

In silico and in vitro metabolism studies support identification of designer drugs in human urine by liquid chromatography/quadrupole-time-of-flight mass spectrometry

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Received: 30 April 2013 / Revised: 7 June 2013 / Accepted: 10 June 2013 / Published online: 25 June 2013
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Abstract Human phase I metabolism of four designer drugs, 2-desoxypipradrol (2-DPMP), 3,4-dimethylmethcathinone (3,4-DMMC), α -pyrrolidinovalerophenone (α -PVP), and methiopropamine (MPA), was studied using in silico and in vitro metabolite prediction. The metabolites were identified in drug abusers' urine samples using liquid chromatography/quadrupole-time-of-flight mass spectrometry (LC/Q-TOF/MS). The aim of the study was to evaluate the ability of the in silico and in vitro methods to generate the main urinary metabolites found in vivo. Meteor 14.0.0 software (Lhasa Limited) was used for in silico metabolite prediction, and in vitro metabolites were produced in human liver microsomes (HLMs). 2-DPMP was metabolized by hydroxylation, dehydrogenation, and oxidation, resulting in six phase I metabolites. Six metabolites were identified for 3,4-DMMC formed via *N*-demethylation, reduction, hydroxylation, and oxidation reactions. α -PVP was found to undergo reduction, hydroxylation, dehydrogenation, and oxidation reactions, as well as degradation of the pyrrolidine ring, and seven phase I metabolites were identified. For MPA, the nor-MPA metabolite was detected. Meteor software predicted the main human urinary phase I metabolites of 3,4-DMMC, α -PVP, and MPA and two of the four main metabolites of 2-DPMP. It assisted in the identification of the previously unreported metabolic reactions for α -PVP. Eight of the 12 most abundant in vivo phase I metabolites were detected in the in vitro HLM experiments. In vitro tests serve as material for exploitation of in silico data when an authentic urine sample is not available. In silico and in vitro designer drug metabolism studies with LC/Q-TOF/MS

produced sufficient metabolic information to support identification of the parent compound in vivo.

Keywords 2-Desoxypipradrol · 3,4-Dimethylmethcathinone · α -Pyrrolidinovalerophenone · Methiopropamine · Metabolite prediction · Liquid chromatography/quadrupole-time-of-flight mass spectrometry

Introduction

An increasing number of new designer drugs arrive annually on the illicit drug market. Between 2005 and 2012, 237 new psychoactive compounds were reported through the early warning system in Europe, and a record number of 73 new substances were notified in 2012 [1, 2]. Analytical laboratories in the fields of forensic and clinical toxicology, as well as customs and criminal investigation, need to tackle this challenge and develop methods for compound identification, even if a certified reference standard is not always available.

In terms of toxicological risk assessment and analytical method development aspect, the knowledge about designer drug metabolism is highly substantial [3–5]. The metabolism of designer drugs is commonly studied in animals, especially rats [3, 5]. In vitro experiments in metabolism studies have become popular among forensic and clinical toxicology research groups [6] and in doping control laboratories [7–10] during recent years. Drug incubation with human liver microsomes (HLMs), S9 fraction, or hepatocytes is an established ethical and cost-effective method in studies of the metabolism of new designer drugs and psychoactive compounds [6]. Their main application to date has been in enzyme kinetics.

High-resolution mass spectrometry (HRMS) applications are today widely employed in drug metabolism studies [11, 12]. Modern quadrupole-time-of-flight (Q-TOF) instruments

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provide high mass resolution and accuracy on a routine basis. Q-TOF instruments operate at high data acquisition speed, and their full-range data acquisition enables retrospective data mining. Q-TOF mass analyzers are frequently used in drug metabolism research in substance identification and in confirmation of nominal mass MS data [11, 13]. Software solutions play a key role in systematic drug metabolism studies by HRMS, and most of the MS manufacturers provide software tools such as MetaboLynx (Waters, Milford, MA, USA) and MetWorks (Thermo Scientific, Waltham, MA, USA) to detect products of common metabolic pathways [14]. Mass defect filtering (e.g., MassLynx, Waters) [15] and MS/MS spectra comparison (e.g., SmileMS, GeneBio, Geneva, Switzerland) [16] software enable nontargeted metabolite screening and have served in the identification of drugs of abuse metabolites. Several software tools for metabolite prediction of xenobiotics are commercially available, three being MetaSite (Molecular Discovery Ltd, Middlesex, UK), MetaDrug (Thomson Reuters, NY, USA), and Meteor (Lhasa Limited, Leeds, UK). Drug metabolite prediction *in silico* is widely used to study the biotransformation of pharmaceuticals [17–21]. Prediction using commercial or self-coded software has not become common in studies of designer drugs or other toxicologically interesting compounds, however.

Here, we studied the metabolism of four structurally different designer drugs: 2-desoxypropiradol (2-DPMP, Daisy), 3,4-dimethylmethcathinone (3,4-DMMC), α -pyrrolidinovalerophenone (α -PVP), and methiopropamine (MPA). At the moment, only 2-DPMP is classified as a controlled drug of abuse in Finland, which makes the other compounds attractive among drug users, as the penalties are less severe than those for drug offenses. Methiopropamine and α -PVP are classified as pharmaceutical compounds, whereas 3,4-DMMC is not yet subject to any legislation and is categorized as a research chemical. 2-DPMP is a phencyclidine-derived compound, and its metabolism has not previously been reported. For the pyrrolidinophenone analog α -PVP, 12 phase I metabolites in rats have been reported [22]. These include hydroxylation, dehydrogenation, ring opening and oxidation reactions, and degradation of the pyrrolidine ring to the corresponding primary amine. These metabolites have not been confirmed from authentic human urine samples. The main human urinary phase I metabolites of the β -keto-structured cathinone 3,4-DMMC have recently been reported by Shima et al. [23]. They identified three metabolites, formed after *N*-demethylation and reduction of the β -ketone, using synthesized standards. In a recent metabolism study of the thiophene derivative of methamphetamine MPA, a nor-metabolite was detected in human urine [24].

We identified the main human urinary phase I metabolites of the designer drugs studied using *in silico* and *in vitro* methods for metabolite prediction. The aim was to produce sufficient metabolic information for urine drug screening to

support the identification of the parent compound. In our previous study, the metabolite prediction software Meteor was found to be useful in assigning metabolites to quetiapine [20]. Here, the ability of the Meteor software to predict the metabolism of designer drugs was evaluated by comparing the results between *in silico* predictions and our own predictions based on known metabolic reactions of structural analogs. The *in vitro* metabolism of 2-DPMP, 3,4-DMMC, α -PVP, and MPA was studied using HLMs to support the *in vivo* metabolite findings and to test whether the HLM experiments produced the main phase I metabolites. Several authentic human urine samples, which had tested positive for the designer drugs studied, were analyzed using liquid chromatography (LC)/Q-TOF/MS, and the metabolites were characterized from their MS/MS spectra without reference standards.

Experimental

Materials

Methanol (LC-MS grade) and isopropanol (analytical grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents (analytical grade) were from Merck (Darmstadt, Germany). Water was purified with a MilliQ Integral5 instrument (Millipore, Billerica, MA, USA). α -PVP (HCl) was obtained from Toronto Research Chemicals (Toronto, Canada), tramadol (HCl) from Nycomed Christiaens (Brussels, Belgium), and dibenzepin (HCl) of pharmaceutical purity from Sandoz (Holzkirchen, Germany). 2-DPMP, 3,4-DMMC, and MPA were obtained from material seized by the Finnish National Bureau of Investigation or from the Finnish Customs. The purity of the substances was determined by LC-chemiluminescence nitrogen detection [25] with a linear gradient separation. The purity of 2-DPMP was 50.7 %, 3,4-DMMC 85.0 %, and MPA 97.6 %, as their hydrochlorides (measurement uncertainty 6.5 %, unpublished data). The unpurified seized drug material (0.1 mM in 0.1 % formic acid + methanol) was analyzed by the LC/Q-TOF/MS method before the *in vitro* incubations to exclude possible contaminating active ingredients. No such substances were found, which would interfere with the metabolic reactions. Thus, the impurity was presumed to be from inactive excipients. HLMs (BD UltraPool™ HLM 150) and NADPH regenerating system solutions A (26 mM NADP⁺, 66 mM glucose-6-phosphate and 66 mM MgCl₂ in H₂O) and B (40 U/mL glucose-6-phosphate dehydrogenase in 5 mM sodium citrate) were supplied by BD Biosciences (Woburn, MA, USA). β -glucuronidase was obtained from Roche (Mannheim, Germany). Mixed-mode solid phase extraction (SPE) cartridges with strong cation exchange and hydrophobic interactions (Isolute HXC-5, 130 mg, 10 mL) were provided by Biotage (Uppsala, Sweden).

Urine samples were either collected at autopsy or they were clinical toxicology cases investigated at our laboratory. The urine samples tested positive for one or more of the designer drugs studied in our routine drug screening by LC-TOF/MS [26]. The positive findings were confirmed and quantified by gas chromatography-MS [27]. Ten 2-DPMP cases (concentrations in urine between 0.29 and 42 mg/L), two 3,4-DMMC cases (0.53 and 1.4 mg/L), eight α -PVP cases (0.08–13 mg/L), and three MPA cases (0.52–19 mg/L) were studied. The urine samples contained several other toxicological findings, including prescription drugs and illicit drugs and their metabolites, as well as alcohol, caffeine, and nicotine. The maximum number of positive findings was 22, and the average and median were 10 and 9, respectively. The predicted metabolites were checked against the in-house toxicology database to exclude potential false-positive findings resulting from other compounds.

Urine sample preparation

Urine samples (1 mL) were hydrolyzed overnight (15 h) at 37 °C, with β -glucuronidase. Solid phase extraction [26] was used for sample preparation. The samples were reconstituted with 150 μ L methanol/0.1 % formic acid (1:9 v/v). The methanol used for washing the SPE cartridge between neutral and basic extractions was evaporated and reconstituted likewise and analyzed by LC/Q-TOF/MS method to find out if any of the most polar metabolites were lost in sample preparation.

In vitro metabolism studies

Phase I metabolism in vitro of the four designer drugs, 2-DPMP, 3,4-DMMC, α -PVP, and MPA, was studied using HLMs. The reaction mixture consisted of 1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, and 3.3 mM magnesium chloride, in 100 mM phosphate buffer at pH 7.4. The final reaction mixture volume was 100 μ L, where the drug concentration was 100 μ M and the protein concentration was 2.0 mg/mL. The reaction was initiated by addition of the microsomes, and the incubation time was 4 h at 37 °C. The reaction was terminated with 100 μ L of ice-cold methanol. The samples were centrifuged (16,000 rpm, 25,000 g-units, 10 min), and the supernatant was stored at –80 °C until analysis. Test samples were prepared in triplicate. In addition to the test samples, a blank sample without the drug, biological control samples without either HLMs or NADPH regenerating solutions, and a chemical control sample without both HLMs and NADPH were prepared to determine any interference resulting from matrix compounds and spontaneously formed metabolites. A standard control sample with a compound of known in vitro metabolism, tramadol, was also included in the test set. The presence of the main in vitro phase I metabolites of tramadol [28] confirmed the incubation conditions.

In silico metabolite prediction

The metabolism of the designer drugs was predicted using Meteor 14.0.0 software (Lhasa Limited, Leeds, UK). Meteor predicts the metabolism of xenobiotics using a rule-based expert system [29]. First, substructures of a query compound labile towards a biotransformation reaction are checked in the knowledge base. Second, an absolute reasoning algorithm evaluates the possibility of that reaction taking place on five likelihood levels: probable, plausible, equivocal, doubted, and improbable [30]. Last, relative reasoning ranks concomitant metabolic reactions to remove the more improbable transformations. The following prediction parameters were used here: metabolism was outlined to include phase I reactions, the maximum number of metabolic steps was set at four, and the maximum number of predicted metabolites was 400. Absolute reasoning likelihood included probable and plausible levels for 3,4-DMMC and α -PVP and was expanded to cover equivocal level for 2-DPMP and MPA. Relative reasoning level was set at one, and mammals were selected as species. Absolute reasoning was set at a higher likelihood level for 3,4-DMMC and α -PVP because at the equivocal level, Meteor predicted over 200 metabolites for these compounds, which was thought to include an excessive number of false-positive predictions. After processing, duplicate metabolites and metabolites with a molecular mass lower than 100 were removed from the results, as they were thought most likely to be false-positive predictions. A database with the molecular formulae of both the origin compound and the predicted metabolites for each designer drug was created in Excel 2003 (Microsoft, Redmond, WA, USA).

Metabolite prediction based on published analogous reactions

The metabolism of the compounds studied was also predicted from the published metabolic reactions of their structural analogs in order to test the ability of the Meteor software to predict designer drug metabolism. The metabolism of 2-DPMP was deduced from the metabolism of phencyclidine-derived designer drugs [3]. Phencyclidines are known to undergo *N*-dealkylation, which in this case was thought to result in opening of the piperidine ring, and further oxidation of the primary alcohol. Those metabolites predicted after β -keto-structured cathinones, especially 4-methylmethcathinone (4-MMC, mephedrone) [3, 31], were added to the list of published metabolites of 3,4-DMMC [23]. The metabolites of α -PVP were taken from the study of Sauer et al. [22] in rats, supplemented with the metabolism of other pyrrolidinophenone-derived designer drugs [3, 5]. The MPA metabolites were based both on the published normetabolite [24] and as predicted by methamphetamine [32], as well as thiophene-structured compounds [33]. Metabolic

reactions were thought to be overlapping, even though this resulted in unreported metabolites. An Excel-based metabolite database of each designer drug was created as above.

Liquid chromatography/quadrupole-time-of-flight-mass spectrometry

The liquid chromatograph was a Waters Acquity Ultra Performance instrument (Milford, MA, USA) including a high-pressure mixing binary-gradient pump with a six-channel solvent degasser, a sample manager, and a thermostated column manager. A Luna PFP(2) column (pentafluorophenyl, 100×2 mm, 3 μm) and a PFP pre-column (4×2 mm, from Phenomenex, Torrance, CA, USA) were used for separation in a gradient mode at 40 °C. The mobile phase components were 2 mM ammonium acetate in 0.1 % formic acid and methanol. The flow rate was 0.3 mL/min, and the pre-run time was 5 min. The gradient started at 5 % of methanol and was increased to 40 % in 5 min, to 75 % in 13.50 min, to 80 % in 16 min, and held at 80 % for 2 min. A partial loop with needle overfill mode was used for injection, and the volume was 3 μL for the urine samples and 7.5 μL for the HLM incubation samples.

The mass spectrometer was a Bruker Daltonics micrOTOF-Q instrument (Bremen, Germany) with an orthogonal electrospray ionization source. The nominal resolution of the instrument was 10,000 FWHM. It was operated in a positive ion mode over a m/z range of 50–800. The drying gas flow was 8.0 mL/min, temperature was 200 °C, and the nebulizer gas pressure was 1.6 bar. The capillary voltage of the ion source was set at 4,500 V and the end plate offset at –500 V. In MS mode, the quadrupole ion energy was 5.0 eV, the collision cell radiofrequency 150.0 Vpp, the quadrupole transfer time 60.0 μs, and pre-pulse storage time 8.0 μs. The spectra time was 0.6 s and the spectra rolling average was set at 2. An external instrument calibration with sodium formate solution (10 mM NaOH in isopropanol and 0.2 % formic acid, 1:1 *v/v*) was performed using ten cluster ions (Na(NaCOOH)_{1–10}), m/z values from 90.9766 to 702.8635. The same calibrant was injected at the beginning and end of each sample run for post-run internal mass scale calibration.

AutoMS(n) methods were built for each designer drug for mass fragmentation. In the AutoMS(n) system, the instrument alternates MS and MS/MS modes in each spectrum. The selection of the precursor ion was based on a preselected mass list and an intensity threshold of 500 counts. If none of the ions from the list were detected, the most abundant ion was fragmented. A precursor ion list of the metabolites detected in the screening mode was constructed for each drug. A smart exclusion technique was used to reduce background noise, and an active exclusion mode allowed the rejection of the precursor ion after three spectra. The voltages for fragmentation were optimized by flow injection of a mixture of the compounds studied (1 μg/mL in methanol) and using an

auto-optimization function. The collision energy was 20–40 eV for ions between m/z 100 and 500, and the energy varied from 80 to 120 % of the set value using collision sweeping. Ion source and transfer parameters were as in the screening mode. Spectra time was shortened to 0.5 s.

Data analysis

DataAnalysis 4.1 (Bruker Daltonics, Bremen, Germany) was employed to process the analysis data. An automatic database search function was created for each designer drug in TargetAnalysis 1.2 (Bruker Daltonics, Bremen, Germany) to find the predicted metabolites. This reverse database search reported hits from the LC/Q-TOF/MS acquisition data within the selected identification criteria: peak area counts of 2,000, a mass tolerance of ±3 mDa, and an isotopic pattern match, mSigma, threshold of 200. The script has been described in more detail by Ojanperä et al. [34].

Rule-based fragmentation prediction software, MS/Fragmenter 12.01 (Advanced Chemistry Development, ACD/Labs, Toronto, Canada), was used to identify and aid in structural determination of the designer drugs and their metabolites. This software was used in our previous studies [20, 35] and is described in more detail in these papers. The function “atmospheric pressure positive ion protonation” was selected as the ionization type, and the number of fragmentation steps was set at five. Other fragmentation parameters outlined aromatic bond cleavage, ring formation, resonance reaction, hydride shift, heterolytic and homolytic cleavages, hydrogen rearrangements, and neutral losses. For some product ions unidentified by the MS/Fragmenter, a DataAnalysis built-in tool for MS spectra interpretation, SmartFormula3D [36], was used to provide additional information in structural determination of the metabolites.

Results and discussion

Table 1 shows the reactions used by the Meteor software for metabolite prediction, the pathways collected from the published analogous reactions, as well as the total number of predicted metabolites, with prediction likelihood levels for each compound.

The identified human urinary metabolites of the designer drugs studied are listed in Table 2 and described below in detail. The structures of the metabolites detected in the human urine samples and in vitro experiments were confirmed by comparing the mass spectra of the metabolites with the product ions identified for the parent compounds in MS/MS spectra using MS/Fragmenter and SmartFormula3D. Fragment prediction for each metabolite was also performed, to determine the site of the metabolic reaction in the molecule and also to differentiate between possible structural isomers. In the analysis

Table 1 List of biotransformation reactions (in alphabetical order) applied to designer drug metabolite prediction based on Meteor software and the analogous reactions found in the literature

2-DPMP	3,4-DMMC	α -PVP	MPA
Meteor (<i>n</i> =42) pro: <i>n</i> =5; pla: <i>n</i> =14; equ: <i>n</i> =23	Meteor (<i>n</i> =69) pro: <i>n</i> =9; pla: <i>n</i> =60	Meteor (<i>n</i> =15) pro: <i>n</i> =1; pla: <i>n</i> =14	Meteor (<i>n</i> =21) pla: <i>n</i> =14; equ: <i>n</i> =7
β -Oxidation of carboxylic acids (pla) Decarboxylation (equ) Hydrolysis of cyclic carboxyamides (equ) Hydroxylation of aromatic methine (equ) Lactams from aza-alicyclic comp. (pla) <i>N</i> -hydroxyl. of secondary amines (equ) Oxidation of primary alcohols (pro) Oxidation of secondary alcohols (pro) Oxidative deamination (equ) Oxidative <i>N</i>-dealkylation (pla) Para-hydroxylation of benzenes (pla) Reduction of aliphatic ketones (pla) Published analogous reactions (<i>n</i> =14) Dehydrogenation Hydroxylation <i>N</i>-dealkylation \rightarrow ring opening Oxidation	5-Hydroxyl. of 1,2,4-subst. benzenes (pla) Hydroxylation of aromatic methyl (pro) Hydroxylation of terminal methyl (pla) Oxidation of primary alcohols (pla) Oxidation of secondary alcohols (pla) Oxidative <i>N</i>-demethylation (pro) Reduction of aliphatic ketones (pla) Published analogous reactions (<i>n</i> =11) Hydroxylation <i>N</i>-demethylation Oxidation Reduction	Hydroxylation of alkyl methylene (pla) Hydroxylation of terminal methyl (pla) Lactams from aza-alicyclic comp. (pla) Oxidation of primary alcohols (pla) Oxidation of secondary alcohols (pla) Oxidative <i>N</i>-dealkylation (pro) Reduction of aliphatic ketones (pla) Published analogous reactions (<i>n</i> =23) Degradation of pyrrolidine ring Dehydrogenation Hydroxylation Ring opening + oxidation	Benzylic hydroxylation (pla) Decarboxylation (equ) Hydroxylation of terminal methyl (pla) <i>N</i> -hydroxylation of secondary amines (equ) Oxidation of primary alcohols (pla) Oxidation of secondary alcohols (pla) Oxidative deamination (equ) Oxidative <i>N</i>-demethylation (pla) Reduction of aliphatic ketones (pla) Published analogous reactions (<i>n</i> =13) Hydroxylation <i>N</i>-demethylation Sulfoxidation

Total number of predicted metabolites and their likelihood levels are in brackets. True identified metabolic reactions are presented in bold *pro* probable likelihood level, *pla* plausible likelihood level, *equ* equivocal likelihood level

of the wash methanol (see “Urine sample preparation”), no designer drug metabolites other than those seen in the urine samples were detected.

2-DPMP

2-DPMP (at *m/z* 252.1747) was found to undergo extensive oxidative metabolism, including aromatic hydroxylation, hydroxylation at the piperidine ring, and oxidation after ring opening. Dehydrogenated hydroxy metabolites were also found, resulting in six identified phase I metabolites in total. The proposed phase I metabolism of 2-DPMP is presented in Fig. 1a. Several characteristic product ions could be identified with the MS/Fragmenter for 2-DPMP and these are presented

in detail in Fig. 2a. Product ions at *m/z* 193.1012, 181.1012, 167.0855, and 91.0542, or their modifications formed in metabolic reactions, could be detected from the MS/MS spectra of the metabolites, making the identification reliable.

Six peaks fitting the exact mass of *m/z* 268.1696, corresponding to a hydroxylated metabolite, were observed in the total ion chromatograms of the urine samples. The site of the hydroxylation reaction was concluded to take place at both aromatic (M1) and piperidine (M2) rings, which could be differentiated by their characteristic product ion spectra. Product ions (exact masses) at *m/z* 197.0961 (C₁₄H₁₃O) and 183.0804 (C₁₃H₁₁O), formed by the addition of a hydroxyl group to the corresponding 2-DPMP product ions (Fig. 2a), indicate

Table 2 Phase I metabolites identified for 2-DPMP, 3,4-DMMC, α -PVP, and MPA in authentic human urine samples. Identification criteria were ± 3 mDa for mass error, mSigma threshold of 200, and peak area counts of 2,000

Metabolite found in human urine in vivo	Metabolic reaction	Formula	Rt (min)	[M+H] ⁺ (m/z)	Found in vitro	Found in silico	Deduced from analogous reactions
2-DPMP		C ₁₈ H ₂₁ NO	7.10	252.1747			
M1 ^a	Hydroxylation (aromatic)	C ₁₈ H ₂₁ NO	5.06 6.30	268.1696		X	X
M2 ^a	Hydroxylation (aliphatic, i.e., piperidine ring)	C ₁₈ H ₂₁ NO	5.86 6.09 6.50 8.68	268.1696			X
M3 ^a	Hydroxylation (aliphatic) + dehydrogenation	C ₁₈ H ₁₉ NO	12.11	266.1539	X	X	X
M4 ^a	2 × hydroxylation (aliphatic) + 1 × dehydrogenation	C ₁₈ H ₁₉ NO ₂	10.46 10.83	282.1489			X
M5	3 × hydroxylation (aliphatic and aromatic) + 1 × dehydrogenation	C ₁₈ H ₁₉ NO ₃	8.34	298.1438			X
M6	2 × hydroxylation (aromatic) + ring opening + oxidation	C ₁₈ H ₂₁ NO ₄	9.97	316.1543			X
3,4-DMMC		C ₁₂ H ₁₇ NO	5.89	192.1383			
M1 ^a	N-demethylation	C ₁₁ H ₁₅ NO	5.51	178.1226	X	X	X
M2 ^a	Reduction	C ₁₂ H ₁₉ NO	5.45	194.1539	X	X	X
M3 ^a	Reduction + N-demethylation	C ₁₁ H ₁₇ NO	4.88 5.06	180.1383		X	X
M4 ^a	Hydroxylation	C ₁₂ H ₁₇ NO ₂	3.18 3.67	208.1332	X X	X	X
M5	N-demethylation + hydroxylation	C ₁₁ H ₁₅ NO ₂	5.88	194.1176		X	X
M6	Reduction + hydroxylation + oxidation	C ₁₂ H ₁₇ NO ₃	4.68	224.1281	X	X	X
α -PVP		C ₁₅ H ₂₁ NO	5.70	232.1696			
M1 ^a	Reduction	C ₁₅ H ₂₃ NO	5.84	234.1852	X	X	
M2	Hydroxylation	C ₁₅ H ₂₁ NO ₂	5.35	248.1645	X	X	X
M3 ^a	Hydroxylation + dehydrogenation	C ₁₅ H ₁₉ NO ₂	12.02	246.1489	X	X	X
M4 ^a	Reduction + hydroxylation + dehydrogenation	C ₁₅ H ₂₁ NO ₂	8.90 9.95	248.1645		X	
M5	Degradation of pyrrolidine ring	C ₁₁ H ₁₅ NO	4.95	178.1226	X		X
M6	Hydroxylation + dehydrogenation + ring opening + oxidation	C ₁₅ H ₂₁ NO ₃	5.97 6.67	264.1594	X		X
M7	Hydroxylation + oxidation	C ₁₅ H ₁₉ NO ₃	10.42	262.1438		X	
MPA		C ₈ H ₁₃ NS	3.13	156.0841			
M1 ^a	N-demethylation	C ₇ H ₁₁ NS	2.79	142.0685	X	X	X

^a Abundant metabolites in human urine

aromatic hydroxylation. The product ion spectra from the M1 peaks were identical; thus, differentiation of the compounds was not possible, nor could the exact site of the hydroxylation be determined. Metabolite M1 was detected in HLM experiments only at trace levels.

In addition to the corresponding product ions of 2-DPMP, the loss of water was detected in the spectra of metabolites

M2 hydroxylated at the piperidine ring. Metabolites M2 were present in relatively high abundance in the urine samples, signifying that hydroxylation at the piperidine ring is the main phase I metabolism route of 2-DPMP. These peaks are possibly a mixture of regioisomers and conformational isomers, forming sum spectra, as the peaks were not fully separated at their baseline. The metabolite M2 eluting at

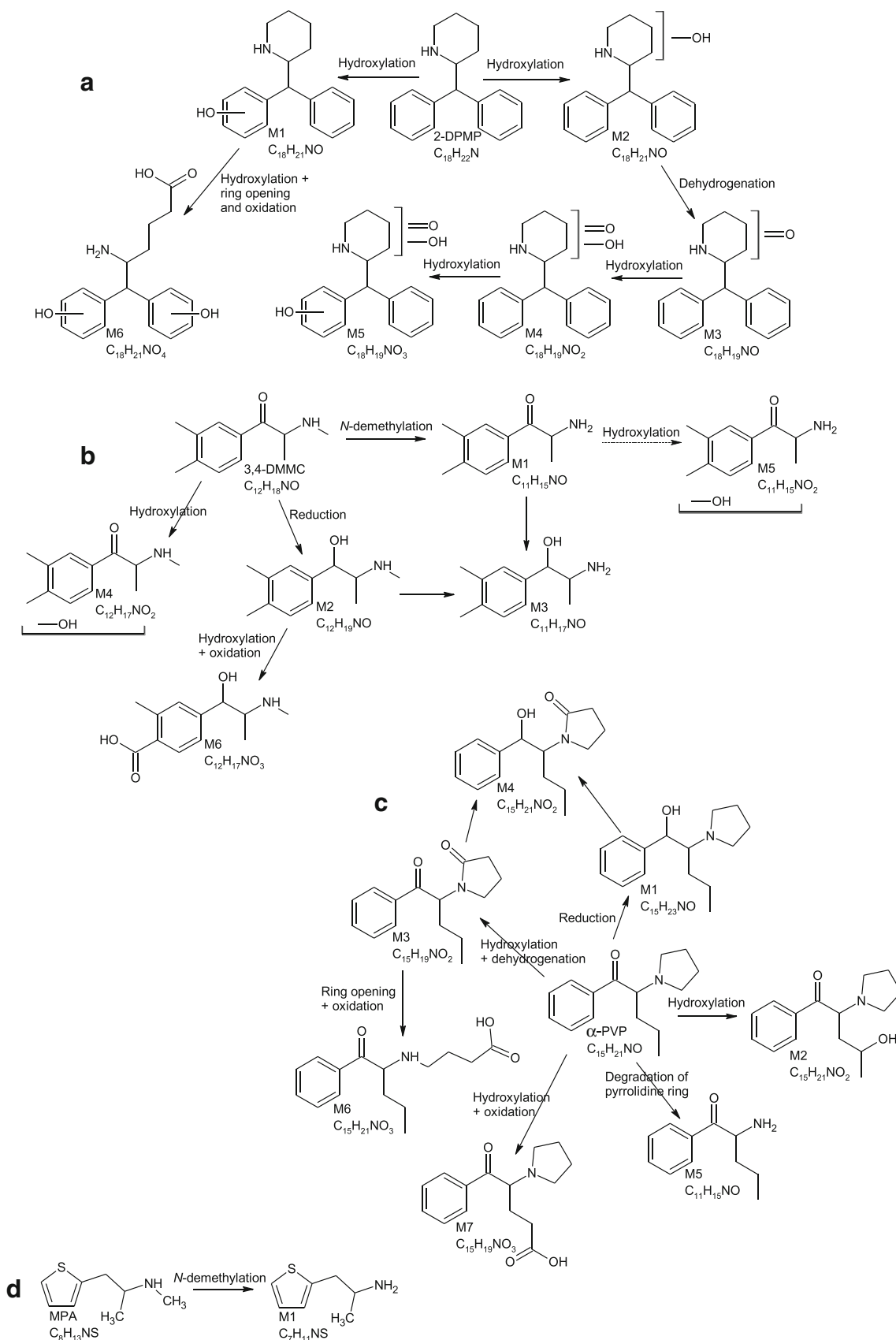


Fig. 1 Proposed phase I metabolism of 2-DPMP, 3,4-DMMC, α -PVP, and MPA in humans. Metabolite M5 of 3,4-DMMC (dashed arrow) was identified solely by exact mass and isotopic pattern comparison

8.68 min was a minor urinary metabolite, but it was present in the HLM samples in rather high abundance. The Meteor software did not predict hydroxylation at carbon atoms in the piperidine ring under the chosen reasoning constraints. However, the α -carbinolamine structure is expressed as a reaction intermediate in the lactam (metabolite M3) formation. In the absence of an intermolecular hydrogen bonding stabilization, this structure is generally not observed in vivo. Because of the relative reasoning filter applied, biotransformations expressing hydroxylation at the alicyclic carbon atoms are discarded. With a lower filter, these metabolites would also have been observed. *N*-hydroxylation at the piperidine nitrogen was predicted for 2-DPMP by Meteor (Table 1) at equivocal likelihood level. *N*-hydroxylated secondary aliphatic amines may react further to produce more complex compounds [37], which means that these metabolites could not be detected in human urine. The presence of this metabolite is therefore rather doubtful.

2-DPMP M2 metabolites were found to undergo dehydrogenation (M3 at m/z 266.1539) to a corresponding lactam or ketone. In the HLM samples, three peaks showing $[M+H]^+$ at m/z 266.1539 at 11.00, 11.47, and 12.11 min were observed. Traces of these two first eluting compounds were also present in the urine samples, and thus, reliably interpretable MS/MS spectra could not be produced to confirm the structures.

The 2-DPMP metabolite M4 (at m/z 282.1489) was detected in rather high abundance. It was formed via two hydroxylation reactions at the piperidine ring, one of the hydroxyl groups subsequently undergoing dehydrogenation. The metabolic reactions of M4 were found to take place only at the piperidine ring, as the product ion at m/z 167.0855 indicated the aromatic rings were not hydroxylated. The loss of H_2O (at m/z 264.1383), followed by the loss of CO (at m/z 236.1434) and NH_3 (at m/z 219.1168), confirmed this presumption. A minor peak of M4, detected at 10.46 min, indicates possible isomerism of this metabolite. Metabolite M5 (at m/z 298.1438) was formed via aromatic hydroxylation of M4. Characteristic product ions for aromatic hydroxylated compounds (see M1 above) were detected for metabolite M5. In addition, product ions formed via loss of H_2O , CO, and NH_3 could be seen, similar to M4, at 15.9949 atomic mass units and higher m/z values. This shows a difference of one hydroxyl group between M4 and M5, located outside the piperidine ring system. M4 and M5 were not detected in in vitro experiments nor were they predicted by the Meteor software.

2-DPMP was found to undergo oxidative *N*-dealkylation resulting in ring opening of the piperidine structure and oxidation of the primary alcohol to a carboxylic acid. Metabolite M6 (at m/z 316.1543) was found to comprise the previous structure and in addition was twice hydroxylated at the aromatic rings. Product ions $[M+H-NH_3]^+$ at m/z 299.1278 and $[M+H-NH_3-CO_2H_2]^+$ at m/z 253.1223, and those identical to other

Fig. 2 Proposed fragmentation schemes identified using fragment prediction for 2-DPMP, 3,4-DMMC, α -PVP, and MPA and the corresponding MS/MS spectra. Product ions denoted with an asterisk (*) were detected in metabolite MS/MS spectra

aromatic hydroxy metabolites, specified this structure. Meteor suggested an oxidative *N*-dealkylation reaction, followed by oxidation of the primary alcohol. Nevertheless, the formation of M6 was not predicted by the software, as the likelihood of sequential hydroxylation reactions in the two aromatic rings is assigned as doubted.

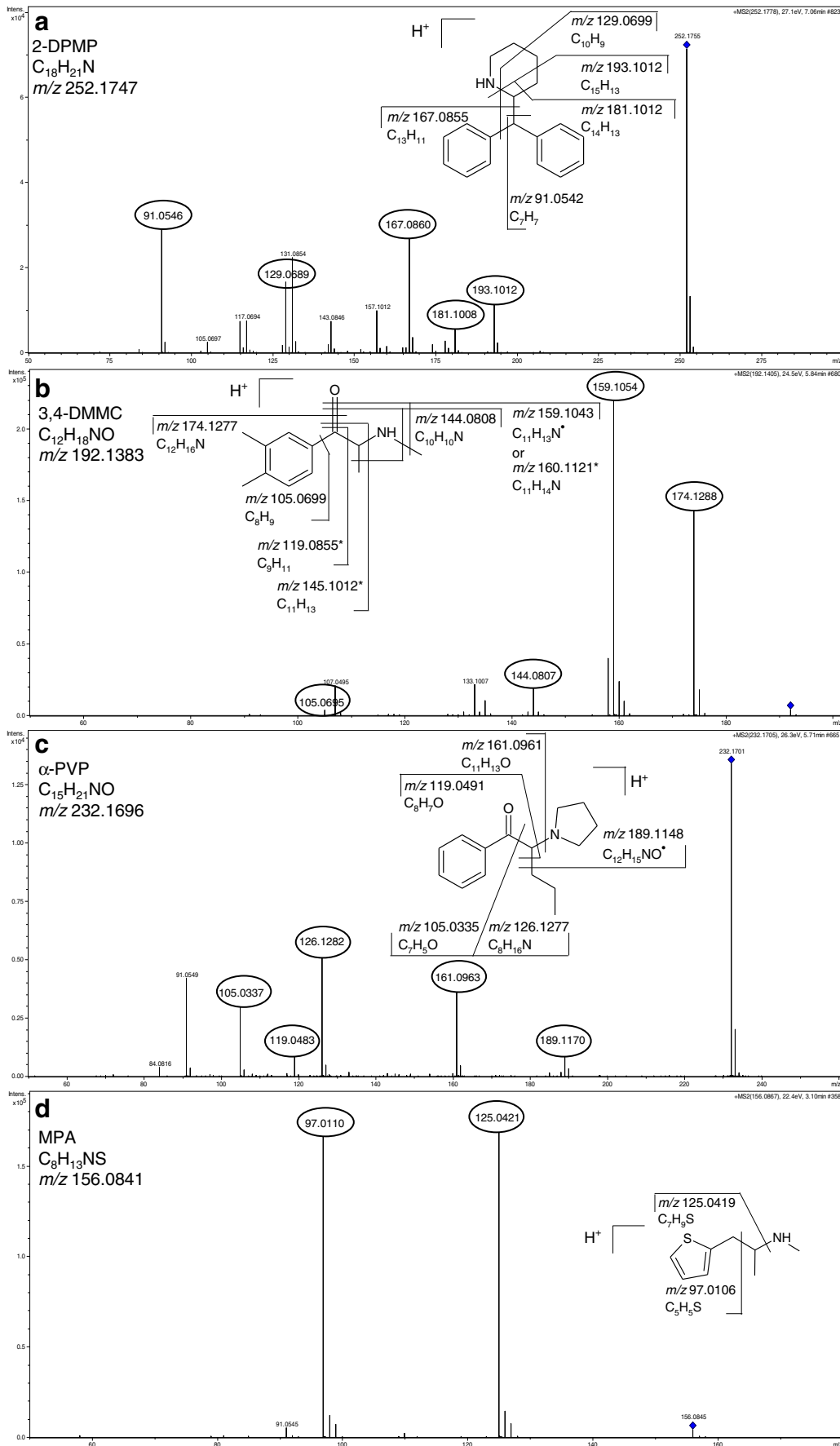
Great individual variation in metabolite abundances was noticed between the 2-DPMP cases studied, which makes the determination of the main metabolites difficult. Metabolites M1 and M2 were identified in nine cases out of ten, indicating hydroxy metabolites would be the primary phase I human metabolites. Metabolites M3–M6 were thought to be minor 2-DPMP metabolites, as the intensities of the compounds were in some cases quite low. The number of other toxicological findings in the urine cases studied was up to 15. Possible drug–drug interactions may have an influence on the metabolic ratios. The urine concentrations of 2-DPMP itself varied greatly as well, and thus, care should be taken when concluding the quantity of the metabolites.

3,4-DMMC

Six metabolites could be identified for 3,4-DMMC in human autopsy urine cases (Table 2). They included the recently reported [23] *N*-demethylated (M1) and reduced metabolites (M2) and the combination of the reactions (M3). Hydroxylated (M4 and M5) and further oxidated (M6) metabolites were detected and identified here as well. Compounds M4 and M6 have previously been reported as putative metabolites, as the identification was based on nominal mass MS/MS data and relative retention times [23]. All metabolites identified here were predicted by the Meteor software. The proposed metabolism of 3,4-DMMC is presented in Fig. 1b.

In the MS/MS spectrum of 3,4-DMMC (at m/z 192.1383), two main peaks formed after fragmentation of the ketone group (at m/z 174.1277), together with further loss of a methyl radical (at m/z 159.1043), were detected (Fig. 2b). Fragmentation of the metabolites mainly followed the path of 3,4-DMMC. An additional loss of water was detected in the MS/MS spectra of hydroxylated and oxidated metabolites. Other characteristic product ions, identified for the metabolites using the MS/Fragmenter, are presented with an asterisk (*) in Fig. 2b.

Two peaks were identified for metabolite M3 (at m/z 180.1383), formed via *N*-demethylation and reduction reactions (Table 2). The product ion spectra of these compounds were identical, indicating the formation of diastereomers [23].



Racemization of the reduced metabolite M2 could not be shown in the analysis of the urine and in vitro samples. However, confirmation of the possible isomerism of M2 would require further analysis under different separation conditions, e.g., with a chiral column.

Two peaks for the hydroxylated metabolite M4 (at m/z 208.1332) were detected in urine and HLM samples (Table 2). Previous studies [23, 29] indicate that the hydroxylation takes place at the aromatic methyl group. In addition to this, Meteor predicted hydroxylations at terminal methyl groups of the side chain (Table 1). The MS/MS spectra of the hydroxy metabolites were identical in both ion quality and abundance, possibly indicating that the hydroxylation reaction takes place at either of the aromatic methyl groups. The prediction likelihood also applies to the aromatic methyl hydroxylation (Table 1). However, hydroxylation of the aliphatic methyl group could not be excluded.

A previously unreported metabolite of 3,4-DMMC, metabolite M5, resulted from *N*-demethylation and hydroxylation (at m/z 194.1176). It eluted at the same time as 3,4-DMMC (at m/z 192.1383); thus, a characteristic MS/MS spectrum of M5 could not be produced, as the separation mass window in the quadrupole had to be wide enough to utilize the isotopic pattern match comparison in the compound identification in MS/MS analysis. The identification of M5 is based on accurate mass measurement only, and further studies should be carried out to confirm its existence.

Metabolite M6 (at m/z 244.1281) was produced via reduction of the ketone group, followed by hydroxylation and oxidation of the aromatic methyl group. The most abundant product ion in its spectrum at m/z 174.0913 corresponded to the structure $C_{11}H_{12}NO$ ($[M+H-H_4O_2-CH_2]^+$). Detection of an ion at m/z 137.0597 (product ion at m/z 105.0699+ O_2 , Fig. 2b.) showed that the position of the carboxylic acid group was in the xylyl methyl. Metabolite M6 was confirmed in only one of the urine samples and was detected in vitro only as trace levels, which would indicate it is a minor metabolite of 3,4-DMMC.

α -PVP

α -PVP (at m/z 232.1696) was metabolized extensively, as seven phase I metabolites were detected in the human urine samples (Table 2). The proposed metabolism is presented in Fig. 1c. Characteristic product ions were identified using fragment prediction (Fig. 2c). α -PVP was found to metabolize by hydroxylation at the propyl side chain (M2 at m/z 248.1645), hydroxylation followed by dehydrogenation at the pyrrolidine ring to form a lactam structure (M3 at m/z 246.1489), and degradation of the pyrrolidine ring to a primary amine (M5 at m/z 178.1226). These metabolites were previously identified in rat urine [22] and were found here in the HLM samples as well. For the hydroxy- α -PVP (M2), the

product ion at m/z 189.1148 ($[M+H-C_3H_7O]^+$) showed that the hydroxylation takes place at the propyl side chain. Metabolite M3 showed a characteristic product ion of a γ -lactam structure at m/z 86.0600 (C_4H_8NO). For metabolite M5, product ions corresponding to the loss of water (at m/z 160.1121) followed by the loss of a propyl side chain (at m/z 118.0651) were identified.

Metabolite M1 (at m/z 234.1852), formed by reduction of the ketone structure to a corresponding alcohol, was the most abundant metabolite of α -PVP in both human urine and in vitro experiments. Loss of water, followed by pyrrolidine ring loss, or propyl side chain loss as a radical cation was identified as an indication of the hydroxylated structure. This metabolic reaction has not previously been reported for α -PVP [22]. Here, it was predicted by the Meteor software. Metabolite M4 (at m/z 248.1645) was found to be derived through a combination of reduction (M1) and lactam formation (M3) reactions. Metabolite M4, which has two chiral atoms, was expressed as potential diastereomers [38, 39], at 8.90 and 9.95 min, with identical MS/MS spectra. The structure of M4 was verified by identification of product ions at m/z 230.1539 from the loss of water and the loss of the lactam ring at m/z 145.1012.

Metabolite M6 (at m/z 264.1594) was formed from metabolite M3 by oxidation after pyrrolidine ring opening. Product ions from the loss of water, the loss of acetic acid, and the loss of aminobutyric acid were identified, demonstrating the proposed structure. Two peaks of M6 with identical MS/MS spectra and ion abundances were detected (Table 2), indicating the possible formation of enantiomers. In the HLM incubations, only one form of this metabolite was produced, as only the first eluting compound was observed.

Hydroxylation followed by oxidation at the propyl side chain produced metabolite M7 (at m/z 262.1438). This proposed structure was based on the detection of benzaldehyde and a loss of propanoic acid from the parent compound. Oxidation of the propyl side chain was not identified for α -PVP in rat urine [22] nor was it reported for other pyrrolidinophenone derivatives [38–44]. Metabolite M7 was predicted by Meteor, but not seen in the in vitro tests.

An additional metabolite of α -PVP, formed via reduction and hydroxylation (at m/z 250.1802), was detected in HLM incubations, but was not present in human urine samples, however.

Four of the seven metabolites identified here for α -PVP were new metabolites, and two of them formed via an unreported metabolic reaction, i.e., reduction of the β -ketone and oxidation of the propyl side chain. Three out of the ten phase I metabolites of α -PVP detected in rat urine [22] were identified here. For the pyrrolidinophenone derivatives studied in rats, reduced metabolites have only been reported as minor metabolites for α -pyrrolidinopropiophenone, PPP [38], 4'-methyl- α -pyrrolidinohexanophenone, MPPH [39], and 4'-

methyl- α -pyrrolidinobutyrophenone, MPBP [43]. Here, the reduced metabolite M1 was found to be as the most abundant metabolite in all eight human urine samples studied. The fact that reduction of the ketone group was not detected in rats may be because of differences between species in the dominant metabolic reactions [45, 46]. Metabolites formed via reduction of the oxo group in humans have been reported for β -keto-structured cathinones [31, 47, 48], however. Based on the findings in this study, reduction of the ketone should be taken into consideration in the identification of human urinary metabolites of pyrrolidinophenone derivatives.

Sauer et al. [22] proposed that the urine screening procedure used for α -PVP, which is metabolized to a great extent in rats, should be based on its metabolites. Although α -PVP also undergoes significant metabolism in humans, the drug itself was the most abundant finding in all urine samples analyzed. Thus, identification of the metabolites in conjunction with α -PVP in human urine makes identification of this compound substantially more reliable.

MPA

In the analysis of three urine cases that tested positive for MPA (at m/z 156.0841), the *N*-demethylated metabolite nor-MPA (M1 at m/z 142.0685) was identified (Fig. 1d and Table 2). The product ions identified were identical for MPA and nor-MPA (Fig. 2d). Traces of MPA hydroxy metabolites could also be seen in the in vitro experiments; however, they could not be identified in human urine. The results are in concordance with the recent paper by Welter et al. [24], who stated that MPA metabolized to only a minor extent, as only one phase I metabolite, nor-MPA, was detected in human urine.

Feasibility of in silico and in vitro experiments in identification of metabolites in vivo

The likelihood level of a metabolic reaction calculated by Meteor was found to be a reasonable indicator of the probability of the prediction. All the reactions identified were either at a probable or plausible level. Four of the five probable reactions, oxidation of primary alcohol, hydroxylation of aromatic methyl, and oxidative *N*-demethylation or *N*-dealkylation (Table 1), were found to occur. The predictions proved to be most successful for the phenethylamine-structured designer drugs: Meteor predicted all the identified metabolites of 3,4-DMMC and MPA, as well as five of the seven metabolites of α -PVP. It improved the identification of the previously unreported metabolic reactions for α -PVP: reduction of the ketone group and oxidation of the propyl side chain, resulting in detection of three new metabolites. The Meteor prediction results were less successful for 2-DPMP, as only two of the six metabolites identified had been proposed. One of the most abundant metabolites of 2-DPMP, M2, was predicted only as an intermediate in the

formation of a lactam structure. It is likely that Meteor finds only a few metabolic reactions from its knowledge base for compounds structurally similar to 2-DPMP, as most of the predictions were created at the equivocal likelihood level. This would explain the low prediction sensitivity [17]. The unpredicted metabolites would probably be achievable by widening the Meteor processing settings. This would, however, also produce a great number of false-positive predictions. The Meteor software has shown a tendency towards overprediction [17], as also discovered in this study. Thus, the relevance of metabolites predicted at a likelihood level lower than “plausible” is at least questionable when the aim is to identify the main urinary metabolites.

In silico metabolite prediction has not previously been applied to designer drugs, and one of the goals in this study was to evaluate the applicability of the Meteor software in this area. Based on the findings in this study, Meteor is a suitable tool for predicting the main human phase I metabolites for amphetamine analogs and phenethylamine analogs. For phencyclidine compounds, or structurally completely novel designer drugs, the prediction results need to be viewed more critically. It is necessary to evaluate the suitability of in silico prediction for each designer drug class individually. The software predictions should always be compared with biological material, in vivo or in vitro samples, to screen for the true-positive metabolites.

Drug metabolism prediction based on published analogous reactions is time consuming and, in the case of structurally new compounds, sometimes uncertain. Although metabolite prediction in silico did not prove here to be better than our own judgment based on the metabolic reactions of structural analogs, it definitely speeds up the creation of the list of possible metabolites to be used in an automated database search for Q-TOF/MS data.

Eight of the 12 most abundant phase I metabolites of the four designer drugs studied in human urine could be detected in the in vitro HLM experiments. A few designer metabolites detected in the HLM samples in minor abundance were not seen in human urine. Thus, the difference between in vitro and in vivo metabolism [6] should be taken into consideration when extrapolating the metabolic data from in vitro experiments to identification of metabolites in vivo. Although in vitro tests do not absolutely predict human in vivo metabolism [6], they definitely serve as material for exploitation of in silico data when an authentic urine sample is not available. The results can be extrapolated for qualitative drug screening analysis in toxicology to support a positive finding of a parent compound. However, to perform in vitro metabolite experiments on designer drugs requires the availability of reference standards for the parent drugs, which limits the usage of the procedure for rare compounds.

The metabolites proposed for the designer drugs studied here do not necessarily cover all the possible phase I metabolites of the compounds. Drug–drug interactions may also

have an influence on the metabolite ratios. A comprehensive designer drug metabolism study on forensic cases is demanding, as information about the possible intake of the drug, or the time since intake, is rarely available. The benefits of *in silico* predictions are in qualitative metabolite identification which was the main objective of this study.

Conclusion

Applying *in silico* and *in vitro* experiments to support identification of designer drug metabolites in drug abusers' urine samples by LC/Q-TOF/MS was both effective and straightforward. The LC/Q-TOF/MS instrumentation used provided sufficient sensitivity for identification of designer drug metabolites in a complex biological matrix that also contained a great number of prescription drugs and street drugs. Compound identification with the automated reverse database search method was feasible, even though the metabolite peaks were partly overlapping. Structural characterization of fragments by accurate mass data, assisted by the MS/MS data interpretation tool MS/Fragmenter, served well in the differentiation between structural isomers. Eleven previously unreported metabolites for the four designer drugs studied, 2-DPMP, 3,4-DMMC, α -PVP, and MPA, were identified here. Six metabolites, including hydroxylated and further dehydrogenated and oxidated compounds, were detected for 2-DPMP, for which the metabolism has not been published earlier. The hydroxy-*N*-desmethyl metabolite was a new product found in this study for 3,4-DMMC. Four of the α -PVP metabolites found here, formed via reduction of the β -ketone and oxidation reactions, were not detected in the earlier metabolism studies in rats. The *in silico* metabolite prediction method proved to be a rapid way to create a list of possible metabolites for a novel designer drug, which can further be screened from *in vitro* incubation samples to identify the true-positive metabolites. These tentatively identified metabolite formulas could then be added to the database used for routine urine drug screening, in order to facilitate designer drug identification in authentic urine samples.

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