

RESEARCH ARTICLE

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Human phase-I metabolism and prevalence of two synthetic cannabinoids bearing an ethyl ester moiety: 5F-EDMB-PICA and EDMB-PINACA

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Abstract

Around 2017, with the appearance of 5F-EDMB-PINACA, synthetic cannabinoids (SCs) carrying an ethyl ester moiety at the linked group started spreading on the market of new psychoactive substances (NPS). In 2020 and 2021, the indole analog of 5F-EDMB-PINACA (5F-EDMB-PICA) and the non-fluorinated analog of this compound (EDMB-PINACA) were analytically characterized. Here, we present suitable urinary markers to prove the consumption of these two ethyl analogs. Ten authentic urine samples for each compound were analyzed by liquid chromatography–quadrupole time-of-flight mass spectrometry (LC–qToF–MS). Anticipated phase-I metabolites detected in urine samples were confirmed in vitro by applying a pooled human liver microsomes (pHLM) assay. Prevalence data were obtained from urines collected for abstinence control and submitted to a screening method for SC metabolites. Ten phase-I metabolites of 5F-EDMB-PICA and 18 of EDMB-PINACA were detected by LC–qToF–MS analysis of authentic urine specimens. The main in-vivo metabolites were built by ester hydrolysis, often coupled to further metabolic processes. Investigation of phase-I biotransformation led to the identification of ester hydrolysis, monohydroxylation, and defluorination products as the most suitable urinary biomarkers for 5F-EDMB-PICA. Metabolites formed by ester hydrolysis coupled to ketone formation and by monohydroxylation are suggested for the detection of EDMB-PINACA. From October 1, 2020 to February 1, 2022, among positive urine samples, 5.4% and 10.1% tested positive 5F-EDMB-PICA and EDMB-PINACA, respectively. Due to common metabolites shared among structurally related SCs, the unequivocal detection of their consumption remains challenging for forensic laboratories and requires sensitive methods to monitor multiple metabolites, ideally including highly specific species.

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KEYWORDS

mass spectrometry, new psychoactive substances, urinary biomarkers

1 | INTRODUCTION

Synthetic cannabinoids (SCs) represent one of the largest groups among the new psychoactive substances (NPS) that are monitored by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA).¹ SCs are often offered as herbal mixtures/blends, labeled as “legal” alternatives to cannabis and mostly act as full agonists at the cannabinoid receptors, while Δ^9 -tetrahydrocannabinol (THC) has a partial agonistic action at these receptors. Since their first detection in ‘Spice’ products in 2008, the EMCDDA has monitored a steadily growing number of SCs.² The number of new compounds per year increased from 1–2 around 2008 to approximately 30 in 2012–2014 and decreased thereafter.¹ The introduction of new compounds on the market is typically followed by scheduling under national or international laws,³ which fuels a cat-and-mouse game between manufacturers of NPS products and legislators. Newer SCs, introduced around 2014 and bearing an indazole core, are characterized by an approximately 5–10 times higher potency at the cannabinoid receptors compared to the first generation of SCs.^{4,5}

Around 2015 and 2016, 5F-MDMB-PINACA (methyl 2-[[1-(5-fluoropentyl)-1H-indazole-3-carbonyl]amino]-3,3-dimethylbutanoate, also called 5F-ADB) and 5F-MDMB-PICA (methyl 2-[[1-(5-fluoropentyl)-1H-indole-3-carbonyl]amino]-3,3-dimethylbutanoate) were two of the most prevalent SCs on the market.^{3,6–9} Since 2017, 5F-EDMB-PINACA (ethyl 2-[[1-(5-fluoropentyl)-1H-indazole-3-carbonyl]amino]-3,3-dimethylbutanoate), the analog of 5F-MDMB-PINACA carrying an ethyl ester moiety at the linked group, was analytically characterized from seized material.¹⁰ The compound started circulating on the market, becoming one of the four SCs more frequently detected by the Drug Enforcement Administration¹¹ and, together with 5F-MDMB-PINACA and 5F-MDMB-PICA, is now a Schedule I substance in the United States.¹²

Since September 2020, ethyl 2-[[1-(5-fluoropentyl)-1H-indole-3-carbonyl]amino]-3,3-dimethylbutanoate (semisystematic name: 5F-EDMB-PICA), the ethyl analog of 5F-MDMB-PICA and indole analog of 5F-EDMB-PINACA, was detected and analytically characterized by the Hungarian Police¹³ and a US-based toxicology service.¹⁴ Herbal products containing 5F-EDMB-PICA have also been seized in China.¹⁵ In 2021, a new SC, ethyl 2-[[1-(pentyl-1H-indazole-3-carbonyl)amino]-3,3-dimethylbutanoate (semisystematic name: EDMB-PINACA), the non-fluorinated analog of 5F-EDMB-PINACA, has been also characterized.¹⁶ Both compounds are not yet explicitly scheduled in the United States, and data on their activity at the cannabinoid receptors is still lacking, although comparable potency has been shown for a few pairs of ethyl and methyl ester SCs.¹⁷

The dynamic of the evolving market together with the wide structural diversity of compounds within the subclass of SCs represents a challenge for forensic toxicology laboratories. To prove their

consumption, targeted methods have to be developed.¹⁸ Indeed, inability to detect these drugs by routine drug tests, together with easy access and availability, low price, perceived legal status, and false assumption of safety, represent the main motivations for SC instead of cannabis, despite the latter has been legalized in some countries.^{1,19,20} Moreover, due to the limited half-life in blood and serum and their extensive metabolism, the proof of consumption often relies on the detection of metabolites in urine, a matrix characterized by a wider detection window.^{21–23} Given the many similarities with previously detected compounds (Figure 1), biotransformation of 5F-EDMB-PICA and EDMB-PINACA can be expected to lead to metabolites identical to those formed after intake of other structurally related SCs.^{6,24} Moreover, it was shown that SCs like 5F-MDMB-PINACA might be subject to transesterification to their respective ethyl ester analogs in the presence of ethanol. On this basis, it was hypothesized that metabolites of the ethyl ester analog could derive from the consumption of methyl ester SCs in combination with ethanol.²⁴ Aside from this, by-products from synthesis or pyrolytic breakdown products can lead to the detection of metabolites in vivo not formed after ingestion of the unchanged parent compound or to a distortion of the metabolic profile.^{25–27}

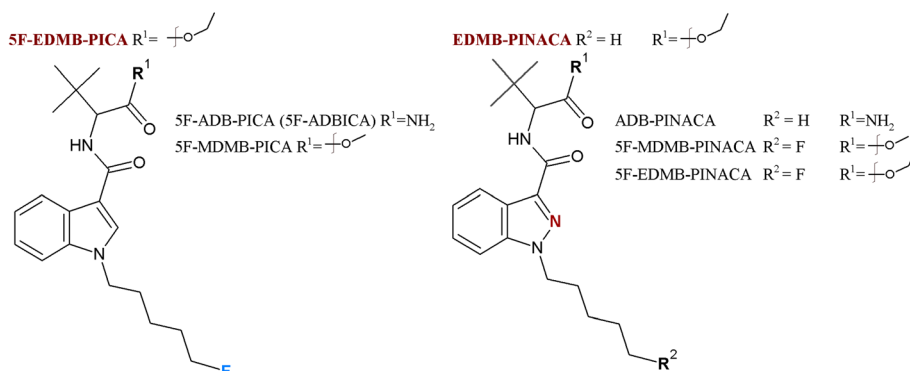
We report on the human phase-I metabolism of EDMB-PINACA and 5F-EDMB-PICA, two structurally related SCs characterized by a 3,3-dimethylbutanoate as well as by an ethyl ester moiety at the linked group, in order to tentatively identify urinary biomarkers useful as unequivocal proof of their consumption. We further describe the prevalence of these compounds in the German market for NPS, on the basis of authentic forensic urine specimens collected in the setting of abstinence control programs.

2 | EXPERIMENTAL

2.1 | Chemicals and reagents

Formic acid (Rotipuran® ≥98%, p.a.), sodium hydroxide (≥99%, p.a., pellets), and potassium hydrogen phosphate (≥99%, p.a.) were obtained from Carl Roth (Karlsruhe, Germany). Acetonitrile (ACN) (LC-MS grade) and ammonium formate 10 M (99.995%) were bought from Sigma-Aldrich (Steinheim, Germany). Isopropanol (Prepsolv®) was obtained from Merck (Darmstadt, Germany). Acetic acid glacial (USP, EP, and JP grades) was purchased from VWR (Darmstadt, Germany). Pooled human liver microsomes (pHLMs; 50 donors, 20 mg/ml protein in 250 mM sucrose), NADPH-regenerating Solutions A and B (reductase activity 0.43 μmol/min * ml), and potassium phosphate buffer 0.5 M (pH 7.5) were purchased from Corning (New York, USA). NADPH regenerating Solution A consisted of 26 mM NADP⁺, 66mM glucose6-phosphate, and 66mM MgCl₂ in water.

FIGURE 1 5F-EDMB-PICA and EDMB-PINACA in comparison with structurally related synthetic cannabinoids [Colour figure can be viewed at wileyonlinelibrary.com]



NADPH-regenerating Solution B consisted of 40 U/ml glucose-6-phosphate dehydrogenase in 5 mM sodium citrate. Roche Diagnostics (Mannheim, Germany) produced the β -glucuronidase (*Escherichia coli* K12) used for conjugate cleavage.

The reference standards for 5F-EDMB-PICA and EDMB-PINACA (purity $\geq 98\%$ for both compounds) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Full analytical data (gas chromatography-mass spectrometry [GC-MS], nuclear magnetic resonance [NMR], and Fourier-transform infrared spectroscopy [FTIR]) on the structural characterization of 5F-EDMB-PICA and EDMB-PINACA have already been provided.^{13,14,16}

Deionized water was prepared using a Medica® Pro deionizer from ELGA (Celle, Germany). Blank urine samples were donated by a volunteer and tested for the absence of SC metabolites prior to use. Mobile phase A (1% ACN, 0.1% HCOOH, and 2 mM NH_4^+HCOO in water) and mobile phase B (0.1% HCOOH and 2 mM NH_4^+HCOO in ACN) were freshly prepared prior to analysis. The sodium formate/acetate clusters solution used for external and internal mass calibration of the quadrupole time-of-flight (QToF)-MS instrument was prepared by mixing 250 ml deionized water, 250 ml isopropanol, 750 μl acetic acid, 250 μl formic acid, and 500 μl sodium hydroxide 1 M.

2.2 | Authentic human urine samples and preparation

For identification of the in vivo phase-I main metabolites of 5F-EDMB-PICA and EDMB-PINACA, liquid chromatography-QToF-MS (LC-QToF-MS) analysis was performed with 10 urine samples obtained from 10 different individuals for each compound. These urine samples were tentatively tested positive for the anticipated metabolites (based on typical metabolic reaction observed for structurally similar compounds). All analyses were conducted in accordance with the inquiry of the respective client (abstinence control). Preparation of specimens was performed as in previously published methods.^{28,29} Briefly, a volume of 0.5 ml of phosphate buffer (pH 6) and 30 μl β -glucuronidase were added to 0.5 ml of urine. A 1 h incubation at 45°C was performed and quenched by addition of 1.5 ml ice-cold ACN; then, 0.5 ml of a 10 M ammonium formate solution were added. The mixture was

shaken (overhead mixing) for 5 min and centrifuged for 10 min (Heraeus Megafuge 1.0, Thermo Scientific, Schwerte, Germany). Then, 1 ml of the organic layer was transferred into a separate vial and evaporated to dryness under a stream of nitrogen. Finally, the samples were reconstituted in 25 μl mobile phases A/B (50/50, v/v) prior to LC-qToF-MS analysis (see Section 2.4). Negative control samples (blank urine) were prepared accordingly.

2.3 | pHLM assay

In vitro phase-I metabolites of 5F-EDMB-PICA and EDMB-PINACA were generated by applying a pHLM assay, which was used for confirmation (in the sense of a plausibility control) of the metabolites detected in the authentic human urine samples. The in vitro assay was performed by adding 0.5 μl of a 1 mg/ml reference standard solution (final concentration of 10 $\mu\text{g}/\text{ml}$ in ACN) to 49.5 μl of a reaction mixture consisting of 2.5 μl pHLM, 2.5 μl NADPH-regenerating Solution A, 0.5 μl NADPH regenerating Solution B, 10 μl phosphate buffer 0.5 M (pH 7.4), and 34 μl deionized water. The reaction was performed during a 30 min long incubation period at 37°C and was terminated by the addition of 150 μl ice-cold ACN. After addition of 25 μl of a 10 M ammonium formate solution, the sample was centrifuged for 4 min at 13,000 rpm. Then, the organic layer was transferred into a separate vial. For LC-qToF-MS analysis (parameters described in Section 2.4), 30 μl of the extracts was evaporated to dryness under a stream of nitrogen and reconstituted with 30 μl mobile phase A/B (50/50, v/v). Two blank pHLM samples, one containing no reference standard (zero-control) and the other one containing no pHLM-enzymes (blank-control), were processed accordingly and served as negative controls. The experiments were performed in triplicates.

2.4 | Identification of tentative main metabolites (LC-qToF-MS)

LC-qToF-MS analysis was performed on an Impact II QToF instrument coupled with an Elute HPLC system (Bruker Daltonik, Bremen, Germany). Chromatographic separation was performed on a Kinetex®

C18 column (2.6 μm , 100 $\text{\AA}$, 100 2.1 mm; Phenomenex, Aschaffenburg, Germany) applying gradient elution as follows: starting condition of mobile phase B was 20%, linearly increased to 50% in 8.0 min, further increased to 60% in 2.0 min, further increased to 95% in 2.0 min, held for 1.0 min, decreased to starting conditions of 20% in 0.1 min, and held for 2.9 min for re-equilibration, resulting in a total run time of 15 min. The flow rate was set to 0.5 ml/min. Autosampler and column oven temperature were set to 10°C and 40°C, respectively. The injection volume was 5 μl . HyStarTM ver. 3.2 and DataAnalysis (DA) ver. 4.2 (Bruker Daltonik, Bremen, Germany) were used for data acquisition and processing, respectively. The MS was operated in positive electrospray ionization (ESI) mode acquiring spectra in the m/z range of 50–650. The dry gas temperature was set to 200°C with a dry gas flow of 8.0 L/min. The nebulizer gas pressure was 2 bar. In a first run, full scan and broadband collision-induced dissociation (bbCID) data were acquired to screen for metabolites. The resulting spectra were compared with a list of hypothetical metabolites, based on the biotransformation of structurally similar SCs. The resulting hits for the molecular ions of anticipated metabolites were further analyzed with defined quadrupole mass selection (± 0.5 Da) and retention time windows. All identified molecular ions of the metabolites were added to an inclusion list and further characterized in MS/MS mode to produce product ion spectra. The collision energy applied for bbCID was 35 ± 7 eV. Full scan and MS/MS data were acquired at an acquisition rate of 2.0 Hz in one run. Nitrogen was used as collision gas. The voltages for the capillary and end plate offset were 2500 and 500 V, respectively. External and internal mass calibration was performed using sodium formate/acetate clusters and high-precision calibration (HPC) mode. Furthermore, precursor ion analysis of the data obtained from full scan and bbCID mode was performed with characteristic fragment ions (e.g., m/z 144.0444 for 5F-EDMB-PICA and m/z 145.0396 for EDMB-PINACA) to detect unexpected metabolites not covered by the inclusion list. For comparison of the relative metabolic profile (to build a metabolic rank in authentic urine samples and pHLM assays), peak area ratios were calculated by dividing the peak area of each metabolite by the peak area of the most abundant metabolite. The following criteria were applied for metabolite identification: mass error of the precursor ion of 5 ppm, a signal to noise ratio of 3:1, and matching isotope pattern of the molecular ion.

3 | RESULTS AND DISCUSSION

3.1 | 5F-EDMB-PICA phase-I metabolism

5F-EDMB-PICA ($[\text{M} + \text{H}]^+$ at m/z 391.2391) was detected in the blank-control as well as in the pHLM assay, eluting at 10 min the LC-qToF-MS conditions previously reported. According to previous analytical data, it showed characteristic fragments at m/z 144.0444, at m/z 116.0495, both corresponding to the indole core, and at m/z 232.1132, which is characterized by the 5fluoro-pentyl chain attached to the indole core.^{13,14} Unchanged 5F-EDMB-PICA was not detected

in any of the analyzed urine samples, and its absence highlights the need for targeting metabolites in urine analysis as a proof of SC consumption.

After enzymatic cleavage of glucuronides, the analysis of the human phase-I metabolism led to the detection of 10 metabolites assigned to the following biotransformations: ester hydrolysis, mono-hydroxylation, defluorination, *N*-dealkylation, pentanoic and propionic acid formation, and combinations of these metabolic reactions (Table 1, Figure 2). In Figure 3, the fragmentation pattern of 5F-EDMB-PICA and of its main metabolites, as shown in authentic urine samples, is displayed.

3.1.1 | Ester hydrolysis and ester hydrolysis plus *N*-dealkylation

M6 (–28.0313 u, $[\text{M} + \text{H}]^+$ at m/z 363.2078) was the most abundant metabolite in all authentic urine samples. Given its protonated mass and the diagnostic product ions m/z 144.0444 and m/z 232.1132, M6 was deemed a product of the terminal hydrolysis of the ethyl group. M6 was absent in the control samples; thus, its formation during sample preparation could be excluded. Ester hydrolysis is a common metabolic step for methyl ester and ethyl ester SCs, leading to identical, often highly abundant metabolites.^{6,24} Particularly, because metabolites with the same masses could be formed by metabolism of 5F-MDMB-PICA, 5F-ADBICA, and 5F-ABICA, the ester hydrolysis product could not be considered a reliable biomarker for the detection of 5F-EDMB-PICA consumption.⁶

Ester hydrolysis biotransformation was also seen in combination with other metabolic reactions, leading to metabolites M1–M4. The protonated mass of M1 ($[\text{M} + \text{H}]^+$ at m/z 275.1390) suggests an *N*-dealkylation (–88.0688 u) plus an ester hydrolysis (–28.0313 u). In accordance with this hypothesis, the indole core fragments m/z 144.0444 and m/z 116.0495 were detected. It must be emphasized that this metabolite is particularly non-specific, as it could be found in any SC bearing an indole group and capable of producing an ester hydrolysis product, like, for example, 4F-MDMB-BICA (IUPAC name: methyl *N*-[[1-(4-fluorobutyl)-1*H*-indol-3-yl]carbonyl]-3-methylvalinate). Interestingly, the intermediate *N*-dealkylation product was not found in vivo but only after pHLM incubation, where it was the most abundant product. As shown for other SCs before, the in vitro metabolic pattern does not always match the pattern in authentic urine specimens, which have to be evaluated to identify the main metabolites excreted renally by humans.

3.1.2 | Monohydroxylation and monohydroxylation coupled to ester hydrolysis

Monohydroxylation ($[\text{M} + \text{H}]^+$ at m/z 407.2341) of the indole core structure represented another main biotransformation pathway of 5F-EDMB-PICA, leading to the formation of the positional isomers M9 and M10. Detection of the specific ion fragment m/z 160.0393 ($+m/z$

TABLE 1 5F-EDMB-PICA human phase-I metabolites detected by LC-qToF-MS analysis of 10 urine samples

ID	RT (min)	Biotransformation	Ranking position	MAR in vivo (%)	Calculated [M + H] ⁺	Formula [M + H] ⁺	Mass error (ppm)	Diagnostic product ions calc. (m/z)	Diagnostic product ions formula	Diagnostic product ions mass error (ppm)	In vitro confirmation via pHLM assay
M00	10	-	-	-	391.2391	C ₂₂ H ₃₂ FN ₂ O ₃ ⁺	-1	116.0495 144.0444 232.1132	C ₈ H ₆ N ⁺ C ₉ H ₆ NO ⁺ C ₁₄ H ₁₅ FNO ⁺	0.5 0.5 -1.9	-
M1	3.3	Ester hydrolysis + N-dealkylation	9	1.1%	275.1390	C ₁₅ H ₁₉ N ₂ O ₃ ⁺	0.1	144.0444 116.0495	C ₉ H ₆ NO ⁺ C ₈ H ₆ N ⁺	0.5 -2.2	✓
M2	4.4	Ester hydrolysis + OH (pentyl)	5	3.45%	379.2028	C ₂₀ H ₂₈ FN ₂ O ₄ ⁺	-3	248.1081 144.0444	C ₁₄ H ₁₅ FNO ₂ ⁺ C ₉ H ₆ NO ⁺	3.9 0.9	✓
M3	4.6	Ester hydrolysis + OH (pentyl)	7	1.62%	379.2028	C ₂₀ H ₂₈ FN ₂ O ₄ ⁺	-0.4	248.1081 144.0444	C ₁₄ H ₁₅ FNO ₂ ⁺ C ₉ H ₆ NO ⁺	0.3 -2	✓
M4	5	Ester hydrolysis + OH (pentyl)	11	0.74%	379.2028	C ₂₀ H ₂₈ FN ₂ O ₄ ⁺	-1.5	248.1081 144.0444	C ₁₄ H ₁₅ FNO ₂ ⁺ C ₉ H ₆ NO ⁺	0.5 -4.2	✓
M5	6.2	Propionic acid	4	21%	375.1914	C ₂₀ H ₂₇ N ₂ O ₅ ⁺	0.6	216.0655 144.0444	C ₁₂ H ₁₀ NO ₃ ⁺ C ₉ H ₆ NO ⁺	1 3.4	-
M6	7.1	Ester hydrolysis	1	100%	363.2078	C ₂₀ H ₂₈ FN ₂ O ₃ ⁺	0.7	232.1132 144.0444	C ₁₄ H ₁₅ FNO ⁺ C ₉ H ₆ NO ⁺	0.9 -2.6	✓
M7	7.1	Pentanoic acid	6	2.4%	403.2227	C ₂₂ H ₃₁ N ₂ O ₅ ⁺	-2.5	244.0968 144.0444	C ₁₄ H ₁₄ NO ₃ ⁺ C ₉ H ₆ NO ⁺	1.2 1.8	✓
M8	7.3	Defluorination	3	28.49%	389.2435	C ₂₂ H ₃₃ N ₂ O ₄ ⁺	0.9	230.1176 144.0444	C ₁₄ H ₁₆ NO ₂ ⁺ C ₉ H ₆ NO ⁺	0.6 0	✓
M9	7.5	OH (aromatic)	2	33.93%	407.2341	C ₂₂ H ₃₂ FN ₂ O ₄ ⁺	0.9	248.1081 160.0393	C ₁₄ H ₁₅ FNO ₂ ⁺ C ₉ H ₆ NO ₂ ⁺	0.7 0.1	✓
M10	8.4	OH (aromatic)	10	0.81%	407.2341	C ₂₂ H ₃₂ FN ₂ O ₄ ⁺	-4.0	248.1081 160.0393	C ₁₄ H ₁₅ FNO ₂ ⁺ C ₉ H ₆ NO ₂ ⁺	0.6 2.1	✓

Abbreviations: LC-qToF-MS, liquid chromatography-quadrupole time-of-flight mass spectrometry; MAR, mean area ratio; OH, monohydroxylation; pHLM, pooled human liver microsomes; RT, retention time.

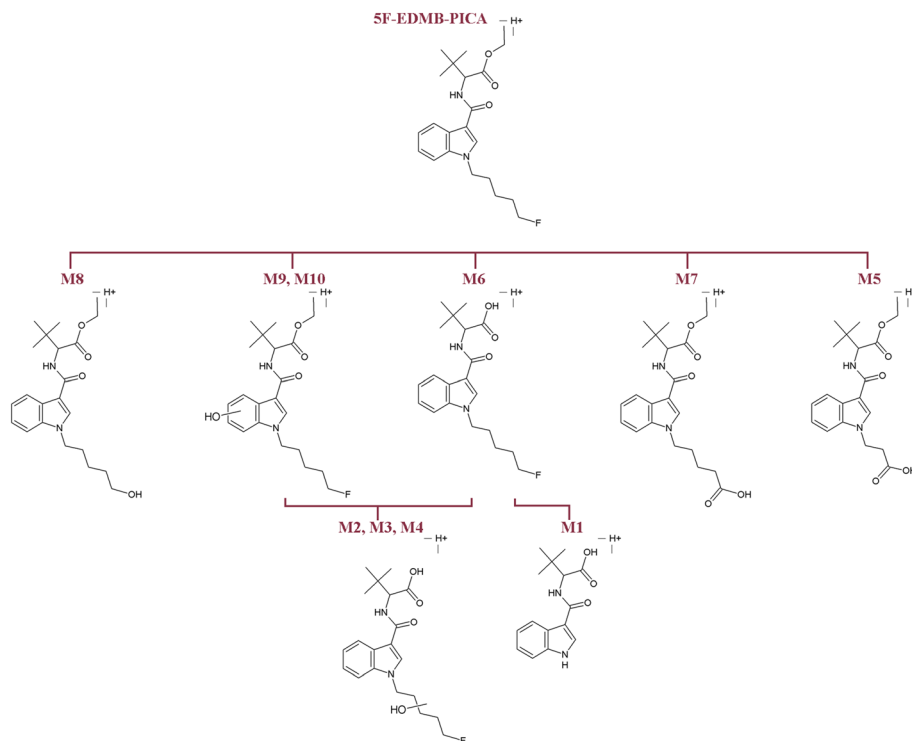


FIGURE 2 Postulated *in vivo* phase-I biotransformation pathways of 5F-EDMB-PICA as investigated in 10 authentic urine samples [Colour figure can be viewed at wileyonlinelibrary.com]

15.9949), coupled to m/z 248.1081 ($+m/z$ 15.9949), indicated a localization of the hydroxyl group at the aromatic structure of the indole moiety (Figure 3). Taking into consideration the mean area ratios (MAR%), M9 was the second most abundant metabolite (MARs, Table 1), whereas M10 showed a low abundance with a MAR of 0.81% overall. M9 shows great potential as a characteristic urinary marker for 5F-EDMB-PICA consumption, as it was detected with relatively high intensities in all the analyzed authentic urine samples (overall rank position 2). Due to its intact ethyl ester moiety and 5-fluoropentyl chain, it can be used to effectively differentiate between other SCs carrying a methyl ester moiety or an amide such as 5F-MDMB-PICA and 5F-ADBICA. No metabolite solely formed by monohydroxylation of the 5-fluoropentyl chain, or involving the linked group, was shown *in vivo*.

However, a monohydroxylation at the 5-fluoropentyl chain, proved by specific ion fragments m/z 144.0444 of the unaltered core and m/z 248.1081, commonly occurred in combination with ester hydrolysis, leading to metabolites M2, M3, and M4 detected with m/z $[M + H]^+$ of 379.2028. M2–M4 are relatively low-abundant in authentic urine samples and also non-specific products, thus not suggested as biomarkers of 5F-EDMB-PICA consumption.⁶

3.1.3 | Hydrolytic/oxidative defluorination

The third most abundant metabolite overall was represented by M8 (-1.9956 u; $[M + H]^+$ at m/z 389.2435). The obtained characteristic fragments corresponding to the unaltered core and to a fragment monitored at m/z 230.1176 suggested a hydrolytic/oxidative

defluorination occurring at the 5-fluoropentyl chain. As also shown for 5F-MDMB-PICA, the potential intermediate, a butanoic acid metabolite ($[M + H]^+$ at m/z 389.2070), could not be found. Defluorination at the 5-fluoropentyl side chain has been reported as one of the main biotransformation steps in the metabolism of SCs²¹ and was also shown *in vitro* for the indazole analog of 5F-EDMB-PICA, 5F-EDMB-PINACA.²⁴ Due to the intact ethyl ester moiety, M8 could also be considered a specific biomarker of 5F-EDMB-PICA, to be monitored in addition to M9. However, in the case that a non-fluorinated analog of 5F-EDMB-PICA is introduced to the market, M8 could be a common metabolite, losing some of its specificity. Moreover, as shown *in vitro* for 5F-EDMB-PINACA, the defluorination product could also be formed from 5F-MDMB-PINACA when ethanol was added.²⁴ Following this scheme, M8 might also be formed after intake of 5F-MDMB-PICA in combination with ethanol, although so far no *in vivo* data exists on this issue.

3.1.4 | Propionic and pentanoic acids

Among the analyzed authentic urinary samples, M5 (-16.0477 u; $[M + H]^+$ at m/z 375.1914) was another frequently found metabolite. The characteristic fragment detected with m/z 216.0655 indicates a metabolic degradation of the 5-fluoropentyl side chain to a propionic acid chain with an intact core and linked group. M5 also built the respective sodium adduct (m/z 397.1734). This was the only metabolite not confirmed by pHLM incubation. The protonated mass of M5 could be confused with other SCs, for example, in the butanoic acid metabolite of 4F-MDMB-BICA, but different characteristic fragments would be expected.

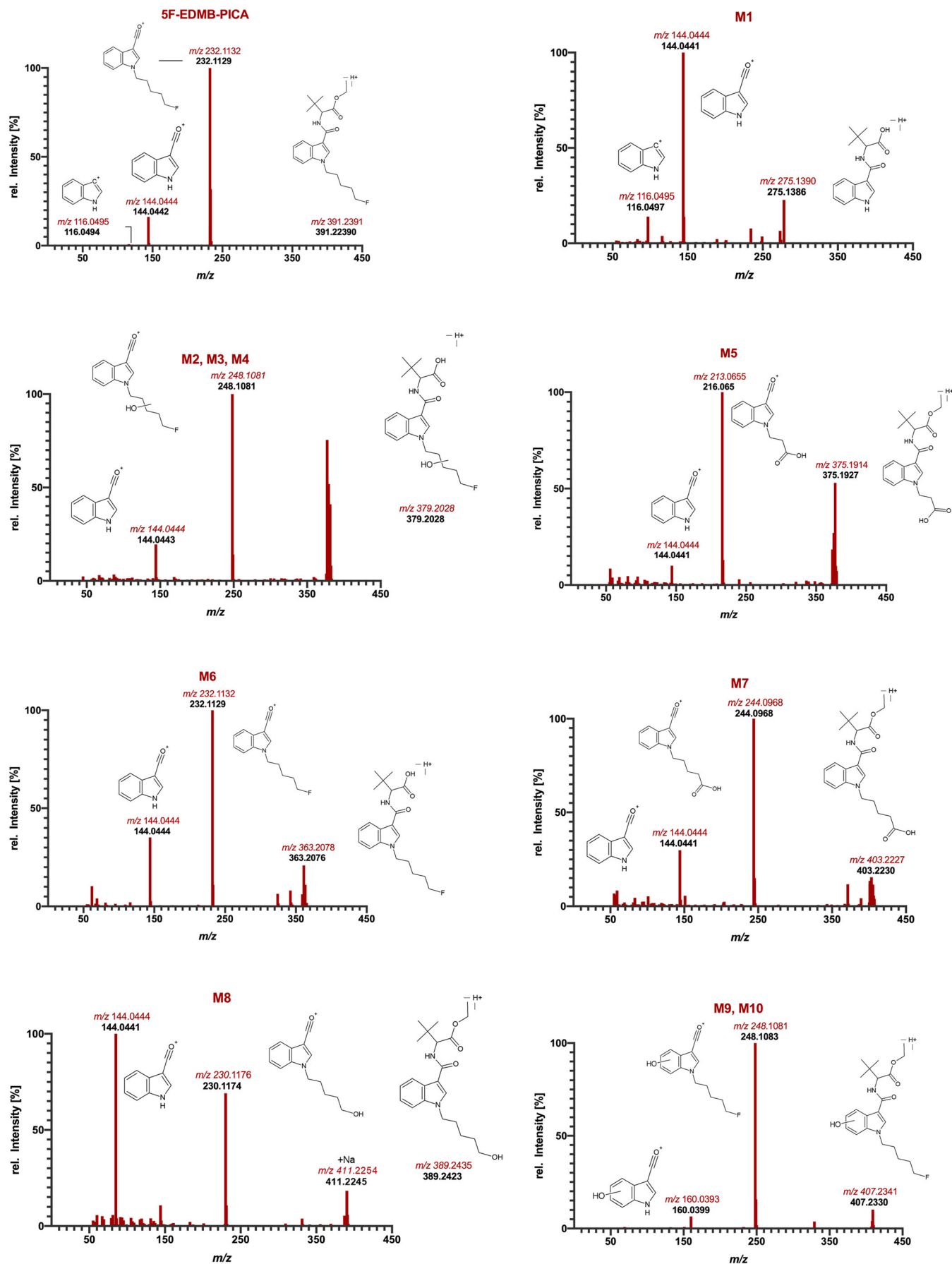


FIGURE 3 Legend on next page.

FIGURE 3 Liquid chromatography–quadrupole time-of-flight mass spectrometry (LC-qToF-MS) spectra (inclusion list) of 5F-EDMB-PICA (M00), taken from the analysis of a reference standard, and its most abundant urinary metabolites, as observed in authentic urine specimens [Colour figure can be viewed at wileyonlinelibrary.com]

The metabolite M7 was formed by metabolic reaction of the 5-fluoropentyl chain to a pentanoic acid and detected with m/z ($[M + H]^+$ 389.2435) and a characteristic fragment ion at m/z 244.0968 (besides the fragment of the unaffected core).

3.1.5 | Urinary biomarkers of 5F-EDMB-PICA

Metabolic reactions involved in the metabolism of 5F-EDMB-PICA were congruent with those described for 5F-MDMB-PICA, with metabolites involving ester hydrolysis, particularly M1, M2, M3, M4, and M6 of the present study, being common to those metabolically formed by 5F-MDMB-PICA.^{6,30} However, some metabolites formed by ester hydrolysis coupled to further reactions (e.g., defluorination, oxidation to the pentanoic acid, or dehydrogenation), as well as the amide hydrolysis metabolite,^{6,30} which were described as metabolites of 5F-MDMB-PICA, could not be detected in the present study and, when detected, might be useful for differentiation. 5F-ADBICA also produced the ester hydrolysis metabolite in vitro, though this was a minor metabolic reaction, while it was shown to be the most abundant metabolite in the here presented study.⁶ Clearly, metabolites of 5F-EDMB-PICA could be differentiated from those built by metabolism of EDMB-PINACA, 5F-MDMB-PINACA, and 5F-EDMB-PINACA considering the protonated masses of metabolites and the fragment ions corresponding to the core. For the unambiguous identification of a 5F-EDMB-PICA uptake, the metabolites M9 (monohydroxylation) and M8 (defluorination) are suggested as specific and highly abundant urinary markers for targeted screening methods (e.g., multiple reaction monitoring with LC-MS/MS). M6 could additionally be targeted, since it appeared as the most abundant metabolite.

3.2 | EDMB-PINACA phase-I metabolism

As shown by the blank control and the pHLM in vitro analyses, EDMB-PINACA ($[M + H]^+$ at m/z 374.2438) (M00) eluted at 11.7 min with the applied LC-qToF-MS method. The parent compound showed characteristic fragments at m/z 145.0396, corresponding to the indazole core structure, m/z 215.1179 (the core structure with the pentyl chain), and m/z 300.2070 (loss of ethyl formate). This is consistent with previous GC and LC-qToF-MS data.¹⁶ No parent substance was found in any authentic urine specimens, as observed for the majority of SCs, with very few exceptions.^{29,31}

The analysis of the human phase-I metabolism of EDMB-PINACA led to the detection of 18 metabolites after enzymatic cleavage of glucuronides, assigned to the following biotransformations: ester hydrolysis, monohydroxylation, dihydroxylation, dehydrogenation, ketone formation, and combinations of these metabolic reactions (Table 2

and Figure 4). In Figure 5, the fragmentation patterns of EDMB-PINACA and its main metabolites are shown.

3.2.1 | Ketone formation plus ester hydrolysis and ketone formation plus monohydroxylation

The most abundant metabolite in all authentic urine samples was M7, detected with m/z ($[M + H]^+$ 360.1918). It presented diagnostic product ions at m/z 145.0396, the unaltered core, m/z 229.0972 and m/z 314.1863. The shift of $+m/z$ 13.9793 with respect to fragments m/z 215.1179 and m/z 300.2070 of the parent compound suggest an oxidation ($+O -2H$) resulting in ketone formation at the pentyl chain, although the exact position remains unknown. The product ion at m/z 85.0648 is consistent with a fragment of the pentyl chain after the ketone formation. Given the protonated mass, M7 was deemed a result of ketone formation and ethyl ester hydrolysis.

The positional isomer of M7, M11 ($[M + H]^+$ at m/z 360.1918), was much less abundant in urine samples (0.82% of M7).

The same fragments seen for the product of ketone formation M7 (m/z 229.0972 and m/z 314.1863) were also observed in minor products of metabolism, M9 and M10, detected with m/z ($[M + H]^+$ 404.2180). Theoretically, a protonated mass of 404.2180, $+m/z$ 29.9724, could also be the result of a dihydroxylation plus dehydrogenation of the parent compound. However, given the characteristic fragments, it is likely that M9 and M10 are formed by ketone formation plus monohydroxylation. The m/z 314.1863, coupled to the protonated mass and to the other fragments, suggests a monohydroxylation occurring at the ethyl group.

Interestingly, the ketone formation from the parent substance without any ethyl ester hydrolysis, $[M + H]^+$ at m/z 388.2230, could not be identified in vivo but only after pHLM incubation.

3.2.2 | Ester hydrolysis and coupled monohydroxylated, dihydroxylated, and/or dehydrogenated products

As already shown for 5F-EDMB-PICA, ester hydrolysis was a prevalent metabolic reaction, leading to the formation of M16 ($-m/z$ 28.0313, $[M + H]^+$ at m/z 346.2125), the second most abundant metabolite, which showed characteristic fragments at m/z 145.0396, m/z 215.1179, and m/z 300.2070.

Ester hydrolysis was coupled to monohydroxylation leading to the positional isomers M3, the third most abundant in all urine samples, M6 and M8 ($[M + H]^+$ at m/z 362.2074). All metabolites presented characteristic fragments for the unaltered core together with m/z 231.1128 ($+m/z$ 15.9949), which indicates a monohydroxylation

TABLE 2 EDMB-PINACA human phase-I metabolites detected by LC-qToF-MS analysis of 10 urine samples

ID	RT (min)	Biotransformation and location	Ranking position	MAR in vivo (%)	Calculated [M + H] ⁺	Formula [M + H] ⁺	Mass error (ppm)	Diagnostic product ions calc. (m/z)	Diagnostic product ions formula	Diagnostic product ions mass error (ppm)	In vitro confirmation via pHLM
M00	11.7	-	-	-	374.2438	C ₂₁ H ₃₂ N ₃ O ₃ ⁺	1.4	215.1179 145.0396 300.2070	C ₁₃ H ₁₅ N ₂ O ⁺ C ₈ H ₅ N ₂ O ⁺ C ₁₈ H ₂₆ N ₃ O ⁺	4.8 7.3 4.6	-
M1	3.2	Ester hydrolysis + diOH (pentyl and pentyl)	6	2.70%	378.2023	C ₁₉ H ₂₈ N ₃ O ₅ ⁺	-0.7	247.1077 145.0396	C ₁₃ H ₁₅ N ₂ O ₃ ⁺ C ₈ H ₅ N ₂ O ⁺	1 1.1	✓
M2	4.3	Ester hydrolysis + OH (pentyl) + deH (EDMB)	8	1.78%	360.1918	C ₁₉ H ₂₆ N ₃ O ₄ ⁺	0	213.1022 145.0396	C ₁₃ H ₁₃ N ₂ O ⁺ C ₈ H ₅ N ₂ O ⁺	-1.1 1.1	✓
M3	4.9	Ester hydrolysis + OH (pentyl)	3	35.84%	362.2074	C ₁₉ H ₂₈ N ₃ O ₄ ⁺	-0.4	231.1128 145.0396	C ₁₃ H ₁₅ N ₂ O ₂ ⁺ C ₈ H ₅ N ₂ O ⁺	-1 0.9	✓
M4	5.0	DiOH (pentyl and ethyl)	13	0.93%	406.2336	C ₂₁ H ₃₂ N ₃ O ₅ ⁺	-1.3	231.1128 316.2020	C ₁₃ H ₁₅ N ₂ O ₂ ⁺ C ₁₈ H ₂₆ N ₃ O ₂ ⁺	-1.2 2.2	✓
M5	5.1	DiOH (pentyl and EDMB)	9	1.71%	406.2336	C ₂₁ H ₃₂ N ₃ O ₅ ⁺	0.1	213.1022 231.1128	C ₁₃ H ₁₃ N ₂ O ⁺ C ₁₃ H ₁₅ N ₂ O ₂ ⁺	-1.6 -0.6	✓
M6	5.3	Ester hydrolysis + OH (pentyl)	5	3.71%	362.2074	C ₁₉ H ₂₈ N ₃ O ₄ ⁺	0.7	231.1128 145.0396	C ₁₃ H ₁₅ N ₂ O ₂ ⁺ C ₈ H ₅ N ₂ O ⁺	1.9 2.5	✓
M7	5.3	Ester hydrolysis + ketone (pentyl)	1	100%	360.1918	C ₁₉ H ₂₆ N ₃ O ₄ ⁺	-1.3	229.0972 85.0648	C ₁₃ H ₁₃ N ₂ O ₂ ⁺ C ₅ H ₉ O ⁺	-1.7 1.3	✓
M8	5.4	Ester hydrolysis + OH (pentyl)	7	1.83%	362.2074	C ₁₉ H ₂₈ N ₃ O ₄ ⁺	1.6	231.1128 145.0396	C ₁₃ H ₁₅ N ₂ O ₂ ⁺ C ₈ H ₅ N ₂ O ⁺	2.2 2.2	✓
M9	5.6	Ketone (pentyl) + OH (ethyl)	10	1.65%	404.2180	C ₂₁ H ₃₀ N ₃ O ₅ ⁺	0.3	229.0972 314.1863	C ₁₃ H ₁₃ N ₂ O ₂ ⁺ C ₁₈ H ₂₄ N ₃ O ₂ ⁺	-1.5 0	✓
M10	5.8	Ketone (pentyl) + OH (ethyl)	12	1.43%	404.2180	C ₂₁ H ₃₀ N ₃ O ₅ ⁺	-0.6	229.0972 314.1863	C ₁₃ H ₁₃ N ₂ O ₂ ⁺ C ₁₈ H ₂₄ N ₃ O ₂ ⁺	-1.6 2.3	✓
M11	6	Ester hydrolysis + ketone (pentyl)	14	0.82%	360.1918	C ₁₉ H ₂₆ N ₃ O ₄ ⁺	1.9	229.0972 145.0396	C ₁₃ H ₁₃ N ₂ O ₂ ⁺ C ₈ H ₅ N ₂ O ⁺	1.1 0.8	✓
M12	7.9	OH (pentyl)	4	16.99%	390.2387	C ₂₁ H ₃₂ N ₃ O ₄ ⁺	-1.9	231.1128 145.0396	C ₁₃ H ₁₅ N ₂ O ₂ ⁺ C ₈ H ₅ N ₂ O ⁺	-1.3 0.3	✓
M13	8.4	Ester hydrolysis + deH (pentyl)	16	0.26%	344.1969	C ₁₉ H ₂₆ N ₃ O ₃ ⁺	1.6	213.1022 145.0396	C ₁₃ H ₁₃ N ₂ O ⁺ C ₈ H ₅ N ₂ O ⁺	1.6 1.9	-
M14	8.5	OH (pentyl)	11	1.57%	390.2387	C ₂₁ H ₃₂ N ₃ O ₄ ⁺	-1.1	231.1128 145.0396	C ₁₃ H ₁₅ N ₂ O ₂ ⁺ C ₈ H ₅ N ₂ O ⁺	-0.1 1.3	✓

(Continues)

TABLE 2 (Continued)

ID	RT (min)	Biotransformation and location	Ranking position	MAR in vivo (%)	Calculated [M + H] ⁺	Formula [M + H] ⁺	Mass error (ppm)	Diagnostic product ions calc. (m/z)	Diagnostic product ions formula	Diagnostic product ions mass error (ppm)	In vitro confirmation via pHLM
M15	9.2	OH (ethyl)	18	0.05%	390.2387	C ₂₁ H ₃₂ N ₃ O ₄ ⁺	1.2	215.1179	C ₁₃ H ₁₅ N ₂ O ₂ ⁺	-0.2	✓
M16	9.3	Ester hydrolysis	2	38.14%	346.2125	C ₁₉ H ₂₈ N ₃ O ₃ ⁺	-1	145.0396	C ₈ H ₅ N ₂ O ⁺	-0.5	✓
M17	9.6	OH (ethyl)	15	0.53%	390.2387	C ₂₁ H ₃₂ N ₃ O ₄ ⁺	-0.6	215.1179	C ₁₃ H ₁₅ N ₂ O ₂ ⁺	-1.5	✓
M18	9.8	OH (methyl)	17	0.17%	390.2387	C ₂₁ H ₃₂ N ₃ O ₄ ⁺	-1.6	145.0396	C ₈ H ₅ N ₂ O ⁺	-0.2	✓
								215.1179	C ₁₃ H ₁₅ N ₂ O ₂ ⁺	-2.3	✓
								145.0396	C ₈ H ₅ N ₂ O ⁺	-0.6	

Abbreviations: deH, dehydrogenation; diOH, dihydroxylation; EDMB: ethyl dimethylbutanoate; LC-qToF-MS, liquid chromatography-quadrupole time-of-flight mass spectrometry; MAR, mean area ratio; OH, hydroxylation; pHLM, pooled human liver microsome; RT, retention time.

at the pentyl side chain. The detected fragment at m/z 213.1022 is likely a product dehydration from m/z 231.1128 (-18.0106 u). Additionally, m/z 316.2020, corresponding to the monohydroxylation of the m/z 300.2070 fragment, and the respective sodium adduct (m/z 384.1894) were detected.

Less abundant metabolites, also involving an ester hydrolysis reaction, were M1 and M13. Given the characteristic ion fragments at m/z 145.0396 and m/z 247.1077, M1 ([M + H]⁺ at m/z 378.2023) was ascribed to an ester hydrolysis reaction coupled to a di-hydroxylation, occurring at the pentyl chain (+ m/z 31.9898 with respect to m/z 215.1179). M13 ([M + H]⁺ at m/z 344.1969) showed m/z 213.1022 as a characteristic fragment, together with m/z 145.0396, allowing to assign the metabolite to an ester hydrolysis reaction coupled to a dehydrogenation (-2.0157 u) of the pentyl chain.

The dehydrogenation product of the parent substance ([M + H]⁺ at m/z 372.2282), still bearing the ethyl moiety at the linked group, could not be identified in vivo but only by pHLM assay.

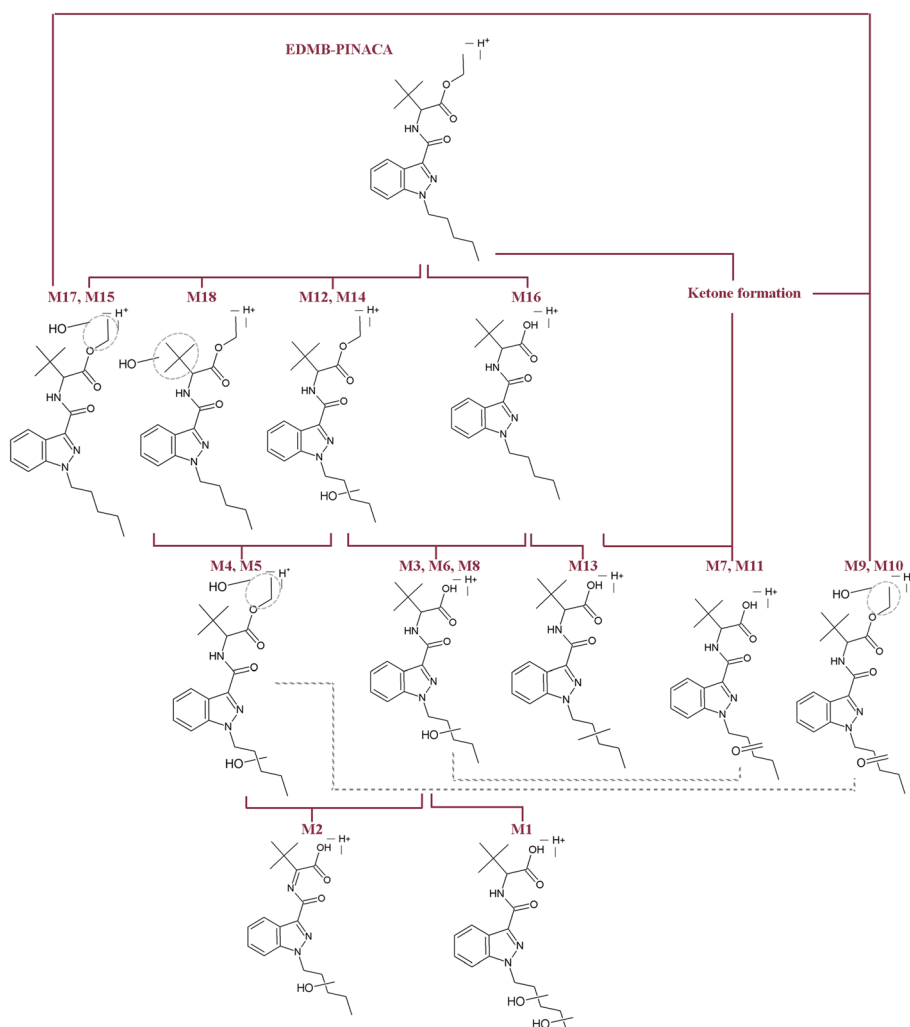
Despite having the same mass as M7 and M11, M2 ([M + H]⁺ at m/z 360.1918) did not show ketone formation at the pentyl chain. Instead, it was characterized by a monohydroxylated pentyl chain (m/z 231.1128) and by m/z 213.1022, corresponding to the dehydrogenated pentyl chain (-2.0157 u with respect to m/z 215.1179). pHLM analysis confirmed the fragments, particularly m/z 231.1128, and also characterized a fragment at m/z 314.1812, which suggests that the dehydrogenation does not involve the carboxylic acid group. Following this hypothesis, M2 would be formed by ester hydrolysis coupled to monohydroxylation at the pentyl chain; m/z 213.1022 would represent a loss of water from the monohydroxylated pentyl chain, and the dehydrogenation could have taken place elsewhere.

Despite their abundance, M16 and M7, as well as those deriving from ester hydrolysis, could not be considered reliable biomarkers for the detection of EDMB-PINACA consumption, because they are not bearing the ethyl ester group. Thus, they cannot be used to unambiguously differentiate from the use of other SCs, such as ADB-PINACA, which is also subject to deamination.³¹

3.2.3 | Mono-hydroxylation

At the fourth rank among urinary metabolites, M12 (+ m/z 15.9949, [M + H]⁺ at m/z 390.2387) was detected. The identification of the characteristic fragment ion m/z 231.1128, together with its dehydration product at m/z 213.1022, coupled to the unaltered indazole core, pointed towards the hydroxylation of the pentyl chain. Furthermore, m/z 316.2020 and m/z 412.2207, the sodium adduct, were identified. Monohydroxylation was responsible for the formation of M14, also hydroxylated at the pentyl chain, as well as of M15, M17, and M18. For M15, M17, and M18, the identification of both the unaltered core (m/z 145.0396) and the pentyl chain (m/z 215.1179) excluded an aromatic and a pentyl chain hydroxylation. M17 showed, as a further characteristic fragment, the non-hydroxylated m/z 300.2070, so that it is likely a product of ethyl hydroxylation. Although characterized by

FIGURE 4 Postulated in vivo phase-I biotransformation pathways of EDMB-PINACA as investigated in 10 authentic urine samples [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1002/dm.3405)]



low abundance, the fragment m/z 316.2020 was detected for M18, suggesting a hydroxylation at the *t*-butyl group.

3.2.4 | Dihydroxylation

Finally, dihydroxylated metabolites M4 and M5 ($+m/z$ 31.9898, $[M + H]^+$ at m/z 360.1918) were detected with lower abundance. Both presented characteristic fragments at m/z 231.1128 and at m/z 213.1022, corresponding to monohydroxylation at the pentyl chain and the loss of water. The fragment m/z 316.2020, identified for M4 but not for M5, indicates that the second hydroxylation might occur at the ethyl group of the ester.

3.2.5 | Urinary biomarkers of EDMB-PINACA

Out of the 18 metabolites detected in vivo, all were confirmed by pHLM assay except one. The comparison between in vitro metabolism and authentic urine specimens once again highlighted the need for

in vivo data, in order to identify the most suitable urinary biomarkers. Indeed, M12 was the most abundant metabolite after pHLM incubation, while showing a MAR of only 17% in vivo. Moreover, the most abundant in vivo metabolite, M7, appeared further down in the in vitro ranking. This might be due to the relatively weak tendency of the in vitro assay, with the here-shown conditions, to perform multiple biotransformations.⁶

Minor metabolites of EDMB-PINACA as well as those only detected by pHLM are shown in the Supporting Information.

Few data are available regarding 5F-EDMB-PINACA metabolism, that was investigated, to the best of the authors' knowledge, only by in vitro assays.

Ester hydrolysis products might be considered non-specific, as they involve the loss of the ethyl ester moiety. Indeed, M2, the ester hydrolysis product, can be formed by other SCs bearing an indazole core and a pentyl chain, like ADB-PINACA.³² Similarly, M3, M6, and M8, formed by ester hydrolysis plus monohydroxylation, had identical masses and fragments to the product of ester hydrolysis coupled to defluorination of 5F-MDMB-PINACA^{33,34} and are thus considered non-specific. Ester hydrolysis coupled to ketone formation at the

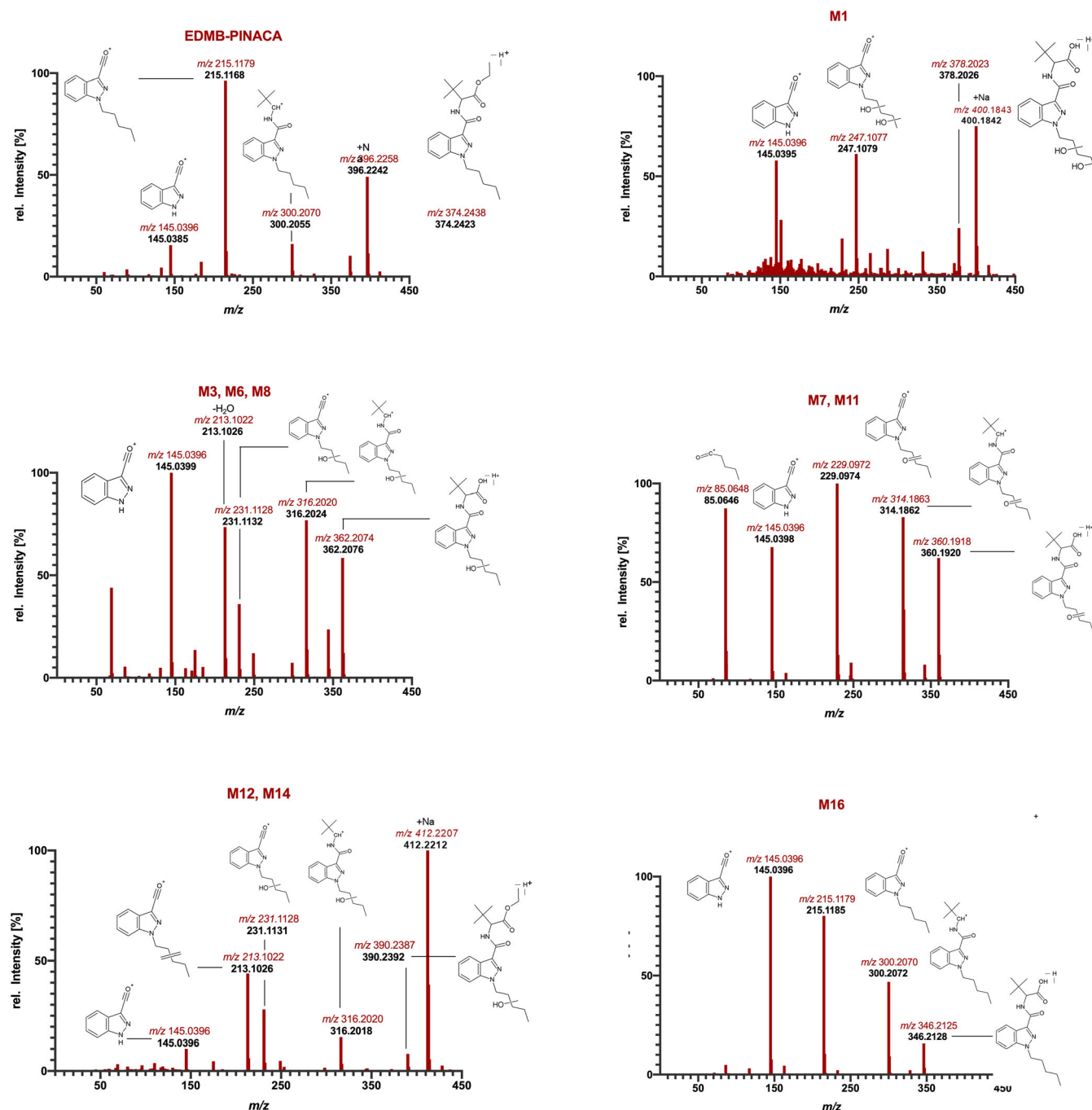


FIGURE 5 Liquid chromatography–quadrupole time-of-flight mass spectrometry (LC-qToF-MS) spectra of EDMB-PINACA (M00), taken from the analysis of a reference standard, and its most abundant urinary metabolites, as observed in authentic urine specimens [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1002/dm.3405)]

pentyl chain might produce metabolites identical to M7, though, to the best of our knowledge, this was not described for 5F-MDMB-PINACA or ADB-PINACA.^{32–34}

Although not characterized by the highest intensities in vivo, M12 is one of the few metabolites retaining the ethyl ester moiety at the linked group. Therefore, this metabolite can be considered as a more specific marker. So far, metabolites of 5F-EDMB-PINACA have not been elucidated by means of in vivo studies, but only by pHLM

incubation.²⁴ The defluorination metabolite of 5F-EDMB-PINACA, as seen by in vitro analysis, would have masses and fragments identical to M12. If these findings would be confirmed by further studies on authentic urine samples, a specific biomarker of EDMB-PINACA consumption, for unequivocal detection in urine specimens, might be difficult to define.

Bearing in mind these limits, the combined monitoring of M7 (possibly non-specific but highly abundant and apparently not formed

by 5F-MDMB-PINACA) in combination with M12 (also possibly non-specific but not in vivo confirmed) can be suggested to prove EDMB-PINACA consumption.

Although the type of metabolic reactions of the two structurally related SCs EDMB-PINACA and 5F-EDMB-PICA was rather congruent, significant differences were found in the biotransformation of the compounds, and the different reactivity between core structures (indole vs. indazole) led to distinct products. For both SCs, ester hydrolysis and monohydroxylation play a major role. However, as already shown for 5F-MDMB-PICA,⁶ 5F-EDMB-PICA is subject to a relatively low number of metabolic transformations compared with its non-fluorinated indazole analog and to fewer types of biotransformation. In accordance with past literature, monohydroxylation primarily involved the aromatic ring for the indole compound, whereas indazoles seem to be monohydroxylated mainly at the pentyl chain.^{21,23,28}

The discrepancy between the cores was also critical for distinguishing similar products generated by different reactions involving the two SCs. For example, the difference in the protonated mass and the characteristic fragments (m/z 144.0444 for 5F-EDMB-PICA and m/z 145.0396 for EDMB-PINACA) allowed to distinguish between the monohydroxylation product (M12) of EDMB-PINACA from the defluorinated one (M8) of 5F-EDMB-PICA.

3.3 | Prevalence in forensic urine samples

An LC-MS/MS screening method for SC metabolites is currently in use at the Institute of Forensic Medicine in Freiburg to analyze urine specimens collected for abstinence control.¹⁸

Since October 2020, some urine samples tested positive for the ester hydrolysis product of 5F-MDMB-PICA but did not fulfill the identification criteria for the detection of the second characteristic product, the monohydroxylated metabolite.⁶ After identification of the main urinary biomarkers of 5F-EDMB-PICA and the integration of the characteristic ion transitions of M8, retrospective analysis the samples (that have been stored at -20°C meanwhile) showed that these samples were instead positive for 5F-EDMB-PICA. Ion transitions for M7 and M12 were used to update the screening method for SC metabolites to detect EDMB-PINACA consumption. From October 1, 2020 to February 1, 2022, $n = 6976$ urine samples were analyzed with the SCs metabolite screening method, of which $n = 1232$ (17.7%) tested positive for SCs.

Among all positive samples, $n = 66$ (5.4%) tested positive for 5F-EDMB-PINACA and $n = 125$ (10.1%) for EDMB-PINACA. Following our data, the consumption of 5F-EDMB-PICA peaked in January 2021, and no sample was detected positive for this compound after August 2021. The first samples positive for EDMB-PINACA were detected in April 2021, peaked right after the decrease of 5F-EDMB-PICA during the summer of 2021, and decreased afterwards (Figure 6).

Their evolution on the market of NPS does not seem to reflect the national legislation, given that both 5F-EDMB-PICA and EDMB-PINACA were already included in the German law on NPS (NpSG) during the period of their occurrence.

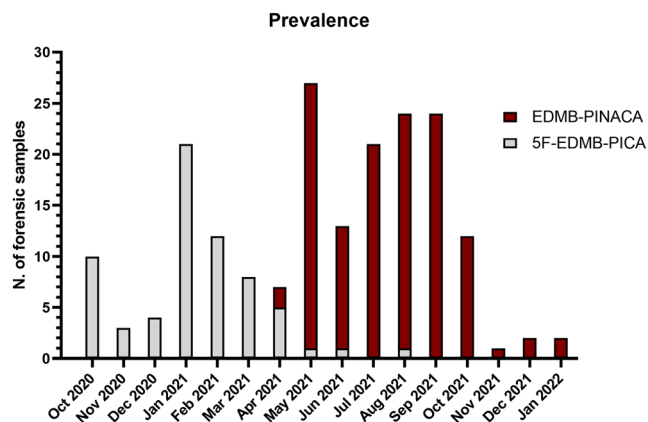


FIGURE 6 Prevalence of 5F-EDMB-PICA and EDMB-PINACA in urine samples collected for abstinence control in Germany [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1002/for.2405)]

Among 5F-EDMB-PICA positive samples, the majority contained more than one SC ($n = 53$; 80%), and among them, MDMB-4en-PINACA (IUPAC name: methyl 3-methyl-*N*-[1-(pent-4-en-1-yl)-1*H*-indazol-3-yl]carbonyl]valinate) ($n = 52/53$, 98.1%), 4F-MDMB-BICA ($n = 32/53$, 60.3%), and ADB-BINACA (IUPAC name: *N*-[1-(amino-3,3-dimethyl-1-oxobutan-2-yl)-1-butyl-1*H*-indazole-3-carboxamide) ($n = 23/53$, 43.4%) were seen particularly often. EDMB-PINACA was less frequently associated with the use of further SCs ($n = 90$, 72%) and also appeared to occur particularly often in combination with MDMB-4en-PINACA ($n = 64/90$, 71.1%) and ADB-BINACA ($n = 36/90$, 40%).

3.4 | Limitations

Several isomeric metabolites with identical fragment ions but different retention times were detected after biotransformation of 5F-EDMB-PICA and EDMB-PINACA. The unambiguous identification of the position of the functional groups introduced by metabolic reactions would require the synthesis of reference material or the isolation of the metabolites of interest for structure elucidation, for example, by NMR spectroscopy. Thus, in the present study, it was not possible to elucidate the exact chemical structures of some metabolites.

As a second limitation, the in vivo MAR% here described that was based on the chromatographic peak areas might not accurately reflect absolute concentrations, given possible differences in ionization efficiency and matrix effects.²⁸

Thirdly, additional metabolites could be found in authentic urine samples or assays relying on human hepatocytes, when the enzymatic cleavage step with β -glucuronidase is omitted.

Concerning the prevalence data presented here, these data cannot be extrapolated to the general population. Indeed, it refers to a very specific population mainly consisting of prisoners and patients in forensic psychiatric hospitals from various regions in Germany.

Finally, instability of SCs metabolites in urine could represent an issue when re-analyzing samples with updated methods. The stability

of the metabolites was not assessed in the present work. However, past studies have demonstrated a long-term stability of SC metabolites at -30°C up to 168 days,³⁵ so that it seems unlikely that a significant degradation took place under the applied storing conditions (-20°C).

4 | CONCLUSIONS

In the present study, the human phase-I metabolism of two SCs bearing an ethyl ester moiety at the linked group is reported, based on LC-qToF-MS data of authentic urine specimens, and confirmed by in vitro analysis. Investigation of phase-I biotransformation of the compounds led to the identification of M9 (monohydroxylation) and M8 (defluorination) as the most specific and suitable urinary biomarkers to detect 5F-EDMB-PICA consumption, in addition to M6 (ester hydrolysis) that was the most abundant metabolite. To detect the consumption of EDMB-PINACA, the monitoring of M7 (ester hydrolysis + ketone formation) and M12 (monohydroxylation) is suggested, although none of these markers is absolutely specific.

As shown by the presented prevalence data, use of the investigated compounds seems to have peaked in the population here-in analyzed in 2021.

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CONFLICT OF INTEREST

Nothing to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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