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Study of the in vitro and in vivo metabolism of 4-HO-MET

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Highlights:

- *in vitro* and in vivo metabolism of 4-HO-MET
- 12 *in vitro* metabolites
- 4 *in vivo* metabolites
- Hydroxylation, carboxylation, deamination and glucuronidation

Abstract

4-Hydroxy-*N*-methyl-*N*-ethyltryptamine (4-HO-MET) is a new psychoactive substance (NPS) of the chemical class of tryptamines. It shows structural similarities to the endogenous neurotransmitter serotonin, and is a serotonergic hallucinogen, affecting emotional, motoric,

and cognitive functions. The knowledge about its biotransformation is mandatory to confirm the abuse of the substance by urine analysis in forensic cases. Therefore, phase I metabolites were generated by the use of the pooled human liver microsomes (pHLM) *in vitro* model and analyzed by high-performance liquid chromatography high-resolution tandem mass spectrometry with information-dependent acquisition (HPLC-IDA-HR-MS/MS). Furthermore, three authentic urine samples was analyzed and results were compared: 12 different *in vitro* and 4 *in vivo* metabolites were found. The predominant biotransformation steps observed *in vitro* were mono- or dihydroxylation of 4-HO-MET, besides demethylation, demethylation in combination with monohydroxylation, formation of a carboxylic acid, deethylation, and oxidative deamination. *In vivo*, monohydroxylation, and glucuronidation were detected. A metabolic pathway based on these results was proposed.

For the analysis of urine samples in forensic cases, the *N*-oxide metabolite and the HO-alkyl metabolite are recommended as target compounds, besides the glucuronides of 4-HO-MET and the parent compound 4-HO-MET itself.

Keywords: 4-HO-MET, NPS, Synthetic Tryptamine, Human Liver Microsomes, HPLC-IDA-HR-MS/MS, Metabolism;

Introduction

Tryptamines are indolealkylamine molecules, which can be divided into the two subclasses: ergolines, such as lysergic acid diethylamide (LSD), and simple tryptamines.[1] Both subclasses have an indole structure bound to an ethylamine group in common.[2-4] There are endogenous tryptamines, such as serotonin (5-HT), which is a monoamine neurotransmitter that is present

the central nervous system and affects mood and behavior [1, 3-5], naturally tryptamines present in plants mushrooms, microbes and amphibians [6], but also synthetic analogs of tryptamines. The last group belongs to the field of new psychoactive substances (NPS). The EU Early Warning System by the European Monitroing Centre for Drugs and Drug Addiction (EMCDDA) is currently monitoring more than 670 NPS. From the 51 newly notified substance in 2017, two were synthetic tryptamines, the so called 1P-ETH-LAD and 5-MeO-pyr-T. [7]

Well known abused natural tryptamines are psilocybin and psilocin that occur in 'magic mushrooms', *N*,*N*-Dimethyltryptamine (DMT) occurring in Ayahuasca, or bufotenine which is present in the skin of some desert toads.[1, 3, 8-11] Furthermore, structurally modified synthetic tryptamines, described by A. and A. Shulgin in their book 'TIHKAL – Tryptamines I Have Known And Loved',[11] have gained interest and have occurred on the designer drug market in the late 1990s. [12] Frequently found modifications are substitutions at the alpha position of the ethylamine group, the nitrogen atom of the head group or at the aromatic ring, mainly at the 4 or 5 position. One example of such a synthetic tryptamine is 4-Hydroxy-*N*-methyl-*N*-ethyltryptamine (4-HO-MET).

Synthetic tryptamines are typically sold as tablets or powders and consumed by various routes of administration, mainly smoking, or orally by swallowing of tablets or dissolving the substance in water or alcohol.[3] Due to the structural similarity of tryptamines to the endogenous monoamine neurotransmitter serotonin, they are referred to as classical serotonergic hallucinogens.[13] Rickli et al. recently reported that synthetic tryptamines bind and activate the 5-HT_{2A} receptor and that 4-HO-MET is a partial 5-HT_{2A} receptor agonist, SERT (serotonin transporter) inhibitor and weak NET (norepinephrine transporter) inhibitor, similar to psilocin.[6] The effect of a 20 mg oral dose of 4-HO-MET has been described by

Shulgin comparable to psilocin. Alterations of color and form, as well as perception modifications of sound and time were described.[11]

Although only a few tryptamines are under international control, several are controlled by the Swiss Federal act on Narcotics and Psychotropic Substances in index d (narcotics and psychotropic substances) and index e (Raw materials and substances with assumed narcotic-like properties).[14] 4-HO-MET was reported for the first time by Sweden to the European Early Warning System in 2007.

There is only minor information available about synthetic tryptamines, especially about 4-HO-MET. In Sweden, a case report of a 4-HO-MET intoxication has been described as well as a phenomenological study of its recreational use.[15, 16] Additionally, screening methods including 4-HO-MET were published,[17, 18] and studies about the metabolism of other synthetic tryptamines, such as *N*,*N*-diallyltryptamine (DALT) and derivatives, 5-methoxy-2-methyl-*N*-allyl-*N*-cyclohexyltryptamine (5-MeO-2-Me-ALCHT), 5-methoxy-2-methyl-*N*,*N*-diisopropyltryptamine (5-MeO-2-Me-DIPT), 5-methoxy-*N*-methyl-*N*-isopropyltryptamine (5-MeO-MiPT), 5-methoxy-*N*,*N*-diallyltryptamine (5-MeO-DALT).[19-24] The knowledge about the biotransformation and the metabolic pathway of drugs is important in terms of toxicological risk assessment and drug abuse confirmation by the presence of identified metabolites in biological samples.[23, 25]

Using *in vitro* pooled human liver microsomes (pHLM) experiments, the aim of this work was to identify phase I metabolites by a high-performance liquid chromatograph high-resolution tandem mass spectrometry method with information-dependent acquisition (HPLC-IDA-HR-MS/MS), thus generating MS/MS spectra for a mass spectrometric screening approach. Furthermore, 4-HO-MET phase I and II *in vivo* metabolism was investigated, using three authentic urine samples from forensic cases, which were tested positive for 4-HO-MET by LC-HR-MS/MS general-unknown screening. Results were compared to the *in vitro* study and

based on these results, a metabolic pathway and possible biomarkers for forensic case work are proposed.

Experimental

Chemicals and reagents

4-HO-MET (>98% purity by GC/MS, HPLC-DAD, LC-HR-MS/MS and ^{1}H-NMR) was provided from the Forensic Institute of Zurich, Switzerland, and the internal standard (ISTD) ethylone-D_5 (100 µg/mL) was obtained from Cerilliant (Round Rock, TX, USA). Pooled human liver microsomes (150 donors, 20 mg/mL), NADPH-regenerating solutions A and B and potassium phosphate buffer (0.5 M, pH 7.4) were purchased from Corning (New York, NY, USA). Superoxide dismutase (6016 units/mg protein) and *Escherichia coli* (*E. coli*) Type IX-A β-glucuronidase were obtained from Sigma Aldrich (Buchs, CH), magnesium chloride from Merk AG (Zug, CH), methanol (MeOH) from Biosolve (Chemie Brunschwig, Basel, CH), acetonitrile (absolute, HPLC grade) from Acros Organics (Chemie Brunschwig, Basel, CH), and formic acid (FA, analytical grade, 98%) from Fluka (Sigma-Aldrich, Buchs, CH). Butyl acetate (99.7%) was purchased from Chromasolv (Sigma-Aldrich, Buchs, CH), di-sodium hydrogen phosphate dihydrate and potassium dihydrogen phosphate, both used to prepare the phosphate buffer at pH 6.8 (0.075 M), were purchased from Merck AG (Zug, CH). Ultrapure water was produced in-house with a Direct-Q water purification system from Millipore (Zug, CH). Various precision pipettes obtained from Gilson (Mettmenstetten, CH), Eppendorf (Schönenbuch, CH), Corning (New York, NY, USA), and Socorex Isba S.A (Ecublens, CH) were used for handling of all solutions and samples. Blank urine was provided by healthy volunteers. Three authentic urine samples from two male and one female person, who had ingested 4-HO-MET for recreational purposes, came available through routine laboratory work in the Institute of Forensic Medicine.

Methods

In vitro assay

Phase I metabolites were generated by microsomal *in vitro* assay using pHLM as previously published [26]. Volumes of 50 µL per sample were prepared with a final 4-HO-MET concentration of either 5, 20, 40, or 100 µM. Potassium phosphate buffer (100 mM final concentration, 10 µL), magnesium chloride (5 mM, 5 µL), superoxide dismutase (200 units/mL, 5 µL), and pHLM (1.0 mg/mL, 2.5 µL) were mixed. Furthermore, NADPH-regenerating solution A (2.5 µL) and B (0.5 µL) were added. Deionized water was used to fill up the volume to 50 µL. Simultaneously, negative control samples, which do not contain pHLM, and blank samples without 4-HO-MET were prepared. All samples were incubated at 37 °C for either 60 or 120 min.

The reaction was stopped by protein precipitation by addition of 50 µL ice cold acetonitrile containing ethylone-D_5 at a concentration of 1.0 µg/mL as ISTD. The samples were centrifuged at 17'000 g and 8 °C for 10 minutes, the supernatant evaporated under nitrogen to dryness and reconstituted in water/acetonitrile (95:5; v/v) containing 0.1% (v/v) formic acid.

Authentic sample

General unknown screening of the authentic urine sample was performed by LC-HR-MS/MS (see below) after sample dilution using information dependent acquisition as previously published [22]. For detection of metabolites by LC-MS/MS, a 30 µL aliquot of the authentic urine sample was diluted with 200 µL reconstitution solution (water/acetonitrile (95:5; v/v) containing 0.1% (v/v) formic acid) and 10 µL ethylone-D_5 solution (1 µg/mL in acetonitrile) was added. An aliquot of blank urine (30 µL) was diluted with 210 µL reconstitution solution without internal standard.

In order to unambiguously confirm the presence of glucuronide metabolites, glucuronidase cleavage was performed using *E. coli* β-glucuronidase. 10 µL ethylone-D_5 solution (1 µg/mL in acetonitrile) was added to 200 µL authentic urine followed by 200 µL of phosphate buffer pH 6.8 containing 5'000 u/mL β-glucuronidase. Samples were incubated for 2 hours at 37 °C. Extraction was performed by adding 1 mL butyl acetate, 10 minutes of shaking and centrifugation (17'000 g at 8 °C for 10 minutes). The supernatant was evaporated under nitrogen at 50 °C and reconstituted in 200 µL water/acetonitrile (95:5; v/v) containing 0.1% (v/v) formic acid.

LC-HR-MS/MS analysis

The LC-MS/MS system for the analysis of the *in vitro* and *in vivo* samples consisted of an Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific, Reinach, Switzerland) coupled to a 5600 TripleTof instrument equipped with a DuoSpray interface and Analyst software 1.7 TF with MasterView™ Software Version 1.1 (SCIEX, Toronto, Canada). Samples were evaluated according to the following parameters: a cut-off mass error of 5 ppm, intensities higher than 500 cps and isotopic range difference of less or equal than 10%. Chromatographic separation was performed with a synergy 2.5 µm Polar-RP 100 Å 100 x 2.0 mm column (Phenomenex, Basel, Switzerland). The mobile phase consisted of water with 0.1% (v/v) formic acid (solvent A) and acetonitrile with 0.1% (v/v) formic acid (solvent B) with a flow-rate of 0.20 mL/min. The following gradient was used: 0 – 1 min: 2.5% B, 1 – 25 min: 2.5 – 30% B, 25 – 26 min: 30 – 97.5% B, 26 – 29 min: 97.5% B, 29 – 29.1 min: 97.5 – 2.5% B, 29.1 – 35 min: 2.5% B. 1 µL of the extract (see above) were injected for analysis of *in vitro* samples, whereas 2.5 µL of the diluted urine samples were injected. All MS and MS/MS experiment were performed in positive ESI mode with an ion source temperature of 650 °C and an ion spray voltage of 5'000 V. The curtain gas was set to 55 arbitrary units (au) and gas 1 and 2 to 55 au. The survey scan was performed in a mass range of 100 – 950 Da

with a orifice potential of 60 V, and an accumulation time of 50 ms. For the nine most abundant signals with an intensity greater than 350 counts, MS/MS was performed. The precursor was selected within a mass tolerance of 25 mDa, accumulation was set to 40 ms, collision energy (CE) was 35 eV with collision energy spread (CES) set to ±15 eV, DP was held at 60 V. The fragment ions were detected from of 50 – 950 Da. Measured precursors were put on an exclusion list for 3 seconds.

In silico predictions

The Biocatalysis/Biodegeneration Database of the Swiss Federal Institute of Aquatic Science and Technology (EAWAG[27]) web tool was used to generate a possible metabolic pathway of 4-HO-MET. It searches for functional groups in the substrate and proposes microbial catabolic reactions based on biotransformation rules found in database or scientific literature. The parameters to perform the prediction were set to consider both aerobic and anaerobic metabolites, display up to seven levels with a maximum of 20 metabolites per level. The received list of proposed metabolites was extended with the identified biotransformation of various tryptamines[20-24, 28-31] applied on the structure of 4-HO-MET. Additionally, the mechanisms of enzymes that are known to be present in pHLM, such as cytochrome P450s and Flavin-dependent monooxygenases[32, 33] were applied to the structure of 4-HO-MET. The structures and exact masses of the assembled list, served as reference for the analysis of the *in vitro* samples. For *in vivo* experiments, phase II metabolites were considered as well.

Results and Discussion

Routine general unknown screening, using LC-HR-MS/MS and previously described [26], of the authentic urine sample was positive for 4-HO-MET. No other drugs of abuse were detected. For the authentic samples, the comparison of positive and blank urine, resulted in 4 *in vivo* metabolites being identified (**M13**-**M15**). In the pHLM samples, endogenous substances were

excluded by comparison of positive, negative control and blank samples and resulted in 12 *in vitro* metabolites (**M1**-**M12**). *In vitro* experiments were performed with four different 4-HO-MET concentrations. In general, metabolites are detected at higher intensities with longer incubation and higher initial concentration of 4-HO-MET. Nevertheless, doubling the initial concentration of 4-HO-MET, does not double the intensity of the metabolites. At concentrations of 5 and 20 µM not all metabolites were detected at significant intensities. Due to the fact, that it is not known what concentrations of the metabolites are expected in case of recreational use of 4-HO-MET, the concentrations were mainly used to increase the dataset for a possible further method development.

Chromatograms of the in vitro samples and of the diluted urine sample are shown in Figure 1A and B, respectively. Product ion mass spectra of 4-HO-MET and the identified metabolites are displayed in Figure 2, and in the Supporting information S.1 and S.2. Table 1 displays the biotransformation observed, retention time, the $[M+H]^+$, and the occurrence of each metabolite.

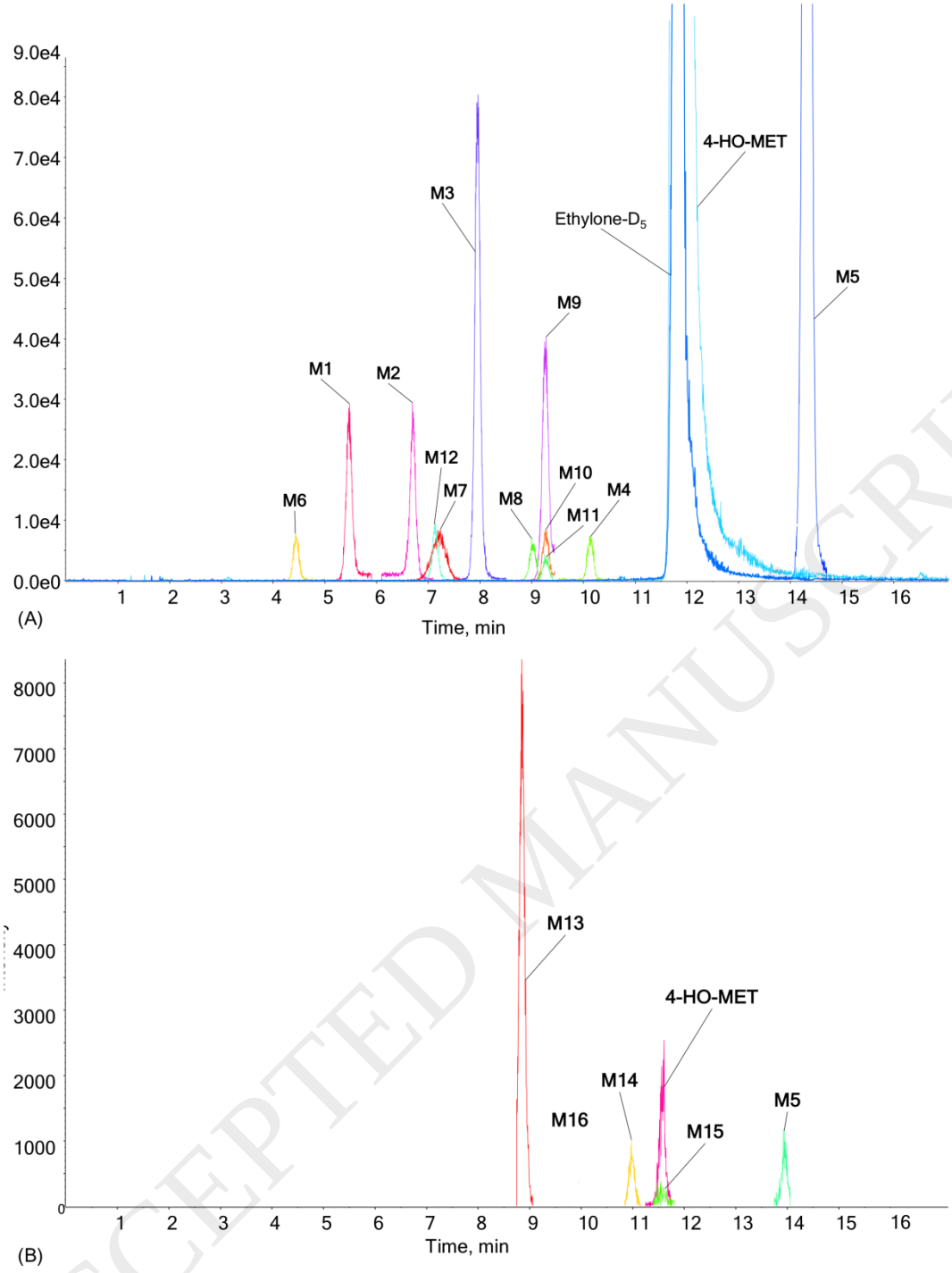

Figure 1: Extracted Ion Chromatogram of LC-HR-MS of identified (A) *in vitro* and (B) *in vivo* metabolites.

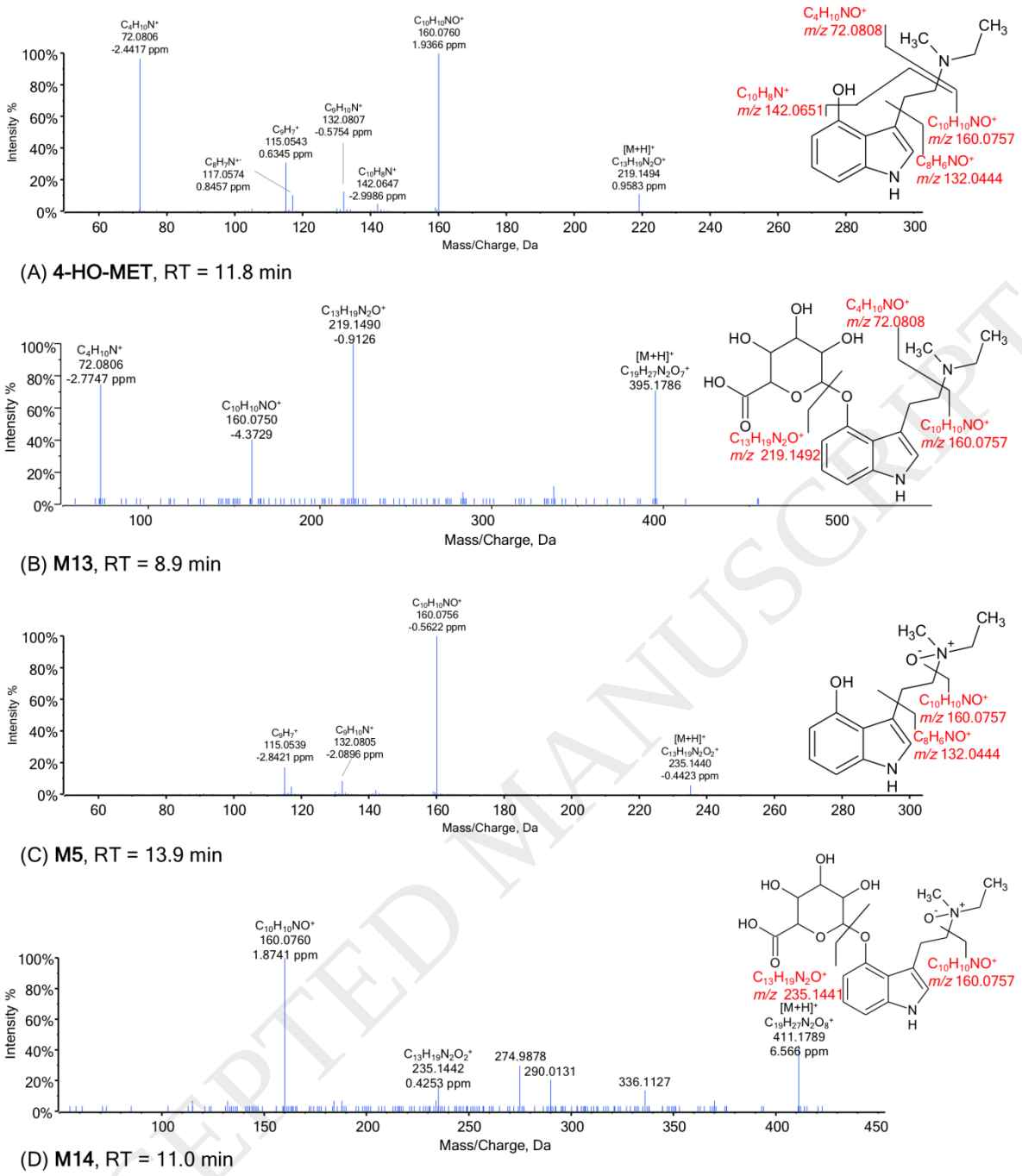

Figure 2: Product ion mass spectra of (A) 4-HO-MET, (B) **M13**, (C) **M5**, and (D) **M14**, found by the IDA experiments. Fragmentation of precursor ions was performed with a CE of 35 eV with CES of ±15 eV.

Name	Biotransformation	RT [min]	Formula	$[M+H]^+$ [*m/z*]	Fragment ions [*m/z*]	Occurrence
4-HO-MET		11.5	$C_{13}H_{18}N_2O$	219.1494	160.0760 72.0806 115.0543	*In vitro* *In vivo*
M1	Hydroxylation	5.5	$C_{13}H_{18}N_2O_2$	235.1441	176.0705 72.0803 148.0392	*In vitro*
M2	Hydroxylation	6.7	$C_{13}H_{18}N_2O_2$	235.1441	176.0708 72.0808 148.0393	*In vitro*
M3	Hydroxylation	7.9	$C_{13}H_{18}N_2O_2$	235.1441	176.0700 148.0385 158.0598	*In vitro*
M4	Hydroxylation	10.1	$C_{13}H_{18}N_2O_2$	235.1441	271.1328 160.0748 188.0927	*In vitro*
M5	*N*-oxide formation	13.9	$C_{13}H_{18}N_2O_2$	235.1441	160.0756 115.0539 132.0805	*In vitro* *In vivo*
M6	Dihydroxylation	4.4	$C_{13}H_{18}N_2O_3$	251.1390	72.0804 164.0338 174.0554	*In vitro*
M7	Dihydroxylation	7.2	$C_{13}H_{18}N_2O_3$	251.1390	176.0700 72.0797 148.0388	*In vitro*
M8	Hydroxylation, N-oxide formation	9.0	$C_{13}H_{18}N_2O_3$	251.1390	176.0705 148.0395 158.0612	*In vitro*
M9	Demethylation	9.3	$C_{12}H_{16}N_2O$	205.1335	160.0751 115.0529 148.0749 58.0653	*In vitro*
M10	Hydroxylation, Demethylation	9.5	$C_{12}H_{16}N_2O_2$	221.1285	176.0694 136.0751 164.0703 58.0650	*In vitro*
M11	Formation of carboxylic acid	9.3	$C_{13}H_{16}N_2O_3$	249.1234	146.0595 160.0755 58.0646	*In vitro*
M12	Desethylation	7.1	$C_{11}H_{14}N_2O$	191.1179	160.0756 115.0537	*In vitro*

					148.0756	
M13	Glucuronidation	8.9	$C_{19}H_{26}N_2O_7$	395.1813	219.1490 160.0750 72.0806	*In vivo*
M14	Glucuronidation of M5	11.0	$C_{19}H_{26}N_2O_8$	411.1762	235.1442 160.0760	*In vivo*
M15	Hydroxylation	11.6	$C_{13}H_{18}N_2O_2$	235.1441	176.0710 72.0805 130.0651	*In vivo*

Table 1: Detected *in vitro* and *in vivo* metabolites, their identified biotransformation, retention times, Formula, monoisotopic mass, the most intense fragment ions, and their occurrence.

Identification of metabolites

The structures of detected metabolites of 4-HO-MET were identified by their precursor ions and product ions formed in the MS/MS experiment. Even-electron ion fragmentation of the selected precursor ions were expected to occur according to Niessen and Correa.[34] The main structural parts of 4-HO-MET are the indole ring, the *N*-containing headgroup, and the linking carbons. The most intense fragment ions of the detected *in vitro* and *in vivo* metabolites are displayed in table 1.

The unmodified parent compound 4-HO-MET resulted in a spectrum shown in Figure 2A. The precursor ion peak of 4-HO-MET was detected at *m/z* 219.1492 ($C_{13}H_{19}N_2O^+$). The fragment ion with *m/z* 160.0757 ($C_{10}H_{10}NO^+$) corresponds to the indole ring and linking carbons and is formed by amine cleavage. The headgroup resulted in a peak at *m/z* 72.0808 ($C_4H_{10}N^+$) also formed by amine cleavage, whereas *m/z* 115.0542 ($C_9H_7^+$) corresponded to the indole ring and linking carbons after the loss of HCN and H_2O.[24]

The identified metabolites followed a fragmentation pattern similar to the one of the parent compound. Each hydroxylation resulted in a mass shift of +15.9949 Da, resulting in a

precursor ion with *m/z* 235.1441 ($C_{13}H_{19}N_2O_2^+$, **M1**, **M2**, **M3**, **M4**, **M15**) for a monohydroxylation and *m/z* 251.1390 ($C_{13}H_{19}N_2O_3^+$, **M6**, **M7**, **M8**) for a dihydroxylation. The loss of a methyl group from the parent compound resulted in a mass shift forming a precursor ion with *m/z* 205.1335 ($C_{12}H_{17}N_2O^+$, -14.0157 Da, **M9**), whereas the loss of an ethyl groups gave a signal at *m/z* 191.1179 ($C_{11}H_{15}N_2O^+$, -28.0313 Da, **M12**). The formation of a carboxylic acid increases the mass of the precursor ion $[M+H]^+$ to *m/z* 249.1234 ($C_{13}H_{17}N_2O_3^+$, +29.9742 Da, **M11**), and the combination of demethylation and hydroxylation results in a precursor ion with *m/z* 221.1285 ($C_{12}H_{17}N_2O_2^+$, +1.9793 Da, **M10**).

Monohydroxylation at the indole ring and linking carbons resulted in a mass shift of the fragment ion with *m/z* 160.0757 ($C_{10}H_{10}NO^+$, 4-HO-MET, **M4**, **M5**, **M9**, **M11**, **M12**, **M13**, **M14**) to *m/z* 176.0706 ($C_{10}H_{10}NO_2^+$, +15.9949 Da, **M2**, **M3**, **M7**, **M8**, **M10**, **M15**) both formed by amine cleavage. When dihydroxylation occurred at the indole ring, as shown in Figure S1 (**M6**), no product ion of *m/z* 176.0706 ($C_{10}H_{10}NO_2^+$) is present, which is observed for the other two dihydroxylated metabolites **M7** and **M8**. The fragment ions with *m/z* 158.0600 ($C_{10}H_8NO^+$, +15.9949 Da, **M2**, **M3**, **M7**, **M8**) and *m/z* 174.0554 ($C_9H_{10}NO_2^+$, +31.9898 Da, **M6**) found for mono- and dihydroxylated compounds, respectively, correspond to the indole ring and linking carbons after the loss of H_2O (-18.0106 Da). Based on these fragment ions, two more fragmentations are noticed if no modification was located at the indole ring or linking carbons. One is the loss of HCN, leading to a peak at *m/z* 115.0542 ($C_9H_7^+$, **M4**, **M9**, **M12**), whereas the other was the radicalic loss of the linking carbons in form of $HCC^{\bullet}$ resulting in a fragment ion with *m/z* 117.0573 ($C_8H_7N^{+\bullet}$, **M4**, **M9**, **M12**).[24] The loss of H_2O (-18.0106 Da) from a compound carrying the monohydroxylation at the ethylamine moiety (**M4**), results in a signal at m/z 217.1335 ($C_{13}H_{17}N_2O^+$). Subsequent radicalic cleavage of the ethyl moiety of the headgroup explains the signal at m/z 188.0944 ($C11H_{12}N_2O^{+\bullet}$). When monohydroxylation occurred on the indole ring, a signal at *m/z*

148.0393 ($C_8H_6NO_2^+$, +15.9949 Da, **M1, M2, M3**, **M7**, **M8**, **M12**) was detected, and for dihydroxylation a mass shift resulting in *m/z* 164.0342 ($C_8H_6NO_3^+$, +31.9898 Da, **M6**) could be observed, both formed by β-cleavage. The fragment ion with *m/z* 72.0808 ($C_4H_{10}N^+$), which was also observed for the unchanged 4-HO-MET, represents the headgroup without any modification (**M1**, **M2**, **M3**, **M6**, **M7**, **M13**, **M15**). Therefore, when a fragment ion with *m/z* 72.0808 was detected for a metabolite, the biotransformation must be located on the indole ring or the linking carbons. For demethylated metabolites, a fragment ion with *m/z* 58.0651 ($C_3H_8N^+$, -14.0157 Da, **M9**, **M10**, **M11**) was observed. This fragment can be detected as well in hydoxylated and oxidized metabolites, indicating the neutral loss of HCOOH. The loss of an ethyl group could not be allocated at this fragment because its expected mass was below the mass spectrometric range (50 – 950 Da). When a carboxyl group was located at the headgroup ($C_{13}H_{17}N_2O_3^+$, *m/z* 249.1234, **M11**), the loss of this group resulted in a signal at *m/z* 203.1164 ($C_{12}H_{15}N_2O^+$, **M11**). For monohydroxylated metabolites ($C_{13}H_{19}N_2O_2^+$, *m/z* 235.1441), the loss of H_2O resulted in a signal at *m/z* 217.1335 ($C_{13}H_{17}N_2O^+$, **M4**, **M15**)

For the evaluation of *in vivo* samples, identical fragment ions as in the *in vitro* samples, are expected to be observed when the same biotransformation step occurred, and hence, similar fragmentation. Therefore, only newly occurring precursor and fragment ions are discussed in detail. In contrast to the *in vitro* experiments, glucuronidation, besides hydroxylation was observed in the authentic urine samples. This resulted in a mass shift of +176.0321 Da (+ $C_6H_8O_6$), and +15.9949 Da, respectively. Therefore, glucuronidation of 4-HO-MET resulted in a peak at *m/z* 395.1813 ($C_{19}H_{26}N_2O_7^+$, **M13**) and glucuronidation of a monohydroxylated metabolite in a peak at *m/z* 411.1762 ($C_{19}H_{26}N_2O_8^+$, **M14**). The fragment ions observed for the glucuronidated metabolites and the respective non-glucuronidated metabolites were the same (see Figure 2B and 2D). In the authentic urine samples, one hydroxylate metabolite was

identified (**M15**), which displayed a precursor ion with *m/z* 176.0706 ($C_{10}H_9NO_2^+$). Due to the presence of the fragment ion with *m/z* of 130.0651, which belongs to the unmodified indole ring, the fragment *m/z* 176.0706, which corresponds to amine cleavage and the simultaneous existence of the fragment ion with *m/z* 72.0808 which is formed by α-cleavage and correlates with an intact headgroup, the position of the hydroxylation is allocated on β-position of the linking carbons.

Microsomal Biotransformation

The pHLM *in vitro* experiment revealed five monohydroxylated metabolites. Three of which (**M1**-**M3**) carrying their modification at the 6-membered ring of the indole structure, which was previously observed for synthetic tryptamines,[20-24] eluting at 5.5, 6.7, and 7.9 min, respectively. The metabolite eluting at 10.1 min (**M4**) was identified as *N*-hydroxymethyl-metabolite of 4-HO-MET. The *N*-oxide metabolite (**M5**) shown in Figure 2C was eluting after the parent compound, which is typical for *N*-oxides and was previously observed for the synthetic tryptamines DALT, 5-MeO-DALT, and 5-MeO-MiPT.[22, 24] Further hydroxylation of **M1**-**M3** and **M5** resulted in metabolites **M6** to **M8**, each carrying at least one hydroxylation at the 6-membered ring of the indole (Figure S.1). **M6**, eluting at 4.4 min, was identified as a di-HO-aryl metabolite of 4-HO-MET. Whereas the peak of **M7**, eluting at 7.2 min, was very broad and the respective mass spectrum was inclonclusive for the site of hydroxylations. It is assumed that **M7** consists of two isobaric di-hydroxylated metabolites, which were not chromatographically separated. Hence no assumption about their structures can be made. At 9.0 min, the HO-aryl-*N*-oxide metabolite of 4-HO-MET (**M8**) was detected. As already observed for the *N*-oxide **M5**, **M8** eluted after the possible corresponding monohydroxylated metabolites **M1**-**M3**. Furthermore, an *N*-demethylated metabolite of 4-HO-MET (**M9**) eluting at 9.3 min, and a HO-aryl-*N*-demethylated metabolite (**M10**) eluting at 9.5 min were detected. **M4** was further oxidized resulting in an *N*-carboxy metabolite of 4-HO-MET (**M11**) eluting at

9.3 min. The last metabolite (**M12**) eluting at 7.1 min was identified as *N*-desethylated 4-HO-MET.

These results coincided with *in vitro* studies about 5-MeO-MiPT[22] as well as with the phase I results of the rat urine analysis of DALT and 5-MeO-DALT.[24]

In vivo Biotransformation

The analysis of authentic urine samples revealed the glucuronidation as important biotransformation occurring *in vivo*. It was detected for the parent compound 4-HO-MET and the metabolite **M5**, resulting in metabolite **M13** (Figure 2B), eluting at 8.9 min, and metabolite **M14** (Figure 2D), eluting at 11.0 min, respectively. The glucuronide cleavage proved these results due to the reduction of this signal and the formation of 4-HO-MET and **M5**. Furthermore, a monohydroxylation, which has not yet been detected *in vitro* was observed *in vivo*. **M15**, eluting at 11.6 min, was identified to carry the monohydroxylation at the linking carbons.

Previous metabolism studies on psilocin, DALT, 5-MeO-DALT, and 5-MeO-MiPT also identified monohydroxylation, especially the formation of an *N*-oxide, and glucuronidation as important biotransformation steps.[21, 22, 24, 30]

Based on these *in vitro* and *in vivo* results, the metabolic pathway shown in Figure 3 was proposed.

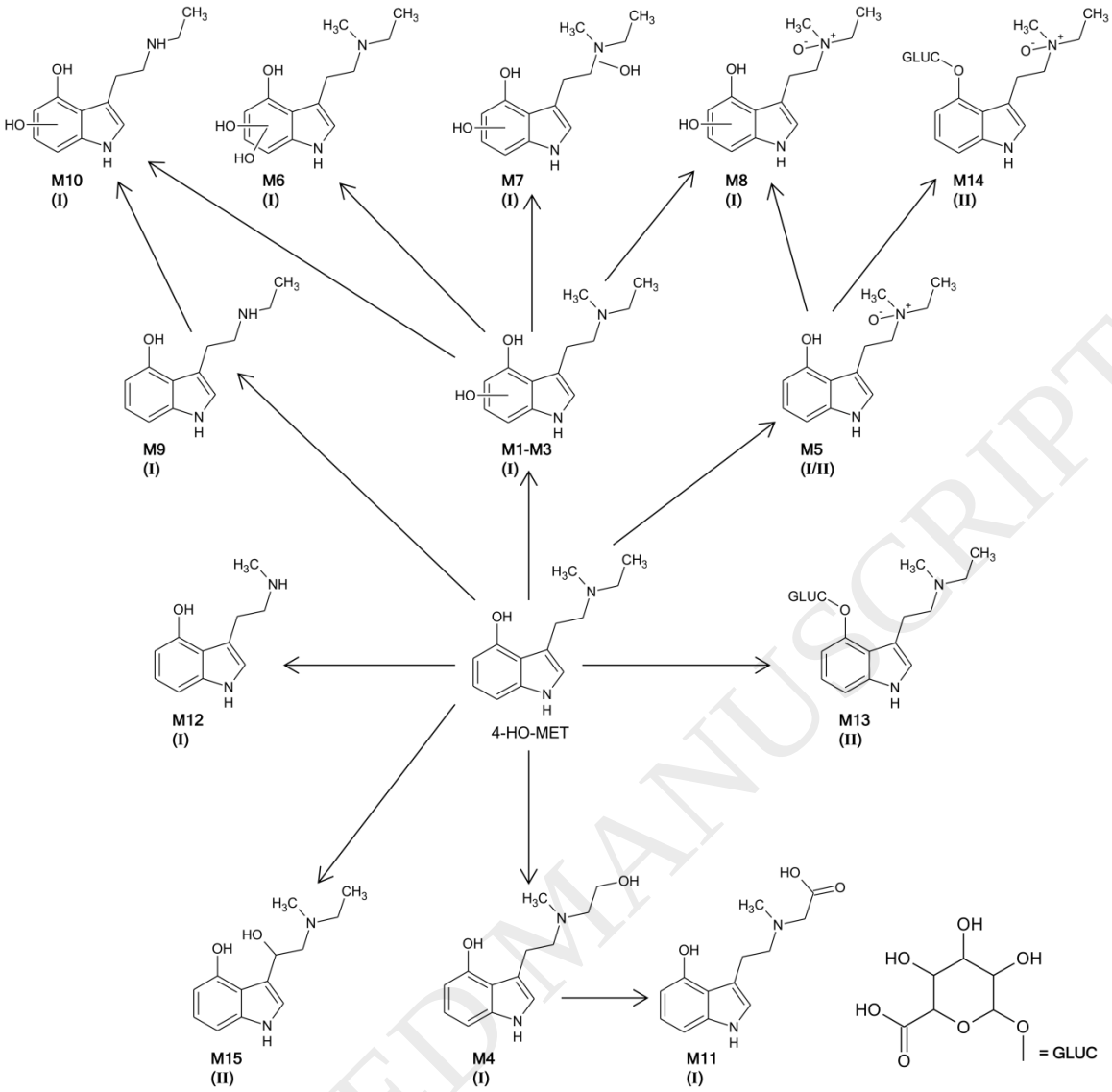

Figure 3: Proposed metabolic pathway of 4-HO-MET including (I) *in vitro* and (II) *in vivo* metabolites. GLUC: glucuronide.

Evaluation of *in silico* predictions

The *in silico* prediction of possible metabolites using software, such as the EAWAG web tool, is very useful to get a first insight into possible biotransformations of a compound. For this study, the EAWAG web too was able to correctly predict the monohydroxylated metabolites **M1**-**M3**, the demethylated compound **M9**, the hydroxylated and demethylated metabolite **M10**, and the desethylated metabolite **M12**. Additional 18 compounds have been

predicted, that have not been detected *in vitro* or *in vivo*. Furthermore, various metabolites were not possible to predict with the EAWAG web tool, namely dihydroxylated and phase II metabolites. Therefore, it is mandatory to extend the reference list by the use of other software systems, theoretical knowledge about the mechanism of present enzymes, or published data of structurally related compounds.

Conclusions

The used pHLM *in vitro* setup allowed to identify 12 different 4-HO-MET metabolites, whereof five occurred in monohydroxylated form (**M1**-**M5**) and three were dihydroxylated (**M6**-**M8**). Furthermore, an *N*-demethylated (**M9**), an *N*-desethylated (**M12**), a demethylated and monohydroxylated (**M10**), and an *N*-carboxy metabolite (**M11**) were identified. The analysis of three *in vivo* urine sample resulted in 4 metabolites of 4-HO-MET. Two were monohydroxylated metabolites (**M5** and **M15**), whereof the *N*-oxide (**M5**) was detected *in vivo* and *in vitro*, and two were glucuronidated (**M13** and **M14**).

Of the four identified *in vivo* metabolites only two could also be detected *in vitro*. The *in vitro* experiments performed in this study only use phase I enzymes. Therefore, it was not possible to predict the two glucuronidated metabolites. The use of pHLM should be seen as a complementary model to *in vivo* human data or other *in vitro* methods, and their results cannot be used alone for biomarker prediction. However, the knowledge of the phase I metabolism of a substance can be used to predict phase II metabolism, for example, when several hydroxylated metabolites are found.

Based on the results from the *in vitro* measurements and the three authentic urine samples, both monohydroxylated metabolites **M5** and **M15**, as well as the glucuronidated metabolites **M13** and **M14** are recommended as biomarkers for the development of mass spectrometric target analysis for authentic urine samples.

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Conflict of interest:

The authors declare no conflict of interest.

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References

1 Hill SL, Thomas SH. Clinical toxicology of newer recreational drugs. *Clin Toxicol (Phila)* 2011; *49*(8):705-19. DOI: 10.3109/15563650.2011.615318.

2 Liechti M. Novel psychoactive substances (designer drugs): overview and pharmacology of modulators of monoamine signaling. *Swiss Med Wkly* 2015; *145*:w14043. DOI: 10.4414/smw.2015.14043.

3 Araujo AM, Carvalho F, Bastos Mde L, Guedes de Pinho P, Carvalho M. The hallucinogenic world of tryptamines: an updated review. *Arch Toxicol* 2015; *89*(8):1151-73. DOI: 10.1007/s00204-015-1513-x.

4 United_Nations_Office_of_Drug_and_Crime. The challenge of new psychoactive substances, 2013; United Nations Office of Drug and Crime: https://www.unodc.org/documents/scientific/NPS_2013_SMART.pdf.

5 Brady ST, Siegel GJ, eds. *Basic Neurochemistry - principles of molecular, cellular and medicinal neurobiology*. 8. Edition ed. 2012, Elsevier Inc.: Waltham, Oxford.

6 Rickli A, Moning OD, Hoener MC, Liechti ME. Receptor interaction profiles of novel psychoactive tryptamines compared with classic hallucinogens. *Eur Neuropsychopharmacol* 2016; *26*(8):1327-37. DOI: 10.1016/j.euroneuro.2016.05.001.

7 EMCDDA. European Drug Report 2018: Trends and Developments. Publications Office of the European Union, Luxembourg., doi:10.2810/800331, 2018

8 Cunningham N. Hallucinogenic plants of abuse. *Emergency Medicine Australasia* 2008; *20*(2):167-174. DOI: 10.1111/j.1742-6723.2008.01070.x.

9 Nichols DE. Hallucinogens. *Pharmacol Ther* 2004; *101*(2):131-81. DOI: 10.1016/j.pharmthera.2003.11.002.

10 Gibbons S. 'Legal highs'--novel and emerging psychoactive drugs: a chemical overview for the toxicologist. *Clin Toxicol (Phila)* 2012; *50*(1):15-24. DOI: 10.3109/15563650.2011.645952.

11 Shulgin A, Shulgin A. TIHKAL. First Edition ed, Berkley, CA: Transform press1997.

12 Meyer MR, Caspar A, Brandt SD, Maurer HH. A qualitative/quantitative approach for the detection of 37 tryptamine-derived designer drugs, 5 beta-carbolines, ibogaine, and yohimbine in human urine and plasma using standard urine screening and multi-analyte approaches. *Anal Bioanal Chem* 2014; *406*(1):225-37. DOI: 10.1007/s00216-013-7425-9.

13 Blough BE, Landavazo A, Decker AM, Partilla JS, Baumann MH, Rothman RB. Interaction of psychoactive tryptamines with biogenic amine transporters and serotonin receptor subtypes. *Psychopharmacology (Berl)* 2014; *231*(21):4135-44. DOI: 10.1007/s00213-014-3557-7.

14 Eidgenössisches_Departement_des_Innern. Verordnung des EDI über die Verzeichnisse der Betäubungsmittel, psychotropen Stoffe, Vorläuferstoffe und Hilfschemikalien. 2017

15 Taljemark J, Johansson BA. Drug-induced acute psychosis in an adolescent first-time user of 4-HO-MET. *Eur Child Adolesc Psychiatry* 2012; *21*(9):527-8. DOI: 10.1007/s00787-012-0282-9.

16 Kjellgren A, Soussan C. Heaven and hell--a phenomenological study of recreational use of 4-HO-MET in Sweden. *J Psychoactive Drugs* 2011; *43*(3):211-9. DOI: 10.1080/02791072.2011.605699.

17 Adamowicz P, Tokarczyk B. Simple and rapid screening procedure for 143 new psychoactive substances by liquid chromatography-tandem mass spectrometry. *Drug Test Anal* 2016; *8*(7):652-67. DOI: 10.1002/dta.1815.

18 Meyer MR, Caspar A, Brandt SD, Maurer HH. A qualitative/quantitative approach for the detection of 37 tryptamine-derived designer drugs, 5 β-carbolines, ibogaine, and yohimbine in human urine and plasma using standard urine screening and multi-analyte approaches. *Analytical and Bioanalytical Chemistry* 2014; *406*(1):225-237. DOI: 10.1007/s00216-013-7425-9.

19 Brandt SD, Kavanagh PV, Dowling G, et al. Analytical characterization of N,N-diallyltryptamine (DALT) and 16 ring-substituted derivatives. *Drug Test Anal* 2017; *9*(1):115-126. DOI: 10.1002/dta.1974.

20 Caspar AT, Gaab JB, Michely JA, Brandt SD, Meyer MR, Maurer HH. Metabolism of the tryptamine-derived new psychoactive substances 5-MeO-2-Me-DALT, 5-MeO-2-Me-ALCHT, and 5-MeO-2-Me-DIPT and their detectability in urine studied by GC-MS, LC-MSn , and LC-HR-MS/MS. *Drug Test Anal* 2017. 10.1002/dta.2197. DOI: 10.1002/dta.2197.

21 Fabregat-Safont D, Barneo-Munoz M, Martinez-Garcia F, Sancho JV, Hernandez F, Ibanez M. Proposal of 5-methoxy-N-methyl-N-isopropyltryptamine consumption biomarkers through identification of in vivo metabolites from mice. *J Chromatogr A* 2017; *1508*:95-105. DOI: 10.1016/j.chroma.2017.06.010.

22 Grafinger KE, Hädener M, König S, Weinmann W. Study of the in vitro and in vivo metabolism of the tryptamine 5-MeO-MiPT using human liver microsomes and real case samples. *Drug Testing and Analysis* 2017. 10.1002/dta.2245:n/a-n/a. DOI: 10.1002/dta.2245.

23 Katagi M, Kamata T, Zaitsu K, et al. Metabolism and toxicologic analysis of tryptamine-derived drugs of abuse. *Ther Drug Monit* 2010; *32*(3):328-31. DOI: 10.1097/FTD.0b013e3181dcb40c.

24 Michely JA, Helfer AG, Brandt SD, Meyer MR, Maurer HH. Metabolism of the new psychoactive substances N,N-diallyltryptamine (DALT) and 5-methoxy-DALT and their detectability in urine by GC-MS, LC-MSn, and LC-HR-MS-MS. *Anal Bioanal Chem* 2015; *407*(25):7831-42. DOI: 10.1007/s00216-015-8955-0.

25 Tyrkkö E, Pelander A, Ketola RA, Ojanperä I. In silico and in vitro metabolism studies support identification of designer drugs in human urine by liquid chromatography/quadrupole-time-of-flight mass spectrometry. *Analytical and Bioanalytical Chemistry* 2013; *405*(21):6697-6709. DOI: 10.1007/s00216-013-7137-1.

26 Grafinger KE, Hadener M, Konig S, Weinmann W. Study of the in vitro and in vivo metabolism of the tryptamine 5-MeO-MiPT using human liver microsomes and real case samples. *Drug Test Anal* 2018; *10*(3):562-574. DOI: 10.1002/dta.2245.

27 EAWAG. Biocatalysis/Biodegeneration Database, Swiss Federal Institute of Aquatic Science and Technology (EAWAG): Dübendorf.

28 Dinis-Oliveira RJ. Metabolism of psilocybin and psilocin: clinical and forensic toxicological relevance. *Drug Metab Rev* 2017; *49*(1):84-91. DOI: 10.1080/03602532.2016.1278228.

29 Hasler F, Bourquin D, Brenneisen R, Bar T, Vollenweider FX. Determination of psilocin and 4-hydroxyindole-3-acetic acid in plasma by HPLC-ECD and pharmacokinetic profiles of oral and intravenous psilocybin in man. *Pharm Acta Helv* 1997; *72*(3):175-84. DOI: 10.1016/S0031-6865(97)00014-9.

30 Hasler F, Bourquin D, Brenneisen R, Vollenweider FX. Renal excretion profiles of psilocin following oral administration of psilocybin: a controlled study in man. *J Pharm Biomed Anal* 2002; *30*(2):331-9. DOI: 10.1016/S0731-7085(02)00278-9.

31 Passie T, Seifert J, Schneider U, Emrich HM. The pharmacology of psilocybin. *Addict Biol* 2002; *7*(4):357-64. DOI: 10.1080/1355621021000005937.

32 Asha S, Vidyavathi M. Role of human liver microsomes in in vitro metabolism of drugs-a review. *Appl Biochem Biotechnol* 2010; *160*(6):1699-722. DOI: 10.1007/s12010-009-8689-6.

33 Guengerich FP. Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem Res Toxicol* 2001; *14*(6):611-50. DOI: 10.1021/tx0002583.

34 Niessen WMA, Correa C. RA. Interpretation of MS-MS mass spectra of drugs and pesticides: John Wiley & Sons, Inc2017.