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ORIGINAL ARTICLE

A validated method for quantitation of psilocin in plasma by LC–MS/MS and study of stability

Rafaela Martin · Jennifer Schürenkamp · Heidi Pfeiffer · Helga Köhler

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Abstract A liquid chromatography–electrospray ionization/tandem mass spectrometry method for the quantitation of psilocin in plasma is presented. Sample workup was performed with mixed-mode solid-phase extraction using ascorbic acid and nitrogen for drying to protect the unstable analyte. Calibration curves were linear from 2 to 100 ng/mL, and no selectivity problems occurred. The limit of detection was 0.1 ng/mL, and the limit of quantitation was 0.34 ng/mL. Recovery was >86% and matrix effects were <110%. Both were reproducible. Interday and intraday precisions at different concentrations were 1.5–4.3% relative standard deviation, bias within ±9%. Processed samples were stable in the autosampler for at least 26 h. Furthermore, the stability of psilocin in blood stored at different temperatures over various periods of time was investigated. Samples stored at room temperature showed a continuous decrease of analyte leading to a loss of about 90% after 1 week. Storage in the fridge improved sample stability significantly. Freezing of blood samples led to a not reproducible loss of psilocin.

Keywords Psilocin · LC–MS/MS · Solid-phase extraction · Validation · Stability

Introduction

Psilocybin and psilocin are the major psychoactive compounds of *Psilocybe*, *Panaeolus*, and *Conocybe* mushrooms, commonly known as "magic mushrooms." Traditionally, they were used for religious ceremonies in Mexico and South America [1, 2]. Nowadays, they are consumed as recreational drugs, and therefore, they are controlled in a lot of countries [3].

After ingestion, psilocybin is rapidly dephosphorylated to psilocin, which is the pharmacologically active substance [4]. It causes hallucinations, thought disorders, and changes in affect and mood [5]. The analysis of psilocin in body fluids is challenging because the analyte is rapidly metabolized and unstable under the influence of light and air [5, 6].

Different techniques for determination of psilocin are used, e.g., high-performance liquid chromatography (HPLC) with electrochemical detection [5, 7, 8], gas chromatography–mass spectrometry [9, 10], and liquid chromatography tandem mass spectrometry (LC–MS/MS) [11–14]. The latter is the most suitable one because it offers best sensitivity and requires no derivatization. Sample workup is performed with microdialysis after freeze-drying, which is very time-consuming [5]. Other methods use liquid–liquid extraction [12, 15], protein precipitation [11, 12], or dilution with water [14]. Simple sample preparations like these bring about the danger of matrix effects and contaminating the measuring instrument, especially if LC–MS is used. Solid-phase extraction (SPE), however, provides cleaner extracts. Published methods using SPE have high limits of detection (0.5–10 ng/mL) and do not take account of the instability of psilocin [9, 13, 16, 17].

This paper describes a fully validated LC–MS/MS method for the analysis of psilocin in plasma with a solid-phase extraction procedure that protects psilocin in an optimal way. Furthermore, our aim was to know what happens to psilocin under different storage conditions between blood collection and arrival in the laboratory, where the blood sample can be centrifuged and the supernatant can be frozen. Stability studies of psilocin were only performed in

R. Martin (✉) · J. Schürenkamp · H. Pfeiffer · H. Köhler
Institute of Legal Medicine, University Hospital Münster,
Röntgenstr. 23,
48149 Münster, Germany
e-mail: rafaela.martin@ukmuenster.de

urine and plasma as well as in aqueous standard solutions [5, 6, 14, 18, 19]. To the best of our knowledge, no studies have so far been published on the stability of psilocin in blood samples.

Materials and methods

Chemicals and reagents

Psilocin and psilocin-d_{10} were obtained as solids from Cerilliant (Round Rock, TX, USA). Ethyl acetate (LiChrosolv) was purchased from Merck (Darmstadt, Germany). Methanol (LC–MS grade) and double-distilled water were from AppliChem (Darmstadt, Germany). All other chemicals were of analytical grade. Drug-free blood and plasma were collected from healthy volunteers. Oasis® MCX solid-phase extraction cartridges (60 mg, 3 mL) were from Waters (Milford, MA, USA).

Standard solutions

Stock solutions of psilocin-d_{10} as internal standard (I.S.) and psilocin at a concentration of 1 mg/mL were prepared in methanol. Using the stock solutions, an I.S. working solution at a concentration of 1 μg/mL in methanol and working solutions of psilocin at concentrations of 0.1 and 1 μg/mL in methanol were further diluted.

LC–MS/MS conditions

A Waters Alliance 2695 Separation Module with a Pursuit C18 (3 μm, 150×2.0 mm, Varian) column was used for chromatographic separation, which was conducted at 40°C. Ten microliters of the extract was used for analysis. Separation was performed with 10 % mobile phase A (methanol with 0.1% formic acid) and 90% mobile phase B (2 mM ammonium acetate buffer with 0.1 % formic acid, pH 3) at a flow rate of 0.2 mL/min for 7 min, switching to 90% A for 4 min to remove lipophilic impurities. The column was equilibrated for 10 min, which resulted in a total run time of 21 min. The retention time of psilocin was 6.0 min. Samples were cooled to 12°C in the autosampler.

A micromass Quattro Micro with positive electrospray ionization was used for mass spectrometry. Optimal operating parameters were as follows: capillary voltage, 0.9 kV; ion source temperature, 120°C; desolvation temperature, 300°C; cone and desolvation gas flow (nitrogen), 50 and 650 L/h, respectively; and collision gas, argon. Cone voltages were 20 V for psilocin and 22 V for psilocin-d_{10}. The MS was run in multiple reaction monitoring mode. The reactions monitored were *m/z* 205>58 (quantifier) and *m/z* 205>160 (qualifier) for psilocin and *m/z* 215>66 (quantifier) and *m/z* 215>164 (qualifier) for psilocin-d_{10}.

Sample preparation

After diluting 0.5 mL plasma with 1.5 mL phosphate buffer (0.1 M, pH 6), 10 μL 0.1 M ascorbic acid for the protection of the unstable analyte during the extraction and 20 μL I.S. working solution were added. The samples were vortex-mixed and centrifuged at 4,000 rpm for 10 min. Oasis MCX columns were conditioned with 1.5 mL methanol and phosphate buffer each. The extraction was performed protected from light. After sample application, the columns were washed with 2 mL water and 2 mL methanol, followed by a drying step of 10 min in a stream of nitrogen with a custom-designed nitrogen-manifold. Then, the columns were washed with 2 mL ethyl acetate and dried again for 5 min with nitrogen. Before psilocin was eluted with 2×1 mL 2% ammonium hydroxide in ethyl acetate, 10 μL 0.01 M ascorbic acid was added to the HPLC vial. Polypropylene needles instead of stainless steel needles were used for elution. The eluate was evaporated to dryness at 40°C in a stream of nitrogen and reconstituted in 100 μL HPLC mobile phase.

Investigation of extraction procedure

Previous methods used vacuum for drying the SPE columns during the extraction procedure [9, 13, 16, 17]. This approach does not take into consideration the known instability of psilocin in the presence of air. Four different calibration curves were made to show the effects of the features of the presented method, which are the addition of ascorbic acid and drying with nitrogen instead of vacuum:

1. With ascorbic acid, drying with nitrogen
2. Without ascorbic acid, drying with nitrogen
3. With ascorbic acid, drying with vacuum
4. Without ascorbic acid, drying with vacuum

The extracts were measured directly after the extraction and a second time 18 h later.

Furthermore, the influence of using polypropylene needles instead of stainless steel needles was examined. Plasma samples were extracted in triplicate with the presented method using polypropylene and stainless steel needles, respectively.

Method validation

Validation was performed according to the criteria of the German Society of Toxicological and Forensic Chemistry guidelines [20] using drug-free plasma. Selectivity was evaluated by extracting six blank plasma samples of

different sources, two blank plasma samples with I.S., and blank plasma samples spiked with drugs expected in forensic samples (tetrahydrocannabinol and metabolites, amphetamines, cocaine and metabolites, morphine, codeine, benzodiazepines, lysergic acid diethylamide, bufotenine). Six calibration curves were constructed for determination of linearity and homoscedasticity by spiking blank plasma with analyte at concentrations of 2, 10, 20, 40, 60, 80, and 100 ng/mL.

Quality control (QC) pools at a low, medium, and high concentration (2, 40, and 100 ng/mL plasma) were prepared. Additionally, QC pools at a very low and a very high concentration (1.2 and 200 ng/mL plasma) were prepared. For extraction of these QCs, the double volume of plasma and one fifth of the volume, as well as a five-fold dilution of plasma, respectively, were used. QCs of each concentration were extracted in duplicate on eight different days. The accuracy of the method was determined by bias (percent), and the interday and intraday precisions were expressed as relative standard deviations (RSD). The limit of detection (LOD) and limit of quantitation (LOQ) were determined with a specific calibration curve using five calibrators in the range of the expected LOD.

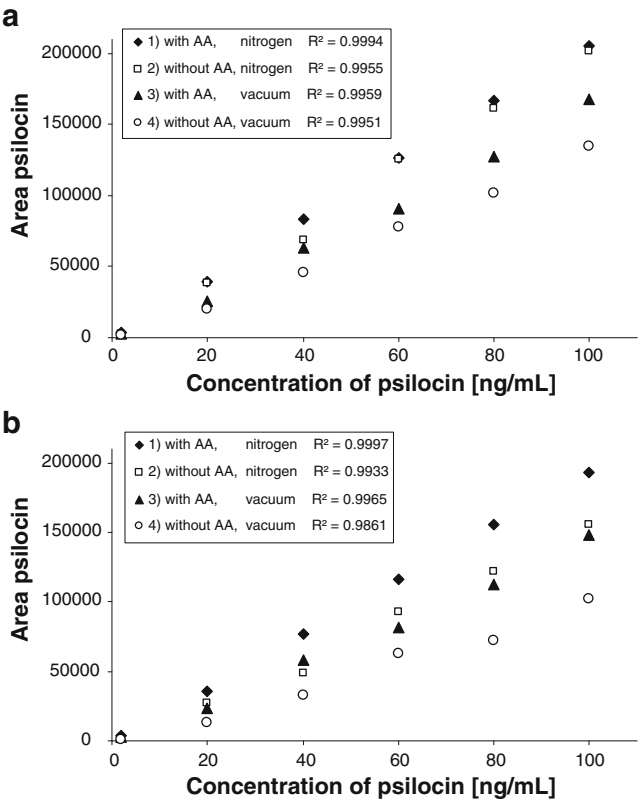

Fig. 1 Calibration curves: concentration of psilocin plotted vs. the absolute area of psilocin peaks. Extraction procedures with or without ascorbic acid (*AA*), drying steps with nitrogen or vacuum. **a** Extracts measured directly after extraction. **b** Extracts measured again after 18 h in the autosampler

Table 1 Accuracy, intraday precision, and interday precision of quality controls at different concentrations

Quality control	Concentration (ng/mL)	Accuracy bias (%)	Intraday precision [RSD (%)]	Interday precision [RSD (%)]
Low	2	1.8	2.7	4.3
Middle	40	−5.1	2.0	2.5
High	100	−4.9	1.7	3.6
Very low[a]	1.2	4.1	1.6	2.4
Very high 1/5[b]	200	−8.8	1.5	2.9
Very high 1:5[c]	200	−4.6	1.7	2.8

[a] The double sample volume (1 mL) was used for extraction

[b] One fifth of the sample volume (0.1 mL) was used for extraction

[c] A five-fold dilution was used for extraction

Recovery was assessed by comparing the peak areas of five different plasma samples spiked before extraction with the ones of five different plasma samples spiked after extraction (spiked extracts) at concentrations of 2 and 100 ng/mL. Matrix effects were investigated by comparing the peak areas of the five spiked extracts mentioned above with the ones of five neat standards at 2 and 100 ng/mL.

The stability of processed samples in the autosampler was tested by repeated injection of six QCs each at two concentrations (2 and 100 ng/mL) and a linear regression analysis of the peak area plotted vs. the injection time. Freeze/thaw stability of spiked plasma samples was evaluated over three freeze/thaw cycles in six-fold at 2 and 100 ng/mL. For evaluation of long-term stability, spiked plasma samples (n=6) were stored at −20°C over a period of 6 weeks and 6 months at concentrations of 2 and 100 ng/mL. Stability samples were compared with six control samples of both concentrations that were extracted directly.

Stability study

For each sample, 1 g blood containing 10 mg sodium fluoride was spiked with 50 ng psilocin. Stability samples were stored dark at room temperature (23°C), in the fridge (4°C) and deep frozen (−20°) in triplicate. Storage periods were 12 h, 1 day, 3 days, and 7 days. Three blood samples that were directly extracted following addition of the analyte were used as control samples. All blood samples were

Table 2 Extraction recovery and matrix effects

Concentration (ng/mL)	Recovery		Matrix effects	
	Mean (%)	RSD (%)	Mean (%)	RSD (%)
2	86.9	5.4	109.7	4.7
100	86.5	2.6	106.2	4.3

Table 3 Freeze/thaw and long-term stability

	Freeze/thaw stability		Long-term stability			
			6 weeks		6 months	
Concentration (ng/mL)	% of controls	90 % CI	% of controls	90 % CI	% of controls	90 % CI
2	93.8	92.8–94.7	95.7	93.2–98.1	60.1	59.7–60.5
100	95.8	94.1–97.4	85.6	84.2–87.1	51.9	51.5–52.4

CI confidence interval

extracted at the same time, as described in the "Sample preparation" section, but with an extended washing procedure because whole blood is a more complex matrix than plasma. Each washing step was performed twice.

Results and discussion

Extraction procedure

The effects of adding ascorbic acid and drying with nitrogen, instead of vacuum, were investigated. Drying with vacuum (curve 3) compared to drying with nitrogen (curves 1 and 2) led to a significant loss of analyte that was even greater if no ascorbic acid was added (curve 4) (Fig. 1a). If the extracts were measured after 18 h in the autosampler (Fig. 1b), the calibration curves without ascorbic acid (curves 2 and 4) showed a decrease of the peak area compared to the measurement directly after the extraction, whereas the ones with ascorbic acid (curves 1 and 3) had nearly the same peak area. Therefore, ascorbic acid is necessary to ensure autosampler stability of psilocin protecting it from oxidation. Besides, the extraction procedure with ascorbic acid and drying with nitrogen (curve 1 in Fig. 1a, b) was the only one to provide calibration curves with acceptable coefficients of determination (R^2) (Fig. 1a, b).

The use of polypropylene needles instead of stainless steel needles during the elution is necessary because steel needles lead to a loss of analyte. The samples eluted with steel needles had only 61% of the mean peak area of the samples with polypropylene needles.

Validation

Selectivity was proven by the lack of response in the blank samples, as well as in the samples spiked with xenobiotics. A linear calibration model without weighting was verified by the Mandel test. The coefficients of determination of all calibration curves were >0.998. Homoscedasticity was shown by the Cochran test. The LOD was 0.1 ng/mL and the LOQ 0.34 ng/mL.

Bias ranged from −9% to 2% for all concentrations. Intraday precision was <3% RSD and interday precision <4.5%. Thus, the acceptance criteria for precision (15% RSD, 20% RSD near LOQ) and accuracy (bias within ±15%, ±20% near LOQ) are fulfilled. All results are summarized in Table 1. The extraction procedure offers high and reproducible recoveries with little matrix effects for both concentrations (see Table 2).

Stability of processed samples in the autosampler is guaranteed for at least 26 h because of the addition of ascorbic acid. Freeze/thaw and long-term stability are assumed if the percentage of the mean concentration in the treated samples compared with the mean concentration in the control samples is within 90–110%. Furthermore, the 90% confidence interval has to be within 80–120 % of the control mean. The acceptance criteria are fulfilled for the freeze/thaw stability (see Table 3). Deep-frozen plasma samples spiked with the low concentration were stable for at least 6 weeks, whereas at the high concentration, a slight decrease could be observed. After 6 months of storage at −20°C, there was a loss of analyte of about 40–50% in plasma samples (Table 3). Holzmann [6] observed a loss of 53% in spiked plasma already after 6 weeks at −20°C.

Stability study

In Germany, sodium fluoride is added as a stabilizer to blood samples (10 mg NaF/g blood) that are taken by the

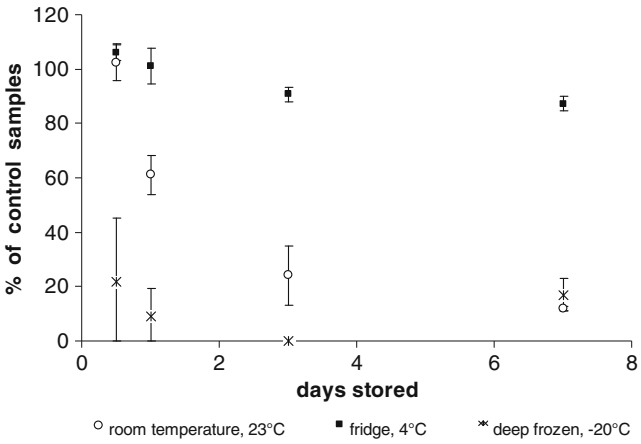

Fig. 2 Mean area ratios of stability samples divided by the ones of control samples (percent) at different temperatures plotted vs. the storage time. *Bars* represent respective standard deviations

police and analyzed for drugs. Therefore, we chose the so-called fluoride blood for the stability study. Since it may take some time from the moment of blood collection at the police station until the samples reach the laboratory for analysis, we investigated the stability of psilocin in fluoride blood samples stored at different temperatures over a period of 12 h to 1 week. The aims were to understand the best storage conditions and to determine the degradation process of psilocin.

The mean area ratios of psilocin and internal standard of the stability samples were compared with the ones of the control samples that were extracted directly after spiking. Psilocin in fluoride blood samples stored at room temperature was stable for only half a day (Fig. 2). After that, there was a continuous loss of analyte, which led to a decrease of the area ratio of nearly 90 % after 1 week. Psilocin was almost stable for over 7 days if fluoride blood samples were stored in the fridge at 4°C. Surprisingly, freezing of blood samples led to a significant loss of analyte that was not reproducible. Up to now, we cannot explain this phenomenon. It may be due to the bursting of erythrocytes when blood freezes, which causes the release of enzymes that probably decompose psilocin.

Conclusions

A quick and sensitive LC–MS/MS method with clean extracts for psilocin quantitation in plasma is presented, which takes into consideration the instability of the analyte. Only little sample material is required, and sample preparation and extraction can be accomplished in less than 2 h. Drying with nitrogen instead of full vacuum is essential for good recovery. Addition of ascorbic acid before and after extraction protects psilocin from oxidation and is important for processed sample stability. The method is fully validated.

On suspicion of psilocin intake, blood samples should not be stored at room temperature after blood collection but be cooled until they reach the laboratory. There they must be centrifuged as soon as possible, to freeze the supernatant. Freezing of blood samples before centrifugation leads to an uncontrollable loss of analyte. Therefore, blood samples must not be frozen.

Conflict of interest The authors declare that they have no conflict of interest.

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