Metabolism of MMB022 and identification of dihydrodiol formation in vitro using synthesized standards

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Abstract
MMB022 (methyl 3-methyl-2-[1-(pent-4-en-1-yl)-1H-indole-3-carboxamido]butanoate) is a new synthetic cannabinoid with an alkene at the pentenyl side chain, a rare functional group for synthetic cannabinoids. Metabolite identification is an important step for the detection of synthetic cannabinoids in humans, since they are generally extensively metabolized. The aims of the study were to tentatively identify in vitro phase I metabolites, to confirm major metabolites using synthesized metabolites, to examine metabolic pathways thoroughly, to study metabolic stability and to suggest metabolites appropriate for urine screening. MMB022 and its synthesized metabolites were incubated with human liver microsomes (HLM) and the supernatants were analyzed by liquid chromatography-quadrupole time-of-flight mass spectrometry. Sixteen metabolites were identified, which were generated via dehydrogenation, dihydrodiol formation, ester hydrolysis, hydroxylation, and combinations thereof. A major biotransformation of the alkene at the pentenyl side chain was confirmed to be dihydrodiol formation. The major metabolites were ester hydrolysis (M15) and dihydrodiol (M8) metabolites, whereas the metabolite derived from the combination of ester hydrolysis and dihydrodiol (M5) was the fourth most abundant metabolite. The metabolic pathways were investigated using synthesized metabolites and revealed that M5 is an end product of the pathways, indicating that it might become a more abundant metabolite in vivo depending on the rate of metabolism in humans. The major pathway of MMB022 to M5 was determined to be via M8 formation. Intrinsic clearance of MMB022 was determined to be 296 mL/min/kg and t1/2 was 2.1 min, indicating a low metabolic stability. M15, M8, and potentially M5 are suggested as suitable urinary targets.

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1 | INTRODUCTION

The emergence and prevalence of new psychoactive substances (NPS) is still a continuing problem in Europe.1,2 NPS can pose a serious health risk to users due to the unknown nature of the potency and toxicity of the newly emerged drugs. To toxicologists, NPS presents a challenge in detection, since these compounds are not in the toxicological databases until their structures are confirmed. The difficulty in detection of NPS is exacerbated when these compounds are extensively metabolized in the body such that parent drugs are not detectable in blood or urine or only detected in small amounts. In such cases, identification of major metabolites prior to drug testing becomes indispensable to prove the use of NPS by detection of metabolites in biological matrices. A class of drugs which is generally known to be extensively metabolized in humans is synthetic cannabinoids.3

MMB022 (AMB-4en-PICA, MMB-4en-PICA, methyl 3-methyl-2-[1-(pent-4-en-1-yl)-1H-indole-3-carboxamido]butanoate) is a new synthetic cannabinoid (Figure 1) that was first reported in August 2018 in Slovenia.4 In Sweden, the cannabinoid is currently scheduled as a substance hazardous to health (Hälsofarlig vara) since November 2018 and was proposed to be reclassified as a narcotic in December 2018 and was proposed to be reclassified as a narcotic in December 2019.5,6 Structurally, MMB022 resembles MMB018 (AMB-PICA) and differs only by the terminal double bond on the pentyl side chain (Figure 1). To date, MMB022 has been identified in seizures in Slovenia (July 2018) and in Germany (January 2019), as well as test purchases in Slovenia (July 2018) and in Germany (January 2019), but not yet reported in biological samples. In comparison, MDMB-4en-PINACA, a synthetic cannabinoid analog with an alkene, has been identified in authentic biological samples,7,8 and has become increasingly prevalent in infused papers at Scottish prisons since June 2019.9 Due to the structural similarity, MMB022 might become one of the next prevalent cannabinoids.

A carbon–carbon double bond on the pentyl side chain is a rare modification among synthetic cannabinoids. It has been reported as a minor component of products with other NPS, analogs, including N-(4-pentenyl)-JWH-122,10 N-(4-pentenyl)-XLR-11 (also known as N-(4-pentenyl)-UR-144),11 and N-(4-pentenyl)-AKB-48.12 However, the first cannabinoids seen as the main components were JWH-0222 and MDMB-4en-PINACA8 and not until the latter became prevalent in 2019 did they receive much attention. Therefore, information on the metabolism of such a double bond is limited. So far, MDMB-4en-PINACA is the only synthetic cannabinoid with an alkene (Figure 1) whose metabolism has been studied.7 In the study, it was suggested that the double bond of MDMB-4en-PINACA undergoes dihydrodiol formation, likely via an epoxide intermediate. Together with ester hydrolysis of the tert-leucinate methyl ester, dihydrodiol formation was considered to be a major metabolic pathway. Due to the structural similarity between MMB022 and MDMB-4en-PINACA, dihydrodiol formation from the alkene and ester hydrolysis of the valinate methyl ester can be expected to be the major metabolic pathways for MMB022. However, in the previous study, formation of dihydrodiol from the alkene was only proposed based on the mass spectral fragmentation of the metabolites, using liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF-MS).7 Although dihydrodiol formation from an alkene is a common biotransformation in metabolism, e.g., ezlopitant alkene (CJ-12,458)13 and α-methylstyrene,14 this assumption is inconclusive given that an alkene can be metabolized to an alcohol,13 and thus further hydroxylation could lead to isomers of the dihydrodiol structure. Therefore, unambiguous identification of the dihydrodiol formation is necessary to confirm the major biotransformation of the alkene in the pentenyl side chain of synthetic cannabinoids.

Consequently, we hypothesize that a major metabolic pathway of a terminal alkene of MMB022 is dihydrodiol formation. Therefore, the aims of the study were to tentatively identify metabolites of MMB022 using human liver microsomes (HLM) and to confirm the structures of major metabolites by synthesizing postulated metabolites as reference materials. Synthesized metabolites, which matched the major in vitro metabolites or were considered to be intermediate metabolites, were further incubated with HLM to elucidate the metabolic pathways. Additionally, the metabolic stability was investigated and suitable urinary marker metabolites were suggested.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

MMB022 was obtained from Cayman Chemical (Ann Arbor, MI, USA). Potassium dihydrogen phosphate was purchased from Sigma-Aldrich (Stockholm, Sweden), dipotassium hydrogen phosphate, magnesium
chloride, and LC–MS grade methanol were obtained from Merck (Darmstadt, Germany). LC–MS grade acetonitrile and formic acid were obtained from Thermo Fisher Scientific (Gothenburg, Sweden). HLM (150 donor pool) were purchased from Bioreclamation IVT (Brussels, Belgium). NADPH regenerating system Solution A and NADPH regenerating system Solution B were purchased from Corning (Corning, NY, USA). Potential metabolites of MMB022 [dihydrodiol (M8, methyl 2-[(1-4,5-dihydroxypentyl)-1H-indole-3-carbonyl] amino-3-methylbutanoate), epoxide (IM2, methyl 3-methyl-2-[(1-[3-oxiran-2-ylpropyl]-1H-indole-3-carbonyl]amino)butanoate), epoxide hydrolysis (IM2, 3-methyl-2-[(1-[pent-4-ethyl]-2H-indole-3-carbonyl]amino)butanoic acid), ester hydrolysis with dihydrodiol (M5, 2-[(1-4,5-dihydroxypentyl)-1H-indole-3-carbonyl]amino)-3-methylbutanoic acid), ester hydrolysis with epoxide (IM1, 3-methyl-2-[(1-[3-(oxiran-2-yl)propyl]-1H-indole-3-carbonyl]amino)butanoic acid)] were synthesized in-house and the structures were confirmed by 1H and 13C NMR. The details of the synthesis can be found in Supplemental 1.

2.2 Human liver microsome incubation for metabolite identification

MMB022 or one of the synthesized metabolites (10 μmol/L), potassium phosphate buffer (100 mM/L, pH 7.4), magnesium chloride (5 mM/L), NADPH regenerating system Solution A (50 μL/mL), and HLM (1 mg/mL) were incubated at 37°C with a final addition of NADPH regenerating system Solution B (10 μL/mL), in duplicate. The incubation volume was 200 μL and the concentrations stated above are all final concentrations. After 60 min, ice-cold methanol (200 μL) was added to terminate the reaction. The sample was gently shaken, placed in a freezer for 10 min, and centrifuged (1.1 × 10^3 g, 15 min, at 4°C). The supernatant (5 μL) was injected into the LC-QTOF-MS system.

Negative controls (HLM without drug) and degradation controls (drug without HLM) were also incubated. A possible epoxide intermediate metabolite IM2 was also incubated without NADPH solutions.

2.3 Metabolic stability

MMB022 was incubated with HLM as described above for metabolite identification with the following changes: the drug concentration was 1 μmol/L; the drug was added last to initiate the reaction; the incubation duration was 0, 2, 4, 6, 8, and 10 min; incubation was performed in triplicate; and the injection volume was 1 μL.

The in vitro half-life (t_{1/2}) of MMB022 was calculated by the formula t_{1/2} = \ln(2)/k, where k is obtained as a slope (−k) from the plot of natural log of percentage of the drug remaining versus time. Intrinsic clearance (CL_{int}, mL/min/kg) was calculated by substituting t_{1/2} into the following formula:15

\[ \text{CL}_{\text{int}} = \frac{\ln 2}{t_{1/2}} \times \frac{1 \text{ mL of incubation}}{45 \text{ mg of microsomes}} \times \frac{20 \text{ g of liver}}{\text{kg of body weight}} \]

where 1 mL of incubation/mg of microsomes was obtained from the experimental condition, while 45 mg of microsomes/g of liver and 20 g of liver/kg of body weight were taken from the previous literature.15

Hepatic clearance (CL_H, mL/min/kg) and hepatic extraction ratio (E_H) were calculated using the following formulae, based on the well-stirred model, without taking into account blood protein and microsome binding:15,16

\[ \text{CL}_H = \frac{\text{Q}_H \times \text{CL}_{\text{int}}}{\text{Q}_H + \text{CL}_{\text{int}}} \]

\[ E_H = \frac{\text{CL}_H}{\text{Q}_H} \]

where 21 mL/min/kg was used for human hepatic blood flow (Q_H).15

2.4 Synthesized reference material analysis

Solutions of synthesized reference material (MMB022 metabolites: dihydrodiol, epoxide, ester hydrolysis, ester hydrolysis with dihydrodiol, ester hydrolysis with epoxide) were made in methanol to the concentration of 100 ng/mL, except for the metabolite with ester hydrolysis with epoxide metabolite to 10 μg/mL, respectively. One μL of each solution was injected into LC-QTOF-MS system.

2.5 LC-QTOF-MS analysis

An Agilent 1290 Infinity UHPLC system (Agilent Technologies, Kista, Sweden) with an Acquity HSS T3 column (150 mm × 2.1 mm, 1.8 μm) and an Acquity VanGuard precolumn, both from Waters (Sollentuna, Sweden), was coupled to an Agilent 6550 iFunnel QTOF mass spectrometer (Agilent Technologies, Kista, Sweden) with a Dual Agilent Jet Stream electrospray ionization source.

Mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) and the samples were run in gradient: 1% B (0–0.6 min), 1–20% B (0.6–0.7 min), 20–85% B (0.7–13 min), 85–95% B (13–15 min), 95% B (15–18 min), 95–1% B (18–18.1 min), and 1% (18.1–19 min). The flow rate was 0.5 mL/min and the column temperature was 60°C.

The mass spectrometer was run in positive electrospray ionization (+ESI) mode with the following parameters: fragmentor voltage, 380 V; gas temperature, 150°C; gas flow, 18 L/min; nebulizer gas pressure, 345 kPa; sheath gas temperature, 375°C; and sheath gas flow, 11 L/min. Automated calibration was in place during data acquisition. For metabolite identification and synthesized reference material analysis, Auto MS/MS acquisition mode was used with scan range, 100–950 m/z (MS) and 50–950 m/z (MS/MS); precursor intensity
threshold, 5000 counts; precursor number per cycle, 5; collision energy (CE), 3 eV at 0 m/z ramped up by 8 eV per 100 m/z. For metabolic stability, full scan mode, 50–950 m/z, was used.

Data were analyzed using Agilent MassHunter Qualitative Analysis software (version B.07.00) with the following parameters: mass error, 30 ppm; absolute peak area threshold, 20,000 counts; maximum number of matches, 20; chromatogram extraction window, 50 ppm. Metabolites were identified based on the following criteria: mass errors of less than 5 ppm for protonated molecules (with the exception of saturated peaks where the mass accuracy is expected to deviate), peak area of greater than 100,000 counts, consistent isotopic pattern, product ion spectrum consistent with the proposed structure, retention time plausible for the proposed structure, and absence of identical peaks in negative controls.

3 RESULTS

3.1 HLM metabolite identification

Incubation of MMB022 with HLM resulted in 16 phase I metabolites (M1–M15), including M7a and M7b coeluting under the employed chromatographic conditions. The metabolites eluted between 3.10 and 8.23 min before the parent drug at 9.90 min. The biotransformation, chemical formula, retention time, exact mass of the protonated molecule, mass error, peak area, and rank based on the peak area can be found for all metabolites in Table 1. Product ion spectra of MMB022 and metabolites are shown in Figure 2 and Supplemental 2.

The detected biotransformations included dehydrogenation, dihydrodiol formation, ester hydrolysis, hydroxylation, and combinations thereof. The top two most abundant metabolites by peak area were ester hydrolysis (M15) and dihydrodiol (M8), while the metabolite in combination of ester hydrolysis and dihydrodiol (M5) ranked fourth (Table 1). It should be noted that although the peak abundance is a useful factor for detection of metabolites, it does not necessarily reflect the concentration of the compounds due to possible differences in ionization efficiencies among compounds. The structures of these abundant metabolites (M5, M8, and M15) were confirmed with the synthesized reference materials based on their retention time (Figure 3) and product ion spectrum. Possible intermediate metabolites, ester hydrolysis with epoxide (IM1), and epoxide (IM2), did not match any of the detected metabolites. Product ion spectra of IM1 and IM2 can be found in Supplemental 2.

When the synthesized metabolites were incubated with HLM, all but M5 underwent further metabolism (Table 2). Potential intermediate metabolites IM1 and IM2 also produced metabolites upon metabolism and their metabolic profiles were similar to those of M5 and M8, respectively. Incubation of the epoxide (IM2) without NADPH resulted in dihydrodiol (M8) and ester hydrolysis and dihydrodiol (M5) metabolites, while the incubation of IM2 without HLM and

| ID | Biotransformation | Chemical formula | RT (min) | Exact mass [M + H]+ (m/z) | Mass error (ppm) | Average peak area (×10³) | Rank |
|----|-------------------|------------------|----------|--------------------------|-----------------|--------------------------|------|
| M1 | Ester hydrolysis + dihydrodiol + O | C_{19}H_{26}N_{2}O_{6} | 3.10 | 379.1864 | −0.38 | 176 | 12 |
| M2 | Ester hydrolysis + dihydrodiol - 2H | C_{19}H_{24}N_{2}O_{5} | 3.77 | 361.1758 | −1.13 | 140 | 15 |
| M3 | Dihydrodiol + O | C_{20}H_{28}N_{2}O_{6} | 3.96 | 393.2020 | −1.05 | 451 | 7 |
| M4 | Dihydrodiol + O | C_{20}H_{28}N_{2}O_{6} | 4.17 | 393.2020 | −0.16 | 1927 | 3 |
| M5 | Ester hydrolysis + dihydrodiol | C_{19}H_{26}N_{2}O_{5} | 4.31 | 363.1914 | 0.07 | 203 | 11 |
| M6 | Dihydrodiol + O | C_{20}H_{28}N_{2}O_{5} | 4.82 | 393.2020 | 0.70 | 273 | 9* |
| M7 | 2O | C_{20}H_{28}N_{2}O_{5} | 5.42 | 375.1914 | −0.12 | 2560 | 2 |
| M8 | Dihydrodiol | C_{20}H_{28}N_{2}O_{5} | 5.58 | 377.2071 | 1.09 | 2560 | 2 |
| M9 | Ester hydrolysis + O | C_{19}H_{24}N_{2}O_{4} | 5.77 | 345.1809 | −1.34 | 630 | 5 |
| M10 | Ester hydrolysis + O | C_{19}H_{24}N_{2}O_{4} | 6.00 | 345.1809 | −1.49 | 582 | 6 |
| M11 | 2O | C_{20}H_{28}N_{2}O_{5} | 6.11 | 375.1914 | 0.46 | 161 | 13 |
| M12 | 2O-2H | C_{20}H_{28}N_{2}O_{5} | 6.54 | 373.1758 | 0.09 | 221 | 10 |
| M13 | 2O | C_{20}H_{28}N_{2}O_{5} | 6.80 | 375.1914 | 0.27 | 402 | 8 |
| M14 | O | C_{20}H_{28}N_{2}O_{4} | 7.32 | 359.1965 | −0.90 | 187 | 14 |
| M15 | Ester hydrolysis | C_{19}H_{26}N_{2}O_{3} | 8.23 | 329.1860 | 10.76 | 8293 | 1 |
| Parent | MMB022 | C_{20}H_{26}N_{2}O_{3} | 9.90 | 343.2016 | 16.49 | 11053 | |

Metabolites in bold indicate those with structures confirmed by synthesized reference materials.
O hydroxylation, 2O dihydroxylation, -2H dehydrogenation
*Indicates the rank based on combined peak area of coeluting peaks.
NADPH still formed M8 although about 20-fold smaller in peak area. Based on these experiments, proposed metabolic pathways of MMB022 are shown in Figure 4.

3.2 | Metabolic stability

The in vitro half-life of MMB022 was calculated to be 2.1 min based on the slope of the plot of natural log of percentage of the drug remaining versus time (Figure 5). Subsequently, CLint, CLu, and \(E_{\text{hl}}\) were estimated to be 296 mL/min/kg, 20 mL/min/kg, and 0.93, respectively. Disappearance of MMB022 as well as formation of major metabolites (M5, M8, and M15) over 10 min is depicted in Figure 6.

4 | DISCUSSION

4.1 | HLM metabolite identification

The analysis of MMB022 (m/z 343.2016) fragmentation in QTOF-MS showed two major fragment ions, m/z 212.1070 and 144.0444, and two minor fragment ions, m/z 158.0600 and 69.0699 (Figure 2). The fragment ion at m/z 212.1070 corresponded to the pentenylindole moiety formed by a cleavage of the amide bond. A further loss of the side chain led to the indole moiety at m/z 144.0444. The ion at m/z 158.0600 was consistent with the indole moiety (m/z 144.0444) with an extra methyl side chain. The ion at m/z 69.0699 represented the pentenyl side chain. These ions were used as the basis for the structural identification of the metabolites.

Two of the major biotransformations were dihydrodiol formation and ester hydrolysis (Table 1). M8 (m/z 377.2071) resulted in product ions at m/z 246.1125, corresponding to the pentenylindole moiety with an addition of two oxygen atoms and two hydrogen atoms, and m/z 144.0444, an unchanged indole group (Figure 2). Based on the mass of M8 and its fragment ions, the biotransformation was elucidated to be dihydrodiol formation from an alkene of the pentenyl side chain. To confirm the structure, the dihydrodiol metabolite reference material (4,5-dihydroxypentyl metabolite) was synthesized and analyzed. The retention time (Figure 3a and 3c) and product ion spectrum matched well with M8, which confirmed M8 to be the dihydrodiol metabolite. Dihydrodiol formation itself is a common reaction observed in synthetic cannabinoid metabolism in vivo and/or in vitro, including, but not limited to, AM2201, JWH-015, JWH-018, JWH-122, JWH-200, JWH-210, PB-22, 5F-PB-22, MDMB-FUBINACA, ADB-FUBINACA, and FUBIMINA. Nevertheless, these are derived from oxidation of aromatic rings rather than from an alkene. The abundance of these dihydrodiol metabolites varies depending on the compounds, and in the cases of AM2201 and ADB-FUBINACA, dihydrodiols were found to be the major metabolites in authentic urine samples.

Likewise, M15 (m/z 329.1860) showed a similar product ion spectrum to MMB022 with fragment ions at m/z 212.1070 and 144.0444, indicating no modification on the pentenyl side chain. The decrease of 14 Da from the parent suggested the metabolite was an ester hydrolysis metabolite. Analysis of the synthesized
ester hydrolysis metabolite confirmed the identity of M15 (Figure 3a and 3d). M5 (m/z 363.1914) exhibited a similar spectrum to that of M8 (Figure 2), suggesting that it had undergone dihydrodiol formation. In addition, the further decrease of 14 Da indicated that it had also undergone ester hydrolysis. A synthesized reference material successfully confirmed M5 to be the metabolite with ester hydrolysis and dihydrodiol (Figure 3a and 3b).

The minor metabolites were analyzed similarly based on the mass and the product ion spectra without synthesized reference materials (Supplemental 2). M3, M4, and M6 (m/z 393.2020) were consistent with M8 with further hydroxylation. The location of hydroxylation was determined to be the valinate ester moiety for M3 (m/z 246.1125 indicating dihydrodiol formation only at the pentenyl indole moiety), the indole moiety for M4 (m/z 262.1074 indicative of dihydrodiol and hydroxylation and m/z 160.0393 indicating hydroxylation of indole) and the pentenyl side chain for M6 (m/z 262.1074 and 144.0444). M1 (m/z 379.1864) showed an almost identical product ion spectrum to M4 and seemed to result from ester hydrolysis of M4. M2 (m/z 361.1758) was consistent with the metabolite with ester hydrolysis, dihydrodiol and dehydrogenation. M9 and M10 (m/z 345.1809) appeared to form from hydroxylation of M15 (ester hydrolysis), and an addition of oxygen was indicated to be at the pentenyl side chain for M9 by the fragment ions at m/z 228.1019 and 144.0444 and at the indole ring for M10 by the ions at m/z 228.1019 and 160.0393.

M14 (m/z 359.1965) was presumably formed by hydroxylation of the pentenyl side chain (m/z 228.1019 and 144.0444). Further hydroxylation of M14 may have resulted in M7a, M7b, M11, and M13 (m/z 375.1914). M7b, M11, and M13 were considered to have the second oxygen atom also at the pentenyl side chain (m/z 244.0968 indicating dihydroxy pentenyl side chain and 144.0444), while M7a was expected to have the second hydroxylation at the valinate ester moiety (m/z 228.1019 and 144.0444). Finally, M12 (m/z 373.1758) showed the product ions at m/z 242.0812 and 144.0444, consistent with one hydroxylation and one carbonylation at the pentenyl side chain.

4.2 Metabolic pathways

Metabolism of synthetic cannabinoids has been studied frequently during the last decade. However, an alkene functional group on the terminal carbons of a pentyl side chain is an uncommon structure for synthetic cannabinoids and, to date, MDMB-4en-PINACA is the only synthetic cannabinoid, for which metabolism of such an alkene group has been studied.7 In the present study, we have demonstrated that similarly to MDMB-4en-PINACA, the double bond of MMB022 was metabolized to a dihydrodiol (M8), whose structure was confirmed by a synthesized reference standard. Likewise, M5 was confirmed to contain a dihydrodiol structure, in addition to ester hydrolysis.

According to the literature, dihydrodiol formation from metabolism of an alkene is via epoxidation catalyzed by CYP enzymes, followed by hydration of the epoxide either spontaneously or facilitated by epoxide hydrolase.13,14 In the present study, the potential intermediate epoxide metabolites (IM1 and IM2) were not directly detected as metabolites. However, the incubation of the epoxide (IM2) resulted in the dihydrodiol metabolite (M8) and M1–M5, while the incubation of M8 also resulted in M1–M5 (Table 2). Similarly, the incubation of ester hydrolysis and epoxide metabolite (IM1) generated ester hydrolysis and dihydrodiol (M5), together indicating that dihydrodiol was formed most likely via these epoxides as intermediates (Figure 4). The reason for not detecting the epoxides in the incubation mixture was possibly because the rate of reaction of hydration was much faster than that of epoxidation. Based on the fact that the incubation of the epoxide (IM2) without NADPH resulted in the dihydrodiol metabolite (M8), the hydration of epoxide was likely facilitated by epoxide hydrolase. Spontaneous hydration, on the other hand, seemed to have little contribution, as the formation of M8 in incubations without HLM and NADPH was approximately 20-fold smaller.

Among the dihydrodiol metabolites followed by hydroxylation, M3 (hydroxylated at the valinate methyl ester) and M4 (hydroxylated at the indole moiety) were formed from incubation of the dihydrodiol metabolite (M8), but M6 (hydroxylated at the pentenyl side chain) was not, suggesting that M6 was formed via a different metabolic
pathway. Thus, M6 was likely to be hydroxylated first followed by dihydrodiol formation (Figure 4). Based on the structure of M6, it could be anticipated that hydroxylation of an already congested pentyl side chain of M8 containing a dihydrodiol group was prevented due to steric hindrance, whereas hydroxylation of the valinate ester or indole group was not hindered by the dihydrodiol group.

The incubation of the ester hydrolysis metabolite (M15) resulted in further dihydrodiol formation (M5). However, surprisingly the incubation of neither M15 nor M5 formed any further metabolites of M5 (Table 2), indicating that M5 was an end product of the metabolic pathways (Figure 4). It implies that M5 could become a more abundant and important metabolite than M15 or M8, depending on the rate of metabolism in humans. M1 and M2, two metabolites easily regarded as further metabolites of M5 based on the structures, were hence formed via the dihydrodiol metabolite (M8) but not from M5.

The major metabolic pathways of MMB022 to the ester hydrolysis and dihydrodiol metabolite (M5) could be estimated from the initial rate of formation of major metabolites and the incubation of the major metabolites with HLM. The dihydrodiol metabolite (M8) had the highest initial rate of formation (Figure 6) and hence it was the major primary metabolic pathway (Figure 4). The pathway of MMB022 to the ester hydrolysis metabolite (M15) was presumably facilitated by carboxylesterases,21 as evidenced by the formation of M5 (ester hydrolysis and dihydrodiol) from the epoxide metabolite (IM2) without NADPH, and it was still a major pathway although slower than the pathway to M8. It follows that the major metabolic pathways to M5 could be via M15. However, the peak area of M5 produced from the incubation of M8 was much greater than that from M15 (Table 2), indicating that the pathway from M8 to M5 was the major pathway to M5 (Figure 4).

### 4.3 | Metabolic stability

MMB022, having an in vitro half-life of 2.1 min, was classified as a high clearance compound according to McNaney et al.22 This is in line with the fact that synthetic cannabinoids are extensively metabolized23 and consistent with the half-life previously determined for other synthetic cannabinoids.23,24
4.4 | Suitable urinary markers

The most important criterion for a urinary marker is that the metabolites are abundant. In this study, the ester hydrolysis (M15) and dihydrodiol (M8) were found to be the two most abundant HLM metabolites. Therefore, these metabolites are suggested as suitable unique urinary metabolites for the purpose of screening for MMB022. The ester hydrolysis with dihydrodiol metabolite (M5) was the fourth most abundant metabolite after 1 h HLM incubation and was found to be formed from further metabolism of M8 and M15. Depending on the extent of metabolism and rate of clearance in vivo, M5 may also be a useful target. It is interesting to note, however, that in the

**FIGURE 4** Proposed metabolic pathways of MMB022. An asterisk indicates the metabolite was confirmed with a synthesized reference material. Brackets indicate potential intermediate metabolites, which were not detected in this study. Bold arrows indicate major pathways. A red cross indicates the end of the pathway [Colour figure can be viewed at wileyonlinelibrary.com]
10-minute metabolic stability incubation, a considerably smaller peak area was observed for M5 in comparison with M8 and M15 (Figure 6). This result seems to indicate that the metabolic transformation of M8 and M15 to M5 is a relatively slow process.

Since none of the metabolites retain the parent drug skeletal structure, their presence does not confirm the consumption of MMB022. However, to our knowledge, these metabolites are not yet reported for other synthetic cannabinoids and thus are deemed suitable for screening purposes. The detection of both M15 and M8 should give more confidence regarding the specificity, as these metabolites together show the full picture of the parent drug skeletal structure.

Finally, the present study was conducted using HLM. Although these two metabolites can be expected to be abundant in human urine samples, authentic human urine samples need to be examined to corroborate the findings of the present study.

5 | CONCLUSION

MMB022 was metabolized by HLM via dehydrogenation, dihydrodiol formation, ester hydrolysis, and hydroxylation. As was hypothesized, it was confirmed, using synthesized reference standards, that the terminal alkene group of the pentenyl side chain underwent dihydrodiol formation. Incubation of the synthesized metabolites revealed major metabolic pathways and that ester hydrolysis with dihydrodiol metabolite (M5) is an end product of metabolism that could potentially become a more abundant and relevant metabolite for drug testing in vivo, if the clearance of intermediate metabolites is low and the formation and clearance of M5 is high. The results illustrate that reference metabolite materials not only are essential for confirmation of metabolite structures in metabolism studies, but also possess the potential to facilitate the understanding of metabolic pathways. The metabolic stability of MMB022 was low, consistent with previously studied synthetic cannabinoids, and the ester hydrolysis (M15) and dihydrodiol (M8) metabolites may be useful urinary target metabolites.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.
REFERENCES

1. European Monitoring Centre for Drugs and Drug Addiction. European drug report 2019: Trends and developments. Luxembourg: Publications Office of the European Union; 2019.

2. Nakajima J, Takahashi M, Seto T, et al. Identification and quantitation of two new naphthylindole drugs-of-abuse, 1-(5-hydroxypentyl)-1h-indol-3-yl)naphthalen-1-yl)methanone (AM-2202) and 1-(4-pentenyl)-1h-indol-3-yl)naphthalen-1-yl)methanone, with other synthetic cannabinoids in unregulated “herbal” products circulated in the Tokyo area. Forensic Toxicol. 2012;30(1):33-44. https://doi.org/10.1007/s11419-011-0130-5

3. Diao X, Huestis MA. New synthetic cannabinoids metabolism and strategies to best identify optimal marker metabolites. Front Chem. 2019;7:1-15. https://doi.org/10.3389/fchem.2019.00109

4. Slovenian Police. Analytical report: MMB-022 (MMB-4en-PICA) (C20H26H2O3). 2018. https://www.policija.si/apps/nfl/response_web/0/Analytical_Reports_final/MBB-022%20(MMB-4en-PICA)-ID-1955-18_report.pdf. Accessed 30 December 2019.

5. Public Health Agency of Sweden. Fjorton nya ämnen klassas som narkotika eller hälsofarlig vara (in Swedish). 2019. https://www.folkhalsomyndigheten.se/nyheter-och-press/nyhetsarkiv/2019/november/fjorton-nya-amnen-klassas-som-narkotika-eller-halsfarlig-vara/. Accessed 11 July 2019.

6. Public Health Agency of Sweden. Tjugotre amnen klassas som narkotika eller hälsofarlig vara (in Swedish). 2019. https://www.folkhalsomyndigheten.se/nyheter-och-press/nyhetsarkiv/2019/december/tjugotre-amen-foreslas-klassas-som-narkotika-eller-halsfarlig-vara/. Accessed 1 January 2020.

7. Watanabe S, Vikingsson S, Åstrand A, Gréen H, Kronstrand R. Bio-transformation of the new synthetic cannabinoid with an alkene, MDMB-4en-PINACA, by human hepatocytes, human liver microsomes, and human urine and blood. AAPS J. 2020;22(1):1-9. https://doi.org/10.1208/s12248-019-0381-3

8. Erol Ozturk Y, Yeter O. In vitro phase I metabolism of the recently emerged synthetic mmb-4en-pinaca and its detection in human urine samples. J Anal Toxicol. 2020. https://doi.org/10.1093/jat/bkaa017

9. Norman C, Walker G, McKirdy B, et al. Detection and quantitation of synthetic cannabinoid receptor agonists in infused papers from prisons in a constantly evolving illicit market. Drug Test Anal. 2020;12(4):538-554. https://doi.org/10.1002/dta.2767

10. Uchiyama N, Kawamura M, Kikura-Hanajiri R, Goda Y. URB-754: a new class of designer drug and 12 synthetic cannabinoids detected in illegal products. Forensic Sci Int. 2013;227(1):21-32. https://doi.org/10.1016/j.forsciint.2012.08.047

11. Kennedy J, Shanks KG, Van Natta K, et al. Rapid screening and identification of novel psychoactive substances using paperspray interfaced to high resolution mass spectrometry. Clin Mass Spectrom. 2016;1:3-10. https://doi.org/10.1016/j.jclims.2016.08.003

12. Moore KN, Garvin D, Thomas BF, Grabenauer M. Identification of eight synthetic cannabinoids, including 5F-AKB48 in seized herbal products using DART-TOF-MS and LC-QTOF-MS as nontargeted screening methods. J Forensic Sci. 2017;62(5):1151-1158. https://doi.org/10.1111/1556-4029.13367

13. Obach RS. Cytochrome P450-catalyzed metabolism of ezlopitant alkene (CJ-12,458), a pharmacologically active metabolite of ezlopitant: enzyme kinetics and mechanism of an alkene hydration reaction. Drug Metab Dispos. 2001;29(7):1057-1067.

14. De Costa KS, Black SR, Thomas BF, Mathews JM. Metabolism and disposition of α-methylstyrene in rats. Drug Metab Dispos. 2001;29(2):166-171.

15. Obach RS. Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: an examination of in vitro half-life approach and nonspecific binding to microsomes. Drug Metab Dispos. 1999;27(11):1350-1359.

16. Nairtomi Y, Terashita S, Kimura S, Suzuki A, Kagayama A, Sugiyaama Y. Prediction of human hepatic clearance from in vivo animal experiments and in vitro metabolic studies with liver microsomes from animals and humans. Drug Metab Dispos. 2001;29(10):1316-1324.

17. Castaneto MS, Wohlfarth A, Desrosiers NA, Hartman RL, Gorelick DA, Huestis MA. Synthetic cannabinoids pharmacokinetics and detection methods in biological matrices. Drug Metab Rev. 2015;47(2):124-174. https://doi.org/10.3109/03602532.2015.1029635

18. Kavanagh P, Grigoryev A, Krupina N. Detection of metabolites of two synthetic cannabinoids, MDMB-FUBINACA and ADB-FUBINACA, in authentic human urine samples by accurate mass LC-MS: a comparison of intersecting metabolic patterns. Forensic Toxicol. 2017;35(2):284-300. https://doi.org/10.1007/s11419-017-0356-y

19. Diao X, Scheidweiler KB, Wohlfarth A, Zhu M, Pang S, Huestis MA. Strategies to distinguish new synthetic cannabinoid FUBIMINA (BIM-2201) intake from its isomer THJ-2201: metabolism of FUBIMINA in human hepatocytes. Forensic Toxicol. 2016;34(2):256-267. https://doi.org/10.1007/s11419-016-0312-2

20. Sobolevsky T, Prasolov I, Rodchenkov G. Detection of urinary metabolites of AM-2201 and UR-144, two novel synthetic cannabinoids. Drug Test Anal. 2012;4(10):745-753. https://doi.org/10.1002/dta.1418

21. Satoh T, Hosokawa M. Structure, function and regulation of carboxylesterases. Chem Biol Interact. 2006;162(3):195-211. https://doi.org/10.1016/j.cbi.2006.07.001

22. McNaney CA, Drexl DM, Hnatyshyn SY, et al. An automated liquid chromatography-mass spectrometry process to determine metabolic stability half-life and intrinsic clearance of drug candidates by substrate depletion. Assay Drug Dev Technol. 2008;6(1):121-129. https://doi.org/10.1089/adt.2007.103

23. Andersson M, Diao X, Wohlfarth A, Scheidweiler KB, Huestis MA. Metabolic profiling of new synthetic cannabinoids AMB and SF-AMB by human hepatocyte and liver microsome incubations and high-resolution mass spectrometry. Rapid Commun Mass Spectrom. 2016;30(8):1067-1078. https://doi.org/10.1002/rcm.7538

24. Watanabe S, Kuzhiumparambil U, Fu S. In vitro metabolism of synthetic cannabinoid AM1220 by human liver microsomes and Cunninghamella elegans using liquid chromatography coupled with high resolution mass spectrometry. Forensic Toxicol. 2018;36(2):435-446. https://doi.org/10.1007/s11419-018-0424-y

SUPPORTING INFORMATION

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

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