MASSACHUSETTS CRIME LAB

STATE POLICE

ANALYSIS OF PSILOCYBIN AND PSILOCIN IN URINE USING SPE AND LC-MS/MS

FORENSICS

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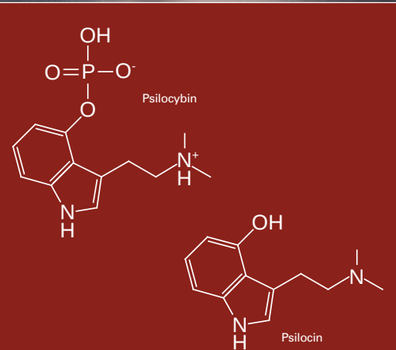
FORENSICS



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INTRODUCTION

Mushrooms that contain or are suspected to contain the hallucinogenic indole alkaloids, Psilocybin and Pscilocin (Figures 1-2) are commonly referred to as "Magic Mushrooms". These include Psilocybe cubensis, P. mexicana, P. subcubensis, P. semilanceata, P. argentipus (Japanese name: Hikageshibiretake). They are naturally occurring and have been used as a god-like traditional medicine for centuries in the religious ceremonies by shamans in Central and South America. Currently, they have been used extensively for recreational purposes as hallucinogenic substances in various countries in Europe, America, and even in Japan. The hallucinogenic ingredients Psilocybin and Psilocin were isolated by Hofmann et al. in 1958. They have structural similarity to the neurotransmitter serotonin, and their highly hallucinogenic potency is thought to occur from their influence on the serotonergic nervous system. The contents of psilocybin and psilocin in these "Magic Mushrooms" have been reported to vary over a wide range from a trace amount to 0.2–0.4% in a dried mushroom. This wide variation has sometimes resulted in hallucinogenic intoxication by overdosing on "Magic Mushrooms".

Previous methods for the analysis of psilocybin and psilocin in biological materials have employed the use of gas chromatography and mass spectrometry. The major problem with this procedure is that the phosphate group (PO4) is cleaved from the parent compound (Psilocybin) leaving only the psilocin. With this data, the analyst is presented with only a value for the total Psilocin content of the sample.

Methods employing high performance liquid chromatography with ultraviolet detection systems have been reported, as have procedures employing high performance liquid coupled to tandem mass spectrometers. These previously reported methods have employed a liquid-liquid extraction prior to injection. This new method utilizes the benefits of solid phase extraction (SPE) to remove interfering materials and present a clean extract for analysis by tandem mass spectrometry.

Although previous SPE methods of analysis have focused their attention upon the metabolite (Psilocin) in biological samples , no single SPE method has been published to date that permits forensic analysts to isolate, and analyze both compounds with the aid of fast chromatography and tandem mass spectrometry. This SPE method coupled with fast LC-MS/MS provides a simple, sensitive, and reproducible quantitative method for the analysis of Psilocybin and Psilocin in a urinary matrix. This procedure should also be of great assistance to those analysts actively involved with the LC-MS/MS analysis of these drugs in biological matrices.

MATERIALS AND METHODS

Reagents

Psilocybin and psilocin were obtained from Alltech (Deerfield, IL), ethyl morphine(IS) from Cerilliant (Roundrock, TX), and formic acid (99%) from Acros Organics via Fisher Scientific (Pittsburgh, PA). All other solvents were HPLC grade or better and were obtained from Fisher Scientific.

Standard stock reference solutions of Psilocybin/ Psilocin (1.0 µg/mL) were prepared in acetonitrile. Calibrators were prepared by spiking drug free urine over the range 10 to 1000 ng/mL. Another stock reference solution was prepared in acetonitrile (1.0 µg/ mL) and was used for the preparation of controls at 10 and 250 ng/ mL. Psilocybin/ Psilocin concentrations in the control specimens were calculated from linear regression of the standard responses based on the peak-area ratios.

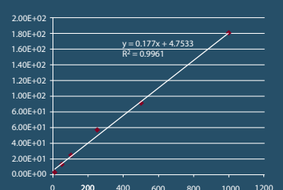
Extraction

Psilocybin/ Psilocin and the internal standard (ethyl morphine) were extracted from buffered (pH 6) urine samples (1 mL) utilizing Clean Screen DAU SPE cartridges (UCT, Inc.). After washing the columns (DI water and methanol (3 mL of each respectively)), samples were eluted with ethyl acetate/2% ammonium hydroxide (3 mL), followed by methanol/ 4% ammonium hydroxide (3 mL). Each eluate solution was evaporated to dryness separately. To the dry eluates, 250 µL of 0.1% aqueous formic acid was added, which was combined prior to the chromatographic analysis.

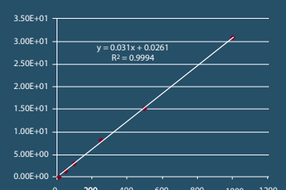
Instrumentation

Liquid chromatography equipment consisted of a Shimadzu Prominence (two pumps LC-20AD, autosampler SIL-20AC, and column oven CTO-20AC) and was performed using Imtakt™ C18 column (50x 2.1mm, 5 µm)at 0.5mL/min. flow, using a gradient program. The mobile phase program: (A) 0.1% aqueous formic acid / (B) acetonitrile containing 0.1% formic acid was started at 5% (B) for 0.5 min, increasing to 90% (B) over 4 min, before returning to 5% (B) and equilibrated for 1 min. The total chromatographic run time was 6 minutes including equilibration.

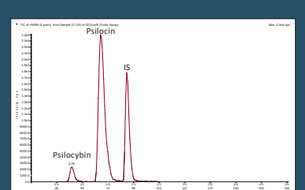
MS/MS analysis was conducted using an Applied Biosystems 3200 Q Trap instrument equipped with ESI source in the positive ion mode. It was operated with multiple reaction monitoring (MRM) under the following conditions: curtain gas 15 psi, collision gas medium, ion spray voltage 5000V, temperature 650°C, ion source gas (1 and 2) 50 psi. The following transitions were monitored (quantification ions underlined): m/z 284.9 → 205.2, 240.0, and for Psilocybin, and m/z 205.6 → 116.1, 115.1 for Psilocin. The internal standard (ethyl morphine) was monitored at the following transitions: m/z 314.2 →152.2, 128.3.



Standard Curve of Psilocybin



Standard Curve for Psilocin



Chromatogram of Psilicybin/Psilocin extracted from urine (10 ng/ mL)

RESULTS AND DISCUSSION

Linearity (r2 >0.99) was achieved from 10 to 1000 ng/ mL using a six point calibration curve, and the limits of detection and quantification were 5 and 10 ng/ mL, respectively. Recovery values for blood and urine (target values: 10 and 250 ng/mL) were greater than 85%. Intra and inter-day precision was less than 5% and 8%, respectively. Ion suppression studies revealed that suppression of monitored ions was less than 6%.

CONCLUSIONS AND CHALLENGES MET

In this methodology, the aim was to develop a SPE procedure in which both psilocybin/psilocin could be efficiently extracted from urine samples. It was concluded that:

1. Psilocin can be extracted from urine at pH 6 and eluted with ethyl acetate containing 2% ammonium hydroxide but psilocybin requires a more polar solvent system i.e. methanol containing 4% ammonium hydroxide.
2. When psilocybin and psilocin are collected together, the psilocin is degraded in the presence of excess base, thus therefore should be collected/ evaporated separately.
3. In terms of LC-MS/MS, psilocin has the same MRM's as Bufotenine. Thus, it must be chromatographically resolved from psilocin. Use of isotope labeled internal standards (or a different compound) is advised.
4. Enzymatic hydrolysis does not degrade Psilocybin but releases Psilocin from its glucuronide.
5. Extracted samples are stable at 10 °C in an LC auto sampler compartment for at least one week.
6. LOD of 5ng/ mL and LOQ of 10 ng/ mL of urine are possible by SPE and LC-MS/MS.