Chapter 84

Assays for Detection of Fungal Hallucinogens Such as Psilocybin and Psilocin

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Abbreviations

5-metoxy-DMT 5-Metoxy-*N*,*N*-dimethyltryptamine
APCI-MS Atmospheric pressure chemical ionization–mass spectrometry
ATR Attenuated total reflectance
DBD-Pro-COCl 4-(*N*,*N*-dimethylaminosulfonyl)-7-(2-chloro formylpyrrolidin-1-yl)-2,1,3-benzoxadiazole
di-TMS-psilocin *N*,*N*-dimethyl-2-(1-trimethylsilyl-4-trimethyl silyloxyindol-3-yl)ethanamine
DNA Deoxyribonucleic acid
DNS-ED 5-Dimethylaminonaphthalene-1-[*N*-(2-aminoethyl)]sulfonamide
ESI-MS Electrospray ionization–mass spectrometry
GC–MS Gas chromatography–mass spectrometry
HPLC High-performance liquid chromatography
HPLC-ECD High-performance liquid chromatography–electrochemical detection
HPLC-FL High-performance liquid chromatography–fluorescence detection
HPLC-MS High-performance liquid chromatography–mass spectrometry
HPLC-MS-MS High-performance liquid chromatography–tandem mass spectrometry
HPLC-UV High-performance liquid chromatography–ultraviolet light detection
IMS Ion mobility spectrometry
LSD Lysergic acid diethylamide
MAO Monoamine oxidase
MSTFA *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide
NMR Nuclear magnetic resonance
PCR Polymerase chain reaction
***p*-DMCA** 4-(Dimethylamino)-cinnamaldehyde
PFP Pentafluorophenyl
R_F Retention factor
SPE Solid-phase extraction
TLC Thin-layer chromatography
tri-TMS-psilocybin Tri(trimethylsilyl) ether derivative of psilocybin
UDP-glucuronosyltransferases Uridine 5′-diphospho-glucuronosyltransferases
UPLC, UHPLC Ultra(high)-performance liquid chromatography

INTRODUCTION

Psilocybin and psilocin are psychoactive indolealkylamines of some therapeutic potential that are currently used for recreational or entheogenic purposes. Hallucinogenic hydroxylated derivatives of tryptamine are synthesized by various widely occurring species of fungi belonging to different genera, including *Psilocybe*; these are psilocybin, psilocin, baeocystin, and norbaeocystin. Bufotenine (5-hydroxy isomer of psilocin) has been found in a species belonging to the *Amanita* genus (*A. citrina*). Psilocybin was, for the first time, isolated from *Psilocybe mexicana* by Hofmann, Heim, Brack, and Kobel (1958). The same authors also identified psilocin along with psilocybin in the fruit bodies of this species, and chemical synthesis confirmed the identification (Hofmann et al., 1959). Baeocystin and norbaeocystin were originally found in mycelial culture of *Psilocybe baeocystis* by Leung and Paul (1968), but the indolealkylamines commonly occur also in other fungi of *Psilocybe* genus. Biosynthesis of psilocin occurs through *N*-methylation of tryptamine and hydroxylation of tryptamine derivatives at position 4 of the indole ring (Agurell & Nilsson, 1968; Gartz, 1989). Psilocin *O*-phosphorylation is the last step of the psilocybin biosynthetic pathway (Agurell & Nilsson, 1968). Aeruginascin (*N*,*N*,*N*-trimethyl analogue of psilocybin) was found along with psilocybin exclusively in one species, *Inocybe aeruginascens* (Jensen, 2004; Jensen, Gartz, & Laatsch, 2006). The chemical structures of all mentioned fungal substituted tryptamines are depicted in Figure 1.

The most popular and richest sources of psilocybin and psilocin are fruit bodies of European species *Psilocybe semilanceata*, called “liberty caps,” and subtropical species *Psilocybe cubensis*. Psilocin and psilocybin are controlled psychoactive substances under Schedule I of the United Nations Convention on Psychotropic Substances (1971). Most of European and non-European countries adapted this classification also with hallucinogenic mushrooms containing these substances, and therefore the

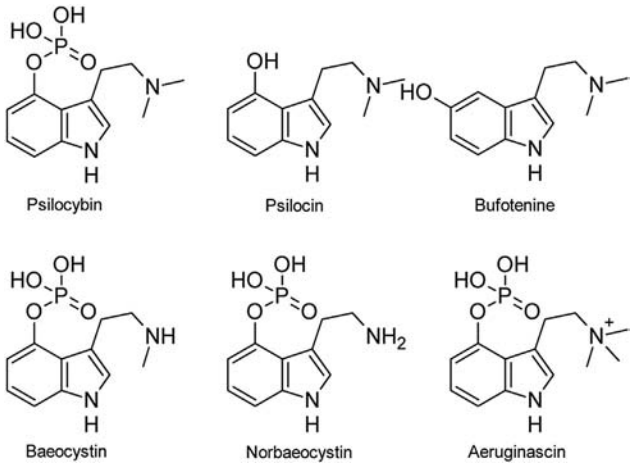

FIGURE 1 Chemical structures of fungal hallucinogens derived from tryptamine. Psilocin and its 5-OH isomer, bufotenine, are fungal hallucinogenic substituted tryptamines. Psilocin is derived from its precursor, psilocybin, through dephosphorylation. Similar phosphorylated compounds differing in a number of *N*-methyl groups were also found in fungi.

cultivation, possession, and sale of the mushrooms are usually specifically prohibited or prohibited by extension. Fruit bodies, if dried at temperatures not exceeding 40 °C, maintain high psilocybin content for a long time. Local jurisdictions consider the procedure of drying as part of psilocybin isolation and narcotic drug production. Therefore, the sale of fresh "magic mushrooms" was legal in several countries (for example, in the United Kingdom until 2005 and in Dutch smart shops until 2008). Since the fungal spores do not contain psychoactive compounds, many business entities still offer them legally for sale, along with all materials and informational support necessary for cultivation of fruit bodies or sclerotia. In addition, the mushrooms that produce hallucinogenic indolealkylamines are available from natural habitats, although many morphologically similar poisonous mushrooms may be easily mistaken for hallucinogenic fungi of *Psilocybe* genus. Fruit bodies and sclerotia of hallucinogenic mushrooms are eaten fresh or dried (Andersson, Kristinsson, & Gry, 2009; Pellegrini et al., 2013; Satora, Goszcz, & Ciszowski, 2005). A typical dose of fresh "liberty caps" is 10–60 specimens (weighing a few grams, containing up to several dozen milligrams of psilocybin) (van Amsterdam, Opperhuizen, & van den Brink, 2011; Andersson et al., 2009; Asselborn, Wennig, & Yegles, 2000; Satora et al., 2005). Such high psilocybin doses are usually not absorbed, since the mushrooms cause nausea and vomiting soon after ingestion. They are consumed raw or are added to hot meals. Dried mushrooms are sometimes smoked. Food products containing "magic mushrooms," such as honey, alcoholic beverage prepared from honey, so-called "blue mead," and chocolates are also available on a drug black market (Bogusz, Maier, Schäfer, & Erkens, 1998; Stenzel, 2008). Less experienced users interested in a purchase of hallucinogenic mushrooms may easily become victims of a deception. For example, in the early 1970, dried fruit bodies of a common champignon (mushroom; *Agaricus bisporus*) spiked with LSD or phencyclidine were sometimes sold as psilocybin-containing mushrooms (Andersson et al., 2009). Inebriation with "magic mushrooms" has become a popular way of spending free time in western countries, particularly among adolescents, students, or subcultures exposed also to other classes of psychoactive substances (van Amsterdam et al., 2011; Andersson et al. 2009; Asselborn et al., 2000; Halpern, 2004; Satora et al., 2005). The fungal hallucinogens were dedicated to prison subculture but they did not become preferred over other drugs, stimulants for example, because of the specific structure of this social group. Members of religious sects (formal churches or informal groups) may be potentially susceptible to abuse of psilocybin, since the compound has been shown to induce mystical-type experiences. Single consumption of the substance may produce acute psychosis or a state close to delirium, and abuse is suspected to precipitate schizophrenia. Alternations of perception and cognition, clouding of consciousness, or panic-like attacks developed in course of intoxication with "magic mushrooms" or due to persistent "flashbacks" may be a cause of traffic accidents. Cases of serious intoxication or even accidental deaths resulting from the psychoactive properties of these fungi are not very common but have been reported (Albers, Köhler, Lehr, Brinkmann, & Beike, 2004; van Amsterdam et al., 2011; Asselborn et al., 2000; Lim, Wasywich, & Ruygrok, 2012; Tiscione & Miller, 2006).

Additionally, there is still scientific interest in these indolealkylamines as hallucinogens and therapeutics. Therefore, analytical methods enabling their detection in fungal tissues, food, parapharmaceutical products, and body fluids have recently been developed. Identification of the mushrooms based on morphological characteristics of the fungal tissues or spores contained in the analyzed material is commonly practiced (Musshoff, Madea, & Beike, 2000; Tsujikawa et al., 2003). However, identification may become difficult when the analyzed material is powdered. By the use of polymerase chain reaction (PCR) techniques, it is possible to amplify even trace amounts of marker fungal DNA, but the developed methods (Lee, Cole, & Linacre, 2000; Linacre, Cole, & Lee, 2002; Maruyama, Yokoyama, Makino, & Goda, 2003; Nugent & Saville, 2004) do not allow the exact concentration of psychoactive indolealkylamines in the analyzed material to be established, and are not suitable for direct analysis of body fluids for the presence of these substances. Highly selective and sensitive radioimmunoassay for psilocin detection has recently been described (Albers et al., 2004). Tiscione and Miller (2006) demonstrated that the fluorescence polarization immunoassay routinely used for detection of amphetamine and methamphetamine gives false-positive results with psilocin. Immunoassays developed for a particular antigen detection generally reveal cross-reactivity with other classes of substances, and it is necessary to confirm the identification of the found compound with other methods. When ingested, psilocybin is quickly converted to psilocin and metabolized mainly to 4-hydroxyindole-3-acetic acid and glucuronides; the presence of these metabolites has been taken into account in the analysis of body fluid samples (Hasler, Bourquin, Brenneisen, Bär, & Vollenweider, 1997; Hasler, Bourquin, Brenneisen, & Vollenweider, 2002). The psilocin elimination half-life after ingestion of a typical psilocybin dose (0.224 mg/kg) was estimated to be around 160 min in human subjects (Hasler et al., 1997). The authors demonstrated that maximal plasma concentration following oral dosing is reached usually after 1.5 h after ingestion (mean 8.2 ng/ml, range 4.8–12.3 ng/ml) (Hasler et al., 1997). In 6–7 h, psilocin may become undetectable in plasma; it is dependent on the exact ingested dose and the analytic method's sensitivity. However, when glucuronide cleavage was performed, the detection of

psilocin in serum collected from intoxicated subject was possible even after 52 h following ingestion (Kamata, Nishikawa, Katagi, & Tsuchihashi, 2006). Urine samples contain detectable amounts of psilocin or its glucuronide typically up to 24 h after ingestion. At a dose of 0.21 mg/kg, the highest concentrations of psilocin (up to 871 ng/ml) were detected within 2–4 h after ingestion (Hasler et al., 2002).

Psilocin concentration in body fluid samples, particularly if not protected from oxidation during analytic procedures, was shown to decrease with time (Martin, Schürenkamp, Pfeifer, & Köhler, 2012). Therefore, routine detection of psilocin in body fluids should be performed rapidly. The best way to reach this goal is by use of high-performance liquid chromatography (HPLC) or ultra-high-performance liquid chromatography (UHPLC) along with a sufficiently sensitive detector system. At present, mass spectrometry is the most frequently utilized for this purpose. Of particular importance are methods enabling simultaneous analysis of several commonly abused substances along with indolealkylamines (Alnajjar, Idris, Multzenberg, & McCord, 2007; Björnstad, Beck, & Helander, 2009; Kikura-Hanajiri, Hayashi, Saisho, & Goda, 2005; Martin, Schürenkamp, Gasse, Pfeiffer, & Köhler, 2013).

PREPARATION OF FUNGAL SAMPLES

The content of psychoactive indolealkylamines in fungi of *Psilocybe* genus is highly variable, dependent on many environmental factors, and usually unpredictable. Moreover, the content of psilocybin and psilocin in fresh mushrooms quickly decreases. The exact concentration of psychoactive substances in fungal material is a meaningful information in forensic sciences. Sample preparation for psilocybin and psilocin detection in fungal material or food products containing the fungi is of great importance for the accuracy of the analysis. Psilocybin and a product of its dephosphorylation, psilocin, are commonly present together in fungal samples. If detection of psilocybin (or other phosphorylated compounds) is necessary, the solvents used for extraction should be carefully chosen, since these compounds are soluble only in highly polar solvents such as water, methanol, ethanol, or water–alcohol mixtures. Mushrooms or mycellium are usually dried and pulverized or lyophilized before extraction. Psilocin and psilocybin are typically extracted with methanol; this procedure additionally prevents enzymatic activity and precipitates proteins. However, methanol is not the best solvent for psilocybin and psilocin, and both compounds were more efficiently extracted from fungal tissues with water–alcohol mixtures (Kysilka & Wurst,1990). Water–ethanol mixture maintains alkaline phosphatase activity (Gartz, 1994; Kysilka & Wurst, 1990) and therefore is not recommended for extraction of phosphorylated indolealkylamines. Kysilka and Wurst (1990) developed a method of psilocybin extraction in a mixture of methanol and water 75:25 (v/v) saturated with potassium nitrate. The same mixture was used for extraction in a study by Maruyama et al. (2003). In their study, Laussmann and Meier-Giebing (2010) used acidified methanol for extraction of dried or fresh fungi containing psilocin and psilocybin. Rat plasma samples were analyzed for the presence of psilocin after the animals were administered water extract prepared from *Gymnopilus spectabilis* (Chen et al., 2011). Acidified water extracts were obtained by several authors (Casale, 1985; Cepas, Silva, & Pérez-Bendito, 1996; Koçak, De Cotiis, & Hoffman, 2010). Less polar organic solvents, such as diethyl ether, ethyl acetate, and chloroform were also used for psilocin extraction (Cepas et al., 1996; Keller, Keller, Tutsch-Bauer, & Monticelli, 2006; Keller et al., 1999; Saito et al., 2004).

Time of extraction depends on the amount of the extracted material, volume of solvent, and technique of extraction. If the extraction is not assisted with any method of agitation or if the samples are only shaken or stirred, the procedure should typically last up to 12–24 h. Sonication, providing more efficient disruption of fungal tissues, shortens the time of extraction with one portion of a solvent to 15–60 min. However it was demonstrated that time extension of the procedure of extraction with methanol to about 12 h (without agitation) provides higher psilocybin recovery when compared to sonication-assisted extraction lasting 15 min (Anastos, Lewis, Barnett, & Sims, 2006). Extraction procedure should be repeated to improve recovery of the analyzed substance and sensitivity of the method. The suspension is then centrifuged, supernatants collected, filtered, and subjected to partial purification or subjected directly to quantitative analysis.

PRELIMINARY QUALITATIVE ANALYSIS

The detection of indolealkylamines in the fungal extracts or fractions obtained from them, for example, if the original fungal material suspected to contain psychoactive indolealkylamines was not fully identified, may be done directly in the fungal tissue, in solution, or after thin-layer chromatography (TLC) of the solution sample using several reagents. Tissue bluing in the air after damage is a result of nonenzymatic or enzymatic oxidation of the indole alkaloids (Bocks, 1967). Psilocybin, psilocin, and baeocystin contained in the extract of *P. baeocystis* appeared as blue spots in TLC (Beug & Bigwood, 1981). If iodine vapors are used for detection, the spot representing psilocin quickly turns blue. The chromatograms were also observed under short-wave ultraviolet (UV) radiation (Stead, Gill, Wright, Gibbs, & Moffat, 1982). Indolealkylamines are sensitive to Erchlich's reagent (van Urk's reagent) containing *p*-dimethylbenzaldehyde acidified with HCl (Beug & Bigwood, 1981; Darby, Ford-Kirkpatrick, Lewis, & Clarke, 1967; Gartz, 1989). Another popular means of psilocin detection on TLC plates is reagent containing 4-(dimethylamino)-cinnamaldehyde (Gartz, Allen, & Merlin, 1994). Diazotized *p*-nitroaniline followed by alkali detects indolealkylamines but not psilocybin (Darby et al., 1967). Indolealkylamines reduce 1% aqueous solution of potassium permanganate (Darby et al., 1967). On TLC chromatograms dipped in a mixture composed of 20% of anhydrous toluene-*p*-sulphonic acid in methanol, spots representing psilocin and psilocybin turn brownish-purple (Beug & Bigwood, 1981). Substituted indole ring of tryptamines, including psilocin, is also sensitive to the Meixner test used for detection of α-amanitin (a sample blotted on a paper and sprayed with concentrated hydrochloric acid gives a blue-colored product in reaction with lignin) (Beuhler, Lee, & Gerkin, 2004). Retention factors (R_F) established for psilocybin, psilocin, or similar compounds subjected to TLC using different systems of solvents and types of plates are presented in Table 1.

Identification based on TLC should be confirmed with other analytical methods. As a technique applicable to qualitative analysis of many drugs of abuse, ion mobility spectrometry (IMS) was also used for psilocin and psilocybin determination (Keller et al.,

TABLE 1 Retention Factors (R_F) Established for Psilocybin, Psilocin, and Other Indolealkylamines Subjected to Thin-Layer Chromatography or Paper Chromatography

Analyzed Material	Type of Plates	System of Solvents	R_F	Type of Reagent, Color of Spots
Beug and Bigwood (1981)				
Psilocybe baeocystis, fruit bodies	Silica gel	Butanol– acetic acid–water 12:3:5 (v/v)	Psilocybin 0.16 Psilocin 0.36	Spots were visualized with Ehrlich's reagent: psilocybin turned pinkish-brown, psilocin–purple. Many other spots became visible.
	Cellulose plates	Butanol–acetic acid–water 12:3:5 (v/v)	Psilocybin 0.48 Psilocin 0.78	
	Silica gel	Butanol–acetic acid–water 2:1:1 (v/v)	Psilocybin 0.21 Psilocin 0.46	
	Silica gel	1.5% ammonia solution in methanol	Psilocybin 0.14 Psilocin 0.45	
	Silica gel	Conc. ammonia–water–propanol 12:188:500 (v/v)	Psilocybin 0.11 Psilocin 0.58	
	Silica gel	Conc. ammonia–water–propanol 10:50:150 (v/v)	Psilocybin 0.16 Psilocin 0.82	
Gartz (1989)				
Psilocybe cubensis, *Psilocybe semilanceata*	Silica gel	Butanol–water–acetic acid –isopropanol 8:5:2:1 (v/v)	Psilocybin 0.16 Psilocin 0.21	Visualization was performed with Ehrlich's reagent–psilocin turned blue.
	Silica gel	*n*-propanol–water–acetic acid 10:3:3 (v/v)	Psilocybin 0.23 Psilocin 0.36	
Darby et al. (1967)				
Standard solutions, a method developed for purposes of forensic investigations	Whatman paper No. 1 dipped in 5% sodium dihydrogen citrate solution and dried	Citrate–butanol system (4.8 g of citric acid dissolved in a mixture composed of 130 ml of water and 870 ml of butanol)	Psilocybin 0.05 Psilocin 0.31 Bufotenine 0.16 5-metoxy-DMT 0.26	Visualization was performed with: • Ehrlich's reagent: psilocybin, psilocin- gray; bufotenine, 5-metoxy-DMT–purple; • Diazotized *p*-nitroaniline followed by alkali: psilocin–gray then purple; bufotenine–orange then purple; 5-metoxy-DMT–orange.
Stead et al. (1982)				
Standard solutions, a method developed for purposes of forensic investigations	Silica gel plates dipped in 0.1 N KOH and dried	Methanol–concentrated ammonia 100:1.5 (v/v)	Psilocin 0.39	Spots were observed under short-wave UV lamp.
		Cyclohexane–toluene–diethylamine 75:15:10 (v/v)	Psilocin 0.05	
		Chloroform–methanol 9:1 (v/v)	Psilocin 0.09	
		Acetone	Psilocin 0.09	

Leung and Paul (1968)							
Mycelia pellets of *P. baeocystis*	Not mentioned	Two dimensional TLC was performed using solvent system A and B	Norbaeocystin–0.17 (solvent system A) Norbaeocystin–0.58 (solvent system B) Baeocystin–0.16 (solvent system A) Baeocystin–0.46 (solvent system B)		Ehrlich's reagent and modified phosphate reagent were used for visualization.		
Jensen (2004)							
Crude or enriched extracts of *Inocybe aeruginascens* and standard solutions of synthetic substances	Silica gel		Visualization was performed with Ehrlich's reagent:				
			Aeruginascin (purple)	Psilocin (violet then bluish violet)	Psilocybin (purple then violet)	Baeocystin (purple then violet)	Norbaeocystin (purple then violet)
	Butanol–acetic acid–water 2:1:1 (v/v)		0.30	0.66	0.42	0.49	0.60
	Butanol–acetic acid–water 24:10:10 (v/v)		0.25	0.62	0.37	0.46	0.56
	Propanol–6% water ammonia solution 5:2 (v/v)		0.08	0.72	0.14	0.12	0.13
	Butanol–acetic acid–isopropanol–water 8:2:3:5 (v/v)		0.31	0.70	0.46	0.55	0.64
	Butanol–acetic acid–isopropanol–water 8:2:1:5 (v/v)		0.35	0.66	0.49	0.54	0.60
	Propanol–concentrated ammonia solution 5:3 (v/v)		0.20	0.92	0.32	0.22	0.18
	Propanol–acetic acid–water 10:3:3 (v/v)		0.25	0.63	0.38	0.51	0.64
	Methanol–water–concentrated ammonia solution 70:30:0.2 (v/v)		0.23	0.32	0.51	0.83	0.93
	Methanol–water–formic acid 80:20:0.2 (v/v)		0.40	0.71	0.56	0.69	0.74

This table presents examples of qualitative determination of indolealkilamines obtained by TLC using different solvent systems and types of a sorbent.

2006, 1999). This technique is simple, fast, and convenient, since time-consuming extraction procedures are not required. Koçak et al. (2010) used reflectance infrared spectroscopic methods (attenuated total reflectance (ATR) and transflection) for qualitative detection of extracted fungal psilocin.

Chromatography coupled with mass spectrometry (GC–MS, HPLC-MS, HPLC-MS-MS) simplifies confirmation of the chemical structure of the analytes, including indolealkylamines of interest and their derivatives.

PURIFICATION AND ISOLATION

The presented methods of psilocybin or psilocin purification were usually developed for purposes of isolation. However, after slight modification, they may be applied for identification, since elimination of lipids simplifies detection, particularly if done by means of GC–MS. Similarly, identification and confirmation of the chemical structure performed with infrared or nuclear magnetic resonance (NMR) spectroscopy is much easier when purified material is analyzed.

Partial purification may be obtained by the use of systems containing several solvents differing in polarity. The most polar phosphorylated indolealkylamines are extracted with polar solvents. For example, psilocybin and aeruginascin could be extracted from *I. aeruginascens* fruit bodies with a mixture composed of methanol–water–formic acid 80:20:0.2 (v/v) (Jensen et al., 2006). The fungal material was extracted with cyclohexane, ethyl acetate, and ethanol before this procedure to eliminate more hydrophobic substances (Jensen et al., 2006). In another method, a mycellial pellet was extracted with petroleum ether using a Soxhlet extractor to eliminate lipids before the extraction of psychoactive alkaloids performed with methanol (Leung & Paul, 1968). Koike, Wada, Kusano, and Nozoe (1981) fractioned methanol extract to aqueous and diethyl ether solutions. Aqueous solution contained psilocybin; the compound could be isolated by means of liquid chromatography using a cellulose column (Koike et al., 1981). A diethyl ether fraction contained lipophilic compounds, namely ergosterol and ergosterol peroxide (Koike et al., 1981). To avoid psilocybin dephosporylation, it is recommended not to exceed the temperature of 40 °C during the procedures of drying and extraction. If necessary, dephosphorylation may be easily obtained in acidified water extracts (pH 4.0) by heating at the temperature of 70–90 °C for 1–2 h (Casale, 1985; Cepas et al., 1996; Koçak et al., 2010). The solution may then be extracted with nonpolar solvents to eliminate lipid impurities. Subsequent alkalization of the solution (pH 8.0) leads to psilocin deionization; such deprotonated psilocin may be extracted using a liquid–liquid extraction procedure with nonpolar solvents (diethyl ether, chloroform, dichloromethane, mixture of isopropanol with dichloromethane 1:3 (v/v), *n*-butyl chloride). Recrystallization of crude diethyl ether extract from chloroform/*n*-heptane 1:3 (v/v) resulted in satisfactory purification of psilocin (Casale, 1985). Psilocin from crude extract may be partially purified by means of ion-exchange chromatography (cation exchange sorbent). Anion exchange sorbent was used in a study by Koike et al. (1981), who fractioned in this way methanol extract (previously evaporated, redissolved in water, and alkalized to pH 9.5). The material eluted from the sorbent with 0.1 M NH_4HCO_3 buffer, pH 9, containing psilocybin and baeocystin, was then subjected to HPLC analysis (Koike et al., 1981). Such procedures, however, are time-consuming, may lead to partial loss of the analyzed substance, and expose indolealkylamines to oxidation.

PREPARATION AND PRELIMINARY PURIFICATION OF BODY FLUID SAMPLES

Samples of body fluids need special treatment, since they contain cellular components, proteins, large concentrations of numerous metabolites, and sometimes only trace amounts of the analyzed indolealkylamines. The presence of the metabolites makes separation and analysis, particularly if done with electrospray ionization–mass spectrometry (ESI-MS), more difficult. Blood samples are centrifuged and the resultant supernatant (plasma) collected for further analysis. Plasma proteins may be precipitated with methanol or a 20% solution of PEG 6000 on ice (Lindenblatt, Krämer, Holzmann-Erens, Gouzoulis-Mayfrank, & Kovar, 1998). Acetonitrile was also used for this purpose (Chen et al., 2011). Hasler et al. (1997) applied a microdialysis technique using polycarbonate membrane probes. Several authors used ascorbic acid to prevent psilocin oxidation in plasma and urine samples (Hasler et al., 1997, 2002; Kamat et al., 2006; Martin et al., 2013). It was necessary to use a precolumn for HPLC separation of the components contained in the protected samples to prevent ascorbic acid and other plasma components loading to the analytic column (Hasler et al., 1997).

Alternatively, preliminary purification may be applied. In a method developed by Saito et al. (2004) plasma was mixed with ethyl acetate and the mixture centrifuged. Separatory funnels or centrifugal tubes in liquid–liquid extraction methods may be replaced with solid-phase columns filled with diatomaceous earth (Asselborn et al., 2000; Lindenblatt et al., 1998). Solid-phase extraction (SPE) is usually performed after separation of precipitated proteins at slightly acidic conditions that are appropriate for enzymatic reaction with glucuronidase and to protonate indolealkylamines of interest, particularly if cation exchange sorbent is used (Lindenblatt et al., 1998; Martin et al., 2013). In case of reversed-phase sorbent, the samples are applied as slightly alkalized water solutions and the analytes eluted with organic solvent (Albers et al., 2004; Bogusz, 2000). Usually it is necessary to initially purify urine samples, since urine matrice is very rich. Solid phase extraction using reversed-phase or mixed-mode SPE columns was typically applied for this purpose by several authors (Alnajjar et al., 2007; Grieshaber, Moore, & Levine, 2001; del Mar Ramirez Fernandez et al., 2007; Sticht & Käferstein, 2000; Tiscione & Miller, 2006). An automated Remedi HS drug profiling system utilizing multicolumn SPE as a method of initial purification appeared to be useful for psilocin detection in urine samples (Sticht & Käferstein, 2000). Before loading to the SPE column, an internal standard is usually added to the sample. SPE methods developed for initial purification of body fluid samples are presented in Table 2.

Plasma, serum, and urine samples treated with glucuronidase yield higher content of psilocin (Hasler et al., 1997, 2002; Grieshaber et al., 2001; Sticht & Käferstein, 2000). In particular, urine samples should be subjected to hydrolysis of psilocin

TABLE 2 Solid-Phase Extraction Methods Used for Psilocin/Psilocybin Determination

Column, Sorbent Type	Sample Preparation	Conditioning	Washing Protocol	Eluent
Diatomaceous Earth				
Lindenblatt et al. (1998)				
Extrelut 3 columns	Plasma sample (2 ml), proteins precipitated with methanol (1.2 ml), pH adjusted to 8.5	–	–	Two 6 ml portions of dichloromethane
Asselborn et al. (2000)				
Chem Elut columns	Plasma, urine, tissue homogenates, pH adjusted to 8.5	–	–	Dichloromethane–isopropanol 85:15 (v/v)
Reversed Phase				
Bogusz (2000)				
Bond Elut C18 cartridges	Blood or urine samples (0.2–1 ml), after centrifugation diluted with 0.01 M ammonium carbonate buffer, pH 9.3 (2 ml)	Before use SPE cartridges were rinsed with methanol (1 ml), water (1 ml), and 0.01 M ammonium carbonate buffer (2 ml).	After application of a sample (2 ml) cartridges were rinsed with 0.01 M ammonium carbonate buffer, pH 9.3 (2 ml) and dried under vacuum.	Methanol–0.5 M acetic acid 9:1 (v/v) (0.5 ml)
Alnajjar et al. (2007)				
C18 cartridges	Urine samples, pH adjusted to 9.5 (0.5 ml)	For conditioning cartridges were rinsed with methanol, water and buffer, pH 9.5 (0.7 ml).	Washing was performed with water (0.5 ml).	85% methanol (20 µl) and water (70 µl)
Mixed-Mode				
Albers et al. (2004)				
Strata X columns, polymeric reversed phase sorbent	Serum or blood samples (1 g) were diluted with 1.5 ml of phosphate buffer, pH 6.	Conditioning was performed with methanol (1.5 ml) and water (1.5 ml).	After washing with two portions of 5% methanol in water (2 × 0.75 ml) the columns were dried under a stream of nitrogen.	Methanol (2 × 0.75 ml)
Martin et al. (2013)				
Oasis MXC SPE cartridges, mixed mode cation exchange sorbent	Plasma samples (1 ml) or urine samples (0.5 ml) were diluted with 0.1 M phosphate buffer, pH 6 (2 ml), protected from oxidation by the addition of ascorbic acid (10 µl of 0.1 M solution).	Conditioning was performed with methanol (1.5 ml) and phosphate buffer, pH 6 (1.5 ml).	After the sample loading columns were washed with phosphate buffer (2 ml), water (3 ml), methanol (2 ml) and dried for 5 min with a stream of nitrogen. The washing was continued with ethyl acetate (2 ml), the columns were dried again before elution.	Two 1 ml portions of mixture composed of 2% ammonium hydroxide in dichloromethane–isopropanol 80:20 (v/v)

Continued

TABLE 2 Solid-Phase Extraction Methods Used for Psilocin/Psilocybin Determination—cont'd

Column, Sorbent Type	Sample Preparation	Conditioning	Washing Protocol	Eluent
Sticht and Käferstein (2000)				
Varian Certify LRC columns, mixed-mode, containing cation exchange and hydrophobic C8 functional groups	Serum or urine samples (0.5 ml) were diluted with 5 ml of phosphate buffer, pH 8.	Conditioning performed with methanol (2 ml), phosphate buffer, pH 8 (2 ml).	Washing performed with water (2 ml), acetate buffer, pH 4 (2 ml), methanol-water 3:7 (v/v) (2 ml), washing followed by drying under vacuum.	Two portions of methanol–ammonium hydroxide 98:2 (v/v) (2 ml + 1 ml) under vacuum
Tiscione and Miller (2006)				
Bond Elut Certify, mixed-mode	Urine samples (5 ml) were diluted with 0.1 M phosphate buffer (5 ml).	Columns were rinsed with methanol (2 ml), 0.1M phosphate buffer under slight vacuum.	Washing done with water (1 ml), 0.01 M acetic acid (0.5 ml) was followed by drying under vacuum, methanol wash (50 ml), and drying again.	Acidic and neutral substances were eluted with acetone–chloroform 1:1 (v/v) (4 ml), basic compounds were eluted with two portions of ethyl acetate–ammonia 98:2 (v/v) (2 × 2 ml).
del Mar Ramirez Fernandez et al. (2007)				
Oasis MCX SPE cartridges, mixed mode cation exchange sorbent	Urine samples (0.5 ml) were diluted with sodium acetate buffer, pH 4 (0.6 ml).	Conditioning performed with methanol (1 ml), water (1 ml), and 0.1 mM sodium acetate buffer, pH 4 (1 ml).	The following washing protocols were tested: • sodium acetate buffer, pH 4, followed by methanol (1 ml portions); • sodium acetate buffer, pH 4, and methanol–water 8:2 (v/v) (1 ml portions); • sodium acetate buffer, pH 4, methanol–water 1:1 (v/v). The third protocol appeared optimal. Drying was performed under vacuum.	Methanol–ammonia 95:5 (v/v) (0.5 ml)
Grieshaber et al. (2001)				
Clean Screen columns	Urine samples (5 ml) were diluted with 0.1 M phosphate buffer, pH 6 (2 ml).The samples were subjected to psilocin glucuronide cleavage, centrifuged and filtered before loading to the SPE column.	Conditioning performed with methanol (3 ml), water (2 ml), and phosphate buffer, pH 6 (2 ml).	Washing carried out with water (3 ml), acetonitrile–water 2:8 (v/v) (2 ml), 0.1 M acetic acid in water (1 ml) was followed by drying under vacuum. Washing was continued with hexane (2 ml), hexane–ethyl acetate 1:1 (v/v) (1 ml), and methanol (1 ml). Before elution of basic compounds the column was dried again under vacuum.	Dichloromethane–isopropanol-ammonia (v/v) 78:20:2 (3 ml)
Cation Exchange Sorbent				
Lindenblatt et al. (1998) (SPE cartridges coupled to analytic column)				
CBA cation exchange sorbent, carboxymethyl functional groups	Plasma samples (0.4 ml), after precipitation of proteins with PEG 6000 on ice, were diluted with lithium acetate solution, pH 6.8.	Cartridges were washed with methanol, acetic acid, and lithium acetate solution, pH 6.8.	Lithium acetate solution, pH 6.8	Methanol

Examples of initial purification of body fluid samples performed with SPE for purposes of psilocybin and/or psilocin detection are presented in this table.

glucuronide before quantitative analysis. Acidic or alkaline conditions were found to be ineffective (Kamata, Nishikawa, Katagi, & Tsuchihashi, 2003). Complete enzymatic hydrolysis of psilocin glucuronide was achieved with *Escherichia coli* β-glucuronidase (Björnstad et al., 2009; Grieshaber et al., 2001; Kamata et al., 2003; Martin et al., 2013). Three other enzymes tested appeared to be less effective (B-1 type bovine liver, H-1 type *Helix pomatia*, *Ampullaria*) (Grieshaber et al., 2001; Kamata et al., 2003). After enzymatic hydrolysis, body fluid samples should be deproteinized with methanol before analysis. In the study by Hasler et al. (2002), urine samples after incubation with β-glucuronidase were freeze-dried and the lyophilisates were extracted with this solvent. It is recommended to hydrolyze serum samples also. In their study, Kamata et al. (2006) used *E. coli* β-glucuronidase for this purpose.

DERIVATIZATION OF A SAMPLE

A derivatization step may be necessary if the GC–MS technique is applied for separation, detection, and quantitative determination, because psilocin is a compound susceptible to oxidation and undergoes decomposition at high temperatures (due to the presence of a phenolic group). Also, psilocybin should be derivatized, since it is a highly polar and unstable compound, easily converted to psilocin. GC–MS analysis of psilocin and psilocybin in methanol extract of *Psilocybe* mushrooms was usually performed after volatilization with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) (Asselborn et al., 2000; Grieshaber et al., 2001; Keller et al., 2006, 1999; Sticht & Käferstein, 2000; Stříbrný, Borovička, & Sokol, 2003). The obtained derivatives, namely di-TMS-psilocin and tri-TMS-psilocybin, are more volatile, less polar, and more stable then the precursor molecules.

Some authors, however, performed derivatization for purposes other than GC–MS analysis. Conjugation of the analytes with fluorescent reagent may be performed if one uses fluorescence detector systems (high-performance liquid chromatography–fluorescence detection (HPLC-FL)). Compounds containing indole structure, including psilocin, emit fluorescent light (of maximum at about 340 nm) upon excitation with 270 nm light (Saito et al., 2004). However the intensity of this fluorescence is not high enough to detect small concentrations in body fluid samples. Derivatization with the fluorescent reagent 4-(*N*,*N*-dimethylaminosulfonyl)-7-(2-chloroformyl-pyrrolidin-1-yl)-2,1,3-benzoxadiazole (DBD-Pro-COCl) was successfully applied for analysis of psilocin (and bufotenine used as an internal standard in this study) (Saito et al., 2004). Fluorescence labeling of psilocybin after derivatization with 5-dimethylaminonaphthalene-1-[*N*-(2-aminoethyl)]sulfonamide (DNS-ED) enables determination with a fluorescence detector (Saito et al., 2005). Derivatization of psilocin with dansyl chloride yields a fluorescent product (Cepas et al., 1996). Dansylated indoles in reaction with bis(2,4,6-trichlorophenyl)oxalate–hydrogen peroxide system produce intensive chemiluminescence, and a method of detection based on this property was developed (Cepas et al., 1996). Derivatization of psilocybin and baeocystin with propyl chloroformate improved identification of the two indole compounds, carried out by means of capillary zone electrophoresis at alkaline conditions (Pedersen-Bjergaard, Rasmussen, & Sannes, 1998).

QUANTITATIVE ANALYSIS

The simplest methods of quantitative determination of psilocin or psilocybin directly in fungal extracts are based on reaction with Ehrlich's reagent. Catalfomo and Tyler (1964) employed an assay utilizing partition paper chromatography and visualization with Ehrlich's reagent. By performing series of dilutions, it was possible to establish the amount of the extract containing the smallest detectable amount of psilocybin (1 μg) (Catalfomo & Tyler, 1964). Psilocybin content determination could be done with colorimetric method based on the reaction with *p*-dimethylbenzaldehyde (Gartz, 1986). These methods, however, are not sensitive enough for detection of psilocybin/psilocin in body fluids where at least nanogram amounts in 1 ml should be detectable. Different more sensitive methods of quantitative determination of psilocybin/psilocin, usually based on gas chromatography and HPLC techniques, have been developed so far. Several examples are presented in Table 3.

Capillary electrophoresis is an analytical technique applicable for both qualitative and quantitative determination of polar and charged compounds such as psilocin and psilocybin. The methods have been developed for detection of these substances in fungal extracts and body fluids. Asselborn et al. (2000) used capillary zone electrophoresis to determinate psilocin and psilocybin in fungal extract. This technique was applied for separation and detection of psilocybin and baeocystin in extracts of *Psilocybe semilancelata* (Pedersen-Bjergaard, Sannes, Rasmussen, & Tonnesen, 1997). Psilocybin could be also detected quantitatively (Pedersen-Bjergaard et al., 1997). Psilocybin and baeocystin derivatized with propyl chloroformate could also be analyzed with capillary zone electrophoresis at alkaline conditions (Pedersen-Bjergaard et al., 1998). The addition of β-cyclodextrin and organic solvents to the electrolyte made possible the satisfactory simultaneous separation of 19 different drugs of abuse, psilocin among them (Alnajjar et al., 2007). Molecule of β-cyclodextrin is cylindrical in shape, and the interior of the molecule is considerably hydrophobic. Thus, the molecule of β-cyclodextrin is able to incorporate other hydrophobic molecules forming inclusion complexes. The migration rate of the complexes is decreased in comparison to the free molecules. Organic solvents additionally modify the rate of formation of such complexes, influencing separation.

Gas chromatography combined with mass spectrometry (usually in the electron ionization mode) is sometimes applied for the detection of psilocin or psilocybin in fungal material or body fluids, despite the thermolability of these compounds (the exemplary conditions of the methods are presented in Table 4). GC–MS methods allow matching of the obtained mass spectra with library mass spectra, simplifying identification. In a GC–MS method developed by Kikura-Hanajiri et al. (2005), a mixture of 18 typical psychoactive phenethylamines, β-carbolines, and indolealkylamines, including psilocin, was successfully separated without previous derivatization. Qualitative detection of psilocin in a urine sample was performed with GC–MS without derivatization (Tiscione & Miller, 2006). Direct psilocin detection in urine

TABLE 3 Sensitivity of Methods Developed for Psilocybin and Psilocin Detection

Type of Method and Authors	Compound	Limit of Detection (Usually Defined as Signal-to-Noise Ratio Equal to 2 or 3)	Limit of Quantitation (Usually Defined as Signal-to-Noise Ratio Equal to 10, or the Lowest Concentration of Standard Solution That Was Calculated to Be within ±20% of the Nominal Value and with a Coefficient of Variation Less than 20%)
Methods of Quantitative Analysis of Fungal Material			
HPLC-UV (Laussmann & Meier-Giebing, 2010)	Psilocin	0.6 ng (6 μg/100 mg of dry weight)	2.2 ng (22 μg/100 mg of dry weight)
	Psilocybin	0.7 ng (7 μg/100 mg of dry weight)	2.8 ng (28 μg/100 mg of dry weight)
Capillary zone electrophoresis (Pedersen-Bjergaard et al., 1997)	Psilocybin	0.0009 mg/ml corresponding to 4.5 μg/100 mg of dry weight	0.0045 mg/ml corresponding to 22.5 μg/100 mg of dry weight
HPLC-ESI-MS-MS (Pellegrini et al., 2013)	Psilocin Psilocybin	0.3 μg/100 mg of dry weight	10 μg/100 mg of dry weight
HPLC-FL (Saito et al., 2005)	Psilocybin	–	0.44 μg/100 mg of dry weight
HPLC-ESI-MS (Saito et al., 2004)	Psilocin	–	0.064 μg/100 mg of dry weight
Methods of Quantitative Analysis of Body Fluid Samples			
HPLC-UV (Anastos et al., 2006)	Psilocin	4.9×10^{-7} M (0.1 μg/ml)	1.2×10^{-6}M (0.24 μg/ml)
	Psilocybin	1.8×10^{-7} M (0.051 μg/ml)	4.0×10^{-7}M (0.11 μg/ml)
UPLC-UV (Chen et al., 2011)	Psilocin	0.05 μg/ml of plasma	0.12 μg/ml of plasma
HPLC-ECD (Hasler et al., 2002)	Psilocin	–	10 ng/ml of urine
GC–MS (Grieshaber et al., 2001)	Psilocin	–	10 ng/ml of urine
Capillary electrophoresis (Alnajjar et al., 2007)	Psilocin	13 ng/ml of urine (direct measurement) 7 ng/ml of urine (with a system of preliminary Sequential Injection -SPE)	–
GC–MS (Albers et al., 2004)	Psilocin	3 ng/g of blood	5 ng/g of blood
HPLC, postcolumn flow injection analysis based on chemiluminescence detection (Anastos et al., 2006)	Psilocin	1.2×10^{-8} M (2.45 ng/ml)	2.5×10^{-8}M (5.1 ng/ml)
	Psilocybin	3.5×10^{-9} M (1 ng/ml)	1.0×10^{-8}M (2.8 ng/ml)
HPLC-APCI-MS (Bogusz, 2000)	Psilocin, psilocybin	2.0 ng/ml of serum	–
HPLC-ESI-MS (Bogusz, 2000)	Psilocin	1.0 ng/ml of serum	–
LC-MS-MS (del Mar Ramirez Fernandez et al., 2007)	Psilocin	0.5 ng/ml of urine	10 ng/ml of urine
	Bufotenine	0.0125 ng/ml of urine	10 ng/ml of urine
LC-MS-MS (Martin et al., 2013)	Psilocin	0.05 ng/ml of plasma or serum, 0.2 ng/ml of urine	0.17 ng/ml of plasma, 0.15 ng/ml of serum, 0.2 ng/ml of urine
	Bufotenine	0.05 ng/ml of plasma, 0.07 ng/ml of serum, 0.1 ng/ml of urine	0.11 ng/ml of plasma, 0.27 ng/ml of serum, 0.14 ng/ml of urine
HPLC-ECD (Hasler et al., 1997)	Psilocin	–	0.8 ng/ml of plasma
LC-MS-MS (Martin et al., 2012)	Psilocin	0.1 ng/ml of plasma	0.34 ng/ml of plasma
Flow injection analysis with chemiluminescence detection (Anastos et al., 2005)	Psilocin	9×10^{-10} M (0.18 ng/ml)	–
	Psilocybin	3×10^{-10} M (0.09 ng/ml)	–

Among the methods developed for psilocin/psilocybin detection, the most sensitive are those based on chemiluminescence detection.

TABLE 4 Gas Chromatography–Mass Spectrometry (GC–MS) Methods Developed for Psilocybin or Psilocin Detection

Capillary Column Parameters	Injection Temperature	Column Heating Program
Kikura-Hanajiri et al. (2005)		
DB5-MS column, 30m × 0.25 mm × 0.25 μm	200 °C	Oven temperature was programmed to be kept at 120 °C during the first 1 min, to increase from 120 °C to 190 °C during the next 20 min, and to increase from 190 °C to 280 °C during the last 5 min.
Stríbrný et al. (2003)		
DB-5 column, 30 m × 0.25 mm × 0.25 μm	250 °C	Gradient heating was applied from 120 °C to 300 °C.
Albers et al. (2004)		
DB-5 MS column, 12 m × 0.2 mm × 0.33 μm	250 °C	Heating started from 140 °C (held for 4 min), temperature increased to 230 °C at a rate of 10 °C/min (held for 1 min), and to 280 °C at a rate of 25 °C/min, and the final temperature was held for 4 min.
Grieshaber et al. (2001)		
DB-5 column, 25 m × 0.32 mm × 0.17 μm	275 °C	Heating started from 70 °C (held for 1 min), then temperature increased to 240 °C at a rate of 20 °C/min and was kept constant for 2 min.
Asselborn et al. (2000)		
HP-Ultra 2 capillary column, 12 m × 0.2 mm × 0.33 μm	260 °C	Heating started from 70 °C (held for 2 min), then the temperature was programmed to increase from 70 °C to 220 °C at a rate of 25 °C/min, then to 255 °C at a rate of 5 °C/min, and to 300 °C, the final temperature was kept constant for 7 min.

This table presents several GC–MS methods of psilocin/psilocybin detection specifying conditions of column heating during elution performed with gas chromatography.

samples containing low amounts of this compound may be impossible (Grieshaber et al., 2001). The requirement of derivatization makes the GC–MS method inconvenient for the detection of psilocin and psilocybin. Heating with MSTFA for 30 min at 70 °C, 80 °C, or 130 °C was typically performed (Albers et al., 2004; Grieshaber et al., 2001; Keller et al., 1999; Sticht & Käferstein, 2000; Stříbrný et al., 2003).

HPLC methods are more convenient for determination of psilocin and psilocybin, since derivatization step may be omitted, separation is usually faster, and there is no need to heat the column, thereby preventing exposure of the labile compounds to high temperatures. Reversed-phase chromatography is generally used for separation with HPLC systems (see Table 5). Beug and Bigwood (1981) combined reversed-phase chromatography with ion-pair chromatography (heptanesulfonic acid was used as an ion-pair reagent). Ion-pair chromatography was utilized for the detection of a psilocin metabolite, namely 4-hydroxy-indole-3-acetic acid, using hexylamine as a counter-ion in a method developed by Hasler et al. (1997). Pentafluorophenyl columns display reversed-phase as well as normal-phase behavior, depending on the content of organic solvent in a mobile phase. Pentafluorophenyl column was applied for separation of psilocin glucuronide in a method developed by Manevsky et al. (2010). Stenzel (2008) performed separation of the compounds contained in the extract, including psilocin, using column of this type. Ion-exchange chromatography is much less frequently used. For example, Laussmann and Meier-Giebing (2010) used a cation exchange column for separation. Anion exchange chromatography was used for psilocybin detection in an HPLC method developed by Koike et al. (1981).

Psilocin is an electrochemically active substance, and therefore electrochemical detection may be performed in the case of HPLC separation, particularly in body fluid samples, due to the high sensitivity and selectivity of the method (Hasler et al., 1997; Kysilka & Wurst, 1990; Lindenblatt et al., 1998). However, despite relatively low psilocybin/psilocin absorbance at UV light, numerous HPLC methods involve UV detection (see Table 5 for authors and details of the methods that they developed). Combined with the Remedi HS system, HPLC-UV detection apparatus equipped with a library of spectra allowed the determination of psilocin quantitatively in urine samples but not in plasma samples (Sticht & Käferstein, 2000). Saito et al. developed methods enabling the detection of fluorescent derivatives of psilocin and psilocybin (Saito et al., 2004, 2005).

Nowadays, the most frequently used HPLC systems allow performance of detection with mass spectrometry and tandem mass spectrometry, enabling confirmation of the chemical structure of the analyzed compound. An HPLC method developed by Bogusz (2000) appeared to be suitable for detection of several common drugs of abuse, including psilocin and psilocybin, in biological samples for the purpose of forensic investigations.

TABLE 5 Examples of High-Performance Liquid Chromatography Methods Developed for Purposes of Psilocybin, Psilocin, and Psilocin Metabolites Detection

Column	Mobile Phase	Flow Rate
HPLC-UV Methods		
Bogusz et al. (1998) (a method developed for psilocin and psilocybin determination in fungal extract)		
Superspher RP 18 column 125 × 4 mm	Acetonitrile–50 mM triethylammonium phosphate buffer, pH 3.0, 3:97 (v/v)	0.7 ml/min
Tsujikawa et al. (2003) (a method developed for psilocin and psilocybin determination in fungal extract)		
Symmetry C18 column, 150 × 2.1 mm, 5 µm	10 mM ammonium formate buffer, pH 3.5–acetonitrile, 95:5 (v/v)	0.2 ml/min
Manevsky et al. (2010) (a method developed for psilocin glucuronide determination in reaction mixture)		
Agilent Zorbax Eclipse C18 Plus column, 250 × 4.6 mm, 5 µm	A was 0.1% formic acid, B was methanol; Gradient elution was carried out: initially with 5% B for the first 5 min, within the next 12 min the content of B increased from 5% to 40%, for the last 8 min the column was eluted with a mixture containing 5% B.	1 ml/min 1 ml/min 1.5 ml/min
Manevsky et al. (2010) (a method developed for psilocin glucuronide separation and detection in reaction mixture)		
Supelco Discovery HS F5 Column, 150 × 4 mm, 3 µm	A was 0.1% formic acid, B was acetonitrile; Gradient elution was performed: initially with 5% B for the first 5 min, within the next 10 min the content of B increased from 5% to 50%, for the last 10 min the column was eluted with a mixture containing 5% B.	0.8 ml/min
Beug and Bigwood (1981) (a method developed for psilocin and psilocybin determination in fungal extract)		
µBondapack C18 column, 300 × 3.9 mm, 10 µm (RP chromatography combined with ion-pair chromatography)	Water–methanol 75:25 (v/v) containing 0.05 M heptanesulfonic acid, adjusted to pH 3.5 with acetic acid	2 ml/min
Anastos et al. (2006) (a method developed for psilocin and psilocybin determination in fungal extract)		
Synergi Max-RP C_{12} column, 150 × 4.6 mm, 4 µm	Methanol-10 mM ammonium formate, pH 3.5, 95:5 (v/v)	0.5 ml/min
Musshoff et al. (2000) (a method developed for psilocin and psilocybin determination in fungal extract)		
LiChrospher 60 RP select B column, 250 × 4 mm, 5 µm	A was composed of 20 mM KH_2PO_4, B was acetinitrile; Gradient elution started from 5% B for 2.5 min, the content of B increased to 25% at 15 min.	1 ml/min
Laussmann and Meier-Giebing (2010) (a method developed for psilocin and psilocybin determination)		
Luna SCX 100A column, 150 × 4.6 mm, 5 µm (cation exchange chromatography)	50 mM KH_2PO_4buffer + 100 mM NaCl, pH 3.0-ethanol 95:5 (v/v)	1.5 ml/min
Koike et al. (1981) (a method developed for psilocybin determination in fungal extract)		
Lichrosorb NH_2 column, 250 × 4 mm (anion exchange chromatography)	0.5 M KH_2PO_4buffer, pH 5.5	–
HPLC-ECD Methods		
Kysilka and Wurst (1990) (a method developed for psilocybin determination in fungal extract, electrochemical and UV detection)		
Column packed with Silasorb SPH C18, 250 × 4 mm, 7.5 µm	Methanol–water–acetic acid 10:90:1 (v/v) for psilocybin determination, 35:65:1 (v/v) for psilocin determination	–

Lindenblatt et al. (1998) (methods developed for psilocin determination in human plasma)		
LiChroCart, Supersher 60 RP select B column, 250 × 4 mm, 5 µm	Buffer composed of 0.1 M sodium acetate, 0.1 M citric acid, 0.03 mM Na_2EDTA, pH 4.1- acetonitrile 83:17 (v/v)	0.7 ml/min
	150 mM KH_2PO_4 buffer, pH 2.3–acetonitrile 94.5:5.5 (v/v) + 160 µmol Na_2EDTA	0.6 ml/min
Hasler et al. (1997) (a method developed for psilocin detection in human plasma)		
Spherisorb RP-8, precolumn 50 × 4.6 mm, analytical column 150 × 4.6 mm, 3 µm	0.3 M ammonium acetate buffer, pH 8.3–methanol 47:53 (v/v)	0.45 ml/min
Hasler et al. (1997) (a method developed for 4-hydroxyindole-3-acetic acid detection in human plasma)		
Lichrospher 100 RP-18 column, 125 × 4.6 mm, 5 µm	70 mM ortho-phosphoric acid containing 5.5% acetonitrile (v/v) and hexylamine (300 µl/l)	1 ml/min
HPLC-FL Methods		
Saito et al. (2004) (a method developed for psilocin determination in fungal extract and rat plasma, derivatization with fluorescent reagent DBD-Pro-COCl, detection at 560 nm, excitation at 440 nm)		
Mightysil RP-18 GP column 150 × 4.6 mm, 3 µm	Water–acetonitrile 73:27 (v/v) containing 0.1% TFA	–
Saito et al. (2005) (for psilocybin determination in fungal extract, derivatization with DNS-ED, detection at 539 nm, excitation at 321 nm)		
Mightysil RP-18 GP column, 150 × 4.6 mm, 3 µm	Isocratic elution with 50 mM ammonium acetate–acetonitrile 77:23 for 22 min; then gradient elution from 77:23 (v/v) to 40:60 (v/v) for 13 min	1 ml/min
HPLC-APCI-MS Methods		
Bogusz et al. (1998), Bogusz (2000) (methods developed for psilocybin/psilocin detection in fungal extract or human body fluid samples)		
Superspher RP 18 column, 125 × 3 mm	Acetonitrile–50 mM ammonium formate buffer, pH 3.0, 5:95 (v/v) Acetonitrile–50 mM ammonium formate buffer, pH 3.0,15:85 (v/v)	0.6 ml/min 0.4 ml/min
HPLC-ESI-MS Methods		
Maruyama et al. (2003) (a method developed for psilocybin and psilocin detection in fungal extract)		
Inertsil ODS-3 column, 250 × 4.6 mm, 5 µm	Mobile phase was composed of acetonitrile in 10 mM ammonium formate buffer, pH 3.0.Gradient elution was performed: within the first 15 min of elution content of acetonitrile increased from 10% to 20% and then decreased to 10% within the next 5 min, and was maintained for the last 5 min.	0.5 ml/min
Kikura-Hanajiri et al. (2005) (a method developed for simultaneous determination of 19 hallucinogenic tryptamines, β-carbolines and phenethylamines, including psilocybin and psilocin, in extracts originating from fungal material or parapharmaceutical products)		
Atlantis dC18 analytic column, 50 × 2 mm, Sentry guard column, 10 × 2 mm, 5 µm	A was composed of 10 mM ammonium formate, pH 3.5-acetonitrile 95:5 (v/v). B was composed of acetonitrile–methanol 7:3 (v/v). A was mixed with B to obtain gradient elution as follows: A:B 100:0 (v/v) at start; A:B 95:5 (v/v) at 15 min; A:B 90:10 (v/v) at 35 min; A:B 73:27 (v/v) at 52 min; A:B 30:70 (v/v) at 60 min.	0.3 ml/min
Saito et al. (2004) (a method developed for fluorescent derivative of psilocin determination in fungal extract and rat plasma)		
Mightysil RP-18 GP column, 100 × 2 mm, 3 µm	50 m mM ammonium acetate–acetonitrile 73:27 (v/v)	0.15 ml/min
Kamata et al. (2006) (a method developed for psilocin determination in human serum samples, detects psilocin glucuronide qualitatively)		
L-column RP-18 column, 150 × 1.5 mm, 5 µm	Acetonitrile–10 mM ammonium formate buffer pH 3.5 12.5:87.5 (v/v)	0.1 ml/min
HPLC-ESI-MS-MS Methods		
Kamata et al. (2003) (a method developed for psilocin determination in human urine samples)		
L-column RP-18 column, 150 × 1.5 mm, 5 µm	Acetonitrile–10 mM ammonium formate buffer pH 3.5 12.5:87.5 (v/v)	0.1 ml/min

Continued

TABLE 5 Examples of High-Performance Liquid Chromatography Methods Developed for Purposes of Psilocybin, Psilocin, and Psilocin Metabolites Detection—cont'd

Column	Mobile Phase	Flow Rate
Martin et al. (2012) (a method developed for psilocin determination in human plasma)		
Pursuit C18 column, 150 × 2 mm, 3 μm	A was composed of methanol and 0.1% formic acid; B was 2 mM ammonium acetate buffer with 0.1% formic acid, pH 3; Elution was performed with mobile phase composed of 10% A and 90% B for 7 min; then the content of A increased to 90% for 4 min to remove lipids.	0.2 ml/min
Martin et al. (2013) (a method developed for psilocin, bufotenine, LSD, and its metabolites in body fluid samples)		
Pursuit C18 column, 150 × 2 mm, 3 μm	A consisted of acetonitrile with 0.1% formic acid; B consisted of 2 mM ammonium acetate buffer with 0.1% formic acid, pH 3.0. Gradient elution was performed: Initially mobile phase was composed of 4% oA and 96% B for 2 min; the content of A increased to 20% within the next 1 min; the content of A increased to 40% within the next 7 min and was maintained for 4 min; for the last 5 min elution was performed with mobile phase composed of 90% A and 10% B to remove the most lipophilic compounds.	0.2 ml/min
Björnstad et al. (2009) (a method developed for determination of 10 psychoactive substances, including psilocin, in human urine)		
Hypersil GOLD analytic column, 100 × 2.1 mm, 5 μm and UNIGUARD C18 precolumn	Elution with linear gradient system from 100% of phase A (1% acetonitrie in 10 mM formic acid) to 100% of phase B (60% acetonitrile in 10 mM formic acid) was performed.	0.2 ml/min
del Mar Ramirez Fernandez et al. (2007) (a method developed for determination of 14 commonly abused substances, including psilocin and bufotenine, in human urine)		
Sunfire C_8 column, 100 × 2.1 mm, 3.5 μm	A was 10 mM ammonium formate buffer, pH 3.5; B was composed of acetonitrile–methanol 2:1 (v/v). Gradient elution started from 2% B at 3.5 min; B content increased to 20% in the next 0.5 min and was maintained for the next 4 min. Between 8 and 11 min the content of B increased linearly to 98%, was maintained for 4 min, and returned to initial value within 0.5 min. Equilibration lasted 6.5 min.	0.3 ml/min
UPLC-UV Methods		
Manevsky et al. (2010) (a method for 4-hydroxyindole glucuronide separation and detection in the presence of psilocin and 1-naphthol)		
Acquity UPLC BEH C18 column, 100 × 2.1 mm, 1.7 μm and a precolumn	A was 0.1% formic acid; B was acetinitrile. Gradient elution was performed as follows: within the first 4 min the content of B increased from 2% to 4%; within the next 0.5 min the content of B increased from 4% to 85%; within the next 0.5 min the content of B decreased from 85% to 2%; for the last 2 min the column was eluted with a mixture containing 2% B.	0.6 ml/min
Chen et al. (2011) (a method developed for psilocin determination in rat plasma)		
C18 column, 100 × 2.1 mm, 2.3 μm	A was 50 mM ammonium acetate in water; B was acetonitrile. Gradient elution was performed as follows: initially the content of B was 10%, then the content of B increased to 25% within the first 2 min and to 55% within the next 3 min and was maintained for the last 5 min.	0.5 ml/min
UPLC-ESI-MS Methods		
Stenzel (2008) (a method developed for psilocybin and psilocin determination in fungal and chocolate extracts)		
Hypersil GOLD PFP column, 100 × 2.1 mm, 1.9 μm	A was water with 0.06% acetic acid; B was acetonitrile with 0.06% acetic acid; C was methanol with 0.06% acetic acid. Gradient elution was carried out. For the first 3 min a mixture composed of 95% A and 5% C was used for elution (within this time psilocybin (Rt 0.64 min) and psilocin (Rt 2.22) were eluted). Then for 1 min other analytes were eluted with a mixture composed of 5% A, 5% B, and 90% C. Mobile phase containing 95% A and 5% C was used for elution for the last 1 min.	1 ml/min

This table groups HPLC methods of psilocybin, psilocin or its metabolites analysis specifying detection system, type of column, composition of mobile phase and type of elution.

Detection was based on mass spectrometry by the use of atmospheric pressure chemical ionization source (APCI-MS) and electrospray ionization source (ESI-MS). ESI-MS improved sensitivity of the method for psilocin measurements (Bogusz, 2000). A similar method allowed detection of psilocin, but not psilocybin, in honey samples containing *Psilocybe* mushrooms (Bogusz et al., 1998). Electrospray ionization type is more suitable and therefore more often used for determination of highly polar molecules such as psilocybin and psilocin (Bogusz, 2000; Björnstad et al., 2009; Kamata et al., 2003, 2006; del Mar Ramirez Fernandez et al., 2007; Martin et al., 2012, 2013; Maruyama et al., 2003; Saito et al., 2004; Stenzel, 2008). Ionization efficiency of the analytes may be influenced by other compounds in the mixture; therefore, preliminary purification steps are usually necessary, and conditions of separation and ionization (cone voltage and collision energy) should be optimized. Electrospray ionization was used in a method developed by Kamata et al. (2003, 2006), who applied HPLC-MS-MS for psilocin and its glucuronide detection in urine and serum samples. HPLC-ESI-MS-MS screening methods developed to detect the presence of several plant-derived psychoactive compounds (psilocin among them) usually required gradient elution for optimal separation (Björnstad et al. 2009; del Mar Ramirez Fernandez et al., 2007; Martin et al., 2013). Manevsky et al. (2010) developed HPLC and UPLC methods combining radiochemical detection with UV detection, for separation and determination of psilocin and its glucuronide to establish kinetics of psilocin glucuronidation and activity of several different UDP-glucuronosyltransferases. Mass spectrometry data allowed detection of psilocin glucuronide precursor ions (Manevsky et al., 2010).

Flow injection analysis coupled with chemiluminescence detection was applied for psilocin and psilocybin determination in methanol extracts of "magic mushrooms" (Anastos et al., 2005). Chemiluminescense was produced in reaction with acidic potassium permanganate and tris(2,2′-bipyridyl)ruthenium(II) (Anastos et al., 2005). Postcolumn analysis with the same system of reagents producing chemiluminescence allowed improvement of sensitivity of the HPLC-UV method (Anastos et al., 2006). Psilocin analysis could be performed by measurements of peroxyoxalate chemiluminescence resulting from the reaction of dansylated psilocin with the bis(2,4,6-trichlorophenyl)oxalate–hydrogen peroxide system (Cepas et al., 1996).

In conclusion, available methods of psilocin concentration measurements allow detection of even trace amounts of this compound (amounts of less than 1 ng in 1 ml are now detectable). This is important, particularly for psilocin determination in body fluid samples for toxicological studies or forensic investigations. Recent findings regarding psilocin metabolism made possible improvement in the methodology of the measurements, since the conjugation of psilocin and its metabolites to glucuronides decreases psilocin concentration in body fluid samples.

APPLICATIONS TO OTHER ADDICTIONS AND SUBSTANCE MISUSE

Some substances are known to potentiate psychedelic effects of indolealkylamines, including psilocybin or psilocin, for example, β-carboline alkaloids, which are potent inhibitors of monoamine oxidase (MAO)-A and which affect synaptic concentrations of catecholamines and serotonin. Tobacco smoke contains β-carbolines, and they are endogenously produced after consumption of alcohol (users commonly combine tobacco smoking and alcohol consumption with "magic mushrooms"; less frequently, cannabis is used). Phenylethylamine present in fungal material or, for example, in chocolates containing psilocybin may play an additional role in the development of psychosis and side effects such as cardiovascular symptoms dependent on a release of catecholamines (Beck, Helander, Karlson-Stiber, & Stephansson, 1998; Lim et al., 2012). Chloral hydrate, a depressant known to induce hypnotic states, is a component of Melzer's reagent, commonly used for mycological identification and microscopic observations of fungal spores. Since chloral hydrate was a popular drug of abuse in the past and was used in a criminal context also, there is a possibility that poor quality fungal material may be treated with this compound to improve its hallucinogenic activity. The addition of other psychoactive substances to *Psilocybe* mushrooms is also highly probable (Andresson et al., 2009). Several authors have developed methods suitable for the simultaneous detection of commonly abused psychoactive compounds, including psilocybin or psilocin (Alnajjar et al., 2007; Björnstad et al., 2009; Kikura-Hanajiri et al., 2005; del Mar Ramirez Fernandez et al., 2007; Martin et al., 2013). The combined administration of several psychedelic compounds may complicate the course of intoxication. Additionally, the co-presence of other substances in the confiscated material simplifies identification of the producer.

DEFINITION OF TERMS

Attenuated total reflectance A spectroscopic technique enabling one to obtain the IR spectrum of the analyzed substance by measurement of intensity of reflected infrared light.

β-Glucuronidase An enzyme catalyzing the cleavage of glucuronides.

Glucuronidation Conjugation of glucuronic acid to xenobiotic or endogenous compounds (with the formation of glycosidic bond) catalyzed by UDP-glucuronyltransferases.

Ion mobility spectrometry Analytical technique enabling the identification of ionized analytes based on differences in gas-phase mobility under an applied electrostatic field and against a flow of a gas.

Indolealkylamines Derivatives of tryptamine substituted at the $-NH_2$ group and indole ring.

Melzer's reagent An aqueous solution of chloral hydrate, potassium iodide, and iodine used for mycological identification.

Matrice A term used in chemical analysis referring to compounds contained in a sample other than the quantified analytes.

Monoamine oxidases Enzymes catalyzing oxidative deamination of monoamines.

Sclerotium A storage structure composed of hardened mycelium, enabling survival when environmental conditions are not optimal, or serving for vegetative reproduction.

Tandem mass spectrometry Mass spectrometry enabling the identification of molecular ions and mass analysis of product ions (after fragmentation).

KEY FACTS ABOUT PSILOCIN/PSILOCYBIN ISOLATION FROM NATURAL SOURCES AND FINDINGS REGARDING THEIR BIOSYNTHESIS

- In 1958, psilocybin was for the first time isolated from *P. mexicana*; it was the first phosphorylated indole compound found in nature.
- In 1959, psilocybin was synthesized, and the chemical structure of the compound isolated from natural sources was confirmed.
- In 1959, psilocin was for the first time isolated and identified (from the same species).
- In 1968, baeocystin and norbaeocystin were found in mycelial culture of *P. baeocystis*; at first they were thought to be precursors of psilocybin.
- In 1968, a biosynthetic pathway of psilocin and psilocybin was established in *P. cubensis* by feeding mycelial culture with radiolabeled precursors; the results revealed that baeocystin and norbaeocytin are by-products of the main biosynthetic pathway leading to psilocybin synthesis.
- Details of the psilocybin biosynthetic pathway are still unknown. Some authors in the 1980s suggested that the pathway may be different in other members of the genus *Psilocybe*.

SUMMARY POINTS

- This chapter reports on progress in the methodology of psilocybin and/or psilocin determination in fungal material and body fluid samples.
- Preliminary screening methods and protocols of qualitative analysis are described separately.
- The methodology of extraction and preliminary purification of analyzed compounds is reported.
- The necessity of special treatment of body fluid samples, taking into account psilocin metabolism and attempting to reduce biomatrice effects and to increase sensitivity, is emphasized.
- The described methods of quantitative analysis involve mainly chromatographic techniques coupled with different detection systems, including advanced mass spectroscopy.

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