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ANALYSIS OF INDOLE ALKALOIDS FROM LEAVES OF *CATHARANTHUS ROSEUS* BY MEANS OF SUPERCRITICAL FLUID CHROMATOGRAPHY/MASS SPECTROMETRY[1]

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ABSTRACT.—An indole alkaloid mixture, obtained from the leaves of *Catharanthus roseus*, was analyzed by means of supercritical fluid chromatography (sfc) in conjunction with ms. Under the conditions employed, a completed chromatogram was obtained within 8 min with an estimated 40 alkaloids being detected when a uv monitor was employed. By using a mass spectrometer in thermospray filament-on mode in conjunction with the uv monitor, an estimated 60 alkaloids could be detected. When the chromatograph was coupled to a mass spectrometer in ei mode, high quality ei mass spectra were obtained, which in turn allowed for the identification of several alkaloids.

Monoterpene indole alkaloids represent a large and diverse family of natural products whose members occur mainly in plants belonging to the families Apocynaceae, Rubiaceae, and Loganiaceae. Separation and characterization of theses alkaloids in plant extracts can be an imposing problem due to the large number and very different amounts of components normally present in an extract. Moreover, the alkaloids possess a wide range of solubilities, molecular weights, and polarities, factors which, although providing the basis for analytical separations, can also limit the utility of various techniques when one considers the overall separation. Tlc has proven to be a useful method for their separation, while use of spray reagents in conjunction with tlc has proven a sensitive method for their detection (1). Reversed-phase hplc using gradient elution has also been used with some success (2,3). Unfortunately, both of these techniques have shortcomings; tlc spots obtained from polar compounds sometimes overlap. Furthermore, identification of unknown alkaloids separated by tlc is not possible using spray detection. Co-elution of components is also common when using hplc for the separation of these compounds (4). Diode array detection is useful for the confirmation of specific alkaloids as well as for studying co-eluting peaks but is of limited use for characterizing unknown components (5).

Traditionally, much structural information has been obtained from ms of individual alkaloids isolated from complex mixtures of plant material using relatively large scale extraction and cc methods. Large scale separations of plant material require a significant number of manipulations which in turn increases the probability of artifact formation. Recently, supercritical fluid chromatography (sfc), a technology well suited to interfacing with a mass spectrometer, has been shown to be more efficient than hplc and has proven effective in analyses of various polar compounds, including mixtures of ergot alkaloids (6). Accordingly, as a potentially useful means of directly obtaining pertinent information on the number and type of monoterpene indole alkaloids in a relatively small amount of complex sample, we sought to evaluate the use of sfc-ms using an alkaloid mixture obtained from the leaves of the medicinal plant *Catharanthus roseus* G. Don. (Apocynaceae). *C. roseus* is one of the most thoroughly investigated of plants with regard to its constituent indole alkaloids, of which more than 70 have been isolated from the whole plant (7). A description of the technique is presented below.

[1]NRCC No. 29148.

RESULTS AND DISCUSSION

The separations obtained on the amino bonded Spherisorb column (Figure 1) afforded a run within 8 min, yielded qualitative information comparable to that obtainable via 2D tlc on Si gel, and indicated the presence of approximately 10 major components and perhaps 30 to 40 minor ones when uv detection was used. Overall, three alkaloids, catharanthine, vindoline, and 3′,4′-anhydrovinblastine, were predominant, with the presence of the latter being noteworthy, as there had been no previous report of its being a major *Catharanthus* alkaloid. In general, peaks were sharp and well shaped with numerous near-baseline separations being noted. More specifically, compounds having very similar structures were well spaced. Desacetoxyvindoline, vindorosine, vindoline, and deacetylvindoline, complex *Aspidosperma* alkaloids differing only slightly from each other in their functional groups, were easily separated from each

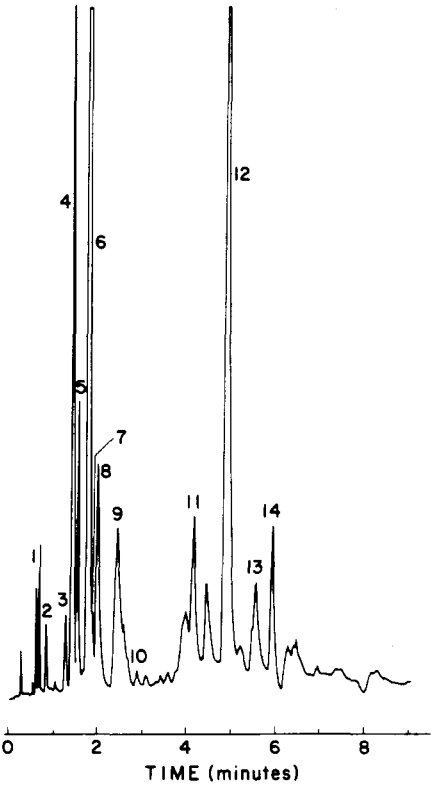

FIGURE 1. Chromatogram (sfc) of *Catharanthus* leaf alkaloids on an amino-bonded Spherisorb column. Alkaloids identified were: 1, lochnerinine; 2, horhammericine; 3, desacetoxyvindoline; 4, catharanthine; 5, vindorosine; 6, vindoline; 7, ajmalicine; 8, vindolinine[2]; 9, epivindolinine[2]; 10, deacetylvindoline; 11, leurosine; 12, 3′,4′-anhydrovinblastine; 13, perivine; 14, vinblastine. Uv detection at 290 nm.

[2]Assignment might be reversed.

other as were the pharmaceutically important bisindoles leurosine, 3′,4′-anhydrovinblastine, and vinblastine which chromatographed as a group, away from the majority of the "monomers". By comparison, the complete and simultaneous separation of these alkaloids could not be achieved via 2D tlc. Although different separations of components by tlc and sfc were observed, there was a general correlation between an alkaloid's R_f value on tlc and its elution time on the amino bonded Spherisorb column. Alkaloids possessing higher R_f values generally eluted earlier than those with lower R_f values.

One of the attractive features of sfc is the use of a large proportion of a highly volatile phase which makes interfacing the chromatograph to a mass spectrometer much less problematic than is the case with hplc. Both ei and thermospray filament-on ionization were successfully employed in the sfc-ms system. As ei mass spectra of indole alkaloids provide significant structural information and can often serve as a "fingerprint" in identifying a known alkaloid, the ability to obtain high quality ei mass spectra of minor components in a complex mixture would constitute a powerful tool in a phytochemist's arsenal. Figure 2 illustrates four representative ei mass spectra obtained via sfc-ms. With the exception of lochnerinine, whose spectrum contained some anomalous peaks (due to the presence of an unidentified alkaloid having $[M]^+$ 338), the spectra were of a quality comparable to those obtained from the pure compounds. The power of the technique was demonstrated by the identification of the minor component desacetoxyvindoline which previously had been identified only in dark-grown seedlings of *C. roseus* (10). Overall, based on comparisons with reference eims obtained from authentic samples, 14 of the components of the leaf mixture were conclusively identified (Figure 1) and estimated, on the basis of preparative isolations performed, to represent more than 95% (by wt) of the alkaloids of the mature leaves. Further identifications of components

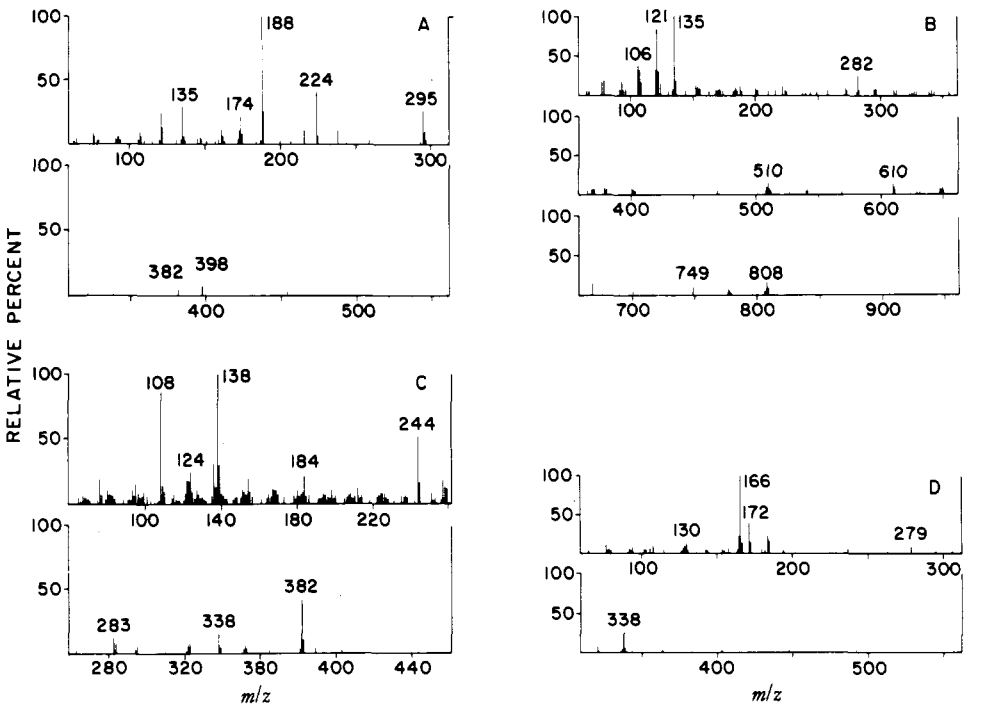

FIGURE 2. Representative ei mass spectra obtained via sfc-ms of *Catharanthus* leaf alkaloids: A, desacetoxyvindoline; B, leurosine; C, lochnerinine; D, perivine.

were not attempted due to lack of reference spectra and fragmentation patterns which were not unambiguously interpretable.

The use of thermospray ionization in the sfc-ms system effectively enhanced the sensitivity for detection of the alkaloids. Because in the case of monoterpene indole alkaloids the ionization technique affords predominantly $[M + 1]^+$ ions with little fragmentation arising, a scan giving rise to a spectrum containing several significant peaks would be indicative of the presence of several compounds of appropriate molecular weights. The spectrum (Figure 3) obtained from the eluent proximate to peak 3 (Figure 1) indicated the presence of components having mol wt of 350 and 352 in addition to desacetoxyvindoline ($[M]^+$ 398). The initial ability to detect 40 or so compounds with a uv detector in conjunction with sfc now has been extended to perhaps 60 or so compounds by using the mass spectrometer equipped with thermospray ionization as a detector. One drawback, however, is that the intensities of peaks observed in the thermospray mass spectrum are not necessarily indicative of the proportions of different alkaloids present, which in turn may cloud the interpretation of the spectrum. On the positive side, the molecular weights of the majority of the leaf components were accessible (data not shown). The use of an ms-ms instrument in conjunction with thermospray ionization in the filament-on mode suggests further interesting possibilities for analyses with sfc-ms systems.

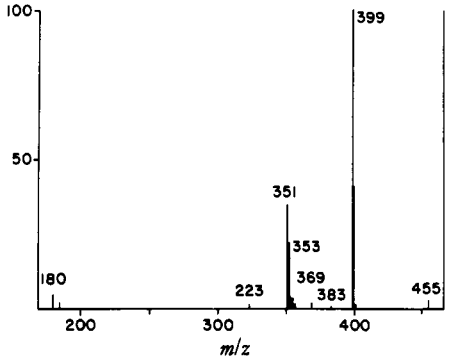

FIGURE 3. Thermospray mass spectrum of eluent from sfc proximate to desacetoxyvindoline peak.

In summary, the effectiveness of sfc-ms in obtaining useful information regarding the numbers and types of monoterpene indole alkaloids present in a complex mixture was demonstrated by the analysis of the leaf alkaloids of *C. roseus*. In principle, this system should prove equally effective in the analysis of indole alkaloids from other species of plants and/or plant cell cultures. Among the benefits noted were high resolution, short run times, and the production of high quality mass spectra.

EXPERIMENTAL

INSTRUMENTATION.—Sfc analyses were performed with a Hewlett-Packard Model 1084B liquid chromatograph modified for sfc (8). An amino-bonded 5 um Spherisorb 100 × 4.5 mm column was used with CO_2 and a 5–15% gradient of MeOH polar modifier as the mobile phase. Eluting peaks were detected at a wavelength of 290 nm. The column temperature was 65°. Electron impact sfc-ms was performed by coupling the chromatograph to a VG model 70/70 mass spectrometer equipped with a moving belt hplc-ms interface. The effluent was deposited on the moving belt by using a Finnigan MAT spray deposition device connected in-line via a "T" piece between the uv detector exit and the back-pressure regulator of the chromatograph (6). The belt speed was 3 cm/sec; the nosetip heater temperature was 250°. The mass spectrometer was scanned from mass 850 to 85 every 2 sec. Thermospray sfc-ms was performed by coupling the chromatograph to a Finnigan MAT model 4000 gc-ms equipped with a modified Finnigan MAT thermo-

spray hplc-ms interface operated in the filament-on mode (9). The thermospray tip temperature was 160°. The mass spectrometer was scanned from mass 150 to 850 every 2 sec.

EXTRACTION AND IDENTIFICATION.—Indole alkaloids were obtained from *C. roseus* plants (commercial variety roseus) by extracting fresh leaves (100 g) with MeOH (400 ml) in a Waring blender. The MeOH solution was separated from the leaves by filtration and concentrated in vacuo. The residue was partitioned between Et_2O (50 ml) and 1 N HCl (100 ml). The aqueous layer was separated, made basic (pH 7.5) with 10 N NaOH, and extracted with EtOAc (2 × 50 ml). The combined EtOAc portion was dried (Na_2SO_4) and the solvent removed in vacuo to yield the leaf alkaloids (240 mg) in free base form as an amorphous tan-colored solid. Although analyses were performed on alkaloids in free base form, they were susceptible to autoxidation and were stored as the HCl salt. Alkaloids were identified by comparison of ei mass spectra obtained via sfc-ms with ei mass spectra previously obtained from authentic samples available from our reference collection.

2D tlc analyses were performed with Whatman K5F 250 μm 20 × 20 cm Si gel plates. The solvents employed were EtOAc-MeOH (4:1) and CH_2Cl_2-MeOH (12:1). Preparative separations of alkaloids were carried out via sequential cc on alumina and Si gel employing as eluent EtOAc-hexane (1:2), EtOAc, or EtOAc-MeOH (4:1) as required.

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