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Note

Qualitative and quantitative determinations of hallucinogenic components of psilocybe mushrooms by reversed-phase high-performance liquid chromatography

R. VANHAELEN-FASTRÉ* and M. VANHAELEN
Pharmaceutical Institute, Free University of Brussels (U.L.B.), Campus de la Plaine 205-4, Boulevard du Triomphe, B-1050 Brussels (Belgium)
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In several countries, the use of hallucinogenic mushrooms, such as Psilocybe species, is on the increase. These mushrooms contain indole alkaloids, mainly psilocybin (4-phosphoryloxy-N,N-dimethyltryptamine), together with lesser amounts of constituents such as baeocystin (4-phosphoryloxy-N-methyltryptamine) and psilocin (4-hydroxy-N,N-dimethyltryptamine).

Several techniques have been used for the determination of psilocin and psilocybin in mushroom extracts, such as paper chromatography[1,2], column chromatography[3,4] and thin-layer chhromatography[5–7], preceding UV spectroscopy or colorimetry. Gas chromatography and mass spectrometry[8] have also been proposed. Gas chromatography requires preliminary derivatization, but high-performance liquid chromatography (HPLC) can be applied after simple extraction.

For the separation of psilocin and psilocybin, Perkal *et al.*[9] developed an HPLC procedure based on ion-exchange chromatography with an aqueous ethanol acidic mobile phase and double detection either by UV absorption or by fluorometry. For the quantitation of psilocin and psilocybin in mushroom extracts, White[10] used as mobile phase an alkaline ammonium nitrate solution in aqueous methanol on a silica column; detection was performed by UV absorption measurement. Christiansen *et al.*[11] modified this procedure to the determination by UV spectroscopy and fluorometry of psilocybin in *Psilocybe semilanceata*. Later, after modification of the mobile phase by introduction of a volatile ammonium salt, acetate or carbamate, they isolated baeocystin on a semi-preparative scale and identified it by mass spectrometry; they also determined psilocybin and baeocystin in mushroom extracts[12]. More recently, these authors[13] completed their study by the simultaneous use of UV, fluorescence and electrochemical detection to check interference with the quantitated peaks of baeocystin, psilocybin and psilocin. However, though this procedure allowed the separation of these alkaloids, the use of a silica column with an aqueous mobile phase leads to inactivation of the packing and to difficulties related to the recovery of column performance after contamination by crude extract constituents.

Because of its high polarity, psilocybin is not sufficiently retained on reversed phase; therefore, Beug *et al.*[14] used HPLC to quantitate psilocin and psilocybin in *Psilocybe baeocystis*, on an octadecyl bonded phase with a paired-ion reagent (hep-

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tanesulphonic acid) in an acidic aqueous methanol mobile phase and UV detection. Later, Wurst *et al.*[15] also used reversed-phase HPLC on an octadecyl bonded phase with an acidic aqueous ethanol mobile phase and double detection by UV spectroscopy and fluorometry for the analysis of psilocybin and psilocin in crude extracts of mushrooms. The isolation of psilocybin and baeocystin on a semi-preparative scale is described. However, as in the Beug method[14], the poor retention of the polar compounds, namely psilocybin, precludes its accurate quantitation in crude extracts.

In this context, the use of reversed-phase HPLC for Psilocybe mushrooms analysis was reinvestigated.

EXPERIMENTAL

Mushroom samples

Dried specimens of *Psilocybe semilanceata* (fruit bodies) were used.

Standards and chemicals

Psilocin and psilocybin were supplied by Sandoz (Basel, Switzerland). All solvents and reagents were analytical grade. Water was twice distilled. 1-Heptanesulphonic acid in acetic solution for preparation of 0.05 *M* solution was obtained from Waters Assoc. (PIC Reagent B-7; Milford, MA, U.S.A.). TLC adsorbents were obtained from E. Merck (Darmstadt, F.R.G.).

Apparatus

The high-performance liquid chromatograph consisted of a Model 6000M (Waters Assoc.) solvent delivery system operating at room temperature, equipped with a variable-wavelength UV detector Model SPD-2AM (Shimadzu, Kyoto, Japan), a septumless injector Model U6K (Waters Assoc.). The system was connected to a HP3392 integrator (Hewlett-Packard, Palo Alto, CA, U.S.A.).

The Bransonic Ultrasonic Cleaner (Model 32,50-55 Kc, Branson, Stamford, CT, U.S.A.) was used to degas mobile phases and to improve mushroom extraction (before preparative layer chromatography).

An AEI MS902 apparatus was used for mass spectrometric measurements.

Standard and sample solutions

Standard solutions. Psilocin and psilocybin were dissolved in ethanol–water (1:1) containing 0.05 *M* 1-heptanesulphonic acid adjusted to pH 3.5 with acetic acid (PIC Reagent B-7, Waters Assoc.); required dilutions were prepared from stock solution with the same solvents mixture.

Sample solutions. Approximatively 20 mg (accurately weighed) of ground dried mushrooms, introduced in a micropercolator, were allowed to macerate, for 2 h, in 3 ml of ethanol–water (1:1) containing 0.05 *M* 1-heptanesulphonic acid adjusted to pH 3.5 with acetic acid (PIC Reagent B-7; Waters Assoc.). After percolation of this first solvent fraction into a 10-ml volumetric flask, extraction was achieved by the addition of fresh solvent so as to obtain a 10-ml volume of final percolate. For HPLC analysis this solution was, if required, diluted twice or four times with the same solvent mixture.

Thin-layer chromatography

Chromatoplates were prepared in the laboratory with a mixture (1:1) of silica gel 60H (Art. 7736, E. Merck) and silica gel $60GF_{254}$ (Art. 7730, E. Merck), *ca.* 3.2 g/plate (10 × 20 cm). Spots, bands of 7 mm width; mobile phase, 1-propanol–water–acetic acid (10:3:3); run, 10 cm; detection: Van Urk spraying reagent (5%-*p*-dimethylaminobenzaldehyde in concentrated hydrochloric acid).

Preparative layer chromatography

Chromatoplates were prepared in the laboratory with a mixture (1:1) of silica gel $60PF_{254}$ with gypsum (Art. 7749, E. Merck) and silica gel $60PF_{254}$ (Art. 7747, E. Merck), *ca.* 23 g/plate (20 × 40 cm); they were prewashed with methanol and dried at 120°C for 30 min just before sample application.

To prepare a sample, 500 mg of ground dried mushrooms were extracted twice with 10 ml of methanol–water (1:1) for 30 min, including each time 10 min sonication. The suspension was filtered through a fritted glass covered with a thin layer of diatomaceous earth. Filtrates were combined, evaporated to dryness and the residue dissolved in 2.5 ml of methanol–water (1:1). Spots, bands of 17 cm width, two chromatoplates; mobile phase, 1-propanol–water–acetic acid (10:3:3); run, 20 cm; detection, UV observation (254 and 365 nm), Van Urk spraying reagent on a marginal band; elution, methanol–water (7:3) after drying of the chromatoplates at ambient temperature for 10 min.

High-performance liquid chromatography

Column, prepacked μBondapak alkylphenyl (mean particle size: 10 μm; 30 cm × 1/4" I.D.) stainless steel column (Waters Assoc.), injection volume, 25 μl. Mobile phases: (a) for identification of psilocin and psilocybin, and quantitation of psilocin, ethanol–water (35:65) containing 0.05 *M* 1-heptanesulphonic acid and adjusted to pH 3.5 with acetic acid (PIC Reagent B-7; Waters Assoc.); (b) for identification and quantitation of psilocybin and baeocystin, water containing 0.05 *M* 1-heptanesulphonic acid and adjusted to pH 3.5 with acetic acid (PIC Reagent B-7; Waters Assoc.). Flow-rate, 1 ml/min; UV detection at 267, 254 or 290 nm.

RESULTS AND DISCUSSION

Extraction procedure

For routine analysis of psychotropic alkaloids in dried mushrooms, an one-step convenient procedure was proposed consisting of a percolation following a short maceration.

Percolation of 20 mg of dried mushrooms with only 5 ml of ethanol–water (1:1) containing 1-heptanesulphonic acid led to the extraction of the major proportion of the alkaloids. Indeed, further extraction with 2 ml of the same solvent mixture contained only 1% of the amount of psilocybin present in the first fraction; all detectable components of the mushrooms seemed to be extracted to a similar extent. Therefore, a 10-ml final volume of percolate was used. No further tedious treatment of the extract (such as centrifugation, evaporation to dryness, filtration) was needed before HPLC or TLC.

Thin-layer chromatography and preparative layer chromatography

As baeocystin was not commercially available, it was isolated by preparative layer chromatography on silica gel. A mobile phase of *n*-propanol–water–acetic acid (10:3:3) led to a better resolution, on both analytical and preparative scale, than mobile phases composed of *n*-butanol, acetic acid and water in the ratio 12:3:5 or 4:2:2, which were, in the opinion of Beug[14], the best among previously described TLC systems (Table I).

Identity of baeocystin

The identity of baeocystin isolated in this way was confirmed by its mass[10,12,15] and UV[12] spectroscopic characteristics, its fluorescence properties[12] and colour reaction with Van Urk reagent (Table I).

High-performance liquid chromatography

The use of reversed-phase chromatography is well known to be very suitable for biological material analysis. However, Psilocybe alkaloids are highly polar molecules, and are therefore retained on the bonded phase hardly at all. The introduction in the mobile phase of paired-ion reagent, including a lipophilic chain, decreased the hydrophilic character of psilocybin and baeocystin. The use of an octadecyl bonded phase with a mobile phase of methanol–water (25:75) containing 1-heptanesulphonic acid, as described by Beug *et al.*[15], led to retention times of 1.94 min for psilocybin and 5.91 min for psilocin. These conditions allowed clear resolution of psilocin, whereas psilocybin remained unresolved from other constituents of the crude extracts. Furthermore, we found that baeocystin was poorly separated from psilocybin (only 0.15 min) under these conditions.

In fact, it was very difficult to quantitate psilocin and psilocybin simultaneously with one isocratic system, because the polarities of these two compounds are very different. To solve this problem, we used two different mobile phases and an ethylphenyl bonded phase as stationary phase. In this case, it exhibited a greater retention power than an octadecyl bonded phase.

The first mobile phase, ethanol–water (35:65) containing 1-heptanesulphonic acid, led to retention times of 8 min for psilocin and 3 min for psilocybin and baeocystin (which were thus unresolved) (Fig. 1). These conditions did not allow accurate determination of psilocybin in mushroom crude extracts, because this alkaloid was insufficiently separated from other polar constituents.

The second mobile phase, water with added 1-heptanesulphonic acid, allowed

TABLE I

TLC R_F VALVES OF PSILOCYBE ALKALOIDS

Adsorbent, silica gel (see Experimental); mobile phase, 1-propanol–water–acetic acid (10:3:3).

Compound	R_F values	*Colour reaction with Van Urk reagent*
Psilocybin	0.30	Purplish-blue
Baeocystin	0.40	Purplish-blue
Psilocin	0.53	Blue

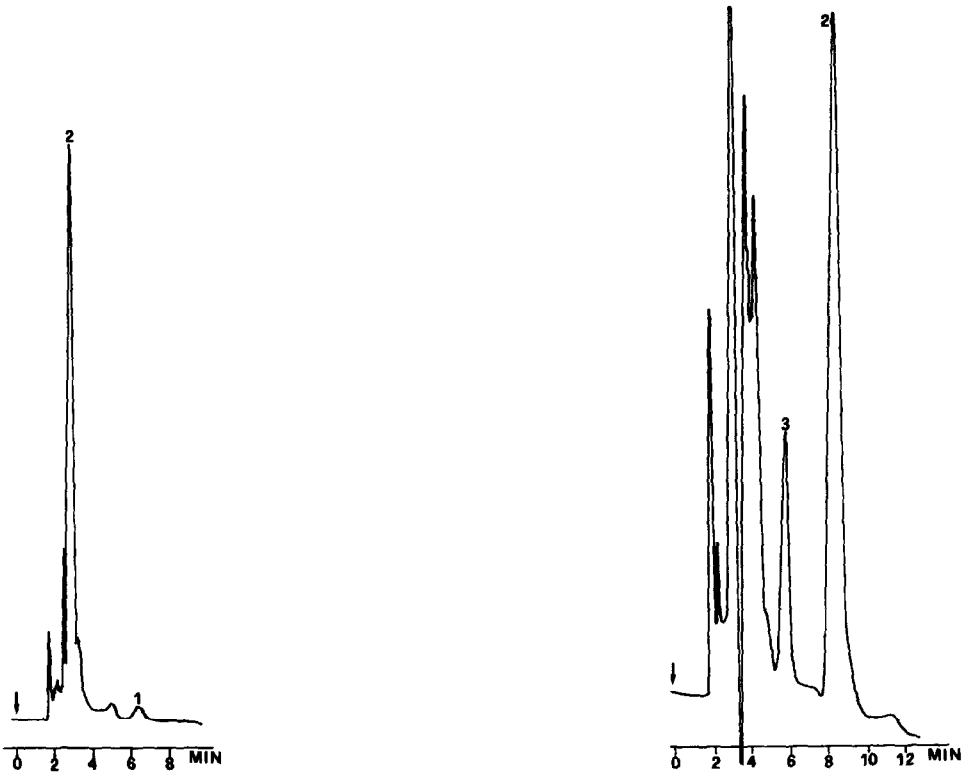

Fig. 1. HPLC chromatogram of *Psilocybe semilanceata* crude extract, on an ethylphenyl bonded stationary phase with a mobile phase of ethanol–water (35:65) containing 1-heptanesulphonic acid, and UV detection at 267 nm. Peaks: 1 = psilocin; 2 = psilocybin and baeocystin.

Fig. 2. HPLC chromatogram of *Psilocybe semilanceata* crude extract, on an ethylphenyl bonded stationary phase with a mobile phase of water containing 1-heptanesulphonic acid, and UV detection at 267 nm. Peaks: 2 = psilocybin; 3 = baeocystin.

the separation of baeocystin (retention time, t_R = 5.5 min) and psilocybin (t_R 8.0 min), which were completely resolved from other constituents of the mushrooms (Fig. 2), whereas psilocin was not eluted under these conditions. In practice, this mobile phase would be more useful than the first because psilocin, particularly in dried mushrooms, is a minor component[9,11,13] compared with psilocybin (Fig. 1).

No significative modification occurred in these separations by modification of the alkyl chain of the counter-ion; 1-heptane-, 1-octane- and 1-pentanesulphonic acids could all be used.

UV absorption measurements at 267, 254 and 290 nm of psilocybin and baeocystin peaks in mushroom extracts, with reference to pure psilocybin and baeocystin chromatographed in the same conditions, confirmed that no substance interferes in the quantitation of psilocybin (Table II); small interferences were noted with baeocystin (at t_R 5.5 min) (Table II). This fact was another reason to consider that the conditions used by Beug *et al.*[14] were unsuitable for psilocybin determination. As expected from the ε (molecular extinction coefficient) values, greater sensitivity was

TABLE II

PSILOCYBIN AND BAEOCYSTIN CONTENTS IN DRIED *PSILOCYBE SEMILANCEATA*

Sample	*Psilocybin content (%) determined by UV detection at*			*Baeocystin content (%) determined by UV detection at*		
	254 nm	*267 nm*	*290 nm*	*254 nm*	*267 nm*	*290 nm*
I	1.19	1.13	1.09	0.37	0.29	0.33
II	1.17	1.12	1.11	0.41	0 37	0.39

observed at 267 nm, which was also the most selective wavelength for baeocystin determination (Table II).

Quantitation

By injection of identical volumes of psilocybin standard solutions, a straight-line calibration graph was obtained over the range 0.25–500 μg/ml; the correlation coefficient was 0.9999, and quantitations were made either by peak area integration or by peak height measurements. Even if 6 ng of psilocybin were still measurable, more precision was offered for injections of at least 25 ng of psilocybin (25 μl of a 1 μg/ml solution). The concentration range 10–20 μg/ml seemed to be better with respect to the stability of the apparatus, reproducibility and column contamination. Under the experimental conditions described, the variation coefficients in the determinations of both psilocybin and baeocystin, calculated over the whole method, extraction and HPLC step, were 3%.

Baeocystin is not commercially available; so it was isolated by preparative layer chromatography for use in peak identification (Figs. 1 and 2). However, as the UV spectra of psilocybin and baeocystin are identical[5,12], baeocystin quantitative determinations were based on peak area measurements with psilocybin as external standard.

Analysis of mushroom specimens

Results of psilocybin and baeocystin determinations obtained for two *Psilocybe semilanceata* samples are summarized in Table II. As expected, their content of psilocin was negligible compared with those of psilocybin and baeocystin.

REFERENCES

1 A. Hofmann, R. Heim, A Brack, H. Kobel, A. Frey, H Ott, T. Petrzilka and F. Troxler, *Helv. Chim. Acta*, 42 (1959) 1557.
2 P. Catalfomo and V. E. Tyler, Jr., *Lloydia*, 27 (1964) 53.
3 J. E. Robbers, V. E Tyler, Jr., and G. M. Ola'h, *Lloydia*, 32 (1969) 399.
4 J. Picker and R. W. Rikards, *Aust. J. Chem.*, 23 (1970) 853.
5 A Y. Leung, A. H. Smith and A. G. Paul, *J. Pharm. Sci*, 54 (1965) 1576.
6 R. Heimm, K. Genest, D. W. Hughes and G. Belec, *J. Forensic Sci. Soc.*, 6 (1966) 192.
7 D. B. Repke and D. L. Leslie, *J. Pharm. Sci.*, 66 (1977) 113.
8 D. B. Repke, D. L. Leslie, D. M. Mandell and N. G. Kish, *J. Pharm. Sci.*, 66 (1977) 743.
9 M. Perkal, Gr. L. Blackman, A. L. Ottrey and L. K. Turner, *J. Chromatogr.*, 196 (1980) 180.
10 P. C. White, *J. Chromatogr.*, 169 (1979) 453.
11 A. L. Christiansen, K. E. Rasmussen and F. Tønnesen, *J. Chromatogr.*, 210 (1981) 163.
12 A. L. Christiansen and K E. Rasmussen, *J. Chromatogr*, 244 (1982) 357.
13 A. L. Christiansen and K. E. Rasmussen, *J. Chromatogr.*, 270 (1983) 293.
14 M. W. Beug and J. Bigwood, *J. Chromatogr.*, 207 (1981) 379.
15 M. Wurst, M. Semerdžieva and J. Vokoun, *J. Chromatogr.*, 286 (1984) 229.