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QUANTITATIVE GAS CHROMATOGRAPHY AND GAS CHROMATO-GRAPHY–MASS SPECTROMETRY OF *CEPHALOTAXUS* ALKALOIDS

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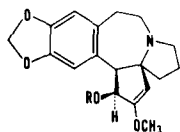
SUMMARY

Plants of the genus *Cephalotaxus* contain many alkaloids, some of which have demonstrated antitumor activity. Analysis of crude alkaloid mixtures by gas chromatography provides quantitation of the active principles and other, non-active, alkaloids. Mass spectrometry is used to identify known alkaloids in extracts and to confirm the presence of previously unknown ones. Such data provide a means for predicting the biological activity of new plant accessions.
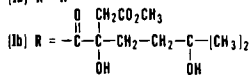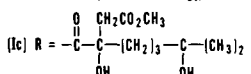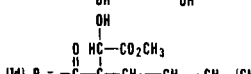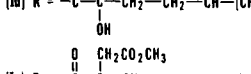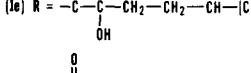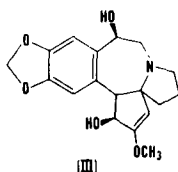
INTRODUCTION

Alkaloids of the genus *Cephalotaxus*, especially esters of cephalotaxine (Ia), have aroused interest since they have exhibited antitumor activity[1,2]. These active esters have been identified as harringtonine (Ib), homoharringtonine (Ic), isoharringtonine (Id) and deoxyharringtonine (Ie). Other alkaloids found in *Cephalotaxus* extracts include acetylcephalotaxine (If), drupacine (II), 11-hydroxycephalotaxine (III), cephalotaxinone (IV), desmethylcephalotaxinone (V)[1-8] and seven alkaloids of the homoerythrina series (VIa and b, VIIa and b, VIIIa and b, and IX)[9,10].

Quantitative analysis of the complex mixtures of alkaloids encountered in *Cephalotaxus* extracts has been based on the amounts of pure compounds that have been isolated. Correlation of antitumor activity with the proportions of alkaloids present in crude preparations made an accurate, rapid analytical method desirable. We report here a gas chromatographic (GC) method that will quantitate and identify almost all the compounds named, particularly the antitumor-active esters (Ib–Ie). If further confirmation is needed, mass spectrometry (MS) of the GC effluents verifies identifications made from retention data.
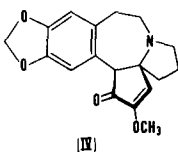
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* The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.
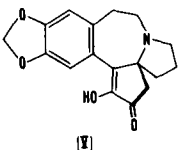
(Ia) R = H

(Ib) R = $-\overset{\overset{\displaystyle O}{\|}}{C}-\overset{\overset{\displaystyle CH_2CO_2CH_3}{|}}{\underset{\underset{\displaystyle OH}{|}}{C}}-CH_2-CH_2-\overset{\overset{}{}}{\underset{\underset{\displaystyle OH}{|}}{C}}-(CH_3)_2$

(Ic) R = $-\overset{\overset{\displaystyle O}{\|}}{C}-\overset{\overset{\displaystyle CH_2CO_2CH_3}{|}}{\underset{\underset{\displaystyle OH}{|}}{C}}-(CH_2)_3-\overset{}{\underset{\underset{\displaystyle OH}{|}}{C}}-(CH_3)_2$

(Id) R = $-\overset{\overset{\displaystyle O}{\|}}{C}-\overset{\overset{\displaystyle HC-CO_2CH_3}{|}}{\underset{\underset{\displaystyle OH}{|}}{C}}-CH_2-CH_2-CH-(CH_3)_2$

(Ie) R = $-\overset{\overset{\displaystyle O}{\|}}{C}-\overset{\overset{\displaystyle CH_2CO_2CH_3}{|}}{\underset{\underset{\displaystyle OH}{|}}{C}}-CH_2-CH_2-CH-(CH_3)_2$

(If) R = $-\overset{\overset{\displaystyle O}{\|}}{C}-CH_3$

(III)

(IV)

(V)
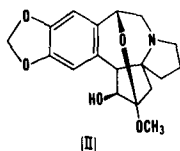
(II)
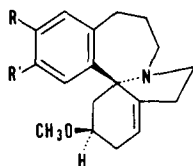
(VIa) R = OCH$_3$; R' = OH
(VIb) R = R' = OCH$_3$
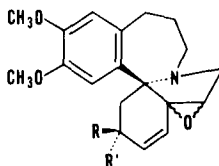
(VIIIa) R = OCH$_3$; R' = H
(VIIIb) R = H; R' = OCH$_3$
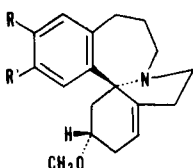
(VIIa) R = OCH$_3$; R' = OH
(VIIb) R = R' = OCH$_3$
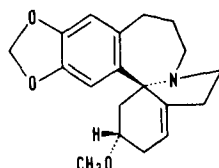
IX

EXPERIMENTAL

Materials

Extractions and preliminary workup of the crude alkaloid extracts have been previously described[7,11]. Pure samples taken from earlier characterization were used

to determine chromatographic response. Methyl lignocerate ($C_{24:0}$) was purchased from Nu-Chek-Prep (Elysian, Minn., U.S.A.) and Regisil RC-1 bis(trimethylsilyl)trifluoroacetamide from Regis Chemical (Chicago, Ill., U.S.A.). The pyridine was redistilled and stored over barium oxide.

Gas chromatography

The gas chromatograph, a Bendix 2600, was equipped with dual, on-column flame ionization detectors. Glass columns (4 ft.) were packed with 3% Dexsil 300 on 100–120 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). GC operating conditions were: inlet heater 245°, detector bath 290°, column oven temperature programmed from 180° to 285° at 2°/min. Analog signals from the electrometers were sampled and digitized by an auto-ranging analog-to-digital converter (Systems Instruments Research) interfaced to Nova 1210 minicomputer (Data General). Collected data were transmitted to a Modcomp II (Modular Computer Systems) computer for determination of peak areas and retention times, further processing and output. Components were quantitated by using the appropriate response factor and the internal standard method[11].

Preparation of alkaloids for gas chromatography

By pipette, 1 ml of internal standard solution (0.50 mg $C_{24:0}$ per ml of hexane) was transferred to a 1/2-dram screw-cap vial. The hexane was removed under a stream of dry nitrogen. About 5 mg of crude alkaloid extract was accurately weighed into the vial and dissolved in a few drops of pyridine. After 150 μl of Regisil RC-1 was added, the solution was heated at 60° for 1 h. Samples (3–5 μl) were then injected into the GC.

Chromatographic response

Chromatographic response factors were determined by analysis of stock solutions of Ia, Ib, Ic, Id, II, III, VIIIa and the internal standard, $C_{24:0}$, in known concentrations. These solutions were prepared so that the relative concentration of each alkaloid to $C_{24:0}$ ranged from 3:1 to 1:3 (roughly 1.5–0.2 mg of alkaloid). Responses were linear over these ranges.

Gas chromatography–mass spectrometry

For GC–MS, the effluent from the chromatograph was directed to a Dupont CEC 21-492-1 mass spectrometer through a jet-type sample enricher.

Spectra were obtained with an ionizing energy of 70 eV, a source temperature of 200°, a source pressure of $1 \cdot 10^{-6}$ torr and a resolution of 1000. Scans were made from m/e 40–700 at 4 sec per decade and were made either on the GC peaks or in an automatically repeating mode at rates up to one every 8 sec. Ion intensities were measured by ion counting techniques. Individual current pulses from the electron-multiplier tube resulting from an ion striking the first dynode were counted on an SSR Model 1120 Amplifier discriminator (Princeton Applied Research) and a 16-bit, 120-MHz counter. Data acquisition (at 5 kHz), peak determination and peak area calculation were controlled by the previously mentioned Nova computer system. The data system assigned the m/e values for the peaks with an electronic mass marker.

TABLE I

CHROMATOGRAPHIC DATA FOR *CEPHALOTAXUS* ALKALOIDS

Peak numbers refer to Fig. 1.

Peak No.	Compound	RRT	Response factor
1	Cephalotaxine (Ia)	0.55	1.41
2	Homoerythrina alkaloids (VIa and b, VIIa and b and IX)	0.67	1.41[*]
3	Cephalotaxinone artifact[**]	0.71	—
4	Drupacine (II)	0.73	1.82
5	Acetylcephalotaxine (If)	0.79 ⎫	
	11-Hydroxycephalotaxine (III)	0.80 ⎬ 1.39[***]	
	Desmethylcephalotaxinone (V)	0.80 ⎭	
6	Homoerythrina alkaloids (VIIIa and b)	0.88	2.63
7	Internal standard ($C_{24:0}$)	1.00	1.00
8	Cephalotaxinone (IV)	1.05	—
9	Unidentified alkaloid	1.24	—
10	Deoxyharringtonine (Ie)	1.76	1.72[*]
11	Isoharringtonine (Id)	1.90	1.92
12	Harringtonine (Ib)	2.00	1.72
13	Homoharringtonine (Ic)	2.14	2.17

   [*] These factors arbitrarily assigned.

   [**] See text for explanation.

   [***] These three compounds not resolved; factor is for 11-hydroxycephalotaxine.

The data system plotted total intensity *versus* spectrum number and provided hard copies of individual normalized spectra.

RESULTS AND DISCUSSION

Chromatographic characteristics of the *Cephalotaxus* alkaloids are given in Table I. In Fig. 1, two typical chromatograms are shown: one of the standards used for response factor calculations (Fig. 1A) and one of a typical plant extract (Fig. 1B) from *C. harringtonia*. It can be seen from Table I and Fig. 1 that almost all the compounds are separated from one another and, particularly, that the biologically active esters are completely resolved under these conditions. GC analysis must be carried out directly after 60 min of heating since solutions allowed to stand 4 or more hours tend to give lower responses for the esters.

The homoerythrina alkaloids are, eluted in two groups: one with relative retention time (RRT) = 0.67 composed of VIa and b, VIIa and b, and IX, and the other with RRT = 0.88 composed of VIIIa and b. Since pure samples of these alkaloids were not available for response determinations, they were assigned a factor equal to that of cephalotaxine. We also did not have enough pure deoxyharringtonine to determine its response, so it was assigned the same response as harringtonine.

Another set of components that exhibit insufficient differences in retention to be resolved are III, If and V (RRT = 0.80). The response factor calculated for III was applied to this peak. Cephalotaxinone (IV) gives rise to only one peak (RRT = 1.05) before treatment with Regisil RC-1. After silylation, however, another peak (a cephalotaxinone artifact, which apparently results from enolization of the ketone
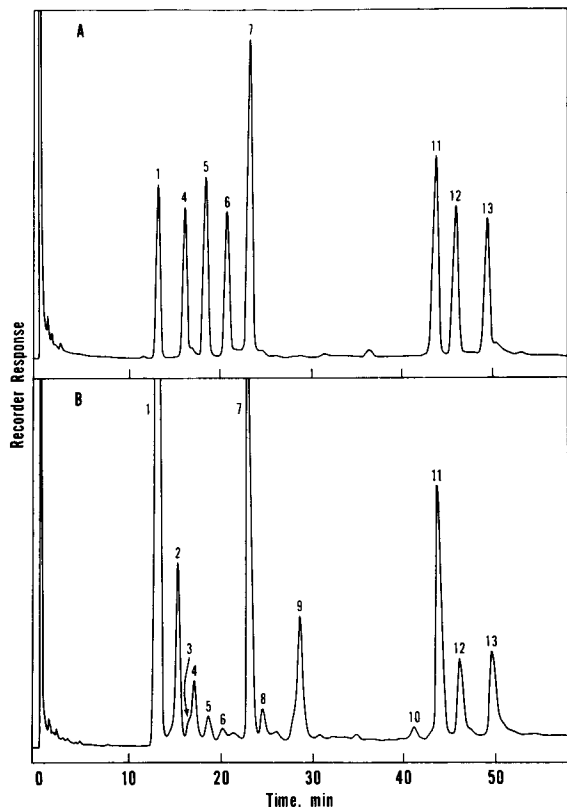
Fig. 1. Chromatograms of standards (A) and a plant extract (B). Peaks are due to: 1, cephalotaxine (Ia); 2, homoerythrina alkaloids (VIa and b, and VIIa and b); 3, cephalotaxinone artifact; 4, drupacine (II); 5, 11-hydroxycephalotaxine (III); 6, homoerythrina alkaloids (VIIIa and b); 7, internal standard ($C_{24:0}$); 8, cephalotaxinone (IV); 9, unidentified alkaloid; 10, deoxyharringtonine (Ie); 11, isoharringtonine (Id); 12, harringtonine (Ib); 13, homoharringtonine (Ic).

TABLE II

GC ANALYSES OF SELECTED *CEPHALOTAXUS* ALKALOID EXTRACTS

| Species | Plant part | Percentage of total alkaloids | | | | | | | |
		Ia	Ib	Ic	Id	Ie	II	III	VIIIa and b
harringtonia var. *drupacea**	Seed	33	8.3	1.2	3.9	0.7	35	9.6	6.5
harringtonia var. *harringtonia***	Root	39	6.6	7.5	19	2.4	3.7	1.1	3.4
harringtonia var. *harringtonia*	Leaf	32	2.4	4.1	13	1.3	5.3	2.6	2.9
harringtonia var. *harringtonia*	Whole plant	45	3.7	8.5	20	1.5	3.7	2.1	2.2
*fortunei****	Leaf	64	0.3	0.1	0.3	—	6.8	1.4	4.2
fortunei	Seed	52	4.4	Trace	0.9	0.4	7.0	2.2	4.6
wilsoniana§	Seed	29	6.1	0.2	0.4	—	0.7	0.9	45
griffithii§§	Root-leaf	40	0.9	1.5	11	4.3	9.0	2.5	1.7

    * Collected in Italy, 1962.

    ** Collected in Maryland, September 1968. Entire plants were collected in Oregon, 1970 (cv. Fastigiata).

    *** Leaf sample from Pennsylvania, 1968. Seed sample from Italy, October 1969.

    § Collected in Taiwan, 1970.

    §§ Collected in India, October, 1972.

and subsequent silylation of the hydroxyl group), appears as a shoulder on the drupacine peak (Fig. 1). For this reason and because it is a relatively minor component, we only report the presence of cephalotaxinone and did not attempt to quantitate it. If quantitation of cephalotaxinone is desired, the alkaloid mixture should be analyzed before silylation. The presence of the artifact introduces a small error for drupacine.

When some typical GC analyses are summarized (Table II), with few exceptions, Ia is the predominant alkaloid. *C. harringtonia* var. *drupacea* may also contain substantial amounts of II, whereas *C. wilsoniana* is particularly rich in homoerythrina alkaloids VIIIa and VIIIb. *C. harringtonia* var. *harringtonia* cv. Fastigiata is consistently rich in the antitumor active esters Ib–Ie.

To study the analytical method further, to help identify components in unresolved mixtures and to obtain data for new compounds —such as the one in

TABLE III

PERTINENT IONS IN THE MASS SPECTRA OF DERIVATIZED (TMS) *CEPHALOTAXUS* ALKALOIDS

Peak numbers refer to Fig. 1. Intensities of ions are in parentheses.

Compound	Peak No.	M^+		$(M - 15)^+$		Other ions
Cephalotaxine (Ia)	1	387	(56)	372	(30)	356 (64), 298 (76), 266 (32), 238 (29), 229 (28), 150 (32), 137 (23), 73 (100).
Drupacine (II)	4	403	(16)	—		262 (74), 228 (18), 172 (32), 159 (14), 154 (30), 138 (12), 103 (62), 73 (100).
11-Hydroxycephalotaxine (III)	5	475	(8)	460	(5)	386 (10), 372 (10), 359 (20), 316 (7), 313 (5), 295 (5), 214 (18), 75 (58), 73 (100).
Desmethylcephalotaxinone (V)	5	371	(100)	356	(43)	343 (13), 329 (12), 320 (22), 314 (19), 254 (22), 78 (11), 75 (41), 73 (64).
Cephalotaxinone artifact	3	385	(32)	370	(100)	354 (8), 298 (2), 296 (6), 281 (3), 75 (20), 73 (44).
Harringtonine (Ib)	12	675	(5)	—		314 (2), 299 (17), 298 (100), 282 (2), 266 (5), 243 (3), 150 (8), 131 (14), 73 (14).
Homoharringtonine (Ic)	13	689	(3)	—		314 (3), 299 (20), 298 (100), 282 (3), 266 (4), 201 (3), 150 (8), 131 (12), 73 (18).
Deoxyharringtonine (Ie)	10	587	(6)	—		314 (3), 299 (23), 298 (100), 266 (9), 159 (3), 150 (10), 141 (3), 75 (4), 73 (18).
Isoharringtonine (Id)	11	675	(5)	—		314 (2), 299 (19), 298 (100), 282 (4), 234 (5), 150 (11), 131 (21), 75 (4), 73 (23).
Homoerythrina alkaloid (VIIa)	2	387	(34)	372	(7)	385 (11), 356 (30), 329 (100), 298 (15), 196 (16), 178 (78), 165 (36), 146 (17), 73 (25)
Homoerythrina alkaloid (VIa)	2	387	(37)	372	(7)	385 (10), 356 (35), 329 (86), 298 (6), 240 (20), 178 (100), 165 (47), 146 (17), 73 (26)

Fig. 1B— mass spectra were taken of the derivatized alkaloids (Table III). Spectra of alkaloids If, IV, VIb, VIIb and IX, which do not add trimethylsilyl (TMS) groups, have already been published[9,10]. We obtained nearly identical spectra of these compounds by GC–MS. Almost all the silylated alkaloids exhibited strong molecular ions and the loss of 15 atomic mass units (a.m.u.) typical for TMS derivatives. Each compound gave a unique spectrum, except for the homoerythrina epimers. Compounds VIb and VIIb had virtually identical spectra[9]. Spectra of the silylated epimers VIa and VIIa differ only slightly in ion intensities except for two characteristic ions: m/e 240 for VIa and m/e 196 for VIIa. Of all the compounds studied, only 11-hydroxycephalotaxine and the esters of cephalotaxine did not give intense molecular ions. The presence of two TMS groups in derivatized 11-hydroxycephalotaxine may reduce stability of the molecule, and although the esters of cephalotaxine did give weak molecular ions, no ions were detected at $(M — 15)^+$. Molecular ions observed in these spectra provide information about the nature of the ester side chains and demonstrate that derivatized alkaloids survive in GC. Spectra of the cephalotaxine esters all contain an intense ion at $m/e = 298$, which is formed by loss of the acyloxy group.

Mass spectra show that certain alkaloids which have similar retention times can be distinguished from others. Of the components eluted at RRT = 0.67, IX has a spectrum that differs from all the others. Compounds VIa and VIIa give similar spectra, but each has a characteristic ion. However, epimer pairs VIb and VIIb exhibit the same spectra. Therefore, the presence of a particular member of an epimeric pair cannot be determined with certainty by this method. By taking spectra of the GC effluent, we can establish the presence or absence of IX, either VIb (and/or VIIb) or VIa and VIIa in the unresolved peak. From the spectra reported here and the spectrum of acetylcephalotaxine[1], the components with RRT ≈ 0.80 can be likewise identified.

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