated completely by the use of the above column chromatographic methods. The technique proved to be readily adaptable to wider use in the separation of pyrrolizidine alkaloids.



I II

Fig. 1. Structure of echimidine (I) and symphytine (II).

EXPERIMENTAL

Plant Materials

Radix consolidae (Symphyti radix) was supplied by Caesar and Loretz (Ham-

burg, G.F.R.). Leaves from *Cynoglossum nervosum* Benth. were kindly provided by the Botanical Garden of Groningen (The Netherlands).

Isolation of alkaloids

Large-scale extraction of alkaloids from *Radix consolidae* was performed by an ion-exchange method[26]. Isolation of alkaloids from leaves of *Cynoglossum nervosum* was performed by a Soxhlet method[16].

Serdoxit was used for reduction of alkaloid N-oxides[27]. As a control in the regeneration procedure on complete removal of dithionite after reduction of Serdoxit, the final aqueous column washings were checked by adding a few drops of the eluate to 1 ml of a 0.01% (w/v) aqueous solution of diazoresorcinol (resazurine; Serva, Heidelberg, G.F.R.). Dithionite in the column effluent caused decolorization or a change from a blue-violet to a red colour, depending on the concentration.

Column parameters

Three column sizes were used:

(A) 15 × 1 cm I.D. with 74–250 μm XAD-2 (purchased from Serva as 300–1000 μm beads, ground and sieved by the authors);

(B) 26 × 1.3 cm I.D.; and

(C) 61 × 2 cm I.D. with 50–100 μm XAD-2 (Serva).

Column B was provided with a cooling jacket through which an ethylene glycol–water mixture was passed from a thermostated water-bath.

Isocratic separation of alkaloids on column A

Before the separation of alkaloids, the column was conditioned by washing with the eluent. Eluents differing in methanol and water contents and adjusted to pH 2.8 and 5.8 were used.

Control of eluted fractions

Monitoring of the separation of XAD-2 pre-purified echimidine and symphytine using column A (low loading) was achieved by the use of an ISCO UA-4 ultraviolet monitor in the transmission mode at a wavelength of 254 nm with a chart speed of 2.5 cm/h.

Determination of tailing factors

For the determination of the tailing factors of peaks after continuous UV monitoring, the first intercept formed by the perpendicular from the top of a peak towards its base was divided by the second intercept followed by multiplication by 100 (ref. 20).

Gradient separation of alkaloids from XAD-2 columns (B and C)

Prior to the elution of alkaloids, the columns were washed with the weakest eluting agent (see below). After draining of approximately 1 ml of methanolic alkaloid extract into the column, gradient elution was performed.

Gradients were established by the addition of a strong eluting mixture consisting of methanol–water–0.5 *M* potassium chloride–hydrochloric acid (pH 2.0) into

a mixing chamber containing a weak eluting mixture consisting of methanol–water–0.5 *M* potassium chloride. The latter chamber was connected to the column.

The flow of the eluent was adjusted to 0.5 ml/min by a pinch-cock, which was placed after the detector in order to prevent the formation of air bubbles in the detector owing to expansion of compressed air in the eluent.

Linear gradient mixing chambers

Perspex linear gradient mixing chambers were used in order to make it possible to apply pressure on to the top of the solutions used by connection with a cylinder of compressed air. The working pressure did not exceed 0.4 bar. For column C a gradient mixer, consisting of two connected identical glass jars and a peristaltic pump for supplying the gradient to the column, was used.

Thin-layer chromatography of alkaloids isolated from collected fractions

To 3-ml fractions eluted from the columns into separate tubes were added 2 ml of saturated sodium carbonate solution (with a few drops of phenolphthalein solution added to the stock solution) and 1 ml of chloroform. After vigorous shaking on a Vortex mixer and after separation of the phases, the upper aqueous phase was aspirated off. If the upper phase was decolorized additional sodium carbonate solution was added.

A 10-μl volume of the chloroform extract of twenty fractions was spotted on to a 10 × 20 cm silica gel GF_{254} TLC plate (Merck, Darmstadt, G.F.R.), followed by development with chloroform–methanol–25% ammonia solution (85:14:1)[28] using a saturated chamber. Alkaloids were detected with an undiluted modification of Dragendorff's reagent (Munier) followed by spraying with a 10% (w/v) solution of sodium nitrite [29,30].

Mass spectrometry

A mass spectrum of the isolated alkaloid from *Cynoglossum nervosum* was recorded on a Finnigan 3300 quadrupole mass spectrometer, equipped with a 6110 data system, using an electron energy of 70 eV, an ionizing current of 100 μA, and a calibrated temperature of the combined electron-impact–chemical-ionization source of 210°.

RESULTS AND DISCUSSION

Neutral pyrrolizidine alkaloids in ammonia solution (pH 9.5) were completely adsorbed to XAD-2 columns and could not be eluted by a basic eluent. Conversion of the alkaloids into the cationic form (NR_3H^+) by means of an acidic eluent caused rapid and complete removal of alkaloids from the column. The separation power of XAD at isocratic conditions at a pH below 2.5 in aqueous solution appeared to be very poor. At higher pH the separation improved but the retention times increased unacceptably. By adding methanol to the acidic eluent, the retention times became shorter, but tailing of the peaks disturbed the separations.

Fig. 2 shows the relationship between tailing and the variation of pH and the methanol content of the eluents. At a higher methanol concentration and at a constant pH the tailing factor decreased. This increased tailing could possibly be induced by

the stronger sorption of the pyrrolizidine nucleus to the adsorber because of the suppression of the dissociation of the tertiary base.

Tailing of peaks could be suppressed by using very low concentrations of alkaloid. Fig. 3. shows the isocratic separation of XAD-2-pre-purified echimidine and symphytine using column A with an eluent consisting of 50% methanol in water at a pH of 3.5 (HCl) at the microgram level.

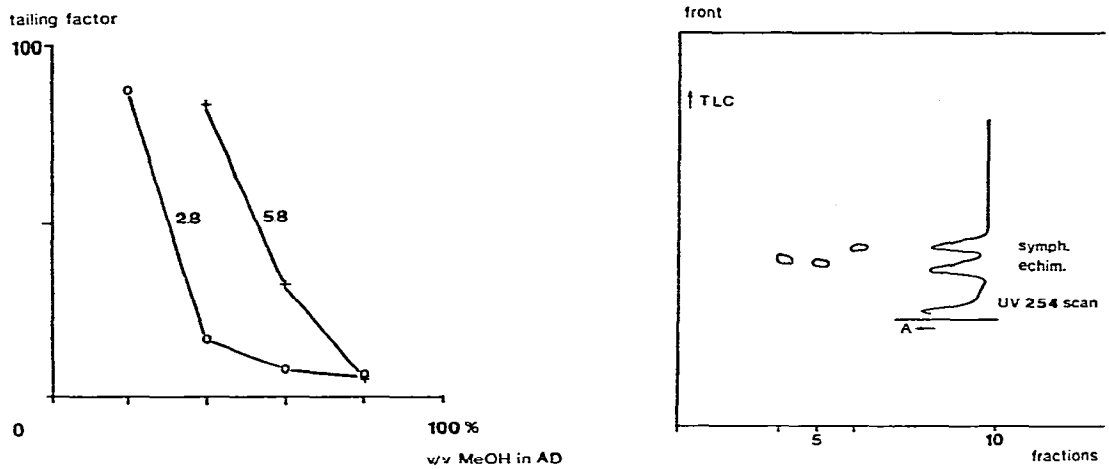


Fig. 2. Influence of methanol concentration and pH (2.8 and 5.8) on peak profile of XAD-2-separated echimidine and symphytine under isocratic conditions.

Fig. 3. Isocratic separation of pre-purified echimidine and symphytine. Conditions: column A; eluent, 50% methanol at pH 3.5 (HCl). The trace of the UV adsorbance changes in the eluent has been drawn on the thin-layer chromatogram which shows the alkaloids in the respective column fractions.

In comparison with Kieselgel TLC plates, the elution behaviour of pyrrolizidine alkaloids eluted from XAD columns could be described as being of the "reversed-phase" type.

The more hydrophilic echimidine was bound less strongly to XAD than was the more hydrophobic alkaloid symphytine, owing to the extra hydroxyl group in the former compound.

Another means of eliminating tailing in the separation of alkaloids at higher concentrations was to use gradient elution, and a further improvement in separation was achieved by cooling the column to 1°. This latter inprovement could possibly be explained by the lowering of diffusion leading to an enhancement of the sorption of alkaloids.

Fig. 4 shows a typical distribution pattern of XAD-2-separated echimidine (fractions 6–14) and symphytine (fractions 14–20) after TLC of collected column fractions.

In a similar experiment with a different batch of *Radix consolidae*, echinatine (fractions 2–11), an unknown alkaloid (fractions 12 and 13), echimidine (fractions 22–25) and symphytine (fractions 27–30) could be isolated. By addition of potassium chloride (0.5 *M*) to the eluent with no other changes in the separation conditions the

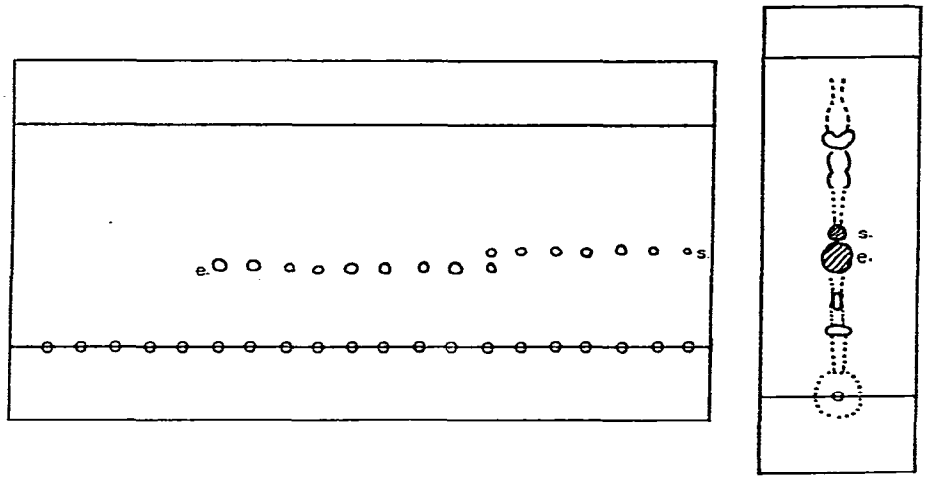


Fig. 4. Separation of 50 mg of a crude *Consolidae radix* extract (right) yielding echimidine (e: fractions 6–14) and symphytine (s: fractions 14–20). Conditions: column B; eluent, 50% methanol; gradient descending to pH 2.0 (HCl); total eluent volume, 400 ml.

separation of echimidine and symphytine could be made even more complete, as shown in Fig. 5. Salting out of the alkaloids from the eluent towards the copolymer could be an explanation for this phenomenon.

Preparative purification of crude extracts from *Cynoglossum nervosum* on column C using a linear gradient consisting of 50% methanol in deionized water with pH decreasing to 2.0 (HCl) with a total gradient volume of 1600 ml at an elution rate

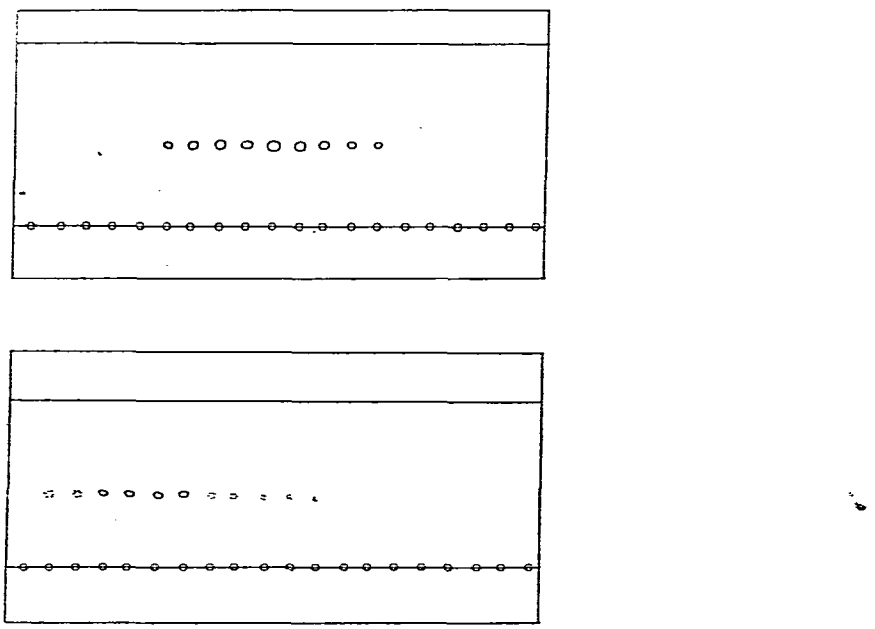


Fig. 5. Separation as in Fig. 4, but with additional 0.5 *M* KCl in the eluent. Upper chromatogram echimidine (fractions 6–14); lower chromatogram, symphytine (fractions 22–32).

of 0.4 ml/min at room temperature gave an almost pure alkaloid in fractions 8–13. In its electron-impact mass spectrum a molecular ion was observed at *m/e* 299, indicating the molecular formula $C_{15}H_{25}NO_5$. The base peak at *m/e* 138 represented the retronecine/heliotridine-type pyrrolizidine nucleus[31].

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