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In vitro, *in vivo* and *in silico* metabolic profiling of α -pyrrolidinopentiothiophenone, a novel thiophene stimulant

Background: Little or no pharmacological or toxicological data are available for novel psychoactive substances when they first emerge, making their identification and interpretation in biological matrices challenging. **Materials & methods:** A new synthetic cathinone, α -pyrrolidinopentiothiophenone (α -PVT), was incubated with hepatocytes and samples were analyzed using liquid chromatography coupled to a Q Exactive™ Orbitrap mass spectrometer. Authentic urine specimens from suspected α -PVT cases were also analyzed. Scans were data mined with Compound Discoverer™ for identification and structural elucidation of metabolites. **Results/conclusion:** Seven α -PVT metabolites were identified in hepatocyte incubations, and in the authentic urine samples, also with an additional monohydroxylated product and a glucuronide of low intensity. α -PVT dihydroxypyrrolidinyl, α -PVT 2-ketopyrrolidinyl, α -PVT hydroxythiophenyl and α -PVT thiophenol had the most intense *in vivo* signals.

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Novel psychoactive substances (NPS) are constantly emerging onto the illicit drug market, making it difficult to identify parent and/or metabolites of these substances as little pharmacological or toxicological data are available. The European Union's Early Warning System (EWS) identified 81 NPS in 2013 and 101 in 2014, bringing the total compounds monitored to over 450 [1]. The term NPS includes a wide range of substances, including synthetic cannabinoids, phenethylamines, cathinones, piperazines, ketamine, tryptamines and other plant-based psychoactive substances (i.e., Kratom, *Salvia divinorum* Epling & Játiva) [2]. Synthetic cathinone abuse may produce severe side effects such as tachycardia, hyperthermia, delusions and violent behavior [2–7]. In 2011 in USA, mephedrone (4-methylmethcathinone), methylone (3,4-methylenedioxymethcathinone) and 3,4-methylenedioxypyrovalerone

(MDPV) were classified as schedule I controlled substances [8]. Ten additional cathinones were added, including 4-methyl-*N*-ethylcathinone, α -pyrrolidinobutiothiophenone (α -PBP) and α -pyrrolidinovalerophenone (α -PVP).

In mid-2012, reports of a new synthetic cathinone called α -pyrrolidinopentiothiophenone (α -PVT) appeared on several online drug forums. Later, α -PVT or 2-(pyrrolidin-1-yl)-1-(thiophen-2-yl)pentan-1-one was identified in illegal products purchased in Japan in 2013 [9] and in Germany in 2014 [10]. α -PVT is a substituted cathinone with structural similarities to schedule I controlled substances MDPV and α -PVP, except the benzene ring is a thiophene ring (Figure 1). The only available reports of α -PVT's effects are described on internet drug forums (e.g., bluelight.org, drugs-forum.com, reddit.com or chemsrus.com). The accounts often document the dose

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Figure 1. Structure of α -pyrrolidinopentiothiophenone and structurally related schedule I controlled substances.

(10–100 mg), route (insufflation or oral), duration (up to 6 h) and side effects (positive: stimulation; negative: psychosis) experienced. However, self-reports are often unreliable as true compound identity is unknown.

As with many NPS, PD and PK profiles are non-existent and controlled administrations or clinical trials are lacking, making identification and interpretation of toxicological findings difficult [11]. Between 2013 and April 2014, risk assessments were only performed on six NPS from the EWS compound list [12]. Pharmacologically, synthetic cathinones interact with monoamine transporters for dopamine (DAT), norepinephrine (NET) and serotonin [13–16]. Mephedrone and methylone increase extracellular dopamine and serotonin similar to 3,4-methylenedioxymethamphetamine [13]. MDPV is a potent DAT/NET transporter blocker, similar to cocaine [14,15]. α -PVP, α -PBP and α -pyrrolidinopropiophenone (α -PPP), three pyrrolidinophenones recently examined by Marusich *et al.* were determined to be DAT/NET transporter blockers with *in vivo* potency increasing with aliphatic chain length (α -PVP > α -PBP > α -PPP) [15]. Due to structural similarity to MDPV and α -PVP, it could be hypothesized that α -PVT would exhibit similar *in vitro* and *in vivo* pharmacological effects. If this is the case, α -PVT could cause severe side effects and pose risks for abuse and addiction. Characterizing NPS metabolic pathways is imperative for PK profiling and understanding PD effects. The metabolic pathways of other α -pyrrolidinophenones, including MDPV, α -PVP, α -PBP, α -PPP, 4-methyl- α -pyrrolidinophenone, 4-methoxy- α -pyrrolidinophenone and 4-methyl- α -pyrrolidinohexiophenone were previously investigated in human liver microsomes (HLM) and rat urine [17–27]. In a recent review of pyrrolidinophenones' pharmacology, Zaitsev *et al.* summarized the main metabolites to include ketone reduction to the corresponding alcohol and oxidation to the 2'-oxo metabolites [28]. In 2014, Takayama *et al.* characterized α -PVT's *in vitro* metabolism with HLM [29] and identified only three metabolites generated by hydroxylations on the pyrrolidine moiety, pyrrolidinoalkyl

moiety and the thiophene ring. *In silico*, *in vitro* hepatocytes and *in vivo* metabolic studies for α -PVT are not available.

As a step toward NPS public education and awareness, complete metabolic profiles are necessary to identify unique metabolites as markers of intake and to link adverse effects to the causative agent. In order to generate the most comprehensive metabolic profile of a substance, *in vitro* human hepatocyte incubations, followed by analysis with liquid chromatography coupled to high-resolution MS (LC–HRMS) and software-assisted data mining were proven to be a promising approach [30–32]. Human hepatocytes offer a more complete metabolic profile than HLM since hepatocytes contain all hepatic enzymes involved in drug metabolism (both phase I and II transformations) as well as endogenous cofactors, provide the natural orientation of membrane enzymes and produce metabolites in concentrations similar to *in vivo* [33]. Analysis by HRMS is becoming more common in metabolic profiling assays and offers the ability to identify expected and unexpected metabolites with exact-mass capabilities. The incorporation of data-mining software decreases data analysis time and offers structural elucidation for a variety of HRMS data file types [34]. *In silico* prediction can assist in metabolite identification by offering accurate mass, fragmentation patterns and distribution constants ($\log D$). Our objectives were to compare *in silico* metabolism predictions to HLM and human hepatocyte incubations analyzed by HRMS with software-assisted data mining for the identification and structural elucidation of α -PVT metabolites. Authentic urine specimens from α -PVT users also were analyzed for α -PVT and metabolites to confirm the metabolites identified in the *in vitro* samples.

Materials & methods

Chemicals & reagents

α -PVT HCl in powder form (5 mg) was obtained from Cayman Chemicals (Ann Arbor, MI, USA) and diclofenac (10 g) from Toronto Research Chemicals (Toronto, Canada). LC–MS grade water, acetonitrile (LC–MS grade) formic acid (LC–MS grade), LC grade methanol, glacial acetic acid, hydrochloric acid and ammonium hydroxide were acquired from Fisher Scientific (Fair Lawn, NJ, USA). Methylene chloride (dichloromethane) was purchased from J.T. Baker (Phillipsburg, NJ, USA). Dibasic and monobasic sodium phosphate and 2-propanol (LC–MS grade) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Water was purified in-house with an ELGA Purelab® Ultra Analytic purifier (Siemens Water Technologies, Lowell, MA, USA). Pooled microsomes ($n = 50$), pooled cryopreserved human hepatocytes ($n = 10$), *In Vitro*–

GRO HT and *InVitro*GRO Krebs–Henseleit buffer were obtained from BioReclamationIVT (Westbury, NY, USA). Solutions A and B (NADPH-regeneration system and Glu-6-phosphate dehydrogenase, respectively) were purchased from BD Biosciences (San Jose, CA, USA). Water bath incubations were performed on a Precision reciprocal shaking bath (Winchester, VA, USA) and hepatocytes were incubated in a FormaTM Steri-CycleTM CO₂ incubator from Thermo Scientific.

In silico metabolite prediction

The structure of α -PVT was imported into MetaSite software (v. 5.0.3; Molecular Discovery, Pinner, UK) and predictions were carried out using the cytochrome P450 (CYP450) liver model, reactivity correction and 39 CYP common biotransformations, including hydroxylation, dealkylation, carbonylation and ketone reduction. The CYP450 liver model provides a consensus of the sites of metabolism predictions from the three major liver isoforms CYP3A4, CYP2D6 and CYP2C9 and flavin containing monooxygenase 3 (FMO3). The computational procedure considers both thermodynamic and kinetic factors by simulating 3D docking of the substrate in the cavity of the CYP proteins. The software provides exact structures for the predicted metabolites, lists the corresponding biotransformation and monoisotopic mass and calculates log D values at pH 4, 7 and 9 as well as a probability score. Compounds with a probability score of 100% were imported into the software to generate a list of second-generation metabolites. Only metabolites >50 Da were considered in the final summary.

Additionally, the *in silico* prediction software generated a list of accurate mass m/z (inclusion list) for first-, second-, and third-generation predicted metabolites. This list was incorporated into the data-dependent MS² (ddMS²) acquisition method operating in positive ESI, but identification of metabolites was not limited to this list. When a mass spectral peak on the inclusion list was detected, an MS/MS spectrum was automatically acquired. This list included 39 CYP and 40 non-CYP-mediated reactions, including oxidation, hydroxylation, carbonylation and glucuronidation. No structure, corresponding biotransformation, log D or prediction score was provided for the predicted m/z values.

Metabolic stability assessment with human liver microsomes

To determine α -PVT metabolism kinetics, 1 μ mol/l drug was incubated with 50-donor-pooled HLM. The reaction mixture consisted of 780 μ l water, 100 μ l 0.5 M potassium phosphate buffer pH 7.4, 10 μ l solution B and 10 μ l α -PVT (100 μ mol/l). After vortexing, 50 μ l HLM (20 mg/ml suspension) was added to the mix-

ture and preincubated for 3 min at 37°C. The reaction was initiated by the addition of 50 μ l solution A. Samples (100 μ l) were collected after 0, 3, 8, 13, 20, 30, 45 and 60 min and the reaction was stopped with an equal volume of ice-cold acetonitrile. The samples were centrifuged at 15,000 g for 5 min at 4°C and supernatant stored at -80°C until analysis. After thawing, HLM samples were diluted 100-fold with mobile phase A (0.1% formic acid in water) before injection (10 μ l) onto the LC–HRMS. The experiment was prepared in duplicate and injected twice, allowing us to examine reproducibility.

Metabolite profiling in human hepatocytes

For metabolic profiling, 10 μ mol/l α -PVT was incubated at 37°C with pooled cryopreserved human hepatocytes. Hepatocytes were washed with *InVitro*GRO HT medium to remove dead cells by centrifugation at 50 g for 5 min at room temperature. Supernatant was aspirated and *InVitro*GRO Krebs–Henseleit buffer was added to the hepatocytes again. After centrifugation and supernatant removal, the cell pellet was resuspended in 2 ml buffer. Cell viability was assessed with Trypan blue exclusion method assuring >80% viability. The reaction mixture consisted of 250 μ l α -PVT in buffer (20 μ mol/l) and 250 μ l cell suspension (2×10^6 cells/ml), yielding a final 10 μ mol/l drug concentration. Samples (500 μ l) were collected after 0, 30 and 120 min according to the HLM half-life derived from the previous HLM substrate depletion experiment. An equal volume of ice-cold acetonitrile was added to the samples immediately to stop the reaction. Diclofenac was incubated in the same manner as α -PVT as a positive control and evaluated for 4'-hydroxydiclofenac and diclofenac acyl glucuronide metabolites to ensure metabolic viability under our experimental conditions. A negative control (parent drug in buffer without hepatocytes) was incubated to rule out nonenzymatic transformation of α -PVT. Samples were stored at -80°C until analysis. After thawing, hepatocyte samples were centrifuged at 15,000 g at 4°C for 10 min to remove cell debris and supernatants diluted 1:5 with mobile phase A before injection (10 μ l) onto the LC–HRMS.

LC–HRMS instrumentation

LC–HRMS was performed on a Thermo Scientific UltimateTM 3000 RSLCnano system coupled to a Thermo Scientific Q ExactiveTM mass spectrometer (Thermo Scientific, Fremont, CA, USA). The UltimateTM 3000 RSLCnano system consisted of a degasser, a tertiary loading pump, a binary eluting pump, a column oven and an RS Autosampler. The Q ExactiveTM was equipped with heated electrospray

ionization source (HESI-II) and operated in positive ionization mode. The spray voltage was 3 kV, capillary temperature 350°C, heater temperature 425°C, S-lens RF level 50, sheath gas flow rate 50, auxiliary gas flow rate 13 and sweep gas 3 (manufacturer's units). Nitrogen was used for spray stabilization, for collision-induced dissociation experiments in the higher-energy collisional dissociation cell, and as the damping gas in the C-trap. The instrument was calibrated in the positive and negative mode every 25 h.

LC–HRMS for HLM incubations

HLM samples were separated on an Accucore™ C₁₈ 100 × 2.1 mm, 2.6 μm column (Thermo Scientific, Fremont, CA, USA) and identically packed defender guard cartridges (10 × 2.1 mm, 2.6 μm) in a thermostatted column oven at 35°C. Gradient elution was performed with mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) at a 0.4 ml/min flow rate. The initial composition (2% B) was maintained for 2 min, then increased from 2 to 10% B over 8 min, increased from 10 to 30% B over 4 min, increased from 30 to 95% B over 2 min, held at 95% for 1 min, returned to initial conditions over 1 min with a 2 min equilibration, yielding a total run time of 20 min. LC conditions and ionization parameters for HLM incubations were the same as our in-house validated stimulant method in urine [35] as the method has proven adequate to detect α-PVT.

The mass spectrometer acquired data using a high-resolution full-scan, data-dependent MS method. The MS acquired full scan MS spectra at a resolution of 35,000 (full width at half-maximum at m/z 200) from m/z 100 to 600. The resolution was not maximal in order to achieve a sufficient number of scan events for each chromatographic peak. Data were acquired in full-scan MS/ddMS² mode: in each cycle, a full MS survey scan was acquired, followed by up to five MS² events. When an ion was detected with an intensity above 8.3×10^4 (5% underfill ratio) in the survey scan, an MS² event was triggered at the peak apex (3–8 s) and the ion was fragmented (isolation window m/z 3). Up to five ions with the most intense signal were fragmented. A 10 s delay was required for the same ion to trigger a new MS² event to provide the opportunity for ions with the same RT and a lower intensity to be fragmented (dynamic exclusion). ddMS² spectra were acquired at a resolution of 17,500 with a stepped normalized collision energy of $50 \pm 30\%$ to produce a high number of fragments.

LC–HRMS for hepatocyte incubations

In order to achieve better separation of metabolites, hepatocyte samples were separated on a Synergi™

Hydro-RP (150 × 2 mm, 4 μm) column (Phenomenex, Torrance, CA, USA) and a C₁₈ end-capped guard in a thermostatted column oven at 30°C. Gradient elution was performed with mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) at a 0.4 ml/min flow rate. The initial composition (2% B) was maintained for 2 min, then increased from 2 to 95% B over 18 min, held at 95% B for 5 min, returned to initial conditions over 1 min with a 4 min equilibration, yielding a total run time of 30 min. For hepatocyte incubations, only one linear gradient was employed, with a longer column and a longer run time than HLM LC conditions to best separate the analytes while preserving good chromatography efficiency, as the aim of the experiment was to detect α-PVT and its metabolites. As we expected to detect a number of compounds with the same accurate mass and similar physical and chemical properties (close retention times), a longer gradient was necessary to provide chromatographic separation. In addition, MetaSite predicted metabolites that may elute after the parent compound, so the longer gradient allowed us to capture those compounds as well.

Hepatocyte samples were analyzed on the Q Exactive™ using two acquisition methods employing full scan and ddMS² with and without precursors' inclusion list. Full scan and ddMS² (without an inclusion list) were acquired as previously described for HLM samples. For full scan and ddMS² with an inclusion list, ddMS² scans were triggered if the precursor ions from the inclusion list were detected above 8.3×10^4 intensity threshold. ddMS² mode without an inclusion list was thus more suitable for unexpected metabolites with high signal intensity, while ddMS² mode with an inclusion list was more suitable for expected metabolites with a low to high signal intensity.

Calculations

In vitro microsomal half-life ($T_{1/2}$) was calculated in Excel by plotting the natural logarithm of % parent compound signal (area) remaining versus time. *In vitro* microsomal intrinsic clearance ($CL_{int, micr}$), intrinsic clearance (CL_{int}), predicted human hepatic clearance (CL_{hep}) and extraction ratio (ER) were calculated in Excel based on the models described in Baranczewski *et al.* [36] and McNaney *et al.* [37], without consideration of plasma protein binding.

Metabolite identification

Raw data files from the hepatocyte incubations (0, 0.5, 2 h in full scan-ddMS² mode with and without an inclusion list) were imported into Compound Discoverer™ software (v. 1.0; Thermo Scientific, Fremont, CA, USA) to identify α-PVT metabolites. The soft-

Table 1. Compound discoverer settings: chromatographic peaks are detected and the mass of the corresponding compound is compared with a list of generated theoretical metabolites; a FISH score is then assigned to determine the percentage of match between theoretical and experimental MS² spectra.

Compound discoverer settings	
Compound generator	
Parent compound	α -PVT (C ₁₃ H ₁₉ NOS)
Phase I transformations	Dehydration, desaturation, hydration, nitro reduction, oxidation, oxidative deamination to alcohol, oxidative deamination to ketone, oxidative debromination, oxidative dechlorination, oxidative defluorination, reduction, reductive debromination, reductive dechlorination, reductive defluorination, thiourea to urea
Phase II transformations	Acetylation, arginine conjugation, glucoside conjugation, glucuronide conjugation, glutamine conjugation, glycine conjugation, GSH conjugation (on bromine), GSH conjugation (on chlorine), GSH conjugation (on fluorine), GSH conjugation 1, GSH conjugation 2, methylation, ornithine conjugation, palmitoyl conjugation, stearyl conjugation, sulfation, taurine conjugation
Maximal number of Phase II reactions	1
Maximal number of reactions	3
Ionization	[M + H] ⁺ , [M + K] ⁺ , [M + Na] ⁺
Expected finder	
Mass tolerance	10 ppm
Intensity tolerance (isotope search)	30%
Minimum peak intensity	200,000
FISH scoring	
Signal/noise ratio threshold	3
Mass tolerance	2.5 mDa
Use libraries	True

α -PVT: α -pyrrolidinopentiothiophenone.

ware detects chromatographic peaks and the mass of the corresponding compound is compared with a list of generated theoretical metabolites (processing settings are summarized in Table 1). A fragment ion search (FISH) score was then assigned to determine the percentage match between theoretical and experimental fragmentation spectra (FISH coverage score = $[\sum_{\text{per all scans}} \text{number of matched fragments}] / [\sum_{\text{per all scans}} \text{number of centroids in the fragmentation scan that are above the signal/noise threshold}] \times 100$). The apex triggering and the dynamic exclusion selected for the ddMS² analysis limit the production of MS² scans of low intensity that can affect the FISH score.

Potential metabolites were considered based on exact mass, MS² fragmentation, isotopic pattern and retention time with respect to the parent compound (as determined by logD from *in silico* predictions). Relative

abundance was considered for ranking and determining the most prominent metabolites. Potential metabolites were further compared with literature reports of structurally similar compounds and *in silico* predictions. Compounds with signal intensity <0.2% parent were judged insignificant and excluded from the analysis.

Authentic specimens

Hepatocyte incubation provides a good representation of hepatic metabolism but cannot mimic all *in vivo* postmetabolism processes (e.g., reabsorption, extrahepatic metabolism, enterohepatic circulation). Authentic blood or urine specimen analysis, when available, is highly valuable to complement *in vitro* experiments. Three authentic human urine specimens suspected to contain synthetic cathinones were analyzed by our in-house validated stimulant solid-phase extraction (SPE)

method in urine [35], and the presence of α -PVT was confirmed and quantified. Additionally, a small volume of the 2 h hepatocyte sample also was extracted in order to determine if the generated metabolites could be successfully recovered. Briefly, the hepatocyte sample was thawed, vortexed, centrifuged and a portion diluted 1:5 with mobile phase A. Diluted hepatocyte sample (50 μ l) was mixed with 1 ml 0.1 M phosphate buffer (pH 6), vortexed and centrifuged. For authentic specimens, 100 μ l urine was fortified with 25 μ l deuterated internal standard solution, mixed with 1 ml 0.1 M phosphate buffer (pH 6), vortexed and centrifuged. Supernatants were loaded onto SOLA CX SPE cartridges (10 mg/1 ml, Thermo Scientific, Fremont, CA, USA) preconditioned with methanol and phosphate buffer. Columns were washed with 1 M acetic acid and methanol before drying at 10 psi for 5 min. Analytes were eluted with two 1 ml aliquots 2% ammonium hydroxide in 95:5 dichloromethane:isopropanol (v/v). Eluents were acidified with 100 μ l 1% HCl in methanol, dried under nitrogen and reconstituted in 200 μ l mobile phase A; extracted hepatocytes were reconstituted in 250 μ l to allow for direct comparison with 1:5 diluted unextracted hepatocyte samples). The extracted samples were analyzed using the same LC–HRMS method as described for the hepatocyte incubations. In addition, samples were analyzed with all-ion-fragmentation (AIF) acquisition (no quadrupole preselection) to identify metabolites whose intensity was not high enough to trigger an MS² event in ddMS² mode. AIF mode operated at a resolution of 35,000 and with a stepped normalized collision energy of 50 $\pm$ 30%. Raw data were processed using Compound Discoverer software with the same conditions as described above. Metabolites were considered identified if exact mass ($\pm$ 5 ppm) and retention time ($\pm$ 0.1 min) matched those found in hepatocytes.

Results

Metabolic stability assessment with human liver microsomes

α -PVT exhibited a 29.9 ± 2.2 min half-life. *In vitro* microsomal CL_{int micr} was 23.3 ± 1.8 μ l/min/mg and CL_{int} was 21.9 ml/min/kg. CL_{hep} was estimated at 10.5 ml/min/kg with a 0.52 ER.

Metabolite profiling in human hepatocytes

4'-hydroxydiclofenac and diclofenac acyl glucuronide were detected in the diclofenac hepatocyte incubations, demonstrating that the hepatocyte system was viable under our experimental conditions. No metabolites were identified in the negative control, demonstrating that nonenzymatic transformation of the parent drug did not occur. α -PVT MS peak area was 6.96×10^9 ,

5.63×10^9 and 4.91×10^9 at 0, 0.5 and 2 h, respectively. Seven phase I α -PVT metabolites were identified after 0.5 and 2 h incubations with mass measurement errors <1.3 (0.5 h) and <0.9 (2 h) ppm in full scan mode. Metabolites were named from M1 to M9 in ascending RT order, including the two additional metabolites identified in authentic urine samples (see below). MS² fragmentation spectra are reported in Figure 2. Metabolites were mainly formed by hydroxylation at the thiophenyl (M2) or the pyrrolidine (M3, M4, M5, M8 and M9) rings or by ketone reduction (M4, M7 and M8). Elemental composition, accurate mass molecular ion, accurate mass diagnostic product ions, MS peak areas and RT of α -PVT and its metabolites are reported in Table 2. M5 (α -PVT dihydroxypyrrolidinyl), M9 (α -PVT 2-ketopyrrolidinyl) and M4 (α -PVT hydroxypyrrolidinylthiophenol) were the metabolites with the most intense signal respectively after 0.5 h incubation. M5, M9 and M2 (α -PVT hydroxythiophenyl) were the metabolites with the most intense signal respectively after 2 h incubation. M8 (α -PVT 2-ketopyrrolidinylthiophenol) signal was the signal of the in-source water loss only, as it represented more than 99% of the total signal.

Metabolic confirmation & profiling in authentic urine samples

Three authentic urine specimens previously tested positive for α -PVT by a validated Q-TOF screening method were selected to evaluate the proposed metabolic profile. The urine samples were analyzed by our in-house validated stimulant SPE method in urine, and the presence of α -PVT and other stimulants were confirmed and quantified [35]. Urine samples were α -PVT-positive at 5, >500 and 251 μ g/l in specimens 1, 2 and 3, respectively. Sample 1 also was positive for 6-APB (8.3 μ g/l), normephedrone (79 μ g/l), 4-methylethcathinone (> 500 μ g/l), α -PVP (> 500 μ g/l) and MDPV (6.8 μ g/l). Samples 2 and 3 also were positive for α -PVP (> 500 μ g/l). α -PVT and all seven metabolites identified in hepatocytes incubations were found in samples 2 and 3 (Figure 3). MS peak areas are reported in Table 2. α -PVT MS peak area was 6.80×10^7 , 9.25×10^9 and 2.74×10^9 in samples 1, 2 and 3, respectively. M5, M2 and M7 were the metabolites with the most intense signal in the three samples. Two additional metabolites were identified in samples 2 and 3: M1 (α -PVT hydroxypropyl) and M6 (α -PVT 2-ketopyrrolidinyl-glucuronide) (Table 2 & Figures 2 & 3). A suggested metabolic pathway is described in Figure 4.

In silico predictions

MetaSite software predicted the formation of 14 metabolites with a probability score above 20% using

Figure 2. α -pyrrolidinopentiothiophenone and metabolites assigned fragmentation pattern.

Table 2. Elemental composition, accurate mass molecular ion, nominal mass diagnostic product ions, MS peak areas and retention time of α -pyrrolidinopentiothiophenone and its metabolites in human hepatocyte incubations and authentic urine specimens.

Peak ID	Biotransformation	Elemental formula	Experimental molecular ion (<i>m/z</i>)	Mass error (ppm)	Diagnostic product ions (<i>m/z</i>)	Peak area, hepatocyte, 0.5 h	Peak area, hepatocyte, 2 h	Peak area, urine #1	Peak area, urine #2	Peak area, urine #3	RT (min)
α -PVT	Parent	C ₁₃ H ₁₉ NOS	238.1260	+0.21	97, 111, 126, 167	5.63 × 10 ⁹	4.91 × 10 ⁹	6.80 × 10 ⁷	9.25 × 10 ⁹	2.74 × 10 ⁹	7.88
M1	Hydroxylation	C ₁₃ H ₁₉ NO ₂ S	254.1206	+1.38	97, 98, 111, 142, 194	ND	ND	ND	3.04 × 10 ⁷	1.27 × 10 ⁷	6.2
M2	Hydroxylation	C ₁₃ H ₁₉ NO ₂ S	254.1207	+0.78	84, 113, 126, 127	2.21 × 10 ⁷	8.14 × 10 ⁷	3.15 × 10 ^{4†}	2.69 × 10 ⁸	2.25 × 10 ⁸	6.88
M3	Hydroxylation	C ₁₃ H ₁₉ NO ₂ S	254.1207	+0.78	97, 98, 111, 142, 167	1.37 × 10 ⁷	2.69 × 10 ⁷	ND	3.64 × 10 ⁷	9.89 × 10 ^{6†}	7.38
M4	Hydroxylation + reduction	C ₁₃ H ₂₁ NO ₂ S	256.1364	+0.63	97, 124, 144, 166, 238	2.41 × 10 ⁷	2.56 × 10 ⁷	ND	6.52 × 10 ⁷	8.14 × 10 ⁶	7.55
M5	Dihydroxylation	C ₁₃ H ₁₉ NO ₃ S	270.1157	+0.32	97, 98, 140, 166, 252	1.05 × 10 ⁸	2.73 × 10 ⁸	2.19 × 10 ^{6†}	8.72 × 10 ⁸	5.45 × 10 ⁸	7.67
M6	Hydroxylation + dehydrogenation + glucuronidation	C ₁₉ H ₂₅ NO ₈ S	428.1369	+1.15	97, 98, 140, 192, 234	ND	ND	ND	1.87 × 10 ⁷	8.49 × 10 ⁶	7.75
M7	Reduction	C ₁₃ H ₂₁ NOS	240.1415	+0.63	97, 126, 151, 179, 222	1.47 × 10 ⁷	5.79 × 10 ⁷	8.27 × 10 ^{4†}	1.92 × 10 ⁸	4.56 × 10 ⁷	8.33
M8	Hydroxylation + dehydrogenation + reduction	C ₁₃ H ₁₉ NO ₂ S	236.1103 [‡]	+0.04	86, 97, 98, 151	8.23 × 10 ⁶	3.63 × 10 ⁷	ND	1.76 × 10 ⁶	6.06 × 10 ⁵	11.07
M9	Hydroxylation + dehydrogenation	C ₁₃ H ₁₇ NO ₃ S	252.1052	+0.14	86, 98, 140, 167	7.29 × 10 ⁷	1.47 × 10 ⁸	1.03 × 10 ^{4†}	3.33 × 10 ⁷	2.18 × 10 ⁷	13.12

Peak area for α -PVT parent at 0 h was 6.96 × 10⁹. Metabolites are ranked by ascending RT.

†No MS2 confirmation.

‡In-source water loss (– *m/z* 18.0105) (>99% of the signal).

ND: Not detected; RT: Retention time.

the CYP450 liver model (P1–P14 in ascending probability score order). Predicted structure, biotransformation, accurate mass, $\log D$ at pH = 4 (closest pH to gradient conditions) and probability score for each molecule are reported in Table 3. Thiophenyl (P1, P7 and P11) and alkyl (P8 and P13) hydroxylation and *N*-dealkylation (P3 and P4) were the most common reactions. P1 (thiophenyl hydroxylation) and P2 (thiophenyl hydroxylation and rearrangement) presented with a probability score of 100%. P3 (*N*-dealkylation and oxidation to carbonyl), P4 (*N*-dealkylation and oxidation to carboxylic acid) and P5 (iminium formation) presented with a score of 57%. Six and two second-generation metabolites were generated from P1 and P2, respectively, as they both showed a 100% score.

MetaSite also generated a list of 237 m/z values of predicted metabolites in HESI+ (theoretical accurate mass). This list was incorporated into the acquisition mode identifying expected metabolites with a low to high signal intensity: ddMS² mode with an acquisition list.

Discussion

Metabolic stability assessment with human liver microsomes

α -PVT can be considered as an intermediate-clearance drug. McNaney *et al.* considers compounds with CL_{int} 15–40 ml/min/kg as intermediate-clearance drugs [37], and Lavé *et al.* classify compounds with an ER between 0.3 and 0.7 as intermediate-clearance drugs [38]. Based on these results, we hypothesize that α -PVT metabolites may be found in urine for a few days after intake. However, CL_{int} and ER are predicted values as the calculations make several assumptions. Hepatic clearance is expected to be sensitive to changes in plasma protein binding, drug metabolism/elimination and hepatic blood flow [39].

Metabolite profiling in human hepatocytes

Takayama *et al.* previously reported formation of M2 and M3 along with another hydroxypyrrolidinopropyl metabolite using HLM, but the last metabolite was not detected in the present hepatocyte incubations [29]. However, it is difficult to make direct comparisons between HLM and hepatocytes results, as metabolites and their relative abundance formed via HLM incubations may not be representative of metabolic profiles produced *in vitro* or *in vivo*. Despite structural similarities to α -PVP (phenyl analog of α -PVT), a few biotransformations were not detected *in vitro*, including *N*-dealkylation and pyrrolidine ring opening and oxidation to corresponding carboxylic acid as detected *in vitro* (HLM) and *in vivo* (urine) in previous

α -PVP studies [40–42]. The differences between α -PVP and α -PVT can be attributed to a number of factors, including the different metabolic systems employed (HLM vs hepatocytes), binding affinity and different structural properties (benzene vs thiophene ring). In hepatocyte incubations experiments, ddMS² mode with an inclusion list identified the same metabolites as ddMS² mode without an inclusion list plus several more, indicating that MetaSite software predicted all the metabolites produced *in vitro* with a high signal intensity and ddMS² mode with an inclusion list allowed identification of lower intensity metabolites.

α -PVT fragmentation pattern

α -PVT fragmentation pattern is reported in Figure 2 (m/z 238.1260, RT: 7.88 min). Major ions were produced by fragmentation on both sides of the α -carbon: fragments m/z 97.0106 and 110.9899 were related to the thiophenyl (thiophenylmethyl cation) and thiophenone (thiophenylmethyldi-neoxonium ion) moieties respectively and fragment m/z 126.1277 was related to the pyrrolidinoalkyl group (butyridenepyrrolidinium ion). Fragments m/z 70.0651 and 84.0808 were produced by the pyrrolidinyl (dihydropyrrolidinium ion) and the methylpyrrolidinyl (methylpyrrolidinium ion) moieties respectively and fragment m/z 167.0525 was produced by the pentothiophenone group (oxothiophenylpentanylium ion).

Hydroxylated metabolites

M2 and M3 (m/z 254.1209, RT: 6.88 and 7.38 min, respectively) were hydroxyl metabolites of α -PVT as suggested by the increase of 15.9949 Da from the parent m/z (Figure 3). Butyridenepyrrolidinium ion (m/z 126.1277) was by far the most intense fragment in M2 MS² spectrum, while the intact thiophenyl fragment was replaced by a hydroxythiophenyl group (m/z 97.0106 + 15.9949 = m/z 113.0055), indicating the formation of α -PVT hydroxythiophenyl. The structure is not stable, however, and resulted in a keto-enol tautomerism in favor of the keto form as suggested by the absence of water loss during fragmentation. The 73% FISH score indicates a good match between the suggested structure of M2 and the theoretical MS² spectrum but the hydroxyl position on the thiophene ring could not be determined by MS. The M3 MS² spectrum included the intact thiophenyl and the hydroxypyrrolidinyl (m/z 88.0757) fragments, while the pyrrolidinoalkyl group was replaced by a hydroxypyrrolidinoalkyl fragment (m/z 126.1277 + 15.9949 = m/z 142.1226), indicating the formation of α -PVT hydroxypyrrolidinyl. The position of the hydroxyl group on the pyrrolidine ring cannot be determined even though the absence of loss of water molecule during fragmentation

Figure 3. Extracted ion chromatograms obtained from hepatocyte incubation with α -pyrrolidinopentiothiophenone (2 h) (A) and from an authentic α -PVT-positive urine sample (specimen #3) (B) (see facing page). α -PVT metabolites are numbered one through nine in ascending order of retention time in hepatocyte incubations and urine samples (M1–M9). The chromatographic peaks with no assignment were not identified as α -PVT metabolites or presented below the signal intensity threshold set for the metabolite identification. M1 and M6 were not detected in hepatocyte incubations. α -PVT: α -pyrrolidinopentiothiophenone.

may indicate the formation of α -PVT 2-hydroxypyrrolidinyl (or α -PVT 5-hydroxypyrrolidinyl), the C–O bond being reinforced by the co-sharing of electrons between the oxygen and nitrogen atoms. Both M2 and M3 were already reported in previous *in vitro* studies using HLM incubations [29].

M5 (m/z 270.1158, RT: 7.67 min) was formed by dihydroxylation of α -PVT as suggested by the addition of 31.9898 Da from the parent drug and the two water molecules loss during fragmentation (Figure 2). *In silico* studies predicted a metabolite with the exact same mass by pyrrolidine ring opening and *N*-butanoic acid formation (Table 3, P4) but the fragmentation pattern was not indicative of the expected acetic acid loss [40]. Therefore, we suggest that both hydroxylation reactions occurred on the pyrrolidine ring as indicated by the presence of the intact thiophenyl fragment (m/z 97.0106), appearing in parent MS² spectrum, the replacement of the pyrrolidinoalkyl fragment by a dihydroxy-substituted group (m/z 126.1277 + 31.9898 = m/z 158.1176) and the formation of butaniminium (m/z 72.0808) and aminothiophenylpentenylium (m/z 166.0685) ions. These two last fragments are noteworthy as the formation of the primary amine (or iminium) structure is favored by hydroxylation at the pyrrolidine ring. These two fragments were not present in parent MS² spectrum. The 60% FISH score indicates a good match between the proposed structure of M5 and theoretical MS² spectra.

Metabolites with β -ketone reduction

The water loss in M7 (m/z 240.1417, RT: 8.33 min) MS² spectrum and addition of 2.0157 Da from parent compound suggested β -ketone reduction (Figure 2). This metabolite was highly expected as ketone reduction was reported as the main α -PVP metabolite in urine samples [40,41] and other β -keto cathinones [43]. Twenty to 30% of signal was produced by in-source water loss (m/z 222.1311). The structure of the most intense M7 fragment at m/z 179.0769 could not be elucidated through the software; nevertheless, the FISH score for the metabolite indicated a good match between the suggested M7 and theoretical MS² spectra (43%). This fragment was not the result of contamination as the full-scan spectrum did not show any co-eluting additional masses in the range selected for M7 fragmentation. Moreover, the same fragment was observed in the MS² spectrum of m/z 222.1311 (in-source water loss

of M7). In a window of 5 ppm, an experimental m/z 179.0769 should have the following elemental composition: C₁₀H₁₃NS. We hypothesized that the fragment was formed by the loss of the hydroxyl group and the alkyl chain. M7 eluted after parent although it is theoretically more polar than α -PVT in these analysis conditions (log D at pH = 4: -1.00 according to MetaSite). However, the ketone reduction remains the most likely reaction, considering the literature and the fragmentation pattern. It is noteworthy that the same retention time order was observed with M3 and M4.

M4 (m/z 256.1366, RT: 7.55 min) was a secondary metabolite produced by both β -ketone reduction, which had led to M7, and pyrrolidine hydroxylation, which also generated M3, as attested by the two water losses during fragmentation and the increase of 18.0106 Da from α -PVT (Figure 2). An intact thiophenyl fragment (m/z 97.0106), an aminothiophenylpentenylium ion (m/z 166.0685) also observed in the M5 MS² spectrum and a thiophenyletheniminium ion (m/z 124.0215) observed in both M3 and M5 fragmentation spectra were produced during M4 fragmentation, demonstrating that hydroxylation occurred on the pyrrolidine ring. The hydroxyl position on the pyrrolidine ring could not be determined.

Metabolites with γ -lactam formation

M9 (m/z 252.1053, RT: 13.12 min) was formed by hydroxylation and dehydrogenation of α -PVT (+13.9793 Da). The MS² spectrum included the intact thiophenyl fragment along with substituted pyrrolidinoalkyl (m/z 126.1277 + 13.9793 = m/z 140.1070) and pyrrolidinyl (m/z 84.0808 + 13.9793 = m/z 98.0600) fragments suggesting an oxidation of the pyrrolidine ring. HRMS allowed the distinction between fragments m/z 98.0600 and 98.0964, the last one being present in parent MS² spectrum (vinylpyrrolidinium ion) (Figure 2). Hydroxylation followed by dehydrogenation to the corresponding ketone is more likely than dehydrogenation of a C–C bond. Moreover, γ -lactam formation is a major metabolic pathway of α -PVP [40,41] and other pyrrolidine compounds [44]. M9 eluted 5.25 min after the parent, supporting the hypothesis of formation of α -PVT pyrrolidin-2-one (or α -PVT pyrrolidin-5-one), a γ -lactam, the carbonyl group being a hindrance to hydrogen bonding with the nitrogen atom and decreasing M9 polarity and alkalinity. M8 (m/z 254.1209, RT: 11.07 min) was

Figure 4. α -pyrrolidinopentiothiophenone suggested metabolic pathway. Plain arrow, observed in human hepatocyte incubations; dashed arrow, observed in authentic urine samples.

a tertiary metabolite produced by β -ketone reduction, pyrrolidine hydroxylation and then dehydrogenation to the corresponding lactam. Interestingly, the M8 signal was drastically reduced due to in-source fragmentation, 99.5% of the signal being produced by the water loss at m/z 236.1104. The MS² spectrum of ion m/z 236.1104 contained an intact thiophenyl fragment (m/z 97.0106), an oxopyrrolidinium (m/z 86.0600) and a methyleneoxopyrrolidinium (m/z 98.0600) ion, which were also present in the M9 MS² spectrum, pointing toward a γ -lactam formation. It also contained a thiophenylpentenylium ion (m/z 151.0576), previously observed in the M7 MS² spectrum and indicating a β -ketone reduction (Figure 2). This metabolite was expected as the reaction is a major α -PVP biotransformation [40].

Metabolic confirmation & profiling in authentic urine samples

The three samples were positive for α -PVT and one to five other cathinones (6-APB, normephedrone, 4-methylethcathinone, α -PVP and MDPV). All seven α -PVT metabolites previously elucidated in hepatocyte incubations contained a thiophene ring and would

not be produced from the other detected cathinones. α -PVT and its seven metabolites were identified in samples 2 and 3 (Figure 3); however, M3 in sample 3 and M7 in both samples were not confirmed as the low signal did not trigger an MS² event in ddMS² mode and the diagnostic MS² ions were not detected in AIF mode. M2, M5, M7, M9 and parent were identified in sample 1 but, similarly, M2, M7 and M9 were not confirmed due to low signal intensity. This is consistent with the lower α -PVT concentration in urine specimen 1. Interestingly, M2 and M4 chromatographic peaks presented with shoulders in authentic samples, suggesting the presence of potential positional isomers. The M2 shoulder peak (M2', RT: 7.03 min) is supposed to be a M2 isomer as it produced the same fragments. Both M2 and M2' were included in the MS peak area calculations (Table 2). The M4 shoulder peak (RT: 7.60 min) was not intense enough to trigger an MS² scan so its identity was not confirmed and was not included in peak area calculations.

In order to protect the instrument, we utilized SPE for the urine samples validated for cathinone parent compounds [35]. The signal intensities of each metabolite were consistent with hepatocyte incubation results

(Table 2). However, relative intensities do not necessarily reflect metabolite concentrations, as ionization, extraction recovery and matrix effect could vary depending on the compound. As certified reference standards are not yet available for these metabolites, recovery and matrix effect cannot be evaluated. The extraction procedure utilized in this study proved useful for the recovery of a wide variety of phenethylamines, pyrrolidinophenones and their respective metabolites (>80% recovery), as seen in our publications [35,45]. A gross estimation of SPE recoveries in hepatocyte samples was made by comparing areas from extracted and unextracted incubations (representing same sample volume). α -PVT, M3, M4 and M7 recoveries ranged from 75 to 95%, while M2, M5, M8 and M9 recoveries ranged from 0.5 to 3.5% (α -PVT extraction recovery in urine is 93.1 – 94.7% [35]). It is important to keep in mind that estimations were made on a single experiment and may also be biased by matrix effect in both SPE and dilution experiments. A strong positive matrix effect in dilution samples and/or a strong negative matrix effect in SPE samples for M2, M5, M8 and M9 could alone explain the low recovery values. Hypothesizing that the extraction recoveries are similar in urine and hepatocyte incubations and regardless of the difference in signal production and matrix effect, M2, M5 and M9 would be the main metabolites of α -PVT *in vivo*. M5, M2, parent and M9 would be respectively the most concentrated α -PVT-related compounds in urine specimens 2 and 3, which present a high α -PVT concentration. M2, M9, M5 and parent would be respectively the most abundant α -PVT-related compounds in urine specimen 1, which presents a low α -PVT concentration. There is currently no mention of α -PVT concentrations in biological matrices in the scientific literature; however, samples 2 and 3 urine concentrations may be indicative of recent drug use or larger doses while sample 1 may not, based on the PK patterns of other cathinones [46].

Two additional metabolites, M1 and M6, were identified in samples 2 and 3 using ddMS² acquisition mode with an inclusion list (Figure 3). M1 (m/z 254.1209, RT: 6.20 min) was formed by hydroxylation, as suggested by the increase of 15.9949 Da from parent (Figure 2). The presence of the intact thiophenyl and pyrrolidine fragments and the substituted pyrrolidinoalkyl fragment (m/z 126.1277 + 15.9949 = m/z 142.1226) indicated a hydroxylation on the alkyl chain. M1 may be the α -PVT hydroxypyrrolidinopropyl metabolite that was previously identified by Takayama *et al.* using HLM incubations [29]. The chromatographic peak presented a small shoulder on both sides, suggesting the presence of 2 positional isomers, but the low abundances did not trigger MS²

events. The shoulder peaks were not included in MS peak area calculations. M6 (m/z 428.1374, RT: 7.75 min) was the glucuronide of M9 as evidenced by the increase by 176.0321 Da from α -PVT and its MS² spectrum, which contained the main M9 fragments along with M9 itself and substituted pyrrolidinoalkyl chain (m/z 126.1277 + 13.9793 + 176.0321 = m/z 316.1391) (Figure 2). Both newly identified metabolites showed only low abundance signals (Table 2).

No other phase II metabolites were detected *in vitro* or *in vivo*. The parent drug is a polar compound and the analyte with the most intense signal detected in urine specimens. The only glucuronide detected in the urine sample was the conjugated form of M9, which is the least polar α -PVT metabolite identified. We did not detect any glucuronidated metabolites in hepatocyte incubations, meaning that glucuronidation does not occur or did not have time to occur in the present 2 h experiments. Identification of diclofenac acyl glucuronide in the positive control samples confirms that the hepatocytes were viable to produce phase II metabolites. The lack of phase II metabolites also was observed with 4-MeO- α -PVP [30].

In silico predictions

MetaSite predicted a list of 14 first-generation and 8 second-generation metabolites, based on a CYP450 liver model (Table 3). A prediction score thus was assigned to each compound depending on the simulations of phase I metabolism. However, *in silico* prediction software is incapable of predicting second- and third-generation metabolites or non-CYP- or FMO3-mediated biotransformations, such as aldehyde oxidase, sulfotransferase etc. In addition, the liver model takes into account the sites of metabolism based on only four metabolic enzymes (CYP3A4, CYP2D6, CYP2C9 and FMO3), potentially introducing a bias in predictions. The predictions should be considered carefully as they might influence the judgment of the operator during metabolite identification. It is noteworthy that P1 and P1–1 to P1–6 are not stable in the conditions of the analysis as they represent the enol form of a keto-enol tautomerism (P2 is the keto P1 form). Similarly, P5 is not stable in aqueous conditions and may lose its pyrrolidine group to produce P9. Both P1 and P5 presented with a high prediction score (100 and 57%, respectively) but were not detected in the present experiments, possibly due to instability. Four metabolites identified in hepatocyte incubations and authentic urine samples matched at least one metabolite predicted by MetaSite software: M1, M2, M3 and M9. M2 matched P2, which presented with a 100% score, and appeared to be, indeed, a major metabolite in *in vitro* and *in vivo* experiments. Lac-

Table 3. *In silico* predictions by MetaSite software for α -pyrrolidinopentiothiophenone metabolism.

First-generation metabolites
	$C_{13}H_{19}NOS$	$C_{13}H_{19}NO_2S$	$C_{13}H_{19}NO_2S$	$C_{13}H_{19}NO_2S$	$C_{13}H_{19}NO_3S$
Biotransformation	Parent	Hydroxylation	Hydroxylation + tautomerism	N-dealkylation	N-dealkylation + hydroxylation
Mass (Da)	237.1187	253.1136	253.1136	253.1136	269.1086
Theoretical log <i>D</i> (pH = 4)	-0.09	-0.05	1.21	-0.3	-0.25
Probability score (%)	NA	100	100	57	57
	$C_{13}H_{18}NOS^+$	$C_{13}H_{17}NO_2S$	$C_{13}H_{19}NO_2S$	$C_{13}H_{19}NO_2S$	$C_9H_{10}O_2S$
Biotransformation	Iminium formation	Carbonylation	Hydroxylation	Hydroxylation	N-dealkylation
Mass (Da)	236.1109	251.0979	253.1136	253.1136	182.0401
Theoretical log <i>D</i> (pH = 4)	1.76	2.49	-0.54	-1.15	1.74
Probability score (%)	57	57	53	40	36
	$C_{13}H_{17}NOS$	$C_{13}H_{19}NO_2S$	$C_{13}H_{19}NO_2S$	$C_{13}H_{19}NO_2S$	$C_{13}H_{19}NO_2S$
Biotransformation	Dehydrogenation	Hydroxylation	Hydroxylation	Hydroxylation	N-oxidation
Mass (Da)	235.1031	253.1136	253.1136	253.1136	253.1136
Theoretical log <i>D</i> (pH = 4)	0.47	-0.64	-0.67	-1.01	2.7
Probability score (%)	36	25	23	22	20

Metabolites are ranked according to their probability score (>20%). P1 and P2 second-generation metabolites were included (when probability score >50%). Theoretical log*D* was indicated at pH = 4 as it was the pH closest to gradient conditions.
 NA: Not applicable.

Table 3. *In silico* predictions by MetaSite software for α -pyrrolidinopentiothiophenone metabolism (cont.).

Second-generation metabolites
	<p>P1-1 $C_9H_{10}O_3S$</p>	<p>P1-2 $C_{13}H_{17}NO_2S$</p>	<p>P1-3 $C_{13}H_{19}NO_3S$</p>	<p>P1-4 $C_{13}H_{19}NO_4S$</p>	<p>P1-5 $C_{13}H_{18}NO_2S^+$</p>
Biotransformation	N-dealkylation	Dehydrogenation	N-dealkylation	N-dealkylation	Iminium formation
Mass (Da)	198.0351	251.098	269.1086	285.1035	252.1058
Theoretical logD (pH = 4)	1.8	0.17	-0.92	-0.45	1.83
Probability score (%)	83.3	83.3	66.7	66.7	66.7
	<p>P1-6 $C_{13}H_{17}NO_3S$</p>	<p>P2-1 $C_{13}H_{19}NO_3S$</p>	<p>P2-2 $C_{13}H_{21}NO_3S$</p>		
Biotransformation	Carbonylation	S-dealkylation	Thioester cleavage		
Mass (Da)	267.0929	269.1086	271.1242		
Theoretical logD (pH = 4)	2.57	-0.88	-0.47		
Probability score (%)	66.7	100	100		

Metabolites are ranked according to their probability score (>20%). P1 and P2 second-generation metabolites were included (when probability score >50%). Theoretical logD was indicated at pH = 4 as it was the pH closest to gradient conditions.
NA: Not applicable.

tam formation is a common reaction for pyrrolidine compounds [43,44] leading to M9 in the present experiments. M9 matched P6, which was predicted with a 57% score. The position of the hydroxyl group in M1 could not be elucidated in the conditions of the analysis and the compound could match either P8 or P13, which were respectively predicted with a 40 and 22% score. Similarly the hydroxylation site of M3 could not be elucidated but the metabolite matched P12, which was predicted with a 23% score and was the only predicted α -PVT hydroxypyrrolidine. N-dealkylation reactions were not detected *in vitro* or *in vivo*. Five metabolites were detected in hepatic or/and urine samples but were not predicted by MetaSite software (score <20%): M4, M5, M6, M7 and M8. M5, the metabolite with the most intense signal in hepatic incubations and urine samples, did not match any predicted metabolite as it was a second-generation metabolite from P12, which presented with a 23% score. M6 did not match any predicted metabolite as it was the result of a phase II reaction (glucuronidation). More interest-

ingly, M4, M7 and M8 did not match any predicted metabolite although ketone reduction is a common biotransformation for β -keto cathinones [40,41,43].

HRMS inclusion list

Beside the list of the 22 most expected metabolites based on the CYP450 liver model, MetaSite generated a more exhaustive list of 237 *m/z* values of predicted metabolites in HESI+ for ddMS² analysis. This list included not only the 39 biotransformations generated by the CYP450 liver model, but also 40 additional non-CYP mediated reactions as well as second- and third-generation metabolites. However, the structure, the corresponding biotransformations and the prediction score assigned to these *m/z* were not provided. No additional metabolites were identified in ddMS² acquisition mode without an inclusion list compared with ddMS² mode with an inclusion list, indicating that the inclusion list contained the *m/z* value of all the metabolites that were detected in hepatic and urine samples. Moreover, the ddMS² mode with an inclu-

sion list allowed the elucidation of a higher number of metabolites, indicating that the list of m/z generated by MetaSite software was crucial in the identification of metabolites with lower signal intensity.

Conclusion

For the first time, a comprehensive metabolic profile of α -PVT was generated using *in vitro* HLM and human hepatocyte incubations, and confirmation in authentic urine specimens with HRMS detection. α -PVT was predicted as an intermediate-clearance drug with a 30 min half-life in HLM. Nine metabolites were elucidated in hepatocyte incubations and urine samples. Observed metabolic patterns were comparable to other pyrrolidinophenones, including hydroxylation, ketone reduction, lactam formation and combinations thereof. In addition to parent drug, analytical methods should monitor M2 (α -PVT hydroxythiophenyl), M5 (α -PVT dihydroxypyrrolidinyl), M7 (α -PVT thiophenol) and M9 (α -PVT 2-ketopyrrolidinyl) to serve as markers for α -PVT intake in forensic and clinical samples. Results were compared with *in silico* predictions by MetaSite software. MetaSite CYP450 liver model appeared to be unsuitable for α -PVT metabolites prediction, as only four of nine predicted metabolites achieved a score higher than 20%. However, the software also generated a more exhaustive list of theoretical m/z values of predicted metabolites in HESI+ that proved crucial for the identification of metabolites with low intensity signal in HRMS experiments using ddMS² acquisition mode with an inclusion list.

Future perspective

In the last several years, more than 450 NPS were identified by the EWS. As the drug market evolves, reference standards for new NPS and metabolites are not always readily and quickly available, making it difficult for forensic and clinical laboratories to maintain vali-

dated analytical assays for identifying and quantifying these markers in biological specimens. The advent of HRMS allowed laboratories to screen for hundreds of additional compounds against NPS libraries. However, knowledge of metabolites can increase detection windows for many drugs. Since controlled administration studies are not yet permissible, *in vitro* studies are the fastest, most robust assays for determining metabolic profiles. Identification of prominent metabolites can assist reference standard manufacturers in deciding which compounds to produce and publishing of product ion spectra can help laboratories confirm NPS intake. Over the next several years, *in silico* predictions and *in vitro* assays will become routine in mapping NPS metabolic pathways. Understanding drug clearance and production of potential toxic metabolites will assist practitioners in understanding the PD and PK profiles of emerging compounds. Knowledge of several drugs in a particular class also will provide insight into how other analogs may behave.

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Executive summary

- Little pharmacological or toxicological data are available for constantly emerging novel psychoactive substances (NPS).
- Characterizing NPS metabolic pathways is necessary for improving identification of NPS intake.
- Human hepatocyte drug incubations, followed by analysis with liquid chromatography coupled to high-resolution MS and software-assisted data mining is a promising approach to generating comprehensive NPS metabolic profiles.

Results

- We generated the metabolic profile for α -pyrrolidinopentiothiophenone (α -PVT), a novel synthetic cathinone and identified seven metabolites *in vitro* and two additional metabolites *in vivo*.
- The primary biotransformations were hydroxylation, ketone reduction, lactam formation and combinations thereof.
- The most intense α -PVT metabolites and suitable markers for documenting α -PVT intake in addition to the parent drug were hydroxythiophenyl, dihydroxypyrrolidinyl, thiophenol and 2-ketopyrrolidinyl α -PVT.

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