QUANTITATIVE ANALYSIS OF PSILOCYBIN AND PSILOCIN IN *PSILO-CYBE BAEOCYSTIS* (SINGER AND SMITH) BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND BY THIN-LAYER CHROMATO-GRAPHY

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SUMMARY

Rapid quantification of psilocybin and psilocin in extracts of wild mushrooms is accomplished by reversed-phase high-performance liquid chromatography with paired-ion reagents. Nine solvent systems and three solid supports are evaluated for their efficiency in separating psilocybin, psilocin and other components of crude mushroom extracts by thin-layer chromatography.

INTRODUCTION

In 1957, the appearance of an article in *Life* magazine, "Seeking the magic mushroom"[1], and publication of *Mushrooms, Russia and History*[2], spurred renewed scientific investigation into the active principles of hallucinogenic mushrooms used for the past 2000 years or more by Mesoamerican Indians. In 1959, Hofmann *et al.*[3] isolated two new compounds, psilocybin (I) and psilocin (II) from *Psilocybe mexicana* Heim and quantified their results by using ultraviolet and infrared spectroscopy. Since then, several workers have reported qualitatively on the presence of psilocybin and psilocin in wild mushrooms from North America and Europe, basing their work on
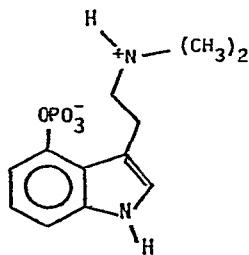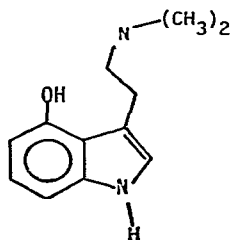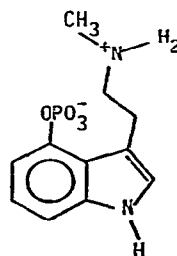
| I | II | III |
| Psilocybin | Psilocin | Baeocystin |

thin-layer chromatography (TLC)[4–9]. Repke *et al.*[10] report on quantitative analysis by gas chromatography–mass spectrometry and White[11] reports on quantitative analysis by normal-phase high-performance liquid chromatography (HPLC). We report here a superior quantitative analytical method involving paired-ion (PIC) reversed-phase HPLC. We also evaluate various TLC materials and mobile phases for their efficacy in separating psilocybin, psilocin and other potentially interesting indoles [including baeocystin (III)] in *P. baeocystis* Singer and Smith[12,13].

EXPERIMENTAL

The HPLC was performed on a Waters 200 series chromatography system (Waters Assoc., Milford, MA, U.S.A.) incorporating a Model 6000A reciprocating pump operating at a flow-rate of 2 ml/min; a model 440 fixed-wavelength absorbance detector at 254 nm; a Model U6K septumless injector; and a Waters 30 cm × 3.9 mm I.D. μBondapak C_{18} column (particle size 10 μm). The solvent was water–methanol (75:25) or water–acetonitrile (75:25) containing 0.05 M heptanesulfonic acid adjusted to pH 3.5 with acetic acid (Waters PIC B-7 reagent). We also used water–methanol (75:25) containing 0.05 M tetrabutylammonium phosphate adjusted to pH 7.5 with phosphoric acid (Waters PIC A reagent). The methanol and acetonitrile were supplied by Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), and the water was purified by using organic removal cartridges and ion exchange, followed by distillation from glass apparatus.

Mushroom samples were collected fresh, promptly freeze-dried, sealed in plastic, and stored either at −5°C or at −60°C until ready for analysis. They were then ground to a fine powder, and portions weighing 250 mg were placed in 10-ml beakers and stirred at room temperature with *ca.* 7 ml of methanol for 12 h. Each mixture was then filtered through a 0.5-μm PTFE filter, the filter was rinsed with additional methanol, and the filtrate was diluted to exactly 10 ml. An injection volume of 10 μl produced satisfactory peak size at 0.1 a.u.f.s.

Recovery of psilocybin and psilocin was tested by injecting 250-mg freeze-dried samples of *Psathyrella foenisecii* (Fr.) Smith with 0.25 to 1.5 ml of methanol containing 1.0 mg/ml of psilocybin and 1.0 mg/ml of psilocin. The methanol was allowed to evaporate overnight, then the samples were placed in the freeze-dryer, freeze-dried as if they were fresh mushrooms, and extracted by using the procedure described for fresh mushrooms. In a test of alternative extraction methods, a duplicate set of "spiked" and "unspiked" *P. foenisecii* was extracted with about 100 ml of methanol in a Soxhlet extractor for 24 h; the extract was then concentrated to 10 ml, filtered and analyzed. Another duplicate set was extracted with 10 ml of methanol under reflux for 24 h, filtered and analyzed. The *P. foenisecii* was used in the "spiking" experiment because it was similar in stature to *P. baeocystis* and it gave a similar chromatogram, except that it contained no psilocybin or psilocin. Recovery of psilocybin and psilocin was also tested by "spiking" *P. baeocystis* with a standard addition of 1.0 mg of psilocybin and 1.0 mg of psilocin per 250 mg of freeze-dried mushroom and then by extracting as for fresh mushrooms.

The instrument was calibrated with psilocybin and psilocin standards obtained from the National Institute on Drug Abuse (Rockville, MD, U.S.A.). A standard solution was prepared containing 0.1 mg/ml of psilocybin and 0.1 mg/ml of psilocin

in methanol; a second standard solution contained 0.01 mg/ml of each. Calibration involved injection of 2 to 25 μl of the 0.1 mg/ml standard and 5 to 10 μl of the 0.01 mg/ml standard, and a calibration curve was prepared. For daily work, the volume of standard solution injected was so chosen as to give a peak area similar to that obtained with the mushroom being analyzed. At least three injections of the standard were averaged for each day's standardization. Peak areas were determined by using the internally programmed area percent method of a Hewlett-Packard 3380A reporting integrator–plotter.

TLC was carried out on three different types (and sizes) of plates: 5 × 20 cm coated with a 0.25-mm layer of silica gel 60F$_{254}$ (Merck, Darmstadt, G.F.R.); 10 × 20 cm coated with a 0.25-mm layer of microcrystalline cellulose (Q2F, Quantum Industries, Fairfield, NJ, U.S.A.); and 5 × 10 cm coated with a 0.25-mm layer of alumina K (Q3F, Quantum Industries). All plates were oven-dried and stored in a desiccator (damp plates resulted in inconsistent results). Psilocybin and psilocin standards were spotted on each plate, together with the methanolic extracts used in the HPLC work.

The plates were developed for 15 cm at room temperature in the dark in either a covered development tank or a half-gallon covered Mason jar with one or more filter-paper wicks to ensure vapor saturation in the tanks. Nine solvent systems were used: (1) butanol–acetic acid–water (12:3:5); (2) butanol–acetic acid–water (2:1:1); (3) pure methanol; (4) 1.5 % conc. aqueous ammonia in methanol (1.5 ml of reagent-grade ammonium hydroxide in 98.5 ml of methanol); (5) conc. ammonia–water–*n*-propanol (12:188:500); (6) methanol saturated with conc. aqueous ammonia in chloroform (5:95); (7) water-saturated *n*-butanol; (8) pure *n*-propanol; and (9) ammonia–water–*n*-propanol (10:50:150). Development times ranged from 1.5 h with methanol to more than 7 h with aqueous ammonia–*n*-propanol.

Developed plates were air-dried and then placed under a short-wave UV lamp (Mineralight, Ultra-Violet Products). Any spots visible to the naked eye or under short-wave UV radiation were encircled with a pencil. Ehrlich's (or Van Urk's) reagent [10 % *p*-dimethylaminobenzaldehyde (*p*DAB) in conc. hydrochloric acid] was freshly prepared and sprayed in an acetone solution (1 part of Ehrlich's reagent to 4 parts of acetone), and color was allowed to develop overnight by wrapping the plate in the paper towel upon which it had been sprayed. On other plates, color was developed by dipping the plates in a 20 % solution of pure anhydrous toluene-*p*-sulfonic acid (*p*TSA) in methanol. The plates were then photographed, and the photographs and plates were stored for future reference.

RESULTS

For quantification of psilocybin and psilocin levels in wild mushrooms, we found that prompt freeze-drying of the fresh-picked carpophores was important. Freeze-dried mushrooms retained their psilocybin and psilocin levels for over 2 years without noticeable loss when stored in a freezer at $-60°C$ or at $-5°C$, whereas dried herbarium material often lost all activity after 1 year.

We obtained quantitative extraction of psilocybin and psilocin from powdered freeze-dried mushrooms after stirring for 12 h in methanol at room temperature. The standard addition of psilocybin and psilocin to *P. baeocystis* after freeze-drying

indicated $90 \pm 5\%$ recovery for both psilocybin and psilocin. The recovery of psilocybin and psilocin "spikes" from *Ps. foenisecii* in four trials was $90 \pm 7\%$ for psilocybin and $60 \pm 8\%$ for psilocin after freeze-drying and room-temperature extraction. Extraction at higher temperature or in a Soxhlet extractor led to partial or complete loss of psilocin although loss of psilocybin was generally less than 20%. The methanolic extracts could be stored in a freezer at $-5°C$ for over 1 year with little change, although storage at room temperature led to complete loss of psilocin and some loss of psilocybin within a few months.

HPLC

A typical HPLC chromatogram obtained from *P. baeocystis* with water–methanol (75:25) with 0.05 M PIC B-7 and a flow-rate of 2 ml/min revealed seven well-resolved peaks (Fig. 1): the retention times were 1.23 min (3.6% of the total area); 1.58 min (49.8%); 1.94 min (psilocybin, 14.9%); 2.20 min (18.9%); 4.1 min (0.1%); 5.21 min (1.7%); and 5.91 min (psilocin, 11%). The amount of psilocybin was found to be 2.0 ± 0.2 mg/g (dry weight), while that of psilocin was 1.3 ± 0.2 mg/g
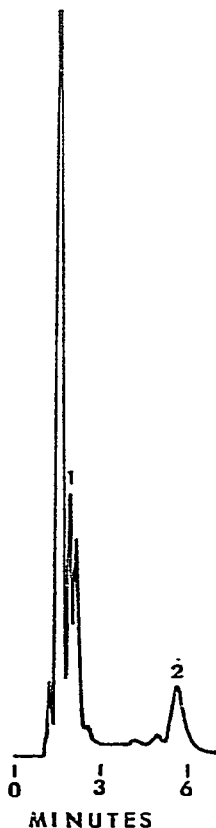

MINUTES

Fig. 1. HPLC chromatogram of a crude methanolic extract of *Psilocybe baeocystis* Singer and Smith on a μBondapak C_{18} column, with water–methanol (75:25) with 0.05 M heptanesulfonic acid at pH 3.5 (PIC B-7) as mobile phase (2 ml/min) and UV detection at 254 nm. Peaks: 1 = psilocybin; 2 = psilocin.

(dry weight). Collections from the same area but made at other times, as well as collections from other areas, showed that both psilocybin and psilocin levels were highly variable: psilocybin levels ranged from 1.5 mg/g (dry weight) up to 8.5 mg/g (average 2.9 ± 1.5 mg/g), but were typically 2.0 ± 0.2 mg/g. Psilocin levels ranged from zero to 5.9 mg/g (average 1.9 ± 1.5 mg/g) and showed no tendency to cluster round any one value.

The quantification of peaks was performed by comparison of integrated peak areas of psilocybin and psilocin standards with those of the wild mushroom. A calibration curve was prepared, and, within the repeatability of the method (±10%), there was a linear relationship between concentration and peak area from 0.2 μg to 3 μg of total psilocybin or psilocin, corresponding to levels in the mushroom (assuming a 10-μl injection volume) from 0.8 to 12 mg/g. The practical detection limit in crude mushroom extracts appeared to be about 0.01 μg of psilocybin or psilocin; this was due to interference by other trace compounds in the extracts. HPLC chromatograms obtained by using water–methanol (75:25) with 0.05 M PIC A reagent showed clear resolution of psilocin, but psilocybin was not resolved from other peaks. No mixture of water–methanol or water–acetonitrile alone gave satisfactory results with the μBondapak C_{18} column. Mixtures of water–acetonitrile with PIC A or PIC B-7 reagent failed to give satisfactory resolution of psilocybin from other peaks in the chromatogram.

TLC

When 20 × 5 cm silica gel plates and butanol–acetic acid–water (12:3:5) were used, $P. baeocystis$ showed three sky-blue spots immediately upon drying: psilocybin at R_F 0.16, psilocin at R_F 0.36 and another spot of identical color at R_F 0.44. Under short-wave UV radiation many other spots were visible, and most of these appeared colored when sprayed with Ehrlich's reagent. Psilocybin appeared as pinkish brown immediately after spraying, then brownish purple (R_F 0.16); psilocin became a darker purple (R_F 0.36). Additionally, our $P. baeocystis$ sample produced the following unidentified colored spots: a light brown spot at R_F 0.13, a brownish orange spot (turning pink with time) between psilocybin and psilocin at R_F 0.28, a light blue spot at R_F 0.31 and an aquamarine blue spot at R_F 0.40. The aforementioned light blue spot above psilocin at R_F 0.44 turned lavender; a yellowish brown spot at R_F 0.48 merged with a spot at R_F 0.50, which was pink-red immediately after spraying, but turned grey later; an orange spot at R_F 0.56; a very light blue spot at R_F 0.61; a strong light purple spot at R_F 0.76; and a yellow-brown spot at R_F 0.83.

When a silica gel plate was developed the same way, but dipped in 20% p-toluenesulfonic acid solution in anhydrous methanol, only psilocybin, psilocin and three other spots (R_F 0.13, 0.34 and 0.76) were visible. All were of a similar color to those obtained with Ehrlich's reagent, but with a brownish cast.

Cellulose plates developed in butanol–acetic acid–water (12:3:5) also showed ample separation of psilocybin and psilocin, but only resolved four of the other spots. Psilocybin had an R_F of 0.48, and psilocin migrated to R_F 0.78.

Alumina plates developed in butanol–acetic acid–water (12:3:5) did not separate psilocybin from psilocin.

Solvent butanol–acetic acid–water (2:1:1), when used with 20 × 5 cm silica gel plates, also effectively separated psilocybin (R_F 0.21) from psilocin (R_F 0.46), as

well as yielding several other spots, but gave less effective separation of other peaks than did butanol–acetic acid–water (12:3:5). Similarly, good separation of psilocybin (R_F 0.14) from psilocin (R_F 0.45) was obtained with 1.5% ammonia solution in methanol, but only four other spots were observed.

Conc. ammonia–water–n-propanol (12:188:500) resolved nine spots in addition to psilocybin (R_F 0.11) and psilocin (R_F 0.58). However, the spots were streaked and were not as cleanly resolved as in butanol–acetic acid–water (12:3:5). Ammonia–water–n-propanol (10:50:150) resolved eight spots in addition to psilocybin (R_F 0.16) and psilocin (R_F 0.82), but also displayed considerable streaking.

Methanol, n-propanol, water-saturated n-butanol and methanol saturated with conc. aqueous ammonia in chloroform (5:95) did not adequately resolve psilocybin and psilocin.

DISCUSSION

The use of HPLC in a paired-ion reversed-phase system provides a quick, accurate method of quantifying psilocybin and psilocin in wild mushrooms. The longer retention time of psilocin in reversed-phase chromatography compared with that in normal-phase chromatography allows one to separate psilocin from other absorbing compounds of similar retention time that otherwise could lead to errors in quantification. Since HPLC does not involve derivatization, there are no problems associated with degradation of psilocybin and/or psilocin leading to errors in quantitation. Degradation is a very real threat in gas chromatography–mass spectrometry, which requires prior derivatization with bis(trimethylsilyl)trifluoroacetamide[10].

The evaluation of TLC supports and solvent systems showed that silica gel was the best stationary phase in all solvent systems. On 20 × 5 cm silica gel plates, development for at least 15 cm with butanol–acetic acid–water (12:3:5) and (2:1:1), 1.5% ammonia solution in methanol, and the two aqueous ammonia–n-propanol systems all clearly separated psilocybin from psilocin. The best overall peak separation was obtained with butanol–acetic acid–water (12:3:5), in which as many as 12 spots were well resolved from psilocybin and psilocin. At present, we are unable positively to identify most of them. It seems likely that, besides psilocybin and psilocin, there are the N-methyl and tryptamine analogues of both these compounds in mushroom extracts. Urea is also present and gives a yellow spot. Neither the pink spot that turns grey nor the aquamarine spot are known, and we are currently attempting to identify these zones.

To summarize, we have found that solvent butanol–acetic acid–water (12:3:5) and silica gel offer an excellent system for the TLC of mushroom extracts containing psilocybin and psilocin. For rapid quantification of psilocybin and psilocin in the extracts, reversed-phase HPLC on μBondapak C_{18}, with 0.05 M PIC B-7 in water–methanol (3:1) as mobile phase, gave excellent results.

ACKNOWLEDGEMENT

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