Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 796 (2003) 421–427
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Optimized glucuronide hydrolysis for the detection of psilocin in human urine samples

Tooru Kamata*, Mayumi Nishikawa, Munehiro Katagi, Hitoshi Tsuchihashi

Forensic Science Laboratory, Osaka Prefectural Police H.Q., 1-3-18, Hommachi, Chuo-ku, Osaka 541-0053, Japan

Received 6 May 2003; received in revised form 15 August 2003; accepted 20 August 2003

Abstract

In order to develop a sensitive and reliable analytical method for psilocin (PC) in urine samples, the hydrolysis conditions including the acid, alkaline and enzymatic hydrolyses have been investigated by monitoring not only PC but also psilocin glucuronide (PCG) by liquid chromatography tandem mass spectrometry (LC-MS-MS); PCG was initially identified in a "magic mushroom (MM)" user's urine by liquid chromatography mass spectrometry (LC-MS) and LC-MS-MS.

The proposed conditions optimized for the hydrolysis are as follows: hydrolysis, enzymatic hydrolysis; enzyme, *Escherichia coli* β-glucuronidase (5000 units/ml urine); incubation, pH 6 at 37 °C for 2 h. The complete hydrolysis of PCG in urine was obtained under these conditions, while the enzymatic hydrolyses with three types of β-glucuronidases originated from bovine liver (Type B-1), *Helix pomatia* (Type H-1) and *Ampullaria* provided uncompleted hydrolysis of PCG. Also, neither the acid nor alkaline hydrolysis was found to be applicable. According to the present method, 3.55 μg/ml of psilocin was detected in the "magic mushroom" user's urine after the enzymatic hydrolysis, though psilocin was not detected without hydrolysis.

Keywords: Glucuronide hydrolysis; Psilocin

1. Introduction

Indole derivatives, psilocin (PC) and its phosphate psilocybin (PB), have structural similarity to a neurotransmitter, serotonin, and thereby exhibit a highly hallucinogenic potency. They are both principally contained in some species of the genus *Psilocybe*, which go under the name of "magic mushrooms (MMs)", including *Psilocybe cubensis*, *Psilocybe mexicana*, *Psilocybe subcubensis*, *Psilocybe semilanceata*, *Psilocybe argentipus* (Japanese name: Hikageshibiretake), etc. and naturally occur throughout the world.

The mushrooms were originally used as godly traditional medicine for centuries in the religious ceremony by the shamans in Central and South America. Nowadays, they have extensively been used for recreational purposes as hallucinogenic substances in various countries including the European and American countries, and even in Japan. It has resulted in increasing abuse of MMs and sometimes in hallucinogenic intoxication by overdosing. Therefore, not only the contained hallucinogenic ingredients PB and PC, but also the fruit body of the MMs are controlled in Japan.

* Corresponding author. Tel.: +81-6-6268-1234; fax: +81-6-6271-8066.
E-mail address: t-kamata@mahoroba.ne.jp (T. Kamata).

1570-0232/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2003.08.030

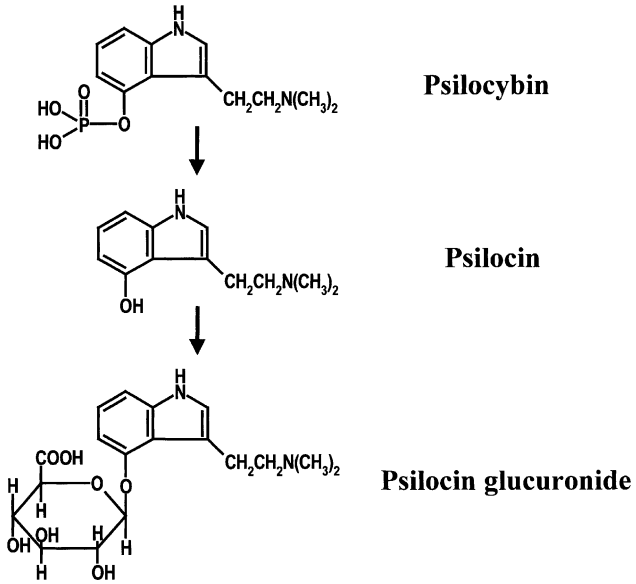

Fig. 1. An expected metabolic pathway for psilocybin. More information appears in the reference [6].

For confirmation of MM intake, the detection of PC in human urine has been generally performed by gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS), because PB is expected to be rapidly and readily dephosphorylated into PC by the intrinsic phosphates in animals [1,2]. PC is also expected to be metabolized by conjugation with glucuronic acid, demethylation, deamination, oxidation, etc. [3], though the metabolic pathways for PC and PB are not currently known. The expected metabolic pathway of psilocybin is shown in Fig. 1. Several publications have reported analyses of urinary PC by GC-MS [4,5] or HPLC-ECD [6] and described that the enzymatic hydrolysis with β-glucuronidase led to the evident increase in the amount of detected urinary PC [4–6], suggesting the fairly high elimination of PC as psilocin glucuronide (PCG) in urine. Thus, the direct analysis of PCG may be promising. Actually, several LC-MS techniques have been developed for the direct determination of glucuronide conjugates [7–13]. Our research group has also reported the application of LC-MS to the direct analyses of glucuronide conjugates of forensic interests including morphine glucuronides [7], oxazepam glucuronide [8] and ethyl glucuronide [11] in body fluids. However, the direct LC-MS analysis of PCG is not practical for the reliable confirmation of the MM intake because the standard PCG is not currently available. The cleavage of PCG into PC by hydrolysis followed by the determination of PC would be currently preferable, and the complete hydrolysis of PCG is, therefore, indispensable for the confirmation of the MM intake by urine analysis.

For the hydrolysis of PCG in urine, some studies have reported the investigation of the conditions [5,6]. However, it was not clarified whether PCG present in urine was completely cleaved or not, because only the released PC was determined before and after the hydrolysis. The optimization of the hydrolysis for the complete cleavage of PCG would be required for a more accurate and sensitive analysis.

In the present study, PCG was initially identified in urine collected from a “magic mushroom user” by employing liquid chromatography tandem mass spectrometry (LC-MS-MS), which allows a more accurate identification, in addition to LC-MS. The optimization of the complete hydrolysis of PCG has been explored by directly monitoring PCG as well as PC throughout the hydrolysis with β-glucuronidase.

2. Experimental

2.1. Materials

PC and PB were obtained from the Ministry of Health, Labour and Welfare of Japan, and those standard stock solutions were prepared in methanol (1 mg/ml). The solutions were stored at −20 °C until used, and adjusted to the appropriate concentration with distilled water or drug-free human urine immediately prior to use.

β-Glucuronidases (EC 3.2.1.31) originating from bovine liver (Type B-1), *Helix pomatia* (Type H-1) and *Escherichia coli* (Type IX-A) were purchased from Sigma (St. Louis, MO, USA), and that from *Ampullaria* from Wako (Osaka, Japan). Enzyme solutions were prepared in 0.5 M acetate buffer (25,000 units/ml) immediately prior to use. Acetonitrile was of HPLC-grade, and the other chemicals used were of analytical grade from Wako. The acetate buffer (0.5 M) used for sample preparation in the enzymatic hydrolysis was prepared by adjusting a 0.5 M sodium acetate aqueous solution to a pH specific for each β-glucuronidase with 3% (v/v) acetic acid.

2.2. Instrumentation

LC-MS and LC-MS-MS were performed on an Agilent 1100 HPLC system (Agilent, Palo Alto, CA, USA) linked to a Quattro LC (Micromass, Manchester, UK) triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface. The analytical column used was an L-column ODS semi-micro column (1.5 mm i.d. × 150 mm, Chemicals Evaluation and Research Institute, Tokyo, Japan). The mobile phase consisted of acetonitrile–10 mM ammonium formate buffer (pH 3.5) (12.5/87.5, v/v), and the flow rate was 0.1 ml/ min.

The operating parameters for mass spectrometry were as follows: capillary voltage, 4.5 kV; cone voltage, 30 V; ion source temperature, 280 °C. Collision-induced dissociation (CID) was performed using argon as the collision gas at a collision energy of 15 V, and ions at *m/z* 205 and 381 were selected as precursor ions for PC and its expected glucuronide conjugate PCG, respectively.

Urinary PC and PCG were monitored by LC-MS-MS in selected reaction monitoring after the sample preparation described below. The reactions monitored were *m/z* 381 > 205 for PCG and *m/z* 205 > 160 for PC, and the integrated peak areas were measured. The PC analysis was validated by analyzing spiked urine at concentration of 1 μg/ml.

2.3. History of urine specimen

The urine specimen used in the present study was collected from a 16-year-old female. She had allegedly taken approximately 9 g of dried MM obtained via Internet. The urine was collected 8 h after intake. The leftover mushrooms were submitted for LC-MS determination, and 0.375 mg/g of PC and 11.2 mg/g of PB were detected in our laboratory. Determinations of PC and PB in the mushrooms were performed by analyzing methanolic extract in selected ion monitoring mode. The ions at *m/z* 205 for PC and *m/z* 285 for PB were selected, and the integrated peak areas of them were compared with those of standards.

2.4. Hydrolyses of urine specimen

For the enzymatic hydrolyses, β-glucuronidase solution was added to 100 μl of each urine specimen adjusted to the appropriate pH (between 5 and 6) with 10% (v/v) acetic acid, and the mixture was incubated at 37 °C. In order to explore the optimum conditions for the complete enzymatic hydrolysis of PCG, four types of β-glucuronidases from different sources including bovine liver, *H. pomatia*, *Ampullaria* and *E. coli* were used. In the pre-experiment, the urine specimen was incubated with each β-glucuronidase (final enzyme concentration being 5000 units/ml urine) at 37 °C for 3 h.

For the acid and alkaline hydrolyses, 100 μl of each urine specimen was mixed with 100 μl of concentrated hydrochloric acid and 20 μl of 10 M potassium hydroxide aqueous solution, respectively. The mixture was incubated at 50 °C for 30 min, and neutralized with 1.2 M hydrochloric acid or 10 M potassium hydroxide aqueous solution.

The incubation mixture was subjected to LC-MS and LC-MS-MS analyses after the sample preparation.

2.5. Sample preparation

A urine sample with/without hydrolysis was deproteinized by adding two volumes of methanol, and

centrifuged at 1500 × *g* for 10 min. The supernatant 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 the 5 μl aliquot was injected into the LC-MS and LC-MS-MS system.

3. Results and discussion

3.1. Identification of psilocin glucuronide (PCG) in urine

A urine specimen from the user (referred to as specimen A) as well as those from drug-free volunteers as blank urine samples were prepared according to the procedure described in Section 2 followed by LC-MS, and the resultant chromatograms from specimen A and the blank urine specimens were compared. A relatively large peak specific to specimen A (referred to as peak A) appeared in Fig. 2 at the retention time of 2.9 min in the extracted mass chromatogram at *m*/*z* 381, which corresponds to the protonated molecule of PCG. The spectrum obtained for peak A was characterized by a predominant ion at *m*/*z* 381 and some less intense ions such as an ion at *m*/*z* 205 corresponding to the protonated molecule of PC. For further examination, the LC-MS analysis at a higher acetonitrile concentration (80%) in the mobile phase was performed, however, no other peak was detected in the extracted mass chromatogram at *m*/*z* 381. On the other hand, free PC was not detected in specimen A.

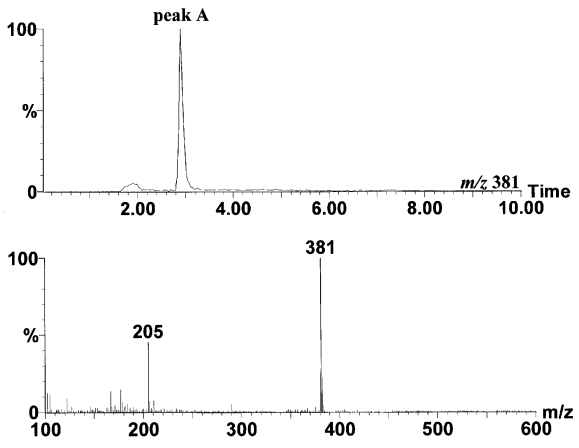

Fig. 2. Extracted mass chromatogram at *m*/*z* 381 obtained from a "magic mushrooms" user's urine without hydrolysis (top) and a mass spectrum for the peak A (bottom).

Additionally, a liquid–liquid extraction with chloroform followed by the LC-MS analysis was carried out for specimen A and no peak corresponding to peak A was detected. This is consistent with the presumption that PCG would not be extracted by chloroform because of its high polarity, suggesting that peak A corresponds to PCG.

For the confirmation of the direct LC-MS identification of PCG, we explored the LC-MS-MS analysis. In the pre-experiment, the collision energy was varied between 10 and 25 eV, and the product ion spectrum derived from the ion at *m*/*z* 381 was measured. As depicted in Fig. 3A–C, the higher voltage of the collision energy produced lower and higher intensities of ions at *m*/*z* 381 and 205, respectively. Finally, a product ion at *m*/*z* 160 also appeared at 20 eV; the ion at *m*/*z* 160 was postulated to be due to $[M\text{-}N(CH_3)_2]^+$ which can be seen in the cleavage of PC. Also, the CID of the ion at *m*/*z* 205 was further accomplished at the collision energy of 15 eV. Fig. 3D shows the product ion spectrum produced from the precursor ion at *m*/*z* 205. The spectrum was characterized by a predominant ion at *m*/*z* 160 and a minor ion at *m*/*z* 205, which well agreed with the product ion spectrum of PC (Fig. 3E) acquired under the same operating condition. These represent the structure of PCG.

Based on the above examination, the compound corresponding to peak A was assigned to PCG, and peak A was used for PCG in the following experiment. In a further experiment for specimen A, none of the specific peaks were observed on the extracted mass chromatograms corresponding to other expected conjugates of PC including the sulfate conjugate (*m*/*z* 285), methyl conjugate (*m*/*z* 219), and glucose conjugate (*m*/*z* 367).

3.2. Re-investigation of operating parameters of MS and MS-MS for PC and PCG

The optimum operating parameters of MS and MS-MS for both PC and PCG were re-investigated using specimen A. As mentioned above, PCG would not be readily extracted by organic solvents. For the simultaneously monitoring both PC and PCG, the specimen was prepared according to the pro-

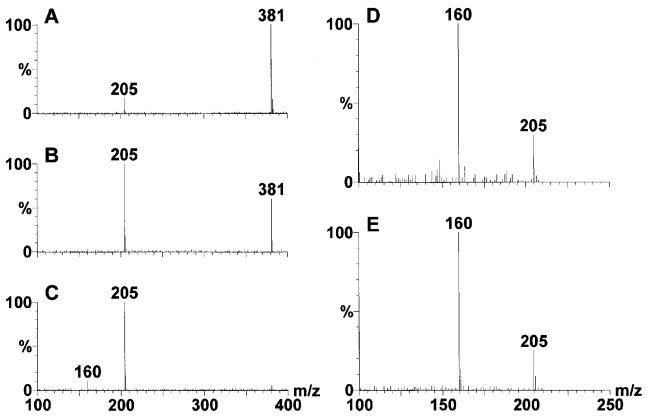

Fig. 3. Product ion spectra of psilocin glucuronide produced from the precursor ions at *m/z* 381 with collision energies of 10 eV(A), 15 eV (B), and 20 eV (C), and product ion spectra of psilocin glucuronide (D) and psilocin (E) produced from the precursor ions at *m/z* 205. Other conditions appear in the text.

cedure described in Section 2. The capillary and cone voltages were varied between 3.0 and 5.0 kV, and 10 and 40 V, respectively, and the intensity of the protonated molecules of PC (*m/z* 205) and PCG (*m/z* 381) were measured by LC-MS. The optimal parameters were set at 4.5 kV and 30 V for the capillary and cone voltages, respectively, where the intensities of both protonated molecules were the highest. Under these conditions, the operating parameters of MS-MS by choosing each protonated molecule as the precursor ions were further explored. The collision energy was varied between 10 and 25 eV, and the intensity of the product ions at *m/z* 160 for PC and 205 for PCG were monitored by LC-MS-MS. The optimal collision energy was set at 15 eV, where the intensities of both product ions were the highest. The final optimized parameters were set as follows: capillary voltage, 4.5 kV; cone voltage, 30 V; collision energy, 15 eV.

3.3. Precision of the present method

The present LC-MS-MS method was validated for determination of urinary PC level. A calibration curve was prepared in the range 0.5–5 μg/ml urine, and there was a linear relationship between concentration and peak area. The intra- and inter-day coefficients of variation were 7.07 and 9.11%, respectively. The lower limit of detection was 0.5 ng/ml urine.

3.4. Optimization of hydrolysis for PCG

With acid hydrolysis, neither PC nor PCG was detected. This could have been caused by the decomposition of PCG without release of PC or by further decomposition of the released PC. With alkaline hydrolysis, no decrease in the concentration of PCG was observed, and no released PC was detected. These results suggest that PCG was not cleaved by incubation with alkaline. On the other hand, it is well known that the hydrolysis rate of glucuronides changes mainly depending on the configuration of the enzyme and substrate [14,15].

As shown in Fig. 4, the hydrolysis with *E. coli* β-glucuronidase was found to produce complete cleavage of PCG and to release the highest amount of PC among the four. These results are quite consistent with the previous report by Grieshaber et al. [5]. Therefore, we chose *E. coli* β-glucuronidase for the hydrolysis of PCG, and the optimum conditions were further explored.

Fig. 5 shows the time-course of the concentration of the remaining PCG and the released PC under the incubation conditions of 37 °C and pH 6 with different amounts of the enzyme. Although 9.1% PCG remained unhydrolyzed with 1000 units/ml enzyme for even 5 h, the complete hydrolysis was achieved by incubation with 5000 units/ml enzyme for 2 h. Also, with 5000 units/ml enzyme, the PC concentration reached the highest level, 3.55 μg/ml urine, after 2 h of incuba-

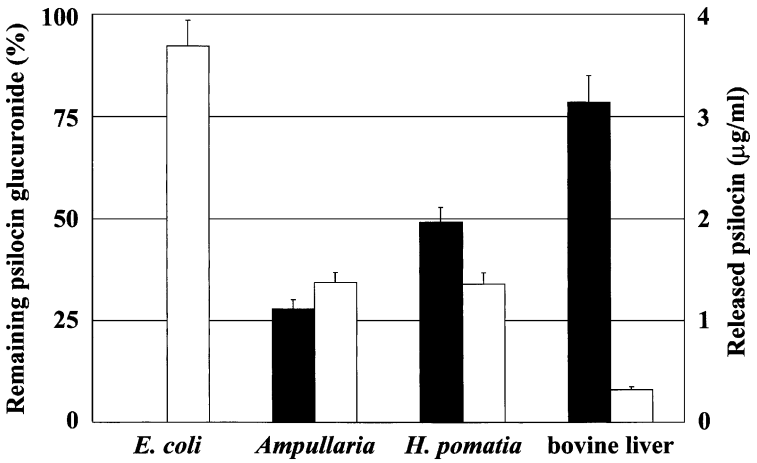

Fig. 4. Comparison of the hydrolysis rates for urinary psilocin glucuronide with four different β-glucuronidases. (■), Remained psilocin glucuronide (%); (□), released psilocin (μg/ml); error bars: standard deviation, hydrolysis conditions appear in the text.

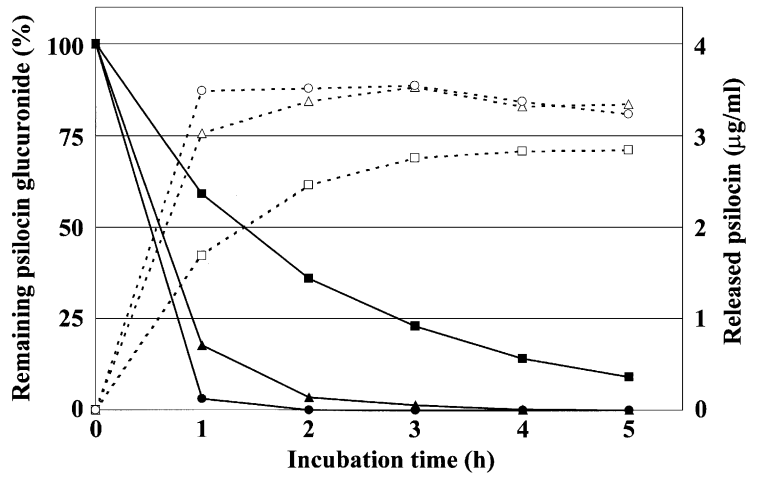

Fig. 5. Time-course of remained psilocin glucuronide (closed symbols) and released psilocin (open symbols) during incubation with *E. coli* β-glucuronidase. Amounts of added enzyme were 1000 units/ml (■, □), 3000 units/ml (▲, △), and 5000 units/ml (●, ○) of urine. Incubation was performed at pH 6 and 37 °C.

tion while an extended incubation time led to a slight loss of PC. For further improvement, the effect of the incubation temperature and urinary pH was examined, however, no significant improvement was achieved.

Based on these results, we have established the optimal hydrolysis conditions for urinary PCG as follows: enzyme, *E. coli* β-glucuronidase; amount of enzyme, 5000 units/ml urine; urinary pH during incubation, pH 6; incubation time, 2 h; incubation temperature, 37 °C.

4. Conclusion

For optimization of the hydrolysis conditions for PCG in human urine, urinary PCG was, for the first time, identified in an preliminary experiment using LC-MS and LC-MS-MS. Also, the optimal hydrolysis conditions were explored by monitoring not only PC but also PCG by LC-MS-MS. The proposed optimal hydrolysis conditions are as follows: hydrolysis, en-

zymatic hydrolysis; enzyme, *E. coli* β-glucuronidase (5000 units/ml urine); incubation, pH 6 at 37 °C for 2 h. These established conditions allow the complete and effective hydrolysis of PCG and, therefore, will contribute toward accurate and sensitive analyses for the confirmation of "magic mushroom" intake in the forensic and clinical science fields.

References

[1] A. Horita, L.J. Weber, Biochem. Pharmacol. 7 (1961) 47.
[2] A. Horita, L.J. Weber, Toxicol. Appl. Pharmacol. 4 (1962) 730.
[3] F. Kalberer, W. Kreis, J. Rutschmann, Biochem. Pharmacol. 11 (1962) 261.
[4] G. Sticht, H. Käferstein, Forensic Sci. Int. 113 (2000) 403.
[5] A.F. Grieshaber, K.A. Moore, B. Levine, J. Forensic Sci. 46 (2001) 627.
[6] F. Hasler, D. Bourquin, R. Brenneisen, F.X. Vollenweider, J. Pharm. Biomed. Anal. 30 (2002) 331.
[7] M. Nishikawa, K. Nakajima, K. Igarashi, F. Kasuya, M. Fukui, H. Tsuchihashi, Jpn. J. Toxicol. Environ. Health 38 (1992) 121.
[8] M. Tatsuno, M. Nishikawa, M. Katagi, H. Tsuchihashi, K. Igarashi, F. Kasuya, M. Fukui, Jpn. J. Toxicol. Environ. Health 38 (1992) 162.
[9] M.J. Bogusz, R.-D. Maier, S. Driessen, J. Anal. Toxicol. 21 (1997) 346.
[10] M.J. Bogusz, R.-D. Maier, M. Erkens, S. Driessen, J. Chromatogr. B 703 (1997) 115.
[11] M. Nishikawa, H. Tsuchihashi, A. Miki, M. Katagi, G. Schmitt, H. Zimmer, T. Keller, R. Aderjan, J. Chromatogr. B 726 (1999) 105.
[12] I. Sundström, U. Bondesson, M. Hedeland, J. Chromatogr. B 763 (2001) 121.
[13] I. Sundström, M. Hedeland, U. Bondesson, P.E. Andrén, J. Mass Spectrom. 37 (2002) 414.
[14] V. Graef, E. Furuya, O. Nishikaze, Clin. Chem. 23 (1977) 532.
[15] T.V. Zenser, V.M. Lakshmi, B.B. Davis, Drug Metab. Dispos. 27 (1999) 1064.