Research Article

Pentylindole/Pentylindazole Synthetic Cannabinoids and Their 5-Fluoro Analogs Produce Different Primary Metabolites: Metabolite Profiling for AB-PINACA and 5F-AB-PINACA

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Abstract. Whereas non-fluoropentylindole/indazole synthetic cannabinoids appear to be metabolized preferably at the pentyl chain though without clear preference for one specific position, their 5-fluoro analogs' major metabolites usually are 5-hydroxypentyl and pentanoic acid metabolites. We determined metabolic stability and metabolites of *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1*H*-indazole-3-carboxamide (AB-PINACA) and 5-fluoro-AB-PINACA (5F-AB-PINACA), two new synthetic cannabinoids, and investigated if results were similar. *In silico* prediction was performed with MetaSite (Molecular Discovery). For metabolic stability, 1 $\mu\text{mol/L}$ of each compound was incubated with human liver microsomes for up to 1 h, and for metabolite profiling, 10 $\mu\text{mol/L}$ was incubated with pooled human hepatocytes for up to 3 h. Also, authentic urine specimens from AB-PINACA cases were hydrolyzed and extracted. All samples were analyzed by liquid chromatography high-resolution mass spectrometry on a TripleTOF 5600+ (AB SCIEX) with gradient elution (0.1% formic acid in water and acetonitrile). High-resolution full-scan mass spectrometry (MS) and information-dependent acquisition MS/MS data were analyzed with MetabolitePilot (AB SCIEX) using different data processing algorithms. Both drugs had intermediate clearance. We identified 23 AB-PINACA metabolites, generated by carboxamide hydrolysis, hydroxylation, ketone formation, carboxylation, epoxide formation with subsequent hydrolysis, or reaction combinations. We identified 18 5F-AB-PINACA metabolites, generated by the same biotransformations and oxidative defluorination producing 5-hydroxypentyl and pentanoic acid metabolites shared with AB-PINACA. Authentic urine specimens documented presence of these metabolites. AB-PINACA and 5F-AB-PINACA produced suggested metabolite patterns. AB-PINACA was predominantly hydrolyzed to AB-PINACA carboxylic acid, carbonyl-AB-PINACA, and hydroxypentyl AB-PINACA, likely in 4-position. The most intense 5F-AB-PINACA metabolites were AB-PINACA pentanoic acid and 5-hydroxypentyl-AB-PINACA.

KEY WORDS: 5-fluoro-AB-PINACA; AB-PINACA; *in silico* prediction; metabolism; synthetic cannabinoids.

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ABBREVIATIONS: AB-PINACA, *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1*H*-indazole-3-carboxamide; CB, Cannabinoid; CL, Clearance; cps, Counts per second; CYP, Cytochrome P450; ER, Extraction ratio; ESI, Electrospray ionization; FDA, Food and Drug Administration; HLM, Human liver microsomes; HRMS, High-resolution mass spectrometry; IDA, Information-dependent acquisition; LC-MS, Liquid chromatography-mass spectrometry; MDF, Mass defect filter; MS, Mass spectrometry; MW, Molecular weight; NADPH, Nicotinamide adenine dinucleotide phosphate reduced form; NPS, Novel psychoactive substances; Q, Qualifier; Q-TOF, Quadrupole/time of flight; T, Target; TOF, Time of flight.

INTRODUCTION

Cannabimimetic synthetic cannabinoids are widespread novel psychoactive substances (NPS) (1), producing adverse effects like seizures, myocardial injuries, strokes, and acute kidney failures (2,3). In an attempt to prevent harm, legal authorities worldwide try to control NPS distribution; however, novel structural classes continuously emerge to circumvent these laws, leading to a plethora of targets (1).

Knowledge of synthetic cannabinoids' metabolism is required for identifying appropriate urinary targets (4), improving urine test interpretation, and revealing potentially toxic metabolites. Further, it is necessary to identify the most prevalent metabolites for synthesis of reference standards for analytical method development. Since the first appearance of synthetic cannabinoids, many metabolism studies with human liver microsomes (HLM), human hepatocytes, and/or authentic specimens elucidated their metabolic pathways (5–22).

The most popular structural modification is fluorine substitution at the 5-pentyl position of pentylindole/pentylindazole synthetic cannabinoids, which generally enhanced potency (3), i.e., JWH-018 to AM2201 (23). New synthetic cannabinoids and their

5-fluoro analogs include JWH-122/MAM2201, AM679/AM694, UR-144/XLR-11, NNEI/5F-NNEI, PB-22/5F-PB-22, APICA/STS-135, AKB48/5F-AKB48, and THJ-018/THJ-2201. Interestingly, similar patterns of major urinary metabolites were noted for these pairs. For JWH-018/AM2201, JWH-122/MAM2201, UR-144/XLR-11, PB-22/5F-PB-22, and AKB48/5F-AKB48, non-fluoro compounds generally appear to be metabolized preferably at the pentyl chain though without clear preference for one specific position, whereas 5-fluoro analogs' major metabolites usually were 5-hydroxypentyl and pentanoic acid metabolites (Supplementary Table 1).

In 2012, *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1*H*-indazole-3-carboxamide (AB-PINACA), a new pentylindazole synthetic cannabinoid, was identified in herbal blends (24). In 2013, its 5-fluoro analog *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(5-fluoropentyl)-1*H*-indazole-3-carboxamide (5F-AB-PINACA) was reported (25). The indazole core structure was common to AKB48; however, the aminooxobutane linked by a carboxamide group was new, as noted in a 2009 Pfizer patent on indazole derivatives with CB₁ agonist activity (26). In January 2015, AB-PINACA became a controlled substance in the USA and 5F-AB-PINACA will be prohibited based on the analog law (27). Takayama *et al.* incubated AB-PINACA with HLM and identified three hydroxylated metabolites (28), while Thomsen *et al.* identified 10 metabolites in HLM, with AB-PINACA carboxylic acid as the major metabolite. The latter also identified carboxylesterase 1 (CES1) as the key enzyme for amide hydrolysis (29). No 5F-AB-PINACA metabolism data are available.

We provide here a comprehensive overview of AB-PINACA and 5F-AB-PINACA metabolism in human hepatocytes, HLM metabolic stability data and an evaluation of *in silico* software to predict major metabolites. We also analyzed two urine specimens from suspected AB-PINACA cases and present suitable urinary markers for urine drug testing methods. Finally, we investigated if the most intense AB-PINACA and 5F-AB-PINACA metabolites fit the suggested pattern for 5-fluoropentyl side chain-containing synthetic cannabinoids.

MATERIALS AND METHODS

Chemicals and Reagents

Cryopreserved human hepatocytes, HLM, thawing and incubation media, and NADPH-regenerating system solutions were purchased from BioreclamationIVT (Baltimore, MD, USA). AB-PINACA and 5F-AB-PINACA were kindly provided by the Drug Enforcement Administration Special Testing and Research Laboratory (Dulles, VA, USA). Liquid chromatography-mass spectrometry (LC-MS)-grade acetonitrile and trypan blue were supplied by Sigma-Aldrich (St. Louis, MO, USA). Potassium phosphate, glacial acetic acid, and formic acid LC-MS grade were obtained from Fisher Scientific (Waltham, MA, USA).

Metabolic Stability Assessment with HLM

HLM suspensions (1 mL, 1 mg/mL, 50-donor pool) consisting of 50 mmol/L potassium phosphate buffer (pH 7.4) and NADPH-regenerating system (glucose-6-phosphate, MgCl₂, and glucose-6-

Fig. 1. Combined extracted ion chromatograms showing the metabolic profiles of AB-PINACA and 5F-AB-PINACA after 3-h human hepatocyte incubation and in authentic urine specimens associated with AB-PINACA intake. Signals in the samples, which were enzymatically hydrolyzed, are shown in red. In case the peak was too little to be visible, the metabolite is given in *parenthesis*. **a** AB-PINACA hepatocyte sample, diluted. **b** AB-PINACA hepatocyte sample, after SLE+ (with and without hydrolysis). **c** Authentic urine #1 after SLE+ (with and without hydrolysis). **d** Authentic urine #2 after SLE+ (with and without hydrolysis). **e** 5F-AB-PINACA hepatocyte sample, diluted. **f** 5F-AB-PINACA hepatocyte sample, after SLE+ (with and without hydrolysis)

phosphate dehydrogenase) were incubated with 1 μmol/L AB-PINACA or 5F-AB-PINACA at 37°C for up to 1 h in a shaking water bath. Organic solvent percentage (methanol and DMSO, respectively) was <1%. One hundred-microliter samples were collected at 0, 3, 8, 13, 20, 30, 45, and 60 min and added to 100 μL ice-cold acetonitrile. Samples were centrifuged in a 5804r bench top centrifuge (Eppendorf, Hamburg, Germany) (15,000g, 4°C, 10 min), supernatant was removed and stored at -80°C. After thawing and vortexing, samples were spun again; 10 μL supernatant was diluted with 990 μL mobile phase A/B (90:10, v/v) and 10 μL injected onto the LC-MS/MS.

The chromatographic system consisted of two LC-20AD_{XR} pumps, a DGU-20A3R degasser, a SIL-20AC_{XR} autosampler, and a CTO-20A column oven (Shimadzu Corp., Columbia, MD, USA). The Kinetex™ C18 column (100 mm×2.1 mm ID, 2.6 μm) was fitted with a KrudKatcher Ultra HPLC in-line filter (0.5 μm×0.1 mm ID) (Phenomenex, Torrance, CA, USA). Mobile phases were 0.1% formic acid in water (A) and acetonitrile (B), and the gradient was 10% B for 0.5 min, ramped to 58% B at 24 min, then increased to 95% B at 24.2 min, and held until 26.2 min. Total run time was 29 min. Column and autosampler temperatures were 40 and 4°C, respectively.

Mass spectrometric analysis was performed on a QTRAP 5500 mass spectrometer (AB SCIEX, Redwood City, CA, USA) with AB SCIEX Analyst software, version 1.6, and positive electrospray ionization (ESI) mode. Two transitions were monitored for AB-PINACA (330.9-215.1; 330.9-286.1) and 5F-AB-PINACA (349.2-233.1; 349.2-304.2). For AB-PINACA, declustering potential was 80 V, entrance potential 10 V, exit potential 10 V (target, T) and 16 V (qualifier, Q), and collision energy 33 eV (T) and 19 eV (Q). For 5F-AB-PINACA, declustering potential was 80 V, entrance potential 10 V, exit potential 10 V (T and Q), and collision energy 20 eV (T) and 32 eV (Q).

Peak areas were plotted against time, and *in vitro* microsomal half-life (*T*_{1/2}) and intrinsic clearance (CL_{int, micr}) were calculated (30). Microsomal intrinsic clearance was scaled to whole-liver dimensions (31), yielding intrinsic clearance (CL_{int}). Human hepatic clearance (CL_H) and extraction ratio (ER) were calculated (30), without considering plasma protein binding.

Metabolite Profiling with Human Hepatocytes

Cryopreserved human hepatocytes (10 donor pool) were thawed and immersed in hepatocyte thawing medium. After centrifugation (50g, 5 min at room temperature), the supernatant medium was aspirated and the remaining cell pellet resuspended in Krebs-Henseleit buffer. Cell viability, assessed with trypan blue (0.4%, v/v) exclusion dye method, was 85%.

AB-PINACA and 5F-AB-PINACA (10 $\mu\text{mol/L}$ final concentration, the stock solution in methanol or DMSO was diluted with water to ensure organic content $<1\%$) were incubated with hepatocytes (5×10^5 cells/ 0.5 mL/well) at 37°C . Diclofenac (CYP2C9 substrate, 10 $\mu\text{mol/L}$) was incubated as a positive control. Reactions were stopped with 0.5 mL ice-cold acetonitrile after 0, 1, and 3 h. After centrifugation, supernatants were removed to separate vials and stored at -80°C . Samples were treated as above, 20 μL supernatant was diluted with 80 μL mobile phase A, and 25 μL injected.

Chromatographic separation was performed on a Shimadzu Prominence™ HPLC system with two LC-20AD_{XR} pumps, a DGU-20A5R degasser, a SIL-20AC_{XR} autosampler, and a CTO-20AC column oven (Shimadzu Corp., Columbia, MD). HPLC column, mobile phases, gradient and temperatures for column oven, and autosampler were identical to those for the half-life determination experiments. Mass spectrometric analysis was achieved on a TripleTOF 5600+ mass spectrometer (AB SCIEX, Redwood City, CA, USA), AB SCIEX Analyst TF v.1.6 software, and MS and MS/MS data acquired in positive electrospray ionization mode with dynamic background subtraction (DBS). For information-dependent acquisition (IDA), any time of flight (TOF)-MS survey scan peak exceeding 500 cps was selected for dependent scan, with isotopes within 3 Da being excluded. Mass tolerance was $\pm 50 \text{ mDa}$. Ten candidate ions were allowed per cycle. TOF-MS was acquired scanning a mass range from m/z 100–950 followed by product ion scanning from m/z 60–950 with 0.1 and 0.075 s accumulation times, respectively. Declustering potential and collision energy were optimized by infusing AB-PINACA and 5F-AB-PINACA and were 80 V and $33 \pm 17 \text{ eV}$, respectively. An automated calibration was run after every third injection.

Mass spectra were analyzed with MetabolitePilot, v.1.5 (AB SCIEX), utilizing different peak-finding algorithms, e.g., common product ion and neutral loss scanning, mass defect filtering, and generic peak finding. The total ion chromatogram peak intensity threshold was 1500 cps, MS peak intensity 400 cps, and MS/MS peak intensity 100 cps, respectively. The mass defect filter window was set to $\pm 50 \text{ mDa}$. The number of unexpected metabolites was 10. Potential metabolites generated by adduct formation or in-source collision-induced dissociation or water loss were eliminated. Potential metabolites were evaluated based on mass measurement error, mass defect, MS/MS fragmentation patterns, and plausible retention time.

Analysis of Authentic Urine Specimens

Two urine specimens from subjects suspected of consuming AB-PINACA were analyzed with and without hydrolysis. After dilution of 250 μL urine with 600 μL 0.4 mol/L ammonium acetate buffer (pH 4.0) and 200 μL acetonitrile, samples were centrifuged (15,000g, 4°C , 5 min), decanted onto Isolute supported liquid extraction (SLE+) cartridges (1 mL, Biotage, Uppsala, Sweden), allowed to equilibrate for 2 min, and eluted with $2 \times 3 \text{ mL}$ ethyl acetate. After drying under nitrogen at 45°C , samples were reconstituted in 250 μL mobile phase A/B (90:10, v/v). Samples were briefly vortexed and centrifuged (11,000g,

4°C , 10 min) and supernatant transferred to autosampler vials.

For hydrolysis, a 250 μL sample was diluted with 600 μL 0.4 mol/L ammonium acetate buffer (pH 4.0), and 40 μL BG100 Red Abalone beta-glucuronidase (15,625 U/mL in water, Kura Biotec, Puerto Varas, Chile) was added. After samples were incubated at 55°C for 1 h, 200 μL acetonitrile was added, followed by the described extraction. As controls, AB-PINACA hepatocyte samples were diluted 1:5 with ammonium acetate buffer and subjected to the SLE+ procedure with and without hydrolysis. Authentic urine specimens and hepatocyte controls were analyzed on the 5600+ QTOF with LC-MS conditions described for metabolite profiling.

We monitored peak areas of metabolites previously identified in hepatocytes with MultiQuant software (AB SCIEX). We also processed urine specimen data with MetabolitePilot data mining procedures (LC threshold 5000 cps). Data were reviewed in two ways. First, the search was limited to previously identified metabolites in hepatocytes. Second, the 20 most intense metabolites (molecular weight $>250 \text{ Da}$, mass measurement error $\leq \pm 5 \text{ ppm}$) were identified, regardless if previously identified in hepatocytes or not.

In Silico Metabolite Prediction

The MetaSite software (version 5, Molecular Discovery, Pinner, UK) is training-independent and accounts for enzyme-substrate interaction by simulating three-dimensional docking of the substrate in the cavity of the specific CYP protein and predicting the reactivity of all sites in the molecule. The MetaSite software was evaluated for accurate prediction of AB-PINACA and 5F-AB-PINACA metabolism. Liver metabolites were predicted from 39 common CYP biotransformations. For 5F-AB-PINACA, aliphatic oxidative dehalogenation, an uncommon biotransformation, also was evaluated. Only metabolites $>150 \text{ Da}$ and with a probability score $>20\%$ were included in the final evaluation.

RESULTS

Metabolic Stability Assessment with HLM

For AB-PINACA, *in vitro* half-life ($T_{1/2}$) was $18.7 \pm 0.4 \text{ min}$, and *in vitro* microsomal intrinsic clearance ($\text{CL}_{\text{int, micr}}$) $0.037 \text{ mL} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, which was scaled to whole-liver dimensions yielding an intrinsic clearance of $35 \text{ mL} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$. Without considering plasma protein binding and with a simplified Rowland's equation (30), we calculated predicted hepatic clearance (CL_H) as $12.7 \text{ mL} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ and extraction ratio of 0.6. For 5F-AB-PINACA, *in vitro* ($T_{1/2}$) was $35.9 \pm 3.0 \text{ min}$, *in vitro* $\text{CL}_{\text{int, micr}}$ $0.019 \text{ mL} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, and intrinsic clearance (CL_{int}) $18 \text{ mL} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$. CL_H was predicted as $9.5 \text{ mL} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ and extraction ratio as 0.5.

Metabolic Profiling with Human Hepatocytes

In the diclofenac control, there was a 90% decrease in peak area after 3 h incubation and a simultaneous increase in 4'-hydroxydiclofenac and diclofenac β -D-acetyl-glucuronide peak areas, demonstrating hepatocyte activity.

Table 1. Metabolite profiling for AB-PINACA with human hepatocytes: retention time, accurate mass protonated molecule m/z , elemental composition, diagnostic product ions, mass error, and MS peak areas (1 and 3 h) of AB-PINACA and its metabolites. MS peak area of AB-PINACA at 0 h was 6.19E+06 cps

Peak ID	Metabolic reaction	RT (min)	Protonated molecule (m/z)	Elemental composition	Diagnostic product ions (m/z)	Mass error (ppm)	MS Area (1 h)	MS Area (3 h)
A1	Dioxidation	9.05	363.2028	$C_{18}H_{26}N_4O_4$	145, 213, 231, 300, 318, 346	0.3	2.35E+04	3.70E+04
A2	Ketone formation+oxidation	9.40	361.1876	$C_{18}H_{24}N_4O_4$	85, 145, 229, 298, 316, 326, 344	1.6	1.77E+04	4.52E+04
A3	Epoxide formation with subsequent hydrolysis to dihydrodiol	9.82	365.2187	$C_{18}H_{28}N_4O_4$	161, 179, 249, 320, 348	1.1	3.61E+04	8.12E+04
A4	Oxidation+glucuronidation	10.47	523.2403	$C_{24}H_{34}N_4O_9$	161, 231, 407, 478, 506	0.9	2.70E+05	3.13E+05
A5	Amide hydrolysis+oxidation+glucuronidation	11.12	524.2239	$C_{24}H_{33}N_3O_{10}$	145, 213, 231, 284, 302, 330, 348, 488, 506	-0.3	2.12E+04	5.00E+04
A6	Oxidation	11.32	347.2089	$C_{18}H_{26}N_4O_3$	145, 213, 231, 284, 302, 330	3.2	7.48E+05	8.44E+05
A7	Carboxylation	11.36	361.1872	$C_{18}H_{24}N_4O_4$	145, 217, 227, 245, 298, 316, 344	0.5	5.42E+04	9.93E+04
A8	Ketone formation	12.00	345.1928	$C_{18}H_{24}N_4O_3$	85, 145, 229, 300, 328	2.0	4.19E+05	8.96E+05
A9	Oxidation	12.23	347.2086	$C_{18}H_{26}N_4O_3$	145, 231, 302, 330	2.4	2.79E+05	3.57E+05
A10	Oxidation	12.30	347.2084	$C_{18}H_{26}N_4O_3$	145, 231, 302, 330	1.8	6.63E+04	9.61E+04
A11	Amide hydrolysis+oxidation+glucuronidation	12.36	524.2243	$C_{24}H_{33}N_3O_{10}$	161, 231, 302, 348, 407, 478, 506	0.8	3.09E+04	6.09E+04
A12	Amide hydrolysis+carboxylation	13.34	362.1716	$C_{18}H_{23}N_3O_5$	145, 199, 217, 227, 245, 298, 316, 344	1.5	2.40E+04	8.35E+04
A13	Amide hydrolysis+oxidation	13.41	348.1926	$C_{18}H_{25}N_3O_4$	145, 185, 213, 231, 284, 302, 330	2.5	1.63E+05	5.27E+05
A14	Oxidation	13.59	347.2081	$C_{18}H_{26}N_4O_3$	145, 231, 302, 330	1.0	2.29E+04	3.58E+04
A15	Amide hydrolysis+oxidation	14.32	348.1919	$C_{18}H_{25}N_3O_4$	145, 213, 231, 302, 330	0.5	6.79E+04	2.36E+05
A16	Amide hydrolysis+ketone formation	14.36	346.1771	$C_{18}H_{23}N_3O_4$	85, 145, 229, 300, 328	2.7	1.38E+05	6.52E+05
A17	Oxidation (at indazole)	15.13	348.1924	$C_{18}H_{26}N_4O_3$	161, 215, 231, 302, 330	0.6	2.05E+04	3.97E+04
A18	Oxidation (at butane moiety)	16.23	347.2085	$C_{18}H_{26}N_4O_3$	145, 215, 272, 284, 302, 330	2.1	2.50E+05	5.01E+05
A19	Internal amide hydrolysis (pentylindazole part)	17.00	233.1285	$C_{13}H_{16}N_2O_2$	145, 215	0.2	1.70E+04	3.27E+04
A20	Amide hydrolysis+oxidation (at butanoic acid)	17.48	348.1922	$C_{18}H_{25}N_3O_4$	145, 215, 231, 284, 302, 330	1.2	2.20E+04	9.12E+04
A21	Amide hydrolysis+glucuronidation	17.71	508.2289	$C_{24}H_{33}N_3O_9$	145, 215, 286, 314, 332, 490	-0.1	2.21E+04	4.63E+04
A22	Amide hydrolysis+oxidation (at indazole)	18.73	348.1924	$C_{18}H_{25}N_3O_4$	161, 231, 302	1.8	1.93E+04	2.36E+04
A23	Parent Amide hydrolysis	19.34 21.85	331.2133 332.1974	$C_{18}H_{26}N_4O_2$ $C_{18}H_{25}N_3O_3$	145, 215, 286, 314 145, 215, 286, 314	1.4 1.7	3.04E+06 4.08E+06	1.91E+06 8.77E+06

AB-PINACA peak area dropped to 49% after 1 h and 31% after 3 h. We identified 23 metabolites with mass measurement errors <3.2 ppm (Fig. 1a, Table I). The dominant biotransformation was terminal carboxamide hydrolysis to AB-PINACA carboxylic acid; other metabolites were generated by hydroxylation at the pentyl side chain, indazole core or butane moiety; ketone formation; carboxylation; epoxide formation with subsequent hydrolysis; combinations of these, and phase II glucuronidation. Figure 2 includes extracted ion chromatograms for all amide hydrolysis metabolites and Fig. 3 hydroxylated and carboxylated metabolites. Based on MS peak areas, the most intense AB-PINACA metabolites were AB-PINACA carboxylic acid (A23, hydrolysis product), carbonyl AB-PINACA (A8), and hydroxypentyl AB-PINACA (A6). AB-PINACA eluted at 19.34 min and metabolites between 9.05 and 21.85 min (Fig. 1a). Table I lists all AB-PINACA metabolites with retention times, observed m/z , metabolic reactions, elemental composition, diagnostic fragment ions, mass error, and MS peak areas at 1 and 3 h.

Results for 5F-AB-PINACA were similar to AB-PINACA. 5F-AB-PINACA peak areas decreased to 65% after 1 h and 18% after 3 h, indicating extensive metabolism. Eighteen 5F-AB-PINACA metabolites generated by the same biotransformations described above were identified with mass measurement errors <2.9 ppm (Fig. 1e). Again, terminal carboxamide hydrolysis was observed; hydroxylation at the pentyl side chain, indazole, and butane substructures; epoxide formation with subsequent hydrolysis; and glucuronidation. Additionally, oxidative defluorination occurred, producing 5'-hydroxypentyl and pentanoic acid metabolites. Extracted ion chromatograms for defluorinated, hydrolyzed, and hydroxylated metabolites are shown in Fig. 4a–c. The three most intense metabolites at both time points were AB-PINACA pentanoic acid (F10) and 5'-hydroxypentyl-AB-PINACA (F11), followed by 5F-AB-PINACA carboxylic acid (F18). Retention times were 7.73 to 18.79 min, with 5F-AB-PINACA eluting at 16.29 min. Table II lists all 5F-AB-PINACA metabolites with retention times, observed m/z , metabolic reactions, elemental composition, diagnostic fragment ions, mass error, and MS peak areas at 1 and 3 h.

Analysis of Authentic Urine Specimens

Comparing diluted and SLE+-extracted hepatocytes proved that all the various AB-PINACA metabolites could be recovered with the SLE+ procedure (Table III, Fig. 1b). Beta-glucuronidase hydrolysis cleaved A4 and A11 glucuronide metabolites, but was less efficient for A21. All metabolites in hepatocytes were identified in authentic urine, except A18 (Table III). The most intense metabolites in both specimens were A16, A23, and A13, followed by A3 and A15. Two hydroxylated AB-PINACA metabolites, A10 and A14, were present only as glucuronides. Enzymatic hydrolysis also was necessary for detection of A3, A9, A17, A19, A22, and A23 (increased by >100%). Comparing urine profiles in Fig. 1c, d and Figs. 2c, d and 3b, c, e, f with hepatocyte profiles, good agreement was observed for metabolite abundances. The only exceptions were A16 and A17 showing higher and A23 showing lower intensities than expected.

Interestingly, a minor signal for AB-PINACA was detected in urine #1, but not in urine #2.

Figure 5 depicts the 20 most intense metabolites in urine with and without hydrolysis; Supplementary Table 2 summarizes biotransformations and ranks metabolites. Eleven and nine metabolites from hepatocytes were among the 20 most intense metabolites in urine #1 and #2, respectively. These were A1 (only urine #1), A2, A3, A6, A7 (only urine #1), A12, A13, A16, A17, A21, and A23. Many additional metabolites not previously identified were products of internal carboxamide hydrolysis (signals a–c, p) or terminal carboxamide hydrolysis with multiple oxidative biotransformations (signals f–i, k–o, r).

In Silico Metabolite Predictions

MetaSite predicted 9 and 10 metabolites with masses >150 Da and a probability score >20% for AB-PINACA and 5F-AB-PINACA, respectively. AB-PINACA metabolites were generated by aliphatic hydroxylation, *N*-dealkylation of the pentyl chain and aminooxobutane moiety, dehydrogenation, and aliphatic carbonylation. 5F-AB-PINACA metabolites were generated by aliphatic and aromatic hydroxylation, *N*-dealkylation of the pentyl chain and aminooxobutane moiety, dehydrogenation, and aliphatic carbonylation. In fact, oxidative defluorination was predicted, but with only 18% probability. Supplementary Table 3 shows predicted metabolites with proposed biotransformation, monoisotopic mass, and calculated log D at pH 4, closest to our gradient conditions. Log D 4 for AB-PINACA and 5F-AB-PINACA were 3.19 and 2.72, respectively.

DISCUSSION

HLM Metabolic Stability

In vitro half-life ($T_{1/2}$) and intrinsic clearance (CL_{int}) estimate the drug's susceptibility to biotransformation, predicting *in vivo* hepatic clearance, *in vivo* half-life, and bioavailability (30). AB-PINACA and 5F-AB-PINACA CL_{int} and predicted extraction ratios were consistent with intermediately fast metabolized drugs (31,32). $T_{1/2}$ and CL_{int} are useful for future predictions of human pharmacokinetics once plasma protein binding and volume of distribution are determined.

Identification of AB-PINACA Metabolites After Human Hepatocyte Incubation

Characteristic Fragments of AB-PINACA

The AB-PINACA product ion spectrum has four characteristic fragments with base ion m/z 215, the unchanged pentylindazole acylium ion, and m/z 145, the indazole acylium ion (Fig. 6). Terminal carboxamide removal yields m/z 286, and cleavage between the carboxamide carbon and nitrogen atom generates m/z 314.

Metabolites Generated by Carboxamide Hydrolysis

Terminal carboxamide hydrolysis, a reaction predominantly catalyzed by carboxylesterase 1 (29), yielded the most

Fig. 2. Combined extracted chromatograms for AB-PINACA metabolites that underwent amide hydrolysis and further biotransformations. Signals in the samples, which were enzymatically hydrolyzed, are shown in *red*. **a** AB-PINACA hepatocyte sample, T3h, diluted: extracted ion chromatogram for AB-PINACA metabolites that underwent amide hydrolysis without oxidation (A23; *black*) or with oxidation (A13, A15, A20, A22; *dark blue*), ketone formation (A16; *light blue*), and carboxylation (A12; *green*). **b** AB-PINACA hepatocyte sample, T3h, diluted: extracted ion chromatogram for glucuronides of AB-PINACA metabolites that underwent amide hydrolysis without (A21; *gray*) or with oxidation (A5, A11; *purple*). **c** Urine of a suspected AB-PINACA case, #1, after SLE+ extraction (with and without hydrolysis); metabolite A21 showed a cluster, as typical for acyl glucuronides, with the highest signal at 17.7 min detected as the aglycone. **d** Urine of a suspected AB-PINACA case, #2, after SLE+ extraction (with and without hydrolysis); metabolite A21 showed a cluster, as typical for acyl glucuronides, with the highest signal at 17.7 min detected as the aglycone.

intense AB-PINACA metabolite at 1 and 3 h, AB-PINACA carboxylic acid (A23), with an absolute MS peak area 10 times higher than the second most intense metabolite A8 (Fig. 1a). AB-PINACA carboxylic acid might not ionize as efficiently in positive mode as other AB-PINACA metabolites, yet still had the highest signal. A23 shared all common fragments with AB-PINACA (Fig. 6) and eluted 2.6 min after it. Nine of the remaining 22 metabolites were second-generation metabolites from AB-PINACA carboxylic acid—four hydroxylated (A13, A15, A20, A22) and two

further glucuronidated (A5, A11), one carbonylated (A16), one carboxylated at the pentyl chain (A12), and one glucuronidated (A21) (Fig. 2a, b).

Investigating the product ion spectra of the six hydroxylated metabolites (selection in Fig. 6) reveals different locations for hydroxyl groups: MS/MS spectra of A13 and A15 (retention time, RT, 13.41 and 14.32 min) show fragments at m/z 145 and 231, indicating pentyl chain hydroxylation. Based on retention time and calculated $\log D_4$ values, A13 is likely hydroxylated at 4'-position, the preferred site, as for JWH-018 (5). F16 in the 5F-AB-PINACA

◀ **Fig. 3.** Combined extracted chromatograms for hydroxylated and carboxylated AB-PINACA metabolites and their glucuronides in hepatocyte samples and authentic urine specimens. Signals in the samples, which were enzymatically hydrolyzed, are shown in red. **a** AB-PINACA hepatocyte sample, T3h, diluted; hydroxylated metabolites in black and glucuronides in green. **b** Authentic urine of a suspected AB-PINACA case, #1, after SLE+ extractions (with and without hydrolysis); hydroxylated metabolites in black and glucuronides in green. **c** Authentic urine of a suspected AB-PINACA case, #2, after SLE+ extractions (with and without hydrolysis); hydroxylated metabolites in black and glucuronides in green. **d** AB-PINACA hepatocyte sample, T3h, diluted; carboxylated metabolites in black and glucuronides in purple. **e** Authentic urine of a suspected AB-PINACA case, #1, after SLE+ extractions (with and without hydrolysis); carboxylated metabolites in black and glucuronides in purple. **f** Authentic urine of a suspected AB-PINACA case, #2, after SLE+ extractions (with and without hydrolysis); carboxylated metabolites in black and glucuronides in purple

profile (RT 13.54 min) was derived by oxidative defluorination, producing 5'-hydroxypentyl AB-PINACA. The A13 and F16 retention times do not match, ruling out the 5'-hydroxypentyl isomer for A13. However, there was a small shoulder on A13 (arrow in Fig. 2a), but it had no product ion spectrum for further interpretation. A15 can be hydroxylated at 1'-, 2'-, or 3'-pentyl position. A20 (RT 17.48 min) was hydroxylated at the butanoic acid moiety (m/z 302) leaving the pentylindazole substructure unchanged (m/z 145, m/z 215). The MS/MS spectrum for A22 showed characteristic fragments at m/z 161, associated with indazole hydroxylation, and m/z 231. A22 eluted last of the six hydroxylated metabolites (18.73 min), consistent with reduced polarity from aromatic hydroxylation as compared to aliphatic hydroxylation. The glucuronides were hydroxylated at the pentyl side chain (A5) or indazole core (A11), as indicated by fragments m/z 145/231 or m/z 161/231, respectively. Positioning of the glucuronic acid is unclear, but chromatogram signals suggest an acyl glucuronide, which can isomerize by intramolecular acyl migration generating clusters (33). Acyl glucuronides are reactive and can irreversibly bind to proteins and nucleic acids, potentially leading to *in vivo* toxicity (34,35).

Ketone formation (A16) occurred at the pentyl chain (m/z 145, m/z 229), but the exact location is unclear. A12 was carboxylated at the pentyl chain and showed intense fragments at m/z 217 and m/z 245, associated with carboxylated pentylindazole and pentylindazole acylium fragments, respectively. AB-PINACA carboxylic acid also can form an ester glucuronide (A21), which was incompletely hydrolyzed by beta-glucuronidase (Fig. 1b, Table III).

Hydroxylated Metabolites

Besides amide hydrolysis, oxidation of the parent molecule occurred at different sites. We identified eight monohydroxylated metabolites including A6, A9, A10, A14, A17, A18, one dihydroxylated (A1), and one further glucuronidated (A4). The pentyl side chain was preferred for hydroxylation (A6, A9, A10, and A14), with product ion spectra showing intense ions at m/z 145 and m/z 231. Exact location assignment was not possible, although the 5'-hydroxypentyl isomer was excluded as analysis of the corresponding peaks in the 5F-AB-PINACA profile suggests a retention time around 11.49 min after AB-PINACA pentanoic acid at 11.41 min. There is a small peak next to the intense A6 peak (arrow in Fig. 3a), but MS/MS was not acquired for structural

elucidation. Considering peak intensities ($A6 \gg A9 \gg A10, A14$) and retention times, we propose A6 as the 4'-hydroxypentyl isomer and A9, A10, and A14 hydroxylated at other possible sites. Only one metabolite, A17, was hydroxylated at the indazole core (m/z 161). The glucuronidated metabolite A4 also was hydroxylated at this site, but the exact position is unclear. A18 was hydroxylated at the aminooxobutane moiety based on the presence of m/z 145, 215, and 302. The dihydroxylated metabolite A1 was hydroxylated at the pentyl chain (m/z 231) and the aminooxobutane moiety (m/z 318).

Ketone Formation, Carboxylation, Epoxide Formation with Subsequent Hydrolysis, and Internal Carboxamide Hydrolysis

Additionally, AB-PINACA underwent other biotransformations. Ketone formation occurred at the pentyl chain (m/z 145, 229) producing the second most intense metabolite, A8, and A2 that was further hydroxylated at the aminooxobutane moiety (m/z 145, 229, and 316). Oxidation of terminal hydroxyl groups led to pentanoic acid metabolites A7 and A12 (m/z 245). AB-PINACA also underwent epoxidation and hydrolysis to form a dihydrodiol (A3), a reaction previously described for AM2201 (18) and PB-22 and 5F-PB-22 (19). The product ion spectrum showed intense fragments at m/z 249 and 320 and minor signals at m/z 161, 179, and 231, all consistent with dihydrodiols. Notably, m/z 145 for an unchanged indazole structure was not found. Lastly, internal carboxamide hydrolysis produced A19, the remaining pentylindazole substructure, in low abundance. However, the carboxamide linkage seems relatively stable and not the preferred biotransformation site.

The Most Intense AB-PINACA Metabolites

Absolute MS peak areas are affected by matrix effects and differing ionization efficiencies for different structures. Nevertheless, comparing peak areas provides an insight into metabolite prevalence. In hepatocytes, the most intense AB-PINACA metabolites were AB-PINACA carboxylic acid (A23), carbonyl AB-PINACA (A8), hydroxypentyl AB-PINACA (A6), carbonyl AB-PINACA carboxylic acid (A16), and a hydroxypentyl AB-PINACA carboxylic acid isomer (A13). Amide hydrolysis, catalyzed by carboxylesterase 1, and oxidation reactions, catalyzed by CYP450 monooxygenases, can both occur, producing a combination of carboxylic acid and hydroxylated metabolites.

In Silico Prediction

In silico prediction can assist in metabolite identification without requiring a reference standard. The MetaSite software predicts metabolites generated by cytochrome and flavin-containing monooxygenase-mediated reactions covering the majority of metabolic reactions for xenobiotics. It does not simulate biotransformations catalyzed by other enzymes, e.g., carboxylesterases, which generated the most intense metabolite A23. Even with this limitation, there is good agreement between the predicted reactions and our hepatocyte findings. The #1 predicted metabolite was 4'-hydroxypentyl-AB-PINACA, which ranked #2 and #3 in the

Fig. 4. Combined extracted chromatograms for 5F-AB-PINACA metabolites after 3 h incubation with human hepatocytes, diluted sample. **a** 5F-AB-PINACA metabolites that underwent oxidative defluorination (*F11*) and subsequent biotransformations like amide hydrolysis (*F16*), carboxylation (*F10*), oxidation (*F5*), glucuronidation (*F7*), and combinations (*F2*, *F4*, *F6*, *F13*). **b** 5F-AB-PINACA metabolites that underwent carboxamide hydrolysis (*F18*) and subsequent biotransformations (*F12*, *F14*, *F17*); *F13* and *F16* were already included in **a**. **c** Hydroxylated (*F8*, *F9*, *F15*) and hydroxylated/glucuronidated (*F3*) 5F-AB-PINACA metabolites

1- and 3 h hepatocyte samples. Although we cannot assign the exact location of the hydroxy group, retention times strongly suggest hydroxylation at 4'-position (A6). With 42% probability, MetaSite predicted a hydroxylated, carbonylated, or dehydrogenated metabolite at 1'-pentyl position as well as depentylation. Carbonyl AB-PINACA (A8) ranking #3/#2 in the hepatocytes (1 h/3 h) might match with the second metabolite, another AB-PINACA hydroxypentyl isomer (A9) ranking #4/#7 might match with the first predicted metabolite. For a final confirmation, the location of the functional group has to be determined. Interestingly, we did not observe *N*-depentylated metabolites—metabolites that were often observed for other synthetic cannabinoids including JWH-018 (14), JWH-250 (13), AM2201 (18), RCS-4 (10), UR-144 (18), and AB-001 (12). Currently, it is unclear

which structural elements favor or prevent *N*-depentylation as many other structurally similar synthetic cannabinoids including RCS-8 (20), AM-694 (11), AKB-48 (8), XLR-11 (21) and PB-22 and 5F-PB-22 (19) did not undergo *N*-depentylation either.

Identification of 5F-AB-PINACA Metabolites After Human Hepatocyte Incubation

Characteristic Fragments of 5F-AB-PINACA

5F-AB-PINACA fragments similar to its non-fluoro analog. The most intense fragment occurs at *m/z* 233, the unchanged fluoropentylindazole acylium ion. This ion can further fragment to *m/z* 213 and 145 corresponding to the

Table II. Metabolite profiling for 5F-AB-PINACA with human hepatocytes: retention time, accurate mass protonated molecule m/z , elemental composition, diagnostic product ions, mass error, and MS peak areas (1 and 3 h) of 5F-AB-PINACA and its metabolites. MS peak area of 5F-AB-PINACA at 0 h was 6.17E+06. All metabolites generated by oxidative defluorination are potentially shared with AB-PINACA

Peak ID	Metabolic reaction	RT (min)	Protonated molecule (m/z)	Elemental composition	Diagnostic product ions (m/z)	Mass error (ppm)	MS Area (1 h)	MS Area (3 h)
F1	Epoxide formation with subsequent hydrolysis to dihydrodiol	7.73	383.2091	C ₁₈ H ₂₇ N ₄ O ₄ F	151, 161, 179, 247, 267, 338, 366	0.6	1.71E+04	2.54E+04
F2	Oxidative defluorination to COOH+oxidation	8.87	377.1826	C ₁₈ H ₂₄ N ₄ O ₅	145, 217, 227, 245, 314, 342	1.7	1.11E+04	1.99E+04
F3	Oxidation+glucuronidation	8.97	541.2310	C ₂₄ H ₃₃ N ₄ O ₉ F	161, 229, 249, 320, 425, 496, 524	1.0	3.50E+04	5.77E+04
F4	Oxidative defluorination to COOH+oxidation	9.13	377.1825	C ₁₈ H ₂₄ N ₄ O ₅	145, 217, 227, 245, 302, 314, 332, 360	1.5	ND	2.19E+04
F5	Oxidative defluorination+oxidation	9.20	363.2030	C ₁₈ H ₂₆ N ₄ O ₄	145, 213, 231, 300, 318, 346	0.8	ND	2.44E+04
F6	Oxidative defluorination to COOH+glucuronidation	9.36	537.2188	C ₂₄ H ₃₂ N ₄ O ₁₀	217, 245, 298, 316, 344, 361, 520	-0.6	ND	2.37E+04
F7	Oxidative defluorination+glucuronidation	9.74	523.2396	C ₂₄ H ₃₄ N ₄ O ₉	145, 213, 231, 302, 330, 347, 478, 506	-0.5	ND	2.19E+04
F8	Oxidation	11.05	365.1984	C ₁₈ H ₂₅ N ₄ O ₃ F	145, 231, 249, 320, 348	0.2	2.68E+04	3.71E+04
F9	Oxidation	11.25	365.1986	C ₁₈ H ₂₅ N ₄ O ₃ F	145, 249, 320, 348	0.6	4.02E+04	4.86E+04
F10	Oxidative defluorination to COOH	11.41	361.1875	C ₁₈ H ₂₄ N ₄ O ₄	145, 217, 227, 245, 298, 316, 344	1.3	1.69E+06	3.61E+06
F11	Oxidative defluorination	11.49	347.2083	C ₁₈ H ₂₆ N ₄ O ₃	145, 213, 231, 302, 330	1.4	1.67E+06	2.31E+06
F12	Amide hydrolysis+oxidation	13.21	366.1829	C ₁₈ H ₂₄ N ₃ O ₄ F	145, 231, 249, 320	1.4	ND	3.34E+04
F13	Oxidative defluorination to COOH+amide hydrolysis	13.39	362.1719	C ₁₈ H ₂₃ N ₃ O ₅	145, 217, 227, 245, 298, 316, 344	2.4	1.88E+05	5.88E+05
F14	Amide hydrolysis+oxidation	13.39	366.1827	C ₁₈ H ₂₄ N ₃ O ₄ F	145, 233, 303, 321, 349	1.1	ND	3.04E+04
F15	Oxidation	13.47	365.1991	C ₁₈ H ₂₅ N ₄ O ₃ F	145, 213, 233, 290, 320, 348	2.0	1.15E+05	1.31E+05
F16	Oxidative defluorination+amide hydrolysis	13.54	348.1928	C ₁₈ H ₂₅ N ₃ O ₄	145, 213, 231, 302, 330	2.9	6.64E+04	3.16E+05
F17	Amide hydrolysis+glucuronidation	15.26	526.2194	C ₂₄ H ₃₂ N ₃ O ₉ F	145, 213, 233, 304, 332, 350	-0.3	1.50E+04 ^a	3.55E+04
	Parent	16.29	349.2038	C ₁₈ H ₂₅ N ₄ O ₂ F	145, 213, 233, 304, 332	1.1	4.03E+06	1.11E+06
F18	Amide hydrolysis	18.79	350.1883	C ₁₈ H ₂₄ N ₃ O ₃ F	145, 213, 233, 304, 332	2.5	1.43E+06	2.22E+06

ND not detected

^a As aglycone

Table III. Analysis of hepatocyte samples and authentic urine specimens associated with AB-PINACA intake: The 3 h hepatocyte sample was analyzed 1:5 diluted, after supported liquid extraction (SLE+) and after enzymatic hydrolysis followed by SLE+. The two authentic urine specimens were analyzed with and without hydrolysis, subjected to SLE+ and analyzed by HRMS. Peak areas were quantified by MultiQuant and are ranked within each sample (in parenthesis)

ID	Metabolic reaction	RT	3 h hepatocyte sample				Urine 1		Urine 2	
			1:5	SLE+	Hydrolysis+ SLE+		SLE+	Hydrolysis+ SLE+	SLE+	Hydrolysis+ SLE+
A1	Dioxidation	9.01	3.70E+04 (18)	2.20E+04 (18)	2.10E+04 (17)	3.60E+06 (9)	3.80E+06 (9)	1.10E+06 (11)	1.10E+06 (11)	1.10E+06 (10)
A2	Ketone formation+oxidation	9.38	4.20E+04 (15)	2.50E+04 (17)	2.70E+04 (15)	6.20E+06 (8)	7.50E+06 (8)	2.30E+06 (7)	2.30E+06 (7)	2.40E+06 (7)
A3	Epoxide formation with subsequent hydrolysis to dihydrodiol	9.8	8.10E+04 (11)	5.70E+04 (11)	6.10E+04 (10)	8.50E+06 (5)	1.70E+07 (4)	1.70E+06 (9)	1.70E+06 (9)	5.20E+06 (5)
A4	Oxidation+glucuronidation	10.45	1.40E+05 (9)	1.00E+05 (9)	ND	2.60E+05 (15)	ND	3.00E+05 (15)	ND	ND
A5	Amide hydrolysis+oxidation+glucuronidation	11.09	4.10E+04 (16)	1.80E+04 (19)	1.30E+04 (20)	7.30E+05 (13)	1.00E+05 (19)	3.60E+05 (13)	3.60E+05 (13)	6.70E+04 (19)
A6	Oxidation	11.29	6.30E+05 (2)	5.00E+05 (2)	5.30E+05 (2)	2.30E+06 (11)	2.90E+06 (10)	1.20E+06 (10)	1.20E+06 (10)	1.30E+06 (9)
A7	Carboxylation	11.34	5.90E+04 (12)	4.50E+04 (13)	4.60E+04 (12)	7.70E+06 (6)	8.50E+06 (7)	2.60E+06 (6)	2.60E+06 (6)	2.40E+06 (6)
A8	Ketone formation	11.98	4.80E+05 (3)	4.00E+05 (3)	4.00E+05 (3)	8.00E+05 (12)	1.00E+06 (17)	5.90E+05 (12)	5.90E+05 (12)	6.00E+05 (15)
A9	Oxidation	12.14	3.20E+05 (6)	2.60E+05 (6)	2.50E+05 (7)	3.20E+05 (14)	1.20E+06 (16)	3.20E+05 (14)	3.20E+05 (14)	8.80E+05 (13)
A10	Oxidation	12.28	8.50E+04 (10)	6.50E+04 (10)	6.40E+04 (9)	ND	1.30E+06 (12)	ND	ND	2.50E+05 (17)
A11	Amide hydrolysis+oxidation+glucuronidation	12.33	2.60E+04 (20)	1.50E+04 (22)	ND	1.30E+05 (18)	ND	4.10E+04 (17)	ND	ND
A12	Amide hydrolysis+carboxylation	13.31	4.70E+04 (14)	4.10E+04 (14)	3.90E+04 (13)	7.70E+06 (7)	9.20E+06 (6)	2.20E+06 (8)	2.20E+06 (8)	2.20E+06 (8)
A13	Amide hydrolysis+oxidation	13.38	3.20E+05 (7)	2.60E+05 (7)	2.70E+05 (6)	1.50E+07 (2)	2.40E+07 (3)	6.90E+06 (4)	6.90E+06 (4)	8.60E+06 (3)
A14	Oxidation	13.55	3.90E+04 (17)	2.80E+04 (16)	2.80E+04 (14)	ND	2.40E+05 (18)	ND	ND	6.80E+04 (18)
A15	Amide hydrolysis+oxidation	14.3	2.30E+05 (8)	2.00E+05 (8)	2.00E+05 (8)	1.10E+07 (4)	1.30E+07 (5)	7.30E+06 (3)	7.30E+06 (3)	8.20E+06 (4)
A16	Amide hydrolysis+ketone formation	14.32	3.90E+05 (5)	3.50E+05 (4)	3.60E+05 (5)	4.70E+07 (1)	6.70E+07 (1)	2.40E+07 (1)	2.40E+07 (1)	3.00E+07 (1)
A17	Oxidation	15.08	2.40E+04 (21)	1.70E+04 (20)	2.50E+04 (16)	1.10E+05 (19)	2.20E+06 (11)	1.80E+04 (19)	1.80E+04 (19)	6.10E+05 (14)
A18	Oxidation	16.19	4.50E+05 (4)	3.40E+05 (5)	3.70E+05 (4)	ND	ND	ND	ND	ND
A19	Internal amide hydrolysis	16.94	2.80E+04 (19)	1.50E+04 (21)	1.70E+04 (18)	2.40E+05 (16)	1.30E+06 (14)	5.90E+04 (16)	5.90E+04 (16)	9.00E+05 (12)
A20	Amide hydrolysis+oxidation	17.43	5.70E+04 (13)	4.90E+04 (12)	5.00E+04 (11)	8.0E+05 ^a	3.1E+05 ^a	2.5E+06 ^a	2.5E+06 ^a	6.2E+05 ^a
A21	Amide hydrolysis+glucuronidation	17.68	1.80E+04 (23)	2.90E+04 (15)	1.40E+04 (19)	3.50E+06 (10)	1.30E+06 (13)	3.00E+06 (5)	3.00E+06 (5)	9.90E+05 (11)
A22	Amide hydrolysis+oxidation	18.67	2.20E+04 (22)	5.70E+03 (23)	3.30E+03 (21)	1.70E+05 (17)	1.30E+06 (15)	2.10E+04 (18)	2.10E+04 (18)	3.20E+05 (16)
	Parent	19.24	1.00E+06	9.00E+05	8.80E+05	1.60E+04	2.60E+04	ND	ND	ND
A23	Amide hydrolysis	21.8	4.90E+06 (1)	4.30E+06 (1)	4.20E+06 (1)	1.10E+07 (3)	3.30E+07 (2)	9.00E+06 (2)	9.00E+06 (2)	2.10E+07 (2)

ND not detected

^a Co-eluting peak complicating peak integration

Fig. 5. Metabolic profiles of the authentic urine specimens #1 (a) and #2 (b) associated with AB-PINACA intake. The 20 most intense metabolites are shown for each urine sample with (red) and without (black) hydrolysis. Only metabolites >250 Da and within ± 5 ppm mass measurement error were included. Metabolites previously identified in hepatocytes are given with their corresponding identification

pentylindazole acylium and indazole acylium ion, respectively. Removal of the terminal carboxamide group of 5F-AB-PINACA yields m/z 304; cleavage between the carboxamide carbon and nitrogen atom generates m/z 332.

Metabolites Generated by Oxidative Defluorination

As described for AM2201, AM694, 5F-PB-22, XLR-11, and MAM2201 [5, 11, 19, 21, 35], oxidative defluorination is common for 5-fluoropentyl-containing synthetic cannabinoids and, as expected, occurred for 5F-AB-PINACA, generating metabolites shared with AB-PINACA. MS/MS spectra and fragmentation patterns are depicted in Fig. 7. The primary metabolite was 5'-hydroxypentyl AB-PINACA (F11), with m/z 145, 231, 302, and 330. The corresponding signal in the AB-PINACA hepatocyte profile was too low to obtain a product ion spectrum for comparison.

F11 was subsequently converted into eight more metabolites (F2, F4–F7, F10, F13, and F16), shown in Fig. 4a. Further oxidation led to F10, AB-PINACA pentanoic acid, the most intense metabolite (Fig. 1e). Both F10 and F11 were further hydroxylated to F2/F4 and F5, respectively, as well as further glucuronidated to yield F6 and F7. All metabolites produced m/z 245 (indazole pentanoic acid acylium) or m/z 231 (hydroxypentyl indazole acylium ion), while simultaneously generating m/z 145, proving that the indazole

structure remained unchanged. F10 (m/z 361.1875, RT 11.41 min) corresponded to A7 in the AB-PINACA profile (m/z 361.1872, RT 11.36 min) showing the same characteristic fragments.

Similar to AB-PINACA, 5F-AB-PINACA's terminal carboxamide group underwent hydrolysis. Oxidative defluorination in combination with carboxamide hydrolysis led to F13 and F16, #4 and #5 in intensity. For both metabolites, the corresponding counterparts can be found in the AB-PINACA profile: F13 (m/z 362.1719, RT 13.39 min) corresponded to AB-PINACA's A12 (m/z 362.1716, RT 13.34 min), and F16 (m/z 348.1928, RT 13.54 min) matched the small shoulder next to the A13 peak. These findings highlight the difficulty in conclusively identifying AB-PINACA or 5F-AB-PINACA intake when metabolites are shared.

Metabolites Generated by Amide Hydrolysis

5F-AB-PINACA carboxylic acid (F18) ranked #3 in the overall profile. Apart from the defluorinated F13 and F16, three more metabolites were generated from F18, further oxidation led to F12 and F14 and glucuronidation to F17. Overall, metabolites generated by amide hydrolysis were of lower abundance than for AB-PINACA (Fig. 4a), suggesting

that the alternative pathway of oxidative defluorination was favored.

Hydroxylated Metabolites

We identified three hydroxylated (F8, F9, F15) and one hydroxylated/glucuronidated (F3) 5F-AB-PINACA metabolites (Fig. 4c). The position of the hydroxyl group was at the pentyl side chain in F8 and F9 (m/z 145, 249), at the aminooxobutane moiety in F15 (m/z 233, 320), or at the indazole moiety in F3 (m/z 161). For both AB-PINACA and 5F-AB-PINACA, only one hydroxylated/glucuronidated metabolite was observed, with hydroxylation at the indazole.

Epoxide Formation with Subsequent Hydrolysis

Similar to AB-PINACA, we identified one metabolite (F1) that underwent epoxidation and internal hydrolysis to form a dihydrodiol. This structure is suggested by fragments m/z 267, 338, 161, 179, and 247 (Fig. 7) and absence of m/z 145, associated with an unchanged indazole structure. The exact location of the two hydroxyl groups is unknown.

The Most Intense 5F-AB-PINACA Metabolites

The most intense 5F-AB-PINACA metabolites were AB-PINACA pentanoic acid (F10), 5'-hydroxypentyl AB-PINACA (F11), and the hydrolysis product 5F-AB-PINACA carboxylic acid (F18). Notably, none are specific for 5F-AB-PINACA, as two are shared with AB-PINACA and the third, strictly speaking, does not contain the complete original structure. To interpret results correctly, it is strongly recommended to monitor a variety of metabolites, to include specific metabolites (even if minor), and to consider metabolite ratios. In the case of 5F-AB-PINACA, hydroxylated 5F-AB-PINACA metabolites would be suitable specific targets, e.g., F15. AB-PINACA pentanoic acid and 5'-hydroxypentyl AB-PINACA clearly dominated 5F-AB-PINACA's metabolic profile, which is in stark contrast to the AB-PINACA profile where these two metabolites were less intense.

In Silico Prediction

Introduction of fluorine into a molecule can change metabolic profiles, dependent on the site of fluorination in relation to sites of metabolic attack in the non-fluorinated analog (36). It is not easy to predict how fluorine substitution alters metabolism as carbon-fluorine bonds are strong, providing increased oxidative stability, but fluoride also is an excellent leaving group, making oxidative defluorination readily achievable.

The MetaSite predictions of the most intense 5F-AB-PINACA metabolites were not as effective as for AB-PINACA because oxidative defluorination was underestimated. 5'-Hydroxypentyl AB-PINACA (F11), the most intense 5F-AB-PINACA metabolite, ranked #16 in the *in silico* prediction. When excluding metabolites generated by amide hydrolysis and oxidative defluorination, the metabolite ranking highest was F15, with hydroxylation at the aminooxobutane moiety. This metabolite might match with the primary predicted metabolite, hydroxylated in 2''-

Fig. 6. Mass spectra and structures for selected AB-PINACA metabolites. Based on analyte intensities in human hepatocyte and authentic urine specimens, the eight most relevant metabolites were chosen. All spectra are from the 3-h hepatocyte sample

position. The two other metabolites scoring 100% probability—dealkylated and dehydrogenated metabolites—were not identified in hepatocyte samples.

Did AB-PINACA Hepatocyte and Urine Metabolic Profiles Match?

The lack of toxicity and safety data for new designer drugs hinders controlled administration studies. Therefore, authentic urine specimens from drug intoxication cases can help confirm *in vitro* or *in silico* metabolism studies.

The non-targeted data mining of two urine specimens from suspected AB-PINACA cases yielded many potential metabolites. After applying general filtering steps (mass measurement error ≤ 5 ppm, $m/z \geq 250$, peak area $\geq 1.0E5$), we still observed more than 100 potential candidates in non-hydrolyzed and hydrolyzed specimens, demonstrating extensive AB-PINACA metabolism. However, many potential metabolites had undergone numerous biotransformations including cleavage and hydrolysis, making them less valuable for forensic interpretations. When substantial parts of the molecule are missing, differentiation between synthetic cannabinoids might be impossible.

Four of the top five major metabolites in hepatocytes were among the top 20 most intense metabolites in the urine specimens (A6, A13, A16, and A23), demonstrating the usefulness of human hepatocytes for predicting synthetic cannabinoid metabolites. Enzymatic hydrolysis of urine specimens significantly increased peak areas for hydroxylated metabolites (A9, A10, A14, A17), hydrolysis products (A21, A23), the dihydrodiol (A3), and hydrolyzed/hydroxylated metabolites (A13, A22). Some differences in metabolites were observed possibly due to different dosages or time points after self-administration. Sampling after 3 h of hepatocyte incubation is considered a relatively early time point, whereas urine collection could have occurred many hours after drug intake, allowing for more extensive metabolism. The most intense urinary metabolites had undergone amide hydrolysis (A23), additional oxidation (A16, A13, A15, a, c), and often glucuronidation, suggesting a later time point in the metabolic process (Fig. 5, Supplementary Table 2).

Did AB-PINACA and 5F-AB-PINACA Fit into the Suggested Metabolism Pattern?

The hepatocyte metabolic profiles of AB-PINACA and 5F-AB-PINACA support the hypothesis that pentyl side chain-containing synthetic cannabinoids are generally transformed without clear preference for a particular molecular site, while their 5-fluoro analogs are usually metabolized to 5-hydroxypentyl and pentanoic acid metabolites. AB-PINACA was primarily hydrolyzed to AB-PINACA carboxylic acid (A23) and metabolized to carbonyl-AB-PINACA (A8) and hydroxypentyl AB-PINACA (A6), likely in 4'-position. The three major 5F-AB-PINACA metabolites were AB-PINACA pentanoic acid (F10), 5'-hydroxypentyl-AB-PINACA (F11),

AB-PINACA **A16** **A23** **A12** **A15** **A13** **A7** **A3** **A8** **A19**

Fig. 7. Mass spectra and structures for selected 5F-AB-PINACA metabolites. Based on analyte intensities in human hepatocyte and authentic urine specimens, the eight most relevant metabolites were chosen. All spectra are from the 3 h hepatocyte sample

and 5F-AB-PINACA carboxylic acid (F18). In order to distinguish between these parent drugs, fluoro-containing metabolites (F18, F15) should be targeted and the ratios of 5'-hydroxypentyl and pentanoic acid metabolites to other hydroxylated metabolites, e.g., 4'-hydroxypentyl metabolite, considered. It remains to be confirmed if the suggested pattern can also outline the general metabolism of future analog pairs. If it does, it will be useful for predicting metabolites of potential new synthetic cannabinoids.

CONCLUSION

We determined the metabolic profile of AB-PINACA and 5F-AB-PINACA after incubation with human hepatocytes using high-resolution mass spectrometry and software-assisted data mining, performed *in silico* prediction, assessed metabolic stability with HLM, and confirmed our findings for AB-PINACA with two authentic urine specimens. Twenty-three metabolites, generated by carboxamide hydrolysis, hydroxylation, ketone formation, carboxylation, epoxide formation with subsequent hydrolysis, and reaction combinations, were identified for AB-PINACA. For 5F-AB-PINACA, 18 metabolites were identified, generated by the same biotransformations and oxidative defluorination producing 5'-hydroxy and pentanoic acid metabolites, which are shared with the non-fluoro analog. In two authentic urine specimens from suspected AB-PINACA cases, we found similar metabolic profiles confirming the usefulness of human hepatocyte experiments.

The analog pair AB-PINACA/5F-AB-PINACA fit the expected pattern based on metabolic profiles of other pentylindole/indazole synthetic cannabinoids and their 5-fluoro analogs. For distinguishing between both parents, fluoro-containing metabolites should be targeted and the ratios of 5'-hydroxypentyl and pentanoic acid metabolites to other hydroxylated metabolites, e.g., 4'-hydroxypentyl metabolite, evaluated.

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Conflict of Interest None

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