Study of the *in vitro* and *in vivo* metabolism of the tryptamine 5-MeO-MiPT using human liver microsomes and real case samples

Katharina Elisabeth Grafinger, Marianne Hädener, Stefan König, Wolfgang Weinmann

Corresponding author: Katharina Elisabeth Grafinger, Institute of Forensic Medicine, Forensic Toxicology and Chemistry, University of Bern, Bühlstrasse 20, 3012 Bern, Switzerland. E-mail: katharina.grafinger@irm.unibe.ch

Running title: *In vitro* and *in vivo* metabolism of 5-MeO-MiPT

Abstract

The synthetic tryptamine 5-methoxy-*N*-methyl-*N*-isopropyltryptamine (5-MeO-MiPT) has recently been abused as a hallucinogenic drug in Germany and Switzerland. This study presents a case of 5-MeO-MiPT intoxication and the structural elucidation of metabolites in pooled human liver microsomes (pHLM), blood, and urine. Microsomal incubation experiments were performed using pHLM to detect and identify *in vitro* metabolites. In August 2016, the police encountered a naked man, agitated and with aggressive behavior on the street. Blood and urine samples were taken at the hospital and his premises was searched. The obtained blood and urine samples were analyzed for *in vivo* metabolites of 5-MeO-MiPT using LC-high resolution MS/MS (LC-HR-MS/MS). The confiscated pills and powder samples were qualitatively analyzed using FTIR, GC-MS, LC-HR-MS/MS and NMR. 5-MeO-MiPT was identified in two of the seized powder samples. General unknown screening detected cocaine, cocaethylene, methylphenidate, ritalinic acid and 5-MeO-MiPT in urine. Seven different *in vitro* phase I metabolites of 5-MeO-MiPT were identified. In the forensic case samples, four phase I metabolites could be identified in blood and seven in urine. The five most abundant metabolites were formed by demethylation and hydroxylation of the parent compound. 5-MeO-MiPT concentrations in the blood and urine sample were found to be 160 ng/mL and 3380 ng/mL, respectively. Based on the results of this study we recommend metabolites 5-methoxy-*N*-isopropyltryptamine (5-MeO-NiPT), 5-hydroxy-*N*-methyl-*N*-isopropyltryptamine (5-OH-MiPT), 5-methoxy-*N*-methyl-*N*-isopropyltryptamine-*N*-oxide (5-MeO-MiPT-*N*-oxide) and hydroxy-5-methoxy-*N*-methyl-*N*-isopropyltryptamine (OH-5-MeO-MiPT) as biomarkers for the development of new methods for the detection of 5-MeO-MiPT consumption, as they have been present in both blood and urine samples.

Introduction

Since their first occurrence as recreational drugs, new psychoactive substances (NPS) have taken over the online drug market. The initial appeal of NPS was that these substances were legally and easily available via the internet[1, 2] and were untraceable by commonly used immunoassay drug tests[3, 4]. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) is monitoring over 620 substances[5], classified into different chemical classes including phenethylamines, cathinone derivatives, synthetic cannabinoids, piperazines and tryptamines [6]. In this last category, 16 tryptamines have been recorded since 2009[7].

The NPS market is constantly changing with alone 101, 98 and 66 new substances emerging in 2014, 2015 and 2016, respectively [5, 7]. As soon as a substance is legally controlled its structure is altered in order to evade legislations once again[2, 8, 9]. These structural alterations can result in a modified pharmacological and toxicological profile [10]. When new substances appear on the market, only little is usually known about their mode of action, metabolism and toxicity, making the assessment of potential harms in humans challenging.

NPS were initially derived from pharmaceutical research. An example of a tryptamine researched and developed for pharmaceutical application is alpha-methyltryptamine (AMT). It was developed in the 1960s as an antidepressant in the Soviet Union and was studied for its clinical value by the Upjohn Pharmaceutical Company. Although it was shown to have no therapeutically applicability AMT became popular in the 1990s as an NPS because of its purported hallucinogenic properties [9, 11]. Tryptamines are originally derived from natural sources such as toads, plants, mushrooms [12], microbes and amphibians [13]. Naturally occurring tryptamines with a classical psychedelic profile are for example *N*,*N*-dimethyltryptamine (DMT), 4-phorphoryloxy-*N*,*N*-dimethyltryptamine (psilocybin) and 5-methoxy-*N*,*N*-dimethyltryptamine (5-MeO-DMT) [14]. First synthetic analogs of tryptamines have become sold on the designer drug market in the late 1990s [14] and have become recently available and more popular on the internet [9]. These derivatives are structurally related to the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) [12]. Serotonin is one of the most important signaling hormones in the body [15] and is involved in the regulation of a variety of functions such as appetite, sex, sleep, cognition and memory, sensory perception, mood, nociception, endocrine function, temperature regulation, motor activity and behavior [16]. It is today's understanding that the hallucinogenic properties of tryptamines lie in the principal structural feature of the tryptamine nucleus [17] and additionally is greatly affected by substitution on the indole ring, the side-chain carbons and alkylation of the side-chain nitrogen [18]. Moreover, the psychoactive effects of hallucinogens are modulated mainly via the 5-HT$_{2A}$ [12, 19, 20] and 5-HT$_{1A}$ receptor [14], with possible additional receptor subtypes being important, which has been shown in an extensive study with 35 psychoactive tryptamine analogs being evaluated for their binding profiles [21]. One of the most common routes of synthesis has been adapted from the method of Speeter and Anthony [17, 22]. For this approach, an indole precursor is acylated with oxalyl chloride followed by the formation of 3-ylglyoxalylamide. Finally reduction with lithium aluminum hydride produces the desired tryptamine compound [22, 23]. Research on synthetic tryptamines has been published by Alexander Shulgin and Ann Shulgin and has been described in the book TiHKAL [24].

The psychedelic and hallucinogenic drug 5-methoxy-*N*-methyl-*N*-isopropyltryptamine (5-MeO-MiPT) is chemically related to the naturally occurring tryptamine 5-MeO-DMT and is an analogue of the synthetic tryptamine 5-methoxy-*N,N*-diisopropyltryptamine (5-MeO-DiPT), also known as Foxy or Foxy methoxy. Therefore, 5-MeO-MiPT is often also referred to as Moxy [13]. 5-MeO-DiPT was placed in Schedule I of the Controlled Substances Act in the United States in 2004 [25]. Studies have suggested that 5-MeO-DiPT has potent neurotoxic effects on the serotonergic neurons [26] which is consistent with behavioral data [27]. It has been clinically shown to promote emotional expression, a talkative uninhibited state and visual and auditory sensory distortion [24, 28, 29].

The synthesis and psychoactive effects of 5-MeO-MiPT were first described by Repke *et. al* [30]. Alexander Shulgin and Ann Shulgin recommend doses of 4-6 mg for oral consumption and higher doses of 12-20 mg for administration via smoking[24]. The attachment of a 5-methoxy group to the tryptamine core has been found to increase potency [9] and to decrease the effects on perceptual changes in vision [30]. *N*-Substitutions have also been reported to have an influence on the *in vivo* potency of tryptamines [30, 31]. It has been demonstrated that 5-MeO-MiPT inhibits the re-uptake of 5-HT, dopamine and norepinephrine, a mechanism of action similar to that of 5-MeO-DiPT. Furthermore, it was shown to have little dopamine, 5-HT and norepinephrine releasing activity [13, 32]. In Switzerland 5-MeO-MiPT is listed in the "Federal Act on Narcotics and Psychotropic Substances" index e, No. 85 [33]. Until now, however, it has not been listed in the neighboring state Germany [34]. Metabolism studies are a valuable tool in forensic science. Data obtained from these studies help to decide on biomarkers not only as target for the development of new bioanalytical methods [35], but also to prove consumption of a drug of abuse.

For the study of NPS metabolism different *in vivo* and *in vitro* models can be used for the formation of phase I and phase II metabolites. *In vivo* experiments involving human subjects are difficult to conduct, due to obvious health risks and ethical questions. Mice, rats and primates can be used as alternative *in vivo* models. However, these also suffer from limitations such as ethical constraints, high costs, time, and species difference to humans [36]. *In vitro* experiments, such as pooled human liver microsomes (pHLM), hepatocytes or perfused liver are adequate alternatives. The pHLM model has been widely used for metabolism studies of different substance classes of NPS [37-39], including synthetic tryptamines [40]. Although pHLM assays do not always produce a metabolite pattern completely congruent with the one observed in human urine and blood, they are still a valuable tool in metabolism studies.

So far, publications available on 5-MeO-MiPT have focused on receptor interaction profiles [12], receptor functional data [41], effects on monoamine neurotransmission in rat brain [42], characterization of the synthetic route [18] and the simultaneous detection of 31 NPS [43]. Studies on the receptor interaction profiles by Rickli *et al.* [12] could show that 5-MeO-MiPT is a near-full (83 %) agonist at the 5-HT$_{2A}$ receptor but had no relevant action at the human monoamine transporters. A case study described the intoxication with 78 mg of 5-MeO-MiPT in combination with 120 mg of methylone which led to adverse effects and hospitalization of the man [44]. Shima *et al.* [45] reported on the detection of three *in vivo* metabolites in two different individuals, one of them being fatal. However, they gave no description of the experimental procedure and how the metabolites were identified. Recently Fabregat-Safont *et al.* [46] identified four 5-MeO-MiPT metabolites in total in serum and urine samples of adult male C57BLJ/6 mice.

This paper presents a detailed analysis of phase I *in vitro* metabolites of 5-MeO-MiPT, using pHLM and phase I and phase II *in vivo* metabolites found in authentic blood and urine samples after 5-MeO-MiPT intoxication. Identification of drugs and metabolites was performed by LC-high resolution MS/MS (LC-HR-MS/MS), quantification of 5-MeO-MiPT in the case samples were performed using LC-MS/MS and chemical analyses of seized material were performed using FTIR, GC-MS, LC-HR-MS/MS and NMR.

Case report

In late August 2016 the police was called by a friend of a man, who supposedly had taken a "LSD-like substance". The police encountered a 32 year old male (height 184 cm, weight 84 kg) standing naked and without orientation on the street. He had several abrasions all over his body and seemed to be in conation (a "bad state"). When approached by the police, the suspect reacted agitated and aggressive. The man was then handcuffed and taken to the hospital. A blood sample was collected one hour and a urine sample two hours after the police appeared at the scene. The second man present at the scene was tested positive for THC and opiates using a lateral flow immuno-test. Both men´s flats were searched and one sachet containing eight yellow candy-like pills (sample 1) and three different powder sachets (sample 2-4) were confiscated. Blood and urine samples of the first suspect and all seized material were sent to the Institute of Forensic Medicine Bern for analysis.

Materials and Methods

Chemicals and Reagents

Sulfosalicylic acid and superoxide dismutase (6016 units/mg protein) were obtained from Sigma Aldrich (Buchs, Switzerland), sodium hydroxide and magnesium chloride from Merck AG (Zug, Switzerland). The following reference standards were obtained from Cerilliant (Round Rock, TX, USA): ecgonine methyl ester-D_3 (0.1 mg/mL in MeOH), tramadol-d_3C_{13} (0.1 mg/mL in MeOH), THC-D_3 (0.1 mg/mL in MeOH) and MBDB-D_5 (0.01 mg/mL in MeOH). The reference standards D,L-amphetamine (HCl, >98.5 %), D,L-methamphetamine (HCl, 98.5 %), D,L-MDMA (HCl, >98.5 %), and 3,6-diacetylmorphine were purchased from Lipomed (Arlesheim, Switzerland), and cocaine-HCl (>99 %) from Siegfried AG (Zolfingen, Switzerland). Methanol (absolute, HPLC grade) was purchased from Biosolve (Chemie Brunschwig, Basel, Switzerland), acetonitrile (HPLC gradient grade, 99.9 %) from Acros Organics (Chemie Brunschwig, Basel, Switzerland), formic acid (analytical grade, 98 %) and ammonium formate from Fluka (Sigma-Aldrich, Buchs, Switzerland). Deuterated chloroform (CDCl$_3$) was obtained from Cambridge Isotopes Laboratories (Tewksbury, MA, USA). Ultrapure water was produced in-house with a Direct-Q water purification system from Millipore (Zug, Switzerland). Blank blood and urine were provided by healthy volunteers and were tested with the presented LC-HR-MS/MS method to ensure the absence of drugs of abuse. Urine samples were collected in plastic containers without adding a preservative. Pooled human liver microsomes (pHLM) (150 donors, 20mg/mL), NADPH-regenerating solutions A/B and 0.5 M potassium phosphate buffer pH 7.4 were purchased from Corning (New York, NY, USA). Precision pipettes from Gilson (Mettmenstetten, Switzerland) and Socorex Isba S.A (Ecublens, Switzerland) were used for handling of all solutions and samples. A reference standard of 5-MeO-MiPT·HCl (>98 %) was kindly provided by the Forensic Institute Zurich, Switzerland and used for pHLM experiments.

In Silico predictions

To assist in the interpretation of the collected mass spectrometric data, *in silico* predictions were performed using EAWAG-BBD Pathway Prediction System (formerly known as UM-BBD/PPS) [47-49]. It predicts plausible pathways for aerobic microbial degradation of chemical compounds using biotransformation rules, based on reactions found in the EAWAG-BBD database and in the scientific literature. This includes a multi-level prediction with testing query compounds against rules, validating product qualifications and displaying all qualified products in one or more plausible pathway branches. It differentiates the likelihood of predicted degradation products based on their aerobic likelihood into very likely (dark green), likely (light green) or neutral (yellow) (for the generated predictions of 5-MeO-MiPT see Supporting Information Figure S1) [50]. A recursive prediction method is used to construct the pathway. It starts from the initial query compound, which is inserted as a SMILES string, followed by further prediction levels until the breadth (row) or depth (levels) reaches chosen cut-off values. Default values, which have been applied, are ten compounds in breadth and six in depth. Once the prediction is completed, an automatic layout algorithm is applied and the graphical pathway is produced by the GraphViz dot program [49]. Predicted degradation products are then compared to full scan LC-HR-MS/MS measurements using Analyst software 1.6 TF with the extracted ion current (XIC) tool.

In vitro metabolism assay for pHLM incubations and sample preparation for screening

Microsomal *in vitro* experiments were performed to generate phase I metabolites. The final reaction volume was 50 µL. Potassium phosphate buffer (100 mM), deionized water, NADPH-regenerating solution A (NADP+ and glucose-6-phosphate) and B (glucose-6-phosphate dehydrogenase), magnesium chloride (5mM), superoxide dismutase (200 units/mL), pHLM (1 mg/mL) and a reference standard of 5-MeO-MiPT (100 mM), were mixed in a reaction tube and incubated for 120 minutes at 37 °C (indicated concentrations are final concentrations). Blanks containing no drug substrate and negative controls containing no pHLM were processed accordingly. Reactions were stopped by the addition of extraction solvents. Experiments were repeated at least 6 times for every MS experiment.

Two different extraction procedures were used in order to take into account potential differences in the extraction behavior of the metabolites. The first procedure consisted of protein precipitation by adding 50 µL ice-cold acetonitrile. The sample was then centrifuged at 17,000 g and 4 °C for 10 minutes. The supernatant was transferred into an autosampler vial, evaporated to dryness at 50 °C under nitrogen and reconstituted in 50 µL of water/acetonitrile/formic acid, (95:5:0.1; v/v/v). The second procedure involved liquid-liquid extraction with 20 % sulfosalicylic acid, acetonitrile and ammonium formate (10 mM) (7:80:13, v/v/v) followed by centrifugation at 17,000 g for ten minutes at 4 °C. The organic phase was transferred into an autosampler vial containing ethylene glycol (5 µL), which was added to avoid evaporation of compounds and further to avoid compounds being attached to the walls of the vial and hence not be reconstituted. Finally the organic phase was evaporated under nitrogen at 50 °C and reconstituted in deionized water and methanol (89:11; v/v).

Preparation of authentic blood sample for general unknown screening using LC-HR-MS/MS

Ten μL of internal standard (ISTD) solution was added to 200 μL of whole blood sample. Protein precipitation was performed by adding acetonitrile in a ratio of 1:3. The sample was then centrifuged at 17,000 g at 4 °C for 10 minutes and the supernatant was evaporated to dryness under nitrogen at 50 °C. The sample was then reconstituted in 200 μL water, acetonitrile, formic acid, (97.5/2.5/0.1; v/v/v) with 2.5 mM ammonium acetate and 2.5 mM ammonium formate.

Preparation of authentic urine sample for general unknown screening using LC-HR-MS/MS

For the preparation of the urine sample our general in-house urine work-up procedure for LC-HR-MS/MS was performed as follows: The urine sample (30 μL) was diluted with 10 μL ISTD solution containing ecgonine methylester-D_3 (500 ng/mL), tramadol-$D_3{}^{13}C_1$ (500 ng/mL) and THC-D_3 (5000 ng/mL) and 200 μL mobile phase of deionized water, acetonitrile, formic acid (97.5:2.5:0.1; v/v/v) with 2.5 mM ammonium acetate and 2.5 mM ammonium formate. The sample was then ready for LC-HR-MS/MS analysis.

Preparation of calibration and quality control samples for the quantification of 5-MeO-MiPT

Working solutions were prepared via dilution of the reference standard solutions in methanol (0.37 mg/mL) and used for preparing calibration and quality control (QC) samples. Calibration and QC working solutions were prepared using separate dilution series and stored at −20 °C in polypropylene tubes. Spiking 190 μL blank blood or blank urine with 10 μL of working solution, created calibration samples with concentrations of 5, 10, 25, 50, 100, 250, 500 ng/mL and QC samples with concentrations of 5, 15, 125 and 400 ng/mL. Linear least-squares regression with a weighting factor of 1/x was used for fitting the calibration curve.

Preparation for the quantification of 5-MeO-MiPT in blood

Protein precipitation of 200 μL calibration, QC and authentic whole blood samples spiked with 10 μL ISTD containing MBMB-D_5 (4000 ng/mL), was performed by adding acetonitrile in a ratio of 1:3 (v/v). After centrifugation of the samples at 17,000 g at 4 °C for 10 minutes, the supernatant was evaporated to dryness under nitrogen at 50 °C. Samples were reconstituted in 200 μL water, acetonitrile and formic acid (97.5/2.5/0.1; v/v/v).

Preparation for the quantification of 5-MeO-MiPT in urine

Sample preparation of calibration, QC and the authentic urine samples was performed by dilution. A 30 μL sample was diluted with 10 μL ISTD containing MBMB-D_5 (4000 ng/mL) and 200 μL mobile phase consisting of deionized water, acetonitrile and formic acid (97.5:2.5:0.1; v/v/v). The sample was then ready for LC-MS/MS analysis.

LC-HR-MS/MS analysis of pHLM, blood and urine samples for phase I and phase II metabolites

The LC-HR-MS/MS system used for the analysis of pHLM samples, blood, urine and seized samples consisted of a Dionex Ultimate 3200 HPLC system (Thermo Fisher Scientific, Reinach, Switzerland) coupled to a 5600 TripleTof System equipped with a DuoSpray interface and Analyst software 1.6 TF with MasterView™ Software Version 1.1 (Sciex, Toronto, Canada). MS data was acquired in positive ionization mode with an ionspray voltage of 5.0 kV and a source temperature of 650 °C. The curtain gas was set to 35 arbitrary units, gas 1 and 2 to 40 arbitrary units. Information dependent data acquisition (IDA) was performed with a survey scan from 100 to 950 *m/z*, triggering the acquisition of product ion mass spectra at a mass range from 50 to 950 *m/z*. Accumulation time was set to 40 ms and 50 ms for dependent and survey scans, respectively. Fragmentation was selected for the nine most abundant precursor ions with a collision energy of 35 eV and a collision engergy spread of ± 15 eV.

The analysis of pHLM samples was performed with the following chromatographic conditions: chromatographic separation was performed on a reversed phase Kinetex C8 column, 2.6 μm, 100 Å, 100 x 2.1 mm (Phenomenex, Aschaffenburg, Germany), at a flow rate of 0.25 mL/min. The mobile phase consisted of water with 0.1 % formic acid (A) and acetonitrile with 0.1 % formic acid (B). The following gradient was used: 0-1 min: 2.5 % B, 1-20 min: 2.5 % to 30 % B, 20-24 min: 30 %-97.5 % B, 24-28 min: 97.5 % B, 28-29 min: 97.5 %-2.5 % B and 29-34 min: 2.5 % B. The injection volume was 2.5 μL.

For the analysis of blood and urine samples the following gradient was used: 0-1 min: 2.5 % B, 1-7 min: 2.5 % to 97.5 % B, 7-11 min: 97.5 % B, 11-11.01 min: 97.5 %-2.5 % B, and 11.01-14 min: 2.5 % B for reequilibration. The flow rate was set at 0.35 mL/min. Chromatographic separation was performed on a Kinetex C8 column, 2.6 μm, 100 Å, 50 x 2.1 mm (Phenomenex, Aschaffenburg, Germany). The mass spectra were acquired in positive mode and Tof-MS and Tof-MS/MS scan modus. A volume of 2.5 μL of the processed, blood and urine sample was injected for analysis.

Quantification of 5-MeO-MiPT in authentic blood and urine samples using LC-MS/MS

An HPLC System 1200 (Agilent, Waldbronn, Germany) coupled to a QTRAP 3200 hybrid mass spectrometer with Analyst 1.5.1 software (Sciex, Toronto, Canada) was used for the quantification of 5-MeO-MiPT in authentic case samples. Following settings were applied: chromatographic separation was performed on a reversed phase Kinetex C8 column, 2.6 μm, 100 Å, 100 x 2.1 mm (Phenomenex, Aschaffenburg, Germany). The mobile phase consisted of water with 0.1 % formic acid (A) and acetonitrile with 0.1 % formic acid (B). The injection volume was 20 μL. The chromatographic gradient was used at a flow rate of 0.35 mL/min: 0-1 min: 2.5 % B, 1-10 min: 2.5 % to 30 % B, 10-12 min: 30 %-97.5 % B, 12-14 min: 97.5 % B, 14-15 min: 97.5 %-2.5 % B and 15-20 min: 2.5 % B. MS data was acquired in positive ionization mode with an ion source voltage of 4500 V, a source temperature of 650 °C, curtain gas was set to 25 arbitrary units, gas 1 and 2 to 40 arbitrary units. Selected reaction monitoring (SRM) experiments were run using parameters presented in Table 1.

Evaluation of extraction efficiency, matrix effects and recovery of 5-MeO-MiPT in pHLM samples and authentic blood samples using LC-MS/MS

Extraction efficiency, matrix effects, and recovery for phLM and blood samples were evaluated according to Matuszewski et al. [51] using three different sets of samples (n = 6, concentration for pHLM samples 100 µM, concentration for blood samples low and high QC level, 15 und 400 ng/mL). MDMB-d$_5$ was used as an internal standard. Set A consisted of pHLM and blood samples which were spiked with the 5-MeO-MiPT reference standard prior to extraction. Set B samples were extracted blank samples spiked with a reference standard of 5-MeO-MiPT after extraction and set C consisted of neat 5-MeO-MiPT reference standard samples prepared in pure HPLC solvent. Measurements were performed on the LC-MS/MS as previously described for pHLM using a selected reaction monitoring (SRM) method with mass spectrometric parameters of 5-MeO-MiPT and MDMB-d$_5$ as described in Table 1. Chromatographic conditions for pHLM samples were identical as for the LC-HR-MS/MS analysis and chromatographic conditions for blood samples were the same as for the quantification of 5-MeO-MiPT. MS settings were the same as for the quantification of case samples. Calculations were based on the mean peak areas obtained for each set, using the following formulae: extraction efficiency = A/B x 100, matrix effect = B/C x 100 and recovery = A/C x 100.

Matrix effects for the urine sample were assessed by comparison of the 5-MeO-MiPT concentration determined by the external seven-point calibration method and a two-point standard addition method. The authentic urine sample was diluted 1:10 (v/v) with blank urine and aliquots thereof were spiked with 300 ng/mL and 600 ng/mL of 5-MeO-MiPT reference standard, respectively. The unspiked sample and the two spiked samples were measured in triplicates using the previously described SRM method. The mean peak area was plotted against concentration and the resulting three-point calibration line was extrapolated to zero response (y = 0). The absolute value of the x-intercept corresponds to the 5-MeO-MiPT concentration present in the tenfold diluted authentic urine sample [52].

Qualitative analysis of seized pills and powder samples

FTIR-UATR

A Fourier-transformation-infrared-spectrometer with a Universal-Attenuated-Total-Reflectance polarization accessory (FTIR-UATR) and Spectrum v2.00 software (Perkin Elmer, Schwerzenbach, Switzerland) was used for the identification of seized pills and powder. Scan range was from 4000 cm^{-1} to 400 cm^{-1} with a resolution of 4 cm^{-1}. Pills were homogenized for 30 seconds in a mortar and placed on the UATR for measurement. Powder samples were directly analyzed without any pre-treatment.

GC-MS

A Clarus 500 Gas-Chromatograph coupled to a Clarus 560S single quadrupole mass spectrometer with Turbochrom software was used (Perkin Elmer, Schwerzenbach, Switzerland). Separation was performed on a capillary 5% phenyl-methyl-polysiloxane column (Perkin Elmer, Schwerzenbach, Switzerland). GC-Interface temperature was set to 280 °C, the source temperature to 250 °C and the electron multiplier voltage to 500 V. MS scan range was from 35-450 amu. GC temperature gradient was the following: 0-3 min: 80 °C, 3-10 min: 15 °C/min to 280 °C and held for 20 min at 280 °C

resulting in a total run time of 20 min. Injection volume was 0.5 μL (splitless) and the carrier gas was helium with a flow rate of 1 mL/min and a split flow of 30 mL/min. Samples were dissolved in methanol with a concentration of 0.05 mg/mL, ultrasonicated and filtered prior to analysis.

NMR Spectroscopy

NMR data of powder sample 2 dissolved in $CDCl_3$ were acquired at room temperature using a Bruker Avance II spectrometer (Bruker BioSpin, Fällanden, Switzerland) operating at a resonance frequency of 400.13 and 100.62 MHz for ^1H- and ^{13}C-nuclei, respectively, and equipped with a 5 mm dual probe (BBI) for inverse detection with a z-gradient coil. The ^1H and ^{13}C NMR spectra were recorded using the standard zg30 and zgpg30 pulse program, respectively, from the Bruker pulse-program library. For the ^1H NMR spectrum a total of 64 transients were collected into 65,536 data points over a spectral width of 25 ppm with an acquisition time of 3.29 s, a relaxation delay of 6 s and a 90°-pulse width of 10 μs. For the ^{13}C NMR spectrum 2048 transients were acquired over a spectral width of 270 ppm, a data size of 65'536 points, an acquisition time of 1.21 s, a relaxation delay of 1 s and a 90°-pulse width of 9.5 μs. Acquisition and processing of both spectra was performed using the Bruker Topspin software version 3.5 and confirmed by calculated spectra (ACD/NMR Predictors software version 2016.1, Advanced Chemistry Development, Inc., Toronto, ON, Canada).
The co-added free induction decays (FIDs) were exponentially weighted with a line broadening factor of 1.0 Hz, Fourier-transformed, and phase- and baseline-corrected. Chemical shifts δ are reported in parts per million (ppm) relative to tetramethylsilane (0.00 ppm).

Results and Discussion

The data obtained were compared to negative and blank samples in the case of pHLM and to blank value blood and urine samples from volunteers. The problem of checking for precursors of metabolites and/or endogenous substances was overcome by comparing potential metabolites found in the case samples to blank blood and blank urine samples.

In total, nine different metabolites of the synthetic tryptamine 5-MeO-MiPT were detected and identified in the *in vitro* microsomal pHLM assay and in the authentic blood and urine samples by LC-HR-MS/MS. Table 2 lists all detected and identified metabolites plus the parent drug along with the molecular formulae and monoisotopic masses. Table 2 also indicates whether the metabolites were detected *in vitro*, in blood or in urine. Figure 1 presents the postulated metabolic pathway of 5-MeO-MiPT. Chromatograms of all three sample types are depicted in the supporting information Figures S1-S3. In all three sample types only phase I metabolites were detected. 5-Hydroxy-*N*-isopropyltryptamine (5-MeO-NiPT, *m/z* 233.1576), 5-hydroxy-*N*-methyl-*N*-isopropyltryptamine (5-OH-MiPT, *m/z* 233.1576), hydroxy-5-methoxy-*N*-methyl-*N*-isopropyltryptamine isomer 1 and 2 (OH-5-MeO-MiPT, *m/z* 263.1754) and 5-methoxy-*N*-methyl-*N*-isopropyltryptamine-*N*-oxide (5-MeO-MiPT-*N*-oxide, *m/z* 263.1754) had the highest L-HR-MS/MS signal intensities. The corresponding product ion mass spectra are presented in Figure 2 and 3.

Identification of metabolites

The fragmentation of the parent compound (precursor ion [M+H]$^+$ *m/z* 247.1805) yielded product ions of *m/z* 174.0918, *m/z* 131.0733, and *m/z* 86.0967 (Figure 2a). These product ions are formed by cleavage of the amine side-chain, simultaneous *O*-demethylation of the 5-methoxy substituent and β-cleavage releasing *N*-methyl-*N*-isopropyl-*N*-ethylamine and α-cleavage producing the iminium ion, respectively. Fragmentation of metabolites follows a similar pattern with *O*-demethylation, *N*-dealkylation and α-cleavage as predominant fragmentation patterns of metabolites. Mass spectra of the parent compound and detected metabolites contained a product ion of *m/z* 115.0545, suggesting an intact indole core structure. This in turn indicates that biotransformation takes place either at the 5-methoxy group or the side-chain. A metabolite product ion of *m/z* 86.0967 suggests that the *N*,*N*-dialkylated amino group remained unaltered during biotransformation, whereas the presence of a product ion of *m/z* 72.0812 indicates that *N*-demethylation has occurred. When a product ion of *m/z* 174.0918 is found in the mass spectrum of a metabolite, then no biotransformation took place on the 3-ethyl-5-methoxy-1*H*-indole moiety.

In vitro metabolism

Seven distinctive phase I metabolites were detected *in vitro* using microsomal pHLM experiments (see detailed list in Table 3). The parent compound 5-MeO-MiPT was identified with the in-house Designer Drug Library (HR-MS/MS) and the reference spectrum of a 5-MeO-MiPT standard. The metabolic steps observed were *N*-demethylation, *O*-demethylation, demethylation and hydroxylation and *N*-oxide formation and hydroxylation of the parent compound and *N*-O-bis-demethylation of the metabolite 5-OH-MiPT. The resulting metabolite 5-hydroxy-*N*-isopropyltryptamine (5-OH-NiPT, *m/z* 218.1420) was only detected *in vitro*. Simultaneous demethylation and hydroxylation of the parent compound produced the metabolite bishydroxy-*N*-methyl-*N*-isopropyltryptamine (Bishydroxy-MiPT, *m/z* 248.1525). The exact site of hydroxylation for the bishydroxy-MiPT metabolite could not be located. This metabolite was present *in vitro* and *in vivo* in urine.

In vivo metabolism

In blood four and in urine seven phase I metabolites were found (see detailed list in Table 4 and 5, respectively). The parent drug 5-MeO-MiPT was found in both, blood and urine. Hydroxylation of the parent compound formed two isomeric metabolites OH-5-MeO-MiPT. For these two isomers of OH-5-MeO-MiPT the site of hydroxylation could be narrowed down to the indole ring. 5-MeO-MiPT-*N*-oxide was formed by *N*-oxidation of the parent compound which is coherent with this metabolite eluting after the parent compound. *N*-oxides typically elute later on reversed-phase columns [53]. Loss of the *N*-methyl-*N*-isopropyl moiety by oxidative deamination and further oxidation of the intermediate aldehyde (5-MeO-indole-3-acetaldehyde, *m/z* 189.0790) to carboxylic acid led to the formation of the metabolite 5-MeO-indole-acetic acid (*m/z* 205.0793).

Studies on 5-MeO-DiPT [54] showed a very similar phase I metabolic pathway for this synthetic tryptamine. The three main routes identified were also *O*-demethylation of the 5-methoxy group, hydroxylation of the aromatic ring and side-chain *N*-dealkylation. In contrast to the present study, they found that phase II metabolites were formed by sulfonation and glucuronidation of the mainly formed metabolites. Shima *et al.*[45] identified three different metabolites (5-OH-MiPT, 5-MeO-NiPT,

6-OH-5-MeO-MiPT) in blood and urine, each specimen from a different individual after intoxication with 5-MeO-MiPT. Additionally we were able to detect and identify 5-MeO-MiPT-*N*-oxide as *in vivo* metabolites in human blood specimen. Further analysis of the urine sample led to the identification of four additional *in vivo* metabolites (compared to the study presented by Shima *et al* [45]). Fabregat-Safont *et al.* [46] recently reported on four different 5-MeO-MiPT metabolites in serum and urine samples of C57BLJ/6 male mice. Two phase I metabolites (5-OH-MiPT and 5-MeO-MiPT-*N*-oxide) and two phase II metabolites (glucuronides) were found. In comparison we were able to detect additional five phase I metabolites in human blood and urine but no phase II metabolites. The presence of two glucuronide metabolites in mice urine but absence of them in human urine is very likely due to interspecies differences- clearance of a free drug has been observed to be faster in smaller animals than in large species[55].

General-unknown screening results

Our routine GC-MS screening of the urine sample detected cocaine and its metabolites, cocaethylene, which is due to the combined consumption of cocaine and alcohol [56] and the cocaine adulterants levamisole, paracetamol, and phenacetin. Furthermore, chinine, nicotine and its metabolite cotinine and caffeine and its metabolites theophylline and theobromine as well as methylphenidate and ritalinic acid were found in the urine sample. The routine LC-HR-MS/MS screening method described earlier, detected in the urine sample cocaine and its metabolites, cocaethylene, methylphenidate and ritalinic acid and 5-MeO-MiPT. Additionally, methylphenidate and its metabolite ritalinic acid, with concentrations of 1.36 ng/mL and 64.1 ng/mL in blood and 671 ng/mL and 2230 ng/mL in urine, respectively, were quantified. In blood the cocaine metabolites benzoylecgonine and ecgonine methylester were found with concentrations of 305 ng/mL and 21 ng/mL and methylphenidate and ritalinic acid with concentrations of 2.6 ng/mL and 60 ng/mL, respectively. The absence of cocaine and the presence of methylphenidate in a subtherapeutic range [57] point towards an earlier consumption. Therefore the conations as well as the disoriented, agitated and aggressive state of the 32 year old man could be solely due to the consumption of 5-MeO-MiPT.

Evaluation of EAWAG-BBD Pathway Prediction System

The software was a helpful tool in the identification of phase I metabolites. Unfortunately it is not able to predict phase II metabolites. Therefore, glucuronides and sulfates needed to be predicted by the authors. Furthermore the prediction system produces chemical structures and likelihoods of possible metabolites. In order to use predicted metabolites for the evaluation of data it is necessary to calculate the molecular formula and monoisotopic mass of possible metabolites. This means the predictions cannot be used without additional effort. Nevertheless, the software was able to predict six of the found nine metabolites.

Quantification of 5-MeO-MiPT in whole blood and urine samples

Quantification of 5-MeO-MiPT in the authentic blood and urine sample was performed using a seven-point calibration (R= 0.994 and 0.998 respectively). In the blood sample a 5-MeO-MiPT concentration of 160 ng/mL was determined. The authentic urine sample exceeded the calibration range and hence was diluted with blank urine (1:10 v/v) and reanalyzed with newly prepared calibration standards and QC samples. The concentration of 5-MeO-MiPT in urine was 3380 ng/mL.

Evaluation of extraction efficiency, matrix effects and recovery

For pHLM samples extraction efficiency, matrix effects and recovery were determined at a concentration of 100 μM for both extraction methods (protein precipitation, liquid-liquid extraction). All values are presented in the supporting information in Table S1. Extraction efficiency, matrix effects and recovery were slightly higher for protein precipitation than for liquid-liquid extraction. Matrix effects were not significant for either extraction method. Furthermore, extraction efficiency, recovery and matrix effects were determined for the extraction of blood samples at concentrations of the QC1 (15 ng/mL) and QC3 (400 ng/mL) samples using the quantifier SRM transition for both analyte and ISTD. Results for the analyte were the following: extraction efficiency was 81 % and 86 %, matrix effects were 94 % and 91 % and recovery was 76 % and 78 % for QC1 and QC3, respectively. In comparison results for the ISTD were: extraction efficiency 72 % and 74 %, matrix effect 110 % and 103 %, and recovery 80 % and 77 % for QC1 and QC3, respectively. No significant matrix effects were observed.

Matrix effects for the urine sample were determined by comparison of the external seven-point calibration method, used for the quantification of 5-MeO-MiPT in urine, and a two-point standard addition method. For the external calibration a 5-MeO-MiPT concentration of 3380 ng/mL was determined and with the two-point standard addition method a concentration of 3604 ng/mL. Hence no relevant matrix effects were observed.

Qualitative analysis of seized pills and powder samples

Four seized samples were analyzed using FTIR, GC-MS and LC-HR-MS/MS. The FTIR analysis results showed that the pills (sample 1) consisted of saccharose. Powder samples 2 and 3 were identified as 5-MeO-MiPT by comparison with a reference standard (see supporting information Figure S5). The FTIR spectrum presented in Figure S5 displays sample 3 (purple, upper spectrum) in comparison with the reference material (5-MeO-MiPT·HCl, black, lower spectrum) with a correlation factor of 0.9848. The GC-MS results confirmed previous FTIR results using SWGDRUG MS Library Version 3.1 (November 29, 2016). Further, powder sample 4 was identified as α-PVP, which is a scheduled synthetic cathinone [58]. The chromatogram of sample 2, 3 of and the 5-MeO-MiPT reference standard showed a single peak at a retention time of 15.15 min. The mass spectrometric fragmentation was characteristic for 5-MeO-MiPT (Figure S3), library search match was 89.6%. Finally, 5-MeO-MiPT with the sum formula $C_{15}H_{22}N_2O$ and a calculated $[M+H]^+$ of m/z 247.1805, was found at m/z 247.1805 in the HR-MS spectrum.

NMR spectroscopy confirmed that the seized powder sample 2 was 5-MeO-MiPT hydrochloride. The ^{1}H NMR spectrum with assigned peaks is shown in Figure S7 and the ^{13}C NMR spectrum in Figure S8 (supporting information). Peak assignment was accomplished based on the observed chemical shift values, splitting patterns and integrated peak intensities and was confirmed by calculated chemical shift data (ACD/NMR Predictors software version 2016.1, Advanced Chemistry Development, Inc., Toronto, ON, Canada) as well as literature data [18]. Splitting of the ^{1}H NMR signal of the *N*-methyl group into a doublet is most likely due to coupling with the N^+H proton, as discussed for other amine hydrochloride salts [59-61]. Observed ^{1}H resonance multiplicities and ^{1}H-^{1}H coupling constants, as well as a comparison between measured and calculated ^{1}H and ^{13}C chemical shift values are given in Table S2 (supporting information).

Conclusion

In the present study, in total nine different metabolites of the synthetic tryptamine 5-MeO-MiPT could be identified, therefrom seven *in vitro,* and two additional *in vivo*. Results from human samples (*in vivo)* showed that in blood four different metabolites and in urine seven distinctive metabolites were present. The parent compound was detectable in blood and urine using LC-HR-MS/MS and was quantified using LC-MS/MS. 5-MeO-MiPT concentrations were 160 ng/mL in blood and 3380 ng/mL in urine. The five most abundant metabolites were formed by demethylation and hydroxylation of the parent compound. For the development of new methods for the detection of 5-MeO-MiPT consumption, the authors recommend 5-methoxy-*N*-isopropyltryptamine (5-MeO-NiPT), 5-hydroxy-*N*-methyl-*N*-isopropyltryptamine (5-OH-MiPT), 5-methoxy-*N*-methyl-*N*-isopropyltryptamine-*N*-oxide (5-MeO-MiPT-*N*-oxide) and hydroxy-5-methoxy-*N*-methyl-*N*-isopropyltryptamine (OH-5-MeO-MiPT) as biomarkers, as they have been present in both blood and urine samples.

Future Perspective

Performing *in vitro* experiments generating phase II metabolites and further kinetic and inhibitory *in vitro* studies to determine the responsible cytochrome P450 (CYP) enzymes appears desirable at this point. In addition, other approaches for generating metabolites such as fungal *in vitro* assays could be cross validated using gained results.

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Tables

Tables 1: Optimized MS/MS parameters for selected reaction monitoring (SRM) experiments.

	Q1 [Da]	Q3 [Da]	DP [V]	EP [V]	CEP [eV]	CE [V]	CXP [V]
Qualifier	247	174.1	37	5	16	27	3
Quantifier	247	159.2	37	5	16	38	3
ISTD	213	136,1	40	4	16	20	4

Q1 *m/z* of the precursor ion
Q3 *m/z* of the fragment ion
DP declustering potential
EP entrance potential
CE collision energy
CXP collision cell exit potential

Table 2: Identified *in vivo* and *in vitro* metabolites of 5-MeO-MiPT in the order of their monoisotopic mass. The presence of a metabolite *in vitro*, *in vivo* (blood) or *in vivo* (urine) is denoted with **+**, the absence with a **-**.

Compound Name	Formula	Mass [Da]	*In vitro* (pHLM)	*In vivo* (urine)	*In vivo* (blood)
5-MeO-indole-3-acetaldehyde	$C_{11}H_{11}NO_2$	189.0790	-	+	-
5-MeO-indole-acetic-acid	$C_{11}H_{11}NO_3$	205.0739	-	+	-
5-OH-NiPT	$C_{13}H_{18}N_2O$	218.1419	+	-	-
5-MeO-NiPT	$C_{14}H_{20}N_2O$	232.1576	+	+	+
5-OH-MiPT	$C_{14}H_{20}N_2O$	232.1576	+	+	+
5-MeO-MiPT (parent drug)	$C_{15}H_{22}N_2O$	246.1732	+	+	+
Bishydroxy-MiPT	$C_{14}H_{20}N_2O_2$	248.1525	+	+	-
OH-5-MeO-MiPT isomer 1	$C_{15}H_{22}N_2O_2$	262.1681	+	+	+
5-MeO-MiPT-*N*-oxide	$C_{15}H_{22}N_2O_2$	262.1681	+	+	+
OH-5-MeO-MiPT isomer 2	$C_{15}H_{22}N_2O_2$	262.1681	+	-	-

Table 3: 5-MeO-MiPT and identified *in vitro* metabolites of 5-MeO-MiPT after 120 minutes incubation with pHLM in the order of the protonated molecule [M+H]$^+$ [*m/z*]. A 34 minute chromatographic method has been applied. The three most abundant fragments are listed with their relative percentage of the base peak as an index number. *: in source fragmentation;

Compound Name	Biotransformation	Formula	[M+H]$^+$ [*m/z*]	Found at Mass [*m/z*]	Error [ppm]	RT [min]	Intensity [cps]	Fragment ions
5-OH-NiPT	*N*,O-bis Demethylation	$C_{13}H_{18}N_2O$	219.1492	219.1491	-0.5	5.09	1078	*210.1483$_{100}$ *195.1223$_{50}$ *177.0542$_{35}$
5-MeO-NiPT	*N*-Demethylation	$C_{14}H_{20}N_2O$	233.1648	233.1649	0.1	10.45	22855	174.0918$_{100}$ 159.0683$_{47}$ 130.0655$_{22}$
5-OH-MiPT	O-Demethylation	$C_{14}H_{20}N_2O$	233.1648	233.1651	0.9	5.68	42457	160.0758$_{100}$ 115.0806$_{17}$ 86.0964$_{56}$
5 MeO-MiPT	Parent drug	$C_{15}H_{22}N_2O$	247.1805	247.1808	1.4	10.83	299435	174.0918$_{100}$ 159.0682$_{53}$ 86.0967$_{60}$
Bishydroxy-MiPT	Demethylation and Hydroxylation	$C_{14}H_{20}N_2O_2$	249.1597	249.1599	0.6	7.59	2995	*249.1606$_{100}$ *195.1753$_{15}$ *160.0759$_{45}$

OH-5-MeO-MiPT Isomer 2	Hydroxylation	$C_{15}H_{22}N_2O_2$	263.1754	263.1753	-0.2	6.97	8782	190.0880 [17] 158.0602 [8] 86.0963 [100]
OH-5-MeO-MiPT Isomer 1	Hydroxylation	$C_{15}H_{22}N_2O_2$	263.1754	263.1756	0.7	8.46	5469	190.0872 [100] 162.0555 [35] 147.0685 [25]
5-MeO-MiPT-N-Oxide	N-Oxidation	$C_{15}H_{22}N_2O_2$	263.1754	263.1752	-0.6	12.72	4105	174.0921 [100] 159.0679 [20] 131.0732 [10]

Table 4: 5-MeO-MiPT and identified *in vivo* metabolites of 5-MeO-MiPT in urine, ordered according to the protonated molecule [M+H]$^+$ [*m/z*]. A 14 minute chromatographic method has been applied. The last four listed substances in grey are due to the consumption of cocaine and methylphenidate and are listed for completeness. For 5-MeO-MiPT metabolites the three most abundant fragments are listed with the relative percentage to the base peak as index number.

Compound Name	Biotransformation	Formula	[M+H]$^+$ [*m/z*]	Found at Mass [*m/z*]	Error [ppm]	RT [min]	Intensity [cps]	Fragment ions
5-MeO-indole-3-acetaldehyde	Oxidative deamination	$C_{11}H_{11}NO_2$	190.0863	190.08630	0.2	3.1	1969	172.0362_{12} 144.0444_{100} 116.0485_{15}
5-MeO-indole-acetic-acid	Oxidative deamination followed by oxidation to carboxylic acid	$C_{11}H_{11}NO_3$	206.0812	206.0810	3.0	2.72	6696	131.0501_{100} 103.0543_{50} 77.0379_{20}
5-MeO-NiPT	*N*-Demethylation	$C_{14}H_{20}N_2O$	233.1648	233.1650	0.6	3.61	40425	174.0907_{100} 159.0671_{47} 131.0728_{18}
5-OH-MiPT	O-Demethylation	$C_{14}H_{20}N_2O$	233.1648	233.1600	0.2	2.62	9438	160.0865_{100} 132.0813_{13} 86.0968_{42}
5-MeO-MiPT	Parent drug	$C_{15}H_{22}N_2O$	247.1805	247.1804	-0.3	3.68	118810	174.0921_{100} 159.0684_{25} 86.0970_{58}
Bishydroxy-MiPT	Demethylation, Hydroxylation	$C_{14}H_{20}N_2O_2$	249.1597	249.1599	0.7	3.16	4057	226.1080_{40} 160.0748_{90} 130.0494_{100}
5-MeO-MiPT-*N*-oxide	*N*-oxide formation	$C_{15}H_{22}N_2O_2$	263.1754	263.1756	0.6	3.87	22010	174.0915_{100} 159.0678_{23} 131.0725_{11}
OH-5-MeO-	Hydroxylatio	$C_{15}H_{22}$	263.175	263.1754	-0.1	3.04	2529	190.08

MiPT	n	N_2O_2	4					83_{18} 178.0447_{25} 86.0957_{100}
Methylphenidate	Parent	$C_{14}H_{19}NO_2$	234.1489	234.1491	0.9	3.75	64070	
Ritalinic acid	Demethylation	$C_{13}H_{17}NO_2$	219.1259	220.1330	-1	3.45	74145	
Cocaine	Parent	$C_{17}H_{21}NO4$	303.1471	304.15430	-0.2	3.93	102407	
Benzoylecgonine	Demethylation	$C_{16}H_{19}NO_4$	289.1314	290.1384	-0.9	3.54	254561	
Cocaethylen	Methylation	$C_{18}H_{23}NO_4$	317.1627	318.1701	0.5	4.21	117211	

Table 5: 5-MeO-MiPT and identified *in vivo* metabolites of 5-MeO-MiPT in blood, ordered according the protonated molecule [M+H]$^+$ (*m/z*). A 14 minute chromatographic method has been applied. The three most abundant fragments are listed with their relative percentage to the base peak as an index number. NA: no MS/MS experiment triggered;

Name	Biotransformation	Formula	[M+H]$^+$ [*m/z*]	Found at Mass [*m/z*]	Errror [ppm]	RT [min]	Intensity [cps]	Fragment ions
5-MeO-NiPT	*N*-Demethylation	$C_{14}H_{20}N_2O$	233.1648	233.1646	-0.8	3.66	29490	174.0915$_{100}$ 159.0686$_{32}$ 130.0649$_{39}$
5-OH-MiPT	O-Demethylation	$C_{14}H_{20}N_2O$	233.1648	233.1645	-1.6	2.62	61561	NA
5-MeO-MiPT	Parent drug	$C_{15}H_{22}N_2O$	247.1805	247.1807	0.7	3.73	210937	174.0909$_{100}$ 159.0668$_{40}$ 86.0959$_{75}$
5-MeO-MiPT-*N*-oxide	*N*-oxide formation	$C_{15}H_{22}N_2O_2$	263.1754	263.1752	-0.6	3.92	11788	174.0910$_{100}$ 159.0666$_{36}$ 130.0654$_{22}$
OH-5-MeO-MiPT	Hydroxylation	$C_{15}H_{22}N_2O_2$	263.1754	263.1750	-1.6	3.12	1257	130.0490$_{100}$ 91.0567$_{62}$ 84.0453$_{65}$

1) **5-MeO-NIPT**
232.1576
$C_{14}H_{20}N_2O$

1) **5-OH-MiPT**
232.1576
$C_{14}H_{20}N_2O$

2) **5-OH-NIPT**
218.1420
$C_{13}H_{18}N_2O$

3) **5-MeO-indol-3-acetaldehyde**
189.0790
$C_{11}H_{11}NO_2$

3) **5-MeO-indol-acetic acid**
205.0739
$C_{11}H_{11}NO_3$

5-MeO-MiPT
246.1732
$C_{15}H_{22}N_2O$

4) **Bishydroxy-MiPT**
248.1525
$C_{14}H_{20}N_2O_2$

1) **5-MeO-MiPT-*N*-Oxide**
262.1681
$C_{15}H_{22}N_2O_2$

1) **OH-5-MeO-MiPT**
262.1681
$C_{15}H_{22}N_2O_2$

2) **OH-5-MeO-MiPT**
262.1681
$C_{15}H_{22}N_2O_2$
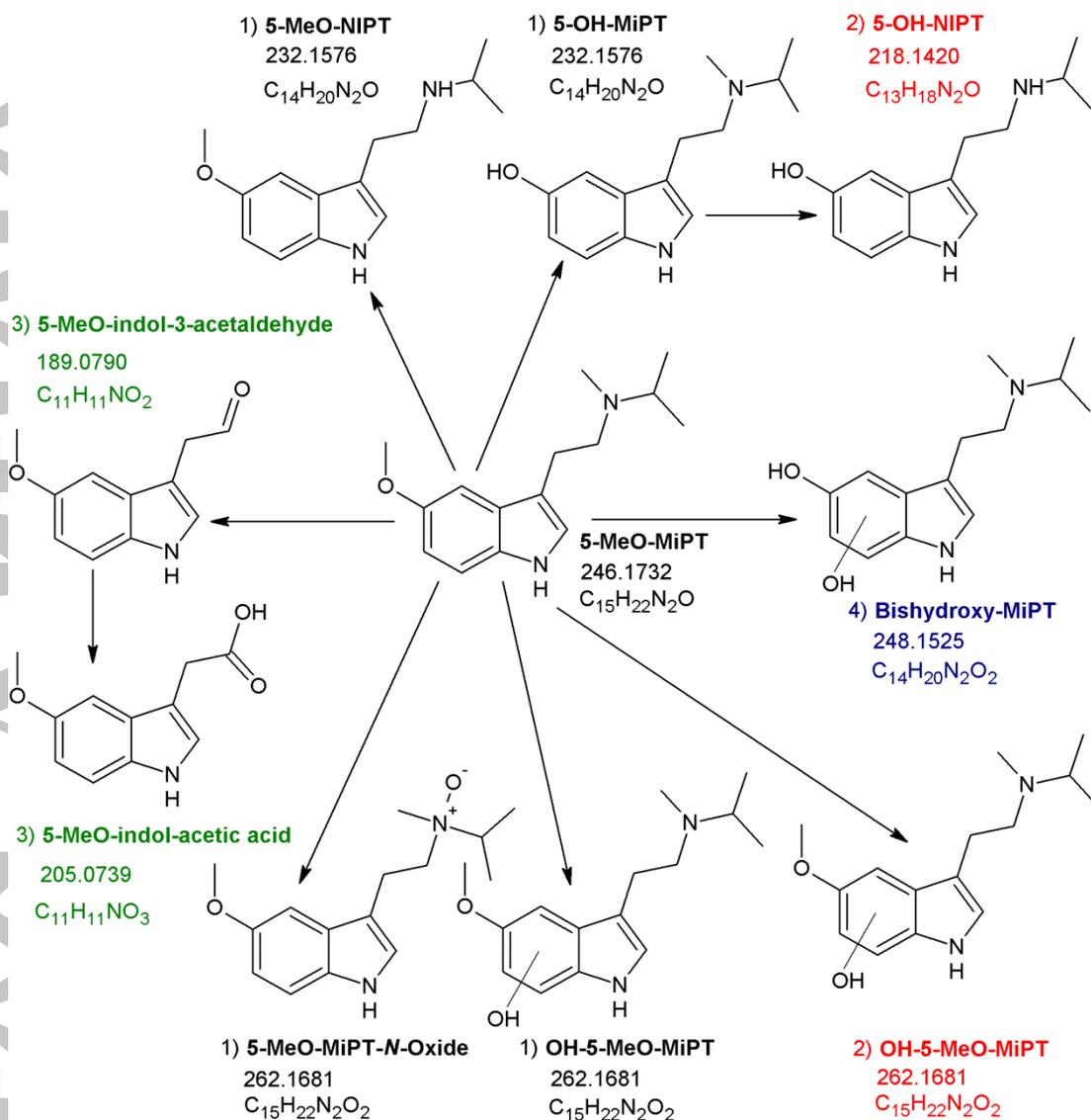
Figure 1 Metabolic pathway of 5-MeO-MiPT, metabolites labelled in black (1) were found in all three sample types, red (2) only in vitro, green (3) in vitro- urine and blue (4) in both in vitro and in vivo-urine.
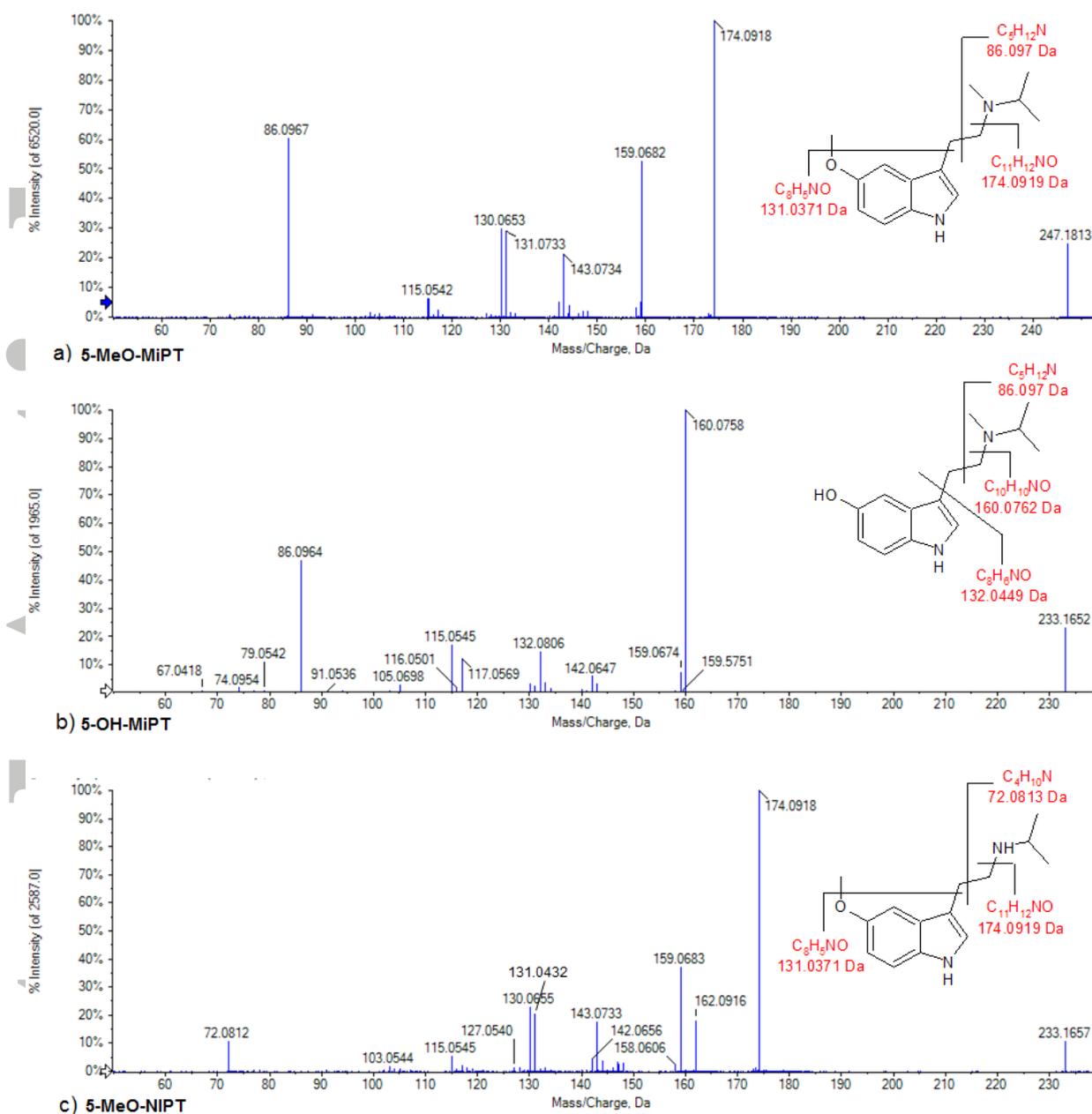
Figure 2 Product ion mass spectra of 5-MeO-MiPT and two of its metabolites 5-OH-MiPT and 5-MeO-NiPT found in vitro by IDA experiments. Fragmentation was selected for the ten most abundant precursor ions with CE of 35 eV with a CES of ± 15 eV.
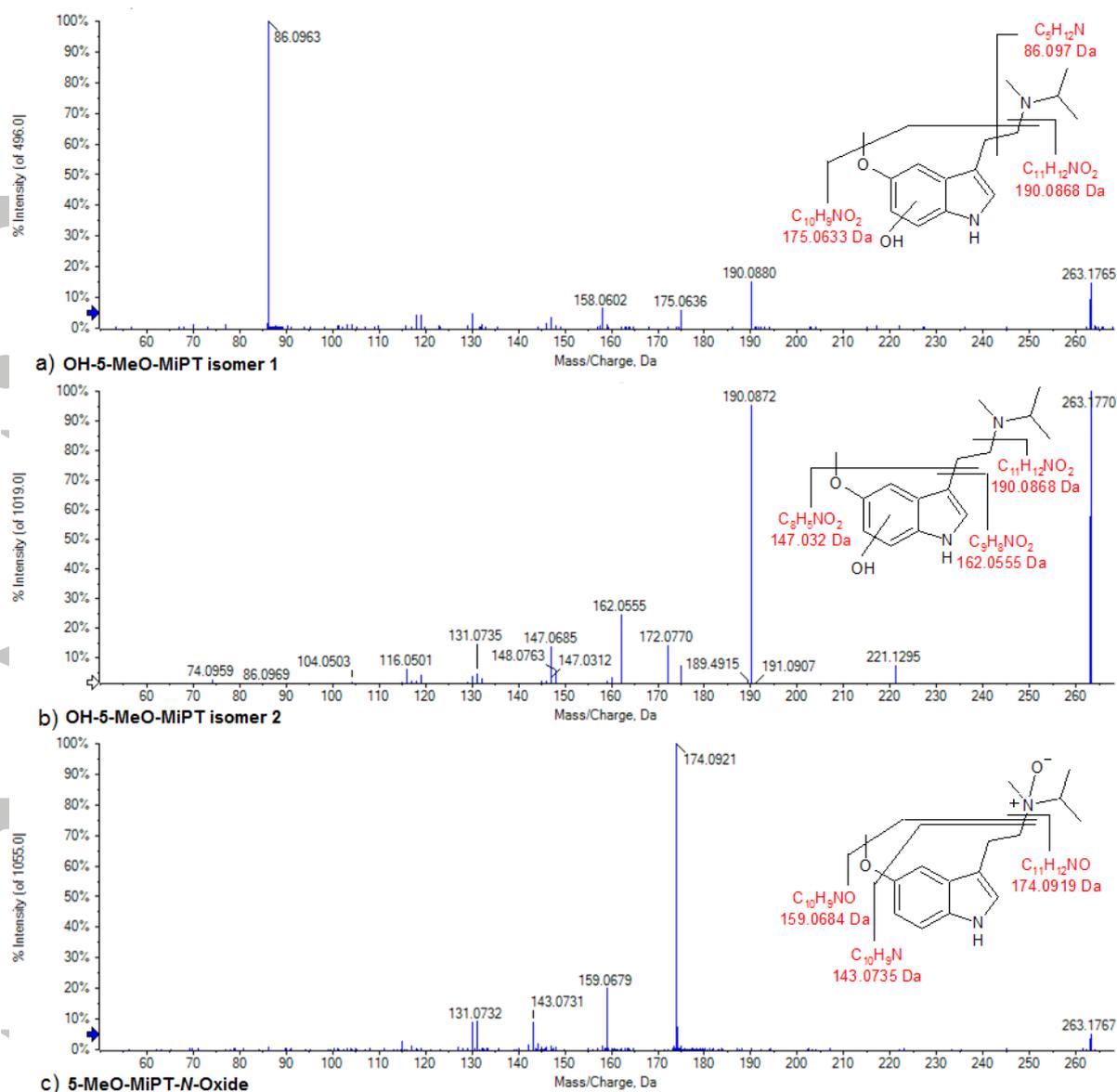
Figure 3 Product ion mass spectra of three metabolites with m/z 263.1754, 5-MeO-MiPT-*N*-Oxide and OH-5-MeO-MiPT isomer 1 and 2. All three metabolites were found in vitro and in vivo in blood but only 5-MeO-MiPT-*N*-Oxide and one isomer of OH-5-MeO-MiPT were found in vivo in urine. Fragmentation was selected for the ten most abundant precursor ions with CE of 35 eV with a CES of ± 15 eV.