In vitro metabolism studies on mephedrone and analysis of forensic cases

Anders Just Pedersen,* Lotte Ask Reitzel, Sys Stybe Johansen and Kristian Linnet

The stimulant designer drug mephedrone is a derivative of cathinone – a monoamine alkaloid found in khat – and its effect resembles that of 3,4-Methylenedioxymethamphetamine (MDMA). Abuse of mephedrone has been documented since 2007; it was originally a ‘legal high’ drug, but it has now been banned in most Western countries.

Using cDNA-expressed CYP enzymes and human liver microsomal preparations, we found that cytochrome P450 2D6 (CYP2D6) was the main responsible enzyme for the in vitro Phase I metabolism of mephedrone, with some minor contribution from other NAPDH-dependent enzymes.

Hydroxytolyl-mephedrone and nor-mephedrone were formed in vitro, and the former was purified and identified by nuclear magnetic resonance (NMR). In four forensic traffic cases where mephedrone was detected, we identified hydroxytolyl-mephedrone and nor-mephedrone again; as well as 4-carboxy-dihydro-mephedrone, which has been previously described; and two new metabolites: dihydro-mephedrone and 4-carboxy-mephedrone. Fragmentation patterns for all detected compounds were determined by a UPLC-QTOF/MSE system, and a fragmentation pathway via a conjugated indole structure was proposed for most of the metabolites. Blood concentrations in the forensic traffic cases ranged from 1 to 51 µg/kg for mephedrone, and from not detected to 9 µg/kg for hydroxytolyl-mephedrone. In one case, urine concentrations were also determined to be 700 µg/kg for mephedrone and 190 µg/kg for hydroxytolyl-mephedrone. All compounds were detected or quantified with an ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) system and an ultra performance liquid chromatography-time of flight/mass spectrometry (UPLC-TOF/MS) system. Copyright © 2012 John Wiley & Sons, Ltd.

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Introduction

In recent years, many new designer drugs have emerged, including a group of cathinone derivatives. One frequently occurring drug of this group is mephedrone, which was first synthesized in 1929 but did not appear as a drug of abuse until 2007. Mephedrone use has now been reported in most parts of the world, especially in the UK[1–4] and Sweden.[2,6,8,9] In Denmark, the drug has been detected in cases of seizures and in forensic traffic cases.

Little is known about the pharmacology of mephedrone. Until present, gas chromatography–mass spectrometry (GC-MS) analysis of human and rat urine has detected unchanged mephedrone, as well as metabolites of the drug, including nor-mephedrone, nor-dihydro mephedrone, hydroxytolyl-mephedrone, nor-hydroxytolyl-mephedrone, and 4-carboxy-dihydro-mephedrone[10] (Figure 1). The enzymes responsible for the metabolism have not been reported. Knowledge of the metabolism is of relevance for assessing the toxicity of a compound; for example, the possible impact of genetic variations and drug-drug interactions.[11] Here we present a study on the cytochrome P450 enzymes responsible for the Phase I in vitro metabolism of mephedrone, with identification of the main metabolite by nuclear magnetic resonance (NMR). Additionally, we present results of some forensic traffic cases involving mephedrone and the identification of two new (in vivo) metabolites. All compounds were detected or quantified by an ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) system and an ultra performance liquid chromatography-time of flight/mass spectrometry (UPLC-TOF/MS) system; additionally, the fragmentation pattern of all compounds was determined by a quadrupole time of flight/mass spectrometry (UPLC-QTOF/MS5) system.

Materials and methods

Reagents

Mephedrone was purchased from Toronto Research Chemicals (Toronto, Canada). Amphetamine-D5 (Cerilliant, Texas, USA) and orphenadrine (GEA, Copenhagen, Denmark) were used as internal standards (IS). Acetonitrile (LC-MS grade) and toluene (LC-MS grade) were obtained from Fisher Scientific (Leicestershire, UK).

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Fully automated SPE of blood and urine

Blood and urine samples were placed in the Tecan robot, where 200 μl of each sample was transferred to a 96-well plate and mixed with 20 μl of IS (amphetamine-D5 0.4 mg/L) solution and 800 μl of 8% ammonium acetate in acetonitrile. The mixtures were centrifuged, and 800 μl of the supernatant was loaded on SPE-columns and washed with 30% MeOH in water. Elutions were performed with 900 μl of acetonitrile containing 8% of 25% ammonium in water. The eluates were evaporated to dryness, reconstituted in 50 μl of 5% MeOH in water containing 1% formic acid, and 7.5 μl and 5 μl of the reconstituted eluate was analyzed by UPLC-TOF/MS and UPLC-MS/MS, respectively. This sample preparation method was validated and used for authentic forensic cases involving mephedrone. When a metabolite was detected in either the forensic samples or in the enzyme experiments described below, an aliquot of the extracted sample was also injected into the UPLC-QTOF/MS system to determine the fragmentation pattern.

Urine samples were extracted both without and following β-glucuronidase/arylsulphatase hydrolysis. Hydrolysis was performed by centrifuging the urine, then mixing 500 μl of the supernatant with 250 μl 1.0 M acetate buffer (pH 5.5) and 25 μl β-glucuronidase/arylsulphatase-solution (diluted 1:3 with water). This mixture was incubated at 40 °C for 18 h.

UPLC-MS/MS

Analysis was performed using an Acquity UPLC BEH C18, 1.7 μm, 2.1 × 100 mm column from Waters (Manchester, UK). The flow rate was 0.6 ml/min and the mobile phase consisted of acidic water (A) and acetonitrile (B) containing 0.05% formic acid at 50 °C. The gradient was programmed as follows: 0–7 min from 100% to 85% A, 7–11 min to 67.5% A, 11–11.10 min to 20% A, 11.10–12 min to 0% A, 12–12.10 min to 100% A, and 12.10–16.5 min at 100% A. The detection was performed by positive electrospray ionization (ESI+) operating in multiple reaction monitoring (MRM) mode. The determination was done by two MRM transitions for the following compounds: mephedrone 178 > 145 (quantifier) and 178 > 160; hydroxytolyl-mephedrone 194 > 146 (quantifier) and 194 > 158; and nor-mephedrone 164 > 146 (quantifier) and 164 > 131. For the remaining metabolites, only the molecular mass was measured: dihydro-mephedrone,180; 4-carboxy-mephedrone, 208; 4-carboxy-dihydro-mephedrone, 210; nor-hydroxytolyl-mephedrone, 180; and nor-dihydro-mephedrone, 166. For the IS (Amphetamine-D5) only one transition was determined 141 > 96. Argon was used as collision gas at 0.45 Pa, and the desolvation gas flow was fixed at 1100 L/hr. The source temperature was set at 120 °C, and the desolvation temperature at 450 °C.

Fully automated SPE extraction was used for sample preparation. Validation of the method was performed following the method of Mateszewski et al.[13] Matrix effect (ME), extraction recovery (RE), and process efficiency (PE) were calculated for mephedrone, hydroxytolyl-mephedrone, and nor-mephedrone. These three parameters were determined in quadruplicate for blood spiked at a low and a high level of the analytes. The linear range was determined for mephedrone and hydroxytolyl-mephedrone and the imprecision and accuracy were calculated from a QC blood sample spiked to 240 μg/kg with mephedrone.

UPLC-TOF/MS

UPLC separation and MS configuration were performed in accordance with our previously published protocols,[14] with the
exception of using a steeper gradient to reduce the run time. The mobile phase consisted of 0.1% formic acid and 100% acetonitrile (B). The gradient was run from 2–98% of solvent B from 0–5.0 min, and the column was then reconditioned with 100% solvent A (5–7 min). The TOF/MS was used in the positive mode, but some metabolized mephedrone samples were also injected in negative mode to ensure that we did not fail to detect any acid or neutral metabolites. The validation was performed for the UPLC-TOF/MS system as described for the UPLC-MS/MS system.

Some supplementary qualitative studies were performed by UPLC-QTOF/MS5 to identify the fragmentation pattern for mephedrone and its metabolites. The collision cell of this instrument was switched between two functions, collision energy of 10 V for no fragmentation, and ramping from 20 V to 40 V to create fragments. The rest of the settings of the detector were as previously published.13 Please notice that all exact and accurate masses presented in this article have a deviation of 0.55 mDa from the true value, as the calculation performed by MassLynx software uses the mass of the hydrogen instead of the proton for the mass calculation of [M + H]+. Because this deviation is also applied during mass axis calibration, there is no negative impact on the presented mass errors.

In vitro metabolism studies

We initially investigated the degradation of mephedrone and the formation of mephedrone metabolites by the following cDNA-expressed P450 CYP isoenzymes: 1A2, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, and 3A4. The reaction mixture consisted of 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 3.3 mM MgCl2, 0.4 U/ml glucose-6-phosphate dehydrogenase, and 0.05 mM sodium citrate in 0.1 M phosphate buffer at pH 7.4. Each enzyme (20 pmol/ml) was incubated with two concentrations of mephedrone (1 mM and 50 μM) in a final volume of 250 μl for 80 min, 37 °C. The incubation was quenched with the addition of 250 μl ice-cold acidic acetonitrile containing 0.5% formic acid and 300 μg/L orphenadrine as an internal standard. The solution was centrifuged for 10 min at 4 °C, and 7.5 μl of the supernatant was analyzed directly by the UPLC-TOF/MS system; a supplementary investigation of fragmentation patterns was also carried out by UPLC-QTOF/MS5.

We also investigated the metabolism of mephedrone by human liver microsomes (UltraPoolTM HLM 150, 1 mg protein/ml) and the S9 Liver fraction. The same experimental conditions were used as with the cDNA-expressed P450 CYP isoenzymes, except that the mephedrone concentration was 1.0 μM and various selective CYP enzyme inhibitors were added to the HLM incubations. The inhibitors and their concentrations are listed in Table 1. We also tested inhibitor combinations of quinidine with (S)-(+)-N-3-benzylnirvanol or omeprazole. At the time points 0, 10, 20, 35, 50, 70, 100, and 140 min, 20 μl aliquots of the incubations were quenched with ice-cold acidic acetonitrile containing 0.5% formic acid and 300 μg/L orphenadrine. Positive and negative control incubations were also prepared by omitting the inhibitor and NADPH regeneration system, respectively. All experiments were performed in triplicate and analyzed by the UPLC-TOF/MS and UPLC-QTOF/MS5 systems.

The main CYP2D6 metabolite of mephedrone (hydroxytolyl-mephedrone) was produced in preparative scale for NMR studies. The same experimental conditions were used as with the cDNA-expressed P450 CYP isoenzymes, except that the mephedrone concentration was 60 μM, and the total incubation volume was scaled up to 24 ml. After quenching and centrifugation, the supernatant was filtered and reduced to 10 ml with a nitrogen flow at 40 °C. A small quantity of this solution of unchanged mephedrone and metabolites was used for the validation of the analytical methods. The rest of the solution was further evaporated, purified with preparative LC, and the dried hydroxytolyl-mephedrone residue was quantified with the NMR method described below.

NMR (determination and metabolite quantification)

Methanol-d4 was used as the solvent for all NMR experiments. NMR data were recorded for the produced hydroxytolyl metabolite as well as for pure mephedrone (1 H-NMR, 13 C-NMR, COSY, and HSQC). A relaxation time of 1 s was adequate to fully relax all protons before the next impulse, which was especially important for the quantification experiment. An appropriate number of scans was set, which was 64 or less in all experiments. The NMR quantification of hydroxytolyl-mephedrone from the upscaled CYP2D6 incubation was performed as described by D. Dagnino et al.16 with toluene as internal standard. A solution of 3.3 μg/ml toluene in methanol-d4 was prepared and the dried residue of hydroxytolyl-mephedrone was dissolved in 600 μL of this solution. 1 H-NMR of the dissolved residue was recorded and hydroxytolyl-mephedrone was quantified by comparing the intensities of the five aromatic protons form tolune with those of the four aromatic protons from the metabolite.

Forensic cases

In our laboratory, mephedrone has been detected in four forensic traffic cases in 2010 and 2011. Blood was available for all cases, and urine was also available in one of the cases. One aliquot of the urine sample was hydrolyzed by β-glucuronidase/arylsulphatase and another was used without this treatment. The four blood samples and the urine sample were subjected to solid phase extraction and analyzed by UPLC-TOF/MS, UPLC-MS/MS, and UPLC-QTOF/MS5 as described above.

Results and discussion

Validation results

With the fully automated SPE extraction followed by analysis with UPLC-MS/MS the following results were obtained: Mephedrone (ME 83% SD 8%, RE 103% SD 8%, and PE 84% SD 8%), hydroxytolyl-mephedrone (ME 83% SD 9%, RE 80% SD 7%, and PE 67% SD 7%), and nor-mephedrone (ME 90% SD 7%, RE 97% SD 7%, and PE 86% SD 4%). For both mephedrone and hydroxytolyl-mephedrone there was a linear range from 1 μg/kg to 500 μg/kg and the imprecision (CV) of the QC blood sample spiked with mephedrone was less than 10%, and the accuracy ranged from 93% to 107%.

For the UPLC-TOF/MS system with the fully automated SPE extraction the same parameters were determined: Mephedrone (ME 75% SD 9%, RE 104% SD 8%, and PE 78% SD 5%),

<table>
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<th>Table 1. Specific inhibitors for cytochrome CYP enzymes and the concentrations (μM) used in the assays</th>
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<td>Inhibitor</td>
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<tr>
<td>Furafylline</td>
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<td>(S)-(+)-N-3-Benzylnirvanol</td>
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hydroxytolyl-mephedrone (ME 54% SD 11%, RE 77% SD 12%, and PE 42% SD 7%), and nor-mephedrone (ME 90% SD 14%, RE 92% SD 12%, and PE 86% SD 11%). Mephedrone and hydroxytolyl-mephedrone had linear ranges from 1 to 400 μg/kg and 1 to 200 μg/kg, respectively. The imprecision (CV) of the QC blood sample was less than 8%, and the accuracy ranged from 85% to 103%.

A ME value of 100% is equal to no matrix effect, a value less than 100% means that there is some suppression of the analytes (ion suppression), and a value higher than 100% is equal to ion enhancement in accordance to the definition of Mateszewski et al. All the ME results from the two different analytical methods are between 54% and 90% for all three analytes. This level is acceptable, even though 54% means that almost half of the ion signal has been suppressed. All RE's from the two different analytical methods are between 77% and 104% for all three analytes. This means that the SPE extraction is effective in relation to recovery of the analytes. The overall process efficiency (PE) is per definition equal to ME × RE. (Please notice that this is not the case with all the results above, as each result is the mean of four replications in two concentrations.) All PE results are between 67% and 86%, except for hydroxytolyl-mephedrone analyzed by UPLC-TOF/MS, where PE is only 42%.

**In vitro metabolism results**

Metabolism of mephedrone by recombinant CYP isoenzymes resulted in the formation of the metabolites hydroxytolyl- and nor-mephedrone (Figure 1, Table 2). The pure CYP enzymes 1A2, 2B6, 2C8, 2C18, 2C19, and 3A4 all formed detectable levels of metabolites, but no significant mephedrone depletion was observed (Figure 2). The metabolites were tentatively identified by UPLC-TOF/MS in positive mode, and no further metabolites were detected in negative mode. CYP2D6 significantly degraded mephedrone (Figure 2), and up-scaling of this experiment produced the hydroxytolyl metabolite in preparative scale, such that $^1$H-NMR of this metabolite could be performed.

The results of the metabolism in HLM are presented in Figures 3 and 4. The substrate depletion curve over time shows that the substrate depletion was not affected by the inhibitors furafylline, (S)-(−)-N-3-benzylnirvanol, omeprazole, or ketoconazole when compared to incubation with no inhibitor (Figure 3). Thus, neither 1A2, 2C19, 2C9, nor 3A4 contributed significantly to in vitro metabolism of mephedrone at the studied concentration level. However, inhibition of CYP2D6 with quinidine significantly reduced the substrate depletion, with only about 20% of the mephedrone having disappeared after 140 min compared to around 60% with no inhibition. The 20% disappearance might have been due to the action of other NADPH-dependent enzymes with activity towards mephedrone, as no substrate degradation was observed with the negative control that lacked NADPH. Inhibition by quinidine alone and combined with (S)-(−)-N-3-benzylnirvanol and omeprazole provided about similar results. Thus, although recombinant CYP enzymes (other than 2D6) were able to form metabolites, the inhibitor experiments using HLM suggested that they made no or at most a negligible contribution to the overall metabolism of mephedrone. In the HLM experiments, only formation of hydroxytolyl-mephedrone was observed (Figure 4). The same pattern as with the degradation of mephedrone was observed: only inhibition of CYP2D6 by quinidine suppressed the formation to an amount approaching the level of the negative control, whereas the other inhibitors hardly influenced the formation. Please notice that there was a minor background peak in all chromatograms (not shown) with the same mass and retention time as the hydroxytolyl metabolite. The contribution from this minor peak at incubation time 0 was subtracted from all the other measurements of the metabolite. This explains why some of the results in Figure 4 are slightly negative, and the formation of metabolite to around 15% is considered an artifact, which is further supported by the size of the error bars. Finally, the incubation with the 59 fraction formed the same hydroxytolyl metabolite, as observed in the HLM incubation.

![Figure 2. Mephedrone remaining after incubation with various recombinant CYP isoenzymes for 80 minutes. SEM (standard error of the mean) marked with bars.](image-url)
Supplementary description of identification of mephedrone and metabolites

In the present study, we observed five of the seven metabolites listed in Figure 1. Only hydroxytolyl- and nor-mephedrone were observed in the in vitro metabolism experiments, but, additionally, three of the other metabolites were observed in the forensic cases. A description of the identification of mephedrone and the observed metabolites by mass spectrometry and NMR is provided below.

Mephedrone

Formula C\textsubscript{11}H\textsubscript{15}NO; HRMS [M+H\textsuperscript{+}] calculated m/z 178.1232, found 178.1228 (−2.2 ppm); Fragments observed with QTOF/MS\textsuperscript{E} (listed in order of descending abundance) m/z 145.0890 (\textsubscript{CH\textsubscript{2}}O\textsuperscript{−} radical loss), 144.0818 (\textsubscript{CH\textsubscript{2}}O loss), 160.1152 (H\textsubscript{2}O loss), and 119.0844 (\textsubscript{C\textsubscript{2}H\textsubscript{5}}NO loss). These fragments of mephedrone have previously been reported from our laboratory.\textsuperscript{[15]} \textsuperscript{1}H-NMR (500 MHz, CD\textsubscript{3}OD) \(\delta\) 7.96 (d, \(J=8.2\ \text{Hz}\), 2 H, Ar-H), 7.42 (d, \(J=8.2\ \text{Hz}\), 2 H, Ar-H), 5.06 (q, \(J=7.3\ \text{Hz}\), 1 H, CH-CH\textsubscript{3}), 2.76 (s, 3 H, CH\textsubscript{3}-NH), 2.45 (s, 3 H, CH\textsubscript{3}-Ar), and 1.57 ppm (d, \(J=7.3\ \text{Hz}\), 3 H, CH-CH\textsubscript{3}). Couplings between the aromatic protons and the coupling between the protons in CH-CH\textsubscript{3} were further confirmed with H-H COSY correlation (not shown). \textsuperscript{13}C-NMR (500 MHz, CD\textsubscript{3}OD) \(\delta\) 197 (C=O), 148–130 (4 different aromatic C), 61 (C=O)-CH-NHCH\textsubscript{3}), 32 (CH\textsubscript{2}-NH), 22(CH\textsubscript{2}-Ar), and 16 ppm (CH\textsubscript{2}-CH-NHCH\textsubscript{3}). All outlined C-H bindings were confirmed by HSQC.

Fragment structures are proposed in Figure 5. The formation of radicals is rarely observed when using the relatively soft ESI\textsuperscript{[17]}.
but the proposed formation of the conjugated indole system is believed to stabilize and thereby favor the radical formation. The spontaneous formation of the indole system in the gas phase has to the authors’ knowledge not been reported before and needs further investigation to be fully verified, but the formation of radicals stabilized by the conjugated indole system has previously been published.\[14,19\] This is by no means a definitive verification of the fragment structures suggested in Figure 5, but it is to the authors’ knowledge the most likely fragmentation pattern, as the six double bond equivalents are hard to fulfill without the formation of a two-ring system.

The fragmentation of mephedrone using a tandem MS method has previously been reported.\[20\] In this study m/z 147.1 was detected instead of m/z 144.0818 and the molecular formula of the m/z 119 fragment was suggested to be C6H7O+.

The exact mass of this fragment is m/z 119.0497. Our accurate mass determination (m/z 119.0844) can exclude this fragment, but no conclusions can be made about which fragment is actually formed in the MS/MS because of the mass accuracy limitations with this method.

### Hydroxytolyl-mephedrone

This metabolite was detected both in vitro and in vivo. Formula C11H15NO2; HRMS [M + H+] calculated m/z 194.1181, found 194.1184 (1.5 ppm); Fragments observed with QTOF/MS\(^5\) (listed in order of descending abundance) m/z 158.0962 (2 × H2O loss), 131.0741 (C2H8O2 loss), 146.0963 (CH3O2 loss), 144.0818 (CH2O2 loss), 130.0655 (C2H7O2 loss), and 145.0868 (CH4O2 loss). \(^1\)H-NMR (500 MHz, CD3OD) \(\delta\) 8.04 (d, J = 8.1 Hz, 2H, Ar-H), 7.58 (d, J = 8.1 Hz, 2H, Ar-H), 4.97 (q, J = 7.3 Hz, 1H, CH-CH3), 4.73 (s, 2H, HO-CH2-Ar), 2.71 (s, 3H, CH3-NH), and 1.54 ppm (d, J = 7.3 Hz, 3H, CH3-H). Couplings between the aromatic protons and the coupling between the protons in CH-CH3 were further confirmed with H-H COSY correlation (not shown). It was not possible to record \(^13\)C-NMR because of the limited amount of the compound. The preparative CYP2D6 incubation of mephedrone produced 94 \(\mu\)g (0.49 \(\mu\)mol) of hydroxytolyl-mephedrone, determined with \(^1\)H-NMR quantification. This is an efficacy of 37% in relation to the amount of mephedrone used for the metabolite production. The calculations were performed from the sum of the intensities of the five toluene protons in relation to the two peaks from the two chemically different aromatic protons. The intensity of the five toluene protons was adjusted to 5 arbitrary units. This gave the two peaks from the metabolite an average intensity of 4.5 arbitrary units, each peak representing 2 protons. The amount of toluene is 0.22 \(\mu\)mol (600 \(\mu\)L * 33.2 \(\mu\)g/ml / 92.14 g/mol), which equals a metabolite amount of 0.49 \(\mu\)mol.

Hydroxytolyl-mephedrone is the major metabolite formed in vitro, and the only one which is formed in adequate amount for \(^1\)H-NMR. The accurate mass of this metabolite was measured to be m/z 194.1184, which is only −2.2 ppm from the exact mass of a hydroxylated mephedrone metabolite (m/z 194.1181). The metabolite had become more polar, which was observed from a relative retention time of 0.73 in relation to mephedrone. The \(^1\)H-NMR chemical shift of the aromatic methyl group was 2.45 ppm for mephedrone. This chemical shift was changed to 4.73 ppm for the hydroxyl metabolite, and the intensity was changed from 3H to 2H. The NMR data excludes all other hydroxylated metabolites, and the metabolite is consistent with the observations previously made by Meyer et al. in human and rat urine.\[10\]

The proposed fragment structures are presented in Figure 6, together with the fragmentation of methcathinone, which we have previously reported.\[15\] As observed with mephedrone itself these two compounds produced odd-electron fragments, i.e. radicals. The suggested formation of an indole structure together with the observed loss of a methyl radical is common to all proposed radical fragmentation pathways, and these processes are believed to drive the formation of the detected radical fragments. The actual order of the formation of the fragments
and which fragments that are formed from each other still remains to be investigated, but the radicals are suggested to lose a hydrogen atom to form an even-electron fragment. This pair of fragments with a mass difference corresponding to a hydrogen atom (1.0078 Da) is typical for this fragmentation pattern, except for cathinone. Our laboratory has previously reported the masses of cathinone and its fragments to be m/z 150.0919 [M + H]+, 132.0823 (H2O loss), 117.0584 (CH3O radical loss) and 105.0712 (CH3NO loss).[13] Again, we suggest that after the water loss the radical formation is driven by the methyl radical loss and probably with the indole formation, but the m/z 117.0584 fragment does not have an alkyl-group from which it can lose a hydrogen atom. This can explain why m/z 116.0500 (CH3O total loss) is not detected. This observation further supports the overall proposed fragmentation pattern with the indole formation, but still, the presented data cannot exclude that other fragmentation processes can take place.

Nor-mephedrone

This metabolite was detected both in vitro and in vivo. Formula C10H13NO; HRMS [M + H]+ calculated m/z 164.1075, found 164.1070 (+3.0 ppm); Fragments observed with QTOF/MS5 (listed in order of descending abundance) m/z 130.0657 (CH3O loss), 131.0712 (CH3O radical loss), and 146.0968 (H2O loss). Because of the minor amounts of this metabolite that were formed in vitro, no NMR data were recorded. The molecular formula determined from the accurate mass excludes all metabolites other than a demethylated form of mephedrone. The formation of nor-mephedrone by N-demethylation is the most likely metabolite, as was also concluded by Meyer et al. when they analyzed rat and human urine by GC-MS.[10] The masses of the fragments formed from nor-mephedrone were similar to those observed with methcathinone, but the retention time for methcathinone relative to mephedrone was determined to 0.82, which is significantly different from that of nor-mephedrone (0.96). Thus, the most likely demethylated metabolite is nor-mephedrone. The fragmentation of this compound is believed to follow a pattern similar to that of methcathinone proposed in Figure 6, beginning with water loss to form C9H9N+, followed by CH3 radical loss to form C8H8N+ and finally hydrogen atom loss to form C7H7N+. If the proposed metabolite structure is right, the formation of the two last fragments is hard to imagine without the formation of the indole ring system, in order to fulfill the six double equivalents.

Dihydro-mephedrone

This metabolite was only detected in vivo. Formula C11H17NO; HRMS [M + H]+ calculated m/z 180.1388, found 180.1388. Fragments observed with QTOF/MS5 (listed in order of descending abundance) m/z 162.1280 (H2O loss), 146.0965 (CH3O loss), and 147.1039 (CH3O radical loss). In relation to mephedrone, the relative retention time of dihydro-mephedrone was 0.98. The accurate mass detection identifies this metabolite as a compound with two more oxygen atoms than mephedrone. Meyer et al. previously identified the metabolite as 4-carboxy-dihydro-mephedrone.[10] The fragmentation pathway of both 4-carboxy-dihydro- and 4-carboxy-mephedrone is not as straightforward as for some of the other metabolites, except for the water loss. The formation of m/z 160.0762 from both carboxy metabolites is more complex and will require some intermediates, because losses must occur from each end of the molecules. Unfortunately, no intermediates were detected that could help solving the fragmentation pathway, and the structures of the metabolites could not be unequivocally identified by its QTOF-fragmentation pathway. In relation to mephedrone, the relative retention times of 4-carboxy-dihydro- and 4-carboxy-mephedrone were 0.87 and 0.92, respectively, supporting the structures of the two metabolites, as the 4-carboxy moiety is expected to make the molecule more polar. Furthermore, the 4-carboxy-dihydro metabolite is slightly more polar than the 4-carboxy metabolite, which is the same pattern observed with mephedrone in relation to dihydro-mephedrone.

Figure 7. Scheme for the proposed fragmentation of dihydro-mephedrone. The pathway with the formation of the dihydro-indole is unlikely because this does not lead to a more conjugated system that generally is considered to drive the radical formation.

4-carboxy-dihydro-mephedrone

This metabolite was only detected in vivo. Formula C11H15NO3; HRMS [M + H]+ calculated m/z 210.1130, found 210.1129 (+0.5 ppm). Fragments observed with QTOF/MS5 (listed in order of descending abundance) m/z 160.0757 (CH3O loss), 192.1020 (H2O loss), 132.0808 (C2H4O loss), and 117.0585 (C3H4O2 radical loss). The accurate mass detection identified this metabolite as a compound with two more oxygen atoms than mephedrone. Meyer et al. previously identified the metabolite as 4-carboxy-dihydro-mephedrone.[10] The fragmentation pathway of both 4-carboxy-dihydro- and 4-carboxy-mephedrone is not as straightforward as for some of the other metabolites, except for the water loss. The formation of m/z 160.0762 from both carboxy metabolites is more complex and will require some intermediates, because losses must occur from each end of the molecules. Unfortunately, no intermediates were detected that could help solving the fragmentation pathway, and the structures of the metabolites could not be unequivocally identified by its QTOF-fragmentation pathway. In relation to mephedrone, the relative retention times of 4-carboxy-dihydro- and 4-carboxy-mephedrone were 0.87 and 0.92, respectively, supporting the structures of the two metabolites, as the 4-carboxy moiety is expected to make the molecule more polar. Furthermore, the 4-carboxy-dihydro metabolite is slightly more polar than the 4-carboxy metabolite, which is the same pattern observed with mephedrone in relation to dihydro-mephedrone.
Concerning occurrence in urine, we found that mephedrone was excreted unchanged along with metabolites, which is in accordance with the findings reported by Meyer et al.\textsuperscript{[10]} Hydroxymethyl-mephedrone was excreted 79% as the β-glucuronide/aryl sulphate conjugate. Nor-mephedrine, dihydro-mephedrone and 4-carboxy-mephedrone were detected both in blood and urine, while hydroxymethyl- and 4-carboxy-dihydro-mephedrone were primarily found in urine.

### Conclusion

We identified CYP2D6 as the main enzyme responsible for the phase 1 \textit{in vitro} metabolism of mephedrone, corresponding to the metabolic process for many similar designer drugs. The structure of the \textit{main in vitro} metabolite, hydroxymethyl-mephedrone, was verified by NMR and was quantified along with mephedrone in four authentic, forensic samples. In these samples, we confirmed the presence of three out of five previously identified metabolites, plus two new metabolites: 4-carboxy-mephedrone and dihydro-mephedrone. The identification of the main liver enzyme for metabolism of mephedrone provides knowledge of possible drug-drug interactions with the compound, and the possibility of enhanced toxicity in subjects lacking the enzyme because of genetic polymorphism (7% of Caucasians). These toxic effects are thought to be expected because mephedrone is also excreted unchanged in the urine, which reduces the tendency to accumulate high levels of the drug despite absent or decreased CYP2D6 function. The detailed studies of spectra of metabolites and the quantification of the main metabolite, hydroxymethyl-mephedrone, in forensic cases provide a background for assessing mephedrone and metabolites in forensic cases.

### References

In vitro metabolism studies on mephedrone and analysis of forensic cases


