Mass Spectrometric Evaluation of Mephedrone In Vivo Human Metabolism: Identification of Phase I and Phase II Metabolites, Including a Novel Succinyl Conjugate

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ABSTRACT

In recent years, many new designer drugs have emerged, including the group of cathinone derivatives. One frequently occurring drug is mephedrone; although mephedrone was originally considered as a “legal high” product, it is currently banned in most Western countries. Despite the banning, abuse of the drug and seizures are continuously reported. Although the metabolism of mephedrone has been studied in rats or in vitro using human liver microsomes, to the best of our knowledge, no dedicated study with human volunteers has been performed for studying the in vivo metabolism of mephedrone in humans. Therefore, the aim of this study was to establish the actual human metabolism of mephedrone and to compare it with other models. For this purpose, urine samples of two healthy volunteers, who ingested 200 mg mephedrone orally, were taken before administration and 4 hours after substance intake. The discovery and identification of the phase I and phase II metabolites of mephedrone were based on ultra-high-performance liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry, operating in the so-called MS² mode. Six phase I metabolites and four phase II metabolites were identified, four of them not previously reported in the literature. The structure of four of the detected metabolites was confirmed by synthesis of the suggested compounds. Remarkably, a mephedrone metabolite conjugated with succinic acid has been identified and confirmed by synthesis. According to the reviewed literature, this is the first time that this type of conjugate is reported for human metabolism.

Introduction

The stimulant designer drug mephedrone (M-CAT, Meow Meow) is a derivative of cathinone, a monoamine alkaloid found in khat, the effects of which resemble that of MDMA (3,4-methylenedioxymethamphetamine; ecstasy). There are several reported fatalities in Europe in which mephedrone intoxication appears to be the sole cause of death (Torrance and Cooper, 2010; Lusthof et al., 2011; Maskell et al., 2011; Adamowicz et al., 2013; Cosbey et al., 2013), and it has also been detected in numerous postmortem biologic samples of fatal cases in which the cause of death was not specifically mephedrone (Torrance and Cooper, 2010; Maskell et al., 2011; Schifano et al., 2012; Cosbey et al., 2013). Abuse of mephedrone has been documented since 2007; it was originally a “legal high” drug, but has now been banned in most Western countries. Therefore, an intake of this drug must be monitored in clinical and forensic toxicology and doping control. The detection of urinary metabolites is the most suitable strategy for this purpose (Maurer et al., 2011).

The metabolism of mephedrone has been studied mainly in rats (Meyer et al., 2010; Martínez-Clemente et al., 2013) or in vitro using either rat liver hepatocytes (Khreit et al., 2013) or human liver microsomes (Pedersen et al., 2013). Some of the metabolites found in these studies have also been found in human urine samples submitted for drug testing; however, to our knowledge, no dedicated study with human subjects has been performed for studying the in vivo metabolism of mephedrone in humans.

Liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) and/or to high-resolution mass spectrometry (MS) is one of the most useful analytical tools for metabolic studies. It allows for the direct and simultaneous detection of both phase I and phase II metabolites. The versatility of LC combined with different analyzers

Abbreviations: ACN, acetonitrile; CID, collision-induced dissociation; HCOOH, formic acid; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MDMA, 3,4-methylenedioxymethamphetamine; MeOH, methanol; MS, mass spectrometry; MS/MS, tandem mass spectrometry; m/z, mass-to-charge ratio; QTOF, hybrid quadrupole time-of-flight; UHPLC, ultra-high-performance liquid chromatography.
favored the development of a large number of analytical approaches for the detection of metabolites (Prakash et al., 2007; Gomez et al., 2014). Among them, ultra-high-performance liquid chromatography (UHPLC) coupled to high-resolution MS, specifically using hybrid quadrupole time-of-flight (QTOF) MS, is one of the most attractive for the open detection of unknown metabolites. This configuration allows for operating in the so-called MS<sup>6</sup> mode (i.e., two accurate mass full spectra are acquired sequentially). The first one is acquired without applying collision energy, providing information about the intact molecules [commonly the (de)protonated molecule] present in the urine sample. The second, applying a collision energy ramp, promotes fragmentation obtaining accurate mass fragment ions useful for metabolite identification. This approach has been successfully applied for the detection of metabolites/transformation products of several xenobiotics such as omeprazole (Boix et al., 2014b), cocaine and benzoylcegonine (Bijlsma et al., 2013), and cannabis (Boix et al., 2014a).

In this study, the human metabolism of mephedrone was evaluated using UHPLC-QTOF MS. For this purpose, urine samples collected at predose and 4 hours after drug administration were analyzed. Potential structures for the detected metabolites were established based on their mass spectrometric behavior. Metabolic pathways found in humans were compared with those obtained with other models such as rats or microsomes.

**Materials and Methods**

**Reagents and Chemicals**

Mephedrone, mephedrone-d<sub>3</sub>, and nor-mephedrone reference standards were purchased from LGC GmbH (Lakenwalde, Germany) and Toronto Research Chemicals (North York, Ontario, Canada). NaBH<sub>4</sub> and succinic anhydride were obtained from Sigma-Aldrich (St. Louis, MO). High-performance liquid chromatography (HPLC)–grade water was obtained from deionized water passed through a Milli-Q water purification system (Millipore, Bedford, MA). HPLC-grade methanol (MeOH), HPLC-grade acetonitrile (ACN), sodium hydroxide, and formic acid (HCOOH) were purchased from ScharLab (Barcelona, Spain). Leucine enkephalin was purchased from Sigma-Aldrich.

**Instrumentation**

An Acquity ultra-performance LC system (Waters, Milford, MA) was interfaced to a QTOF mass spectrometer (QTOF Xevo G2; Waters Micromass, Manchester, UK) using an orthogonal Z-spray electrospray interface. The LC separation was performed using an Acquity ultra-performance LC BEH C18 1.7-μm particle size analytical column of 100 × 2.1 mm (from Waters) at a flow rate of 0.3 mL/min. The mobile phases used were H<sub>2</sub>O (A) and MeOH (B), both with 0.01% (v/v) HCOOH. The proportion of MeOH was linearly increased as follows: 0 minute, 10%; 14 minutes, 90%; 16.01 minutes, 10%; and 18 minutes, 10%. The injection volume was 20 μL. Nitrogen (Praxair, Valencia, Spain) was used as both the drying and nebulizing gas. The gas flow rate was set at 1000 L/h. The resolution of the time-of-flight mass spectrometer was approximately 20,000 at full width half maximum at a mass-to-charge ratio (m/z) of 556. MS data were acquired over a m/z range of 50–1000 in a scan time of 0.3 seconds. The microchannel plate (MCP) detector potential was set to 3700 V. Capillary voltages of 0.7 kV and −2.0 kV were used in positive and negative ionization modes, respectively. A cone voltage of 15 V was applied. The collision gas was argon (99.995%; Praxair). The interface temperature was set to 600°C and the source temperature to 130°C. The column and autosampler temperatures were set to 40°C and 5°C, respectively.

For MS<sup>3</sup> experiments, two acquisition functions with different collision energies were created: the low-energy function with a collision energy of 4 eV, and the high-energy function with a collision energy ramp ranging from 15 to 30 eV. For MS<sup>5</sup> experiments, two acquisition functions with different collision energies were also performed, using a cone voltage of 15 V and collision energies of 10, 15, and 20 eV. In those cases in which a single abundant product ion was obtained, a MS<sup>3</sup> experiment was performed by selecting the product ion generated in-source at high cone energy (30 V) and acquiring the product ion scan of this in-source fragment.

**Data Processing**

MetaboLynx XS software was used to process QTOF MS data obtained from metabolic studies. This software compares extracted ion chromatograms of a positive sample with a control sample for detecting, identifying, and reporting differential ions/chromatographic peaks that would correspond, in principle, to metabolites. In metabolites studies (Boix et al., 2013, 2014a, b), MetaboLynx XS proved to be highly useful for the investigation of both expected and unexpected metabolites/transformation products.

Acquisitions were performed in centroid, in both positive and negative ion modes. For all compounds detected by MetaboLynx, the accurate mass of protonated/deprotonated molecules was determined on the basis of averaged spectra obtained in the survey scan. Then, possible elemental compositions were calculated using the Masslynx elemental composition calculator with a maximum deviation of 2 mDa from the measured accurate mass. The maximum and minimum parameters were restricted considering the elemental composition and structure of mephedrone (C<sub>11</sub>H<sub>15</sub>NO) and the possibility of phase II metabolism, such as glucuronidation, as follows: C 0–25, H 0–50, N 0–2, O 0–12, and S 0–1. The applied double-bond equivalent filter was set between −0.5 and 50. To calculate the elemental composition of fragment ions, parameters settings were restricted as a function of the calculated elemental composition of the (de)protonated molecule, whereas no restrictions were applied for neutral losses. In addition, the option “even-electrons ions only” was selected for the precursor ion, and “odd- and even-electrons ions” for the fragment ions.

**Reduction of Reference Material: Synthesis of Reference Standards for M3 and M5**

Qualitative reduction of reference material was performed based on a procedure described elsewhere (Pozo et al., 2012), in which the carbonyl group is converted in an alcohol moiety. Briefly, 100 μL of reference material (both mephedrone and nor-mephedrone) at 100 μg/ml was evaporated to dryness. After addition of 2 ml methanol and 100 mg NaBH<sub>4</sub>, the mixture was maintained under agitation for 3 hours. After evaporation of the solvent, the residue was dissolved in 1 ml of mobile phase. The presence of the reduced metabolites was confirmed by UHPLC-QTOF MS analysis using the above-described experimental conditions.

**Synthesis of Nor-Mephedrone Succinate (M4)**

Nor-mephedrone conjugated with succinic acid was qualitatively synthesized by mixing 100 μL nor-mephedrone at 100 μg/mL with 100 mg succinic anhydride in acetone. The mixture was heated at 60°C for 3 hours. After evaporation of the solvent, the residue was dissolved in mobile phase. Residues of insoluble salts were removed by centrifugation. The presence of the succinate metabolite was confirmed by UHPLC-QTOF MS analysis using the above-described experimental conditions.

**Urine Samples**

Urine samples were obtained predose and after 4 hours from two volunteers that were administrated with a single oral dose of 200 mg mephedrone in the Institut Hospital del Mar d’Investigacions Mèdiques Clinical Research Unit. Subjects were two Caucasian healthy male recreational users of psychostimulants and hallucinogens (ages 29 and 36 years). Both participants were extensive-intermediate CYP2D6 metabolizers. They were free of any medicine or drugs of abuse. A rapid urine test for main drugs of abuse was negative before administration. Subjects signed an informed consent and the protocol was authorized by the local ethics...
Sample Treatment

One milliliter of the urine sample was diluted 2-fold with Milli-Q water and centrifuged at 10,000 rpm for 7 minutes. After that, 20 μl was directly injected in the LC-QTOF MS system.

Results

Mass Spectrometric Behavior of Mephedrone

Similar to other nitrogen-containing stimulants, mephedrone (C_{11}H_{15}NO) is easily ionized in positive ionization mode producing an abundant [M + H]^+ ion. The ions formed in the collision-induced dissociation (CID) of mephedrone are shown in Table 1. Some ions resulting from neutral losses of water and a methyl group are common for similar molecules, such as amphetamine and ephedrine derivatives (Deventer et al., 2009; Bijlsma et al., 2011). However, the observed MS/MS behavior of mephedrone was to some extent unexpected. Nitrogen-containing stimulants usually lose the amine group (normally as ammonia or methylamine) in one of the first fragmentation steps (Deventer et al., 2009; Bijlsma et al., 2011). Contrary to this behavior, the neutral loss of methylamine is poor (2%; see Table 1) for mephedrone. In fact, the loss of the nitrogen atom seems to be unfavored because all of the ions with abundances higher than 20% still preserve the nitrogen (Table 1). Two losses of radical CH$_3^+$ were observed without losing the amine. This behavior can be considered as exceptional because the formation of odd electron ions by losses of radicals is uncommon in CID (Thurman et al., 2007).

This unusual behavior may be explained by an in-source rearrangement previous to the fragmentation (Fig. 1, top). This rearrangement would form an indole ring by reaction between the nitrogen atom and the C$_2$, with the help of the carbonyl function at C$_1$. The stability provided by the indole would explain the unusual behavior of mephedrone including the abundant loss of water (coming from the ketone) and the subsequent losses of CH$_3^+$. The analysis of mephedrone-d$_3$ confirmed that both methyl groups could be lost, although the loss of the C$_3$ methyl group seems to be favored because the ion at m/z 148 is more abundant than the ion at m/z 145 (Fig. 1, bottom).

Detection of Mephedrone Metabolites

The strategy applied in this study (dilution of the sample, acquisition in UHPLC-QTOF MS, and processing by MetaboLynx software) to urine samples collected before and after administration of 200 mg mephedrone allowed for the detection of the parent compound and 10 metabolites (Table 1).

The mass accuracy obtained provided only one feasible formula for each metabolite at the selected narrow-mass window (2 mDa). Among the 10 metabolites found, 4 were detected only in the positive ionization mode, similar to the behavior observed for mephedrone. Six of the metabolites were also detected in negative ionization mode (Table 1), suggesting the presence of an acidic moiety.

The product ion scan of each detected metabolite was acquired and the results are shown in Table 1. Based on the comparison between these results and the CID behavior of mephedrone (see above), a putative structure for each metabolite was established (described below).

In those cases in which a phase II metabolite was predicted (M4, M8, M9, and M10) or a single abundant product ion was obtained (M2 and M7), an MS$^3$ experiment was performed by selecting the product ion generated in-source at high cone voltage and acquiring the product ion scan of this in-source fragment. This allowed us to garner more knowledge or to obtain additional relevant information about the unconjugated metabolite. The results of these experiments are shown in Table 1.

Mass Spectrometric Results for Mephedrone Metabolites

Metabolite M1. The molecular formula obtained for metabolite M1 (C$_{10}$H$_{14}$NO) suggested the loss of a methyl group from mephedrone. Because N-demethylation is a common metabolic pathway of N-methyl stimulants, N-demethylmephedrone was postulated as a feasible structure for M1.

As expected, the product ion spectrum followed behavior similar to the observed for mephedrone. Thus, the expected neutral loss of ammonia was substantially less abundant than the losses of water and CH$_3^+$ (Table 1). This fact can be explained by the same rearrangement postulated for mephedrone (Supplemental Fig. 1).

The structure of M1 was confirmed by comparison with the reference material (Fig. 2, bottom).

Metabolite M2. The molecular formula of M2 (C$_{10}$H$_{14}$NO$_2$) indicated a double oxidation from M1. In addition, M2 was also detected in negative ionization mode (Table 1), suggesting the presence of an acidic moiety. Therefore, the oxidation of one of the methyl groups of M1 to carboxylic acid was the most feasible mechanism to produce M2.

The product ion spectrum provided information about the location of the carboxylic function. The product ion spectrum of M2 was dominated by the ion at m/z 119.0497 (Table 1). This ion can be obtained after the neutral loss of glycine (Supplemental Fig. 2), suggesting a proximity between the amine and the acidic groups. In addition, the formula of this ion (C$_7$H$_5$O$_2$) implied that the aromatic zone of the mephedrone remains intact.

This fact was confirmed by the fragmentation of the ion C$_7$H$_5$O$_2$ generated in-source. This fragmentation followed the expected behavior for a CH$_4$PhCO$^-$ ion. It was dominated by the tropium ion (m/z 91.0552) and its subsequent fragmentation (Supplemental Fig. 2). In addition, an apparent neutral loss of 10 Da was observed. This fact can be explained by the addition of water in the collision cell after the CO loss as it was reported for similar ions (Beuck et al., 2009).

Based on this information, N-demethylmephedrone-3-carboxylic acid was selected as the potential structure for M2.

Metabolite M3. The molecular formula obtained for M3 (C$_{11}$H$_{15}$NO) indicated a reduction step from mephedrone.

The product ion spectrum exhibited an abundant neutral loss of water and subsequent losses of CH$_3^+$ and methylamine (Table 1). This behavior is similar to the one observed for ephedrine derivatives that have a hydroxyl group in C$_7$. Therefore, the reduction of the ketene moiety would hamper the rearrangement postulated for mephedrone, decreasing the number of nitrogen-containing product ions (Supplemental Fig. 3).

The structure of this metabolite as 1-dihydromephedrone was confirmed by comparison with synthesized material (Fig. 2, middle).

Metabolite M4. Metabolite M4 exhibited a molecular formula containing three carbon atoms more than mephedrone (C$_{13}$H$_{17}$NO$_2$). This fact suggested that M4 was conjugated by phase II metabolism. A diacetyl metabolite with the same formula was reported in the in vitro metabolism of mephedrone (Khreit et al., 2013). However, M4 showed ionization in both positive and negative modes (Table 1), indicating the presence of an acidic moiety in M4. This result is in disagreement with the diacetyl metabolite.

The product ion spectrum of M4 was one of the most complex among the detected metabolites (Fig. 3A). It showed that the molecule could be divided in two parts: one fragment at m/z 164 (C$_{10}$H$_{14}$NO) and the other at m/z 101 (C$_9$H$_7$O$_2$). The fragment C$_9$H$_7$NO has the same molecular formula as M1 (i.e., nor-mephedrone). Therefore, the fragment at m/z 101 provided information about the phase II metabolite. The molecular
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Rt (min)</th>
<th>Ionization Mode</th>
<th>m/z</th>
<th>Error (Da)</th>
<th>Formula</th>
<th>Precursor Ion</th>
<th>Product Ion</th>
<th>Abundance (%)</th>
<th>Error (Da)</th>
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<td>164 147.0803</td>
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<td>C_{10}H_{12}NO</td>
<td>194 138.0666</td>
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<td>180 162.1276</td>
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<td>166 148.1122</td>
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(continued)
formula was in agreement with a succinyl conjugate. The product ion spectra could be explained by this conjugation as shown in Fig. 3C. The structure of M4 as N-succinyl nor-mephedrone was confirmed by comparison with synthetic material as shown in Fig. 3B.

Metabolite M5. Metabolite M5 showed only positive ionization with a molecular formula of C_10H_15NO. This formula suggested a demethylation followed by a reduction of the keto function. Therefore, 1-dihydro-nor-mephedrone was selected as the most feasible candidate for M5.

This hypothesis was supported by the product ion spectrum. Similar to M3, the absence of the ketone functionality seems to hamper the rearrangement observed in mephedrone. In agreement with this, the product ion spectrum of M5 was dominated by subsequent neutral losses of water and ammonia (Supplemental Fig. 4).

The proposed structure was confirmed by the reduction of nor-mephedrone, as can be seen in Fig. 2 (top).

### Table 1—Continued

<table>
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<tr>
<th>Metabolite</th>
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<th>Formula</th>
<th>Precursor Ion</th>
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ESI, electrospray ionization; Rt, Retention time.
metabolites and the other coming from the incorporated hydroxyl group. In addition, an abundant neutral loss of formaldehyde was also observed (Table 1; Supplemental Fig. 5). This neutral loss of 30 Da has been reported for hydroxymethyl metabolites in compounds with stable rings, such as steroids (Pozo et al., 2008). Therefore, 4'-hydroxymethyl-mephedrone was proposed for M6 in agreement with previously published studies (Meyer et al., 2010; Khreit et al., 2013; Martínez-Clemente et al., 2013; Pedersen et al., 2013).

Fig. 1. Proposed fragmentation pathway for mephedrone reference standard (top), product ion spectrum at 20 eV for mephedrone (middle), and product ion spectrum at 20 eV for mephedrone-d3 (bottom).
Metabolite M7. The molecular formula of M7 (C₁₁H₁₃NO₃) suggested a double oxidation from mephedrone. Similar to M2, the oxidation of a methyl group to carboxylic acid seems to be the most feasible mechanism to obtain M7. This fact was also supported by the negative ionization observed for M7 (Table 1).

In contrast with M2, the product ion spectrum of M7 did not show the ion C₈H₇O⁻ (m/z 119.0500) indicative of an intact benzylic area. These losses could be explained after the rearrangement described for mephedrone if the structure of M7 is 4'-carboxymephedrone (Supplemental Fig. 6) as previously reported (Khreit et al., 2013; Pedersen et al., 2013; Martínez-Clemente et al., 2013).

Metabolite M8. M8 showed a molecular formula of C₁₇H₂₃NO₈, which implied an increase of C₆H₈O₆ from hydroxylmephedrone. Therefore, hydroxylmephedrone-3-O-glucuronide was proposed as a feasible structure for M8.

The product ion spectrum confirmed the presence of the glucuronide moiety by the typical loss of 176 Da in positive ionization mode (Table 1). Therefore, hydroxylmephedrine-3-O-glucuronide was proposed as a feasible structure for M8.

The product ion spectrum of the in-source fragment at m/z 194 (produced after the neutral loss of the glucuronide and corresponding to the unconjugated metabolite) showed an abundant ion at m/z 119.0497 (Table 1) corresponding to CH₃-Ph-CO⁻. As commented for other metabolites, this ion is indicative of unaltered benzylic zone in M8. Therefore, C₃ seems to be the most suitable hydroxylation site. Similarly to M2 in which a carboxylic acid was proposed in C₃, M8 also showed the ions corresponding to the loss of CO and the subsequent gain of a water molecule (Supplemental Fig. 7), supporting the assignation. Therefore, a C₃ hydroxylation previous to glucuronidation is proposed as the most feasible metabolic pathway to obtain M8.

Metabolite M9. The molecular formula for M9 (C₁₇H₂₁NO₉) also suggested a glucuronide conjugate. In that case, the phase I metabolite seemed to be a double oxidized metabolite.

The product ion spectrum of M9 after the loss of glucuronide moiety was identical to the one observed for M7 (Table 1; Supplemental Fig. 8). Therefore, the glucuronide conjugate of 4'-carboxymephedrone was proposed as the structure of M9. Based on the structure of M7, N-glucuronidation seems to be the most feasible metabolic pathway to obtain M9 (4'-carboxymephedrone-N-glucuronide).

Metabolite M10. Similarly to M8 and M9, the molecular formula of M10 (C₁₆H₂₀NO₈) indicated a glucuronidation. In that case, the phase I metabolite seemed to be a double oxidized metabolite.

The product ion spectrum of M10 after the loss of glucuronide moiety was identical to the one observed for M7 (Table 1; Supplemental Fig. 8). Therefore, the glucuronide conjugate of 4'-carboxymephedrone was proposed as the structure of M9. Based on the structure of M7, N-glucuronidation seems to be the most feasible metabolic pathway to obtain M9 (4'-carboxymephedrine-N-glucuronide).
The product ion spectrum of M10 exhibited an abundant ion at \( m/z \) 119.0478 (C₈H₇O). As described for other metabolites such as M2 and M8, this ion is indicative of an intact aromatic area (Supplemental Fig. 9). Following the considerations described for these metabolites, hydroxylation in C3 of nor-mephedrone and subsequent glucuronidation was proposed as a potential metabolic pathway to obtain M10 (hydroxyl Fig. 9). Following the considerations described for these metabolites, hydroxylation in C3 of nor-mephedrone and subsequent glucuronidation was proposed as a potential metabolic pathway to obtain M10 (hydroxyl
nor-mephedrone-3-O-glucuronide). The C3-hydroxylated metabolite precursor of M10 might also be proposed as an intermediate metabolite in the generation of M2 (Fig. 4).

**Discussion**

The study of mephedrone metabolism by combination of UHPLC-QTOF MS and MetaboLynx after dilution of the sample allowed for the detection of mephedrone and 10 metabolites in human urine. The metabolic pathway proposed based on our results is summarized in Fig. 4.

The accurate mass measurements played a critical role in properly interpreting the fragmentation of mephedrone and proposing suitable structures for detected metabolites. For example, mephedrone and most of its metabolites show a product ion with nominal m/z 119. In a previous report, this ion was assigned to CH$_3$PhCO$,^{+}$ suggesting a cleavage of the C$_1$-C$_2$ bond of the molecule (Martínez-Clemente et al., 2013). However, accurate mass measurements revealed that it corresponds to a formula of C$_9$H$_{11}$, indicating that the expected cleavage is not produced. By contrast, several metabolites produced the ion CH$_3$PhCO$,^{+}$, which was confirmed by the subsequent loss of CO and the ion at m/z 109 corresponding to [CH$_3$PhCO – CO + H$_2$O]$^{+}$. The presence of this ion would imply a structural change in the surroundings of the nitrogen atom. Therefore, discerning between both C$_9$H$_{11}$ and C$_8$H$_7$O formulae for the ion at m/z 119 was found to be compulsory for structural elucidation.

In summary, six phase I and four phase II mephedrone metabolites were detected and elucidated in human urine. Phase I reactions included N-demethylation, reduction of the keto function, hydroxylations in C$_3$ and in the benzylic carbon, and oxidation of C$_3$ and the benzylic methyl to carboxylic acid (Fig. 4). Our data confirmed the presence of some compounds that were previously described as mephedrone metabolites in humans or other species (e.g., M1, M3, M5, M6, M7, and M9) (Meyer et al., 2010; Khreit et al., 2013; Martínez-Clemente et al., 2013; Pedersen et al., 2013). However, it is remarkable that structures for M2, M4, M8, and M10 are reported for the first time in this study.

Regarding phase II metabolism, three metabolites conjugated with glucuronic acid (M8, M9, and M10) were found. More surprising was the detection of a conjugate with succinic acid (M4). To the best of our knowledge, this is the first report of such a conjugate in human urine. Succinylation in drug development falls into the prodrug category. Succinic acid derivatives are attached to target drugs to produce more desirable physical and biologic properties, with the goal that the resulting conjugates are degraded by cellular enzymes to release the target drugs in vivo. In the context of physiologic compounds, biogenic...
amines such as octopamine, dopamine, and serotonin are typically deactivated by acetylation in insects, nematodes, and other invertebrates. Succinylation, in addition to acetylation, was recently reported for octopamine and tyramine ascorcidoses in nematodes, raising the question of to what extent this metabolic reaction may represent a general pathway of biogenic amine metabolism (Artyukhin et al., 2013). In the context of our study, succinic acid was conjugated to nor-mephedrine. The hydrolysis of the conjugate would release nor-mephedrine, a metabolite which pharmacology activity is unknown but expected to be lower than the corresponding to mephedrine.

The structure of four of the detected metabolites (M1, M3, M4, and M5) was confirmed by comparison with synthesized material. All four were correctly assigned based on their mass spectrometric behavior. Synthesis of the remaining metabolites would be required to ultimately confirm their structure.

In vitro studies suggest that mephedrone metabolic disposition is regulated by the highly polymorphic isoenzyme of CYP2D6 (Pedersen et al., 2013). The quantification of metabolites identified in this report is of importance since it is unknown i) the in vivo relevance of CYP2D6 polymorphism in mephedrone disposition and ii) the metabolic pathways regulated by this isoenzyme to interpret metabolic profiles in the context of forensic toxicology.

In the near future, research is needed to evaluate the behavior of unchanged mephedrone and the 10 reported metabolites in urine samples collected from a larger population, ideally after administration of different doses, to determine which should be the most appropriate biomarkers when investigating drug use.

**Authorship Contributions**

**Conducted experiments:** Pozo, Ibáñez, Lahoz-Beneytez, Farré, Papaseit, de la Torre.

**Performed data analysis:** Pozo, Ibáñez, Sancho.

**Wrote or contributed to the writing of the manuscript:** Pozo, Ibáñez, de la Torre, Hernandez.

**References**


SUPPLEMENTAL DATA

MASS SPECTROMETRIC EVALUATION OF MEPHEDRONE IN VIVO HUMAN METABOLISM: IDENTIFICATION OF PHASE I AND PHASE II METABOLITES INCLUDING A NOVEL SUCCINYL CONJUGATE

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Drug Metabolism and Disposition
Supplemental Figure 1. MS/MS spectrum at 20eV for metabolite 1 (N-demethyl mephedrone) and proposed fragmentation pathway.
Supplemental Figure 2. MS/MS spectrum at 20eV for metabolite 2 (*N*-demethylmephedrone-3-carboxylic acid) and proposed fragmentation pathway.
Supplemental Figure 3. MS/MS spectrum at 20eV for metabolite 3 (1-dihydro mephedrone) and proposed fragmentation pathway.
Supplemental Figure 4. MS/MS spectrum at 20eV for metabolite 5 (1-dihydro nor-nemphedrone) and proposed fragmentation pathway.
Supplemental Figure 5. MS/MS spectrum at 20eV for metabolite 6 (4'-hydroxymethyl-mephedrone) and proposed fragmentation pathway. * Peak do not observed in the spectrum.
Supplemental Figure 6. MS/MS spectrum at 20eV for metabolite 7 (4'-carboxy-mephedrone) and proposed fragmentation pathway.
Supplemental Figure 7. MS/MS spectrum at 20eV for metabolite 8 (hydroxyl-mephedrone-3-O-glucuronide) and proposed fragmentation pathway.
Supplemental Figure 8. MS/MS spectrum at 20eV for metabolite 9 (4’-carboxy-mephedrone-N-glucuronide) and proposed fragmentation pathway.
Supplemental Figure S9. MS/MS spectrum at 20eV for metabolite 10 (hydroxyl nor-mephedrone-3-O-glucuronide) and proposed fragmentation pathway.