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QUANTITATIVE ANALYSIS OF DIHYDROERGOTOXINE ALKALOIDS BY GAS CHROMATOGRAPHY AND GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A rapid, sensitive and specific method for the analysis of the dihydroergotoxine alkaloids (dihydroergocornine, dihydroergocryptine and dihydroergocristine) by gas chromatography and gas chromatography–mass spectrometry is described. The method is based on the quantitative thermal decomposition of the compounds in the injection port of the gas chromatograph, using a 3% SE-30 column with a nitrogen detector. The sensitivity is about 1–10 ng. Gas chromatography–mass spectrometry was used for the structure elucidation of the thermal decomposition products of the dihydroergotoxine alkaloids and for the determination of these compounds at the picogram level.

INTRODUCTION

Dihydroergotoxine (DET) is a mixture of dihydroergocornine (DECO), dihydroergocryptine (DECY) and dihydroergocristine (DECI). The methanesulphonate salt of dihydroergotoxine, Hydergine, is used in the treatment of peripheral and cerebral vascular diseases. It has an α-adrenergic blocking action and produces a generalized peripheral vasodilatation without lowering the blood pressure in normotensive patients, but it may cause a reduction in blood pressure when administered to hypertensive patients. DET is obtained by hydrogenation of the ergotoxine fraction of the Claviceps alkaloids. This fraction is composed of ergocornine, ergocryptine and ergocristine, in various ratios depending on the species of ergot. The isolation and the physical and chemical characteristics of the DET alkaloids were first described by Stoll and Hofmann[1] in 1943. Since then, many attempts have been made to develop sensitive and specific analytical methods for the identification and determination of the ergot and dihydroergot alkaloids.

The DET alkaloids can be separated by paper[2,3], thin-layer (TLC)[4–7], liquid[8,9] and gas chromatography (GC)[10] or by counter-current distribution[11]. Most methods proposed for the determination of the DET alkaloids are based on separation by

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TLC and quantitation of the separated alkaloids either directly by UV or UV–visible spectrophotodensitometry or fluorodensitometry[12–14] or indirectly by eluting the active substance from the layer and measuring the UV absorption or photometrically by Van Urk's colour reaction[15–17].

All of these thin-layers methods are time consuming and, except for the fluorodensitometric detection[14], are insensitive. Recently, a rapid GC method[10] for the separation and determination of the DET alkaloids has been developed, using an all-metal injector and column system with flame ionization detection. The procedure is based on the quantitative decomposition of the compounds, catalysed by a metal surface, and the subsequent separation and detection of the peptide moieties formed from various DET alkaloids. However, 5 μg of each compound is required for adequate quantitation.

In this paper we describe a rapid, sensitive and specific procedure for the analysis of DET alkaloids by GC and combined GC–mass spectrometry (GC–MS). The method is based on a quantitative, non-catalysed, thermal decomposition of the DET alkaloids into a peptide moiety and dihydrolysergic acid amide (DLAA) in the injection port of the gas chromatograph. A normal all-glass system and a selective and sensitive nitrogen detector are used, and a sensitivity of about 1–10 ng for the various DET alkaloids is obtained. With the aid of the combination of GC and chemical ionization (CI) mass fragmentography, a 10-fold increase in sensitivity compared with the GC method used is obtained. Electron impact (EI) and chemical ionization mass spectrometry were used to elucidate the mechanism of the thermal decomposition of the DET alkaloids.

EXPERIMENTAL

Reagents

All solvents were of analytical-reagent grade and were obtained from Merck (Darmstadt, G.F.R.). The methanesulphonates of dihydroergocornine, dihydroergocryptine and dihydroergocristine were obtained from United Pharmaceutical Works (Prague, Czechoslovakia).

For the liberation of the bases of the DET alkaloids from their methanesulphonates, the strongly basic anion exchanger Dowex 1-X2 (100–200 mesh; Fluka, Buchs, Switzerland) in the hydroxide form was used in a methanolic medium. The hydroxide form of the anion exchanger was prepared by eluting a chromatographic column (20 × 2 cm) packed with about 10 g of Dowex 1-X2 with 2.5 ml 1 *N* sodium hydroxide solution until the eluate was alkaline to phenophthalein, followed by rinsing with water until the eluate was neutral; the product was stored under methanol until required.

A stock solution of a 1:1:1 mixture of the DET alkaloids was prepared by dissolving 30.0 mg of each of the methanesulphonates in 5.00 ml of methanol in a 15-ml glass-stoppered tube. After adding 200 mg of air-dried anion exchanger, swirling on a Vortex–Genie mixer for 10 min and allowing the resin to settle down, the solution was used for the preparation of the calibration standards.

An internal standard (I.S.) solution of 5.00 mg/ml of codeine hydrochloride was prepared by dissolving 250.0 mg of codeine hydrochloride (Merck), in 50.0 ml of methanol. Calibration standards containing 0.50 mg of I.S. and 0.10, 0.20, 0.50,

1.00, 2.00, 3.00, 4.00 and 5.00 mg/ml respectively of each of the methanesulphonates of the DET alkaloids were prepared by diluting mixtures of 100 μl of I.S. and aliquots of the DET stock solution to 1.00 ml with methanol in 1-ml Reacti-vials (Pierce, Rockford, Ill., U.S.A., Cat. No. 13221). These solutions were stable for at least 4 weeks when stored in a refrigerator. Volumes of 1 μl were injected into the gas chromatograph.

Samples

A commercial dihydroergotoxine methanesulphonate sample was dissolved in methanol to a final concentration of each component of about 0.2% (w/v). A dihydroergotoxine methanesulphonate formulation (oral tablets of 1.5 mg, sublingual tablets of 0.25 mg, oral solutions of 1.00 mg/ml and injection solutions of 0.30 mg/ml) was extracted either directly (solution) or after powdering and homogenization (tablet) with three portions of 20 ml of chloroform, the combined extracts were filtered, evaporated to dryness under reduced pressure in a Büchi Rotavapor and the residue was dissolved in a suitable volume of methanol to a final concentration of each component of about 0.07% (w/v).

After applying about 100 mg of Dowex 1-X2 (OH^-) anion exchanger, aliquots of these methanolic solutions were treated as described for the preparation of the calibration standards.

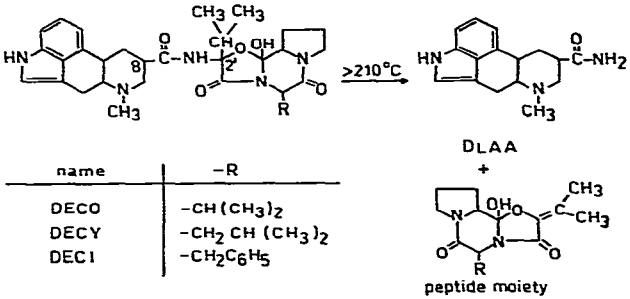
Gas chromatography

A Varian Model 2100 gas chromatograph equipped with a phosphorus/nitrogen detector containing a rubidium sulphate salt tip and a Varian Model A25 1-mV recorder were used. A Varian CDS 101 Chromatography Integrator was employed for the measurement of peak retention times and peak areas.

A U-shaped glass column (125 cm × 3.8 mm I.D.) packed with 3% SE-30 on Supelcoport, 80–100 mesh (Supelco, Bellefonte, Pa., U.S.A.), was found to be the most suitable. The following GC operating parameters were used: column temperature, isothermal at 200° for 21 min, then programmed from 200° to 270° at 10°/min (for routine measurements the chromatograph is programmed after two runs); detector temperature, 250°; injector temperature, 225°; nitrogen flow-rate, 26 ml/min; hydrogen flow-rate, 34 ml/min; air flow-rate, 180 ml/min; amplifier range, 10^{-12} A/mV; electrometer attenuation setting, ×2–128; recorder chart speed, 2 mm/min.

Gas chromatography–mass spectrometry

Mass spectrometry was performed on a Finnigan 1015D GC–MS system equipped with an EI source and on a Finnigan 3200F GC–MS system equipped with a CI source. Both mass spectrometers were coupled to a Finnigan 6000 computer. The GC conditions were as stated under *Gas chromatography* but the carrier gas was helium for the EI instrument and methane for the CI mass spectrometer. The separator oven and transfer line were kept at 260°. The CI source temperature was regulated at 130° while the source pressure was maintained at 900 μmHg by the methane column flow.



name	-R
DECO	$-CH(CH_3)_2$
DECY	$-CH_2CH(CH_3)_2$
DECI	$-CH_2C_6H_5$

Fig. 1. Structures of the dihydroergotoxine alkaloids and their thermal decomposition products.

RESULTS

Gas chromatography

The GC analysis of the DET alkaloids in their base or salt forms cannot be achieved because of their low volatility and thermal breakdown in the gas chromatograph. Hence the determination of these alkaloids by GC is possible only if a complete and reproducible decomposition is observed.

Using a suitable injection temperature, the DET alkaloids bases were each split reproducibly into two components. The structures and the decomposition of these compounds into a peptide moiety and dihydrolysergic acid amide (DLAA) are

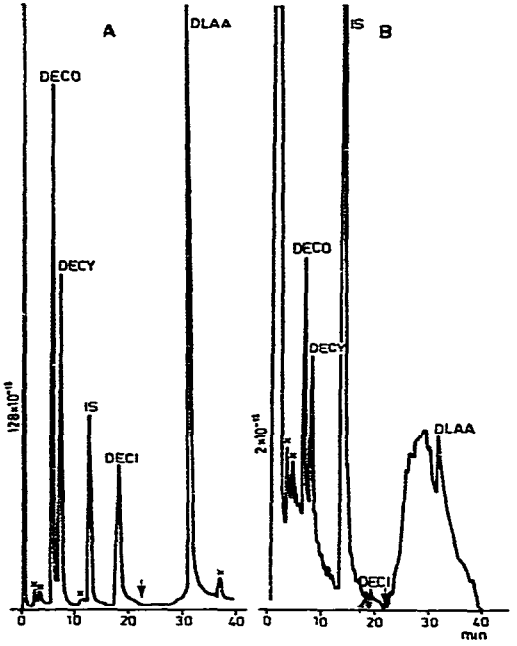


Fig. 2. Gas chromatograms of calibration standards containing (A) 0.5 μg/μl of I.S. and 1 μg/μl of DECO, DECY and DECI, and (B) 0.05 μg/μl of I.S. and 0.01 μg/μl DECO, DECY and DECI. Volume injected: 1 μl. X = unknown thermal decomposition products. Arrows indicate programmed at 10°/min from 200° to 270°.

TABLE I

RELATIVE RETENTION TIMES ON 3% SE-30 OF THE DECOMPOSITION PRODUCTS OF DIHYDROERGOCORNINE, DIHYDROERGOCRYPTINE, DIHYDROERGOCRISTINE AND THE INTERNAL STANDARD

Compound	*Relative retention time*
Internal standard	1.00 (retention time = 12.67 min)
Dihydroergocornine	0.46
Dihydroergocryptine	0.57
Dihydroergocristine	1.42
Dihydrolysergic acid amide	2.42

shown in Fig. 1. These decomposition products were confirmed with the aid of GC–MS (see below). As these peptide moieties are different (Fig. 1), after GC separation they can be used for the analysis of the corresponding DET alkaloids. Figs. 2a and 2b show the GC analysis of calibration standards containing 1 and 0.01 μg/μl, respectively, of each of the DET alkaloids. The relative retention times of these compounds were measured (Table I).

To establish the optimal conditions for the thermal decomposition, the influence of the injection temperature on the separation of the DET alkaloids was investigated. Fig. 3 shows gas chromatograms for the separation of these compounds at injection temperatures of 200° (a), 225° (b), 250° (c) and 275° (d). From Fig. 3 it

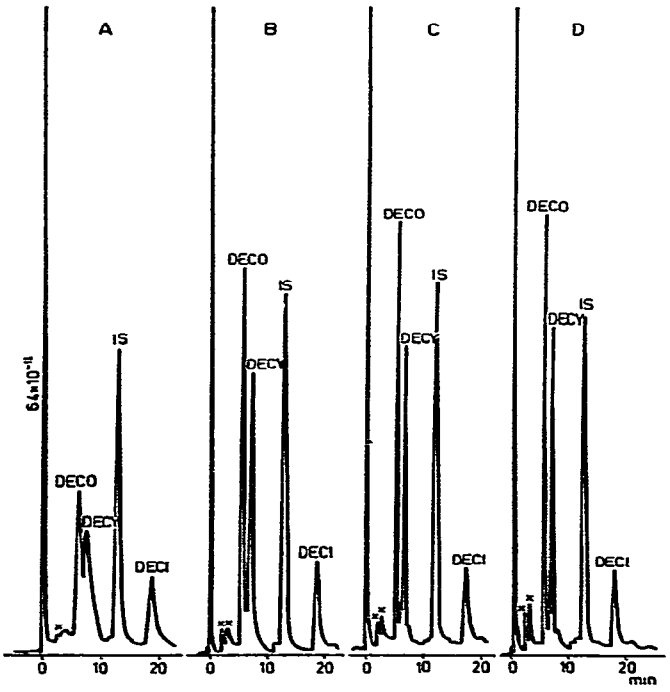


Fig. 3. Gas chromatograms of the separation of the DET alkaloids at injection temperatures of (a) 200°, (b) 225°, (c) 250° and (d) 275°. Injected: 1 μl of the calibration standard containing 0.5 μg/μl of I.S., DECO, DECY and DECI. X = unknown thermal decomposition product.

can be seen that a satisfactory separation and a minor further breakdown of the alkaloids were obtained by using an injection temperature between 225° and 250°. In further experiments a temperature of 225° was employed.

Fig. 4 shows the gas chromatograms of a dihydroergotoxine methane sulphonate solution before (a) and after (b) the addition of the strongly basic anion exchanger Dowex 1-X2. This figure clearly demonstrates that the analysis of these alkaloids in their salt forms cannot be performed without excessive decomposition.

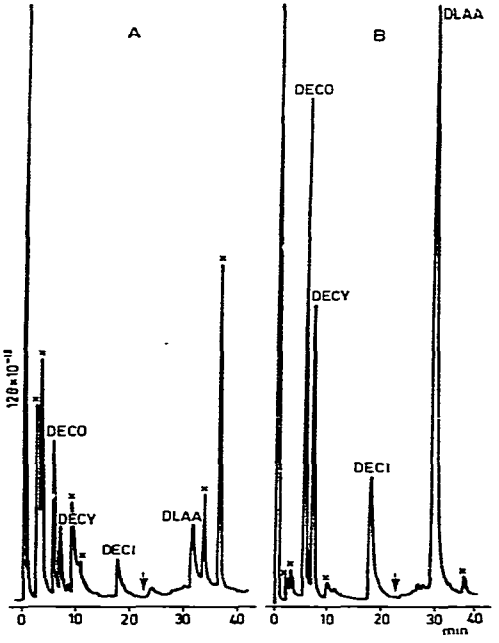


Fig. 4. Gas chromatograms of a dihydroergotoxine methane sulphonate solution containing 1 μg/μl of DECO, DECY and DECI before (a) and after (b) addition of the basic anion exchanger Dowex 1-X2. Injected volume: 1 μl. X = unknown decomposition product. Arrows indicate programmed at 10°/min from 200° to 270°.

After the GC analysis of the calibration standards, calibration graphs for the three DET alkaloids were constructed by plotting the peak-area ratios of dihydroergocornine, dihydroergocryptine, dihydroergocristine and the I.S. against the concentration of these alkaloids in the various calibration standards (Fig. 5). It can be seen that the calibration graphs for the three DET alkaloids show a linear relationship from 0.10 up to 5.00 mg/ml. One of the calibration standards was chromatographed several times daily prior to the determination of the unknown samples; the peak areas of the DET alkaloids and the I.S. were recorded and the ratios were compared with the calibration graphs in order to check the condition of the column and the detector.

The reproducibility of the method was determined by four replicate analyses on several days of five calibration standards, and the results are summarized in Table II. The limit of sensitivity for the DET alkaloids varied from 1 to 10 ng and was derived from the GC response of a calibration standard containing 0.01 μg/μl of each alkaloid (Fig. 2).

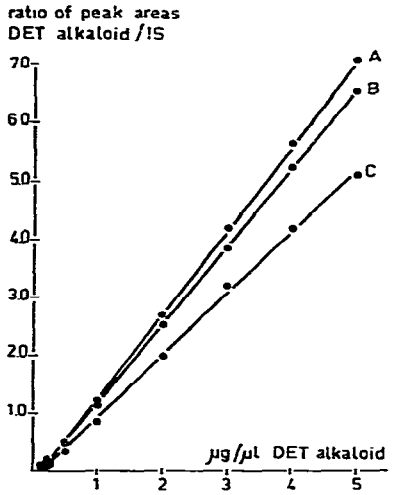


Fig. 5. Calibration graphs for (A) DECO, $y = 1.443\,x - 0.155$, $r = 0.99$; (B) DECY, $y = 1.3367\,x - 0.130$, $r = 0.99$; (C) DECI, $y = 1.0943\,x - 0.153$, $r = 0.99$. Concentration of I.S.: 0.5 µg/µl.

To evaluate the accuracy of the method, synthetic samples were assayed. These samples were prepared by adding 10.0 mg of each of the dihydroergotoxine methanesulphonates to about 2 g of tablet powder, containing all tablet ingredients except the DET alkaloids. The mean recovery in three experiments was 99.0 ± 1.5% for DECO, 98.5 ± 1.8% for DECY and 98.2 ± 2.4% for DECI.

The GC method was applied to the determination of the DET alkaloids in commercial samples and pharmaceutical preparations. The mean dihydroergotoxine methane sulphonate content of five commercial samples was 95.2 ± 2.9%, including a mean DECO content of 30.9 ± 0.8%, a mean DECY content of 32.8 ± 2.1% and a mean DECI content of 31.5 ± 3.6%. In the analysis of three portions of ten tablets of 1.5 mg obtained from two manufacturers, a mean dihydroergotoxine methane sulphonate content of 92.9 ± 3.5% of the declared value, including a mean DECO content of 32.8 ± 1.8%, a mean DECY content of 32.3 ± 2.5% and a mean DECI content of 27.8 ± 3.5%, was found.

Gas chromatography–mass spectrometry

The decomposition products of the DET alkaloids were established by means

TABLE II

RESULTS OF REPRODUCIBILITY STUDIES

Concentration of compound (µg/µl)	*Standard deviation (%)* *		
	Dihydroergocornine	*Dihydroergocryptine*	*Dihydroergocristine*
0.10	6	11	16
0.20	3	6	6
0.50	2	2	4
1.00	1	1	3
3.00	1	2	3

* Relative standard deviation (%) = $(\sigma/\bar{X}) \cdot 100$ of the peak area ratio of DET alkaloid to I.S.

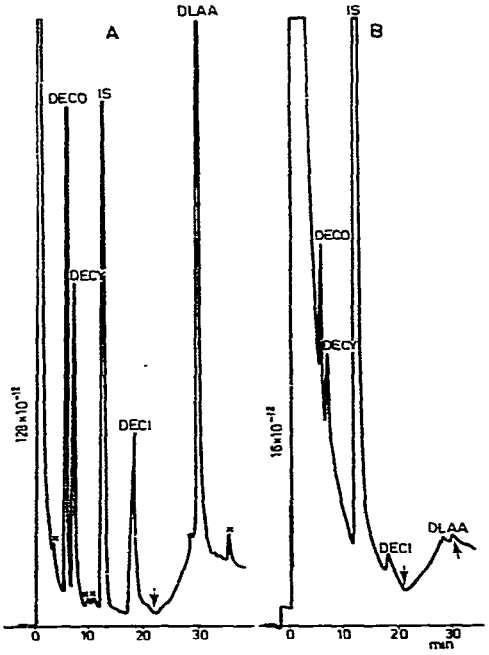


Fig. 6. Gas chromatograms of calibration standards containing (A) 1 μg/μl and (B) 0.1 μg/μl of the DET alkaloids using flame-ionization detection. Injected volume: 1 μl. Arrows indicate programmed at 10°/min from 200° to 270°.

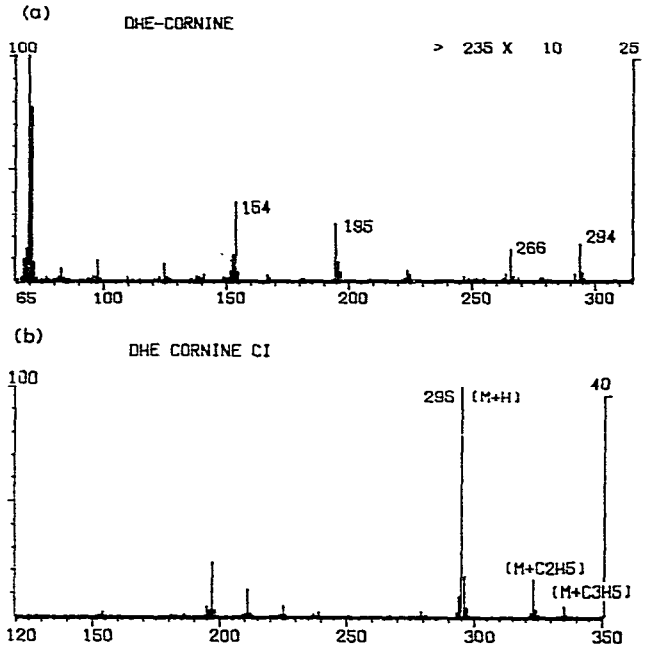


Fig. 7. (a) EI mass spectrum of the DECO peptide moiety. The masses indicated in the spectrum are explained in Fig. 8. (b) The methane CI spectrum of the DECO peptide moiety.

of EI and CI mass spectrometry. The thermal degradation of these substances involves a cleavage of the $NH–C_2^1$ peptide bond and a hydrogen transfer from the peptide moiety to the DLAA, as indicated in Fig. 1. The peptide moiety is characteristic of the DET alkaloids. In the gas chromatograph these moieties are eluted at their respective retention times before the DLAA molecule (Fig. 2).

Figs. 7a and 7b show the EI and CI spectra of the DECO peptide moiety. The ion at *m/e* 294 (Fig. 7a) is the molecular ion of the peptide and corresponds to the quasi-molecular ion at *m/e* 295 (Fig. 7b). The ions above *m/e* 295 are the (M+

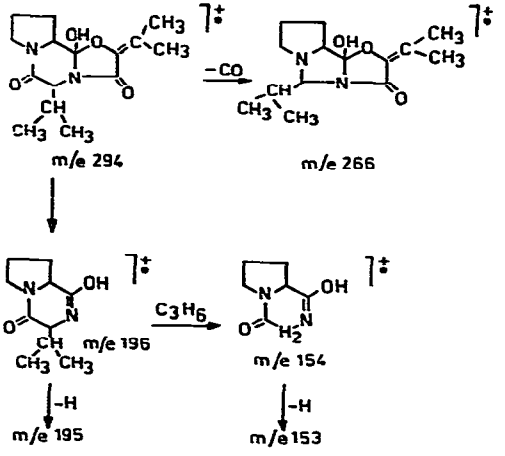


Fig. 8. The fragmentation scheme for the DECO EI mass spectrum.

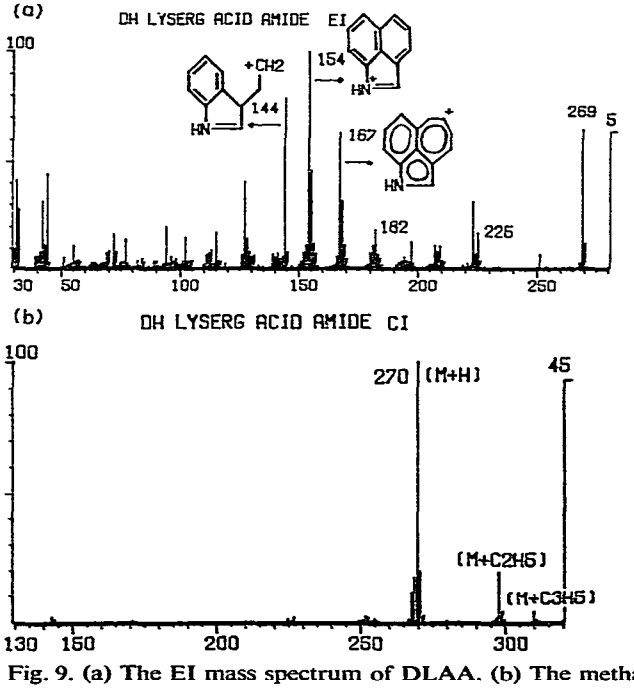


Fig. 9. (a) The EI mass spectrum of DLAA. (b) The methane CI mass spectrum of DLAA.

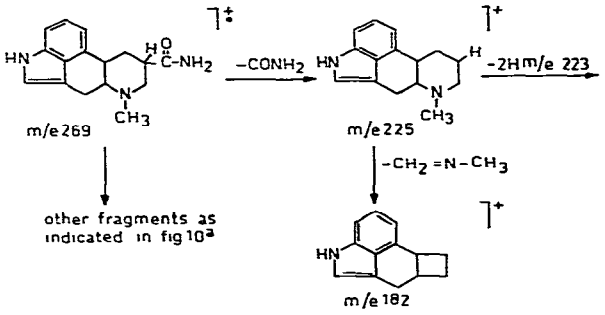


Fig. 10. The fragmentation scheme for the DLAA EI spectrum.

$C_2H_5)^+$ and the $(M+C_3H_5)^+$ ions usually found in CI with methane as reagent gas. The additional structural information as obtained from the EI spectrum is summarized in Fig. 8.

Figs. 9a and 9b show the EI and CI spectra of DLAA. The ion at *m/e* 269 (Fig. 9a) represents the molecular ion; in Fig. 9b the quasi-molecular ion at *m/e* 270 and the C_2H_5 and C_3H_5 addition products are shown. The major fragments of the EI spectrum are indicated in Fig. 10. These fragments agree with those observed by Voigt *et al.*[18] except for the mass difference of 2 a.m.u. due to differences in the ergotoxine and the dihydroergotoxine alkaloids. The fragments indicated in Fig. 9a are identical with those described by Voigt *et al.*

Similar results were obtained for dihydroergocryptine and dihydroergocristine. The major fragments and their intensities are compared with fragments of dihydroergocornine and are listed in Table III for both EI and CI.

In addition to the identification studies, we used the CI instrument for mass fragmentography. In Fig. 11 the computer-added trace of the three *m/e* values 295, 309 and 343 for the quasi-molecular ions of the DET alkaloids and the *m/e* value 300 of the I.S. codeine is shown. The amount per DET alkaloid injected into the gas chromatograph was 500 pg.

TABLE III

MASSES AND RELATIVE INTENSITIES OF THE MAJOR FRAGMENTS OF THE PEPTIDE MOIETIES

Ionization	*Fragment**	*Dihydroergocornine***	*Dihydroergocryptine***	*Dihydroergocristine***
EI	M^+	294 (1.7)	308 (1.9)	342 (3.8)
	$(M-CO)^+$	266 (1.4)	280 (0)	314 (0.6)
	$(M-C_5H_6O_2)^+ (= F_1)$	196 (8)	210 (10)	244 (11)
	$(F_1-H)^+$	195 (26)	209 (20)	243 (10)
	$(F_1-R)^+$	154 (35)	154 (37)	154 (32)
	$(F_1-R-H)^+$	153 (12)	153 (5)	153 (3)
CI	$(M+H)^+$	295 (100)	309 (100)	343 (100)
	$(M+C_2H_5)^+$	323 (16)	337 (19)	371 (17)
	$(M+C_3H_5)^+$	335 (5)	349 (6)	383 (6)

* R is the substituent group indicated in Fig. 1.

** The numbers in parentheses are the relative abundances of the respective *m/e* values.

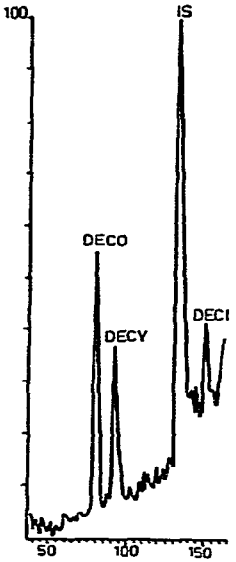


Fig. 11. Computer-added mass fragmentogram traces of a DET sample containing 500 pg of each compound.

DISCUSSION

The GC procedure described here is more specific and sensitive than previously reported chromatographic methods, the lowest measurable concentration of the dihydroergotoxine alkaloids varying from 1 to 10 ng. The method is also less time consuming, as the analysis of these alkaloids can be performed in about 35 min.

The results demonstrate that the most important steps in the procedure for DET are the conversion of the salt forms of the alkaloids into their free base forms with the aid of the strongly basic anion exchanger Dowex 1-X2 (Fig. 4) and the selection of a suitable injection temperature (Fig. 3).

As the "peptide moieties" of the DET alkaloids (Fig. 1) have a nitrogen content varying from 8.2 to 9.5%, it is obvious that a nitrogen detector should be used with these compounds. Using this detector, a minimal solvent response and a selective and sensitive response for the nitrogen-containing compounds is observed in the analysis of methanolic DET solutions (Fig. 2). Our GC method can, of course, also be performed with a flame-ionization detector, as shown in Fig. 6. However, as would be expected with this detector, a much higher solvent response, a decreased sensitivity and a lack of selectivity are obtained in the assay of the DET alkaloids. From the results obtained in the analysis of a number of commercial samples and pharmaceutical preparations, it can be concluded that our method is suitable for detecting and determining the DET alkaloids in these products.

Compared with the GC method previously reported by Szepesi and Gazdag[10], the procedure described here has the advantages of much higher sensitivity and selectivity. The favourable results obtained with the standard glass column system suggest that the thermal decomposition of the DET alkaloids is not catalysed by a

metal surface. By using mass spectrometry we could identify the thermal degradation products.

Although the structure of the peptide moiety could not be established with certainty, the point of cleavage at the NH–C_2^1 bond is certain, which is in contrast to the suggested CO–NH cleavage proposed by Szepesi and Gazdag[10]. The formation of a double bond between C_2^1 and the isopropyl group instead of the dioxocyclobutane ring proposed by Hofmann[19] is speculative but seems possible considering the relative mass spectral stability of the peptide moiety.

Using the CI mass spectrometer in the mass fragmentography mode, we were able to detect even smaller amounts of the DET compounds. Quantification could be performed easily at the 500-pg level.

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