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The Quantitation of Psilocybin in Hallucinogenic Mushrooms Using High Performance Liquid Chromatography

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ABSTRACT: A method using high performance liquid chromatography (HPLC) with an acetonitrile, water, and phosphoric acid mobile phase and a bonded cyano-amino-type polar phase column has been developed for the rapid, selective, and accurate quantitation of psilocybin in dry mushroom material. A simple one-step procedure is used for the quantitative extraction of psilocybin in under 60 min. The 267:254 nm absorbance ratio is used as a check on peak purity for the psilocybin response.

KEYWORDS: forensic science, chromatographic analysis, psilocybin, high performance liquid chromatography, psychedelic mushrooms, mycophilia renaissance, absorbance ratio

In the United States, there has been a marked increase in the use of mushrooms containing the hallucinogenic drug psilocybin (4-phosphoryloxy-*N,N*-dimethyltryptamine). The psychedelic effects of this drug are well documented [*1*], and possession of it is controlled by federal law [*2*]. The abuse of this hallucinogen is reported to be on the rise in many other countries as well [*3-6*]. Its current popularity in this country can be related to low price, abundant availability, and the belief that mushrooms growing in the woods are a "natural" high. Several drug-oriented publications are now carrying advertisements for psilocybin mushroom home growing kits, as well as feature articles on psychedelic mushroom use. The news media have recently reported the arrest of a major distributor of psilocybin mushrooms [*7*]. This "mycophilia renaissance" is also indicated by a twofold increase in submissions of hallucinogenic mushroom samples to the Drug Enforcement Administration's forensic science laboratory system during 1982. The forensic chemist must be able to identify and accurately quantitate the controlled drug present in these samples. The quantitative analysis is particularly important where a jurisdiction, such as New York or Vermont, bases an indictment upon total hallucinogen content rather than on the aggregate weight of the exhibit.

Several spectrophotometric and chromatographic techniques, and various extraction procedures, have been used for the analysis of psilocybin in mushrooms. Recently published extraction procedures have used:

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1. A weighed amount of material, ground with sand to a fine powder and roller-mixed with methanol for 24 h [*3*].

2. A weighed amount of material passed through a (No. 22) sieve and homogenized in a 3-mL volume of methanol for 2 min with a Polytron-Kinematica homogenizer [*4*].

3. A weighed amount of ground mushroom material placed into a rotary mixer and extracted with a 10% 1*N* ammonium nitrate in methanol solution for 30 min. After centrifugation and separation of the supernatant, the residue is reextracted for 30 min with 2 mL of the extraction solution. The two extracts are then combined and diluted for analysis [*5*].

4. A weighed amount of freeze-dried material (ground to a fine powder) stirred at room temperature in methanol for 12 h [*8*].

The extraction method using a homogenizer was originally reported for mushroom samples with a very low psilocybin content (0.01 to 0.20%). Christiansen et al [*5*] believe that this method would not be adequate for the extraction of mushrooms with a much higher psilocybin content (such as Norwegian *Psilocybe semilanceata*). The remainder of these procedures appear to be time-consuming or tedious or both.

The use of gas chromatography (by means of derivatization) has been reported for the quantitation of psilocybin [*9*]. However, most of the recent approaches to assaying psilocybin in mushroom material have involved the use of high performance liquid chromatography (HPLC). These include:

1. A reverse phase paired-ion system using a microbondapack C-18 column with a phosphate buffered methanol/water/cetrimonium bromide mobile phase with ultraviolet detection at a wavelength of 280 nm [*3*].

2. A reverse phase paired-ion system, using a microbondapack C-18 column and a water/methanol mobile phase containing heptanesulfonic acid, with ultraviolet detection at a wavelength of 254 nm [*8*].

3. An adsorption or ion-exchange system or both using a Partisil 5 silica column and an ammonia-buffered methanol/water/ammonium nitrate mobile phase, with ultraviolet detection at a wavelength of 254 nm and fluorimetric detection at 335 nm with excitation at 267 nm [*5*].

4. An ion-exchange system using a Partisil SCX-10 column (in a column oven at 50°C) and an ammonium phosphate/potassium chloride buffered methanol/water mobile phase, with ultraviolet detection at 267 nm and fluorimetric detection at 335 nm with excitation at 267 nm [*4*].

Of the systems described, only Perkal's [*4*] offers baseline resolution of psilocybin from mushroom coextractives. That method, however, makes necessary the use of fluorescence detection and a liquid chromatograph column oven. We have developed an HPLC system using a bonded cyano-amino-type polar phase column, and a water/acetonitrile/phosphoric acid mobile phase, with ultraviolet detection. This method provides for the rapid, selective, and accurate quantitation of psilocybin in dry mushroom material, following a simple one-step extraction procedure. In addition, the dual parameters of retention time and absorbance ratio provide a check on peak purity, as well as greatly enhancing the specificity of the analysis [*10,11*].

Experimental Procedure

The chromatographic system consisted of a Model 6000-A pump (Waters Associates, Milford, MA), a Model U6K septumless injector (Waters), a Model 770 variable ultraviolet (UV) detector at 267 nm (Schoeffel Instruments, Westwood, NJ), a Model 440 fixed UV detector at 254 nm (Waters), and a 4.6 by 25 cm stainless steel column, prepacked with Partisil-10 PAC (Whatman, Clifton, NJ).

The mobile phase used was 5% acetonitrile, 94.5% water, and 0.5% phosphoric acid, adjusted to pH 5.5 to 6.0 with 2*N* sodium hydroxide. The solution was filtered and degassed by passing it through a 0.45-μm filter (Rainin Instruments Co., Woburn, MA). A flow rate of 2.0 mL/min was used.

The acetonitrile used was distilled in glass, Omnisolv (MCB, Cincinnati, OH). All other chemicals were of reagent grade. The psilocybin and psilocin drug standards were obtained from Sandoz Pharamecuticals, Hanover, NJ.

All glassware was rinsed well with distilled water and alcohol before being used.

Mushroom samples were prepared by reducing a representative portion of the caps and stipes in size, then pulverizing the material to a fine powder in a micromill (Chemical Rubber Co., Cleveland, OH). (If the mushrooms are not dry enough on receipt to be finely powdered, then a very gentle drying procedure is recommended, such as that described by Heim et al [*12*], which uses a vacuum oven at 40°C.) An accurately weighed amount of the powdered material, about 0.6 g, was transferred to a glass-stoppered Erlenmeyer flask. A 10.0-mL volume of methanol was added, and the flask was placed in an ultrasonic water bath (Triple J/Ultrasonic Industries, Port Richey, FL) for a period of 50 min. The extract was then transferred to a glass-stoppered centrifuge tube and centrifuged until the supernatant liquid was clear. Ten microlitres of this extract were injected into the HPLC system.

The efficiency of the procedure for extraction of psilocybin from mushroom material was studied by determining the average percent of the drug extracted from three different samples at varying lengths of time in the ultrasonic bath.

Psilocybin was quantitated by comparing average peak heights of sample drugs versus standards, based on dual injections of each, using ultraviolet detection at 254 nm. The concentration of the standard psilocybin solution, prepared in methanol, was 0.2 mg/mL. Injection volume was 10 μL.

The precision of the chromatographic response was examined by making five quantitative determinations of a single sample extract. The precision of the extraction procedure was examined by analyzing five weighed portions of one exhibit (composite material). Each portion was extracted and quantitated by the procedure described.

The absorbance ratio data were obtained by measuring the peak height response of more than 30 injections of standard material and mushroom extracts, using the variable UV wavelength detector at 267 nm, in series with the fixed UV detector at 254 nm.

Results and Discussion

For the analysis of hallucinogenic mushroom exhibits, it is desirable to have a simple, rapid, and quantitative extraction procedure for psilocybin. The use of a Soxhlet extractor is not only time-consuming, but has been reported to cause a substantial loss of psilocybin and psilocin because of breakdown [*8*]. We have found that treatment of finely powdered mushroom material with methanol, in an ultrasonic water bath, breaks up the mushroom tissue matrix sufficiently to allow over 95% extraction of psilocybin into the solvent in less than an hour. The temperature of the extraction solvent (methanol) was found to rise to about 45°C during the 1-h treatment. Standard psilocybin, dissolved in methanol, was similarly treated to see if any breakdown would occur under these conditions. None was detected.

The amount of psilocybin extracted as a function of treatment time is shown in Fig. 1. Since it was felt that "spiking" the mushroom material with standard would not be a true test of the ability of the method to extract active components from the tissue matrix, an alternate procedure was used to achieve as nearly as possible 100% recovery. The methanol-insoluble residue from each sample, after 1 h of treatment in the ultrasonic water bath, was washed well and extracted for an additional 15 h with methanol by stirring. The number of milligrams of psilocybin recovered in each sample following this additional extraction was added to that obtained after the initial 1-h extraction. These totals were assumed to represent 100% extraction amounts for the purpose of the recovery study.

This extraction procedure has been used in our laboratory for over a year. The psilocybin content of mushroom exhibits was found to vary over a wide range (0.1 to 1.5%). This is to be expected, since the amount of active constituents present in mushrooms depends on such fac-

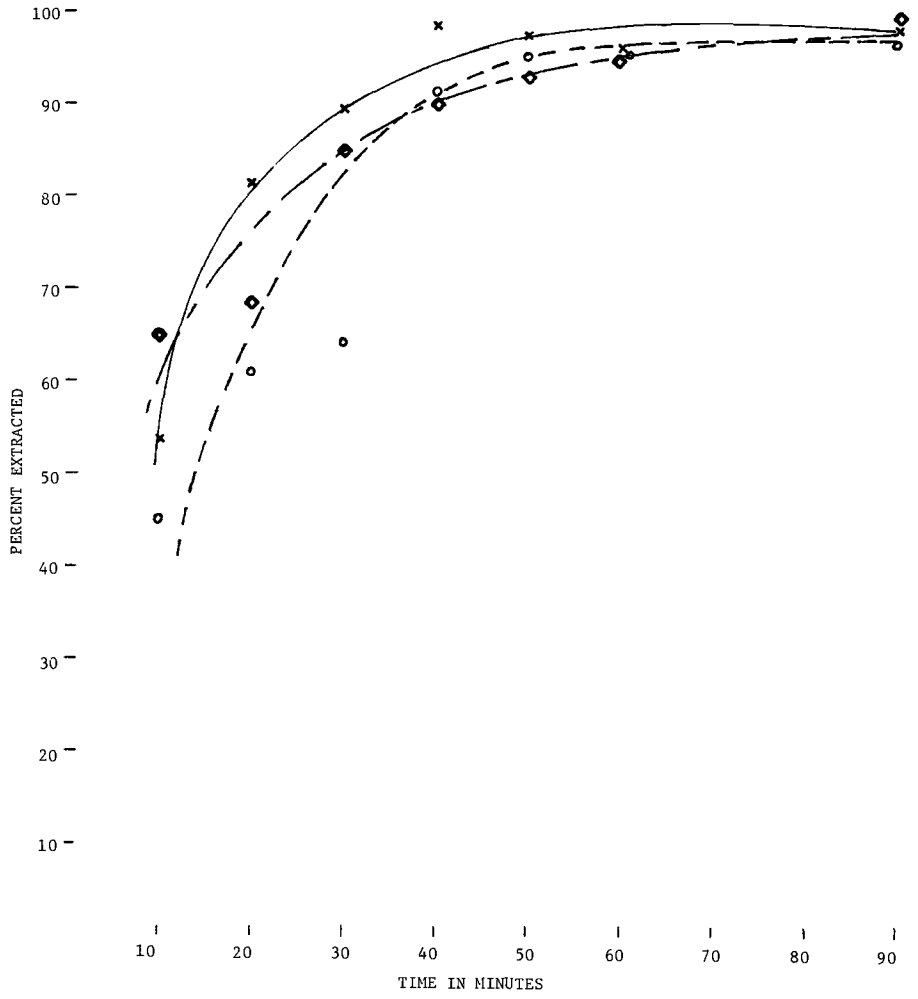

FIG. 1—*Percentage of psilocybin extracted into methanol versus time treated in ultrasonic water bath. Sample 1 is* —×—×—×—, *Sample 2 is* —○—○—○—, *and Sample 3 is* —◇—◇—◇—.

tors as climatic conditions, species, time of collection, preservation of material, and possibly the availability of soluble nitrogen and phosphorus in the soil [*12,13*].

Standard chromatograms of psilocybin and psilocin are shown in Fig. 2. Since psilocin is reported to be present in hallucinogenic mushrooms in only trace amounts [*5,14*], we have concentrated on obtaining separation of psilocybin from other coextracted components. A reverse phase HPLC procedure that will achieve baseline resolution of psilocin is currently being investigated for use in exhibits where that compound is present as the major hallucinogen.

A representative chromatogram of a mushroom extract is shown in Fig. 3. The quantitative determination of psilocybin was based on peak height measurements of the UV trace at 254 nm.

The relationship of detector response with psilocybin concentration was found to be linear over the range 0.03 to 0.60 mg/mL.

The purity of the psilocybin response of the mushroom extracts was checked by comparing the 267:254 nm absorbance ratio for each sample to that of standard material. These ratios were found to be in good agreement; the mean value for the 267:254 absorbance ratio of psilocybin was 1.40, with a relative standard deviation of 1.14%.

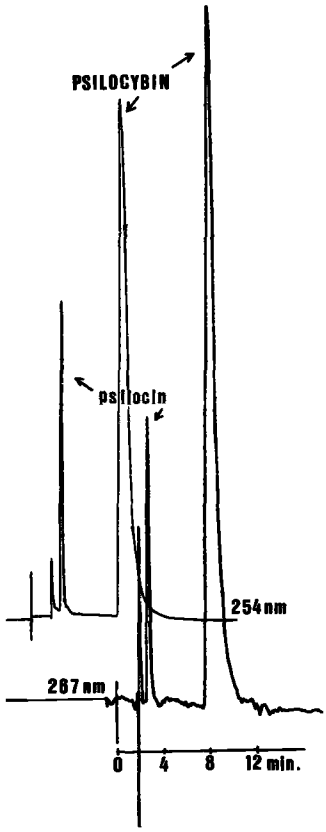

FIG. 2—*Liquid chromatogram of psilocybin and psilocin: detection by ultraviolet absorbance at 267 and 254 nm.*

The relative standard deviations of the chromatographic response and of the extraction procedure were found to be 1.06 and 3.75%, respectively.

After prolonged use, column performance may degrade. A 50-mL 0.1*N* phosphoric acid wash, followed by a 50-mL acetonitrile/water (50:50) wash, was found to work well in restoring the efficiency of the column.

The separation mechanism for psilocybin possibly involves a combination of ion exchange, reverse phase, and ion pair chromatography. Ion exchange could occur between the quaternized NH groups on the Partisil-10 PAC column and the phosphate moiety on the psilocybin molecule. It has been reported that in an acidic aqueous mobile phase, the NH groups are quaternized [*15*]. When the phosphoric acid concentration in the mobile phase is increased from 0.5 to 1%, the retention time of psilocybin decreases. This effect is consistent with an ion exchange mechanism where increasing the concentration of a counter-ion, such as phosphate, results in decreased retention. In addition, ion exchange could occur between the protonated amine group on the psilocybin molecule and the weakly acidic silanol sites on the column. When the amount of acetonitrile in the mobile phase is increased from 5 to 50%, there is a small decrease in retention for psilocybin. This is consistent with reverse phase chromatography. The Partisil-10 PAC packing has short CH_2 chains [*16*], which can explain why the reverse phase effect is small. Finally, ion pairing may be occurring between the protonated amine group on the psilocybin molecule and phosphate ions in the mobile phase.

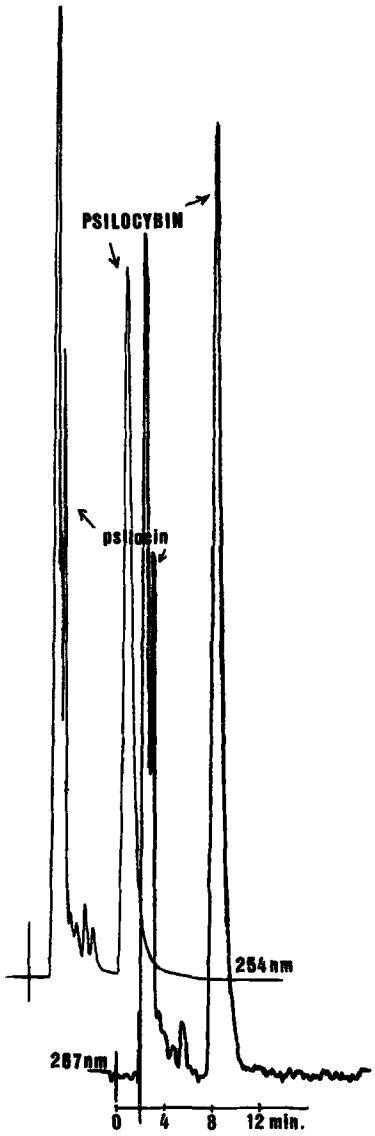

FIG. 3—*Liquid chromatogram of mushroom extract: detection by ultraviolet absorbance at 267 and 254 nm.*

Conclusion

An HPLC system using an acetonitrile/water/phosphoric acid mobile phase, with a Partisil-10-PAC column, has been developed for the quantitation of psilocybin in hallucinogenic mushrooms. A simple and rapid procedure is presented for the extraction of psilocybin from dry mushroom material using an ultrasonic water bath. At least 95% of the drug is extracted into methanol in less than an hour. The 267:254 nm absorbance ratio is used as a check on peak purity, as well as aiding in the qualitative identification of psilocybin when combined with the parameter of retention time.

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