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# Analysis of Psilocybin and Psilocin in Mushroom Extracts by Reversed-Phase High Performance Liquid Chromatography

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**ABSTRACT:** A method has been developed for the analysis of psilocybin and psilocin in dry and preserved mushrooms using reversed-phase high performance liquid chromatography. A mobile phase of phosphate buffered methanol/water/cetrimonium bromide allows good separation of the two hallucinogens.

**KEY WORDS:** toxicology, psilocybin, psilocin, chromatographic analysis

The hallucinogenic drugs psilocybin (Fig. 1, I) and psilocin (II) are found as naturally occurring indoles in several mushroom species [*1,2*]. The hallucinogenic effects of these compounds are similar to those produced by lysergic acid diethylamide (LSD) and mescaline [*3*] and therefore possession of these drugs is controlled. Recently, in New Zealand, there have been a number of forensic science cases involving the illegal possession of hallucinogenic mushrooms.

Until recently, psilocybin and psilocin have been analyzed by thin-layer chromatography [*2*] and ultraviolet (UV) [*4*] and infrared (IR) spectroscopy [*5*]. All these methods require pure samples. The drugs are highly polar and therefore not suitable for direct analysis by gas chromatography. Derivatization followed by gas chromatography/mass spectrometry has been developed for drug quantification [*6*]. The method is specific and sensitive but requires equipment not always available to the forensic scientist and is not suitable for the analyses of sugar-preserved mushrooms, which have been encountered.

A more recent approach has involved the use of high performance liquid chromatography (HPLC), which allows elution of highly polar, nonvolatile substances without derivatization. Psilocybin and psilocin have been separated on a normal phase silica column with a buffered methanol/water solvent [*7*]. Although this method is simple and resolves the two indoles, the use of a silica column has disadvantages as the columns are susceptible to contamination from polar materials and solvents containing water shorten column life [*8*].

A reversed-phase column is more useful because it is versatile and less stringent in terms of requirements for sample size and solvent purity. It was decided, therefore, to develop a method for the quantification of these indole alkaloids on a reversed-phase column.

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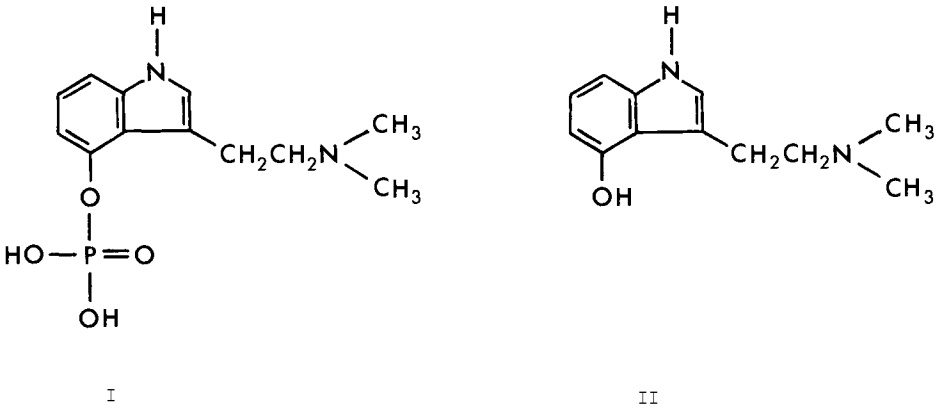


FIG. 1—*Structure of psilocybin* (I) *and psilocin* (II).

## Experimental Procedure

### *High Pressure Liquid Chromatograph*

All components for the high pressure liquid chromatograph were obtained from Waters Associates and included their Model 6000A pump, Model U6K injector, Model 440 UV detector fitted with a 280-nm converter, and a 30-cm μBondapak C18 column.

### *Reagents*

The cationic detergent cetrimonium bromide (cetyltrimethylammonium bromide) was obtained from BDH. Paired ion chromatography (PIC) reagents, in buffered solutions, were supplied by Waters Associates except tetrahexyl-ammonium bromide, which was obtained from Eastman. Samples of pure psilocybin and psilocin were obtained from Sandoz. Other reagents were of analytical grade. All HPLC solvents were filtered through a 0.5-μm filter (Millipore FHUP 04700) to remove particulate matter; this procedure also served to degas the liquids. Any precipitate formed in the cetrimonium bromide solvent was removed by further filtration.

### *Mushroom Extracts*

A weighed amount of mushroom material, about 0.3 g, was oven-dried at 40°C for 16 h and then ground with sand to a fine powder and roller-mixed with methanol for 24 h. The mixture was filtered and the residue washed with methanol. The combined eluants were reduced in volume and reconstituted in 10 mL of methanol. The extract was filtered through a 0.45-μm filter (Millipore FHLP 01300) prior to the injection of 10- to 20-μL aliquots into the HPLC.

For the mushroom material preserved in sugar, about 3 g was roller-mixed directly with methanol with no initial drying period. The remaining extraction procedure was as above. Where doping was performed, 1 mL of a 3.1 mg/5 mL solution of psilocybin in methanol was added to the sugar-preserved sample before it was shaken with methanol.

### *Solvents*

The solvent used for drug quantification was a 40:60:0.15 (v/v/w) methanol/buffer/cetrimonium bromide combination. The aqueous portion was phosphate-buffered to a pH

of 7 to 7.5 with approximately 0.25% disodium phosphate ($Na_2HPO_4$) (w/v) and 0.15% monobasic sodium phosphate ($NaH_2PO_4 \cdot 2H_2O$) (w/v). For measurement of the effect of pH on the rate of drug elutions the methanol/buffer/cetrimonium bromide (40:60:0.15, v/v/w) solvents were prepared at various acidities. The buffer pH was altered by combining various amounts of two solvents containing either 0.4% (w/v) $Na_2HPO_4$ or 0.4% (w/v) $NaH_2PO_4 \cdot 2H_2O$.

## Results

Figure 2 shows a typical chromatogram obtained from a mushroom extract with the methanol/buffer/cetrimonium bromide mobile phase. Since there was psilocin but no psilocybin detected in the mushrooms, a sample of those mushrooms preserved in sugar was doped with psilocybin. Recovery of the psilocybin was 95%. Figure 3 shows that psilocybin in an extract is also well resolved.

Comparison of peak heights with the external standard showed the concentration of psilocin to be 0.28% of the dry weight for the mushrooms not preserved in sugar.

Consistent with other HPLC methods [*7,8*], the retention volume of both drugs can be increased by increasing the amount of aqueous phase in the solvent.

### *Effect of Buffer*

It was found that a minimum phosphate concentration of 0.3% (w/v) was required to give a single peak for psilocybin and a stable baseline. Variation of phosphate concentration above this level resulted in no change to the rate of elution or peak shape for either drug. When an ammonium acetate/acetic acid buffer was used, over the same pH range as for the phosphate buffer, the elution of psilocin could not be delayed sufficiently to resolve it from the polar contaminants in a mushroom extract.


FIG. 2—*Chromatogram of extract from dry mushrooms, on a 30-cm μBondpak C18 column with a 40:60:0.15 (v/v/w) methanol/phosphate buffer (at pH 7.2)/cetrimonium bromide eluant.*

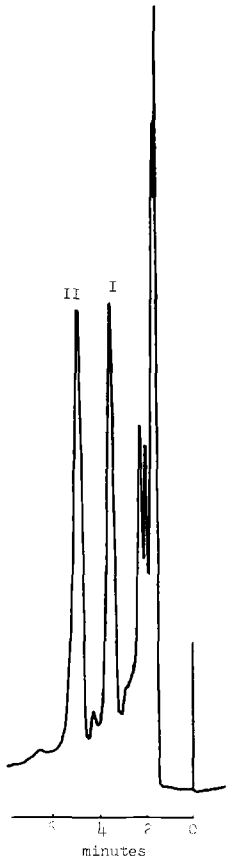


FIG. 3—*Chromatogram of psilocin* (I) *and psilocybin* (II) *in an extract prepared from mushrooms preserved in sugar and doped with psilocybin. Conditions were as for Fig. 2.*

*Effect of pH*

Methanol/buffer/cetrimonium bromide solvents were prepared at various acidities to measure the effect of pH on the rate of drug elution. Retention volume data for standard samples are given in Table 1.

The results show that elution of the two drugs can be brought together by increasing the pH of the solvent. At a pH of not less than 7, both drugs in a mushroom extract are resolved from the solvent front and interfering peaks, and quantification is possible.

*Linearity*

Various concentrations of psilocybin and psilocin were prepared over the range detected in the mushroom extracts. The detector response, for a constant volume injected, was linear for both drugs over this range. After three weeks of continual use it was observed that the peak shape for psilocybin had broadened and was delayed. Column performance was fully restored by washing with water, 50:50 methanol/water, methanol, methylene chloride, hexane, and then back to methanol via methylene chloride for a final wash.

TABLE 1—*Variations of the retention volumes of psilocybin and psilocin as the pH of the mobile phase is varied.*[a]

| Ratio (w/w) $HPO_4^{2-}/H_2PO_4^-$ | Retention Volume, mL | | pH of Aqueous Phase |
|---|---|---|---|
| | Psilocybin | Psilocin | |
| 0:1 | 13.8 | 2.6 | 4.6 |
| 1:4.4 | 13.8 | 2.9 | ... |
| 1:2 | 13.2 | 3.8 | ... |
| 1:1 | 7.8 | 4.7 | 7.2 |
| 2:1 | 8.1 | 6.5 | 7.5 |

[a]Chromatography on a 30-cm μBondpak C18 column with mobile phase consisting of 40:60:0.15 v/v/w of methanol/0.4% aqueous phosphate/cetrimonium bromide.

## Discussion

Initial attempts at the separation of psilocybin and psilocin simply using buffered acetonitrile/water solvent systems were unsatisfactory. Because of the ionic nature of the psilocybin, it was not possible to sufficiently resolve it from both the solvent front and interfering components of the mushroom extract. Variation of solvent pH or polarity altered the elution pattern of psilocin but gave no change to that of psilocybin. Sample clean-up did not improve the chromatograms.

Paired-ion chromatography has been applied to the HPLC analysis of readily ionizable compounds [*9–14*]. The technique involves the addition of a large organic counterion in the mobile phase that is capable of forming a reversible ion-pair complex, which partitions between the stationary and aqueous phases [*8*]. Since psilocybin and psilocin exist as zwitterions, both cationic and anionic ion-pairing reagents were added to the mobile phase in an attempt to alter the rate of drug elution from a reversed-phase column. Alkyl sulfonates with chain lengths C5 to C8 were used as anionic ion-pairing reagents and tetrapropyl-, tetrabutyl-, and tetrahexyl-ammonium ions served as cationic analogues. Although there was a marked increase in the retention volume for psilocin with both cationic and anionic reagents, there was no significant change in that for psilocybin, suggesting that either no complex was formed or that the complex was not partitioning between the mobile and the C18 column phases.

A longer chain quaternary amine, cetrimonium bromide, has been associated with the reversed-phase HPLC of ionic compounds, especially sulfonic acids, for several years and has been used successfully for the separation of food dyestuffs [*11,12*].

When a methanol/buffer/cetrimonium bromide solvent was prepared with the aqueous portion phosphate-buffered, good separations of psilocin and psilocybin were achieved. The mushrooms were dry on receipt (weight loss after oven drying was 5%) and could not be botanically identified beyond the genus *Psilocybe*. However, the psilocin level detected (0.28%) for the mushrooms not preserved in sugar was similar to the psilocin concentration obtained by Repke et al [*6*] for a sample of *Psilocybe cubensis*.

Solvent pH and phosphate concentration were found to have lower limits, and the presence of phosphate was essential to delay the elution of psilocin. It would appear that the phosphate ions are necessary as counterions to allow psilocin to partition between

the stationary and mobile phases and that optimal pH alone is not sufficient for good separation with this solvent system and a reversed-phase column.

### *Mechanism*

Two proposals have been accepted that explain the effect of long-chain alkyl ions on the elution of ionized solutes. The first mechanism postulates that the solute molecule forms an ion pair with the counterion. This "neutral" complex can be reversibly bound to the stationary phase. The alternative theory postulates an ion-exchange mechanism where the counterion is absorbed onto the stationary phase with its ionic group oriented outwards. The solute molecules are subsequently exchanged on this surface [*13–16*].

It now seems likely that a mixed mechanism is involved.[2] Several equilibria are possible between the stationary phase, counterions, and solute ions, and these equilibria are altered by the chain length of the counterion. It appears that elution of solute ions with a short-chain alkyl counterion (up to C6) is essentially via an ion-pairing mechanism whereas for long-chain alkyl counterions (greater than C10, C12) it is via an ion-exchange mechanism.

For the elution of psilocybin, there was a marked change when the counterion was changed from tetrahexyl-ammonium bromide (C6) to cetrimonium bromide (C16), suggesting a change in mechanism over this range. For the alkyl chain length less than 6, the elution of psilocybin could not be delayed sufficiently from the solvent front.

It was found that the system took about 60 min to come to a steady state. If psilocybin was injected into the HPLC prior to this, multiple broad peaks were obtained. This suggests that there is a critical equilibrium established between the stationary column phase and the mobile phase containing counterion, independent of the presence of any sample. This is supportive of a predominantly ion-exchange mechanism.

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