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RESEARCH ARTICLE

The metabolism of the synthetic cannabinoids ADB-BUTINACA and ADB-4en-PINACA and their detection in forensic toxicology casework and infused papers seized in prisons

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Abstract
Early warning systems detect new psychoactive substances (NPS), while dedicated monitoring programs and routine drug and toxicology testing identify fluctuations in prevalence. We report the increasing prevalence of the synthetic cannabinoid receptor agonist (SCRA) ADB-BUTINACA (N-[1-amino-3,3-dimethyl-1-oxobutan-2-yl]-1-butyl-1H-indazole-3-carboxamide). ADB-BUTINACA was first detected in a seizure in Sweden in 2019, and we report its detection in 13 routine Swedish forensic toxicology cases soon after. In January 2021, ADB-BUTINACA was detected in SCRA-infused papers seized in Scottish prisons and has rapidly increased in prevalence, being detected in 60.4% of the SCRA-infused papers tested between January and July 2021. In this work, ADB-BUTINACA was incubated with human hepatocytes...
Synthetic cannabinoid receptor agonists (SCRAs) are a class of new psychoactive substances (NPS) that bind to and activate the cannabinoid receptors, CB1 and CB2.1–3 SCRs elicit a range of psychoactive and harmful physiological effects with many, but not all, appearing to be mediated by binding to, and activation of, the CB1 receptor.4–6 SCRs have been implicated in many non-fatal poisonings and drug-related deaths worldwide,7,8 and in some jurisdictions, their prevalence is particularly high in vulnerable groups such as rough-sleepers and prison populations.9–16 To date, SCRA production has been based primarily in the People’s Republic of China, from where substances are distributed to other jurisdictions via international mail and freight distribution networks. Legislative controls in China are known to have a significant influence on the appearance of specific SCRs on the global illicit drugs market, and the most prevalent substances often emerge simultaneously in different jurisdictions.15–17 In some cases, a particular SCRA will appear and will initially seem to have little impact on the illicit market. The substance may then rapidly increase in prevalence, seemingly without warning. One such example is MDMB-4en-PINACA (methyl 3,3-dimethyl-2-[1-(pent-4-en-1-yl)-1H-indazole-3-carboxamide]-butanoate). This potent, efficacious, and prevalent SCRA with a benzyl tail moiety (N-[1-amino-3,3-dimethyl-1-oxobutan-2-yl]-1-benzyl-1H-indazole-3-carboxamide) was first reported in Europe via the European Monitoring Centre for Drugs and Drug Addiction Early Warning System (EMCDDA-EWS) on September 19, 2019, following the seizure of a powder in Sweden on July 24, 2019.22 It was subsequently identified on October 14, 2019, in a test purchased sample analyzed and reported by the RESPONSE project, coordinated by the Slovenian National Forensic Laboratory,23 and in the United States (reported as ADB-BINACA) in a seizure received for testing in July 2020.24 The name ADB-BINACA was first used in the scientific literature25 to describe a tert-leucinamide indazole-3-carboxamide SCRA with a benzyl tail moiety (N-[1-amino-3,3-dimethyl-1-oxobutan-2-yl]-1-benzyl-1H-indazole-3-carboxamide). A commercially available reference material of this substance uses the name ADB-BINACA26; therefore, in this study, we use the name ADB-BUTINACA to clearly differentiate the two compounds. The second compound, ADB-4en-PINACA (Figure 2: N-[1-amino-3,3-dimethyl-1-oxobutan-2-yl]-1-[pent-4-en-1-yl]-1H-indazole-3-carboxamide), was first reported in the United States on March 4, 2021, in plant-like material seized in the United States (reported as ADB-BINACA) in a seizure received for testing in July 2020.24 The name ADB-BINACA was first used in the scientific literature25 to describe a tert-leucinamide indazole-3-carboxamide SCRA with a benzyl tail moiety (N-[1-amino-3,3-dimethyl-1-oxobutan-2-yl]-1-benzyl-1H-indazole-3-carboxamide). A commercially available reference material of this substance uses the name ADB-BINACA26; therefore, in this study, we use the name ADB-BUTINACA to clearly differentiate the two compounds. The second compound, ADB-4en-PINACA (Figure 2: N-[1-amino-3,3-dimethyl-1-oxobutan-2-yl]-1-[pent-4-en-1-yl]-1H-indazole-3-carboxamide), was first reported in the United States on March 4, 2021, in plant-like material seized on January 7, 2021.27 It was subsequently reported in Europe via the
EMCDDA-EWS on 23 March 2021 in Hungary, relating to a sample seized in late January 2021.28

The synthesis and in vitro pharmacological evaluation of ADB-BUTINACA and ADB-4en-PINACA has been reported recently.20,29–31 Both have been shown to be potent (low nanomolar) and efficacious CB1 agonists using a range of in vitro bioassays.

In this study, we present information on the synthesis and analytical characterization of ADB-BUTINACA, ADB-4en-PINACA, and a dihydrodiol metabolite of ADB-4en-PINACA. We discuss the metabolism of ADB-BUTINACA and identification of its metabolites using human hepatocytes (HHeps) and the detection of ADB-BUTINACA and its metabolites in authentic blood and urine samples from 13 routine toxicology cases in Sweden in 2019. We compare the metabolites detected to those recently reported following incubation of ADB-BUTINACA with human liver microsomes (HLM) and in four authentic urine samples originating from Singapore.32 We also report the detection of ADB-BUTINACA within an established drug prevalence monitoring program in Scotland, operating in partnership with the Scottish Prison Service.13–15 In addition, we detail the metabolites identified following incubation of ADB-4en-PINACA with HHeps, with the aim of identifying suitable analytical targets to aid their detection in toxicological samples and better establishing their prevalence in toxicology casework and mandatory drug testing (MDT) programs in prisons.

The Scottish prisons drug prevalence monitoring program utilizes unattributable or non-judicial drug seizures, including SCRA-infused papers which are often detected by prison staff screening incoming prison mail using ion mobility spectrometry.13,14 We present the latest prevalence data for SCRA-infused papers in seized samples in Scottish prisons. Mail containing SCRA-infused papers is currently the most common route of entry of these drugs into Scottish prisons, and this method of supplying SCRA is increasingly reported in prisons in other jurisdictions.15,16,21 Some prison authorities, including in Sweden, now regularly photocopy mail, providing prisoners with the copy and retaining/destroying the original; however, this approach varies due to specific legal issues around opening and retaining prisoner mail in

**FIGURE 1** Proposed metabolic pathways of ADB-BUTINACA. All metabolites were detected after duplicate 5-h incubations of ADB-BUTINACA with human hepatocytes. Metabolites marked with a yellow circle and a red square were detected in urine and blood, respectively, from authentic forensic toxicology cases [Colour figure can be viewed at wileyonlinelibrary.com]
different jurisdictions. We provide an assessment of the potency of these two SCRAMs using an alternative in vitro bioassay to those already reported\textsuperscript{20,30,31} and compare their potency directly with that of MDMB-4en-PINACA, in order to discuss their relative potential for harm.

2 | MATERIALS AND METHODS

2.1 | Material

2.1.1 | Analysis of seized prison samples and preparation of seizure-based reference standards from seized prison samples

Methanol, dichloromethane (HPLC Grade), and water (LC–MS grade) were purchased from Fisher Scientific, UK; bupivacaine and formic acid were obtained from Sigma Aldrich, Poole, UK.

2.1.2 | Synthesis of (S)-ADB-BUTINACA and (S)-ADB-4en-PINACA (Manchester Metropolitan University, UK)

All reagents used in the synthesis were of commercial quality (Sigma-Aldrich, Gillingham, UK or Fluorochem Limited, Hadfield, UK) and used without further purification. Further information on the chemicals used is provided in Section S2.2.

2.1.3 | Synthesis of (S)-ADB-4en-PINACA and (S)-ADB-4en-PINACA dihydrodiol metabolite (Linköping University, Sweden)

All reagents used in the synthesis were of commercial quality, purchased from Sigma-Aldrich (Stockholm, Sweden), and used without further purification. Further information on the chemicals used is provided in Section S2.3.
2.1.4 | Toxicological analysis and metabolism studies

Sodium acetate, acetic acid, and LC-MS grade methanol were obtained from Merck (Darmstadt, Germany). Ethanol was from Kemetyl AB (Jordbro, Sweden). LC-MS grade acetonitrile, formic acid, Williams E medium, L-glutamine, and HEPES buffer were obtained from Thermo Fisher Scientific (Gothenburg, Sweden). Cryopreserved primary human hepatocytes (LiverPool, 20 donor pool) and InvitroGro HT thawing medium were purchased from Bioreclamation IVT (Brussels, Belgium). β-Glucuronidase/arylsulfatase was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Amphetamine-d₃, diazepam-d₅, mianserin-d₅, and phenobarbital-d₅ were purchased from Cerilliant (Round Rock, USA) and prepared in LC-MS grade acetonitrile from Thermo Fisher (Gothenburg, Sweden).

2.2.2 | Preparation of seizure-based reference standards from seized infused papers (University of Dundee, UK)

ADB-BUTINACA and ADB-4en-PINACA were preliminarily identified in seized infused paper samples using GC-MS. They were initially identified by matching mass spectra to entries in spectral libraries (ADB-BUTINACA) and/or spectral interpretation followed by confirmatory analysis by ultra-high pressure liquid chromatography coupled to high resolution accurate mass spectrometry (UHPLC-HRAM-MS, ADB-BUTINACA, and ADB-4en-PINACA). In the absence of in-house synthesized or purchased reference standards at the time of first identification, “seizure-based reference standards” were created by extraction and crystallization of the substances from seized infused papers identified through routine GC-MS screening. ADB-BUTINACA was extracted from infused paper sample FL21/0015, consisting of five pieces of blank white paper seized on January 20, 2021 (see Table S1 for all information related to the seized samples reported in this study). One of the five pieces of paper (355 mg), selected at random, was cut into small pieces and extracted by ultrasonication for...
20 min using dichloromethane. The paper was removed, and the dichloromethane allowed to evaporate. In total, 5.8 mg of crystalline material was recovered, indicating a minimum concentration of SCRA in the paper of 1.7% w/w, or 0.17 mg/g paper assuming that not all of the crystalline material could be recovered from the beaker and that the recovery from the paper may have been less than 100%. ADB-4en-PINACA was extracted from infused paper sample FL21/0026 which consisted of three sheets of A4-size white paper with some blank ink printing. The paper cut from one of these sheets (1.448 g) was cut into small sections, avoiding any printed areas, and extracted and crystallized as described for FL21/0015. In total, 103 mg of crystalline material was recovered, indicating a minimum concentration of substance in the paper of 7.1% w/w or 0.71 mg/g paper. The recovered materials were characterized by GC-MS, UHPLC-HRAM-MS with photodiode array (PDA) detection, and NMR. We refer to these reference materials as seizure-based reference standards (>98% purity as measured by HPLC-PDA and GC-MS) because ADB-BUTINACA and ADB-4en-PINACA are chiral molecules, and their chirality in the recovered material has not been determined. These seizure-based reference standards were used to confirm the identification of ADB-BUTINACA and ADB-4en-PINACA in subsequent seized samples and facilitated rapid evaluation of their in situ detectability within prisons using ion mobility spectrometry until the synthesized (S)-enantiomer reference materials supplied by Manchester Metropolitan University became available.

The GC-MS method used to characterize the materials is as described above for seized samples. UHPLC-HRAM-MS analysis was carried out using a Thermo Scientific Vanquish UHPLC coupled to a VF-D11-A Diode Array Detector and Thermo Scientific Exemplar 120 Orbitrap. Samples were run using Thermo Scientific Excalibur software (version 4.4.16.14) and Thermo Scientific Exemplar 120 Tune Application (version 2.0.182.25). Samples were processed using Thermo Scientific Freestyle software (version 1.7.73.12). The mobile phases used were (A) LC-MS grade water with 0.01% formic acid and (B) acetonitrile with 0.01% formic acid. The gradient used was 98:2 A:B from 0.00 min to 2:08 at 7.00 min, 2:08 A:B from 7.00 to 8.05 min, and 98:2 A:B from 8.05–9.00 min. The flow rate was 0.5 ml/min, and 2 μl of sample was injected onto a BEH C18 50 × 2.1 mm, 1.7 μm particle size column (Waters Corporation, Milford, MA, USA). The orbitrap was operated in positive electrospray ionization mode (sheath gas: 60 psi, auxiliary gas flow: 15 psi; ion transfer tube: 320°C, vaporizer: 375°C; capillary voltage at 3.5 kV). Full scan analysis for the high-resolution determination of molecular mass was carried out with a resolution of 30,000 and a scan range of 80–800 Da. Data-dependent MS/MS (ddMS²) was performed with a resolution of 15,000 using 4 data dependent scans with an isolation window of 1.5 m/z in stepped collision energy mode using normalized collision energies (%) of 15, 30, and 45. NMR spectroscopy was performed using a Bruker AVANCE III HD500 MHz spectrometer (Bruker, Billerica, MA, USA) running under TopSpin v.3.2.5 and equipped with a QCI-F cryo-probe at a sample compartment temperature of 20°C. Samples were prepared in CDCl₃ (~10 mg/ml).

2.2.3 | Reference standard synthesis and analytical characterization (Manchester Metropolitan University, UK)

(S)-ADB-BUTINACA and (S)-ADB-4en-PINACA were synthesized using previously reported synthetic methods.¹⁰,²⁹ Full synthesis details and analytical characterization of the intermediates and final products are provided in Section S2.2. GC-MS analysis was performed using an Agilent 7890B GC and a 5977B mass selective detector (Agilent Technologies, Wokingham, UK). The mass spectrometer was operated in the electron ionization mode at 70 eV. Separation was achieved with a capillary column (HP-5MS, 30 m length, 0.25 mm i.d., 0.25 μm film thickness) with helium as the carrier gas at a constant flow rate of 1.2 ml/min. The following oven temperature program was used: 50 to 290°C at 30°C/min, hold for 4 min for a 12 min total runtime. A 2 μl aliquot of the samples was injected with a split ratio of 50:1. The injector and the GC interface temperatures were both maintained at 280°C, respectively. The MS source and quadrupole temperatures were set at 230°C and 150°C. Mass spectra were obtained in full scan mode (40–550 amu).¹³H NMR and ¹³C[¹H] NMR spectra (10.0 mg/1.0 ml in CDCl₃) were acquired on a JEOL JMN-EC500 (JEOL, Tokyo, Japan) NMR spectrometer operating at a proton resonance frequency of 400 MHz, referenced to the residual solvent peak (CDCl₃: ¹H NMR δ = 7.26 ppm, ¹³C[¹H] NMR δ = 77.16 ppm, respectively). Infrared spectra were obtained in the range 4,000–400 cm⁻¹ using a Thermo Scientific Nicolet iS10 ATR-FTIR instrument (Thermo Scientific, Rochester, USA).

2.2.4 | Reference standard synthesis and analytical characterization (Linköping University, Sweden)

(S)-ADB-4en-PINACA and a dihydrodiol metabolite of (S)-ADB-4en-PINACA, N-[S]-1-amino-3,3-dimethyl-1-oxobutan-2-yl]-1-(4,5-dihydroxypentyl)-1H-indole-3-carboxamide, were synthesized and characterized using NMR and liquid chromatography-mass spectrometry (LC-MS) as described in Section S2.3. HPLC-MS was performed on a Waters system equipped with 2 × 151 HPLC pump, 2,998 photodiode array detector, 2,424 ELD detector and a C18 column (XBridge, 3.5 μm, 4.6 × 50 mm), and with mobile phase: water phase A: acetonitrile: water 5:95, 10 mM NH₄OAc; organic phase B: acetonitrile: water 90:10, 10mM NH₄OAc.¹³H and ¹³C-NMR spectra were recorded on a Varian Mercury 500/125 MHz instrument using solvent as reference (CDCl₃: δH = 7.26 ppm, δC = 77.16 ppm; CD,O: δH = 3.30 ppm, δC = 49.00 ppm). A seizure-based reference standard for ADB-BUTINACA (purity 96.6% according to quantitative NMR) was kindly provided by the National Forensic Centre (NFC), Linköping, Sweden, and the certificate of analysis is provided in Section S2.4. We refer to this material as a seizure-based reference standard as the chirality of the ADB-BUTINACA was not determined but was assumed to be the (S)-enantiomer. This was later verified by the inclusion of an (S)-ADB-BUTINACA reference standard purchased from Cayman.
Chemicals in the CB1 receptor activation assay experiment and comparison of the resulting bioassay potency and efficacy data to that obtained for the reference standard provided by NFC

2.3 | Toxicological analysis and metabolite identification studies

2.3.1 | Cryopreserved pooled primary human hepatocyte (HHep) incubations

Hepatocyte incubation and metabolite identification experiments were performed according to a published procedure with slight modifications. Briefly, 5 μmol/L ADB-BUTINACA (obtained from the National Forensic Centre, Sweden) and (S)-ADB-4en-PINACA (synthesized at Linköping University, see above) were incubated with HHeps (1 x 10^5 cells in 100 μl) in a total volume of 100 μl. ADB-BUTINACA was incubated for 1, 3, and 5 h in duplicate during the fall of 2020 and (S)-ADB-4en-PINACA for 0.5, 1, and 3 h during the spring of 2021. Ice-cold acetonitrile (100 μl) was added for quenching the incubation reaction. The injection volume for LC-QTOF-MS analysis was 5 μl. Negative controls (HHeps without drug) and degradation controls (drug without HHeps) were also incubated until the end of the experiment.

2.3.2 | LC-QToF-MS analysis for metabolite identification studies

The samples were analyzed by a LC-QTOF-MS system comprising of an Agilent 1290 Infinity UHPLC system (Agilent Technologies, Kista, Sweden) with an Acquity HSS T3 column (150 mm x 2.1 mm, 1.8 μm; Waters, Sollentuna, Sweden) fitted with an Acquity VanGuard precolumn (Waters, Sollentuna, Sweden) coupled to an Agilent 6550 iFunnel QTOF mass spectrometer (Agilent Technologies, Kista, Sweden) with a Dual Agilent Jet Stream electrospray ionization source. Mobile phases (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile were used in gradient mode: 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); 1% B (18.1–19 min). The flow rate was 0.5 ml/min, and the column temperature was 60°C. Mass spectrometric data were acquired in positive mode using Auto MS/MS acquisition with the following parameters: scan range, 100–950 m/z (MS) and 50–950 m/z (MS/MS); precursor intensity threshold, 5,000 counts; precursor number per cycle, 5; fragmentor voltage, 380 V; collision energy (CE), 3 eV at 0 m/z ramped up by 8 eV per 100 m/z; 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. Data analysis was performed by Agilent MassHunter Qualitative Analysis software (version B.07.00) with the following parameters: mass error, 20 ppm; absolute peak area threshold, 20,000 counts; maximum number of matches, 20; chromatogram extraction window, 50 ppm. Criteria for metabolite identification were as follows: mass errors of less than 5 ppm for protonated molecules (with the exception of saturated peaks and small peaks where the mass accuracy is expected to deviate), 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.

2.3.3 | Toxicological samples: Case information and analysis

The urine and blood samples reported in this study originated from 13 routine forensic toxicology cases where ADB-BUTINACA was detected during routine analysis using previously published methods. The samples were obtained between March 3, 2020, and June 1, 2020. The use of case samples in studies such as this was approved by the Swedish Ethical Review Authority (Approval Numbers 2018/186-31 and 2020-07238). Of the 13 cases reported, six cases were from suspected petty drug offenses, and in two of these cases, both blood and urine samples were collected, whereas only urine was collected in the rest. Of the remaining cases, six were from prisoners who were suspected of consuming synthetic cannabinoids, and the last case was a post-mortem case where femoral blood and urine were obtained.

2.3.4 | Analysis of urine and blood samples for metabolite investigations

A total of 250 μl blood or 250 μl urine was mixed with 250 μl 1 mol/L sodium acetate buffer (pH 5) and 25 μl internal standard (Amphetamine-d8 150 ng/ml, Diazepam-d5 100 ng/ml, Mianserin-d3 50 ng/ml, and Phenobarbital-d5 2 μg/ml in acetonitrile) and 1.5 ml diethyl ether were added to the vials and mixed for 10 min at 1000 rpm. The samples were then centrifuged for 10 min at 5,000 rpm after which they were placed in –80°C for 15 min. The diethyl ether was transferred to new tubes and evaporated to dryness under nitrogen using a TurboVap LV Evaporator at 40°C. The samples were reconstituted in 100 μl methanol, and 5 μl was injected into the LC-QTOF-MS system and run using the same gradient and settings as the hepatocyte samples. Data analysis was performed using Agilent MassHunter Qualitative Analysis software (version B.07.00). In the analysis, only the formulas corresponding to the metabolites found in the hepatocyte runs for ADB-BUTINACA were investigated using the “Find by formula function” with the following parameters: mass error, 10 ppm; absolute peak area threshold, 4,000 counts. The peaks were also matched according to retention time.

2.4 | Measurement of in vitro CB1 potency

The activation of the CB1 receptor by ADB-BUTINACA (seized sample supplied by the National Forensic Centre, Sweden and the reference standard purchased from Cayman Chemicals) and ADB-4en-PINACA (synthesized at Linköping University) were performed as previously
JWH-018 and MDMB-4en-PINACA were analyzed in the same concentration (EC50) values, and efficacy was calculated using 95% confidence intervals. Differences in efficacy and potency (Log EC50 values) of the drugs were compared to that of JWH-018 using a Brown-Forsyth and Welch ANOVA with Dunnett T3 correction for multiple tests.

Prior to the dose-response assays, the cells were cultured at a concentration of 3 x 10^5 cells/ml in DMEM/Ham's F12 without phenol red supplemented with 15 mM HEPES, L-glutamine, and protease-free BSA with a final BSA concentration of 0.1%. Pre-incubation with coelenterazine (2.5 μM) on a rotating wheel at room temperature was performed for 3 h while protected from light. The dose-response assays were performed in 96-well-plates (OptiPlate-96, white opaque microplates from PerkinElmer), where each drug was analyzed in triplicate, in a eightfold serial dilution at eight different concentrations starting with 60 μM. JWH-018 and MDMB-4en-PINACA were analyzed in the same concentration range to serve as CB1 receptor agonist references. Positive controls for coelenterazine cell loading, digitonin (67 μM) and ATP (7 μM), negative controls (cells without drugs), and blank wells (wells without cells) were used. Using a TECAN Spark 10 M (Tecan, Switzerland), the receptor activation at each drug concentration was determined. The first 10 baseline readings were recorded, after which 50 μl of cells were dispensed into each well (15,000 cells/well) and luminescence was registered for an additional 190 reads (approximately 25 s). The experiments for each substance were repeated using at least three different independent experiments. Organic solvent was kept below 1% in the wells containing drugs. The area under the curve for the luminescence data from each well was calculated, and the response signals were normalized to the signal of three independent wells containing JWH-018 at 60 μM and denoted as 100% activity. GraphPad Prism version 8.3.0 for Windows (GraphPad Software, La Jolla, CA, USA) were used to determine the half maximal effective concentration (EC50) values, and efficacy was calculated using 95% confidence intervals (profile likelihood) and curve fittings (non-linear fit, three parameters). Differences in efficacy and potency (Log EC50 values) of the drugs were compared to that of JWH-018 using a Brown-Forsyth and Welch ANOVA with Dunnett T3 correction for multiple tests.

3 | RESULTS

3.1 | Human hepatocyte metabolite identification: ADB-BUTINACA

ADB-BUTINACA and its presumed metabolites were initially detected in blood and urine samples collected for routine forensic toxicology casework in Sweden. In vitro metabolite identification studies were performed to investigate ADB-BUTINACA metabolism; to identify metabolites observed in the case samples that were likely to be present due to the consumption of ADB-BUTINACA; and to identify characteristic metabolites for use in targeted analytical screening methods for future analyses, to help track prevalence of use.

ADB-BUTINACA (NFC) was incubated with HHeps and resulted in the identification of 21 phases I and II metabolites (B1–B21). The metabolites eluted between 4.08 and 9.66 min, with the parent drug eluting at 8.71 min. The biotransformation, chemical formula, retention time, exact mass of the protonated molecule, mass error (minimum and maximum), peak area, and rank based on the peak area can be found in Table 1. In Table 1, we also compare our metabolite identification data to the metabolites recently reported by Sia et al.32 following the incubation of ADB-BUTINACA with human liver microsomes (HLM). The proposed metabolic pathway for ADB-BUTINACA, based on our Hhep incubation data, is provided in Figure 1. Extracted mass chromatograms and product ion spectra for the parent compound and metabolites are shown in Section S3.1.

The observed biotransformations included dehydrogenation, dihydrodiol formation, amide hydrolysis, hydroxylation, glucuronidation, and combinations thereof (see Table 1 and Section S4). The two most abundant metabolites in the HHeps incubations were the dihydrodiol metabolite formed via biotransformation of the indazole moiety (B4, tR = 4.51 min m/z 365.2188) and a mono-hydroxylated metabolite formed via oxidation of the n-butyl chain, exact position unknown (B9; tR = 5.52 min, m/z 347.2086). Metabolite B9 was one of five observed mono-hydroxylated metabolites formed on the n-butyl tail moiety, the others being B8, B10, B12, and B14. The detection of these five metabolites was reproducible, being observed across all time course samples across the HHeps incubations (duplicate samples taken at three time points). One explanation is that the two peaks identified at 5.52 min (B9) and 5.59 min (B10, m/z 347.2079) represent a single metabolite giving rise to a split chromatographic peak due to column overloading; however, there is nothing to explain why this would happen to this peak alone and not the other chromatographic peaks present. Alternatively, when the tail moiety is hydroxylated in the two or three position, a second chiral center is formed introducing diastereomers, which could potentially be at least partially chromatographically separated.

While dihydrodiol formation (possibility via epoxide formation, followed by epoxide hydrolysis) on the benzene ring of the indazole has been reported previously in indazole-containing valinamide SCRs incubated with HHeps (e.g., ADB-FUBINACA38 THU-018 and THU-2201,39 and AB-FUBINACA40), it has been commonly reported as a relatively minor metabolite.

Two other relatively abundant metabolites featuring mono-hydroxylation on the indazole ring are reported in this study, one of which is a phase 1 metabolite (B16, ranked third in abundance, as estimated by peak area) and the other a phase II glucuronidated metabolite (B5, ranked sixth in abundance, as estimated by peak area). The
| Met # | Reported met # | Biotransformation | Chemical formula | Exact mass [M + H]^+ (m/z) | Mean RT (min) | Mass error (ppm) | Peak area (× 10^7) | Rank # |
|-------|----------------|-------------------|------------------|-----------------------------|--------------|----------------|-------------------|--------|
| B1    | M1             | Di-hydroxylation (n-butyl tail + tert-butyl) | C_{18}H_{26}N_{4}O_{4} | 363.2023 | 4.08 | -2.31 | 0.75 | 33 | 51 | 70 | 76 | 14 |
| B2    | -              | Mono-hydroxylation (tert-butyl) + Gluc | C_{24}H_{34}N_{4}O_{9} | 523.2397 | 4.53 | -1.75 | 0.46 | 102 | 15 | 11 | 20 | 7 |
| B3    | -              | Di-hydroxylation (indazole ring) + Gluc | C_{18}H_{26}N_{4}O_{4} | 359.2342 | 4.28 | -2.06 | -0.94 | 20 | 53 | 55 | 60 | 62 | 16 |
| B4    | M2             | Di-hydrodiol (indazole ring) | C_{18}H_{26}N_{4}O_{4} | 365.2188 | 4.51 | 0.23 | 5.52 | 409 | 450 | 660 | 693 | 996 | 998 | 1 |
| B5    | -              | Mono-hydroxylation (indazole ring) + Gluc | C_{24}H_{34}N_{4}O_{9} | 523.2397 | 4.53 | -1.75 | 0.46 | 102 | 11 | 11 | 20 | 7 |
| B6    | M3             | Di-hydroxylation (indazole ring + tert-butyl) | C_{18}H_{26}N_{4}O_{4} | 363.2026 | 4.99 | -1.19 | -0.45 | 6 | 11 | 11 | 21 | 21 | 21 |
| B7    | -              | Di-hydroxylation + dehydrogenation (n-butyl tail) | C_{18}H_{26}N_{4}O_{4} | 361.1869 | 5.31 | -2.15 | 0.27 | 30 | 61 | 69 | 59 | 65 | 15 |
| B8    | M5             | Mono-hydroxylation (n-butyl tail) | C_{18}H_{26}N_{4}O_{3} | 347.208 | 5.37 | -2.44 | 0.71 | 98 | 100 | 77 | 90 | 116 | 122 | 10 |
| B9    | M6             | Mono-hydroxylation (n-butyl tail) | C_{18}H_{26}N_{4}O_{3} | 347.2086 | 5.52 | 0.23 | 2.55 | 487 | 525 | 433 | 488 | 747 | 792 | 2 |
| B10   | -              | Mono-hydroxylation (n-butyl tail) | C_{18}H_{26}N_{4}O_{3} | 347.2079 | 5.59 | -1.08 | 0.48 | 251 | 215 | 103 | 108 | 150 | 128 | 9 |
| B11   | M8             | Mono-hydroxylation + dehydrogenation (n-butyl tail) | C_{18}H_{26}N_{4}O_{3} | 345.1919 | 5.95 | -0.81 | 0.8 | 114 | 105 | 74 | 94 | 235 | 241 | 6 |
| B12   | M9             | Mono-hydroxylation (n-butyl tail) | C_{18}H_{26}N_{4}O_{3} | 347.2067 | 5.97 | -2.94 | -0.23 | 34 | 32 | 30 | 24 | 56 | 55 | 18 |
| B13   | -              | Amide hydrolysis A + Mono-hydroxylation (n-butyl tail) + dehydrogenation (carboxamide) | C_{18}H_{26}N_{4}O_{3} | 346.175 | 6.12 | -3.12 | -1.01 | 6 | 23 | 33 | 34 | 54 | 56 | 19 |
| B14   | -              | Mono-hydroxylation (n-butyl tail) | C_{18}H_{26}N_{4}O_{3} | 347.2073 | 6.17 | -1.79 | -0.57 | 61 | 63 | 56 | 48 | 110 | 111 | 11 |
| B15   | -              | Amide hydrolysis A + Mono-hydroxylation (n-butyl tail) | C_{18}H_{26}N_{4}O_{3} | 348.1915 | 6.41 | -1.52 | -0.9 | 6 | 12 | 53 | 51 | 60 | 58 | 17 |
| B16   | M11/M14        | Mono-hydroxylation (indazole ring) | C_{18}H_{26}N_{4}O_{3} | 347.2085 | 6.69 | -0.61 | 1.7 | 247 | 285 | 140 | 168 | 421 | 421 | 3 |
| B17   | -              | Amide hydrolysis B (carboxamide) | C_{18}H_{26}N_{4}O_{3} | 347.2083 | 6.88 | -0.42 | 2.32 | 392 | 403 | 159 | 216 | 392 | 400 | 4 |
| B18   | -              | Mono-hydroxylation (tert-butyl) | C_{18}H_{26}N_{4}O_{3} | 345.192 | 6.97 | -1.58 | -0.36 | 51 | 50 | 46 | 56 | 108 | 113 | 12 |
| B19   | M13            | Mono-hydroxylation + dehydrogenation (tert-butyl) | C_{18}H_{26}N_{4}O_{3} | 330.1812 | 9.54 | -1.67 | 1.1 | 46 | 43 | 49 | 69 | 154 | 173 | 8 |
| B20   | -              | Amide hydrolysis A + dehydrogenation (carboxamide) | C_{18}H_{26}N_{4}O_{3} | 332.1971 | 9.66 | -0.85 | 0.91 | 145 | 137 | 206 | 248 | 261 | 268 | 5 |

Note: Metabolite numbering (Met #) refers to the numbering of chemical structures provided in Figure 2.
suggested metabolite structures are based on evaluation on LC-QTOF-MS data and previous studies of the biotransformation of similar SCRAs.\textsuperscript{33,34} Comparison of retention times of the metabolites observed in the HHeps incubation samples with those obtained from the analysis of high purity synthesized reference standards under the same conditions or NMR is required for unequivocal structural confirmation.

### 3.2 Detection of ADB-BUTINACA and its metabolites in routine toxicology casework

The results of the analysis of blood and urine samples from authentic forensic toxicology cases are provided in Table 2. The data for human urinary/blood metabolites and metabolites identified following incubation of ADB-BUTINACA with HHeps were generally in good agreement. In total, of the 21 metabolites found following incubation with HHeps, 14 were detected in case samples, including 9 of the 10 most abundant metabolites observed in the HHeps experiments. The eight metabolites found in the urine samples across the four authentic cases (three from living individuals and one post-mortem case) were all detected in the hepatocyte incubations (Table 1). In urine samples from cases 1–3, the dihydrodiol metabolite (B4) and a mono-hydroxylated metabolite (B9) were the most abundant metabolites detected (based on peak area response, which may not reflect the actual proportions of compounds present). This was also observed in the 5-h hepatocyte incubations and overall abundance in the HHeps incubations. The parent drug, ADB-BUTINACA, was not detected in any of these three urine samples. Interestingly, in the single post-mortem urine sample reported here, the mono-hydroxylated metabolite where hydroxylation occurs on the indazole moiety (B16) rather than the butyl tail, was the most abundant urinary metabolite detected, followed by the dihydrodiol metabolite (B4), with biotransformation also occurring on the indazole moiety. The parent drug, ADB-BUTINACA was detected in the single post-mortem urine sample reported here, but at a very low proportion in comparison to metabolites B16 and B4, based on peak area response. ADB-BUTINACA was detected in all blood samples included in this study, and in 10 of the 11 samples from the living individuals, it was the most abundant substance present, based on peak area response (Table 2). Several mono-hydroxylated metabolites were detected in significant proportions in blood (B8–10, B16, and B18) compared to ADB-BUTINACA, as well as metabolite B11, formed by a combination of mono-hydroxylation and dehydrogenation. In the blood sample from the single PM case reported here, the mono-hydroxylated metabolites B8 and B16 were the most abundant, based on peak area. If detected, metabolites formed via amide hydrolysis (B13, B20, and B21) were present in low relative abundance in comparison with other metabolites in the urine and blood samples reported here. The amide hydrolysis metabolites would be common to the tert-leucine methyl ester analog of ADB-BUTINACA, MDMB-BUTINACA.

#### 3.3 Human hepatocyte metabolite identification: ADB-4en-PINACA

ADB-4en-PINACA and its likely metabolites have not yet been detected in routine forensic toxicology casework in Sweden. Incubation of the (S)-ADB-4en-PINACA reference standard (synthesized at Linköping University) with HHeps for 3 h resulted in the formation of 11 phases I and II metabolites (E1–E11). The metabolites eluted between 3.42 and 10.58 min, and the parent drug eluted at 9.54 min. The biotransformation, chemical formula, retention time, exact mass of the protonated molecule, mass error (minimum and maximum), peak area for all metabolites, and rank, based on the peak area, can be found in Table 3. The proposed metabolic pathway for ADB-4en-PINACA is shown in Figure 2. Product ion spectra of ADB-4en-PINACA and its proposed metabolites are provided in Section S3.2.

The detected biotransformations included dehydrogenation, dihydrodiol formation, amide hydrolysis, hydroxylation, glucuronide formation, and combinations thereof (Table 3; Figure 2). The three major in vitro phase I metabolites based on their measured peak areas were the dihydrodiol metabolite (E3, \( t_R = 4.76 \) min, \( m/z \) 377.2184), formed by hydrolysis of the terminal alkene group of the pentenyl side chain, most likely via epoxidation, as observed previously for MDMB-4en-PINACA\textsuperscript{35}; a mono-hydroxylated metabolite (E7, \( t_R = 6.41 \) min, \( m/z \) 359.2081) with oxidation elsewhere on the pentenyl moiety, leaving the double bond intact; and an amide hydrolysis metabolite (E11, \( t_R = 10.58 \) min, \( m/z \) 344.1966) (Table 3). The structure of the major dihydrodiol metabolite (E3) was confirmed with the synthesized reference material based on a comparison of its retention time and product ion spectrum match (see Section S3.2). The amide hydrolysis product (E11) is common to the previously reported abundant ester hydrolysis metabolite of MDMB-4en-PINACA\textsuperscript{35} but is likely to be formed to a lesser extent from ADB-4en-PINACA due to the greater resistance of the amide to metabolism compared to the methyl ester moiety as observed for other similar analog pairs.\textsuperscript{38,39} Therefore, it is not considered a suitable urinary marker to demonstrate ADB-4en-PINACA consumption. Metabolites E3 and E1 are therefore suggested as the most suitable urinary markers to identify ADB-4en-PINACA consumption (especially as the identity of E3, the dihydrodiol metabolite, was confirmed using a synthesized reference material; Section S3.2). Other metabolites, which retain both the amide moiety on the headgroup and the double bond on the pentenyl chain, could also be considered as urinary markers (e.g., E4, E6, E7, E8, E9, and E10) although this cannot yet be supported by comparison to in vivo data from authentic toxicology casework.

#### 3.4 Detection and prevalence of ADB-BUTINACA and ADB-4en-PINACA in seized samples from Scottish prisons

As previously reported\textsuperscript{13–15,19} SPS and LRCFS test unattributable and non-judicial drug seizures in Scottish prisons for the presence of...
TABLE 2  ADB-BUTINACA and its metabolites detected in authentic forensic toxicology casework samples

| Met #   | Mean RT (min) | Peak area ($\times 10^3$) | Case 1 | Case 2 | Case 3 | Case 4 | Case 5 | Case 6 | Case 7 | Case 8 | Case 9 | Case 10 | Case 11 | Case 12 | PM | PM |
|---------|---------------|----------------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|-----|-----|
|         |               |                            | U      | B      | U      | B      | B      | B      | B      | B      | B      | B      | B      | B      | B   | B  |
| ADB-BUTINACA | 8.71         | -                           | 221    | -      | 15     | -      | 48     | 226    | 779    | 292    | 1761   | 128    | 405    | 974   | 520 | 86 | 18 |
| B1      | 4.08          | -                           | -      | 17     | -      | 33     | -      | -      | 17     | 17     | -      | -      | 11     | 5     | 4   | 49 |
| B4      | 4.51          | 1.151                       | 11     | 2.178  | 11     | 2.671  | -      | 13     | 40     | -      | 26     | -      | 72     | 127   | 90  | -  | 737|
| B8      | 5.37          | 47                          | 27     | 16     | -      | -      | 11     | 42     | 69     | 945    | 639    | -      | 58     | 770   | 286 | 633| 455|
| B9      | 5.52          | 334                         | 40     | 274    | -      | 317    | 5      | 26     | 52     | 140    | 207    | 27     | 256    | 534   | 354 | 65 | 323|
| B10     | 5.59          | -                           | -      | -      | -      | -      | -      | -      | 21     | 39     | 1.211  | 359    | -      | -     | -   | -  | -  |
| B11     | 5.95          | -                           | -      | -      | -      | -      | -      | -      | -      | -      | 193    | 487    | -      | 7     | 53 | 47 | -  |
| B12     | 5.97          | -                           | -      | -      | -      | -      | -      | -      | -      | -      | -      | -      | 11     | -     | -   | -  | -  |
| B13     | 6.12          | 156                         | -      | 49     | -      | 65     | -      | 5      | 25     | 15     | -      | 21     | 27     | 21    | 16  | 516|
| B14     | 6.17          | -                           | -      | -      | -      | -      | -      | -      | 6      | -      | 5      | 5      | 8      | 4     | -   | -  | -  |
| B16     | 6.69          | 119                         | 51     | 144    | 11     | 139    | 15     | 51     | 119    | 417    | 368    | 31     | 346    | 653   | 516 | 459| 2,934|
| B18     | 6.88          | -                           | 10     | -      | -      | -      | -      | 10     | 171    | 448    | -      | 50     | 99     | 43    | -   | -  | -  |
| B19     | 6.97          | -                           | -      | -      | -      | -      | -      | -      | 4      | 11     | -      | -      | 17     | 8     | 5   | 5  | -  |
| B20     | 9.54          | -                           | -      | -      | -      | -      | -      | -      | 4      | 10     | 68     | -      | 14     | 76    | 5   | 5  | -  |
| B21     | 9.66          | -                           | -      | -      | 130    | -      | -      | -      | -      | -      | -      | -      | -      | -     | -   | -  | 10 |

Note: Met # refers to metabolites described in Table 1 and Figure 2; metabolites B2, B3, B5, B6, B7, B15, and B17 were not detected in any of the case samples. All other case samples were collected ante-mortem.

Abbreviations: B, blood sample; PM, post-mortem toxicology case; U, urine sample.
| Met # | Name                                                                 | Chemical formula | Exact mass [M + H]^+ (m/z) | Mean RT (min) | Mass error (ppm) | Peak area (×10^3) | Rank # |
|-------|----------------------------------------------------------------------|------------------|----------------------------|--------------|------------------|--------------------|--------|
|       |                                                                     |                  |                            |              |                  | ½ h Rep 1 ½ h Rep 2 1 h Rep 1 1 h Rep 2 3 h Rep 1 3 h Rep 2 |        |
| ADB-4en-PINACA |                                                                     | C_{19}H_{26}N_{4}O_{2} | 343.2150                  | 9.54         | 3.17 9.19        | 11,598 19,124 13,658 14,698 5,186 8,462 |        |
| E1    | Dihydrodiol (N-pentenyl tail) + Monohydroxylation (tert-butyl)    | C_{19}H_{28}N_{4}O_{5} | 393.2126                  | 3.42         | −0.86 −2.4       | 71 54 50 48 88 43 | 11     |
| E2    | Dihydrodiol (N-pentenyl tail) + Gluc                              | C_{25}H_{36}N_{4}O_{10} | 553.2500                  | 4.05         | −0.41 −2.02      | 63 68 79 81 182 139 | 5      |
| E3    | Dihydrodiol (N-pentenyl tail)                                     | C_{19}H_{26}N_{4}O_{4} | 377.2184                  | 4.76         | 0.05 1.83        | 749 793 836 874 1,445 1,277 | 1      |
| E4    | Dihydroxylation (N-pentenyl tail, alkene intact)                  | C_{19}H_{26}N_{4}O_{4} | 375.2021                  | 5.27         | −0.59 −2.62      | 65 91 102 113 84 147 | 7      |
| E5    | Amide hydrolysis + Dihydrodiol (N-pentenyl tail)                  | C_{19}H_{27}N_{3}O_{5} | 378.2014                  | 5.60         | −0.14 −3.88      | 26 n.d. 31 27 95 67 | 9      |
| E6    | Dihydroxylation + dehydrogenation (N-pentenyl tail)              | C_{19}H_{24}N_{4}O_{4} | 373.1863                  | 5.71         | −0.19 −1.84      | 73 60 69 72 127 106 | 6      |
| E7    | Monohydroxylation (N-pentenyl tail)                               | C_{19}H_{26}N_{4}O_{3} | 359.2081                  | 6.41         | 0.47 1.31        | 1,072 1,412 1,195 1,262 1,073 994 | 2      |
| E8    | Monohydroxylation (indazole ring)                                | C_{19}H_{26}N_{4}O_{3} | 359.2079                  | 7.30         | 0 −2.32          | 119 155 246 147 190 139 | 4      |
| E9    | Monohydroxylation (tert-butyl)                                    | C_{19}H_{26}N_{4}O_{3} | 359.2077                  | 7.52         | −0.23 −4.42      | n.d. 130 n.d. 109 100 107 | 8      |
| E10   | Dehydrogenation (N-pentenyl tail)                                | C_{19}H_{24}N_{2}O_{2} | 341.1973                  | 8.97         | −0.45 −2.63      | 52 79 72 73 78 57 | 10     |
| E11   | Amide-hydrolysis                                                  | C_{19}H_{25}N_{2}O_{3} | 344.1966                  | 10.58        | 0.46 1.42        | 77 92 130 141 382 522 | 3      |

Abbreviation: nd, not detected.
SCRAs and other psychoactive substances. Samples may be seized following in situ detection in incoming mail using ion mobility spectroscopy screening\textsuperscript{13,14} or may be recovered via cell and prisoner searches. Samples have been collected from 11 of the 15 prisons in Scotland to date and testing has been carried out almost continually since June 2018. The number of detections of individual SCRAs over time, up to July 1, 2021, is shown in Figure 3. Although the number of samples seized and found to contain SCRAs varies for each quarter, the data indicate the changing availability and prevalence of individual SCRAs in Scottish prisons over time. The data have also been shown to be representative of the wider transnational SCRA market.\textsuperscript{15}

Although ADB-BUTINACA was first detected in drug seizures and forensic toxicology casework in Sweden in late 2019, it was not detected in infused papers seized in Scottish prisons until January 14, 2021. In the absence of a primary reference standard, preliminary identification of ADB-BUTINACA was initially achieved by comparison of the EI mass spectrum to available mass spectral libraries, previously published spectra,\textsuperscript{23,24} and comparison of its retention time and mass spectra to an analytically characterized seizure-based reference standard prepared as described earlier. Such seizure-based reference standards have proven useful in the context of a prison market where “new” SCRAs are detected in infused paper samples, allowing rapid confirmation of the drugs present. Their use to set up new detection algorithms for in situ screening of mail in prisons using IMS ensures that such methods remain effective. Once available, the identification of ADB-BUTINACA in seized samples was confirmed by comparison to an in-house synthesized reference standard of known purity (>98%, prepared by Manchester Metropolitan University). In a matter of weeks after its first detection in Scottish prisons, ADB-BUTINACA became one of the most prevalent SCRAs detected in seized samples submitted for laboratory analysis. Of the 332 seizures tested between the January 14, 2021 and June 30, 2021, 208 (62.7%) contained suspected infused papers and the remainder were greetings cards suspected to be infused with other controlled substances, blotters, tablets and capsules, powders, textiles, foils, e-cigarette components/ e-liquids, and herbal material. Of the 208 paper seizures, 126 (60.6%) were found to contain at least one SCRA. Of the 126 SCRA positive paper containing seizures identified between January 14, 2021 and June 30, 2021, 76 (60.3%) contained ADB-BUTINACA, 86 individual papers in total (see Table S1 for further sample and analytical details).

While ADB-BUTINACA is most commonly detected in infused papers as the only SCRA present, it has also been detected in combination with MDMB-4en-PINACA; Cumyl-4CN-BINACA (Cumyl-4CN-BUTINACA) and MDMB-4en-PINACA; 4F-MDMB-BICA, 5F-EMB-PICA and MDMB-4en-PINACA; and ADB-HEXINACA.

Although at the time of writing, ADB-4en-PINACA and its metabolites have not yet been detected in forensic toxicology samples in Sweden, ADB-4en-PINACA has been detected in infused samples seized in Scottish prisons, albeit at relatively low prevalence (see section Table S1 for sample information and analytical data). It was first detected in two visually distinct infused papers in a single seizure (sample FL21/0022) collected in December 2020. Between December 2020 and 30 June 2021, it has been detected in a further 12 seizures, with the latest seizure being from February 2021.

In the context of an increase in the prevalence of tert-leucinamide (ADB-type) indazole-3-carboxamide SCRAs as a result of recent or imminent legislative controls on tert-leucine methyl ester (MDMB-type) indole- and indazole-3-carboxamide, it is noteworthy that ADB-
HEXINACA (ADB-HINACA, N-[1-amino-3,3-dimethyl-1-oxobutan-2-yl]-1-hexyl-1H-indazole-3-carboxamide), has also been detected recently in three samples seized in Scottish prisons between April and May 2021 (see Table S1). ADB-HEXINACA was first reported in the UK in an herbal sample of “spice” sold in Manchester and analyzed on April 29, 2021. ADB-HEXINACA was detected for the first time in the United States, in an herbal sample received on April 12, 2021. Identification of ADB-HEXINACA in the samples reported in this study was confirmed by comparison of the GC-MS data (retention time and EI mass spectra) with an ADB-HEXINACA reference standard synthesized and characterized by Manchester Metropolitan University, and confirmatory analysis by UPLC-HRAM-MS (see Section S4). ADB-HEXINACA was detected in two separate infused papers (one containing ADB-HEXINACA and ADB-BUTINACA in late April 2021 and in a single seizure of more than 700 individual 5 mm² unmarked white blotter tabs intercepted in incoming mail in May 2021 (see Table S1 for further sample information and for an image of the blotters). This latter seizure represents the first time that a SCRA has been detected in blotters in Scottish prisons, most likely designed for sublingual use. Further discussion and evaluation of ADB-HEXINACA in infused paper and blotters are, however, beyond the scope of this study.

### 3.5 Evaluation of the in vitro CB2 activity of ADB-BUTINACA and ADB-4en-PINACA

The chirality of the ADB-BUTINACA seizure-based reference standard obtained from NFC was unknown, and it has been established that chirality greatly affects SCRA potency, with the (S)-enantiomer being significantly more potent than the (R)-enantiomer of all SCRs tested to date. To address this, an (S)-ADB-BUTINACA reference standard purchased from Cayman Chemicals was compared with the ADB-BUTINACA obtained from NFC using the CB2 receptor activation assay in the same experiment. The results of the comparison are provided in Section S4. In summary, there was no significant difference in potency and efficacy between the two materials, leading to the conclusion that the seizure-based reference standard from NFC comprised (S)-ADB-BUTINACA. The CB2 activity (in vitro efficacy relative to JWH-018 and in vitro potency) of (S)-ADB-BUTINACA and (S)-ADB-4en-PINACA were determined and compared with (S)-MDMB-4en-PINACA, previously one of the most prevalent SCRs in Scottish prisons (Figure 3) and in many other jurisdictions. The data are provided in Table 4, alongside previously reported in vitro potency data using other methods, and are summarized graphically in Figure 4. ADB-BUTINACA and ADB-4en-PINACA were confirmed to be potent CB2 receptor agonists with almost identical in vitro potency (mean EC50 values of 11.5 and 11.6 nM, respectively), and the data were comparable to data from other studies using different in vitro methods. They were found to be slightly less potent than MDMB-4en-PINACA (EC50 = 4.3 nM) but within the same order of 117 114

| Substance | Emax | 95% CI | P-value |
|-----------|------|--------|---------|
| (S)-MDMB-4en-PINACA | 1.17 | <0.0001 | 4.3 |
| (R)-MDMB-4en-PINACA | 1.14-1.20 | 0.003 | 0.005 |

| Substance | Emax | 95% CI | P-value |
|-----------|------|--------|---------|
| ADB-BUTINACA | 1.24 | <0.0001 | 11.6 |
| ADB-4en-PINACA | 1.22-1.28 | 0.004 | 0.008 |

| Substance | Emax | 95% CI | P-value |
|-----------|------|--------|---------|
| JWH-018 | 1.12 | <0.0001 | 38.2 |
| Ref | 1.06-1.10 | 0.012 | 1.57 |

**Note:** P-value using Brown-Forsythe and Welch ANOVA tests with JWH-018 as a reference; (i) b-recruitment complementation assay; (ii) Mini-Gaussia assay; (iii) [35S]-GTPγS receptor. As the ADB-BUTINACA standard used originated from seized material from NFC, (96.6% purity) for which the chirality present had not been definitively identified and enantiopurity had not been determined, it was compared to the ADB-BUTINACA reference standard synthesized by Manchester Metropolitan University, (98.9% purity) and confirmatory analysis by UPLC-HRAM-MS (see Section S4). The measured potency of the two substances was not significantly different (p = 0.07). The full data for the comparison are provided in Section S4. It is therefore assumed that the ADB-BUTINACA in the seized material is (S)-ADB-BUTINACA although enantiopurity has not been determined for either of the reference standards. Comparison to potency values reported in the literature using alternative in vitro bioassays is also provided.
magnitude, and more potent than the positive control compound, JWH-018 ($EC_{50} = 28.2$ nM).

4 | DISCUSSION

ADB-BUTINACA is likely to have emerged onto the illicit drug market in late 2019, when it was first detected in Sweden in a seized sample and where its possible metabolites were identified in the routine forensic toxicology casework samples reported here. ADB-BUTINACA appears to have had minimal market impact at that time until it rapidly increased in prevalence in early 2021. The identification of ADB-BUTINACA was first reported in the United States (as ADB-BINACA) on November 18, 2020, in a hand-rolled cigarette containing plant-like material seized on July 17, 2020, more than 6 months after the first detection in Europe. ADB-BUTINACA was first detected in seized infused papers in Scottish prisons in January 2021 (more than a year after the first detection in Sweden) and was one of the most prevalent SCRA compounds detected in the monitoring program by the end of 2021 Q2. ADB-4en-PINACA appears to be less prevalent, having been only detected in 14 paper samples in the drug monitoring program in Scottish prisons, in only five toxicology samples reported by NPS Discovery in the United States up to the end of 2021 Q3 and with no reported detections in seized sample data reported by the National Forensic Laboratory Information System (NFLIS) in the United States by the end of 2021 Q1 (latest data available at the time of writing). Experience has shown that SCRA compounds which emerge but have limited initial impact (for example ADB-4en-PINACA and ADB-HEXINACA) may rapidly increase in prevalence at a later date and so their prevalence should be actively monitored across multiple jurisdictions.

The compound-by-compound legislative approach adopted by the international community and unilaterally in source countries such as China has most likely driven the market to produce new SCRA compounds. It can also drive the reappearance of existing but less prevalent SCRA compounds, as the more prevalent substances come under legislative control. In 2015, the tert-leucinamide indole and indazole-3-carboxamide SCRA compounds ADB-PICA, 5F-ADB-PICA, and ADB-PINACA were controlled unilaterally by China, followed by ADB-CHMINACA and ADB-FUBINACA in 2018 and which were subsequently internationally controlled in 2019. Between 2017 and 2021, an increasing number of tert-leucine methyl ester (MDMB-type) indole- and indazole-3-carboxamide SCRA compounds, such as 5F-MDMB-PINACA (5F-ADB), 5F-MDMB-PICA (5F-MDMB-2201), 4F-MDMB-BINACA, and MDMB-4en-PINACA (5Cl-ADB-A) appeared on the illicit market and were rapidly unilaterally and/or internationally controlled or are in the process of being controlled. The market has often responded by introducing (or re-introducing) SCRA compounds with similar structures, synthetic routes, production costs, and potency, but producers may have adopted the approach of releasing fewer compounds at any one time to protect others from compound-by-compound control.

MDMB-4en-PINACA, ADB-4en-PINACA, and ADB-BUTINACA were all shown to have similar high potency in agreement with previously published data. Variability in the user experience and the harms related to the use of SCRA compounds may not be affected by the different substances present but is more likely to be due to the unpredictable dose consumed, the mode of use, the impact of polydrug use, and personal factors related to the health of the person using the SCRA(s). As demonstrated by this and many other studies, tert-leucinamide (ADB-type) indole-3-carboxamide SCRA compounds have similar potency to their tert-leucine methyl ester (MDMB-type) analogs and so their appearance on the SCRA market is not unexpected. Recently, the Chinese government announced far-reaching analog controls on SCRA compounds, covering most of the most potent and currently most prevalent substances, and the market response is yet to be seen. New SCRA compounds purporting to circumvent the new legislation are already apparently being advertised on vendor sites and are being discussed on drug user forums, and at least some are expected...
to have significantly lower CB1-related potency. With the increasing prevalence of ADB-BUTINACA and the recent appearance of ADB-4en-PINACA in drug seizures, it is important that forensic toxicology laboratories include these two SCRAs and their characteristic metabolites in targeted and semi-targeted test methods.

In this study, we found a reasonable correlation between the metabolites detected in the HHeps incubations and the metabolites detected in authentic blood and urine samples. Sai et al. reported 40 phase I metabolites following incubation of ADB-BUTINACA with HLM, with 15 metabolites reported in the study representing more than 94% of the chromatographic peak area. We estimate that 11 of these 15 metabolites have also been identified in this study. It is notable that there is a significant difference in the relative proportions of some metabolites (calculated using peak area) between the studies. Most significantly, we report that the dihydrodiol metabolite (B4), where biotransformation occurs on the indazole ring most likely via formation of an epoxide intermediate, is the most prevalent ADB-BUTINACA metabolite in the 3- and 5-h HHep incubation samples. This is in contrast to the study of Sai et al. using HLM, where the dihydrodiol was reported as a very minor metabolite (M2, 0.8% of total metabolite peak area) following incubation with HLM for 2 hours. This highlights an important difference between the two in vitro metabolism platforms for this SCRA, their use in metabolite identification studies, and their application to forensic toxicology casework.

A mono-hydroxylated metabolite, with biotransformation occurring on the n-butyl tail (M6), was the most abundant metabolite in the HLM-based study and was ranked as the second most abundant in vitro metabolite in this HHeps-based study (B9).

In the HHLM-based study, two phase I mono-hydroxylated metabolites, where biotransformation occurs on the indazole ring, were reported (M11 and M14). In our HHeps-based study, we report one phase I (B16, mono-hydroxylation at the indazole ring) and one phase II metabolite (B5, mono-hydroxylation at the indazole ring and subsequent glucuronidation). Additionally, a number of other amide hydrolysis products were noted in the current study (B13, B15) including hydrolysis of the amide in the carboxamide linker (B17) which were not reported in the HLM-based study.

For the detection of ADB-BUTINACA consumption, based on the available in vitro data and data from the analysis of authentic blood and urine samples reported in this study, we recommend the use of the parent drug and the mono-hydroxylated metabolites B9 and B16 (m/z 347.2087) as biomarkers of ADB-BUTINACA consumption in blood. The dihydrodiol metabolite B4 (m/z 365.2188), where hydroxylation has occurred on the indazole moiety, was detected in the highest abundance in urine samples collected from the living (n = 3) and was the second most abundant in the single post-mortem urine sample and is therefore recommended as a urinary biomarker, although to the best of the authors knowledge, this metabolite is not yet commercially available. Interestingly, despite being detected as the most abundant metabolite (M2) in four of the seven urine samples analyzed, this metabolite was not recommended for use as a urinary biomarker by Sai et al. It was not detected in the remaining three urine samples reported, a potential indication of differences in metabolic enzyme expression between individuals. In addition to metabolite B4, we recommend the mono-hydroxylated metabolites B8 and B9 (mono-hydroxylation on the n-butyl tail) and B16 (mono-hydroxylation on the benzene ring of the indazole) as urinary biomarkers (m/z 347.2087). A reference material for the n-butanoic acid metabolite of ADB-BUTINACA is commercially available; however, in this study, the potential corresponding metabolite (B7) was present only as a minor metabolite in the HHep incubations and was not detected in any of the authentic blood or urine samples. Sai et al. did not detect the n-butanoic acid metabolite of ADB-BUTINACA following incubation with HLM but did detect it at low abundance compared to other metabolites in three out of seven of the authentic urine samples tested. We therefore do not recommend the n-butanoic acid metabolite of ADB-BUTINACA as a urinary biomarker.

Cryopreserved pooled primary human hepatocytes are often considered to be the “gold standard” for in vitro NPS metabolism studies. They are intact cells with the full range phase I and II metabolism enzymes, cofactors, binding proteins, and transporters. Although considerably more expensive than other available in vitro metabolism platforms such as HLM, HepaRG cells, s9 fraction, zebrafish and the fungus c. elegans, they have been shown to more closely mimic in vivo metabolism, a view supported by this study in the case of ADB-BUTINACA metabolism.

To identify ADB-4en-PINACA consumption, we suggest the use of the dihydrodiol metabolite E3 (m/z 377.2184) and E7 (m/z 359.2081) with mono-hydroxylation on the n-pentenyl chain, as suitable urinary markers. Although these metabolites have been identified by in vitro metabolism study using HHeps, the findings cannot, yet, be supported by comparison to in vivo data from authentic toxicology casework. Other in vitro metabolites reported in this study may, therefore, also be suitable urinary biomarkers of ADB-4en-PINACA consumption (Table 3). In line with other similar SCRAs, the parent compound is likely to be a biomarker of consumption in blood, but again, this is not currently supported by in vivo data. At the time of writing, there have been no published studies on the in vivo or in vitro metabolism of ADB-HEXINACA, and the detection of this drug or its metabolites in toxicological samples has not yet been reported, but such research is recommended.

## Conclusion

We have demonstrated that the SCRA market continues to evolve: ADB-BUTINACA is currently increasing in prevalence and other tert-leucinamide compounds such as ADB-4en-PINACA and ADB-HEXINACA have recently emerged on the SCRA market but are currently less prevalent. In addition to adding characteristic ions for ADB-BUTINACA, ADB-4en-PINACA, and their metabolites to targeted and semi-targeted analytical methods, clinical and forensic toxicologists are advised to continue to monitor early warning system data and seizure data related to populations where SCRA use is prevalent, for example, prison drug seizure testing programs. Drug analysts and toxicologists should remain vigilant to the emergence of other
new tert-leucinamide indole and indazole-3-carboxamide SCRAs and should be aware of the likelihood of the emergence of new substances sold as SCRAs with alternative scaffolds specifically designed to evade the SCRA analog controls recently introduced by the Chinese government.

In vitro biotransformation and pharmacokinetics studies should, where possible, be pre-emptively performed to identify the most suitable urinary markers for emerging or prophetic compounds, which should be used to monitor appearance and any increase in prevalence on the illicit market. In this way, market changes can be identified quickly, and the relative harms assessed and laboratory-based and in situ detection methods, particularly in prisons, can be kept up to date and fit for purpose.

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CONFICT OF INTEREST
The authors do not report any conflict of interest.

DATA AVAILABILITY STATEMENT
The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher’s website.

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