Journal of Natural Products
Vol. 54, No. 6, pp. 1558-1563, Nov-Dec 1991

CAPILLARY GAS CHROMATOGRAPHIC ANALYSIS OF INDOLE ALKALOIDS: INVESTIGATION OF THE INDOLE ALKALOIDS PRESENT IN *TABERNAEMONTANA DIVARICATA* CELL SUSPENSION CULTURE

DENISE DAGNINO,* JAN SCHRIPSEMA, ANJA PELTENBURG, ROBERT VERPOORTE,

Biotechnology Delft Leiden, Project Group Plant Cell Biotechnology, Division of Pharmacognosy, Center for Bio-Pharmaceutical Sciences, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands

and KEES TEUNIS

Department of Organic Chemistry, Wageningen Agricultural University, Dreijenplein 8, 6703 HB Wageningen, The Netherlands

ABSTRACT.—A capillary gas chromatographic analysis is described by which non-derivatized indole alkaloids and indole-related compounds can be separated and identified when the system is coupled to a mass spectrometer. By the use of this analysis some phenolics and sterols could also be separated. The phenolics coniferyl alcohol and sinapyl alcohol and the sterols campesterol and stigmasterol were identified in *Tabernaemontana divaricata* cell suspension cultures.

Several methods are available for the analysis and identification of known indole alkaloids. Analysis of complex mixtures is frequently done by tlc, through comparison of R_f values in different solvent systems, and by comparison of specific color reactions of components of the mixture with reference compounds (1). Tlc remains one of the preferred methods for qualitative analysis of known compounds since it requires neither sophisticated equipment nor extensive sample preparation.

For quantitative analysis, hplc systems linked to a uv detector are commonly used (2). Coupling to a photodiode array uv detector makes it possible to combine the information over retention times and the uv spectrum of each compound and in some cases it also enables the quantification of overlapping peaks.

Capillary gc analysis has been described for several classes of alkaloids. A major advantage of gc over the above-mentioned methods is its enhanced sensitivity and high resolution. Another is its easy coupling to a mass spectrometer, which allows the identification of new and minor compounds of a mixture without laborious isolation procedures, which makes it a particularly attractive method when no decomposition due to the high temperatures applied in gc occurs.

The number of articles describing capillary gc analysis of underivatized alkaloids is continuously increasing. The number of references of underivatized pyrrolizidine alkaloids by far exceeds the one found for other classes of alkaloids. With pyrrolizidine alkaloids, gc is used alone (3) or in combination with ms (4–6) and Ft-ir spectroscopy (7) to quantify and elucidate the structure of new compounds. The analysis of tropane alkaloids (8,9), steroidal alkaloids (10,11), quinazoline alkaloids (12), *Lupine* alkaloids (13), diterpenoid alkaloids (14), and *Lycopodium* alkaloids (15) has been described.

A more recent method is the use of supercritical fluid chromatography (sfc) coupled to a mass spectrometer (ms) (16). Sfc-ms seems promising for the future because of its high resolution and the relatively high stability of compounds under sfc conditions; it also allows the identification of new compounds. Wide application of the method is now limited by the unavailability of the necessary apparatus.

One of the first attempts to separate underivatized indole alkaloids by gc was made by Lloyd *et al.* (17) in 1960 using a packed column. Many other published reports have been reviewed by Verpoorte and Baerheim Svendsen (18). More recently a method has

been described for the separation and identification of vindoline and ajmalicine by capillary gc-ms (19).

In our laboratory the alkaloid extracts of *Tabernaemontana divaricata* (L.) R. Br. ex Roem. et Schult. (Apocynaceae) cell suspension cultures are routinely analyzed by hplc coupled to a photodiode array (2). By this method the major compounds of the alkaloid extract of *T. divaricata*, usually *O*-acetylvallesamine, voaphylline, and apparicine, can be quantified. We are now interested in a more detailed investigation of the biosynthesis and catabolism of these compounds. Considering the above-mentioned advantages of capillary gc over the other techniques it seemed interesting to develop a method to separate complex mixtures of indole alkaloids. This paper describes a capillary gc analysis of underivatized indole alkaloids.

EXPERIMENTAL

MEDIUM EXTRACT.—Culture medium was harvested by filtration through a Miracloth filter from a 14-day-old culture of *T. divaricata* maintained in Murashige and Skoog medium (20) without growth regulators. The medium (pH 5.2) was extracted with CH_2Cl_2 (21). The extract was redissolved in MeOH and used for gc, gc-ms, and hplc analysis.

GC AND GC-MS ANALYSIS.—Gc analysis was performed in a Packard 438A gas chromatograph equipped with a fused silica CP Sil 5 cb capillary column (10 m × 0.22 mm i.d., film thickness 0.13 μm, Chrompack) and with a flame ionization detector (fid). N_2 was used as carrier gas (50 kPa), and the injection split ratio was 1 to 50. The injection temperature was 220°. Column temperature was programmed to rise from 100° to 175° at 15°/min and then to 230° at 5°/min, this temperature being held for 15 min. The detector temperature was 240°. The integrator used was a Shimadzu C-R3A Chromatopack.

Gc-ms data were obtained on a Packard model 438A gas chromatograph equipped with a fused silica CP Sil 5cb capillary column (10 m × 0.22 i.d., film thickness 0.13 μm) and interfaced with a Finnigan MAT 700 Ion Trap detector (ITD, software version 3.0). The temperature program used was the same as described above. He was used as a carrier gas (100 kPa). The transfer line temperature was 250°. The scan range was 40–449 u and the scan time 1 sec.

A Hewlett-Packard 5970B MSD combined with an HP 5890A gas chromatograph was also used. Gc conditions were similar to those described above.

HPLC-UV ANALYSIS.—Hplc analysis was carried out as described previously (2).

RESULTS AND DISCUSSION

The efficiency of the gc system in separating alkaloids was tested by injecting authentic samples. Compounds injected include various classes of indole alkaloids with mol wt varying from 264 (apparicine) to 704 (conoduramine). Quinoline alkaloids were also injected to test the system's ability to separate indole-alkaloid-related compounds.

Table 1 lists the retention times obtained for the alkaloids injected. These varied from 12 to 50 min. No further attempts were made to decrease these retention times; the elution of the main compounds of interest had been achieved and we wished to minimize the chance of artifact formation due to high analysis temperatures. To confirm that the peaks observed in the chromatograms corresponded to the compound injected, gc-ms analysis of some of the compounds was carried out. Table 1 indicates the compounds whose identity was confirmed through gc-ms.

As expected, not all compounds injected were analyzable under these conditions. In particular the dimeric indole alkaloid conoduramine (MW 704) and the quaternary indole alkaloid serpentine could not be detected after injection. Also reserpiline had an exceptionally high retention time (50 min). Although its structure was not confirmed by ms, the compounds of the same and related biosynthetic classes ajmalicine and yohimbine had the next highest retention times (26.3 and 26.1 min, respectively). Cinchonine and cinchonidine normally present in *Cinchona* extracts could not be separated under these conditions. For the indole alkaloids tested a nearly base line separation was obtained, and the system was thus considered suitable for testing extract sam-

TABLE 1. Retention Times of Indole and Related Alkaloids.

Compound	Biosynthetic class	MW	Rt[a]	Mass spectra[b]
Ajmalicine	C2	352	26.3	y[d]
Appericine	A2	264	12.3	y
Aspidospermine	P2	354	15.4	y
Cinchonidine	—[c]	294	14.0	n
Cinchonine	—[c]	294	14.0	n
Conoduramine	C5-I1	704	—	—
Conopharyngine	I1	398	24.0	y
Coronaridine	I1	338	14.8	y
10-Hydroxycoronaridine	I1	354	20.5	n
Corynantheal	C1	294	14.2	n
Dregamine	C5	354	20.4	n
Ibogaine	I1	310	18.1	y
Ibogaline	I1	340	22.8	y
Ibogamine	I1	280	14.4	y
Pericyclivine	C4	322	16.3	n
Perivine	C5	338	18.0	n
Quebrachamine	P1	282	12.1	y
Quinidine	—[c]	324	17.1	n
Quinine	—[c]	324	12.3	n
3-Isoreserpiline	C2	412	50.0	n
Serpentine	C2	348	—	—
Tabersonine	P2	336	13.6	y
Tubotaiwine	A3	324	12.7	n
O-Acetylvallesamine	A2	382	18.2	y
Voaphylline	P1	296	14.6	y
Voacangine	I1	368	18.9	y
Vincamine	E1	354	16.1	y
Vobasine	C5	352	18.8	y
Yohimbine	C3	354	26.1	y

[a]Retention times are given in min.
[b]Structure confirmed by ms.
[c]Indole-related alkaloids.
[d]y = yes, n = no.

ples. Figure 1 illustrates the system's ability to separate a mixture of 13 indole alkaloids.

Figure 2 shows the chromatogram obtained by hplc analysis of the medium extract of *T. divaricata* cell suspension culture. Two main peaks can be distinguished, both having an indole chromophore. Previous analysis of the medium extract has shown that the peaks correspond to voaphylline and *O*-acetylvallesamine (22). The third compound known to be present in medium extracts, appericine, co-elutes with *O*-acetylvallesamine, but because of its different chromophore, its presence can easily be confirmed by uv detection at 310 nm.

Figure 3 shows a chromatogram obtained by gc analysis of the same extract. As expected from the results obtained by the injection of reference compounds, the gc system developed was able to separate in a short time (19 min) the indole alkaloids present in crude *T. divaricata* cell culture medium extracts. The good separation allowed also the detection of some minor alkaloids in the mixture; these were not detectable by hplc-uv because of their low concentration or overlap with the major peaks.

Gc-ms analysis of the extract confirmed the presence of voaphylline, appericine, and *O*-acetylvallesamine, the same compounds found in the hplc analysis of the extract (Figure 2). Besides these major components of the medium, mass spectra of 16 other

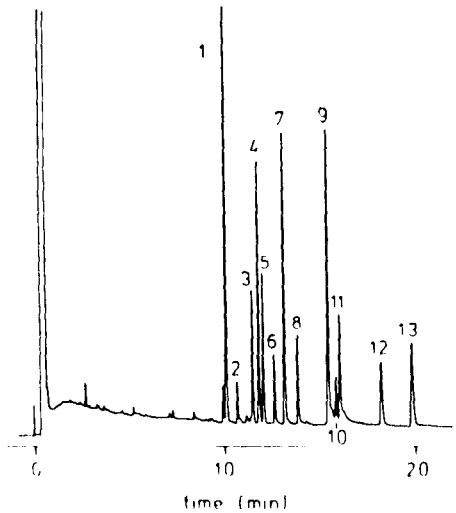

FIGURE 1. Gas chromatogram of a mixture of indole alkaloids: 1, apparicine; 2, tubotaiwine; 3, tabersonine; 4, ibogamine; 5, voaphylline; 6, coronaridine; 7, aspidospermine; 8, pericyclivine; 9, perivine; 10, vobasine; 11, voacangine; 12, ibogaline; and 13, ajmalicine. The column used was different from the one where the data from the table were obtained; gc conditions were the same as described in Experimental.

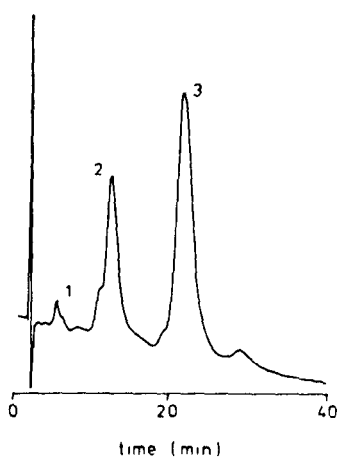

FIGURE 2. Hplc chromatogram obtained by detection with uv (280 nm) of a medium extract of *Tabernaemontana divaricata* cell suspension culture showing a mixture of coniferyl and sinapyl alcohol (1), voaphylline (2), apparicine (3), and *O*-acetylvallesamine (3) as main components of the mixture. The peak of apparicine can be distinguished at 310 nm.

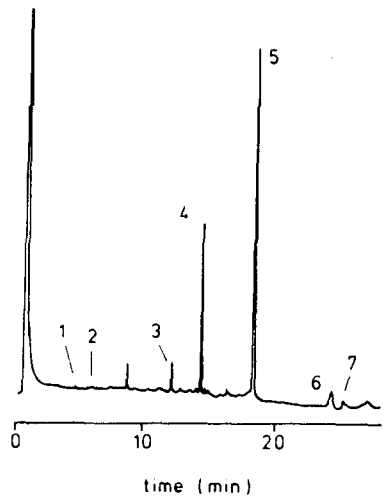

FIGURE 3. Gas chromatogram of a medium extract of *Tabernaemontana divaricata* cell suspension culture. Coniferyl alcohol (1), sinapyl alcohol (2), appariçine (3), voaphylline (4), *O*-acetylvallesamine (5), campesterol (6), and stigmasterol (7) have been identified.

minor indole alkaloids were obtained, many of them showing fragmentation patterns similar to those of the main components. The identification of the minor alkaloids is at present under way.

A comparison of the gc and hplc chromatograms indicates that the same ratio of areas of the compounds are obtained with both analytical methods, although accurate quantifications through gc can only be obtained by determining the correction factor for each of the components of the mixture. The response of the fid for *O*-acetylvallesamine was investigated and was shown to be linear between 1 and 1000 pmol.

By further analysis of the mass spectra it was possible to identify two phenolics, coniferyl alcohol and sinapyl alcohol, whose chromophore had already been detected in the hplc analysis of the extract (Rt 6 min). The sterols campesterol and stigmasterol, which were transparent to the uv detection used, were also detected by gc-ms. The identity of these compounds was confirmed by injection of reference compounds.

The applicability of capillary gc for the separation and identification of crude mixtures of indole alkaloids has thus been demonstrated.

ACKNOWLEDGMENTS

We thank the Fundação BIO-RIO through which an extension of the grant from the Brazilian National Council of Research (CNPq) to Denise Dagnino was made possible.

LITERATURE CITED

1. T.A. van Beek, R. Verpoorte, and A. Baerheim Svendsen, *J. Chromatogr.*, **298**, 289 (1984).
2. R. van der Heijden, P.J. Lamping, P.P. Out, R. Wijnsma, and R. Verpoorte, *J. Chromatogr.*, **396**, 287 (1987).
3. E. Roeder and V. Neuberger, *Dtsch. Apoth. Ztg.*, **39**, 1991 (1988).
4. C. Bicchi, A. D'Amato, and E. Cappelletti, *J. Chromatogr.*, **349**, 23 (1985).
5. C. Bicchi, R. Caniato, R. Tabacchi, and G. Tsoupras, *J. Nat. Prod.*, **52**, 32 (1989).
6. L.A. Pieters, T. Hartmann, J. Janssens, and A.J. Vlietinck, *J. Chromatogr.*, **462**, 387 (1989).
7. C. Bicchi, P. Rubiolo, and C. Frattini, *J. Chromatogr.*, **473**, 161 (1989).
8. M. Ylinen, T. Naaranlahti, S. Lapinjoki, A. Huhtikangas, M.L. Salonen, L.K. Simola, and M Lounasmaa, *Planta Med.*, **52**, 85 (1986).

9. L. Witte, K. Mueller, and H.A. Arfmann, *Planta Med.*, **53**, 192 (1987).
10. W.M.J. van Gelder, *J. Chromatogr.*, **331**, 285 (1985).
11. W.M.J. van Gelder, H.H. Jonker, H.J. Huizing, and J.J.C. Scheffer, *J. Chromatogr.*, **442**, 133 (1988).
12. I. Laakso, P. Virkajarvi, H. Airaksinen, and E. Varis, *J. Chromatogr.*, **505**, 424 (1990).
13. C.R. Priddis, *J. Chromatogr.*, **261**, 95 (1983).
14. G.D. Manners and M.H. Ralphs, *J. Chromatogr.*, **466**, 427 (1989).
15. R.V. Gerard and D.B. MacClean, *Phytochemistry*, **25**, 1143 (1986).
16. J. Balsevich, L.R. Hogge, A.J. Berry, D.E. Games, and I.C. Mylchreest, *J. Nat. Prod.*, **51**, 1173 (1988).
17. H.A. Lloyd, H.M. Fales, P.F. Highet, W.J.A. VandenHeuvel, and W.C. Wildman, *J. Am. Chem. Soc.*, **82**, 3791 (1960).
18. R. Verpoorte and A. Baerheim Svendsen, "Chromatography of Alkaloids, Part B," Journal of Chromatography Library, Vol. 23B, Elsevier, Amsterdam, 1984, Chapter 17.
19. M. Ylinen, P. Suhonen, T. Naaranlahti, S.P. Lapinjoki, and A. Huhtikangas, *J. Chromatogr.*, **505**, 429 (1990).
20. T. Murashige and F. Skoog, *Physiol. Plant.*, **15**, 473 (1962).
21. J. Schripsema and R. Verpoorte, *Planta Med.*, in press.
22. R. van der Heijden, A. Hermans-Lokkerbol, R. Verpoorte, and A. Baerheim Svendsen, *J. Chromatogr.*, **396**, 410 (1987).

Received 18 March 1991