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The Detection of Psilocin in Human Urine*

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ABSTRACT: Pharmacokinetic studies of psilocybin in humans have shown the rapid dephosphorylation of psilocybin to psilocin with further conversion to 4-hydroxy-tryptophole (4HT) and 4-hydroxyindole-3-acetic acid (4HIAA) in plasma. Our study shows that psilocin also undergoes conjugation and can be found in the urine as the psilocin-glucuronide conjugate. Recoveries after enzymatic hydrolysis of the urine with β-glucuronidase (*Helix Pomatia* or *E. Coli*) when compared to non-hydrolyzed urine confirmed the presence of the glucuronide. Detection of psilocin from hydrolyzed and extracted samples was optimized for GC/MS by derivatization with MSTFA. The method developed allows for the detection of psilocin in urine with a limit of quantitation of 10 ng/mL, based on 5 mL of spiked urine. Using this method, our laboratory has confirmed the presence of psilocin in 6 out of 8 urine samples, with concentrations ranging from 10 ng/mL to greater than 200 ng/mL. Before implementation of the hydrolysis and derivatization steps, our limit of detection was 200 ng/mL, based on spiked urine standards. No case samples were positive without hydrolysis and derivatization.

KEYWORDS: forensic science, forensic toxicology, psilocybin, psilocin, GC/MS, urine drug analysis

Psilocybin is an indole derivative of tryptamine found in over twenty species of mushrooms. People wanting to experience the hallucinogenic effects of its de-phosphorylated and pharmacologically active metabolite, psilocin (alternate spelling psilocyn), seek mushrooms containing psilocybin. Users will eat the mushrooms either raw or dried, or, drink the liquid after steeping the fungus in hot water. Psilocybin and psilocin are DEA schedule I drugs.

The rapid and complete de-phosphorylation of psilocybin to psilocin upon ingestion has been established (1,2). A study on the pharmacokinetics of psilocin in rats showed that ^{14}C-labeled psilocin is excreted unchanged in rat urine, as well as in the form of 4-hydroxyindole-3-acetic acid (4HIAA) (3). Although hydrophilic radioactive metabolites were found in the urine, they were not identifiable as glucuronides after treatment with glucuronidase. More recently, Hasler et al. (4) discussed a new metabolite found in plasma, 4-hydroxytryptophole (4HT). The metabolic profile of psilocybin suggested by the authors reiterates the possibility that the phenolic hydroxyl group of psilocin may form a glucuronide conjugate to facilitate excretion; however, such a conjugate had not been identified.

Extraction of psilocin and its metabolites from biological matrices has proved difficult. Manual solid phase extraction (SPE) of psilocin in plasma using reverse phase material (RP18), strong cation exchange (SCX), amino bonded or CBA cation exchange sorbent (carboxymethyl functional group) has produced variable results, purportedly due to the increased oxidation sites on the large surface area of the columns (4,5). Extraction recovery was improved with liquid/liquid extraction, on-line SPE or microdialysis. Hasler et al. (4) found psilocin was easily oxidized in plasma, lowering recovery and creating degradation products. This was resolved by addition of >10mM ascorbic acid to the plasma. These studies (4,5) used high performance liquid chromatography, electrochemical detection (HPLC-ECD) for the analysis. A recent article on solid dose analysis of *Psilocybe subcubensis* mushrooms (6) reported a gas chromatography/mass spectrometry (GC/MS) method for the detection of psilocin after derivatization with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA).

This laboratory routinely receives requests for psilocin analysis from military criminal investigative services. Until recently, the method employed by the laboratory for the detection of psilocin in urine gave a limit of detection of 200 ng/mL, based on 5 mL of spiked urine. Analysis was by GC/MS after SPE for basic drugs using a mixed resin extraction column (7). No derivatization was performed. No cases were positive by this method, even after psilocybin ingestion was admitted. As a result, the method was modified to address the metabolism of psilocybin and improve analytical sensitivity. The following is a report of this modified method.

Experimental

Materials

Psilocin and psilocin-d_{10} in 100 μg/mL methanolic solutions were purchased from Radian International (Austin, TX) and Sigma (St. Louis, MO). The β-glucuronidases (*Helix Pomatia,* type HP-2, and *E. Coli,* type IX-A), bufotenine monooxalate salt:monohydrate, proadifen, and glacial acetic acid (99+ %), were purchased from Sigma. N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) was purchased from Pierce (Rockford, IL). Ammonium hydroxide (trace metal grade) and all other reagents (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA). Clean Screen® CSDAU extraction columns (ZCDAU020) were purchased from United Chemical Technologies, Inc., (Bristol, PA).

Method

Standard methanolic solutions of psilocin were prepared at concentrations of 0.01 mg/mL and 0.001 mg/mL in volumetric glassware. A methanolic solution of proadifen was prepared at a con-

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centration of 0.01 mg/mL for use as internal standard. Four calibrators were prepared in urine at concentrations of 10, 25, 100, and 200 ng/mL. To 5 mL of case samples, psilocin calibrators and a negative urine blank, 100 μL of internal standard, 2 mL of 100 mM pH 6 phosphate buffer and β-glucuronidase (*E. Coli*) were added. The β-glucuronidase was added at a concentration equivalent to 12 500–25 000 U for 5 mL of urine (i.e. 2500–5000 U/mL). All samples were vortexed and pH tested. The pH was adjusted to 6.0 ± 0.5 by dropwise addition of 1M acetic acid if necessary. All samples were placed in a 45°C water bath for 1.5 h, followed by centrifugation at 3000 rpm for 10 min. The supernatant was removed and extracted for basic drugs using a modified version of United Chemical Technologies, Incorporated's, "Therapeutic and abused drugs in urine for GC or GC/MS confirmations using: 200 mg Clean Screen® extraction column," (7). The extraction was performed on a vacuum manifold with 10 mL columns. The columns were conditioned with 3 mL methanol, followed by 2 mL of deionized water and 2 mL of 100 mM pH 6 phosphate buffer. The samples and calibrators were loaded on the columns and eluted. The columns were washed with 3 mL deionized water, 2 mL of 20% acetonitrile and 1 mL of 100 mM acetic acid, then dried under full vacuum for 3 min. A column rinse of 2 mL hexane, followed by 3 mL of hexane/ethyl acetate (50/50) and 3 mL methanol, was employed to remove the acid/neutral drugs. The columns were dried under full vacuum for 3 min. Two mL of freshly prepared methylene chloride/isopropanol/ammonium hydroxide (78/20/2) were eluted through the columns and collected. The eluate was dried at 35°C under a steady stream of nitrogen. The residue was reconstituted with 50 μL of MSTFA and transferred to autosample vials for analysis by GC/MS.

Analysis

One microliter of sample was injected on a Hewlett-Packard 5890/5970 GC/MS system (Palo Alto, CA) equipped with a DB-5 25 m × 0.32 mm × 0.17 μm thick column (J&W Scientific, Folsom, CA). Helium was used as the carrier gas at a constant pressure of 5 psi. The injection port was fitted with a 4 mm split/splitless injection liner with a small glass wool plug at a temperature of 275°C. The oven equilibration time was 1.0 min with an initial temperature of 70°C, raised to 240°C at a rate of 20.0°C /min and held for 2 min. The transfer line temperature was 290°C. The solvent delay was set for 4 min. PFTBA was used as the tune calibrator with "autotune" set as the tune parameters.

Samples were screened by selective ion monitoring (SIM) and confirmed by full scan spectral match. SIM injection was split, with TMS-psilocin ions 58, 290, 348 (7.0 to 9.0 min) and ISTD ions 86, 165 (9.0 min to end) monitored. Scan injection was splitless with all masses between 40 and 500 amu monitored.

Results and Discussion

Without derivatization, psilocin ions were detectable only at concentrations of 200 ng/mL or higher by GC/MS. Even at these high concentrations, chromatography was poor, with wide, inconsistent peak shapes. However, derivatization with MSTFA increased sensitivity over tenfold, lowering the limit of quantitation (LOQ) to 10 ng/mL from 200 ng/mL. The upper limit of linearity (LOL) was 200 ng/mL. The derivatization is simple, fast, and does not require heat. Derivatization with MSTFA creates a trimethylsilyl derivative of psilocin with a molecular weight of 348. The major fragments after electron ionization (EI) have molecular weights of 290 and 58 (Fig. 1).

Three internal standards were investigated throughout the development of the method. Deuterated psilocin (psilocin-d_{10}) was used as internal standard initially, however, validation studies used proadifen as internal standard. Recovery and linearity of psilocin were not affected by the use of proadifen as internal standard. Psilocin-d_{10} is preferred since it derivatizes and has the same properties as psilocin. Bufotenine, an isomer of psilocin, was also investigated as an internal standard. Comparison studies with bufotenine added either before or after extraction showed effective extraction recovery of the internal standard. Bufotenine derivatizes to the same molecular weight trimethylsilyl derivative, producing the same ions of interest (348, 290, and 58). This method separated TMS-psilocin and TMS-bufotenine effectively, with a retention time difference of 0.14 min. Psilocin-d_{10}, proadifen, and bufotenine were all effective internal standards for this method and do not adversely affect the recovery of psilocin. A 100 ng/mL extracted psilocin standard, with both bufotenine and proadifen added as internal standards, is shown in Fig. 2. Internal standard concentrations were 200 ng/mL. Analysis was by SIM, with the 58, 290, and 348 ions selected for TMS-psilocin and TMS-bufotenine, and 86 and 165 for proadifen.

Control urine obtained after consumption of various commercially available mushrooms, including portabella, champignon, shiitake, oyster, and kikurage, was evaluated by the method presented. As expected, none of these specimens contained psilocin.

Eight investigative cases were screened for psilocin in urine by the method presented. Six of the cases were confirmed positive for psilocin, with concentrations ranging from 30 ng/mL to >200 ng/mL. The positive samples were tested in three ways: without hydrolysis, with β-glucuronidase from *Helix Pomatia,* and with acid hydrolysis. For *Helix Pomatia* enzyme, the samples were brought to a pH of 5.0 with 2.0 M acetate buffer, then incubated at 45°C for 1.5 h. The pH of the supernatant was brought to 6.0 ± 0.5 with 1 N KOH. Extraction and analysis proceeded as in the presented method. For acid hydrolysis, 1 mL of concentrated HCl was added to the samples and incubated at 120°C for 0.5 h. The samples were

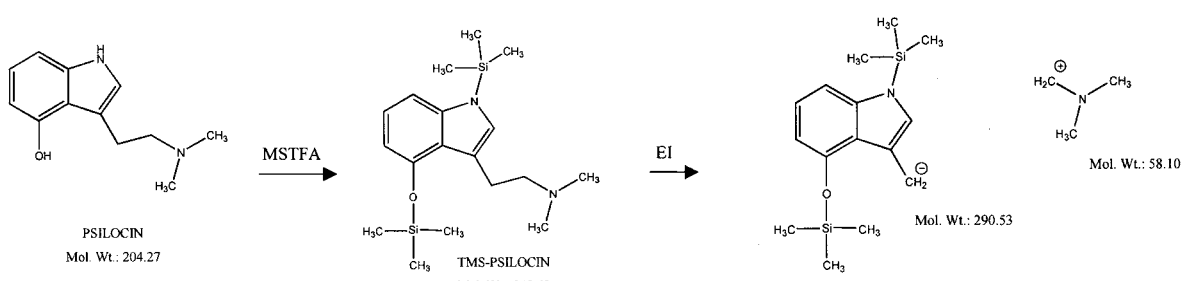

FIG. 1—*Formation and mass fragmentation of TMS-psilocin.*

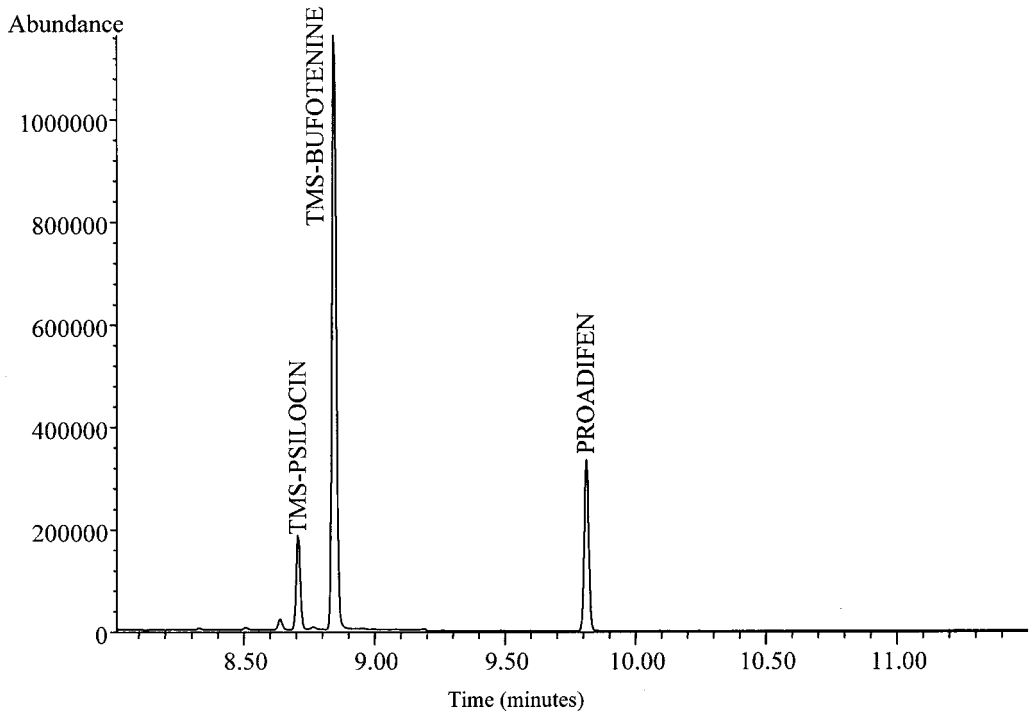

FIG. 2—*Chromatogram of an extracted 100 ng/mL psilocin calibrator with bufotenine and proadifen as internal standards.*

TABLE 1—*Psilocin concentrations in 6 cases.*

Specimen Number	Unhydrolyzed*	Hydrolyzed* (*H. Pomatia*)	Hydrolyzed* (*E. Coli*)
1	<LOQ	10	30
2	41	56	60
3	<LOQ	35	58
4	<LOQ	130	>200 (LOL)
5	<LOQ	31	46
6	<LOQ	32	44

* Concentration in ng/mL.

brought to pH 6.0 ± 0.5 with 500 mM phosphoric acid. Extraction and analysis proceeded as in the method presented.

Acid hydrolysis destroyed the psilocin, as the standards were not identifiable. The unhydrolyzed and *Helix Pomatia* hydrolysis gave quantifiable results and are compared with the *E. Coli* results in Table 1. The samples which were not hydrolyzed but derivatized show only one sample which was above the LOQ at 41 ng/mL. All other samples were less than the LOQ of 10 ng/mL. Thus, out of the six positive cases, only one (#4) would have been detected with hydrolysis (*E. Coli* only) but without derivatization. None of these cases would have been positive without hydrolysis and without derivatization. Only one case (#2) would have been positive without hydrolysis but with derivatization. This case is significant in that the hydrolyzed samples gave only slightly higher recoveries of psilocin, possibly indicating recent use of psilocybin, with psilocin eliminated by first pass metabolism. Enzymatic hydrolysis by either *Helix Pomatia* or *E. Coli* is acceptable. *E. Coli* was chosen as our enzyme of choice since recovery is slightly better, and the pH does not need to be adjusted between incubation and extraction.

Conclusion

Psilocybin and psilocin appear to be excreted as psilocin glucuronide conjugates, as hydrolysis with either *E. Coli* or *Helix Pomatia* β-glucuronidase will break the conjugate. After hydrolysis, extraction of this basic drug is achieved with solid phase extraction utilizing a hydrophobic cation exchange sorbent. The compound is thermally labile and not suitable for mass spectrometry, unless derivatized to a more stable compound. This method identifies psilocin as its trimethylsilyl derivative by GC/MS with a LOD of 10 ng/mL.

Urine is an ideal matrix for detection of psilocin, with the glucuronide conjugate stabilizing the drug from rapid decomposition. Psilocin levels are very low in blood and quickly deteriorate without large amounts of preservative (4,5). Detection of psilocin in urine is indicative of psilocybin or psilocin use.

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