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Determination of psilocybin in hallucinogenic mushrooms by reversed-phase liquid chromatography with fluorescence detection

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Abstract

The determination of psilocybin was carried out by reversed-phase liquid chromatography (HPLC) with fluorescence (FL) detection. Psilocybin was labeled with 5-dimethylaminonaphthalene-1-[*N*-(2-aminoethyl)]sulfonamide (DNS-ED) at 60 °C for 4 h in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) as the activation reagent. The resulting derivative was separated on a Mightysil RP-18 GP column (150 mm × 4.6 mm, i.d. 3 μm) with the mixture of 50 mM ammonium acetate ($AcONH_4$) and CH_3CN, and detected at 539 nm (excitation at 321 nm). The structure of the derivative was identified by HPLC–ESI-MS. A good linear relation of the calibration curve of psilocybin was observed under the proposed conditions for labeling, separation and detection. The quantification limit was 4.4 ng in 1 mg dried mushroom. The proposed procedure was successfully used for the determination of psilocybin in real samples. The contents of psilocybin in six magic mushrooms by the proposed HPLC–FL method were less than 20.0 ng in 1 mg dried samples.

Keywords: Psilocybin; Hallucinogenic mushroom; Fluorescence labeling; Reversed-phase HPLC

1. Introduction

Psilocin and its phosphoryloxy derivative, psilocybin, are biologically synthesized in mushrooms belonging to the genera of *Psilocybe* [1,2]. Psilocin and psilocybin have been identified worldwide in a variety of mushrooms, which are called "magic mushrooms". The ingestion of small amounts of the hallucinogens induces ecstasy. The hallucinogenic activity of psilocybin is relatively lower than that of psilocin. However, psilocybin is easily converted to psilocin (a major active constituent) in biological systems. Thus, the development of a determination method of psilocybin is important, the same as that of psilocin. In Japan, magic mushrooms involving hallucinogenic activity were ruled illegal on 6 June 2002. In order to carry out a screening of hallucinogenic mushrooms, a suitable method is required that allowed the determination of the mushroom components to be easily done and with a great deal of specificity and sensitivity. The trace analysis of the hallucinogens is thus of great interest.

Psilocybin in fungus material was quantified by high-performance liquid chromatography (HPLC) coupled with ultraviolet (UV) [3–7], native fluorescence (FL) [8–10] and electrochemical (EC) detection [10,11]. Gas chromatography (GC)–mass spectrometry (MS) has also been introduced in order to increase the sensitivity and selectivity for the determination of psilocybin in a mushroom sample [12–14]. The quantification range of psilocybin was 100–900 ng in a 1 mg dried mushroom. The sensitivity seems to be insufficient for the trace analysis of actual samples. Furthermore, the GC method required volatilization with *N*-methyl-*N*-(trimethylsilyl)-2,2,2-trifluoroacetamide (MSTFA), etc., before analysis. The GC method seems not to be recom-

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mended for psilocybin analysis because of the easy dephosphorylation to psilocin at high temperature [15]. As a result, the precise and reliable determination of trace amounts of psilocybin in biological specimens may be fairly difficult.

This paper describes the development of a determination method for psilocybin with increased sensitivity. In this research, the determination of psilocybin is carried out by HPLC with fluorimetry (FL) after derivatization with a fluorescence labeling reagent, 5-dimethylaminonaphthalene-1-[*N*-(2-aminoethyl)]sulfonamide (DNS-ED) [16–18]. The proposed method was used for the analysis of magic mushrooms.

2. Experimental

2.1. Materials and reagents

Psilocybin was synthesized according to a previous report [19]. 5-Dimethylaminonaphthalene-1-[*N*-(2-aminoethyl)] sulfonamide (DNS-ED) was purchased from Molecular Probes (USA). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide·hydrochloride (EDC) was supplied by Wako Pure Chemicals (Tokyo, Japan). 1-Methylimidazole was obtained from Kanto Chemicals (Tokyo, Japan). 3-Indoxyl phosphate·disodium salt (3-IP, ICN) was used as an internal standard (IS). Acetonitrile (CH_3CN), methanol (MeOH) and *N*,*N*-dimethylformamide (DMF) were of special reagent grade (Wako Pure Chemicals). Deionized and distilled water was used in all the experiments. All other chemicals were of analytical-reagent grade and used without further purification.

2.2. HPLC–ESI-MS

A Hewlett-Packard 1100 series HPLC (Wilmington, DE, USA) coupled to a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) fitted with an ESI source was used. Psilocybin labeled with DNS-ED was separated on a Mightysil RP-18 GP column (100 mm × 2 mm, i.d. 3 μm) using the mixture of 50 mM ammonium acetate ($AcONH_4$)–CH_3CN (73:27) as the eluent. The flow rate of the mobile phase was 0.15 mL/min. The ESI capillary temperature and capillary voltage were 200 °C and 4.0 V, respectively. The tube lens offset was set at 15.0 V. The sheath gas flow rate, aux gas flow rate, and spray voltage were 70 arb (1 L/min), 10 arb (3 L/min), and 4.00 kV, respectively. All spectra were obtained in the positive ion mode over a mass range of m/z = 150–800 and at a scan speed of 0.5 scan/s.

2.3. HPLC–FL

A Shimadzu (Kyoto, Japan) HPLC system that consisted of two LC-10AD pumps, an SCL-10A system controller, an auto-injector (SIL-10A_{XL}), and a degasser (DGU-12A) was used. The analytical column for reversed-phase chromatography was a Mightysil RP-18 GP column (150 mm × 4.6 mm, i.d. 3 μm; Kanto Chemical). The column was maintained at 40 °C by a CTO-10A column oven (Shimadzu). The effluent was monitored with a RF-10A_{XL} FL detector equipped with a 12 μL flow cell (Shimadzu). The flow rate of the mobile phase was 1.0 mL/min.

Psilocybin in 10 μL of 100 mM 1-methylimidazole buffer (pH 7.0) containing 100 μM 3-IP (IS) reacted with 10 mM DNS-ED in 40 μL DMF in the presence of 10 μL of 100 mM EDC dissolved in 100 mM 1-methylimidazole buffer (pH 7.0). After the reaction at 60 °C for 4 h, the solution was diluted 20 times with the mobile phase, i.e., 50 mM $AcONH_4$–CH_3CN (77:23), separated on a Mightysil RP-18 GP column and detected at 539 nm (excitation at 321 nm). The elution conditions were as follows: an isocratic elution of 50 mM $AcONH_4$–CH_3CN (77:23) for 22 min and then a linear gradient elution from 50 mM $AcONH_4$–CH_3CN (77:23) to 50 mM $AcONH_4$–CH_3CN (40:60) for 13 min.

2.4. Calibration curve

A volume of 1 mM psilocybin in MeOH was diluted to fixed concentrations (31.25–500 μM, 8.88–142.1 μg/mL) with 100 mM 1-methylimidazole buffer (pH 7.0) containing 3-IP (IS) (100 μM, 21.42 μg/mL); then 10 mM DNS-ED in DMF (40 μL) and 100 mM EDC in 0.1 M 1-methylimodazole buffer (pH 7.0) (10 μL) were added to each solution (10 μL). After heating at 60 °C for 4 h, the reaction solutions were diluted 20 times with 50 mM $AcONH_4$–CH_3CN (77:23), and then an aliquot (5 μL) was subjected to HPLC–FL. The peak area ratios of the analyte to IS were plotted versus each concentration of psilocybin.

2.5. Determination in magic mushroom

A dried magic mushroom was pulverized in a glass mill. A volume of 1 mL MeOH containing 20 μM (4.28 μg/mL) 3-IP (IS) was then added to 20 mg of the powder. The solution was sonicated for 30 min at a temperature lower than 50 °C. The suspension was then centrifuged at 3000 rpm for 5 min. The solution was separated, and the residues were extracted two times with 1 mL MeOH under the same manner. The combined MeOH extracts were evaporated under reduced pressure. The resulting residues were redissolved in 200 μL of 100 mM 1-methylimidazole buffer (pH 7.0) and filtered through a 0.2 μm membrane filter (Millex-LG). Then, 10 μL of filtrate was reacted with 40 μL of 10 mM DNS-ED in DMF and 10 μL of 100 mM EDC in pH 7.0 buffer. After heating at 60 °C for 4 h, the reaction solutions were diluted 20 times with 50 mM $AcONH_4$–CH_3CN (77:23), and then an aliquot (5 μL) was subjected to HPLC–FL.

3. Results and discussion

3.1. Optimization of labeling and separation

Based on previous research concerning the reaction of DNS-ED and nucleotides [16,17], the reaction of psilocybin with DNS-ED seems to proceed in the presence of an activation reagent such as EDC (Fig. 1). Thus, the reaction conditions affecting the reaction rate were optimized. The effect of the reaction temperature was initially studied. Fig. 2 shows the time courses of the labeling reaction at each temperature (40–60 °C). The reaction effectively proceeded with elevated temperature. The maximum yield was obtained at 60 °C in 4 h and then gradually decreased. The concentration of the labeling reagent is another important factor for the reaction. As shown in Fig. 3, the reaction yields were drastically increased with the change in the DNS-ED concentration over 10 mM. The existence of EDC is essential for activating the phosphroyloxy group in psilocybin. Based on the results shown in Fig. 4, the EDC concentration of more than 100 mM is required to obtain a constant yield. Consequently, the labeling reaction of psilocybin was carried out with 10 mM DNS-ED at 60 °C and 4 h in the presence of 100 mM EDC. This type of reaction sometimes interfered with by moisture in the reaction medium. Therefore, the influence of water in the reaction medium was tested. The interference was not identified even with a 30% concentration of water in the medium (Fig. 5). Thus, the volume ratio of DMF and 100 mM methylimidazole buffer (pH 7.0) was determined to be 2:1.

Under the recommended conditions, psilocybin was efficiently labeled with DNS-ED (Fig. 1). The peak of the resulting derivative was separated by an ODS column with the mixture of 50 mM $AcONH_4$ and CH_3CN as the mobile phase. When the separation condition was adopted for the mushroom sample, many peaks appeared on the chromatogram. There-

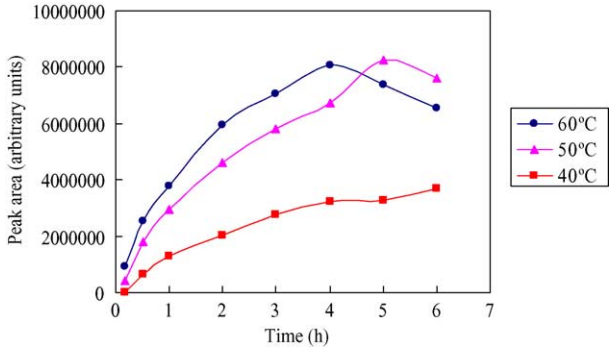

Fig. 2. Time courses of labeling reaction of psilocybin with DNS-ED at each temperature: DNS-ED, 10 mM; EDC, 100 mM. The other conditions are given in Section 2.

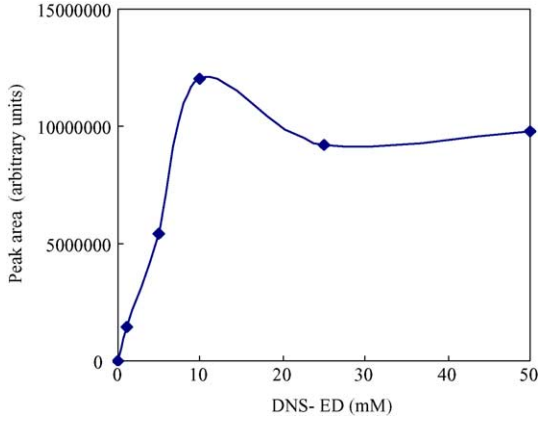

Fig. 3. Effect of DNS-ED concentration on the labeling reaction: EDC, 100 mM; reaction temperature, 60 °C; reaction time, 4 h. The other conditions are given in Section 2.

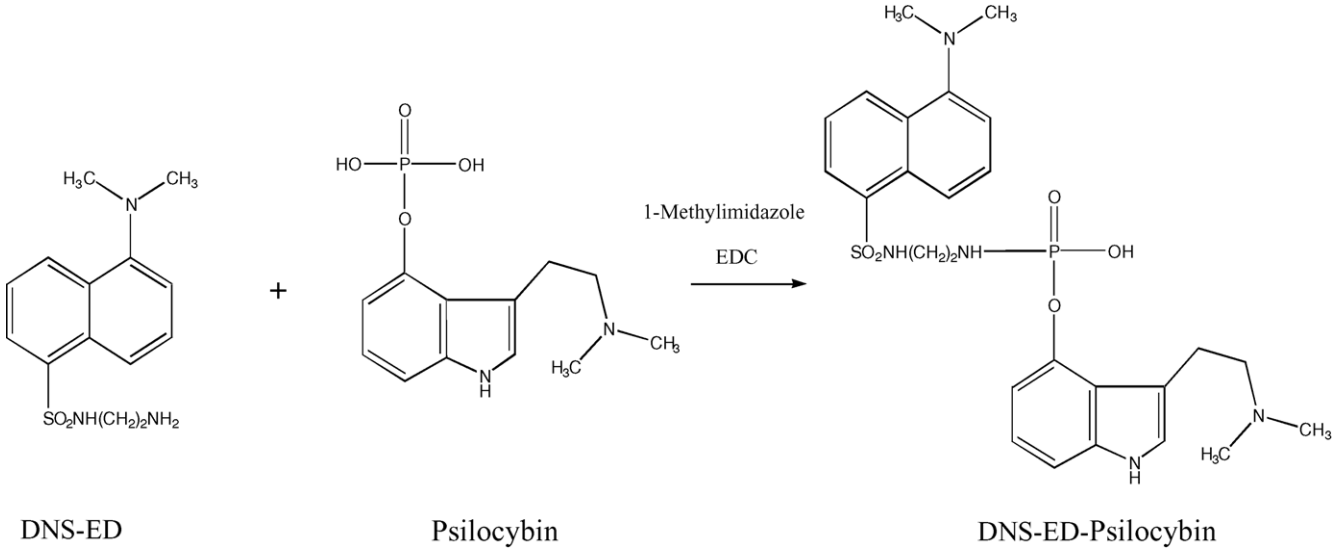

Fig. 1. Labeling reaction of psilocybin with DNS-ED in the presence of EDC.

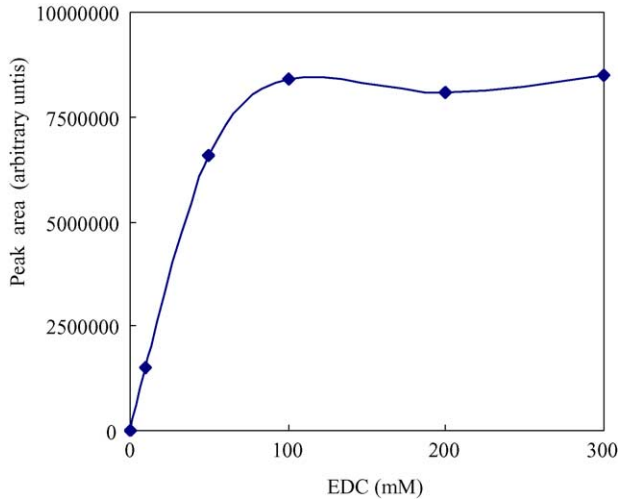

Fig. 4. Effect of EDC concentration on the labeling reaction: DNS-ED, 10 mM; reaction temperature, 60 °C; reaction time, 4 h. The other conditions are given in Section 2.

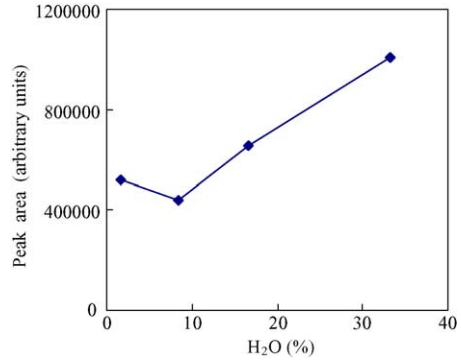

Fig. 5. Influence of water in the reaction medium: DNS-ED, 10 mM; EDC, 100 mM; reaction temperature, 60 °C; reaction time, 4 h. The other conditions are given in Section 2.

fore, the addition of an internal standard (IS) seems to be essential to obtain reproducible results. However, the compounds possessing the phosphoryloxy group in the structure were very few. 3-IP was selected after searching reagent catalogs. The derivative of 3-IP completely separated the derivatives of psilocybin and endogeneous substances in the mushroom. Thus, 3-IP was used as the IS for the determination of psilocybin in real samples.

3.2. Determination of psilocybin in magic mushroom by HPLC–FL

Initially, a suitable organic solvent was selected for the extraction of psilocybin in the magic mushrooms. Since psilocybin is a hydrophilic compound, water and water miscible organic solvents seem to be suitable for its extraction. Although water was a good solvent for the extraction of psilocybin in the samples, many interfering substances were also extracted. MeOH extracts the endogenous substances together with psilocybin, however, the number was relatively lower than that from water. Since psilocybin was efficiently extracted with MeOH, this solvent was selected for the extraction of the mushroom samples.

Fig. 6 shows a typical chromatogram obtained from the extraction solution of the magic mushroom. The derivatives of psilocybin and IS appeared at approximately 27 and 32 min, respectively. The two large peaks at around 12 and 20 min were due to the reagents used. The other unknown peaks seem to be due to endogenous and reactive substances in the sample. However, the structural elucidation of each peak could not be carried out.

The calibration curve was obtained by plotting the peak area ratios of IS versus the injected amounts of psilocybin.

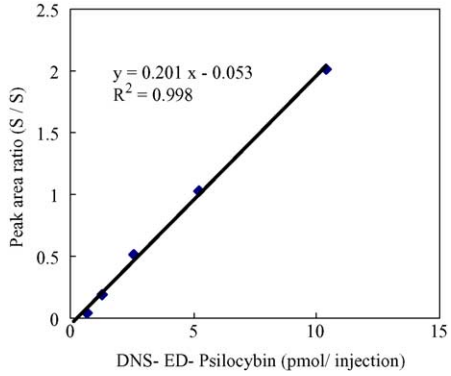

Fig. 7. Calibration curve of psilocybin by HPLC–FL.

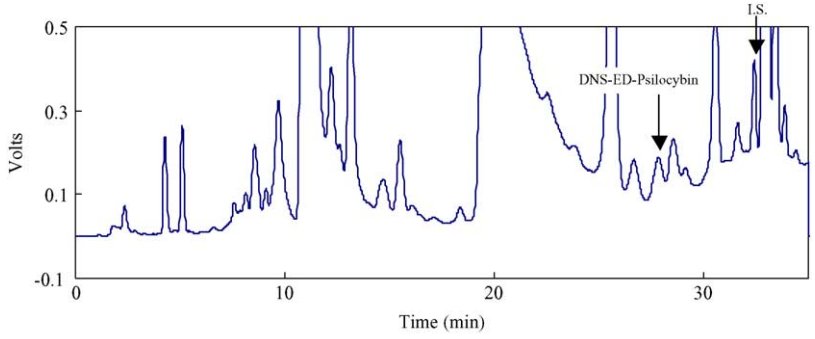

Fig. 6. Chromatogram of magic mushroom sample after labeling with DNS-ED. The labeling and HPLC–FL conditions are given in Section 2.

As shown in Fig. 7, a linear relation was observed in the range of 0.65–10.4 pmol ($r^2 = 0.998$). Furthermore, the slope of the calibration curve of psilocybin spiked into a pulverized mushroom was almost comparable to the standard psilocybin. The coefficient of variation (CV (%), $n = 5$) for the 0.37 ng (1.3 pmol), 0.74 ng (2.6 pmol) and 1.5 ng (5.2 pmol) injections on the calibration curve were 5.7, 3.2 and 1.5%, respectively. The accuracy (defined as recovery (%)) and precision (defined as CV (%)) of psilocybin spiked in a magic mushroom were determined before the real sample analysis. When 4.4, 20.2 and 40.4 ng were added to 1 mg of mushroom, the recovery (%) and CV (%) were 93 and 12% (4.4 ng spiked), 96 and 6.3% (20.2 ng spiked), and 98 and 3.9% (40.4 ng spiked), respectively. The quantification limit (signal-to-noise ratio of 10) of the proposed method was 4.4 ng in 1 mg (dry weight) mushroom, and the sensitivity was about two orders of magnitude higher than the UV, EC and native FL detections. These results suggest that the proposed procedure is applicable to actual samples. Thus, the amount of psilocybin in each magic mushroom was determined using this procedure.

The concentration of psilocybin in some mushroom samples was determined under the proposed extraction, labeling, separation, and FL detection conditions. Table 1 shows the amounts and scientific names of the six tested mushrooms.

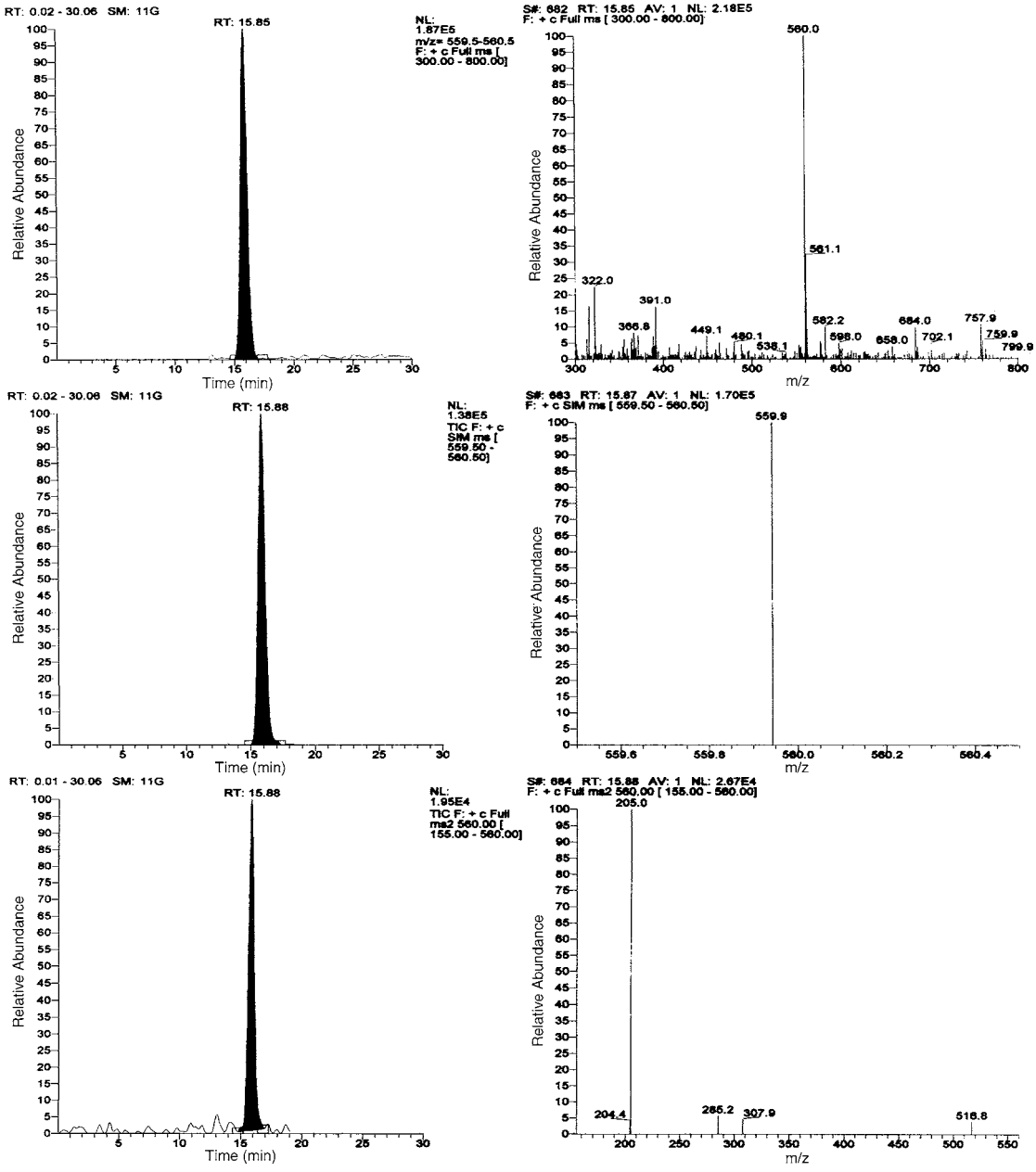

Fig. 8. MS chromatograms and MS spectra of psilocybin after labeling with DNS-ED. Top panels: left, total ion chromatogram; right, mass spectrum of psilocybin. Middle panels: left, SIM chromatogram; right, SIM mass spectrum of psilocybin at $m/z = 560$. Bottom panels: left, SRM chromatogram; right, product ion mass spectrum of psilocybin at $m/z = 205$. HPLC–MS conditions are given in Section 2.

Table 1
Psilocybin content in magic mushrooms

No.	Scientific name	ng/mg (dry weight)
MM-1	Putative *Psilocybe cubensis*	8.0
MM-2	*Psilocybe cubensis*	4.8
MM-3	*Panaeolus cyanescens*	Trace[a]
MM-4	*Psilocybe cubensis*	n.d.[b]
MM-5	*Psilocybe cubensis* (cultured)	20.0
MM-6	Putative *Psilocybe cubensis* (cultured)	Trace[a]

[a] Less than quantification limit, 4.4 ng/mg (dry weight).
[b] Not detected.

The amounts were varied in each sample (∼20.0 ng/mg). The reason for the difference is not obvious, but this may be due to the mushroom species and/or decomposition during storage.

Since a large variation in the psilocybin amounts of the mushroom samples was observed in the HPLC–FL method, the structural elucidation of the derivative was carried out by HPLC–MS. In this analysis, an isocratic elution utilizing 50 mM $AcONH_4$–CH_3CN (73:27) was adopted to shorten the elution time. Fig. 8 shows the MS chromatograms and MS spectra of the DNS-ED derivative of the psilocybin standard. The derivative was eluted at around 16 min, and the protonated-molecular ion ($m/z = 560$) corresponding to DNS-ED-psilocybin was identified in the MS spectra. Similar MS results were also obtained from the reaction solution of the mushroom sample. Thus, psilocybin seems to be actually labeled with DNS-ED to produce the corresponding derivative. When the protonated-molecular ion $[M + H]^+$ ($m/z = 560$) was used as the precursor ion for the MS–MS analysis, the product ions, i.e., $m/z = 285$ and 205, were identified from the derivatives. These ions seem to be due to the cleavage of DNS-ED and the phosphate ester moieties. Based on these results, psilocybin seems to be determined not only by HPLC–FL but also by HPLC–MS. Therefore, the determination of psilocybin by the SIM and the SRM mode was attempted similar to the HPLC–FL analysis. Since the calibration curves showed a good linearity, the HPLC–MS method may be applicable to the determination of psilocybin. However, its sensitivity was less than the HPLC–FL method. Consequently, the determination by HPLC–MS was not attempted in this study.

4. Conclusion

This paper described the determination of psilocybin in magic mushrooms using HPLC–FL method. The amount in the magic mushrooms varied in each sample. The difference might be due to the mushroom species and/or the condition of the sample. However, the exact reason is not obvious at this time.

Psilocybin has been quantified by HPLC coupled with UV, EC and native FL detection. GC–MS and HPLC–MS have recently been introduced in order to increase the sensitivity and selectivity for the determination of psilocybin in a mushroom sample [12–15]. UV detection is neither sensitive nor selective. Although the selectivity increases with the native FL detection after HPLC separation, the sensitivity seems not to be good enough for a real sample analysis. The GC–MS methods [12–14] require the volatilization treatment with TMS and MSTFA, etc. On the other hand, psilocybin was sensitively and selectively determined by HPLC–APCI-MS [16] and HPLC–ESI-MS [15,20]. These methods seem to be applicable to real samples, however, the GC–MS methods are not recommended due to the possible cleavage of the phosphoryloxy group in the psilocybin structure at high temperature. HPLC–MS is one of the most suitable methods. However, many unknown hydrophilic substances exist in the real samples. Therefore, many peaks corresponding to these substances appeared on the mass chromatogram. Furthermore, psilocybin is a hydrophilic and amphoteric compound and is not retained on the common ODS columns. Therefore, the separation of psilocybin from endogenous substances is very difficult. It is one of the major reasons for the determination difficulty of psilocybin in real samples. In contrast, the present method for the determination of psilocybin is based upon HPLC–FL detection after derivatization with DNS-ED. The derivatization with DNS-ED increased the hydrophobicity and thus increased the retention time. Possible interference by endogenous substances is thus avoidable by the derivatization. Furthermore, the detection sensitivity is about two orders of magnitude higher than that of the native FL detection. Consequently, the present method may be one of good methods for the determination of psilocybin. The application of the proposed method to various specimens is currently underway in our laboratory and will be reported elsewhere.

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References

[1] J. Bigwood, M.W. Beug, J. Ethnopharmacol. 5 (1982) 287–291.
[2] M. Wurst, M. Semerdzieva, J. Vokoun, J. Chromatogr. 286 (1984) 229–235.
[3] F. Musshoff, B. Madea, J. Beike, Forensic Sci. Int. 113 (2000) 389–395.
[4] R. Kysilka, M. Wurst, J. Chromatogr. 464 (1989) 434–437.
[5] K. Tsujikawa, T. Kanamori, Y. Iwata, Y. Ohmae, R. Sugita, H. Inoue, T. Kishi, Forensic Sci. Int. 138 (2003) 85–90.
[6] S. Borner, R. Brenneisen, J. Chromatogr. 408 (1987) 402–408.
[7] I.S. Lurie, D.A. Cooper, I.S. Krull, J. Chromatogr. 629 (1993) 143–151.
[8] M. Perkal, G.L. Blackman, A.L. Ottrey, L.K. Turner, J. Chromatogr. 196 (1980) 180–184.
[9] A.L. Christiansen, K.E. Rasmussen, F. Tønnesen, J. Chromatogr. 210 (1981) 163–167.

[10] A.L. Christiansen, K.E. Rasmussen, J. Chromatogr. 270 (1983) 293–299.
[11] M. Wurst, R. Kysilka, T. Koza, J. Chromatogr. 593 (1992) 201–208.
[12] D.B. Repke, D.T. Leslie, D.M. Mandell, N.G. Kish, J. Pharm. Sci. 66 (1977) 743–744.
[13] O. Beck, A. Helander, C. karlson-Stiber, N. Stephansson, J. Anal. Toxicol. 22 (1998) 45–49.
[14] T. Keller, A. Scineider, P. Regenscheit, R. Dirnhofer, T. Rucker, J. Jaspers, W. Kisser, Forensic Sci. Int. 99 (1999) 93–105.
[15] M.J. Bogusz, J. Chromatogr. B 748 (2000) 3–19.
[16] S. Sonoki, A. Sanada, S. Hisamatsu, J. Liq. Chromatogr. 17 (1994) 1057–1064.
[17] S. Sonoki, J. Lin, S. Hisamatsu, Anal. Chim. Acta 365 (1998) 213–217.
[18] T. Toyo'oka (Ed.), Modern Derivatization Methods for Separation Sciences, Wiley, Chichester, UK, 1999.
[19] O. Shirota, W. Hakamata, Y. Goda, J. Natl. Prod. 66 (2003) 885–887.
[20] A. Benson, A. Sabucedo, J. Almirall, K.G. Furton, Am. Chem. Soc. 223 (2002) U-72 (abstract).