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## RESEARCH ARTICLE

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# In vitro metabolic profile of mexedrone, a mephedrone analog, studied by high- and low-resolution mass spectrometry

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## Abstract

Mexedrone is a synthetic cathinone structurally related to mephedrone, which belongs to the class of *N*-alkyl cathinone derivatives, whose metabolic profile has not been fully clarified yet. This study considers the in vitro phase I metabolism of mexedrone, to pre-select the most appropriate marker(s) of intake. Mexedrone was incubated in the presence of either human liver microsomes or single recombinant CYP450 isoforms. The metabolic profile was outlined by ultra-high-performance liquid chromatography coupled to both high- and low-resolution mass spectrometry. In detail, the phase I metabolic profile of mexedrone was initially defined by a time-of-flight analyzer, while the chemical structures of the detected metabolites and the potential presence of minor metabolites were subsequently studied by tandem mass spectrometry, using a triple quadrupole analyzer. The main phase I metabolic reactions were hydroxylation and *N*- and *O*-dealkylation. The CYP450 isoforms most involved were CYP2C19, responsible for the formation of both hydroxylated and dealkylated metabolites, followed by CYP2D6 and CYP1A2, involved in the hydroxylation reactions only. Finally, a significant fraction of mexedrone unchanged was also detected. Based on this evidence, the most appropriate markers of intake are mexedrone unchanged and the hydroxylated metabolites.

## KEYWORDS

in vitro metabolism, mephedrone analogs, mexedrone, novel psychoactive substances, synthetic cathinones

## 1 | INTRODUCTION

Synthetic cathinones (SCs) are a class of novel psychoactive substances (NPS) derived from cathinone, a natural alkaloid found in the khat shrub (*Catha edulis* plant).<sup>1</sup> Cathinone exerts similar effects to amphetamine derivatives on central stimulation.<sup>2–4</sup> SCs were developed from natural cathinone as potential therapeutic agents

in the early 1920s.<sup>5,6</sup> Indeed, they were initially used as appetite suppressants and for the treatment of chronic fatigue. They were withdrawn from the market due to their side effects, along with addiction and abuse among users.<sup>4,7</sup> Since the mid-2000s, SCs have appeared on illicit drug markets as “recreational drugs,” illicitly synthesized to circumvent the current legislation on drugs of abuse.

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SCs are known and sold in the United States as “bath salt,” or in Europe as “plant food” or “legal highs,”<sup>4</sup> labeled “not for human consumption”<sup>8–10</sup> to prevent any legal action. SCs have recently been available on the internet/darknet or into the smart shops. Thanks to the ease of purchase, SCs have become widespread since the 2010s,<sup>11</sup> becoming the second-largest group of NPS monitored by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA).<sup>12</sup> Like other NPS, their growing diffusion poses health risks and unknown potential side effects. The abuse of SCs lead to various psychiatric manifestation, sympathomimetic toxicity, and can even lead to fatal intoxication.<sup>4</sup>

Chemically, SCs are a class of psychoactive substances structurally related to amphetamines, with a carbonyl group in the  $\alpha$  position, which configures this class of substances as phenethylamine derivatives, often called as “natural amphetamines,” with stimulant effects similar to those of other amphetamine derivatives.<sup>3,13,14</sup> SCs act on monoamine reuptake transporters, increasing the level of monoamine in the brain and the release of monoamine transporters.<sup>15–19</sup> Since SCs act as stimulant drugs, they have also been prohibited by the World Anti-Doping Agency (WADA). It appears that SCs are abused by athletes to enhance their performance, as they were detected by law enforcement in a 2013 seizure of substances sold for doping purposes. The authorities sent to WADA the list of the 80 seized substances, including a surprising number of cathinone-related drugs. Consequently, SCs were included in 2014 in section S6 “Stimulants” of the WADA prohibited list (“cathinone and its analogs, e.g., mephedrone, methedrone, and  $\alpha$ -pyrrolidinovalerophenone”).<sup>20</sup> Consequently, the detailed knowledge of their metabolic profile, whose first step is generally represented by in vitro metabolism studies, allows to pre-select the most suitable markers of intake, to ensure their detection in biological fluids.<sup>21</sup>

Mephedrone first appeared in 2007 in Israel and then in Finland in 2008,<sup>1,6</sup> it became rapidly popular because of its availability on the web market, lower cost, and higher efficacy than conventional stimulants: it has now become one of the most seized SCs.<sup>19,22–24</sup> Consistent with the growing popularity of mephedrone, an increasing number of fatal and non-fatal intoxications have been reported since 2008.<sup>25–28</sup> These adverse effects have led to the ban of mephedrone in several Countries,<sup>4</sup> and in 2015 it was listed in schedule 2 of the Convention on Psychotropic Substances of 1971.<sup>29</sup> Following this ban, a number of mephedrone derivatives soon appeared on the market as legal substitutes.<sup>24,30</sup> On this trend, mexedrone was presented as a substitute for mephedrone, through extensive web advertising that began 2 years before its market launch. Mexedrone, is indeed a derivative of mephedrone, a SC structurally related to the class of *N*-alkyl compound, or compounds with an alkyl or halogen substituent on the aromatic ring.<sup>24</sup> Mexedrone was first introduced in 2015 through a specific website, reachable at “<https://mexedrone.com>” (now offline) and soon its effects were described in online forums.<sup>31</sup> The structure of mexedrone was first identified and characterized in 2016, in a powder purchased online with other new SCs.<sup>32</sup> Its activity on monoamine transporter (i.e., DAT, NET, and SERT) was also assessed.<sup>31</sup> Mexedrone was first reported in France in 2016 by a

young woman in possession of a powder, who was involved in a case of hospitalization for SC addiction.<sup>33</sup> The first confirmed case of actual mexedrone intake was reported in the post-mortem biological sample of a 27-year-old man in 2016, again in combination with other drugs.<sup>34</sup>

The effects of mexedrone were first reported in 11 analytically confirmed cases by the West Midlands Poisons Unit of the City Hospital of Birmingham (UK) from biological samples collected between December 2015 and July 2016, where mexedrone was either ingested or smoked. The cases were characterized by multidrug intake and the most common effects were agitation, sinus tachycardia, and psychosis, confirmed in a case in which only the consumption of mexedrone was detected.<sup>35</sup> Furthermore, mexedrone is a weak monoamine transporter uptake blocker and a weak serotonin releasing agent,<sup>31</sup> which may lead users to increase the dose to achieve the desired effect, with a potential increase in fatal intoxications or unknown side effects.

To the best of our knowledge, no data are presently available on the metabolism of mexedrone, making its detection in biological fluids a challenge for forensic laboratories. This study aimed to define the phase I biotransformation pathways of mexedrone. For this purpose, due to the strict regulations for conducting controlled administration studies in humans and animals, we have followed the same experimental strategy already followed in similar studies,<sup>36–40</sup> focusing our attention on the in vitro metabolic profile of mexedrone, studied using human liver microsomes and isolated CYP450 recombinant isoforms. The metabolic products were studied by both a targeted and untargeted approach, using liquid chromatography coupled to either low resolution (triple quadrupole) and high resolution (time of flight) mass spectrometry.

## 2 | EXPERIMENTAL

### 2.1 | Chemicals

Mexedrone (1-mg powder) was purchased from LGC standards (Settimo Milanese, Milan, Italy) and stocked in methanol solution (final concentration 1 mg/ml). Methamphetamine (1-mg/ml solution used as internal standard) was purchased from Sigma-Aldrich (Milan, Italy). The reagents and solvents, all of analytic grade, were from Sigma-Aldrich (Milan, Italy), that is, formic acid, sodium phosphate, sodium hydrogen phosphate, potassium carbonate, and potassium hydrogen carbonate.

Ultra-purified water was by Milli-Q system (Millipore, Vimodrone, Milan, Italy). The human liver microsomes (HLM, from 20 Caucasian male and female donors of different ages), the single recombinant enzymatic isoforms (i.e., CYP1A2, CYP2C19, CYP2C9, CYP3A4, CYP3A5, and CYP2D6), and all the reagents used for the in vitro metabolism experiments (i.e., sodium phosphate buffer, NADPH regenerating system [sol A and sol B] containing, NADP<sup>+</sup>, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase) were supplied by Corning Incorporated (Milan, Italy).

## 2.2 | Protocol for the in vitro metabolism studies

The incubation conditions for mexedrone were optimized from protocol already in use in our laboratory to perform metabolism studies of similar substances.<sup>36,37</sup> We assessed different mexedrone concentrations (1, 5, 10, 20, 30, 40, 60, 80, 100, 150, and 200  $\mu\text{M}$ ) and enzymatic proteins concentrations (HLM either CYPs at 0.1, 0.2, 0.5, and 1.0 mg/ml) as well as incubation times (30 min, 1, 2, 4, 8, 12, and 24 h), to reach the most repeatable conditions and the highest enzymatic-assisted synthesis of phase I metabolites. All incubations were performed in phosphate buffer (0.1 M, pH 7.4). Samples were pre-warmed at 37°C for 5 min before HLM or CYPs were added to start the phase I reactions. The final incubation medium contains 40  $\mu\text{M}$  of mexedrone, 0.5 mg/ml of protein s (HLM or CYPs) 0.5 mg/ml, 3.3mM of magnesium chloride, 1.3 mM of NADP<sup>+</sup>, 3.3 mM of glucose-6-phosphate, and finally 0.4 U/ml of glucose-6-phosphate dehydrogenase. Samples were incubated at 37°C for different incubation times. After incubation, 250  $\mu\text{l}$  of acetonitrile were added to stop the phase I reactions. The samples were then transferred to an ice bath (-24°C) for protein precipitation in the assay medium. The precipitate was subsequently separated from the supernatant by centrifugation at 21.000 g (15.000 rpm) at room temperature for 10 min.

Each set of assays also included a negative control sample without HLM or CYPs to monitor the potential non-enzymatic reactions and a negative control sample that contained all the reaction mixture components without mexedrone. Each incubation was processed in triplicate.

## 2.3 | Sample pre-treatment

The sample pre-treatment protocol was developed by evaluating different solvents (i.e., chloroform, ethyl acetate, *tert*-butyl methyl ether), solvents volume (3, 5, 7 ml), and pH values (5, 7.4, 9). Supernatant from the in vitro studies was added with ultra-purified water to a final volume of 1 ml and next spiked with 50  $\mu\text{l}$  of the solution of the internal standard methamphetamine (final concentration of 250 ng/ml). The samples were then added with 100  $\mu\text{l}$  of buffer and the appropriate volume of extraction solvent. After 20 min of mild stirring, the samples were centrifugated at 3000 rpm for 4 min and transferred to an ice bath. The organic layer was then collected into a 10-ml glass tube and evaporated until dry, under gentle nitrogen flow at room temperature. The final residue was dissolved in 50  $\mu\text{l}$  of the mobile phase and analyzed.

## 2.4 | Instrumental conditions

### 2.4.1 | Untargeted high-resolution mass spectrometry

Samples from in vitro metabolism studies were analyzed using an Agilent 1290 infinity II series UHPLC instrument equipped with a: Zorbax C18 (10 cm  $\times$  2.1 mm, 1.8  $\mu\text{m}$ ) coupled with orthogonal acceleration time-of-flight mass spectrometer 6,545 (Agilent Technologies)

equipped with an electrospray ionization (ESI) source operating in positive mode. The solvents used were ultrapure water (eluent A) and acetonitrile (eluent B), both containing 0.1% formic acid. The flow rate was set to a constant flow rate of 400  $\mu\text{l}/\text{min}$ . The elution gradient started at 2% of B and was increased to 30% in 4 min, then to 80% in 2 min and finally to 95% in 1 min for 2 min. The column was flushed for 2 min at 100% B and finally re-equilibrated at 2% B for 2 min.

The mass spectrometric experiments were carried out in positive ionization using full scan as acquisition mode. Nitrogen was used as the drying and nebulizing gas. The drying gas flow was 15 L/min, and sheath gas flow of 10 L/min the temperature was set at 320°C. The nebulizer gas pressure was 45 psi. The applied capillary, nozzle, and fragmentor voltages were 3500, 1000 and 175 V, respectively. Mass spectra data were collected from m/z 50 to 1000 at 9300 transients per second. All other parameters were automatically optimized by the daily performed instrument autotuning procedure. The mass calibration was executed daily at the beginning of every analytical session, using a calibration solution provided by the manufacturer. Purine with an  $[\text{M} + \text{H}]^+$  ion at m/z 121.0509 and an Agilent proprietary compound (HP0921) yielding an ion at m/z 922.0098 were simultaneously introduced via a second orthogonal sprayer, and these ions were used as internal calibrants along with all the analysis. All aspects of instrumental control, tuning, method setup and parameters, sample injection, and sequence operation were controlled by the Agilent Technologies Mass Hunter software version B.08.00.

### 2.4.2 | Targeted tandem mass spectrometry (MS/MS)

Samples were analyzed using an Agilent 1200 series HPLC instrument coupled with an API4000 triple quadrupole mass spectrometer (ABSciex, Monza, Italy) with an ESI source operated in positive ionization.

The chromatographic separation was performed by using a SUP-ELCO C18 column (15 cm  $\times$  2.1 mm  $\times$  2.7  $\mu\text{m}$ ). The mobile phases were ultrapure water (eluent A) and acetonitrile (eluent B), both containing 0.1% formic acid. The gradient program started at 5% B and increasing to 60% B in 6 min, after 3 min, to 100% B in 1 min. The column was flushed for 2 min at 100% B and finally re-equilibrated at 5% B for 3 min. The flow rate was set to 250  $\mu\text{l}/\text{min}$ .

Mass spectrometric detection was carried out in positive mode. The capillary and declustering voltages were set at 5000 and 60 V, respectively. The source temperature was set at 500°C. Curtain gas, ion source gas 1 (auxiliary gas), and ion source gas 2 (nebulizer gas) pressures were set at 25, 35, and 40 psi, respectively. Product ion scan and multiple reaction monitoring (MRM) were used as acquisition modes. The collision-induced dissociation (CID) was performed using nitrogen as collision gas at 5.8 mPa, obtained from dedicated nitrogen generator system Parker-Balston model 75-A74, giving gas purity 99.5% (CPS Analytica, Milan, Italy). All aspects of instrument control, method setup parameters, sample injection, and sequence operation were controlled by Analyst software version 1.6.1 ABSciex.

### 3 | RESULTS AND DISCUSSION

#### 3.1 | Mass spectrometric behavior of mexedrone

The MS and MS/MS parameters were optimized by infusing a standard methanolic solution of mexedrone, at a concentration of 10 µg/ml. The experiments were carried out first using full scan as acquisition mode, to obtain information on the ionization behavior of mexedrone, and then in product ion scan, at different collision energies (20, 25, 30, 35, and 40 eV) to characterize the specific fragmentation pathways. The molecular ion was identified using positive ionization at  $m/z$  208, no adducts or in-source fragmentation were detected. The source parameters were then optimized to obtain the maximum signal for the molecular ion selected. Regarding the fragmentation behavior, at a collision energy of 20 eV, dominant signals were found at  $m/z$  176, 158, and 149 likely generated after the loss of the methoxyl group followed by the loss of the ketone group and amino group. Whereas, at collision energies higher than 40 eV, abundant product ions were found at  $m/z$  91 consistent with the tropylium ion and at  $m/z$  119 consistent with the para methyl-phenol derivative (see Figure 1). The fragmentation pathway identified is in accordance with the information reported in literature.<sup>31</sup> Figure 1 reports the product ion spectra at 20 eV and 40 eV and the fragmentation pathway of mexedrone.

#### 3.2 | Optimization of chromatographic conditions

The chromatographic conditions for the parent compound and its metabolites were selected by analyzing the samples obtained after incubation of mexedrone in the presence of HLM. Different elution gradients were tested; optimal separation and chromatographic

retention were obtained by starting with a low percentage of the organic phase (eluent B, 98–95%) and increasing linearly to 60% of eluent B with 3 min of isocratic to achieve optimal chromatographic separation of the three metabolites formed.

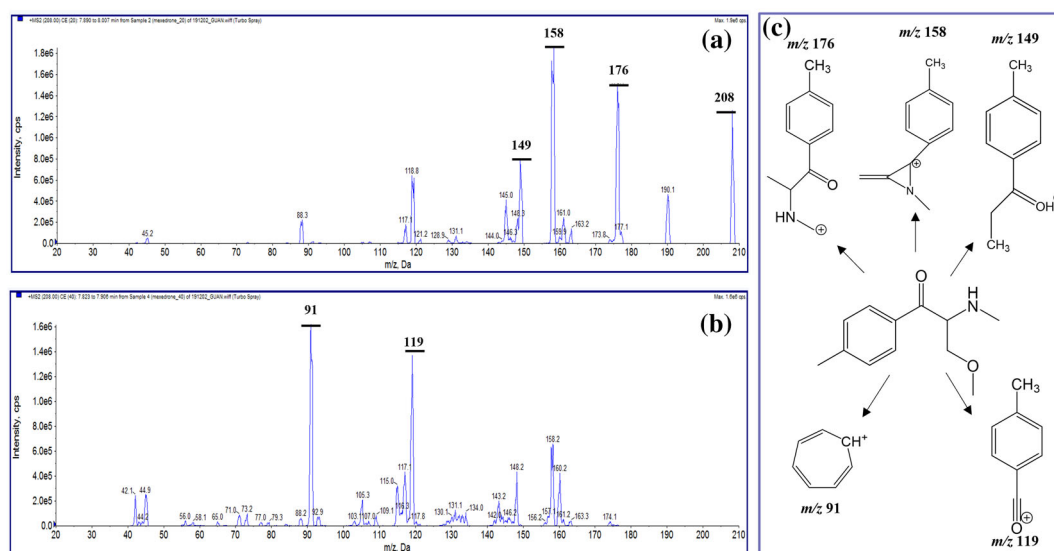
#### 3.3 | Sample pre-treatment

To optimize the pre-treatment protocol, the efficiency of the extraction procedure was assessed using different extraction solvents (*tert*-butyl methyl-ether, chloroform, and ethyl acetate) and at different pH (5.0, 7.4, 9.0, and 11). The best results in terms of recovery (higher than 70%) and cleanliness of the extract were obtained using ethyl acetate in alkaline conditions (pH 9) for both the parent compound and the metabolites. However, mexedrone and the demethylated metabolites were extracted only in small amounts, due to their more pronounced properties as weak bases. The extraction carried out at pH 11 clearly showed the signal of mexedrone, a low signal for the two dealkylated metabolites, while no signal was detected for the hydroxylated metabolite.

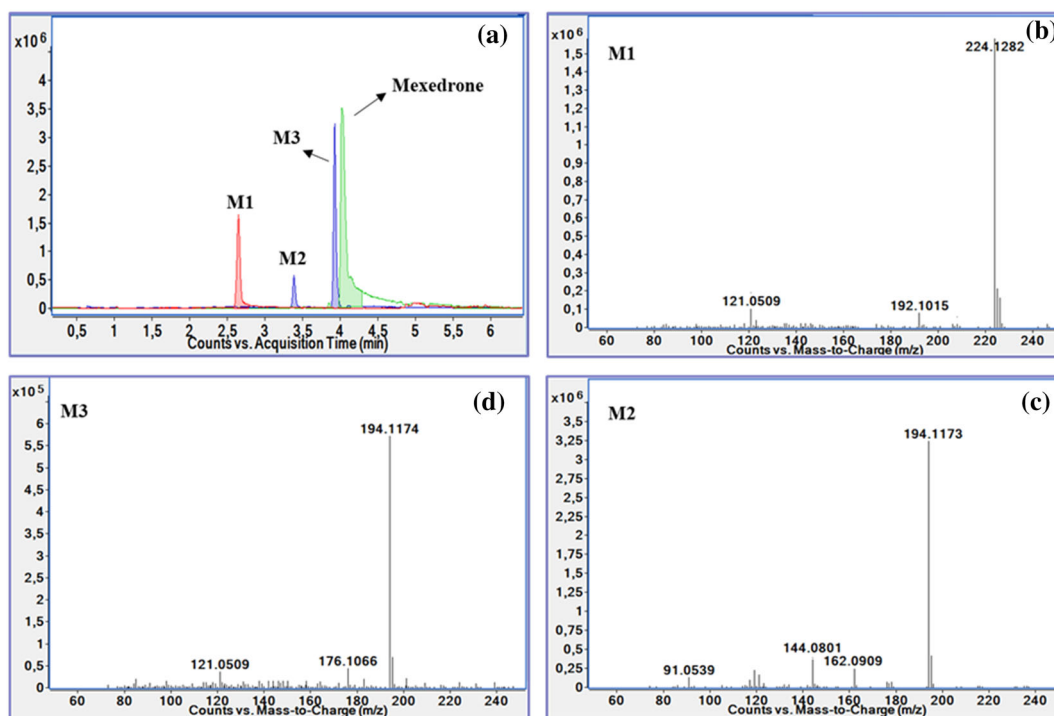
#### 3.4 | In vitro investigation

##### 3.4.1 | Identification of metabolites

After 4 hours of incubation at 37°C, the parent compound and three metabolic products (M1, M2, and M3) not detected in the sample incubated in the absence of HLM, were identified by time-of-flight system operating in full scan as acquisition mode (see Figure 2 and Table 1 for elemental composition and molecular ion of mexedrone



**FIGURE 1** Mass spectra of mexedrone obtained operating in product ion scan mode with precursor ion set at  $m/z$  208, dominant signal underlined. The most representative spectra were at collision energies of 20 eV (a), and at 40 eV (b). The proposed fragmentation pattern of mexedrone was reported in panel (c) [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**FIGURE 2** (a) Extracted ion chromatogram of a representative sample after 4 h of incubation with HLM hydroxylated (M1) and two dealkylated M2 and M3. The extracted mass spectra of M1 (b), M2 (c), and M3 (d) were reported [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

**TABLE 1** Mass spectra parameters for mexedrone and M1-M3 metabolites, including  $[M + H]^+$ , elemental composition,  $m/z$  error ( $\Delta$ ppm) precursor and product ions, and relative collision energies

Untargeted (HRMS, Q-TOF)				Targeted (MS/MS; QqQ)		
Compound	$[M + H]^+$ ( $m/z$ )	Elemental composition	$\Delta$ ppm	Precursor ion ( $m/z$ )	Product ions ( $m/z$ )	Collision energy (eV)
Mexedrone	208.1332	$C_{12}H_{17}NO_2$	0.79	208	<b>176; 158; 149; 119; 91</b>	20; 20; 20; 40; 40
M1 (hydroxyl-)	224.1281	$C_{12}H_{17}NO_3$	0.76	224	<b>192; 174; 135; 107</b> <i>158; 119; 91</i>	20; 20; 40; 40 20; 20; 40
M2 (O-dealkyl-)	194.1175	$C_{11}H_{15}NO_2$	0.12	194	<b>176; 119; 91</b> <i>162; 144</i>	20; 40; 40 20; 20
M3 (N-dealkyl-)	194.1175	$C_{11}H_{15}NO_2$	0.52	194	<b>162; 149; 144; 119; 91</b> <i>176; 158</i>	20; 20; 20; 40; 40 20; 20

Note: The detected product ions are in bold; the undetected product ions are in italics.

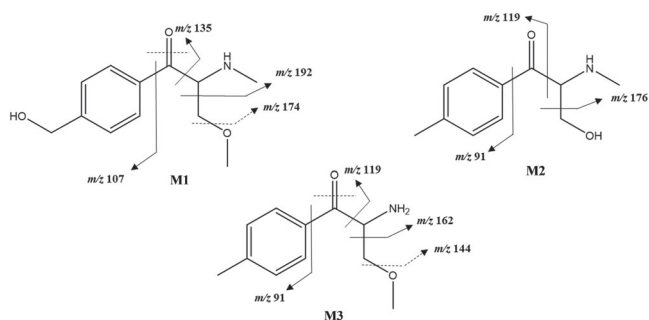
and its metabolites). The three metabolites identified were the following:

- M1 at  $m/z$  224.1281, with elemental composition  $C_{12}H_{17}NO_3$ , compatible with the hydroxylation of mexedrone.
- M2 and M3 with the same  $m/z$  194.1175 and elemental composition  $C_{11}H_{15}NO_2$ , compatible with the *N*/*O*-dealkylation of mexedrone.

These metabolic reactions (i.e., hydroxylation and *N*/*O*-dealkylation) are very similar to those already described for similar SCs in vitro

(e.g., mephedrone).<sup>41,42</sup> However, while the carboxylation of the hydroxy-tolyl portion was reported as the principal urinary marker of intake of mephedrone,<sup>43,44</sup> this metabolic reaction did not seem to take place in our experimental conditions. The extracted ion chromatograms and the related MS spectra for the metabolites of mexedrone are reported in Figure 2.

Samples were then analyzed by a triple quadrupole system operating in MRM mode to hypothesize the structure of the metabolites identified after the analysis with HRMS. The characteristic ion transitions used to set up the MRM acquisition method were obtained by selecting the characteristic fragmentation routes (structural markers)



**FIGURE 3** Proposed structures and mass spectra pattern for hydroxylated (M1), the *O*-dealkylated (M2) and the *N*-dealkylated (M3) metabolites of mexedrone with characteristic diagnostic ion transitions, the dashed line fragmentations

found in the product ion spectrum of mexedrone (see again Figure 1), assuming a similar fragmentation behavior also for the phase I metabolites. The structural elucidation of the phase I metabolites detected by the HRMS analysis was then conducted based on the presence or absence of the representative structural markers, as detailed below (see again Table 1 for the characteristic ion transitions selected for mexedrone and its metabolic products).

In details, the phase-I metabolic products isolated in our experimental conditions were the following:

- M1, with characteristic ion transitions at  $m/z$  192,  $m/z$  174,  $m/z$  135, and  $m/z$  107. These ion transitions differ from those of mexedrone ( $m/z$  176,  $m/z$  158,  $m/z$  119, and  $m/z$  91) by 16 Da (Figure 1c), indicating the insertion of a hydroxyl group. More specifically, the presence of the ions at  $m/z$  107 and 135 and the absence of the product ions at  $m/z$  91 and  $m/z$  119 indicate a hydroxyl group on the tolyl portion of the molecule, confirming the results reported in studies carried out on mephedrone.<sup>41–43</sup>
- M2, with the same ion transitions of mexedrone at  $m/z$  91,  $m/z$  119, and  $m/z$  176 and molecular ion and elemental composition that indicate a dealkylation of the structure. The ion transition at  $m/z$  176 and the absence of the product ions at  $m/z$  144 and  $m/z$  162 indicate the presence of the *N*-alkyl group in the structures. The *O*-dealkylation was subsequently attributed to this metabolite.
- M3, with the same precursor ion of M2 and ion transitions at  $m/z$  91,  $m/z$  119,  $m/z$  149. The presence of the specific ion transitions at  $m/z$  144 and at  $m/z$  162 and the absence of the product ions at  $m/z$  158 and  $m/z$  176 indicate the loss of the methyl group of the amine. Therefore, *N*-dealkylation of mexedrone was attributed to this structure.

The results are shown in Figure 3, with the proposed structures of the above metabolites and their fragmentation pathways. The mass spectrometric parameters for targeted and untargeted analysis, that is,  $[M + H]^+$ , elemental composition,  $m/z$  error ( $\Delta$ ppm), selected precursor and product ions, and relative collision energies, are reported in Table 1.

**TABLE 2** CYP450 isoforms incubated with mexedrone standard solution

Isoforms	M1	M2	M3
CYP3A4	–	–	–
CYP3A5	–	–	–
CYP1A2	+	–	–
CYP2C9	+	+	+
CYP2D6	+	–	–

Note: “+”: detection of a specific metabolite. “–”: non-detection of metabolite after incubation with the single recombinant CYP450 isoforms.

### 3.4.2 | Individual contribution of the different CYP isoforms

The relative contribution of individual CYP450 isoforms to the phase I metabolic reactions of mexedrone identified was also evaluated. The mexedrone solution (40  $\mu$ M) was incubated with five different CYP isoforms (i.e., CYP1A2, CYP2C9, CYP3A4, CYP3A5, and CYP2D6) at a concentration of 1 mg/ml, added to the reaction medium separately. The results showed that CYP3A4 and CYP3A5 were not involved in mexedrone metabolism, while CYP1A2 and CYP2D6 formed only the hydroxylated metabolite M1 (see Table 2). The isoform CYP2C9 was involved in the formation of all the metabolites (M1–M3). These results are in agreement with those obtained for similar SCs<sup>45,46</sup> with exception of mephedrone, for which the role of CYP2D6 in the formation of demethylated products has been reported.<sup>47</sup> These results suggest that all metabolites may be subjected to wide inter-individual variability. For indeed, CYP2C9, that is, the isoform most involved in mexedrone metabolism, is highly polymorphic, and some of its isoforms are characterized by a marked decrease in enzymatic activity.<sup>48</sup> This fact could influence individual metabolic profiles and could have clinical relevance in the case of drug–drug interactions. Nonetheless, based on the experimental evidence here presented, mexedrone unchanged and its metabolites M1 and M3 appear to be the best candidate markers to detect mexedrone intake, although their actual diagnostic value has to be confirmed by controlled in vivo administration studies.

## 4 | CONCLUSION

The phase I metabolic pathway of mexedrone was defined through in vitro studies carried out employing both HLM and five different CYP450 isoforms. The main metabolic reactions were dealkylation and hydroxylation. Three different in vitro metabolites, potentially candidates as markers of intake, were identified by untargeted analysis of incubated samples by UHPLC-QTOF. The analysis carried out in parallel by LC-QqQ confirmed that the detected metabolites are hydroxy-mexedrone (M1), *O*-dealkyl-mexedrone (M2), *N*-dealkyl-mexedrone (M3).

The formation of M1 involved three CYP450 isoforms (i.e., CYP2C9, CYP2D6, and CYP1A2) while M2 and M3 were formed only after incubation with CYP2C9. These results suggest that the formation of M2 and M3 may show a broad interindividual variability due to the influence of allelic variants that, in the case of low activity isoforms, may markedly reduce their formation.

Therefore, based on our *in vitro* observation, the metabolites M1, M3, and mexedrone itself could be pre-selected as markers of intake. Nevertheless, being the carboxylated metabolite the main marker of intake of the structurally related analog mephedrone, we suggest to also include it in the acquisition methods. Further studies are needed using *in vivo* models to confirm the *in vitro* observation and to verify the formation of the carboxylate metabolites for mexedrone defining the windows of detection of mexedrone and its metabolites.

#### DATA AVAILABILITY STATEMENT

All data not specifically included in the manuscript are available on request

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