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Note

Preparative ion-pair high-performance liquid chromatography and gas chromatography of pyrrolizidine alkaloids from comfrey

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Interest in methods for the analysis of pyrrolizidine ester alkaloids (PAs) has increased considerably since it was discovered that many of them possess hepatotoxic properties[1] and that some of them may have potential use as cytostatic agents in cancer therapy[2].

For this analysis, namely thin-layer chromatography (TLC)[1–5], column chromatography[6–10] and counter-current distribution[9] have been used, and to a lesser extent paper electrophoresis[9,11] and gas chromatography[12–16]. However, detailed studies of the gas chromatographic separation of mono- and diester alkaloids in complex mixtures have not so far been published. In our laboratory, a rapid derivatization procedure for PAs prior to analytical and capillary gas chromatographic (GC) separation was developed. The PA derivatives gave mass spectra that were valuable in the qualitative evaluation of the separated PAs.

A search for faster column chromatographic separations of PAs led to the development of high-performance liquid chromatographic (HPLC) methods[17–20]. An extension of these methods to preparative HPLC has so far only been utilized for the separation of macrocyclic PAs from *Senecio vulgaris*[21], but the method was laborious and expensive.

In this paper we describe a cheap and convenient method for the preparative ion-pair HPLC separation of mono- and diester PAs from comfrey, based on the ion-pair separation described previously for TLC[5].

EXPERIMENTAL

Pyrrolizidine alkaloids from ground comfrey roots were isolated as described previously[22]. All reagents and solvents were of analytical-reagent grade.

For preparative ion-pair HPLC a 53 × 2.5 cm I.D. stainless-steel column (Waters Assoc., Milford, MA, U.S.A.) was packed with dry silica gel 60 (230–400 mesh) from Merck (Darmstadt, G.F.R.) under continuous vibration with an engraving instrument. After connection of this column to a Waters Preparative LC/System 500 A, flushing with an eluent consisting of 0.075 *M* lithium chloride in chloroform–methanol (85:15) was carried out until the refractive index (RI) remained constant. Then the column was loaded with a solution of 750 mg of root extract in 10 ml of the eluent and the alkaloids were separated at a flow-rate of 50 ml/min.

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Fractions that coincided with monitored RI peak profiles were collected and the eluent was removed *in vacuo* at 50°C by means of a rotary evaporator. The alkaloids were dissolved in chloroform, leaving the remaining lithium chloride behind.

Approximately 1 mg of the HPLC-purified alkaloid (after evaporation of chloroform in a stream of air) was dissolved in 30 μl of chloroform–dimethyl sulphoxide (1:1) and derivatized with 75 μl of a freshly prepared mixture of hexamethyldisilazane and trimethylchlorosilane (10:1) at room temperature for 5 min in a PTFE-lined Sovirel tube. Whenever small amounts of water were still present in the samples prior to derivatization, 1 ml of dimethoxypropane was added as a water scavenger and removed again by passing a stream of nitrogen into the derivatization tube.

Separation of the trimethylsilyl (TMS) ethers was achieved in a Packard 429 gas chromatograph equipped with a flame-ionization detector on a 1400 × 0.4 cm I.D. glass column packed with 4% OV-17 on Chromosorb W HP (100–120 mesh) under the following conditions: nitrogen flow-rate, 25 ml/min; detector block temperature, 300°C; injector temperature, 250°C; initial column temperature, 220°C, increased at 3.5°C/min to a final temperature of 270°C.

Combined GC–mass spectrometry (MS) was performed on a Finnigan 3300 quadrupole mass spectrometer equipped with a standard chemical ionization source and a 6110 data system at an electron energy of 70 eV and an ionizing current of 100 μA. In this instance a wide-bore 2500 × 0.05 cm I.D. CpSil 5 column (Chrompack, Middelburg, The Netherlands) was used under the following conditions: initial column temperature, 200°C, increased at 4°C/min to a final temperature of 330°C. Mass spectra were taken with a 1.87-sec cycle time and data acquisition was started after 1.85 min.

RESULTS AND DISCUSSION

Fig. 1 illustrates the preparative ion-pair HPLC separation of the pyrrolizidine alkaloids of comfrey roots. The elution pattern resembled the pattern found previously for ion-pair TLC[5]. Lycopsamine/intermedine (peak A) was eluted first, followed by acetyllycopsamine/acetylintermedine (peak B) and symphytine isomers (peak C), as confirmed by comparison with reference substances. No recycling was needed for adequate separation, resulting in short analysis times.

After evaporation of the eluent *in vacuo* the alkaloids were easily separated from the remaining lithium chloride by dissolving them in chloroform. The combined evaporated organic phases were purified by distillation using a Hempel column and checked for purity and deviation from the original 85:15 of the chloroform to methanol ratio by gas chromatography. By addition of more of one of these solvents the correct 85:15 ratio was obtained again. This recycling of solvents and the low cost of the silica gel columns made the entire method cheap in comparison with reversed-phase separations. Another feature of the described system over reversed-phase systems is that alkaloids are simply isolated by evaporation of solvent only, so no basification and extraction of eluted fractions from water–methanol mixtures, which can cause hydrolysis of ester alkaloids, is needed.

The alkaloids from the collected fractions A, B and C were derivatized and separated by gas chromatography on a packed OV-17 column. The short derivatization time at room temperature with the chosen mild silylation agent did not give a

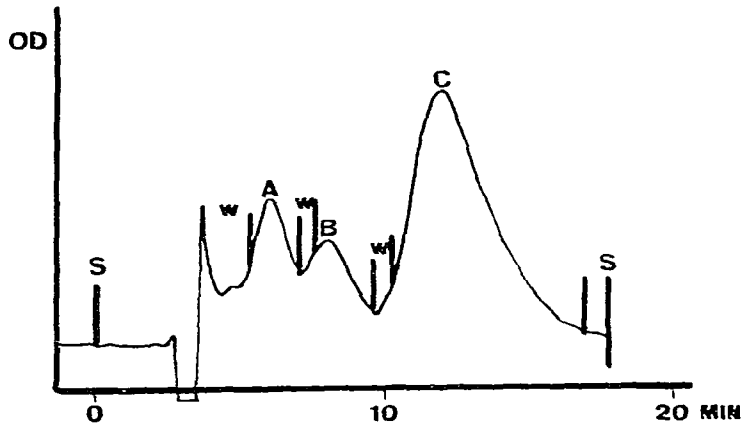


Fig. 1. Preparative HPLC of pyrrolizidine alkaloids from comfrey as ion pairs. Column, 53 × 2.5 cm I.D., packed with silica gel 60 (230–400 mesh); eluent, 0.075 *M* lithium chloride in chloroform–methanol (85:15); flow-rate, 50 ml/min. OD = optical density; S = injection (750 mg of alkaloid in 10 ml of the eluent); W = waste; A = lycopsamine + intermedine; B = acetyllycopsamine + acetylintermedine; C = symphytine and/or isomers.

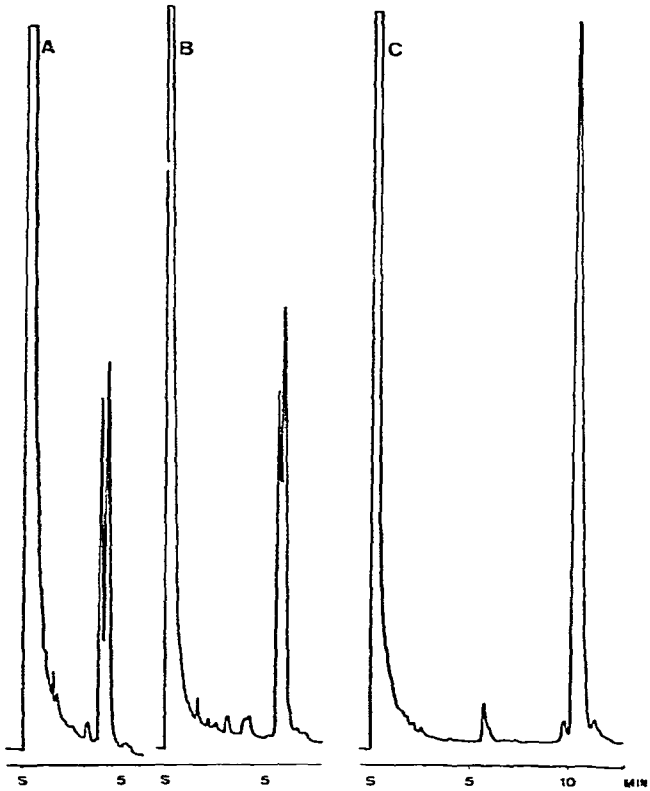


Fig. 2. GC analysis of TMS ethers of the pyrrolizidine alkaloid fractions A–C that eluted from the preparative HPLC as ion pairs (compare with Fig. 1).

complete reaction with the hydroxyl groups of the esterifying acids, as will be shown later. However, this did not affect the separation patterns when the derivatization time did not exceed 30 min. A longer derivatization period gave rise to the appearance of additional peaks, probably because of a higher degree of derivatization of the alkaloids.

Fig. 2 shows the GC separation of the alkaloids in the various HPLC fractions. The double peaks are due to the presence of stereoisomeric forms of the alkaloids. The separation of these isomers by means of affinity chromatography will be described elsewhere.

A total separation of lycopsamine/intermedine and acetyllycopsamine/acetylintermedine together with a partial separation of the isomers of symphytine could be shown by capillary GC on CpSil 5 columns (Fig. 3) in combination with MS. The mass spectra of the isomers were similar in their fragmentation, as would be expected, although the relative intensities of fragments could differ (Fig. 4). The fragmentation pattern of lycopsamine/intermedine (L/I) was comparable to that of indicine N-oxide in the pyrrolizidine form (INOPYZ) except for the ion at *m/e* 261, which was absent in the L/I mass spectra[16]. This peak should be indicative of the fully silylated esterifying dihydroxy acid. The peak at *m/e* 117 (relative intensity 51.5%) shows, however, that the secondary hydroxyl group was silylated (compare Table I).

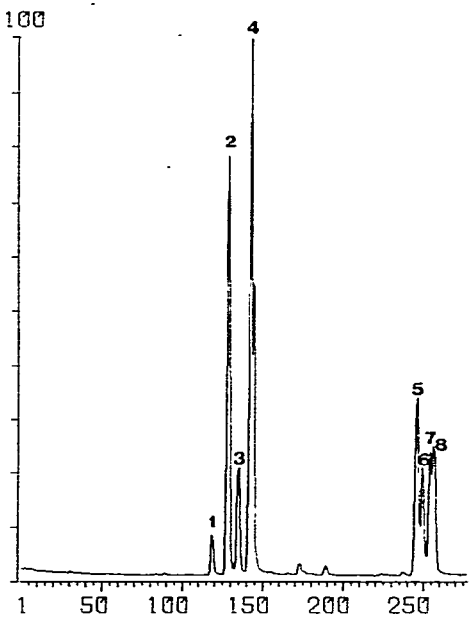


Fig. 3. Wide-bore capillary GC of TMS ethers of pyrrolizidine alkaloids from comfrey root on CpSil 5. 1, 2 = Lycopsamine/intermedine; 3, 4 = acetyllycopsamine/acetylintermedine; 5, 6, 7, 8 = symphytine and isomers.

This, albeit incomplete, silylation was enough to ensure sufficient volatility. Peaks in the mass spectra of the distinct TMS-alkaloids at *m/e* 210, 180 and 220, gave full information of the substitution level at C-7: TMS, acetyl, or tiglic/angelic acid respectively, for L/I, acetyl-L/I and symphytine isomers (Figs. 4 and 5).

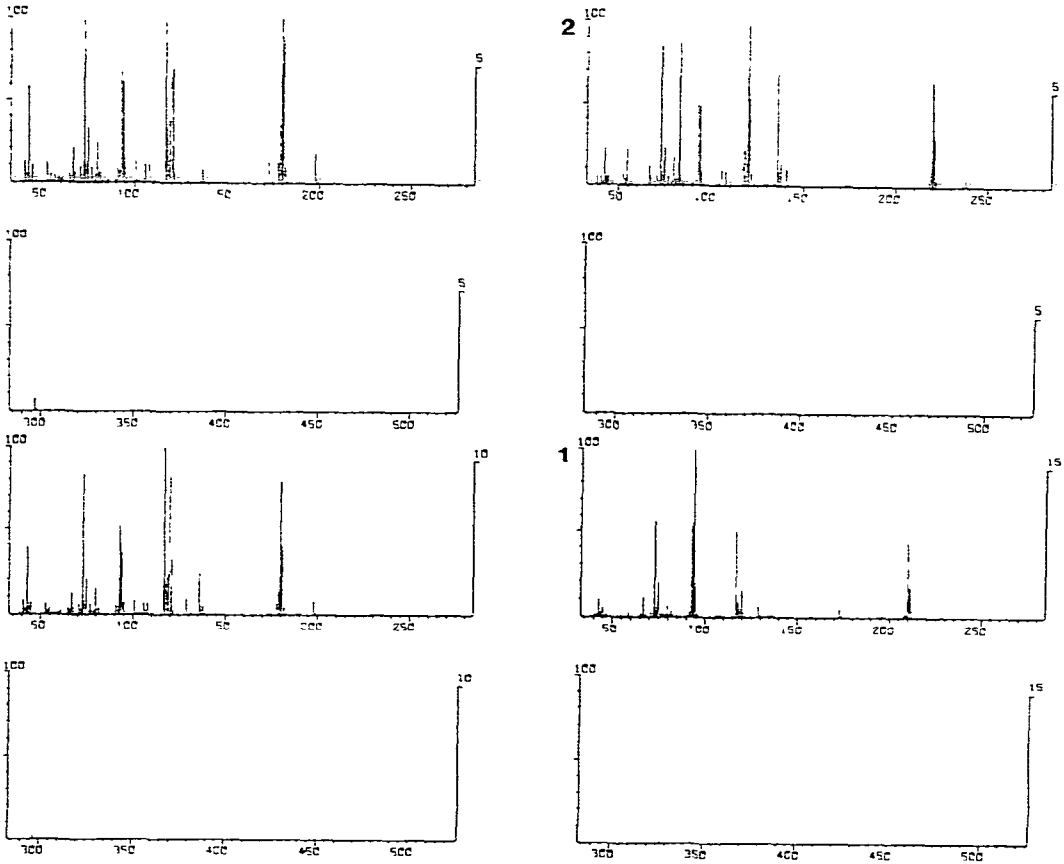
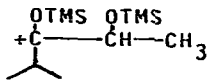
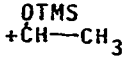


Fig. 4. Mass spectra of diastereoisomeric TMS-acetyllycopsamine or TMS-acetylintermedine (GC peaks 3 and 4, respectively, in Fig. 3).

Fig. 5. Mass spectra of TMS_2-lycopsamine (1) or diastereoisomer and TMS-symphytine (2) or isomer. Compare also with Fig. 4.

TABLE I

FRAGMENTS IN THE MASS SPECTRA OF INDICINE N-OXIDE IN THE PYRROLIZIDINE FORM (INOPYZ) AND LYCOPSAMINE/INTERMEDINE (L/I) TRIMETHYLSILYL DERIVATIVES, SHOWING THE DIFFERENCE IN DERIVATIZATION SITE

Fragment	*m/e*	*Relative abundance (%)*	
		INOPYZ	L/*I*
	261	7.64	–
	117	30.10	51.5

We believe that these methods can contribute to the faster and easier analysis of pyrrolizidine alkaloids from different plant sources, which is of importance for pharmacognostic screenings of plants that show hepatotoxic properties and for chemosystematics.

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