Journal of Chromatography B 1083 (2018) 189–197

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Metabolism of the synthetic cannabinoids AMB-CHMICA and 5C-AKB48 in pooled human hepatocytes and rat hepatocytes analyzed by UHPLC-(IMS)-HR-MS^E

Marie Mardal[a,*], Petur Weihe Dalsgaard[a], Bing Qi[b], Christian Brinch Mollerup[a], Pieter Annaert[b], Kristian Linnet[a]

[a] Section of Forensic Chemistry, University of Copenhagen, Department of Forensic Medicines, Frederik V's vej 11, 2100 København Ø, Denmark
[b] Drug delivery and Disposition, KU Leuven, Department of Pharmaceutical and Pharmacological Sciences, O&N2, Herestraat 49, 3000 Leuven, Belgium

A B S T R A C T

The main analytical targets of synthetic cannabinoids are often metabolites. With the high number of new psychoactive substances entering the market, suitable workflows are needed for analytical target identification in biological samples. The aims of this study were to identify the main metabolites of the synthetic cannabinoids, AMB-CHMICA and 5C-AKB48, using an *in silico*-assisted workflow with analytical data acquired using ultra-high-performance liquid chromatography–(ion mobility spectroscopy)–high resolution–mass spectrometry in data-independent acquisition mode (UHPLC-(IMS)-HR-MS^E). The metabolites were identified after incubation with rat and pooled human hepatocytes using UHPLC-HR-MS^E, followed by UHPLC-IMS-HR-MS^E. Metabolites of AMB-CHMICA and 5C-AKB48 were predicted with Meteor (Lhasa Ltd) and imported to the UNIFI software (Waters). The predicted metabolites were assigned to analytical components supported by the UNIFI *in silico* fragmentation tool. The main metabolic pathway of AMB-CHMICA was *O*-demethylation and hydroxylation of the methylhexyl moiety. For 5C-AKB48, the main metabolic pathways were hydroxylation(s) of the adamantyl moiety and oxidative dechlorination with subsequent oxidation to the ω-COOH. The matrix components in the metabolite spectra were reduced with IMS, which improved the accuracy of the spectral interpretation; however, this left fewer fragment ions for assigning sites of metabolism. Meteor was able to predict the majority of the metabolites, with the most notable exception being the oxidative dechlorination and, consequently, all metabolites that underwent that transformation pathway. Oxidative dechlorination of ω-chloroalkanes in humans has not been previously reported in the literature. The postulated metabolites can be used for screening of biological samples, with four-dimensional identification based on retention time, collision cross section, precursor ion, and fragment ions.

1. Introduction

Synthetic cannabinoids are members of a group of new psychoactive substances (NPSs) that mimic the effect of tetrahydrocannabinol (THC) through agonistic effects on the cannabinoid receptors. The synthetic cannabinoids have a low metabolic stability, so their main analytical targets in biological samples are often metabolites [1,2]. A new trend for the synthesis of synthetic cannabinoids is the incorporation of heavier halogens onto the molecule. Substitution of the ω-carbon of JWH-018 with chloride or bromide results in a compound with similar affinity to cannabinoid receptors to that seen with JWH-018, in the low nanomolar range [3]. For example, *N*-(1-adamantyl)-1-(5-chloropentyl)-1H-indazole-3-carboxamide (5C-AKB48) is a ω-chlorinated derivative of AKB48 and 5F-AKB48 that has been detected in herbal smoking mixtures [4]. Methyl 2-{[1-(cyclohexylmethyl)-1H-indol-3-yl] formamido}-3-methylbutanoate (AMB-CHMICA) is the valinate-analogue of the synthetic cannabinoid MDMB-CHMICA, which has been associated with several drug intoxications [5]. The EC_{50} values of AMB-CHMICA for the CB_1 and CB_2 receptors were measured at 3.5 and 12 nM, respectively, making this compound a potent synthetic

Abbreviations: 5C-AKB48, *N*-(1-adamantyl)-1-(5-chloropentyl)-1H-indazole-3-carboxamide; AMB-CHMICA, methyl 2-{[1-(cyclohexylmethyl)-1H-indol-3-yl]formamido}-3-methylbutanoate; CCS, collision cross section; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid; IMS, ion mobility spectrometry; IS, internal standard (5F-APP-PICA); KHB, Krebbs-Hanseleit buffer; NPS, new psychoactive substance; pHH, pooled human hepatocytes; THC, tetrahydrocannabinol; TWIMS-QTOF, travelling wave IMS – quadrupole time-of-flight; UHPLC-HR-MS, ultra-high performance liquid chromatography–high resolution mass spectrometry

* Corresponding author.
E-mail address: marie.mardal@sund.ku.dk (M. Mardal).

https://doi.org/10.1016/j.jchromb.2018.03.016
Received 29 December 2017; Received in revised form 19 February 2018; Accepted 9 March 2018
Available online 10 March 2018

cannabinoid [6]. Metabolites of synthetic cannabinoids also have affinity for cannabinoid receptors [7], indicating that the main metabolites of emerging synthetic cannabinoids should be included in biological risk assessments. For these reasons, metabolites should be included in systematic toxicological screenings of biological samples, and even if they have no biological activity, they can aid in determination of the parent drug. One metabolite profiling method is incubation of NPSs with primary hepatocyte suspensions [8–11], as good agreement exists between the *in vivo* and *in vitro* analytical targets. Primary hepatocytes contain the metabolic enzymes as well as the necessary metabolic cofactors used for the evaluation of hepatic metabolism.

Ultra-high performance liquid chromatography–high resolution mass spectrometry (UHPLC-HR-MS) is a well-established technique for sensitive and comprehensive toxicological screening of biological samples for substance abuse [12–14]. The sensitivity of commercial UHPLC-HR-MS instruments allows the detection of low-abundance metabolites and the detection of metabolites with a wide range of physiochemical properties. Another useful technique is ion mobility spectrometry (IMS) with travelling wave IMS, which allows the separation of ions based on the collision cross section (CCS) and can be experimentally determined by the drift time of a molecule in a gas phase in the presence of an electric field. Ion mobility is largely independent of chromatography and matrix, making CCS-libraries suitable for sharing among laboratories [15] and for prediction of values [16]. The combination of IMS with UHPLC and HR-MS allows for an orthogonal separation of analytes between the ionization source and mass analyzer. This additional separation prior to MS analysis can further separate matrix components from analytes of interest, while it adds an additional identification parameter when screening biological samples to help filter out false positive results. The power of UHPLC-IMS-HR-MS^E for transformation product characterization, as well as targeted screening in complex matrices, was recently tested for the feed additive ethoxyquin in fish feed [17].

Prediction software can reduce the workload of these types of metabolite profiling studies and can help filter out false-positives in targeted, suspect, and non-targeted screening approaches [18,19]. Target identification for the screening of biological samples for suspected NPS use has been achieved with smartCYP [20], which predicts cytochrome *p*450-mediated metabolism, and MetaSite [11], Metaprint2D [21], or Nexus Meteor [19,22], which predicts both phase I and phase II metabolites. Retention times and CCS predictions are achieved by artificial neural networks [16,23], which provide excellent reduction of false positives when screening complex samples against a large library. However, these are barely able to distinguish small molecule isomers in routine workflows. The collision-induced dissociation of drugs follows a number of rules, which allows the prediction of fragment ions in UHPLC-(IMS)-HR-MS. The most feasible analytical target can be selected from mass spectral information using the *in-silico* fragmentation offered by external programs such as ACD/MS fragmenter [18], and this feature has become embedded in recent years in HR-MS data analysis software, such as UNIFI (Waters) or Compound Discoverer (Thermo Scientific) [24]. Given the large number of NPSs entering the drug market and the need for analytical target identification, laboratories require workflows that are suitable for even higher throughput than is presently available for metabolite identification. Incorporation of reasonable *in-silico*–assisted workflows can increase the output of metabolite identification workflows.

The aims of this study were to identify the main metabolites of AMB-CHMICA and 5C-AKB48 after incubation with pooled human hepatocytes and rat hepatocytes, and to apply an *in-silico*–assisted workflow to acquired UHPLC-(IMS)-HR-MS^E data. The acquired data were further used to assess the potential added benefits of IMS and *in-silico* tools for metabolite identification workflows.

2. Materials and methods

2.1. Chemicals and reagents

AMB-CHMICA, 5C-AKB48, and 5F-APP-PICA (internal standard, IS) were kindly provided and purity tested by the Slovenian National Forensic Laboratory. Diclofenac, Percoll, ammonium formate, dexamethasone, and leucine enkephalin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypan blue solution (0.4%), fetal bovine serum, *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (HEPES), penicillin-streptomycin (10,000 IU/mL), L-glutamine, Dulbecco's modified Eagle's medium, William's E medium, and sodium pyruvic acid were purchased from Lonza Westburg BV (Leusden, the Netherlands). Sodium chloride, potassium chloride, calcium chloride, magnesium chloride, sodium bicarbonate, and glucose were purchased from VWR (Darmstadt, Germany). Methanol, acetonitrile, ultrapure water, PBS × 10, and formic acid (LC-MS grade) were obtained from Fisher Scientific (Leicestershire, UK). The CCS major mix IMS/Tof calibration kit for the CCS and MS calibration was purchased from Waters (Manchester, UK).

The pooled human hepatocytes (pHH) were kindly provided by KaLy-Cell (Plobsheim, France), and stored in liquid nitrogen until use.

2.2. Isolation of rat hepatocytes

Rat hepatocytes were isolated from a 196 g Wistar rat by following a two-step perfusion protocol, as previously reported [25]. After the isolation, the hepatocytes were centrifuged for 3 min at 50*g* at 4 °C, and aspirated. The resulting hepatocytes were resuspended in a first seeding medium consisting of Williams E medium with 100 IU/mL penicillin, 5% fetal bovine serum, 100 µg/mL streptomycin, 4 mg/L insulin, 2 mM L-glutamine, and 1 µM dexamethasone. The cell viability was determined as 96% using the trypan blue exclusion method.

2.3. Metabolite profiling of AMB-CHMICA and 5C-AKB48

2.3.1. Preparation of Krebs-Hanseleit buffer

Krebs-Hanseleit buffer (KHB) for metabolite profiling was prepared as follows: a mixture containing 118 mM NaCl, 5 mM KCl, 1.6 mM $CaCl_2$, 2.5 mM $MgCl_2$,12 mM $NaHCO_3$, 5 mM glucose, 13 mM HEPES, and 5 mM sodium pyruvate was prepared with MilliQ water. This solution was then sparged with 95% CO_2/5% O_2 for 5 min, and the pH was adjusted to 7.4 with NaOH.

2.3.2. Rat hepatocyte suspension

The suspension of rat hepatocytes was diluted in KHB to 1×10^6 hepatocytes/mL. Methanolic AMB-CHMICA, 5C-AKB48, or diclofenac (positive control) was added to a final concentration of 10 µM; the maximum organic content of the incubation was 0.3%. The incubations were performed in quadruplicate for each condition in 24-well plates, at 300 rpm and 37 °C, on a Thermostar shaker (BMG Labtech, Offenburg, Germany). Controls without the addition of hepatocytes were run simultaneously to correct for hydrolysis products and artifacts. Aliquots of 50 µL were collected after 0, 1, and 3 h of incubation and mixed by aspiration with 50 µL of an ice-cold methanolic solution of IS (100 ng/mL) and frozen until analysis [8].

2.3.3. Pooled human hepatocytes

Cryopreserved pooled human hepatocytes were rapidly thawed in a 37 °C water bath and mixed with 50 mL Percoll®: PBS (×10):thawing medium (10% v/v fetal bovine serum, 1 µM dexamethasone, 4 µg/mL insulin, 100 IU/mL penicillin, 2 mM L-glutamine, 100 µg/mL streptomycin diluted in Dulbecco's modified Eagle's medium) (28.8:3.2:68% v/v/v) and centrifuged at 168 *g* for 20 min to remove dead hepatocytes. After aspiration of the supernatant, the hepatocytes were cleaned by resuspending the pellet in 20 mL thawing medium, centrifuging for