Journal of Chromatography, 244 (1982) 357–364
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,929

ANALYSIS OF INDOLE ALKALOIDS IN NORWEGIAN *PSILOCYBE SEMILANCEATA* USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

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(Received March 25th, 1982)

SUMMARY

High-performance liquid chromatography has been used as separation method before spectroscopic investigations of isolated indole alkaloids in *Psilocybe semilanceata* mushrooms. By using a volatile buffer the mobile phase was easily removed by freeze-drying, and the residue introduced directly into a mass spectrometer. For final identification of baeocystin, it was necessary to investigate the isolated compound by mass spectrometry, UV spectroscopy and fluorescence detection. The mushrooms were found to contain up to 0.34% baeocystin. The amount of baeocystin was considerably higher in the cap than in the stipe. The content of psilocybin, however, was of the same order of magnitude in both parts of the mushroom. Rather large amounts of psilocybin were also found in dried, old mushrooms, which showed that the mushroom can still be potent after long storage.

INTRODUCTION

During the last few years ingestion of *Psilocybe semilanceata* (Fr. ex Secr.) Kummer as a narcotic has been rather popular among groups of young people in Norway. Norwegian *P. semilanceata* mushrooms have been found to contain 0.2–2.0% of the hallucinogenic indole alkaloid psilocybin (4-phosphoryloxy-N,N-dimethyltryptamine), analyzed by a recently developed high-performance liquid chromatographic (HPLC) method[1]. Because of the high psilocybin content the mushroom has recently been put on the Norwegian list of narcotic drugs. Traces of psilocin (4-hydroxy-N,N-dimethyltryptamine) has been detected in some mushrooms.

Another interesting indole alkaloid, baeocystin (4-phosphoryloxy-N-methyltryptamine), was first reported by Leung and Paul[2], who isolated this compound and its demethyl counterpart (norbaeocystin) from *P. baeocystis* grown in submerged cultures. Baeocystin was subsequently detected in a Pacific Northwest variety of *P. semilanceata*[3], and White[4] provisionally identified baeocystin in English *P. semilanceata*.

0021-9673/82/0000–0000/$02.75

An HPLC chromatogram of Norwegian *P. semilanceata* extract[1] revealed at least four distinct peaks, one of which was psilocybin; the others were at that time unknown. The purpose of the present investigation was to identify and quantify more of these compounds, which may be the previously mentioned monomethyl and/or demethyl analogue of psilocybin.

Isolation of baeocystin for spectroscopic identification has previously been carried out by preparative thin-layer chromatography (TLC) and classical LC[2–4]. HPLC can also be a valuable tool in separation of components before mass spectral (MS) analysis. By using a volatile buffer the mobile phase can easily be removed by freeze-drying and the residue introduced directly into the mass spectrometer. A simple modification of our HPLC method, by changing to a volatile buffer system, is described. The influence and use of various volatile salts were studied, as well as several methods for identifying the isolated compound.

Finally a quantitative study on the content of baeocystin in Norwegian *P. semilanceata* was carried out. The stipe and cap of some mushrooms were analyzed separately with regard to both psilocybin and baeocystin content. To check the potency as a function of storage time, some herbarium specimens were analyzed for their psilocybin content.

EXPERIMENTAL

Chemicals

Psilocin and psilocybin were supplied by Sandoz (Basel, Switzerland). Analytical grade methanol, ammonium nitrate, ammonium carbamate and ammonium acetate were obtained from E. Merck (Darmstadt, G.F.R.). Analytical grade ammonium formate was purchased from Fluka (Buchs, Switzerland).

Apparatus

A Spectra-Physics Model 3500 liquid chromatograph equipped with a rotary valve injector and a UV detector (Model 225) was used. The injector valve was equipped with a 10-μl loop for quantitative analyses and a 100-μl loop for preparative analyses. The column was a 25 cm × 4.6 mm I.D. stainless-steel tube, slurry-packed with small-particle silica (6-μm Partisil 5; Whatman, Maidstone, Great Britain). Separation of the components was achieved with methanol–water–1 *M* ammonium salt (220:70:10) for analytical purposes and (240:50:10) for preparative purposes. The 1 *M* ammonium salt solutions were buffered to pH 9.6 with 2 *N* ammonia.

Lyophilization was performed on a Hetosicc Freeze dryer CD 13-1 (Heto, Birkerød, Denmark) equipped with a separate Hetofrig Freezing bath. Electron impact mass spectra were obtained with a VG Micromass 7070F mass spectrometer via direct inlet. The probe temperature was 220°C and the ion source potential was 70 eV. A Pye Unicam SP8-100 ultraviolet spectrophotometer was used to determine the UV absorption spectra.

Quantitative analysis

Analysis of the psilocybin content was carried out as described before[1]. The dried mushrooms were extracted twice with a total of 5.00 ml 10% 1 *M* ammonium nitrate in methanol. The mobile phase was methanol–water–1 *M* ammonium nitrate,

pH 9.6 (220:70:10). Quantitation of psilocybin was based on peak height measurements, while determination of the baeocystin content was based on peak area measurements with psilocybin as external standard. The peak areas were measured by the product of peak height and the width at one-half the peak height.

Purification of samples for spectroscopic investigation

Dried mushrooms (300 mg) were extracted once with 4 ml hot methanol, and 100 μl of this extract were injected into the liquid chromatograph. Separation of the components was achieved with methanol–water–1 *M* ammonium salt pH 9.6 (240:50:10). The following salts were tested: ammonium formate, ammonium acetate and ammonium carbamate. After eight to twelve injections of the sample, the collected fractions were concentrated on a rotary evaporator and freeze-dried twice overnight. The residues were dissolved in methanol and evaporated to dryness under a stream of nitrogen.

RESULTS AND DISCUSSION

Preliminary investigations for preparing samples suitable for MS analysis

Isolation of the mushroom components was first carried out by a TLC method as described elsewhere[4]. Mixtures of methanol, water and dilute acetic acid were used as extraction solutions. Low extraction yields, however, indicated that the highly polar indole alkaloids were strongly adsorbed to silica, which complicated further spectroscopic investigations.

Because of these unsuccessful results, HPLC was proposed as isolation procedure. The previously developed HPLC method employed ammonium nitrate in the mobile phase. The presence of salt was essential for separation, but this salt is nonvolatile and difficult to remove after fraction collection. A possible way to solve this problem was to use a volatile buffer system, capable of maintaining the high resolution. Salt-free indole alkaloids could then be isolated simply by lyophilization of the sample after chromatography.

Chromatography and freeze-drying

Wheals[5] reported that ion exchange is probably the dominant process occurring when basic drugs are separated on silica with aqueous buffered solutions at high pH. The surface hydroxyl sites (SiOH) on silica will ionize and create active sites for which the protonated amine and any cations in solution will compete. Sugden *et al.*[6] reported that a change in the counter anion had little effect on k' values, but on the other hand a more significant difference in retention was observed on changing the added cation.

Changing from ammonium nitrate as the buffer salt of the eluting solvent to similar concentrations of ammonium formate, ammonium acetate and ammonium carbamate respectively had no effect on the chromatography of a mushroom extract. The selectivity was the same, and only minor differences in retention and efficiency could be observed. Fig. 1 shows a chromatogram of a methanolic mushroom extract with ammonium acetate as salt in the mobile phase.

Preparative liquid chromatography was performed with mobile phases containing ammonium acetate and ammonium carbamate, respectively. Individual peaks

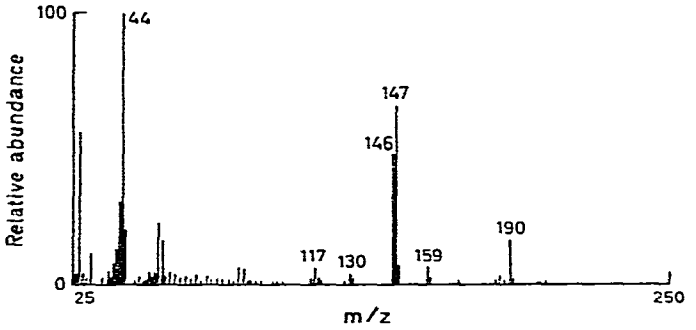

Fig. 1. HPLC chromatogram of a methanolic extract of Norwegian *P. semilanceata*. Peaks: 1, 2 = unknown; 3 = baeocystin; 4 = psilocybin. Mobile phase: methanol–water–1 *M* ammonium acetate, pH 9.6 (240:50:10); flow-rate, 1 ml/min. Detector wavelength: 254 nm. Injected volume: 100 µl.

Fig. 2. Mass spectrum of isolated peak 3 (baeocystin).

were collected, pooled and lyophilized. The pooled fractions had to be lyophilized twice overnight to get satisfactory mass spectra. The yield of psilocybin was in the range 100–200 µg, while the yield of baeocystin was 10–30 µg.. Both ammonium acetate and ammonium carbamate were suitable in this investigation. It has been reported that formate salts do, however, attack stainless steel[7], and ammonium formate was therefore omitted in further work.

Mass spectrometry

The mass spectrum of isolated psilocybin was identical to that obtained for standard psilocybin and showed peaks at *m/z* 204, 160, 159, 146, 130, 117 and 58 (base peak). Psilocybin gives a spectrum identical to that of psilocin, which indicates that dephosphorylation takes place at elevated temperature. The peak at *m/z* 204 was due to the dephosphorylated species.

The mass spectrum of isolated peak 3 (Fig. 1) is shown in Fig. 2. This is consistent with published data for the dephosphorylated species of baeocystin[2], showing peaks at *m/z* 190, 160, 159, 147, 146, 130, 117 and 44 (base peak).

UV spectroscopy

To determine whether the isolated peak 3 is baeocystin or its dephosphorylated species, some further investigations were necessary.

UV spectra of isolated psilocybin and peak 3, together with standard psilocybin and psilocin, are shown in Fig. 3. The UV spectra of the two isolated com-

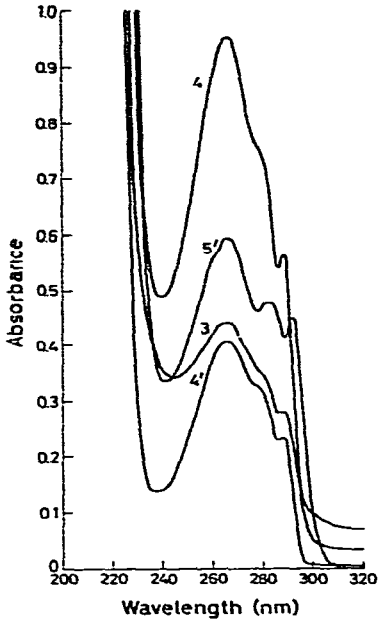

Fig. 3. Ultraviolet absorption spectra of baeocystin, psilocybin and psilocin in methanol. 3 = Isolated baeocystin; 4 = isolated psilocybin; 4′ = psilocybin standard (15 μg/ml); 5′ = psilocin standard (20 μg/ml).

pounds were similar and were identical with that of standard psilocybin, showing absorption maxima at 219, 266 and 288 nm and a shoulder at 280 nm, indicating that they both have the same chromophore, a 4-phosphoryloxytryptamine nucleus. A slightly different chromophore such as that present in the dephosphorylated species, psilocin, has distinctly different absorption maxima[2]. As can be seen in Fig. 3, the UV spectrum of standard psilocin shows maxima at 221, 266, 282 and 292 nm. This indicates that the compound in peak 3 is phosphorylated, therefore baeocystin.

Fluorescence detection at 335 nm (excitation at 267 nm) of a Norwegian *P. semilanceata* extract after HPLC separation[1] showed that only psilocybin and the compound in peak 3 fluoresce strongly at this wavelength. The ratio between the two peaks was similar to that obtained by UV detection at 254 nm. The dephosphorylated species of psilocybin, psilocin, displays only weak fluorescence at this wavelength[8]. This, together with the fact that the compound is soluble in water, further confirms that its identity is baeocystin.

Quantitation of baeocystin

The UV spectrum of baeocystin is identical with that of psilocybin[2]. No baeocystin standard was commercially available, and quantitative analyses of baeocystin were based on peak area measurements with psilocybin as external standard.

A selection of mushrooms analyzed earlier for their psilocybin content[9] were investigated with regard to their baeocystin content. Quantitative data for the psilocybin and baeocystin contents are given in Table I. The mushrooms were found to

TABLE I

QUANTITATIVE HPLC DATA FOR PSILOCYBIN AND BAEOCYSTIN IN A RANGE OF DRIED MUSHROOM SAMPLES

Content (%, w/w)		*Content (%, w/w)*	
Psilocybin	*Baeocystin*	*Psilocybin*	*Baeocystin*
0.55	0.05	1.13	0.28
0.58	0.05	1.34	0.11
0.65	0.09	1.34	0.29
0.70	0.11	1.53	0.18
0.72	0.09	1.66	0.13
0.82	0.14	1.68	0.29
0.84	0.11	1.96	0.29
1.00	0.13	1.96	0.34

contain up to 0.34% baeocystin. The amount of baeocystin seemed to increase with increasing amount of psilocybin.

Analysis of the stipe and cap

Quantitative data for the psilocybin and baeocystin contents in ten different mushrooms, the stipe and cap being analyzed separately, are given in Table II. The mushrooms were divided into groups based on their total dry weight. Only minor differences were observed in percentage psilocybin content between the two parts of the mushroom. The amounts of psilocybin in the stipe and cap, respectively, did not seem to be correlated with the mushroom weight or the total per cent of psilocybin in the mushroom. The baeocystin content is, however, considerably higher in the cap than in the stipe. Typical chromatograms of the stipe and cap are shown in Fig. 4. As can be seen, the stipe contains only slight amounts of baeocystin compared to psilocybin.

TABLE II

QUANTITATIVE HPLC DATA FOR PSILOCYBIN AND BAEOCYSTIN IN SEVERAL SAMPLES OF DRIED MUSHROOMS, THE STIPE AND CAP BEING ANALYZED SEPARATELY

Total dry weight	*Psilocybin content (%, w/w)*			*Baeocystin content (%, w/w)*		
(mg)	*Stipe*	*Cap*	*Total*	*Stipe*	*Cap*	*Total*
<20	0.70	0.49	0.59	–	<0.05	<0.05
<20	0.45	0.36	0.39	–	<0.05	<0.05
20–40	0.58	0.72	0.69	<0.05	0.27	0.22
20–40	0.15	0.19	0.17	–	<0.05	<0.05
20–40	0.73	0.69	0.70	<0.05	0.22	0.13
40–60	0.39	0.47	0.44	<0.05	0.05	<0.05
40–60	0.51	0.70	0.63	<0.05	0.19	0.12
40–60	0.61	0.82	0.74	<0.05	0.21	0.13
>60	1.05	1.10	1.08	0.06	0.16	0.13
>60	1.03	0.78	0.83	0.14	0.28	0.26

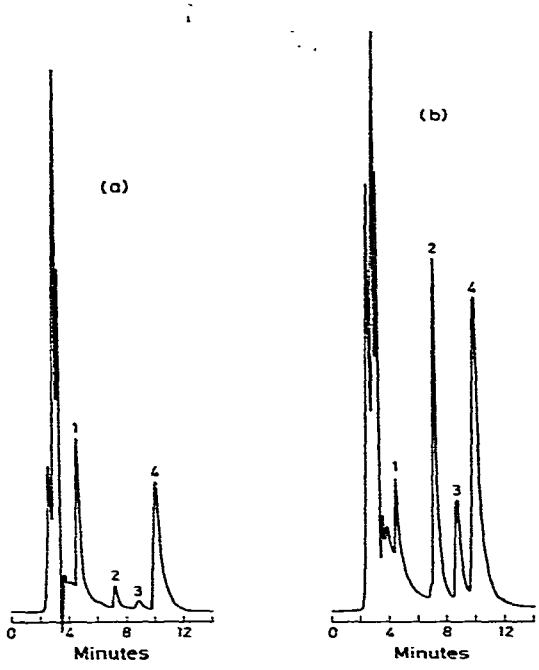

Fig. 4. HPLC chromatogram of the stipe (a) and cap (b) of a Norwegian *P. semilanceata* mushroom. Peaks: 1, 2 = unknown; 3 = baeocystin; 4 = psilocybin. Mobile phase: methanol–water–1 *M* ammonium nitrate, pH 9.6 (220:70:10); flow-rate, 1 ml/min. Detector wavelength: 254 nm. Injected volume: 10 µl.

Analysis of herbarium specimens

Some dried, old *P. semilanceata* mushrooms have been investigated for their content of psilocybin. The results are given in Table III.

The per cent of psilocybin decreased with increasing age of the mushroom. But even after 30 years storage, detectable quantities of psilocybin were still found. Dried mushrooms up to nine years old contained more than 0.5% psilocybin, which shows that the mushrooms can still be potent after long storage. Various amounts of

TABLE III

QUANTITATIVE DATA FOR PSILOCYBIN IN STORED SAMPLES OF *P. SEMILANCEATA*

Years of storage	*Psilocybin content (%, w/w)*	*Years of storage*	*Psilocybin content (%, w/w)*
4	1.42	13	0.64
4	0.75	14	0.54
5	0.63	22	0.29
6	0.61	26	0.12
6	0.55	27	0.15
9	0.87	29	0.38
13	0.41	29	0.10
13	0.65	30	0.05

baeocystin were detected in all mushrooms analyzed, except for the very old herbarium samples. The content of baeocystin seemed to decrease with increasing mushroom age.

CONCLUSIONS

This study shows that HPLC is suitable as a separation method before further spectroscopic investigations of the isolated components. By using a volatile buffer system, salt-free indole alkaloids are isolated simply by lyophilization of the sample after chromatography. The amounts of psilocybin and baeocystin obtained in this way are sufficient to give excellent UV and mass spectra.

The Norwegian *P. semilanceata* mushrooms contain up to 0.34% baeocystin, considerably more in the cap than in the stipe. Herbarium samples have a rather large content of psilocybin, which shows that the mushroom can still be potent after long storage.

ACKNOWLEDGEMENT

We gratefully acknowledge the skilful technical assistance of Mr. Finn Tønnesen.

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