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Liquid Chromatography-Mass Spectrometric and Liquid Chromatography-Tandem Mass Spectrometric Determination of Hallucinogenic Indoles Psilocin and Psilocybin in "Magic Mushroom" Samples*

ABSTRACT: Accurate and sensitive analytical methods for psilocin (PC) and psilocybin (PB), tryptamine-type hallucinogens contained in "magic mushrooms," were investigated using liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS-MS). The chromatographic separation on an ODS column and mass spectral information gave complete discrimination between PC and PB without derivatization. The mass spectrometric detection had a high sensitivity, and the tandem mass spectrometric detection provided more specificity and accuracy, as well as high sensitivity. The detection limits ranged from 1 to 25 pg by LC-MS in the selected ion monitoring mode, and the intra- and inter-day coefficients of variation were estimated to be 4.21–5.93% by LC-MS-MS in the selected reaction monitoring mode. By applying the present LC-MS-MS technique to four real samples, the contents of PC and PB were found to vary over a wide range (0.60–1.4 and 0.18–3.8 mg/g dry wt. for PC and PB, respectively) between samples.

KEYWORDS: forensic science, psilocin, psilocybin, magic mushrooms, liquid chromatography-mass spectrometry, liquid chromatography-tandem mass spectrometry

"Magic mushrooms (MMs)," which is the street name for mushrooms containing hallucinogenic indole alkaloids, psilocin (PC) and its phosphate ester, psilocybin (PB) (Fig. 1), include *Psilocybe cubensis*, *P. mexicana*, *P. subcubensis*, *P. semilanceata*, *P. argentipus* (Japanese name: Hikageshibiretake), etc. 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. This has led to the placement of the fruit body (including its powder) of the mushrooms containing PC and/or PB on a contraband list in June 2002 in Japan.

The hallucinogenic ingredients PC and PB were isolated by Hofmann et al. in 1958 (1). They have structural similarity to the neurotransmitter serotonin, and their highly hallucinogenic potency is thought to occur from their influence on the serotoninergic nervous system. The contents of PC and PB in MMs have been reported to vary over a wide range from a trace amount to 0.2–0.4% in a dried mushroom (2), and this wide variation has sometimes resulted in hallucinogenic intoxication by overdosing on MMs. PC and PB identification is important for forensic toxicology. Thus, simple, sensitive, and accurate analytical methods, which allow us to determine PC and PB in mushroom samples, have been in high demand.

The determination of PC and PB has often been studied by thin-layer chromatography (3–10), high performance liquid chromatography (HPLC) with ultraviolet detection (5,6,8,11–16), electrochemical detection (8,11,13,17,18) and fluorescence detection (11), gas chromatography (GC) with flame ionization detection and infrared spectrometry (19), ion-mobility spectrometry (20), and gas chromatography-mass spectrometry (GC-MS) with/without trimethylsilyl derivatization (7,9,10,19–25). However, the above-mentioned HPLC and GC systems show less specificity compared with mass spectrometry. Also, the time-consuming derivatization step is indispensable for the discrimination between PC and PB by GC-MS because PB is readily pyrolyzed to form PC through the high temperature injection port without derivatization (2), though a rapid GC-MS method for identification of only PC has been reported (25). On the other hand, liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS-MS) have advantages because of their applicability to thermolabile compounds and inherent high sensitivity. These results suggest the superiority of the LC-MS and LC-MS-MS techniques for the simultaneous determination of PC and PB. The LC-MS and LC-MS-MS are also becoming popular in forensic toxicology. Only a few reports, however, have briefly mentioned LC-MS for PC and PB (14,26), and a detailed investigation of the analytical conditions has never been reported.

In this study, LC-MS and LC-MS-MS conditions for the simultaneous determination of PC and PB were investigated. The

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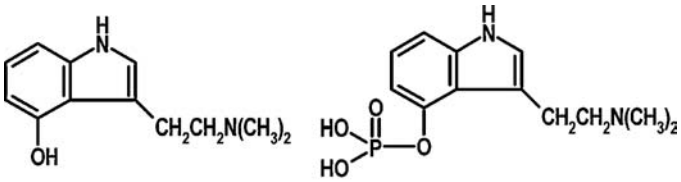

Psilocin **Psilocybin**

FIG. 1—*Chemical structures of psilocin and psilocybin.*

developed methods also were applied to some mushroom samples, and the large variation in the PC and PB contents between samples was confirmed.

Experimental

Chemicals

PC and PB were provided by the Ministry of Health, Labour and Welfare of Japan. Standard stock solutions were prepared in methanol (1 mg/mL). The solutions of these drugs were stored at −20°C until used and adjusted to the appropriate concentration with distilled water or methanol immediately prior to use. Acetonitrile was of HPLC-grade, and the other chemicals used were of analytical grade from Wako (Osaka, Japan). The 10 mM formate buffer used for the mobile phase was prepared by adjusting a 10 mM ammonium formate aqueous solution to pH 3.5 with formic acid.

The quantitated mushroom samples were as follows: two "native" *P. argentipes* (Samples 1 and 2) were collected in Kyoto, Japan, and a dried fruit body (Sample 3) of *P. subcubensis* and a powder sample in a plastic capsule (Sample 4) were exhibits seized in Osaka, Japan.

Instrumentation

LC-MS was carried out on a ZMD (Waters, Milford, MA, USA) equipped with an Alliance 2690 pump (Waters). The column used was an L-column ODS (1.5 mm i.d. × 150 mm) (Chemicals Evaluation and Research Institute, Tokyo, Japan). The mobile phase consisted of acetonitrile—10 mM formate buffer (pH 3.5) (12.5/87.5, v/v). Electrospray ionization (ESI) (positive mode) was selected, and the operating conditions for the analysis were as follows: flow rate, 0.1 mL/min; capillary voltage, 4.0 kV; cone voltage, 33 V; ion-source temperature, 300°C.

LC-MS-MS was accomplished on a Quattro LC (Micromass, Manchester, UK) equipped with an Agilent 1100 pump (Agilent Technologies, Palo Alto, CA, USA) under the same chromatographic conditions and operating parameters, except that the ion-source temperature was set at 280°C. Argon was used as the collision gas at a collision energy of 15 eV. Ions of m/z 205 and m/z 285 were selected as precursor ions for PC and PB, respectively.

The instrumental analyses were optimized and validated by analyzing standard solutions where the concentrations of the added PC and PB were 1 and 5 μg/mL, respectively. Quantitation of the hallucinogens in the mushroom samples was performed by LC-MS-MS in the selected reaction monitoring (SRM) mode. The monitored reactions were m/z 205 > 160 for PC and m/z 285 > 240 for PB, and the integrated peak areas were measured.

Sample Preparation

Approximately 25 mg of mushroom samples were cut into small pieces, after air-drying if necessary, and then soaked in 2 mL of methanol at 25°C overnight in glass test tubes. The powder sample was directly soaked in the same manner. A 50-mL aliquot of the methanolic extract was evaporated to dryness under a gentle stream of nitrogen at 40°C, the residue was dissolved in 100 μL of the mobile phase, and a 5-μL aliquot each was injected into the LC-MS and LC-MS-MS systems.

Results and Discussion

Sample Preparation (Extraction of PC and PB from Mushrooms and Clean Up)

As a pre-experiment, refluxing with methanol at 50°C and sonication was performed. However, no significant improvement in the extraction was obtained in our investigation, and therefore the extraction was then carried out by soaking the mushrooms in methanol.

To remove the other ingredients in the mushrooms from the methanolic extracts, liquid-liquid extraction (LLE) and solid-phase extraction (SPE) were explored: LLE with chloroform under an acidic or alkaline condition was performed after dilution of the methanolic extracts with water. SPE techniques also were examined using three types of SPE cartridges (the reversed-phase-, cation-exchange-, and strong cation-exchange-type cartridges). However, with either LLE or SPE, no significant improvement in cleanup was achieved. Thus, we finally used the methanolic extracts without further cleanup for the following steps.

LC-MS

The spectral profile and sensitivity of the ESI MS are known to be largely dependent on the capillary and cone voltages. In order to optimize the capillary and cone voltages for analysis of PC and PB, the capillary and cone voltages were varied between 2 and 5 kV, and 10 and 40 V, respectively. In this study, 4.0 kV for the capillary voltage and 33 V for cone voltage were chosen, where the highest intensity of the protonated molecule ($[M + H]^+$) at m/z 285 for PB and a sufficiently high intensity of $[M + H]^+$ at m/z 205 for PC were observed in their mass spectra.

Additionally, the chromatographic conditions were optimized for the separation of PC and PB using the ODS column. PB was not retained under the neutral condition of the mobile phase, while the acidic condition restrained the dissociation of the phosphoric acid moiety in PB, achieving an appropriate retention. The mobile phase was then optimized to be 12.5% of acetonitrile concentration in 10 mM ammonium formate (pH 3.5), which gave the retention times of 4.9 min for PC and 2.9 min for PB (Figs. 2*A* and 3*A*).

The spectra of both PC and PB were characterized by each predominant $[M + H]^+$ ion at m/z 205 for PC and m/z 285 for PB (Figs. 2*B* and 3*B*). The detection limits of PC and PB were estimated to be 25 pg and 2500 pg in the scan mode, and 1 pg and 25 pg in the selected ion monitoring mode, respectively. The intra- and inter-day coefficients of variation obtained at the sample concentration of 1 μg/mL for PC and 5 μg/mL for PB were 6.04 and 7.58% for PC, and 5.77 and 8.19% for PB, respectively. The resultant data suggest superiority in the sensitivity and precision for the analysis of PC and PB in comparison with the data obtained from the pre-experiments by GC-MS in our laboratory (no data shown).

Based on the above investigation, the LC-MS technique, which allows the rapid, sensitive, and accurate determination of PC and PB, has been developed.

In addition, based on the optimized conditions, the methanolic extracts of mushrooms were subjected to the LC-MS analysis. Not

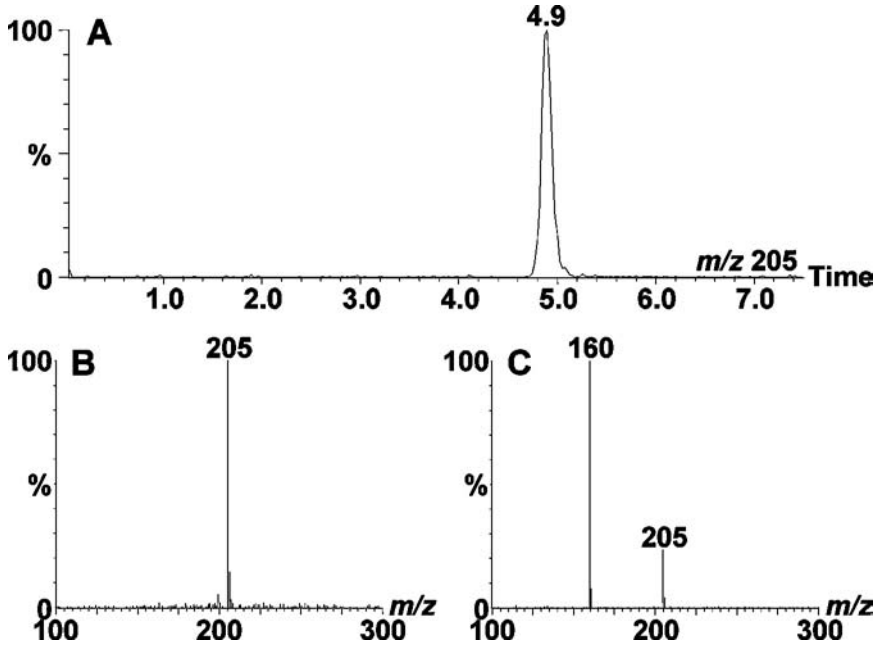

FIG. 2—*Extracted mass chromatogram at m/z 205 (A) and mass spectrum of psilocin (B) from the LC-MS analysis and product ion spectrum of psilocin (C) from the LC-MS-MS analysis. Precursor ion selected for the LC-MS-MS analysis was m/z 205. The other conditions are listed in the text.*

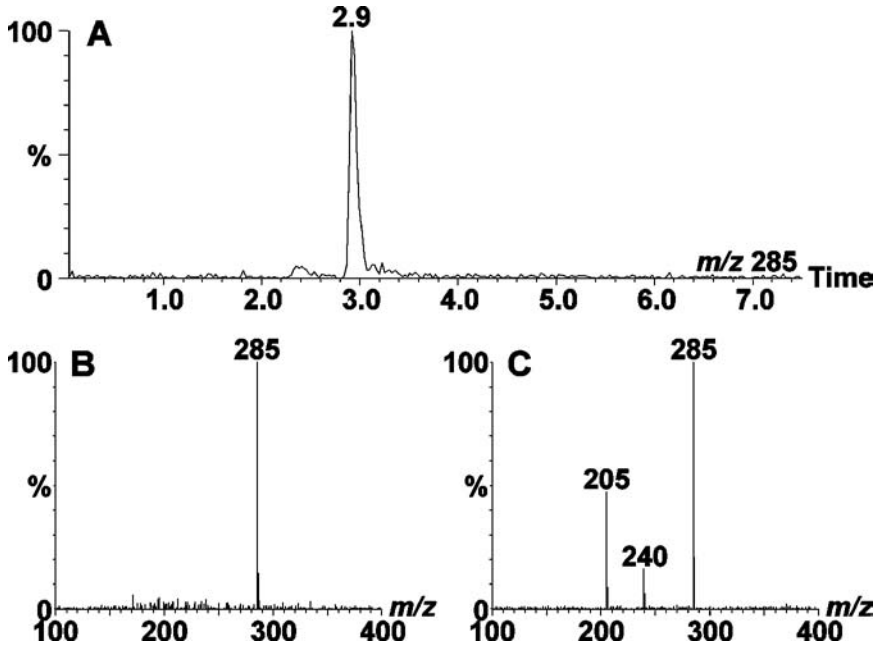

FIG. 3—*Extracted mass chromatogram at m/z 285 (A) and mass spectrum of psilocybin (B) from the LC-MS analysis and product ion spectrum of psilocybin (C) from the LC-MS-MS analysis. Precursor ion selected for the LC-MS-MS analysis was m/z 285. The other conditions are listed in the text.*

only the resultant extracted mass chromatograms at *m/z* 205 and 285, but also the total ion chromatogram, showed no large impurity peak in the mushroom sample (Fig. 4). This is probably caused by the fairly low ionization property of other ingredients in the sample under the developed conditions, suggesting that LC-MS would be advantageous for the analysis of the mushroom samples.

LC-MS-MS

It is well known that the ESI mass spectra often provide an abundant intensity of $[M+H]^+$and few fairly low intensities for fragment ions, which is favorable for a quantitative analysis, but disadvantageous for identification. On the other hand, LC-MS-MS gives a more characteristic product ion spectrum which reflects well the molecular structure of the analyte, which is favorable for identification. Thus, for the more accurate and reliable identification of PC and PB, the LC-MS-MS technique was selected, and the analytical parameters were investigated further.

To optimize the MS-MS conditions, the collision energy was varied between 10 and 25 eV. At the collision energy of 15 eV, the highest intensities of some specific product ions at *m/z* 160 due to $[M\text{-}(CH_3)_2N]^+$ for PC, and at *m/z* 240 and 205 due to

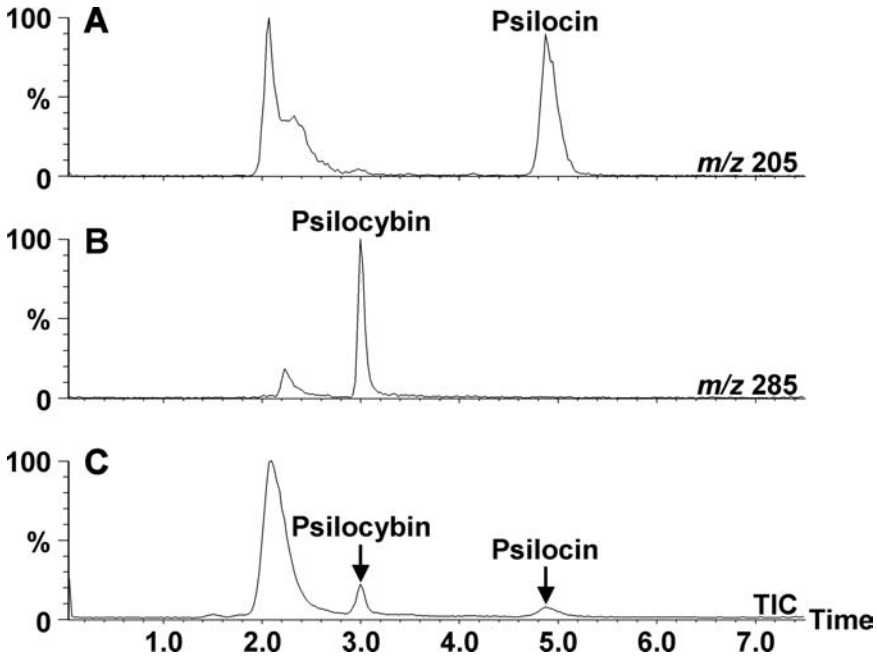

FIG. 4—*Extracted mass chromatograms at m/z 205 (A) and 285 (B) and total ion chromatogram (C) from the LC-MS-MS analysis of the methanolic extract of the "magic mushroom." The analytical conditions are described in the text.*

$[M\text{-}(CH_3)_2N]^+$ and $[M\text{-}H_2PO_3 + 2H]^+$, respectively, for PB were obtained (Figs. 2*C* and 3*C*). Thus, the finally selected conditions of MS-MS were as follows: capillary voltage, 4.0 kV; cone voltage, 33 V; collision energy, 15 eV.

The detection limits of PC and PB were calculated to be 50 pg and 5000 pg, respectively, in the scan mode. When the SRM technique was applied, in which the reactions m/z 205 > 160 for PC and m/z 285 > 205 for PB were monitored, the limits were 2 pg and 40 pg. The intra- and inter-day coefficients of variation (n = 3 per day) obtained at the sample concentration of 1 μg/mL for PC and 5 μg/mL for PB in the SRM mode were 4.21 and 5.70% for PC and 4.27 and 5.93% for PB, respectively.

In the present study, LC-MS-MS had a higher precision but lower sensitivity than LC-MS. This was probably because the sample used for the investigation was a standard aqueous solution which would produce a low background. Compared with the LC-MS, the LC-MS-MS analysis usually provides a higher sensitivity and precision because of the lower background noise from the sample matrices. This will be advantageous especially for the analysis of a biological sample. In a future study on the analysis of PC and PB in biological fluids including urine and blood, the developed LC-MS-MS technique will be a more powerful tool than LC-MS.

Quantitation of PC and PB in Various Kinds of Mushroom Samples

The LC-MS-MS analysis in the SRM mode was applied to the quantitation of PC and PB in four mushroom samples. The standard aqueous solution samples, in which the concentration of the added PC and PB were varied, were analyzed using LC-MS-MS in the SRM mode, and calibration curves were constructed by plotting the peak area versus the concentration of PC and PB. The calibration curves showed a good linearity throughout the range from 0.05 to 5 μg for both PC and PB. Fresh and wet samples (Samples 1 and 2) were dried before the sample preparation, and the other samples were directly subjected to the sample preparation. The PC and PB contents estimated using the calibration curves ranged from 0.60 to 1.4 mg/g dry wt. of PC and from 0.18 to 3.8 mg/g dry wt. of PB (Table 1).

TABLE 1—*Results of the quantitative analysis.*

Sample No.	Form	Species	Hallucinogen Content (mg/g dry wt.) PC	PB
1	Raw mushroom	*P. argentipes*	0.69	3.8
2	Raw mushroom	*P. argentipes*	0.60	3.2
3	Dried mushroom	*P. subcubensis*	1.0	1.5
4	Brown powder	Unknown	1.4	0.18

Chamakura described that the Mexican sacred mushrooms contained trace amounts of PC and 2–4 mg/g of PB (2). Koike et al. also reported that the PB content of *P. argentipes* was 0.03–5.5 mg/g (4). Our quantitative values for Samples 1 and 2 showed good agreement with the previously reported PB content. Keller et al. reported that *P. subcubensis* contained 0.2 mg/g (cap) and 0.3 mg/g (stem) of PC and 8.6 mg/g (cap) and 8.0 mg/g (stem) of PB (20). The PC/PB ratio of Samples 3 and 4 was much larger than their reported ratio. From these findings, it may be expected that the PC/PB ratio increases through the change in PB to PC during storage of the mushrooms. In addition, our study suggested a wide range of variation of the hallucinogen contents between the species or forms of the samples. It is likely that the acute intoxication by the "MMs" has been due to the wide variation in the hallucinogen contents and resultant overdoses.

Conclusions

Analytical methods for PC and PB in mushroom samples by LC-MS and LC-MS-MS were optimized. Overnight soaking in methanol was preferable for the satisfactory extraction of PC and PB from the mushroom samples. LC-MS on an ODS column with an

acidic mobile phase achieved a rapid, sensitive and accurate determination. LC-MS-MS provided characteristic product ion spectra reflecting their chemical structures and more accurate determination. Application of the present LC-MS-MS technique to some mushroom samples revealed that a wide range of variation for PC and PB existed between samples.

In the present study, mushroom sample was used. However, the developed LC-MS and LC-MS-MS techniques will be a powerful tool, especially in the forensic toxicological study for the analysis of PC and PB in biological fluids. The overnight soaking was employed for quantitative extraction in the present method. However, in order to perform a faster qualitative analysis, a shorter extraction time would be substituted for the present extraction procedure. For the purpose of a high throughput analysis of biological samples, a faster chromatographic separation with a short column or by capillary electrophoresis also would be preferable.

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