Proposal of 5-methoxy-*N*-methyl-*N*-isopropyltryptamine consumption biomarkers through identification of *in vivo* metabolites from mice.

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Abstract

New psychoactive substances (NPS) are a new breed of synthetically produced substances designed to mimic the effects of traditional illegal drugs. Synthetic cannabinoids and synthetic cathinones are the two most common groups, which try to mimic the effects of the natural compounds $^{9}\Delta$-tetrahydrocannabinol and cathinone, respectively. Similarly, synthetic tryptamines are designer compounds which are based on the compounds psilocin, *N*,*N*-dimethyltryptamine and 5-methoxy-*N*,*N*-dimethyltryptamine found in some mushrooms. One of the most important tryptamine compounds found in seizures is 5-methoxy-*N*,*N*-diisopropyltryptamine, which has been placed as controlled substance in USA and some European countries. The control of this compound has promoted the rising of another tryptamine, the 5-methoxy-*N*-methyl-*N*-isopropyltryptamine , which at the time of writing this article has not been banned yet. So, it is undeniable that this new substance should be monitored.

5-methoxy-*N*-methyl-*N*-isopropyltryptamine has been reported by the Spanish Early Warning System and detected in our laboratory in two pill samples purchased in a local smart shop. This has promoted the need of stablishing consumption markers for this compound in consumers' urine. In the present work, the metabolism and pharmacokinetic of 5-methoxy-*N*-methyl-*N*-isopropyltryptamine has been studied by an *in vivo* approach, using adult male mice of the inbred strain C57BLJ/6. The use of ultra-high performance liquid chromatography coupled to high resolution mass spectrometry allowed the identification of four metabolites. After the pharmacokinetic study in serum and urine, the *O*-demethylated metabolite and the non-metabolised parent compound are proposed as consumption markers in hydrolysed urine.. Data reported in this work will help hospitals and forensic laboratories to monitor the consumption and potential intoxication cases related to this tryptamine.

Keywords

5-MeO-MiPT, tryptamines, *in vivo* studies, metabolite identification, high resolution mass spectrometry, new psychoactive substances

1. Introduction

The term "new psychoactive substances" (NPS) includes synthetic compounds, but also plant or fungal substances that are used as recreational drugs by elicitation of a psychoactive response, and that are not classified as illegal substances [1]. Synthetic cannabinoids and synthetic cathinones make up most of the NPS described for the EMCDDA in its 2016 report [2]. These compounds try to produce the same psychoactive effects than $^{9}\Delta$-tetrahydrocannabinol (THC), a natural compound found in cannabis (*Cannabis sativa*), or the natural alkaloid cathinone found in khat (*Catha edulis*) [3], respectively. Other widely consumed natural products are the "magic mushrooms" belonging to the genera *Psilocybe*, *Conocybe* and *Hygrocybe* [1]. These fungi, especially *Psilocybe cubensis*, contain high concentrations of psilocybin (4-phosphoryloxy-*N*,*N*-dimethyltryptamine) and psilocin (4-hydroxy-*N*,*N*-dimethyltriptamine), natural tryptamines with hallucinogenic and sedative effects [4]. Other natural tryptamines currently consumed are DMT (*N*,*N*-dimethyltryptamine) and 5-MeO-DMT (5-methoxy-*N*,*N*-dimethyltryptamine), both of them present in the popular "ayahuasca" [4,5]. Several new tryptamines, structurally similar to natural ones, have been detected in the last few years. One of the most popular synthetic tryptamine is 5-MeO-DiPT (5-methoxy-*N*,*N*-diisopropyltryptamine), and its analogues DiPT (*N*,*N*-diisopropyltryptamine) and 4-OH-DiPT (4-hydroxy-*N*,*N*-diisopropyltryptamine).

The compound 5-MeO-DiPT (also known as "Foxy" or "Foxy Methoxy") was reported for the first time in 1999, and in 2003 the DEA reported law enforcements seizures for this compound [6]. Some studies revealed that this tryptamine is a high affinity inhibitor of serotonin, dopamine and norepinephrine transporters [7], but it also acts as a toxin of the serotonergic cells in the brain [8–11]. After the inclusion of 5-MeO-DiPT and its natural analogue 5-MeO-DMT in the list of controlled substances in USA and some

European countries [12,13], another tryptamine has been recently identified, 5-MeO-MiPT (5-methoxy-*N*-methyl-*N*-isopropyltryptamine), which is a 5-MeO-DMT derivative that changes a *N*-methyl group by *N*-isopropyl. This compound, as well as its derivatives MiPT and 4-OH-MiPT, were already described by Alexander Shulgin in his book [14]. **Figure 1** shows the structure of natural tryptamines and their synthetic analogues, including 5-MeO-DiPT and 5-MeO-MiPT. The last one has been reported in intoxication episodes [15,16]. In Spain, it was reported by the Early Warning System (EWS) in 2016, with its first detection taking place in April 2015 [17].

It seems evident that 5-MeO-MiPT should be monitored in seizures and possible intoxication cases. Identification of NPS is commonly an analytical challenge because NPS structures are continuously changing, needing complex and complementary techniques for their structural elucidation [18,19]. In addition, the detection of these compounds in biological samples with the aim of determining the origin of an intoxication/consumption is troublesome. The main handicap lies on the establishment of the target compound to be monitored, which in many cases is unknown to the analyst. Several works developing methodologies for the determination of NPS in blood and urine have been published, searching for the parent compound [21–24]. However, prior to monitoring NPS in biological fluids, drug metabolism studies are commonly required. These studies allow to identify the major metabolites and the most specific ones, thus establishing the potential consumption biomarkers.

Undoubtedly, the most useful experiments would be with humans, but these are in most cases rather problematic, as they are limited to intoxication cases [25] and/or require the participation of healthy volunteers [26], with the subsequent risk. At this point, *in vitro* experiments, using microsomes or cell cultures, and *in vivo* experiments, using animal models, are the most common way of establishing potential consumption markers. The

in vivo approach has been successfully used in different NPS metabolite identification studies [27–31]. For the tryptamine derivative 5-MeO-DiPT, its metabolites have been studied using the *in vitro* approach with rat liver microsomes [32] and rat and human hepatocytes [33], as well as using *in vivo* experiments with urine of rats [34] and human volunteers [35]. These experiments have allowed monitoring the consumption of this substance throughout the analysis of biological samples, searching for the markers previously established [36].

Our research started when the 5-MeO-MiPT was identified in two pill samples purchased in a local smart shop, at the same time than the Spanish EWS reported its presence, which encourage us to study its metabolism (no data was found in literature). *In vivo* experiments were carried out with adult male mice of the inbred strain C57BLJ/6. Ultra-high performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS) was used for metabolite structural elucidation. Using different approaches for metabolite identification, two Phase I and two Phase II metabolites were tentatively identified. The identified Phase I metabolites resulted from demethylation of the methoxy group and the putative formation of the *N*-oxide, while the Phase II were the glucuronide conjugation of the demethylated metabolite, and the glucuronide of the hydroxyindole metabolite.

2. Experimental procedures

2.1. Reagents and chemicals

5-MeO-MiPT reference standard was purchased from Cayman Chemical (Ann Arbor, MI, USA). HPLC-grade water was obtained by purifying demineralised water using a Milli-Q system from Millipore (Bedford, MA, USA). HPLC-grade methanol (MeOH), HPLC-grade acetonitrile (ACN), acetone, sodium chloride (NaCl), dimethyl sulfoxide (DMSO), formic acid (HCOOH), hydrochloric acid (HCl 37%) and sodium hydroxide (NaOH) were purchased from Scharlau (Scharlab, Barcelona, Spain). Diamino hydrogen phosphate ($(NH_4)_2HPO_4$) was purchased from Merck (Darmstadt, Germany). β-glucuronidase from *E. Coli K12* (80 U/mg at 25 ºC) was purchased from Roche (Indianapolis, IN, USA). Leucine enkephalin was acquired from Sigma-Aldrich (St. Louis, MO, USA).

1 M $H_2PO_4^-/HPO_4^{-2}$ buffer was prepared by dissolving the corresponding amount of $(NH_4)_2HPO_4$ in Milli-Q water and adjusting the pH to 7 with HCl.

2.2. Legal high samples containing 5-MeO-MiPT

Two different pills, *Estrella* (which means "Star" in Spanish) and *Corazon* ("Heart") (**Figure 2**) were bought in a local smart shop through its webpage. Both samples were analysed in order to identify the NPS present in their composition. The tryptamine 5-MeO-MiPT was tentatively identified in both samples and unequivocally identified after purchasing its reference standard.

2.3. Animal experiments

In vivo experiments were performed using C57BLJ6 adult male mice (Janvier, France). The study protocol was approved by the ethical committee of Generalitat Valenciana

(Ref. 2015/VSC/PEAI00055). Animals were caged in groups of 3-4 mice and they received intra-peritoneal (i.p.) injections of the drug solution or the vehicle (control). Different groups of animals were used to obtain blood and urine samples.

150 µL of blank solution (0.9% of NaCl and 1% of DMSO) were injected to control group, while for metabolism experiments, 5-MeO-MiPT was added to blank solution in order to obtain a dose of 0.27 mg/kg in a 150 µL injection. This dose was estimated based on a rough quantification of the 5-MeO-MiPT in pill samples by area comparison with the 5-MeO-MiPT standard, considering a standard human consumer of 75 kg.

For obtaining blood samples, four groups of three animals were injected with the 5-MeO-MiPT solution. Ten, 20, 40 and 60 min after drug administration, animals were quickly sacrificed by cervical dislocation and decapitated to obtain large volumes of blood. For the control group (three animals), blood was collected in the same way, 60 min after vehicle injections. Blood of each animal was allowed to coagulate and centrifuged. The serum from the animals of each group (either experimental or control) was mixed, and aliquots were frozen for subsequent use. In conclusion, a total of five serum samples (3 animals per sample) was obtained, one control and four obtained at different times after 5-MeO-MiPT administration.

For urine samples, four groups of three animals were injected with 5-MeO-MiPT solution, and four groups of three animals with the blank solution. Urine from control and 5-MeO-MiPT groups were collected at 1, 2, 3, 4, 5, 6 and 24 h after the administration of the drug or the vehicle solution. To do so, the three mice were put together in a previously sterilized, regular home cage with a meshwire platform that allowed safely collecting urine and avoiding those areas were excrements were present. The sample obtained at a given time point after drug or vehicle injection, was centrifuged, and the aliquots of the urine were frozen for its subsequent use. In

summary, seven control samples and seven 5-MeO-MiPT samples, obtained at different times after vehicle/drug administration (respectively), were collected.

2.4. Sample treatment

For the pill samples, the same extraction procedure described in literature for extracting NPS from legal highs samples was used [18]. Approximately 50 mg of sample was weighted in 2 mL propylene tubes and 1 mL of acetone was added. Extraction was performed under sonication during 15 min. After centrifugation during 15 min at 12000 rpm, supernatant was 10^4-fold diluted with H_2O:MeOH 90:10 and injected into the UHPLC-HRMS system.

For the blood samples, 300 µL of ACN were added to 100 µL of serum in 2 mL propylene tubes. Extracts were shaken during 1 min in a vortex in order to insolubilize the proteins and centrifuged at 12000 rpm during 10 min. Supernatant was collected and evaporated until dryness under gentle nitrogen stream at 40 ºC. Solid residue was dissolved with 100 µL H_2O:MeOH (90:10) and 20 µL were injected into the UHPLC-HRMS.

For urine samples, two different procedures were used in order to evaluate Phase I and Phase II metabolites. For Phase I studies, the procedure used was adapted from the literature [35–37]. Briefly, 200 µL of mice urine were hydrolysed with of 10 µL of β-glucuronidase, buffering the sample with 100 µL of a phosphate buffer adjusted to pH = 7. After incubating for 1 hour at 55 ±2 ºC, samples were frozen for 3 hours in order to remove proteins and lipids by precipitation. Finally, samples were centrifuged at 12000 rpm during 15 min and 20 µL of supernatant were injected into the UHPLC-HRMS system. For Phase II metabolites identification, 100 µL of mice urine was diluted with

100 µL Milli-Q water and frozen in order to remove lipids and proteins. After that, sample was centrifuged at 12000 rpm during 15 min and 20 µL were injected.

2.5. Instrumentation

Sample analysis were performed using an Acquity UPLC liquid chromatography system (Waters, Mildford, MA, USA) interfaced to a XEVO G2 QTOF hybrid quadrupole-time of flight (QTOF) mass spectrometer (Waters Micromass, Manchester, UK) with an orthogonal Z-spray electrospray (ESI) operating in positive and negative ionisation mode. A CORTECS C18 100 x 2.1 mm 2.7 µm particle size analytical column (Waters) was used to perform chromatographic separation, with a flow rate of 0.3 mL/min. Mobile phases were H_2O with 0.01% HCOOH (A) and MeOH with 0.01% HCOOH (B). The mobile phase gradient was performed as follows: 10% of B at 0 min, 90% of B at 14 min linearly increased, 90% of B at 16 min, and finally 10% B at 18 min in order to return to initial conditions. The injection volume was 5 µL for MS^E acquisition, and 20 µL for MS/MS experiments. The column temperature was set to 40ºC. The TOF resolution was ~20000 at FWHM at *m/z* 556 in positive ionisation mode. The range acquired by the MS system was from *m/z* 50 to 1000. A capillary voltage of 0.7 kV and a cone voltage of 20 V for positive ionisation, and a capillary voltage of -1.5 kV and a cone voltage of 20 V for negative ionisation were used during all the chromatographic run. Nitrogen (Praxair, Valencia, Spain) was used as desolvation and nebulizing gas. The desolvation gas flow was set at 1000 L/h. Argon 99.995% (Praxair) was used as a collision gas. The interface temperature was set to 600ºC and the source temperature to 120ºC. For MS^E experiments, two acquisition functions with different collision energy were created. The low energy function (LE) used a collision energy of 4 eV in order to obtain information about the (de)protonated molecule and adducts (if exist), while the

high energy function (HE) applied a collision energy ramp from 15 to 40 eV, in order to promote collision-induced fragmentation of the compounds. Calibration of the mass-axis was performed daily from *m/z* 50 to 1000 using a 1:1 mixture of 0.05M NaOH:5% HCOOH, diluted 1:25 with ACN:H_2O 80:20 mixture. For accurate mass measurement, a 2 µg/mL leucine enkephalin solution in ACN:H_2O 50:50 with 0.1% HCOOH was used as lock-mass, pumped at a flow rate of 20 µL/min. The leucine enkephalin (de)protonated molecule (*m/z* 556.2771 for positive ionisation, and *m/z* 554.2515 for negative) were used for recalibrating the mass axis and ensure an accurate mass during all the chromatographic run. MS data were acquired in continuum mode using MassLynx data station operation software version 4.1 (Waters), and processed with UNIFI scientific information system version 1.8 (Waters).

3. Results and discussion

3.1. Identification of 5-MeO-MiPT in legal highs samples

The suspect screening of the legal highs samples (*Estrella* and *Corazon*) retrieved the 5-MeO-MiPT as a potential candidate to be present in both pills. After an accurate fragment evaluation, the compound was tentatively identified in the two samples. **Figure 3A** shows the LE and HE spectra (**Left**) of the chromatographic peak at 3.24 min observed after analysis of *Estrella* pill. The extracted ion chromatograms (EIC) of the protonated molecule and the collision induced dissociation (CID) fragments are also showed (**Right**). The tentative identification of the compound was based on the fragmentation observed. After this careful evaluation, the analytical reference standard of 5-MeO-MiPT was bought to unequivocally confirm the identity of the compound based on retention time and fragmentation (**Figure 3B)**.

3.2. Mass spectrometric behaviour of 5-MeO-MiPT

Initially, an accurate study of the CID fragments of the parent compound was performed in order to understand the differences on the fragmentation pathways of the potential metabolites respect the parent compound. 5-MeO-MiPT was observed only in positive ionisation mode (*m/z* 247.1810) as protonated molecule ($[M+H]^+$) due to the presence of amine moieties.

The fragmentation spectrum showed five fragment ions (**Figure 3A)**. The most intense fragment ion was observed at *m/z* 174.0916 ($C_{11}H_{12}NO^+$), corresponding to the neutral loss of the *N*-isopropylmethylamine (73.0892 Da). The second most intense fragment, at *m/z* 86.0972 ($C_5H_{12}N^+$), corresponded to the *N*-isopropylmethylamine with an additional *N*-methylene coming from the alkyl chain bonded to the amine with the indole ring. This fragment has its complementary ion fragment corresponding to the molecule

without the functionalised amine at *m/z* 159.0683 ($C_{10}H_9NO^{+\cdot}$). In this case, the fragment corresponded to a radical ion originated by a homolytic cleavage. This fragment is the precursor ion of another radical fragment at *m/z* 131.0732 ($C_9H_9N^{+\cdot}$), originated by a 27.9949 Da neutral loss (corresponding to a CO molecule). Finally, fragment at *m/z* 143.0733 ($C_{10}H_9N^{+\cdot}$) would come from fragment ion at *m/z* 174 after a methoxide radical loss ($CH_3O^{\cdot}$). It can be observed than fragments involving radicals are quite less intense that full-paired electron fragments. The complete fragmentation pathway of 5-MeO-MiPT can be found in Supplementary Information (**SI.1**).

The accurate fragmentation study revealed that the protonation centre would be the *N*-isopropylmethylamine instead of the indole ring, as the fragmentation of the molecule starts on the functionalised amine.

3.3. Analytical strategy for detecting 5-MeO-MiPT metabolites in mice serum and urine

The detection of 5-MeO-MiPT metabolites was performed by 3 different approaches, using UNIFI scientific information system for data processing and compound elucidation. The first approach consisted on the comparison between the blood and urine samples collected after injection with 5-MeO-MiPT, and the control samples, obtained from mice injected with blank solution. Only the compounds which were present in 5-MeO-MiPT samples in a 5:1 ratio with respect to blank samples were considered as potential metabolites. This ratio was experimentally stablished in order to avoid false-positives (endogenic compounds that could be proposed as potential metabolites) or false-negatives (no detection of a potential metabolite due to its confusion with an endogenic compound present in blanks). For urine samples, each 5-MeO-MiPT sample had its corresponding control sample collected at the same time. In the case of blood samples, blank sample collected at 60 min was used as reference for

277 compound discrimination in all blood samples. Obviously, control samples were
278 processed with the same sample treatment than drug samples. Once identified the
279 potential metabolites, metabolite structure was determined based on the observed
280 fragmentation.
281 In the second approach, 5-MeO-MiPT metabolites were searched based on expected
282 biotransformations in a two-step strategy for urine samples. The first step was to search
283 for Phase I metabolites in the hydrolysed urine. In this step, potential Phase I
284 metabolites were searched by applying Phase I biotransformations (such as cleavages,
285 oxidations, reductions…) to the elemental composition of 5-MeO-MiPT. Once
286 identified all the Phase I metabolites, Phase II metabolites were searched in the diluted
287 non-hydrolysed urine. Now, conjugations with glucuronides or sulphates of the
288 elemental composition of the parent compound and the elucidated Phase I metabolites
289 were searched.
290 Finally, in the third approach, the common fragmentation pathway and neutral loss
291 search strategies were applied, assuming that some metabolites would share common
292 fragments and/or neutral losses with the parent compound.
293 In the case of serum, due to the low amount obtained, the hydrolysis step could not be
294 performed.
295 The combination of the three different approaches allowed the identification of two
296 Phase I and two Phase II metabolites. The four identified metabolites were detected only
297 in positive ionisation mode. In negative ionisation mode, no additional metabolites were
298 identified.
299
300 *3.4. 5-MeO-MiPT Phase I metabolites*
301 *Demethylated metabolite (Metabolite 1)*

The Metabolite 1 was detected at *m/z* 233.1649 ($[M+H]^+$, $C_{14}H_{21}N_2O^+$, at chromatographic retention time (rt) of 2.49 min), which was the result of the loss of a methyl group respect the 5-MeO-MiPT. The fragments observed (**Figure 4A**, **Table 1**) were similar to the observed for 5-MeO-MiPT. The fragments at *m/z* 86.0961 ($C_5H_{12}N^+$) and *m/z* 160.0751 ($C_{10}H_{10}NO^+$), and the corresponding neutral loss of the N-isopropylmethylamine (73.0892 Da), were also observed for the 5-MeO-MiPT. This fragmentation would suggest that the demethylation point was not located in the functionalised amine but in the methoxy group. Additionally, the fragment at *m/z* 142.0648 ($C_{10}H_8N^+$) was also observed for the 5-MeO-MiPT, with a difference of 1 Da. This fragment ion was obtained after the homolytic fragmentation of a methoxy loss instead of the heterolytic fragmentation of a water loss observed for the parent compound. Fragment at *m/z* 132.0799 ($C_9H_{10}N^+$) has also the same 1 Da difference respect to the corresponding 5-MeO-MiPT fragment, produced by heterolytic/homolytic fragmentation. The other two minor fragments at *m/z* 117.0573 ($C_8H_7N^{+\cdot}$) and *m/z* 115.0536 ($C_9H_7^{+\cdot}$) were not observed for 5-MeO-MiPT.

After this accurate fragmentation analysis, a plausible fragmentation pathway for the Metabolite 1 was proposed (**Figure 4B**). The aromaticity of the indole ring, and the multiple resonance structures that could be formed allow the fragmentation of the molecule by the groups linked to the indole ring even though the protonation only occur in the amine moiety.

<u>*N-oxide metabolite (Metabolite 2)*</u>

Metabolite 2 presented its $[M+H]^+$ at *m/z* 263.17531 ($C_{15}H_{23}N_2O_2^+$, rt 4.30 min), corresponding to a hydroxylation. This compound presented four fragment ions at *m/z* 174.0913 ($C_{11}H_{12}NO^+$), *m/z* 159.0685 ($C_{10}H_9NO^{+\cdot}$), *m/z* 143.0731 ($C_{10}H_9N^{+\cdot}$) and *m/z*

131.0723 ($C_9H_9N^{+\cdot}$) (**Table 1**), all of them shared with the 5-MeO-MiPT. These fragments would suggest the hydroxylation to occur in the amine group. This is in accordance with the neutral loss of 89.0841 Da observed (263→174), which corresponds to an elemental composition of $C_4H_{11}NO$. According to literature, the hydroxylation in an alkylic chain would produce the loss of a water molecule during CID fragmentation [38]. However, this fragment was not observed, indicating that the hydroxylation point would be the nitrogen atom. The formation of *N*-oxides in tryptamine analogues has been described in literature [28,39]. On this way, the putative structure of Metabolite 2 would be the *N*-oxide of 5-MeO-MiPT. MS^E fragmentation spectrum of Metabolite 2 and the proposed fragmentation pathway for its structure can be found in Supplementary Information **SI.2**.

3.5. 5-MeO-MiPT Phase II metabolites

Glucuronide conjugation of Metabolite 1 (Metabolite 3)

Once identified the two Phase I metabolites, Phase II metabolites were investigated. A drug-unique peak in raw urine and serum samples was detected, fitting with the glucuronide conjugate of Metabolite 1 (*m/z* 409.1965, $C_{20}H_{29}N_2O_7^+$, rt 1.30 min). The position of the glucuronide conjugation was determined based on the observed fragmentation (**Table 1**). The "key" fragment was observed at *m/z* 336.1061 ($C_{16}H_{18}NO_7^+$), corresponding to the neutral loss of *N*-isopropylmethylamine (73.0892 Da), also present for 5-MeO-MiPT and Metabolite 1. This fragment indicates that the glucuronide conjugation should have occurred in the hydroxyl group of the indole ring, discarding the formation of an *N*-glucuronide. Therefore, this compound would correspond to the glucuronide of Metabolite 1. The fragment present at *m/z* 233.1637 involved the loss of the glucuronide, releasing the non-conjugated part ($C_{14}H_{21}N_2O^+$).

The other two observed fragments at *m/z* 160.0750 ($C_{10}H_{10}NO^+$) and *m/z* 86.0963 ($C_5H_{12}N^+$) were shared with Metabolite 1, as expected. For further information about the MS^E fragmentation spectrum and the proposed fragmentation pathway, consult Supplementary Information **SI.3**.

Glucuronide conjugation of hydroxyindole metabolite (Metabolite 4)

The second Phase II metabolite would correspond to a glucuronide conjugation of a non-detected Phase I metabolite, an indole-hydroxylated metabolite (*m/z* 439.20534, $C_{21}H_{31}N_2O_8^+$, rt 1.72 min). The exact position of the hydroxyl group was not determined, but it was enclosed in the indole ring according to its fragmentation. Fragment at *m/z* 366.1159 ($C_{17}H_{20}NO_8^+$) corresponded to the well-known *N*-isopropylmethylamine loss (73.0892 Da), indicating that the glucuronide, and thus the hydroxylation, was present in the indole ring. Another familiar fragment was at *m/z* 86.0961 ($C_5H_{12}N^+$), corresponding to the functionalised amine. Fragment at *m/z* 190.0855 ($C_{11}H_{12}NO_2^+$) corresponded to the loss of the glucuronide conjugation of fragment at *m/z* 366. The other two fragments at *m/z* 175.0634 ($C_{10}H_9NO_2^+$) and *m/z* 158.0592 ($C_{10}H_8NO^+$) were also justified based on the proposed metabolite structure (**Table 1**). In supplementary information **SI.4**, the MS/MS fragmentation spectra of this metabolite and the proposed fragmentation pathway can be consulted. In this case, MS^E fragmentation spectrum was not clean enough for observing the product ions of Metabolite 4, thus MS/MS experiments were carried out to better match the fragmentation observed to the metabolite structure.

In order to enhance the confidence on the fragmentation observed in MS^E, MS/MS spectra were also acquired for Metabolite 1, Metabolite 2 and Metabolite 3 (**SI.5** to **SI.8**).

After the identification of the four metabolites previously described, a plausible metabolic pathway of the 5-MeO-MiPT was proposed (**Figure 5**).

3.6. Implications of the obtained results

A total of four 5-MeO-MiPT metabolites were elucidated in serum and urine: two Phase I and two Phase II metabolites. The two Phase I metabolites tentatively identified corresponded to the *O*-demethylated (Metabolite 1) and the *N*-oxide (Metabolite 2). Our study reveals that 5-MeO-MiPT is mostly metabolized as demethylation of the methoxy group in the indole ring. On the one hand, this tendency is similar to the one reported for the analogue 5-MeO-DiPT, where the demethylated metabolite was the most significant transformation product detected [30,32,33]. On the other hand, the *N*-deisopropylated metabolite described for 5-MeO-DiPT has not been detected for 5-MeO-MiPT, surely due to the change in the amine functionalisation. Moreover, the metabolite 2 detected in this work, corresponding to the oxidation in the amine moiety as N-oxide, has not been reported for 5-MeO-DiPT. Nevertheless, this biotransformation has been recently found for the tryptamine *N*,*N*-diallyltryptamine (DALT) [28,39]. The metabolism study of DALT derivatives revealed a vast number of biotransformations. However, in the case of 5-MeO-MiPT, only the formation of the amine oxidation as *N*-oxide has been found.

Regarding Phase II metabolites, the glucuronide of the demethylated Phase I metabolite (Metabolite 3), and the glucuronide of the hydroxyindole metabolite (Metabolite 4), were detected. The no-detection of the hydroxyindole Phase I metabolite in the hydrolysed urine could indicate that the response of this metabolite was too low to be detected or that the corresponding glucuronide is not hydrolysed under the conditions used, as it has been observed for two biomarkers of testosterone consumption [40].

In this study, a 5-MeO-MiPT dose of 0.27 mg/kg was injected to mice in order to elucidate metabolites and to study their pharmacokinetics in serum and urine. This dose, as explained previously, was based on a rough quantification of the 5-MeO-MiPT detected in the two pill samples, and a typical human consumer weighing 75 kg. However, metabolism studies of 5-MeO-DiPT [32] and DALT derivatives [28,39] in male Wistar rats referred much higher doses (10 mg/kg and 20 mg/kg, respectively) that used in this work. Surely, the use of higher doses facilitates the detection of higher number of metabolites, but it is possible that several of the identified metabolites at high-dose metabolism studies in rats cannot be found in human urine after consumption of a typical dose. In front of this dilemma, we preferred to use realistic doses, based on legal highs analyses or experiences described by consumers in order to obtain putative biological consumption markers, despite that some metabolites observed at higher dose can be ignored. On the basis of our results, Metabolite 1 and its glucuronide (Metabolite 3), in addition to the parent compound, might be candidates as consumption markers, an issue that will be discussed in the next subsection.

3.7. Prevalence and detectability of 5-MeO-MiPT metabolites in serum and urine

The pharmacokinetic study of 5-MeO-MiPT revealed that most of the parent compound is demethylated and conjugated as glucuronide in the first 20 min, as shown in **Figure 6A**. It should be remarked that experiences were performed in mice, which metabolism is faster than human. To have representative curves between percentage of each compound and excretion time, the response of each individual compound was related with the total response of all detected compounds by assuming that each compound gave the same response in the instrument. This approach was used due to the non-availability of standards for the metabolites detected. This figure shows that in

bloodstream, the parent 5-MeO-MiPT has a drop of 75% in the first 30 min, while Metabolite 3 presents its maximum response at 20 min. For Metabolite 1 and Metabolite 4, the response in bloodstream did not exceed 6% respect the initial 5-MeO-MiPT. At 60 min, most of 5-MeO-MiPT was removed from bloodstream by urine excretion or metabolic pathways, while there was still around 30% of Metabolite 3.

Regarding hydrolysed urine, Metabolite 1 presented the highest response, while 5-MeO-MiPT only represented 38% respect to Metabolite 1 (**Figure 3B**). Metabolite 2 was minor (around 2%) and could not be detected at 3 hours. An important decrease between 3-4 hours was observed in the concentration of 5-MeO-MiPT and Metabolite 1. Nevertheless, after 6 hours, Metabolite 1 was still detected, at a concentration of 30 % respect to its concentration at 1 hour. As expected, Metabolite 3 and Metabolite 4 were not observed due to the enzymatic hydrolysis of glucuronides. In this way, Metabolite 1 would be the most suitable biological consumption marker of 5-MeO-MiPT.

In relation to the diluted raw urine (**Figure 3C**), the major compound found at 1 hour, in terms of response, was the parent 5-MeO-MiPT. Metabolite 3 represented around 80%, whereas Metabolite 1 was 50%. However, Metabolite 4 did not exceed the 2% of the response, being not detected at 4 hours. The elimination of 5-MeO-MiPT and its metabolites revealed that Metabolite 3 concentration remains constant for the first 2 hours, while the 5-MeO-MiPT and Metabolite 1 show a decrease. Similarly to hydrolysed urine, the most important concentration fall was observed between 3 and 4 hours. At 6 hours, the concentration of 5-MeO-MiPT represent around the 5% of its initial concentration, and Metabolite 3 the 10%.

Once evaluated the pharmacokinetics of 5-MeO-MiPT, plausible consumption biomarkers can be proposed. The consumption of this tryptamine can be monitored in hydrolysed urine by the parent compound and the *O*-demethylated metabolite

(Metabolite 1). The detection of these biomarkers should be performed in hydrolysed urine, as the glucuronide cleavage of Metabolite 3 would increase the concentration of Metabolite 1. Both compounds were still detected after 24 hours of drug administration, as it can be seen in **Figure 7**, demonstrating that the proposed biomarkers reveal the consumption of 5-MeO-MiPT. Despite Metabolite 1 is the most abundant one in hydrolysed urine, it should not be used as 5-MeO-MiPT consumption marker individually. The detection of Metabolite 1 without the simultaneous detection of 5-MeO-MiPT would generate a reasonable doubt about which is the tryptamine that have been consumed: 5-MeO-MiPT or the synthetic analogue of psilocin, 4-OH-MiPT. Thus, the presence of both 5-MeO-MiPT and Metabolite 1 after 24 hours in urine would reveal the consumption of this tryptamine.

4. Conclusions

Due to the difficulties associated to the metabolism studies in humans, in this work the *in vivo* approach has been applied using adult male mice of the inbred strain C57BLJ/6 as model. This has allowed the establishment of potential consumption markers of the synthetic tryptamine 5-MeO-MiPT. By the use of UHPLC-HRMS, two Phase I and two Phase II metabolites have been elucidated. The accurate-mass data acquired under MS^E mode allowed the tentative identification of three metabolites, but elucidation of the fourth one required MS/MS experiments in order to obtain cleaner spectra. Nevertheless, all the metabolites were additionally confirmed by MS/MS experiments. After evaluating the results, the most important metabolite found was the *O*-demethylated (Metabolite 1) and its glucuronide (Metabolite 3).

The pharmacokinetic study revealed that 5-MeO-MiPT was rapidly metabolized, being almost completely removed in bloodstream after 60 min. In the case of urine samples, an important decrease of 5-MeO-MiPT and the two major metabolites was observed between 3 and 4 hours, although these three compounds were still detected after 6 hours. The parent tryptamine 5-MeO-MiPT and the *O*-demethylated metabolite were both detected in hydrolysed urine collected 24 hours after administration. Both compounds are proposed as biological markers for monitoring 5-MeO-MiPT consumption in hydrolysed consumer's urine, discarding the *O*-demethylated glucuronide because it would be transformed in Metabolite 1 after the hydrolysis step. The detection of the parent compound is crucial for differentiating the consumption of two chemically-related tryptamines, 5-MeO-MiPT and the psilocin analogue 4-OH-MiPT. Data reported in this work will be useful for developing analytical methodologies for 5-MeO-MiPT consumption detection in hospitals and research centres.

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