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Note

Gas chromatographic–mass spectrometric analysis of major indole alkaloids of *Catharanthus roseus*

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During the 1980s, a considerable amount of research has been devoted to studies of the production of medicinally useful indolic alkaloids in cell and tissue cultures of *Catharanthus roseus*[1]. The principal targets of this research are vincristine and vinblastine, which are used in cancer chemotherapy and serve as precursors for some semisynthetic derivatives[2]. The above bisindole alkaloids are biosynthesized by combining catharanthine and vindoline (Fig. 1), which belong to the *Iboga* and *Aspidosperma* groups of monoterpene indole alkaloids, respectively. The third related alkaloid group abundant in *C. roseus* is the *Corynanthé* family, of which ajmalicine is the most abundant compound[3]. In the plant *Aspidosperma* alkaloids exist predominantly in the green parts, whereas the other types are also found in the roots[4], and it seems that only trace amounts of the end products of the *Aspidosperma* pathway are found in the cell and tissue cultures[5]. Consequently, screening of product yield from the different pathways is one of the basic routines included in these studies.

As the compounds are polar and not very volatile, high-performance liquid chromatographic (HPLC) methodology has predominated[6]. The regular target compounds catharanthine, vindoline and ajmalicine may be analysed simultaneously by using gradient elution and UV detection in HPLC[4]. However, this approach is not particularly sensitive and although electrochemical detection has improved sensitivity[7], it does not tolerate a true gradient elution and only a few alkaloids are properly

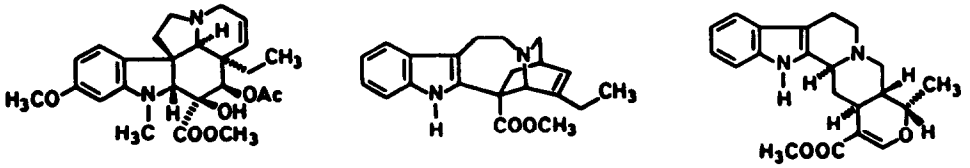

Fig. 1. Structures of the major indole alkaloids of *Catharanthus roseus*.

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quantified per isocratic run. A special problem is the separation of vindoline from ajmalicine and some other compounds that tend to co-migrate.

The commercial availability of an extensive choice of stationary phases in fused-silica capillary gas chromatographic (GC) columns has greatly enhanced the scope of the GC analysis of a variety of drugs and natural compounds, including amines and alkaloids[6,8,9]. This, and the observation that vindoline appeared to be analysable also by using packed-column GC[10], led us recently to try to detect it by capillary GC. We were able to identify it from a plant cell sample using GC–mass spectrometry (MS) after careful purification[5]. This prompted us to develop a GC–MS method for the simultaneous direct detection of vindoline and ajmalicine and for the detection of catharanthine as a pentafuoropropionate derivative from *C. roseus* cells after a simple purification procedure.

EXPERIMENTAL

Preliminary GC experiments with fused-silica capillary columns (OV-1, 15 and 25 m × 0.32 mm I.D., 0.1-μm coating; Nordion Instruments, Helsinki, Finland) were carried out with a Hewlett-Packard HP 5790A instrument with flame-ionization detection (FID). The final GC–MS was performed using the longer of the columns and an HP 5790A–Jeol JMS 300 integrated system. Splitless (60 s) injection was used, and the oven temperature was programmed from the initial 130°C (2 min) to 280°C at 10°C/min with injector and detector temperatures of 250 and 300°C, respectively. The MS results were processed with the JMA 2000 data analysis system included in the instrument.

Trials on the silylation and acylation of catharanthine were performed under a wide range of reaction conditions using the following reagents: N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide, N,O-bis(trimethylsilyl) trifluoroacetamide, N-methyl-N-trimethylsilyl trifluoroacetamide, N-trimethylsilylimidazole, pentafluoropropionic anhydride (PFPA), methyl chloroformate, heptafluorobutyric anhydride and trifluoroacetic anhydride. The final reproducible acylation used was achieved when a sample was dissolved in 500 μl of toluene and 50 μl of pyridine and 50 μl of PFPA were added. The reaction mixture was then maintained at 60°C for 30 min and washed with 1 ml of 5 M ammonia solution. A 2-μl volume of the organic solution was used directly for GC–MS, which was performed with a combination of HP 5890 and VG Trio-2 instruments, but the conditions for the analysis were as described above for the underivatized alkaloids.

The *C. roseus* cell material used and the procedures for alkaloid purification have been described previously[11]. Analytical-reagent grade chemicals were obtained from the usual commercial sources and the alkaloid standards were those described earlier[4].

RESULTS AND DISCUSSION

Preliminary GC experimentation with standard samples of ajmalicine, vindoline and catharanthine using FID indicated that the first two are volatile and detectable under the conditions specified above (data not shown), but catharanthine gave no analytically useful signal under any conditions. Subsequently, ajmalicine and vin-

doline standards were subjected to GC–MS to obtain the mass spectra (not shown) that were utilized in analysing the plant cell samples.

It was essential to use a thin (0.1-μm) stationary phase film in order to keep these relatively involatile analytes moving in the column at reasonable temperatures and to achieve comparatively good separations and peak shapes. The use of helium instead of nitrogen as the carrier gas was also beneficial, as the poorer diffusibility of the latter required strict control of the gas stream.

Fig. 2 shows a typical semipreparative HPLC trace when alkaloids are isolated from cultured *C. roseus* cells using the previously described method[11]. When a narrow middle fraction of eluate from the peak with a retention time of 17 min was analysed by GC–MS, in essence a single total ion current (TIC) peak was observed at 17 min 40 s (Fig. 3) and its mass spectrum (Fig. 4A) was almost identical with that obtained for a standard sample of ajmalicine. There was no distinct HPLC peak at 16 min, where vindoline should appear (Fig. 2), but when the eluate of the zone indicated in Fig. 2 was collected and the existence of the molecular ion for vindoline (*m/z* 456) was monitored, it could be seen in the gas chromatogram at 16 min 15 s (Fig. 3), and the mass spectrum (Fig. 4B) corresponded to that of standard material[5]. As would be expected from the wide sampling range used for vindoline and from the high baseline of the HPLC trace (Fig. 2), the sample contained various other compounds, including ajmalicine, as indicated by the TIC graph (Fig. 3).

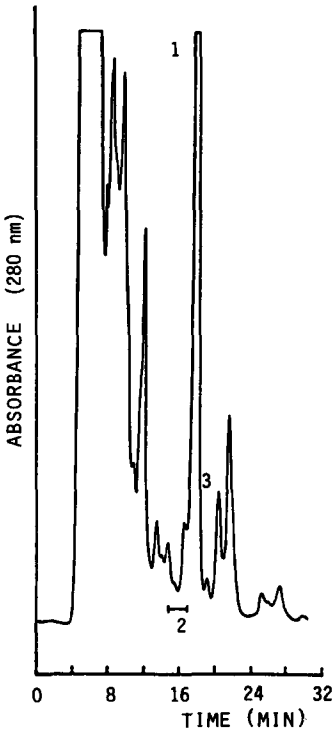

Fig. 2. Semi-preparative HPLC of *C. roseus* cell culture extract. The eluate constituents used for GC–MS analysis of ajmalicine (1), vindoline (2) and catharanthine (3) are indicated.

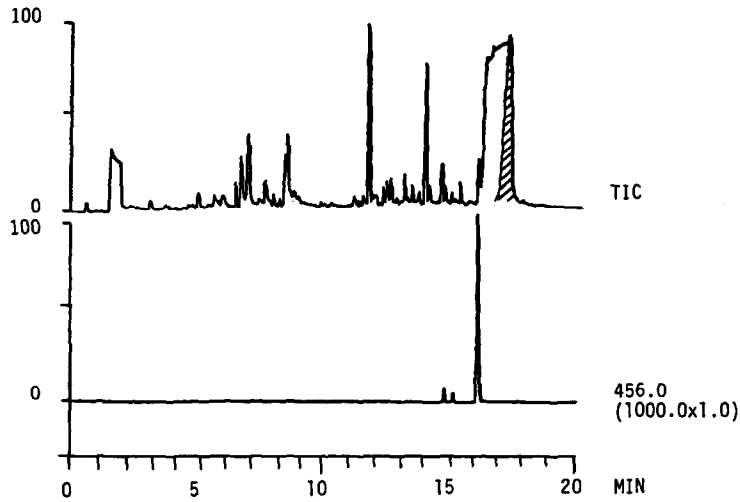

Fig. 3. Top: superimposed TIC GC–MS traces from ajmalicine (shaded area) and vindoline zones of the HPLC trace in Fig. 2. Bottom: ion monitoring trace indicating the existence of vindoline molecular ions (m/z 456) in the vindoline zone of Fig. 2.

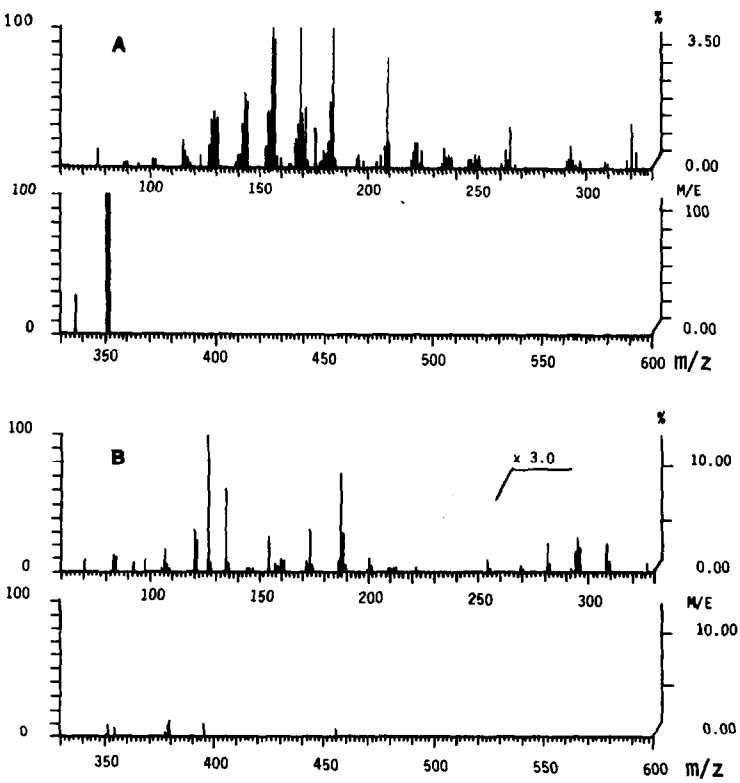

Fig. 4. Mass spectra of (A) the ajmalicine and (B) the vindoline peaks indicated in Fig. 3.

As the direct GC analysis of catharanthine was unsuccessful, a method for preparing a volatile derivative was developed as described under Experimental. When an aliquot of sample eluate from the HPLC (peak at 21 min (Fig. 2) was processed accordingly, all the TIC peaks present in previous standard runs were present (Fig. 5A). Peak 1 at 12 min 40 s represents the main PFP derivative, for which the mass spectrum is given in Fig. 5B. The smaller peak 2 preceding the main one at 12 min 10 s in Fig. 5A is obviously due to a stereoisomeric PFP derivative, as it gives a mass spectrum identical with that in Fig. 5B. The other TIC peaks in Fig. 5A were also seen in standard graphs, and their mass spectra suggest that they are related to the derivatization reagents.

As indicated by the complexity of the TIC graph in Fig. 3, a vast number of compounds could be resolved by GC from a relatively narrow zone of the HPLC eluate. Comparable complexity was also observed for other eluate portions when they were screened by GC. The mass spectra of many of the TIC peaks in Fig. 3 and in the

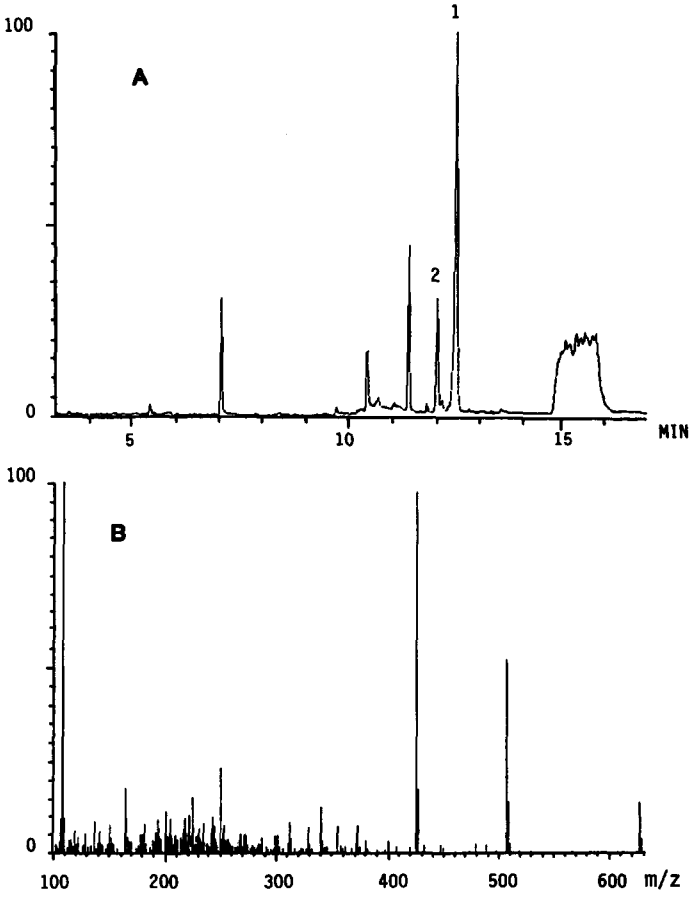

Fig. 5. (A) TIC GC–MS trace after PFP derivatization of the catharanthine zone in Fig. 2 and (B) the common mass spectrum obtained for peaks 1 and 2.

graphs from other eluate portions suggested them to be related to the target alkaloids, but the lack of reference compounds prevented their further identification.

Although the example of catharanthine revealed that direct GC–MS may not be considered a universal method for analysing indole alkaloids, we conclude that according to the present results many such alkaloids may be added to the list of amines and alkaloids[6,8,9] that can be analysed by modern capillary GC techniques.

ACKNOWLEDGEMENTS

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