SHORT COMMUNICATION

5F-MDMB-PICA metabolite identification and cannabinoid receptor activity

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Funding information
Linköpings Universitet; VINNOVA; Strategic Research Area in Forensic Sciences (Strategiområdet forensiska vetenskaper); Vinnova (the Psychomics project)

Abstract
According to the European Monitoring Center for Drugs and Drug Addiction (EMCDDA), there were 179 different synthetic cannabinoids reported as of 2017. In the USA, 5F-MDMB-PINACA, or 5F-ADB, accounted for 28% of cannabinoid seizures 2016–2018. The synthetic cannabinoid, 5F-MDMB-PICA, is structurally similar to 5F-MDMB-PINACA with an indole group replacing the indazole. Limited data exist from in vivo or in vitro metabolic studies of these synthetic cannabinoids, so potential metabolites to identify use may be missed. The goals of this study were to (a) investigate 5F-MDMB-PICA and 5F-MDMB-PINACA in vitro metabolism utilizing human hepatocytes; (b) to verify in vitro metabolites by analyzing authentic case specimens; and (c) to identify the potency and efficacy of 5F-MDMB-PICA and 5F-MDMB-PINACA by examining activity at the CB1 receptor. Biotransformations found in this study included phase I transformations and phase II transformations. A total of 22 5F-MDMB-PICA metabolites (A1 to A22) were identified. From hepatocyte incubations and urine samples, 21 metabolites (B1 to B21) were identified with 3 compounds unique to urine specimens for 5F-MDMB-PINACA. Phase II glucuronides were identified in 5F-MDMB-PICA (n = 3) and 5F-MDMB-PINACA (n = 5). For both compounds, ester hydrolysis and ester hydrolysis in combination with oxidative defluorination were the most prevalent metabolites produced in vitro. Additionally, the conversion of ester hydrolysis with oxidative defluorination to pentanoic acid for the first time was identified for 5F-MDMB-PICA. Therefore, these metabolites would be potentially good biomarkers for screening urine of suspected intoxication of 5F-MDMB-PICA or 5F-MDMB-PINACA. Both 5F-MDMB-PICA and 5F-MDMB-PINACA were acting as full agonists at the CB1 receptor with higher efficacy and similar potency as JWH-018.

KEYWORDS
5F-MDMB-PICA, CB1 activity, hepatocyte metabolism, MDMB-2201, synthetic cannabinoids
INTRODUCTION

The emergence of novel psychoactive substances (NPS) has had a detrimental global impact. Synthetic cannabinoids are some of the first NPS that increased dramatically in the early century and started the epidemic seen today. Substances such as "Spice" or "K2" were commonly found at gas stations or smoke shops across the United States and in vending machines in Japan. These substances were frequently found to contain the JWH series of synthetic cannabinoids with JWH-018 being the most predominant. Since their arrival, the number of synthetic cannabinoids has grown rapidly. According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), as of 2017 there had been 179 different synthetic cannabinoids reported to them. In the United States, there were 24,501 identifications of synthetic cannabinoids reported to National Forensic Laboratory Information System (NFLIS) in 2017. The synthetic cannabinoid, 5F-MDMB-PINACA or 5F-ADB, accounted for 28% of those identifications in 2017.

The synthetic cannabinoid, 5F-MDMB-PICA, first identified in herbal incense packages by Risseeuw et al, is structurally similar to 5F-MDMB-PINACA but the indazole has been replaced by an indole group. It has gained popularity in recreational use and between 2016 and 2018, there have been 340 reports containing 5F-MDMB-PICA according to NFLIS. In 2018, it was placed as a schedule I substance in the United States. The fluoropentyl side chain of 5F-MDMB-PICA, shared with 5F-MDMB-PINACA, indicates high potency at the CB1 receptor. This was verified by Banister et al using the FLIPR™ assay. 5F-MDMB-PICA was shown to be a full agonist at the CB1 receptor with an EC50 concentration of 0.45 nM, similar to 5F-MDMB-PINACA (0.59 nM). In contrast, Noble et al showed 5F-MDMB-PICA to have an EC50 value of 3.3 nM and an efficacy three times that of the full agonist JWH-018 using a β-arrestin-based assay. Our study aligns with previous studies, using a complimentary aequorin-luminescence-based approach measuring the intracellular Ca2+ changes.

Parent structures of synthetic cannabinoids are often difficult to detect in biological matrices, so metabolism studies are needed to improve detection of newly emerging synthetics. Mogler et al used pooled human liver microsome assays to identify phase I metabolites of 5F-MDMB-PICA. Authentic urine samples (n = 24) were also analyzed and 12 phase I metabolites were identified with the ester hydrolysis metabolite being the most abundant.

Although there is information available involving in vivo and in vitro metabolism studies of 5F-MDMB-PICA and 5F-MDMB-PINACA, potential metabolites have not been identified using human hepatocytes. While human liver microsomes are beneficial for discovering metabolites, they are typically unable to produce some phase II metabolites, unlike human hepatocytes which can help characterize both phase I and phase II metabolites. Also metabolites produced by human hepatocyte incubations tend to have concentrations more similar to those observed in vivo.

The specific goals of this study were to (a) investigate the metabolism of 5F-MDMB-PICA and confirm 5F-MDMB-PINACA metabolism utilizing human hepatocyte incubations to identify in vitro metabolites; (b) to examine authentic case specimens that involved these synthetic cannabinoids in order to verify metabolites identified by hepatocyte incubations; and (c) to identify the potency and efficacy of 5F-MDMB-PICA and 5F-MDMB-PINACA on the activation of the CB1 receptor.

MATERIALS AND METHODS

2.1 Chemicals and reagents

A MilliQ Gradient 10 production unit from Millipore (Billerica, MA, USA) was used for in-house ultra-pure water production. 5F-MDMB-PICA and 5F-MDMB-PINACA were purchased from Chiron (Trondheim, Norway). JWH-018 was purchased from THC Pharm (Frankfurt am Maine, Germany). InVitro Gro HT and cryopreserved mixed gender human hepatocytes LiverPool™ (20-donor) were acquired from Bioreclamation IVT (Brussels, Belgium). HEPES buffer, L-glutamine, DMEM/HAM’s F12, and Williams E buffer were acquired from ThermoFisher (Gothenburg, Sweden). Coclenterazine h was obtained from Nanolight® Technology (Pinetop, AZ, USA). Acetonitrile, formic acid, and water liquid chromatography–mass spectrometry (LC–MS) grade from Fisher Scientific (Gothenburg, Sweden). Ammonium formate, digitonin, protease-free BSA, and ATP were purchased from Fluka (Sigma-Aldrich, Stockholm, Sweden). The β-glucuronidase/arylsulfatase (Helix pomatia) mixture was from Roche (Mannheim, Germany).

2.2 Human hepatocytes incubation

Thawed cryopreserved human hepatocytes were added into InVitro Gro HT Medium. The cell solution was centrifuged at 100 g for 5 minutes at room temperature. The supernatant was discarded and the pellet was re-suspended in a mixture of 2mM Williams E medium supplemented with L-glutamine and 20mM HEPES buffer. An additional washing was performed. The Trypan Blue (0.4% v/v) exclusion method was utilized for the cell viability (91%) determination. 5F-MDMB-PICA and 5F-MDMB-PINACA were incubated with hepatocytes in 96-well-plates (10^5 cells/0.1 mL/well) with a final concentration of 5 μM/L. Ice-cold acetonitrile was used to end incubations after 1, 3, and 5 hours. Baseline (t0) samples contained hepatocytes and acetonitrile before drugs were added. Positive controls consisted of CYP substrates: caffeine, bufpropion, diclofenac, omeprazole, dextromethorphan, chlorozoxazone, and midazolam. Negative controls contained incubations without drug standards. Degradation controls were incubations without addition of hepatocytes.

2.3 Authentic urine sample preparation

Post-mortem (n = 3) and ante-mortem (n = 1) urine samples were analyzed with and without hydrolysis. Urine (100 μL) with 300 μL of 1 M sodium acetate buffer (pH 5) was incubated for 2 hours at 40°C with 10 μL of glucuronidase/arylsulfatase (4.5 U/mL and 14 U/mL,
respective) in autosampler vials for hydrolysis. For non-hydrolyzed samples, 10 μL of the sodium acetate buffer was added instead of enzyme. After incubation, unopened vials were placed in the autosampler for analysis.

2.4 | Authentic urine samples

Urine from three cases that presented with positive results for 5F-MDMB-PICA in blood were analyzed for metabolites to corroborate the hepatocyte findings. Two cases were autopsy cases that are described in 2.4.1 and 2.4.2 and the third was from a suspected DUIJD (no case history available). In addition, an autopsy case positive for 5F-MDMB-PINACA was analyzed. The study was approved by the regional ethics committee in Linköping (2018–186/31).

2.4.1 | Case 1

A 47-year-old male with a history of drug abuse and diagnosed with type I diabetes was found lying on the floor of his bathroom. The post-mortem examination revealed pulmonary edema (combined weight 1745 g), fatty liver, and underweight with a BMI of 15.9. Toxicalogical analysis showed no ethanol but 0.45 part per thousand of acetone in femoral blood as well as 0.74 part per thousand in urine. Vitreous glucose was 78.4 mmol/L and the femoral blood had an elevated BHB at >1000 μg/g. The only exogenous compound found was 0.28 ng/g 5F-MDMB-PICA in femoral blood. The investigation concluded that the cause of death was diabetic ketoacidosis.

2.4.2 | Case 2

A 49-year-old male with a history of alcohol and drug abuse was found dead outside. The post-mortem examination was unremarkable and no underlying pathology was found. The toxicalogical analysis revealed no ethanol but 0.13 part per thousand of acetone in femoral blood as well as 0.74 part per thousand in urine. Glucose was negative but the femoral blood had an elevated BHB at >1000 μg/g. The only exogenous compound found was 0.32 ng 5F-MDMB-PICA/g femoral blood. The investigation concluded that the cause of death was ketoacidosis possibly with a contribution from his drug use.

2.5 | Liquid chromatography and high resolution mass spectrometry for metabolite identification

An Agilent 1290 infinity ultra-high performance liquid chromatography system coupled with an Agilent 6550 iFunnel quadrupole time-of-flight (QTOF) mass spectrometer equipped with a Dual Agilent Jet Stream electrospray ionization source (Santa Clara, CA, USA) was used for analysis. Agilent MassHunter software was used for data acquisition and analysis.

Chromatographic separation was achieved using an Acquity HSS T3 column (150 mm x 2.1 mm, 1.8 μm) from Waters (Sollentuna, Sweden). Column temperature was maintained at 60°C. Injection volume was 5 μL. Mobile phases were 0.05% formic acid in 10mM ammonium formate (mobile phase A) and 0.05% formic acid in acetonitrile (mobile phase B). Gradient elution at 0.5 mL/min was initiated with 99% A and a 0.6-minute hold, switched to 80% A at 0.7 minutes, then ramped to 20% A over 13 minutes followed by a 3-minute rinse of high organic (5:95, A:B) and a 2-minute re-equilibration (99:1, A:B) for a total runtime of 19 minutes.

Auto tandem mass spectrometry (MS/MS) acquisition was performed in positive mode with the following parameters: MS range 100 to 950 m/z, threshold of 5000 for precursor selection, drying gas temperature at 150°C, drying gas flow at 18 L/min, sheath gas temperature at 375°C, sheath gas flow at 11 L/min, nebulizer at 50 psi, and fragmentor at 380 V. During acquisition, automated calibration was in place.

2.6 | Metabolite identification

Data from hepatocyte incubations and urine samples was processed using a Personal Compound Database and Library (PCDL) generated in-house using MassHunter PCDL Manager. Potential metabolites for 5F-MDMB-PICA and 5F-MDMB-PINACA were predicted using the following biotransformations either alone or in combination: phase I transformations (amide hydrolysis, butanoic acid formation at the indole/indazole side chain, carboxylation, dehydrogenation, dihydrodiol formation, dihydroxylation, ester hydrolysis, ketone formation, N-dealkylation, hydroxylation, oxidative defluorination, oxidative defluorination to pentanoic acid, propionic acid formation at the indole/indazole side chain) and phase II transformations (glucuronidation and sulfation). Criteria for metabolite identification were as follows: mass error of less than ±5 ppm for protonated molecule, MS/MS spectra showing plausible product ions, retention time feasible for the structures, appropriate peak shape, and absence in negative controls.

2.7 | Receptor activation

Analysis of receptor activation was carried out on AequoScreen recombinant CHO-K1 cell lines purchased from Perkin Elmer (Groningen, Netherlands) expressing the human CB1 receptor (ES-110-A) according to the manufacturer specifications. Specifically, cells have been quickly thawed in BSA medium that was previously warmed to 37°C. Cells were centrifuged at 150 g for 5 minutes at room temperature and the cells were re-suspended with BSA medium (1 mL) in order to have 3x10^5 cells/mL in a 20 mL tube. Coelenterazine was added to achieve a final concentration of 2.5 μM in BSA medium. The cells were incubated at room temperature in the dark during rotation for three hours. 5F-MDMB-PICA and 5F-MDMB-PINACA were prepared in 96-well plates at descending concentrations using serial dilutions. The concentrations were 20 000, 4000, 800, 160, 32, 6.4, 1.28, 0.256, 0.0512, and 0.01024 ng/mL in each well. JWH-018 was also analyzed as a reference agonist for the CB1 receptor. Digitonin was used as a positive control for the coelenterazine cell loading and blank wells with no drug used as negative controls. The activation at each drug concentration was
determined using a Tecan Spark™ 10 M (Männedorf, Switzerland) as 50 μL cell suspension was added to the well (15 000 cells). The Spark 10 M reading protocol was set to 200 luminosity readings and the cells were added to each well at reading cycle #10 (baseline) and luminescence registration was conducted for ~25 seconds. Data was fitted to the Hill equation using GraphPad Software, Prism version 8. Statistical differences were investigated using one-way ANOVA.

3 | RESULTS

3.1 | Metabolic profile of 5F-MDMB-PICA

From hepatocyte incubations and urine samples, 22 metabolites (A1 to A22) were tentatively identified for 5F-MDMB-PICA in this study (Figure 1a and 1b) with mass errors within ±3.27 ppm. These metabolites are summarized in Table 1 in retention time order. The ranks of the tentative metabolites can also be seen in Table 1 as determined by the abundance in the 3-hour incubation and by the abundance in urine samples. Metabolites were formed via carboxylation (A16) or hydroxylation on the aliphatic chain (A20) or aromatic ring (A18) followed by glucuronidation (A4); N-dealkylation (A15) followed by hydroxylation on the aromatic ring (A3); oxidative defluorination (A19) followed by conversion to pentanoic acid (A17); oxidative defluorination with conversion to propionic acid (A14). Oxidative defluorination pathway also subsequently underwent hydroxylation on the aromatic ring (A5, A9) followed by N-dealkylation (A3) or glucuronidation (A1). The ester hydrolysis (A21) metabolite was further metabolized via hydroxylation (A8, A10, A13), glucuronidation (A12), or dehydrogenation (A22) followed by N-dealkylation (A2), or ester hydrolysis with oxidative defluorination (A11) followed by dehydrogenation (A7), or conversion to pentanoic acid (A6). The proposed metabolic pathway is depicted in Figure 2. The two most abundant metabolites after the 5-hour incubation with hepatocytes were ester hydrolysis (A21) and ester hydrolysis with oxidative defluorination (A11). The Case 4 ante-mortem urine had the most metabolites observed (A2, A3, A6, A8-10, A12-A14, A17-A19, and A21-A22), while Case 1 had a couple of metabolites (A6 and A21) and Case 2 only had a single metabolite identified (A21). The ester hydrolysis product was the only metabolite present in all incubation time points and authentic urine specimens. 5F-MDMB-PICA was not detected in any of the urine specimens, but was dominant in all hepatocyte incubations. The mass spectra of 5F-MDMB-PICA and the 9 most abundant metabolites present in urine from Case 4 are shown in Figure S3 in the Supporting Information. 5F-MDMB-PINACA tentatively identified metabolites (Table S1), proposed metabolic pathway (Figure S1), and mass spectra from metabolites found in authentic (Figure S3) are provided in the Supporting Information.

3.2 | Receptor activation

Dose response curves for the full concentration range used are shown in Figure 3 after normalization to the digitonin response. Both 5F-MDMB-PICA and 5F-MDMB-PINACA are full agonists reaching 83% and 88% of the digitonin signal, respectively. Compared to JWH-018, they were significantly more effective in activating the CB1 receptor, reaching an efficacy of 129% (5F-MDMB-PICA, p < 0.05) and 136% (5F-MDMB-PINACA, p = 0.02) of JWH-018. In Table 2, the effective concentration values (EC) expressed both in ng/mL and M including the 95% confidence intervals are shown for JWH-018, 5F-MDMB-PICA, and 5F-MDMB-PINACA. There was no significant difference in comparison of EC50 values of 5F-MDMB-PICA, 5F-MDMB-PINACA and JWH-018 using one-way ANOVA, p = 0.05.
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<th>Retention Time (min)</th>
<th>Molecular Weight (formula)</th>
<th>Mass Error (ppm)</th>
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<th>Peak Area (Urine Samples)</th>
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<td>Case 2 Hydrolyzed Non</td>
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P=Parent compound, NA=not applicable
4 | DISCUSSION

As seen in Figure 3, both compounds have comparable potency to JWH-018, but both have slightly higher efficacy. The EC50 for 5F-MDMB-PINACA and 5F-MDMB-PICA are comparable to each other as was also reported by Banister et al.9 However, our EC50 values are around 60x higher than those reported by Banister et al.9 Noble et al report an EC50 value for 5F-MDMB-PICA 8x lower than in the present study.8 Noble et al8 also report 5F-MDMB-PICA to be 13x more potent than JWH-018 while our results indicate similar potency9. In our study, 5F-MDMB-PICA was also found to have an Emax 30% higher than that of JWH-018. This result differs from what

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<th>Compound</th>
<th>EC50</th>
<th>95% Confidence Intervals</th>
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<td>10.37</td>
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has been reported by Noble et al, with an E_{\text{max}} 3 times higher than that of JWH-018. The differences between our results and those reported by Noble et al could be attributed to the different techniques and intracellular pathways used to determine receptor activity between this study and that of Noble et al, which monitored the interaction between δarr2 and CB1 and CB2 receptors.  

This study is aequorin-luminescence based on Ca^{2+} flux. This method is based on the activation of the calcium sensitive aequorin/aequorin system where coelenterazine is converted by the aequorin upon activation. After the G-protein activation, the second messenger phospholipase C is activated, with succeeding diacylglycerol and inositoltriphosphate production and intracellular calcium ions release. 

The calcium release activates the aequorin/aequorin system and with coelenterazine as substrate luminescence is produced. That said, substantial differences were also seen when comparing our results to those of Banister et al even though they also used an assay based on Ca^{2+} flux (FLIPR™). Taken together, our results as well as those reported by Banister et al and Noble et al illustrate that efficacy and potency estimates are method specific and highlight the need to use several different assays to describe the potency of a CB1 agonist.

Although the cause of death in both Case 1 and Case 2 was attributed to diabetic ketoacidosis, the presence of synthetic cannabinoids cannot be excluded as a contributing or underlying factor. Hess et al presented a fatality that involved several synthetic cannabinoids where cause of the death was also assumed to be from diabetic ketoacidosis. 

The decedent in their study and from the present study (Case 1) had a history of diabetes. However, the decedent in Case 2 had no history of diabetes which is similar to what was reported by Demirci et al.  

5F-MDMB-PICA is a full agonist to the CB1 receptor and may produce severe adverse side effects such as hyperglycemia. In cases of ketoacidosis with or without history of diabetes, comprehensive toxicology screens may be valuable in determining root cause.

For both compounds, ester hydrolysis and ester hydrolysis in combination with oxidative defluorination were the most prevalent metabolites produced in vitro. At least one of these biotransformations were present in each of the case samples presented.

4.1 Authentic urine

In the post-mortem urine samples, a total of two metabolites were tentatively identified (A6 and A21). The ester hydrolysis (A21) was identified in the Case 2 sample following hydrolysis, indicating cleavage of the glucuronide. In the ante-mortem urine samples, 14 metabolites (A2, A3, A6, A8-A10, A12-A14, A17-A19, A21, and A22) were found, with two metabolites (A8 and A14) not produced in vitro. Variation in the numbers of metabolites in the three different samples is difficult to interpret as the time of administration is unknown but the low number of metabolites in the post-mortem samples suggest administration shortly prior to death. Evidence of phase II hydrolytic cleavage was evident in most metabolites, especially with metabolites A3, A9, A19, and A22 as they were identified only after hydrolysis. In all three cases, ester hydrolysis was the most abundant metabolite, which is consistent with the study by Mogler et al. Further, the metabolites produced in the in vitro and in vivo samples aligned with the 12 tentatively identified by Mogler et al, with the exception that the amide hydrolysis was not detected in the present study. However, A6 was not identified by Mogler et al. This metabolite was present in two of the three urine cases presented, with A6 being the most abundant metabolite found in Case 1. The corresponding metabolite for 5F-MDMB-PICA (B7), was found in all incubations of the present study and was the second most abundant metabolite found in Case 3. Yeter et al ranked this metabolite the second most abundant metabolite found in both hydrolyzed and non-hydrolyzed authentic urine samples (n = 30). Based on the structural similarity to 5F-MDMB-PINA and urine findings of the present study, A6 is likely to be a major metabolite in urine for 5F-MDMB-PICA. To the authors’ knowledge, this is the first report identifying A6 as a suitable marker for screening. The ranking for hepatocyte incubations and urine samples share the same metabolite for the top ranking (A21). Although A6 is not in the top 3-5 metabolites in hepatocyte incubations, due to its abundance in two of the three urine specimens and previous data about the corresponding metabolite for 5F-MDMB-PICA, it is given the second top ranking for urine samples.

5 CONCLUSIONS

Based on the aequorin-luminescence method used for this study, the EC_{50} at the CB1 receptor for 5F-MDMB-PICA and 5F-MDMB-PICA were found to be comparable to each other and JWH-018. The EC_{50} value of 5F-MDMB-PICA was found to be 27.6 nM, 8 to 60 times lower than indicated by other assays, indicating that 5F-MDMB-PICA might be less potent than previously believed. For both compounds, ester hydrolysis and ester hydrolysis in combination with oxidative defluorination were the most abundant metabolites produced in vitro. Additionally, we described the conversion of ester hydrolysis with oxidative defluorination to pentanoic acid (A6) for the first time for 5F-MDMB-PICA. At least one of these biotransformations were present in each of the case samples presented and are in agreement with previous literature. Therefore, these metabolites would be potentially good biomarkers for screening urine of suspected intoxication of 5F-MDMB-PICA or 5F-MDMB-PICA. However, these metabolites are not specific to the consumption of 5F-MDMB-PICA or 5F-MDMB-PICA, i.e., they can be formed from drugs of similar structures. Therefore, based on both the hepatocyte incubation abundance and presence in authentic urines, ester hydrolysis (A21) and ester hydrolysis with oxidative defluorination to pentanoic acid (A6) are recommended to be monitored for the purpose of screening/confirmation. As the parent drug was not present in the urine samples analyzed, metabolites may be of utmost importance when determining drug intake.

ACKNOWLEDGEMENTS

This research was funded by Vinnova (the Psychomics project) and Strategic Research Area in Forensic Sciences (Strategiområdet forensiska vetenskaper) at Linköping University.
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SUPPORTING INFORMATION

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

How to cite this article: Truver MT, Watanabe S, Åstrand A, et al. 5F-MDMB-PICA metabolite identification and cannabinoid receptor activity. Drug Test Anal. 2019. https://doi.org/10.1002/dta.2688