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Article

Identification of AKB-48 and 5F-AKB-48 Metabolites in Authentic Human Urine Samples Using Human Liver Microsomes and Time of Flight Mass Spectrometry

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The occurrence of structurally related synthetic cannabinoids makes the identification of unique markers of drug intake particularly challenging. The aim of this study was to identify unique and abundant metabolites of AKB-48 and 5F-AKB-48 for toxicological screening in urine. Investigations of authentic urine samples from forensic cases in combination with human liver microsome (HLM) experiments were used for identification of metabolites. HLM incubations of AKB-48 and 5F-AKB-48 along with 35 urine samples from authentic cases were analyzed with liquid chromatography quadrupole tandem time of flight mass spectrometry. Using HLMs 41 metabolites of AKB-48 and 37 metabolites of 5F-AKB-48 were identified, principally represented by hydroxylation but also ketone formation and dealkylation. Monohydroxylated metabolites were replaced by di- and trihydroxylated metabolites within 30 min. The metabolites from the HLM incubations accounted for on average 84% (range, 67–100) and 91% (range, 71–100) of the combined area in the case samples for AKB-48 and 5F-AKB-48, respectively. While defluorinated metabolites accounted for on average 74% of the combined area after a 5F-AKB-48 intake only a few identified metabolites were shared between AKB-48 and 5F-AKB-48, illustrating the need for a systematic approach to identify unique metabolites. HLMs in combination with case samples seem suitable for this purpose.

Introduction

Synthetic cannabinoids like tetrahydrocannabinol (THC), the active ingredient in marijuana and hashish, are psychoactive drugs showing affinity for the CB1 and CB2 receptors. They were first marketed as herbal products under the name 'Spice' and were discovered on the European market in 2008. Present, at least 200 different analogs have been identified (1).

As a consequence of frequent scheduling of synthetic cannabinoids, rapid introduction of novel analogs has been observed. A recent trend is the introduction of halogenated analogs (2) such as 5F-AKB48, 5F-PB22, AM2201, XLR-11 and THJ-2201 which are fluorinated counterparts of AKB48, PB22, JWH-018, UR-144 and THJ-018, respectively.

Synthetic cannabinoids are normally screened in urine or blood using immunoassays or chromatography combined with mass spectrometry. Urine is an important matrix as it is non-invasive and can be assumed to provide the longest window of detection after a suspected intake. This was shown for AM-2201 by Hutter *et al.* after self-administration (3) but further studies are needed before this can be accepted as a general statement. Parent cannabinoids have rarely been detected in urine and if so only at low levels (4–13) so confirmation of drug intake relies on the detection of drug metabolites.

Metabolites of halogenated analogs pose a particular challenge in the detection of a suspected intake as the halogen atom, being an excellent leaving group, might be substituted during liver metabolism. This loss produces metabolites identical to those that could be expected after an intake of the non-halogenated analog. This phenomenon was illustrated by Wohlfarth *et al.* (14) comparing the metabolites from PB22 to those of 5F-PB22 and Sobolevsky *et al* comparing the metabolites from APICA and 5F-APICA (STS-135) (15). When compounds are scheduled individually, like in Sweden, it is necessary to distinguish between intakes of different analogs. Therefore, it is important to verify that metabolites used as markers of intake are unique to the synthetic cannabinoid analog of interest.

Information needed to identify unique metabolites as potential markers for a specific synthetic cannabinoid intake can be obtained by incubating the drug with human liver microsomes (HLMs) (4, 15–22). HLMs are vesicle-like structures formed by pieces of endoplasmic reticulum from broken up hepatocytes, usually from multiple donors, and contain functional liver enzymes such as the cytochrome P450 (CYP) enzymes and uridine 5′-diphospho-glucuronosyltransferase (UGT). HLMs are frequently used in the pharmaceutical industry to study the metabolism of drug candidates as well as potential drug–drug interactions. Another approach is to use freshly frozen human hepatocytes (14, 23–27). The hepatocytes possess a more complete metabolic system compared with HLMs and therefore their metabolism should be more representative of the metabolism *in vivo*. However, as the preparations of hepatocytes used are pooled from a smaller number of individuals, such as three (24, 26, 27) or ten (14), compared with HLMs (in this study 150 donors of mixed gender), the effects of specific enzyme variants might be overlooked.

Data from HLM or hepatocyte incubations in combination with large data sets of authentic cases are necessary for identification of target metabolites for a suspected drug intake because controlled pharmacokinetic studies are usually absent.

The *in vitro* metabolism of a number of synthetic cannabinoids have been described, including AB-FUBINACA (20), AB-PINACA (20), ADB-FUBINACA (20), AB-001 (11), AKB-48 (25), 5F-AKB-48 (16), AM-2201 (4, 21), AM-694 (10), APICA (15), CP 47,497 (18), HU-210 (19), JWH-018 (9, 12, 13, 21, 22), JWH-073 (9, 12, 22), JWH-081 (12), JWH-122 (12), JWH-200 (17), JWH-201 (6), JWH-210 (12), JWH-250 (12, 28), JWH-251 (6), PB-22 (14, 20), 5F-PB-22 (14, 20), RCS-4 (8, 12, 24), RCS-8 (26), STS-135 (15, 23), UR-144 (4, 29) and XLR-11 (27) but only a limited number of reports describing metabolites identified in authentic cases were found (4, 6, 8–12, 16, 21, 28, 29). Identified metabolites included classic CYP modifications such

as hydroxylations, oxidations, dehydrogenation and dihydrodiol formation but also dealkylation at nitrogen and oxygen atoms (4, 8, 9, 11, 13, 15–17, 24, 26, 28, 29), ring formation (27), ring cleavage (17) and sulfation (24). The metabolites were mainly present as glucuronides (4, 5, 8, 21, 30). For most synthetic cannabinoids, neither the metabolism nor any metabolites present in urine have yet been described.

The synthetic cannabinoid in focus of this article is AKB-48 (aka APINACA) and its halogenated analog 5F-AKB-48 (aka 5F-APINACA). The earliest reports of AKB-48 on the illegal market are from seizures in the USA in 2010 (25) but use of AKB-48 and 5F-AKB-48 have also been reported in Europe (31) and Asia (32).

The metabolism of AKB-48 was investigated by Gandhi *et al.* (25) using hepatocytes. They report mono-, di- and trihydroxylated metabolites with hydroxylations on the adamantyl group and the pentyl side chain as the major metabolites but also report glucuronidated metabolites and ketone formation. The metabolism of 5F-AKB-48 was studied by Holm *et al.* (16) using HLMs as well as analyzing one single authentic urine sample. They report metabolites similar to those of Gandhi *et al.* (25), with or without defluorination after HLM incubation. Additionally, they also report dealkylation of the pentyl chain as a route of metabolism. In the urine sample, the major metabolite was adamantyl-pentyl-dihydroxylated with defluorination and pentyl-oxidized, possibly as a carboxylic acid.

The aim of this study was to generate metabolites from AKB-48 and 5F-AKB-48 using HLMs and to identify unique and abundant markers of intake by retrospective data mining in case samples.

Experimental

All metabolites and internal standards were purchased as 1.0 mg of powder or in solution from Cayman Chemicals (LGC Standards, Boras, Sweden). Ultrapool 150 HLMs, NADPH regenerating system and UGT assay mix were obtained from Corning (Amsterdam, The Netherlands).

Optima-grade acetonitrile, formic acid, methanol, water and 2-propanol for the liquid chromatography quadrupole tandem time of flight mass spectrometry (LC–QTOF-MS) mobile phases and wash solutions were purchased from Fisher Scientific (Gothenburg, Sweden). Acetonitrile gradient grade used in the sample preparation was purchased from Merck (VWR, Stockholm, Sweden). Ammonium formate and ammonium acetate were purchased from Sigma–Aldrich (Stockholm, Sweden). β-Glucuronidase (*E. coli*) was purchased from Roche (Mannheim, Germany). High-purity water used in the sample preparation was produced in-house in a MilliQ Gradient production unit from Millipore (Billerica, MA, USA).

LC–QTOF-MS analysis

In this study, a previously described confirmation LC–QTOF-MS assay measuring 38 metabolites from 15 parent drugs (33), with slight modification, was used to identify the metabolites from AKB48 and 5F-AKB48. Since publication, four new reference substances have been added to the personal composed database library (PCDL), AKB48 *N*-(4-hydroxypentyl), AKB48 *N*-(5-hydroxypentyl), AKB48 *N*-pentanoic acid and 5F-AKB48-(4-hydroxypentyl).

Six-hundred microliters of the urine samples were incubated with 30 μL of β-glucuronidase solution (140 U/mL in 50% glycerol) for 20 min at ambient temperature. The samples were then extracted using salting-out liquid–liquid extraction (SALLE). Then, 400 μL of acetonitrile containing the internal standards were added followed by 200 μL of 10 M ammonium acetate. After centrifugation 100 μL of the acetonitrile phase was transferred to vials for LC–QTOF-MS analysis.

LC–QTOF-MS analysis was performed on an Agilent 6540 quadrupole tandem time-of-flight mass spectrometer in combination with an Agilent 1290 Infinity LC instrument (both from Agilent, Kista, Sweden). Mobile phase A consisted of 0.05% formic acid in 10 mM ammonium formate and mobile phase B of 0.05% formic acid in methanol. Gradient elution was used (2% B was held for 0.5 min followed by an immediate change to 50% B. A linear gradient to 70% B for 11.5 min was followed by a wash with 100% B for 1.5 min and re-equilibration at 2% B for 1.5 min for a total run time of 15 min) at a flow rate of 0.6 mL/min on an ACE Excel 2 C18-AR (100 × 2.0 mm, 2 μm) maintained at 60°C. High resolution mass spectrometry (MS) data (m/z range 50–1,000, 2 spectra/s) was used to measure peak areas and to trigger data-dependent acquisition of tandem mass spectrometry (MS–MS) product ion spectra (m/z range 50–500, 3 spectra/s, 20 V collision energy).

Incubations with HLMs

AKB-48 or 5F-AKB-48 (1 μg/mL) were incubated with HLMs (500 μg/mL) in the presence of co-factors, buffers and reagents necessary to activate both the CYP and UGT enzymes including Tris–HCl (50 mM, pH 7.5), NADP+ (1.3 mM), glucose-6-phosphate (3.3 mM), glucose-6-dehyrdogenase (0.4 units/mL), MgCl (11.3 mM), alamethicin (25 μg/mL) and uridine 5′-diphospho-glucuronic acid (2 mM).

Samples were removed from the incubation mixture immediately after mixing (<2 min) and after 15, 30 and 60 min, respectively. The reaction was stopped by the addition of 0.8 parts of acetonitrile containing the internal standards and 0.5 parts 10 M ammonium acetate. After vortex mixing and centrifugation (17530 RCF, 5 min, 4°C), the acetonitrile phase was injected on the LC–QTOF-MS.

Case samples

A total number of 35 authentic cases from screening for drugs of abuse using the LC–QTOF-MS method described above (33) previously found positive for one or more of AKB48 *N*-(4-hydroxypentyl), AKB48 *N*-(5-hydroxypentyl), AKB48 *N*-pentanoic acid and 5F-AKB48-(4-hydroxypentyl) at a cutoff of 2 ng/mL were selected for this study. The identifications were made by matching retention time accurate mass and MS–MS spectra to those of the reference compounds AKB48 *N*-(4-hydroxypentyl) and AKB48 *N*-(5-hydroxypentyl). The raw data files were reprocessed for identification of AKB-48 and 5F-AKB-48 metabolites.

Data analysis

Based on knowledge from earlier publications (7, 11) on AKB-48 and 5F-AKB-48 metabolism, a list of targets (sum formulas) were prepared representing hydroxylations, oxidations to form keto-groups, glucuronidation, N-dealkylations and combinations of the above. Mass chromatograms were extracted from all HLM

incubations and the selected case samples for the targets and peaks were identified. If available, product ion spectra were analyzed to confirm identity.

In the analysis of LC–QTOF-MS data, thresholds for mass error and peak area were used for identification. For identification as a potential metabolite a mass error <5 ppm of the expected mass and a peak area of at least 100 000 arbitrary units (AU) in authentic urine samples and at least 30 000 AU after HLM incubation were needed. After the initial data analysis, peaks identified in only one sample and peaks with a total area in all authentic urine samples combined less than 1 000 000 AU (100 000 AU after HLM incubations) were removed.

The authentic urine samples were divided into three groups (AKB-48 only, 5F-AKB-48 only and both) based on the presence of the metabolites from AKB-48 and/or 5F-AKB-48 identified in the HLM incubations.

Results

The metabolites of AKB-48 and 5F-AKB-48 in HLMs and authentic case samples were characterized using high sensitivity LC–QTOF-MS measurements in positive ionization mode.

Characterization of metabolism in HLM

The retention times and peak areas of the metabolites found after HLM incubation (<2–60 min) are presented in Table I. For AKB-48 and 5F-AKB-48, 41 and 37 metabolites were identified, respectively. The metabolites of AKB-48 included mono-, di- and trihydroxylations, alone or in combination with keto-groups or N-dealkylations. Most abundant were two di-OH metabolites (one adamantyl-pentyl-hydroxylated metabolite and one diadamantyl-hydroxylated metabolite) at 4.09 and 8.34 min, respectively, as well as one tri-OH metabolite (diadamantyl-pentyl-hydroxylated) at 3.00 min. The major metabolites of 5F-AKB-48 were a 5F-mono-OH metabolite (adamantyl-hydroxylated) at 7.38 min and a 5F-di-OH metabolite (diadamantyl-hydroxylated) at 5.46 min, which represent more than 74% of the combined area. For both substances monohydroxylated metabolites were most abundant at 15 min, while di- and trihydroxylated metabolites were more abundant at later time points. The distribution between mono-, di- and trihydroxylations over time is shown in Figure 1.

Besides hydroxylation the most common modification was oxidation possibly generating keto-groups, aldehydes and/or carboxylic acids. A few N-dealkylations were also observed but only one glucuronide metabolite at low intensity from AKB-48 was identified.

Out of 35 metabolites of 5F-AKB-48 with the pentyl side chain (no N-dealkylation), 31% lacked the fluoride atom making up ~9% of the combined area. Interestingly a majority of the defluorinated metabolites (7 out of 11) were unique for 5F-AKB-48 and were not produced when AKB-48 was the substrate.

Identification of metabolites in authentic urine samples

The metabolites found in the case samples are shown in Table II and Supplementary Table SI. Based on metabolite patterns observed in the authentic urine samples and HLM incubations of AKB-48 and 5F-AKB-48, samples were divided into three groups.

In ten cases where the metabolite pattern indicated a pure AKB-48 intake a total of 25 probable metabolites were detected, chromatograms are provided in Figure 2. Of these, 18 metabolites (72%) were identified in the HLM incubations with AKB-48, representing on average 84% (range, 67–100%) of the combined area. In 21 cases where the metabolite pattern indicated a pure 5F-AKB-48 intake 26 probable metabolites were detected. Of these, 17 metabolites (65%) were identified in the HLM incubations with 5F-AKB-48, representing on average 91% (range, 70–100%) of the combined area. In the remaining four cases metabolites from both AKB-48 and 5F-AKB-48 were identified, indicating an intake of both drugs.

Four abundant and unique metabolites of AKB-48, suitable as markers of drug intake, were identified (see Supplementary Figure S1A–D). One mono-OH metabolite (adamantyl-hydroxylated) at 10.54 min, one di-OH + keto metabolite (diadamantyl-hydroxylated pentyl-oxidized) at 3.08 min and two di-OH metabolites (one adamantyl-pentyl-hydroxylated metabolite and one diadamantyl-hydroxylated metabolite) at 4.55 and 8.34 min, respectively.

Four abundant and unique metabolites of 5F-AKB-48, suitable as markers of drug intake, were identified (see Supplementary Figure S1E–H). The most abundant metabolite of 5F-AKB-48 was a di-OH + keto metabolite (adamantyl-pentyl-hydroxylated pentyl-oxidized) at 4.02 min. Other metabolites included a 5F mono-OH metabolite (adamantyl-hydroxylated) at 7.38 min and a 5F di-OH (diadamantyl-hydroxylated) at 5.46 min. In one subject the most abundant metabolite (63% of combined area) was a di-OH (adamantyl-pentyl-hydroxylated) at 4.19 min. All these metabolites were unique for either AKB-48 or 5F-AKB-48 and suitable as markers of a drug intake, although traces of the di-OH + keto metabolite could be detected in some of the cases consistent with an AKB-48 intake.

The mono-OH (adamantyl-hydroxylated) at 10.54 min, an abundant metabolite of AKB-48 but not present after HLM incubation with 5F-AKB-48, co-elutes with 5-OH pentyl AKB-48. 5-OH pentyl AKB-48 was identified by comparison of spectrum and retention time from a certified reference compound (Figure 3B). The mono-OH (adamantyl-hydroxylated) was identified by the product ion spectrum (see Figure 3A). The later spectrum contains the fragment m/z 135 which is the base peak of 5-OH pentyl AKB-48. The most likely explanation for this fragment is that both mono-OH (adamantyl-hydroxylated) and 5-OH pentyl AKB-48 are produced from AKB-48. The other fragments in the spectrum are in accordance with the previous reports by Gandhi *et al.* (25).

Fragmentation patterns

The most prominent fragments of typical metabolite product ion spectra are found in Table III (examples of spectra are shown in Supplementary Figure S1). Most of the predominant fragments in the observed spectra from AKB-48 and 5F-AKB-48 metabolites originated from the adamantyl group. The parent compounds and metabolites with an unmodified adamantyl group produce a single high intensity fragment at m/z 135 (see Figure 3A) and monoadamantyl-hydroxylated metabolites produce two fragments at m/z 151 (intact) and m/z 133 (after dehydration, see Figure 3B). Diadamantyl-hydroxylated

Table I
Metabolites Identified After HLM Incubations

Type	RT	A[a]	P[b]	F[c]	AKB-48 (min)				5F-AKB-48 (min)			
					<2	15	30	60	<2	15	30	60
AKB-48 metabolites								Area of mass chromatogram				
Keto	4.10		NA				37	50				
	9.02		NA			92	114	66				
	10.62	0	0	Ada		249	241	121				
	10.88	0	0	Pentyl		764	396					
	11.56	0	0	Pentyl		245	125	55				
Mono-OH	10.42		[d]		412							
	11.32	0	1	–	45	1,055	502	117				
	11.64	0	1	–		194	107					
Mono-OH + glucuronid	7.44		NA			67	89	157				
Mono-OH + keto	4.29	1	0	Pentyl		2,315	3,910	3,716				
	4.51	1	0	Pentyl		163	266	305				
	4.72		NA				50	69				
	5.01	1	0	Pentyl		33	70	60				
	7.39	1	0	Ada		69	176	232				
	10.82	1	0	Ada		361	481	233				
Di-OH	4.09	1	1	–		14,478	23,723	25,716				
	4.33	1	1	–		509	903	1,071				
	4.55	1	1	–		2,359	4,181	4,554				
	4.84		NA			60	128	132				
	5.05	1	1	–		270	431	443				
	6.19	2	0	–		530	949	1,339				
	8.12	2	0	–		323	549	707				
	8.34	2	0	–		11,775	17,871	16,838				
Di-OH + keto	3.08	2	0	Pentyl		311	1,151	2,536				
	3.49	2	0	Pentyl			52	168				
	4.41	1	1	Ada			37	87				
Tri-OH	2.45		NA				31	70				
	2.71	1	2	–			66	163				
	2.85	1	2	–			57	126				
	3.00	2	1	–		1,979	5,692	11,566				
	3.28	2	1	–		608	2,072	3,960				
	3.66	2	1	–		66	217	382				
	5.97	3	0	–		120	320	583				
5F-AKB-48 metabolites								Area of mass chromatogram				
5F keto	6.65		NA							31		39
	7.65	0	0	Ada						654	555	201
	8.51	0	0	Ada						408	161	36
5F mono-OH	7.38	1	0	–					3,176	54,681	36,177	8,873
	7.87	1	0	–						378	370	148
	9.78	0	1	–					64	1,310	256	
	10.04	0	1	–						409	97	
	10.11	1	0	–					55	717	174	
	12.04		NA							83	42	
Mono-OH + keto	4.34	1	0	Pentyl						152	70	
	10.2		[f]							336	399	291
5F mono-OH + keto	3.74		NA							34	94	111
	4.09		NA							56	105	141
	4.61		NA							44		74
	4.78	1	0	Ada						219	616	954
	7.75	1	0	Ada						956	1,282	683
Di-OH	4.19	1	1	–						4,346	6,291	5,738
	4.42	1	1	–						162	270	262
5F di-OH	3.67	1	1	–						2,372	3,512	3,550
	3.79	1	1	–						3,064	5,408	4,884
	3.91	2	0	–						1,628	2,996	3,778
	4.09	2	0	–							151	110
	4.40		NA								96	91
	5.46	2	0	–					51	38,997	52,912	51,239
Di-OH + keto	4.02	1	1	Pentyl						147	293	403
Tri-OH	3.05	2	1	–						247	899	2,460
5F tri-OH	2.78	2	1	–						750	2,323	5,290
	3.77	3	0	–						396	1,052	1,967
	4.00	3	0	–						228	663	1,191
	4.46		NA								47	104

(*continued*)

metabolites produce three fragments, m/z 167 (intact) as well as m/z 149 and m/z 131 (dehydration). When multiple fragments of the adamantyl group are formed (from mono- and diadamantyl-hydroxylated metabolites), the relative intensities of the fragments within the spectrum vary between different metabolites.

Table I Continued

Type	RT	A[a]	P[b]	F[c]	AKB-48 (min)				5F-AKB-48 (min)			
					<2	15	30	60	<2	15	30	60
Common metabolites								Area of mass chromatogram				
Mono-OH	10.54		[e]		496	44,044	32,771	12,186	700	2,672	490	
Mono-OH + keto	4.87	1	0	Pentyl		65	166	307				48
Di-OH + keto	2.71		NA				39	103				50
	3.22	2	0	Pentyl			99	254		40	55	67
Tri-OH	2.27		NA				105	264			33	78
N-dealk	7.30	0	0	–		133	150	133		173	196	135
N-dealk mono-OH	2.53	1	0	–		49	84	118		58	92	118

NA, lacking product ion spectra.
[a]Number of OH on adamantyl.
[b]Number of OH on pentyl.
[c]Position of the keto group or glucuronide (Ada, adamantyl, Pentyl, pentylindazole-moiety).
[d]Identified by reference standard as 4-OH pentyl.
[e]Two peaks co-eluting. From AKB-48 it is 1, 0, –; from 5F-AKB-48 0, 1, – spec 5-OH pentyl.
[f]Identified by reference standard as 5-pentanoic acid.

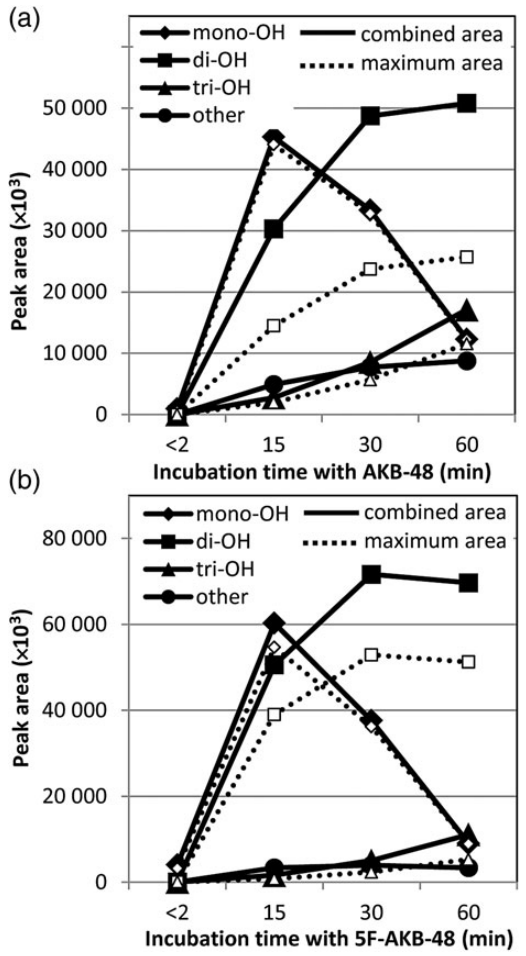

Figure 1. Metabolite distribution over time using HLMs for AKB-48 (a) and 5F-AKB-48 (b). Filled symbols with solid lines represent combined area of all metabolites of the corresponding type. Unfilled symbols with dashed lines represent the highest area of a single metabolite of the corresponding type.

The relative intensity of the adamantyl-fragments with-in the spectra decrease with the number of hydroxylations on the adamantyl. With two or more hydroxylations on the adamantyl group, the fragments formed by loss of the adamantyl-group become important for interpretation of the spectra (m/z 215 and 233 for AKB-48 and 5F-AKB-48 metabolites with an unmodified pentylindazole-moiety, respectively). Also the ions 232 and 251 for AKB-48 and 5F-AKB-48 metabolites, respectively, appear in the spectrum. The origin of these ions is not yet identified. Gandhi *et al.* (25) suggested that they are formed by the loss of the adamantyl-group without the nitrogen and Holm *et al.* (16) suggested that they are formed by migration of a hydroxyl-group from the adamantyl ring.

The introduction of a keto-group modifies the spectra as could be expected by increasing the m/z value of the corresponding fragments while N-dealkylation has no effect on the spectra at all, as the predominant fragments originate from the adamantyl group.

The information provided by the spectra was limited in terms of the position of the modifications. It was only possible to deduce if a modification was located on the adamantyl group or on the pentylindazole-moiety. Gandhi *et al.* (25) described a fragment at m/z 145 corresponding to an unmodified indazole moiety and did not report any metabolites with modifications on the indazole ring. However, that fragment was not detected in this study.

Discussion

In this study, a previously described method for targeted drug screening in human urine by LC–QTOF-MS was adapted for investigation of a broad spectrum of metabolites. Access to data dependent acquired LC–QTOF-MS data allowed reprocessing of previously analyzed case samples for identification of synthetic cannabinoid metabolites retrospectively.

In this study, several abundant and unique metabolites suitable as markers of AKB-48 and 5F-AKB-48 intake were identified. The case samples could be divided into three distinct groups of AKB-48 and/or 5F-AKB-48 intake based on results from HLM incubations as shown in Table II.

Characterization of metabolism in HLM

Both AKB-48 and 5F-AKB-48 exhibited a similar metabolism signified by rapid formation of monohydroxylated metabolites which were transformed into di- and trihydroxylated metabolites after just 15–30 min of HLM incubation. A few abundant

Table II

Metabolites Identified in Authentic Urine Samples

Type	RT	Cases with possible intake of AKB-48										Both drugs			
		#15	#16	#20	#21	#23	#27	#28	#29	#31	#32	#10	#12	#17	#25
Peaks identified after HLM incubations with AKB-48															
Keto	9.02												I	I	
Mono-OH	11.32	II		I	I	I	I	I	II	II	II				II
Mono-OH + keto	4.29	X	II	II	II	II	II	II	X	X	II	I	I	I	II
	10.82	II	I		I	I	I	I	I	II	I				I
Di-OH	4.09	X		I	I	II	I	I	X	II	II	I			
	4.55	X		II	II	II	II	II	X	X	II	II	II	I	II
	8.34	X	II	II	II	II	II	I	II	II	I	II	II		I
Di-OH + keto	3.08						X		X	X	II				X
	4.41	I				I			I	II		I			
Tri-OH	2.45						I		I	II	I				I
	2.71								I	II	I				II
	2.85								I	I	I				I
	3.00						II		X	X	II				
	3.28						II		X	X	II				II
	3.66	I							I	I		I			
Peaks identified after HLM incubations with both drugs															
AKB mono-OH	10.54	X	II	II	II	II	II	II	X	X	II	I	X	X	X
N-dealk	7.30					I					I				II
N-dealk + OH	2.53						I	II	II						X
Peaks identified after HLM incubations with 5F-AKB-48															
5F mono-OH	7.38											I	X	X	X
	10.11												I	I	II
Mono-OH + keto	10.22												II	X	X
5F	7.75											I	II	II	X
mono-OH + keto															
Di-OH	4.19												II	II	X
5F di-OH	3.67												I	I	II
	3.79												II	II	X
	3.91												I		II
	5.46											I	X	X	X
Di-OH + keto	4.02	II				I		I	I	II		II	X	X	X
Tri-OH	3.05														II
5F tri-OH	2.78														X
	3.77											I	II	II	II
	4.00												I	I	

Type	RT	Cases with possible intake of 5F-AKB-48																				
		#1	#2	#3	#4	#5	#6	#7	#8	#9	#11	#13	#14	#18	#19	#22	#24	#26	#30	#33	#34	#35
Peaks identified after HLM incubations with AKB-48																						
Keto	9.02																	I				
Mono-OH	11.32																					
Mono-OH + keto	4.29													I								
	10.82																					
Di-OH	4.09																					
	4.55						I			I				I					I	I		I
	8.34																					
Di-OH + keto	3.08																	I	I	II		II
	4.41																					
Tri-OH	2.45																					
	2.71																					
	2.85																		I	I	I	I
	3.00																		I	I		I
	3.28																		I	I		I
	3.66																					
Peaks identified after HLM incubations with both drugs																						
AKB mono-OH	10.54	I	I	I	I	II	II	I	I	I	I	I	I	X	II	II	II	II	II	II	X	II
N-dealk	7.30						I							I					I			
N-dealk + OH	2.53																	II		I	II	I
Peaks identified after HLM incubations with 5F-AKB-48																						
5F mono-OH	7.38	II	II	I	II	X	X	I	I	X		II	II	X	II	II	II	X	X	II	X	II
	10.11					I	I							I				I	I	I		I
Mono-OH + keto	10.22				II	II	II	I		I	II	II	I	II	II		II	II	X		II	
5F	7.75		I		I	II	II			II		II		X	II	I	II	I	II	I	I	I
mono-OH + keto																						
Di-OH	4.19	I	I	I	I	II	X	I	I	II		I	I	II	I	X	I	II	II	I	II	II
5F di-OH	3.67					I				I				I				I	I			
	3.79	I	II	I	II	II	X	I		II			I	II	I		I	I	II	II	II	I
	3.91									I		I		I				I	I		I	
	5.46	II	II	II	II	X	X	II	I	X		II	II	X	II	II	II	X	X	II	X	II

(*continued*)

Table II Continued

		Cases with possible intake of AKB-48										Both drugs										
Type	RT	#15	#16	#20	#21	#23	#27	#28	#29	#31	#32	#10	#12	#17	#25							
Di-OH + keto	4.02	X	X	X	X	X	X	X	II	X	X	X	X	X	X	II	X	X	X	X	X	X
Tri-OH	3.05																		I		I	
5F tri-OH	2.78																	II	X	II	II	
	3.77		I		I	II	II			I		II		II	I		I	I	I	I	I	
	4.00					I	I					I							I			

The table shows the peak area of extracted mass chromatograms for the parent mass using the following symbols: I, peak area between 100k and 500k; II, peak area between 500k and 2,500k; X, peak area above 2,500k.

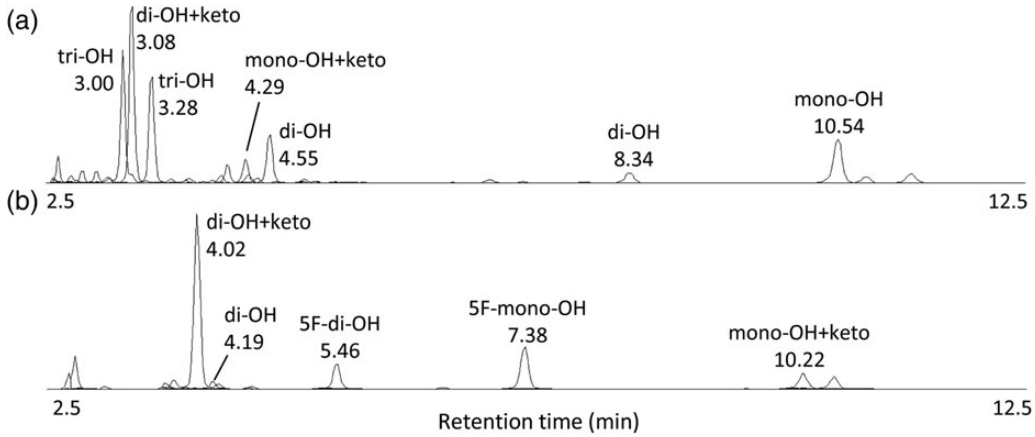

Figure 2. Combined mass chromatograms from typical cases where the metabolite pattern indicated an intake of AKB-48 (a) and 5F-AKB-48 (b). Peaks with an area above 10^6 are included and peaks with an area above 2.5×10^6 are annotated along with suitable markers for drug intake.

metabolites represented the major part of the metabolites found and interestingly most metabolites, both halogenated and other, were specific to either AKB-48 or 5F-AKB-48.

Many metabolites of AKB-48 and 5F-AKB-48 were detected in this study, but metabolites not included in the target list might have been overlooked since only previously known modifications and combinations thereof were targets (16, 25).

In this study, a higher number of metabolites of AKB-48 after HLM incubation were identified compared with Gandhi *et al.* (41 compared with 17). Their top four metabolites include one each of the mono-, di- and triadamantyl-hydroxylated metabolites and one adamantyl-pentyl-hydroxylated metabolite (25). In this study, the same type of top metabolites was identified with the exception that the tri-OH was a diadamantyl-pentyl-hydroxylated metabolite (Table I). A higher number of metabolites of 5F-AKB-48 compared with Holm *et al.* were also identified (37 compared with 4). They reported mono-, di- and trihydroxylations, mainly on the adamantyl ring (16), which is similar to the top metabolites in this study (Table I).

Potential metabolite markers were identified based on their product ion spectra. Definite structure confirmation will require NMR spectroscopy but that is beyond the scope of this study.

Identification of metabolites in authentic urine samples

In the case samples slightly fewer metabolites were identified than after HLM incubation and although the relative abundance of the different metabolites differed between case samples and HLM incubations on average 89% (range, 67–100) of the combined area in the case samples represented metabolites identified after HLM incubation, including all of the most abundant metabolites. The data from probable metabolites found in case samples but not identified after HLM incubation should be interpreted with caution. These metabolites could potentially come from other sources than AKB-48 and 5F-AKB-48 such as impurities in the drug formulation, intake of another drug (both illicit and prescribed), food intake and/or endogenous sources.

The main differences between the case samples and the HLM incubations were that metabolites containing keto-groups and N-dealkylations represented a larger part of the combined area in the case samples (48% compared with 5% and 7% compared with 0.2% for keto-groups and N-dealkylations, respectively).

Holm *et al.* (16) reported metabolites after suspected intake of 5F-AKB-48 in one subject. The major metabolite was a di-OH + keto (adamantyl-pentyl-hydroxylated pentyl-oxidized) which is in agreement with our findings (Table II). To our knowledge, metabolites of AKB-48 in authentic case samples have not previously been reported.

There were substantial differences in the relative distribution of metabolites between subjects for both AKB-48 and 5F-AKB-48 as shown in Figure 4. The observed differences might be due to a number of factors such as dose and time of last intake, multiple intakes, presence of other drugs (legal or illicit) and individual differences in metabolic ability.

Due to differences between subjects, several metabolites are probably needed as markers for reliable identification of an intake of structurally related synthetic cannabinoids. In this study, several metabolites suitable as such markers were identified as suggested above (Supplementary Figure S1 and Supplementary Table SII).

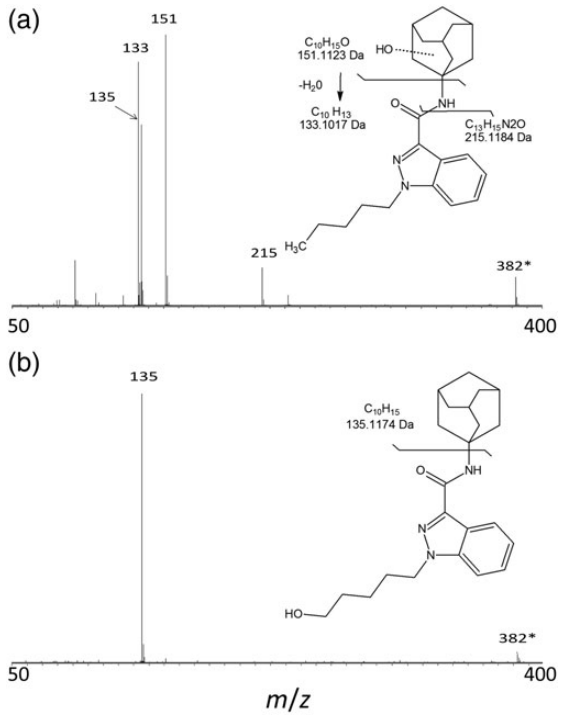

Figure 3. Spectra of metabolites co-eluting at 10.54 min monohydroxyl on adamantyl from AKB-48 (a) and 5-OH pentyl AKB-48 from 5F-AKB-48 (b).

Table III
Fragmentation patterns

Metabolite	Mass	A[a]	P[b]	F[c]	Pos	Fragments			
Keto		0	0	Pentyl	0,0,P	135			
		0	0	Ada	0,0,A	151	133	215	
5F keto		0	0	Ada	0,0,A	233			
Mono-OH	381.24	1	0	–	1,0,–	151	133	135[d]	215
		0	1	–	0,1,–	135			
5F mono-OH	399.23	1	0	–	1,0,–	151	133	233	
		0	1	–	0,1,–	135			
Mono-OH + keto	395.22	1	0	Pentyl	1,0,P	151	133		
		1	0	Ada	1,0,A	135			
		0	1	Pentyl	0,1,P	215	119	232	165
5F mono-OH + keto	413.21	1	0	Ada	1,0,A	233	119	165	250
Di-OH	397.24	2	0	–	2,0,–	215	149	[e]	
		1	1	–	1,1,–	151	133		
5F-di-OH	415.23	2	0	–	2,0,–	233	149	[e]	
		1	1	–	1,1,–	151	133		
Di-OH + keto	411.22	2	0	Pentyl	2,0,P	167	149	131	229
		1	1	Pentyl	1,1,P	151	133		
		1	1	Ada	1,1,A	231	213	248	
Tri-OH	413.23	3	0	–	3,0,–	215	232		
		2	1	–	2,1,–	167	213	149	
		1	2	–	1,2,–	133	151		
5F tri-OH	431.22	3	0	–	3,0,–	233	250	182	
		2	1	–	2,1,–	167	249	149	131
N-dealk		0	0	–	0,0,–	135			
N-dealk mono-OH		1	0	–	1,0,–	151	133		

[a]Number of OH on adamantyl.
[b]Number of OH on pentyl.
[c]Position of the keto group or glucuronide (Ada = adamantyl, Pentyl = pentylindazole-moiety).
[d]This fragment could be from co-eluting mono-OH 0,1,–.
[e]Intensity of fragments 167, 131 and 233/251 vary between metabolites.

Due to the diverse chemical properties of synthetic cannabinoids and their metabolites limitations of analytical methodology will affect the analytical outcome in studies such as the present.

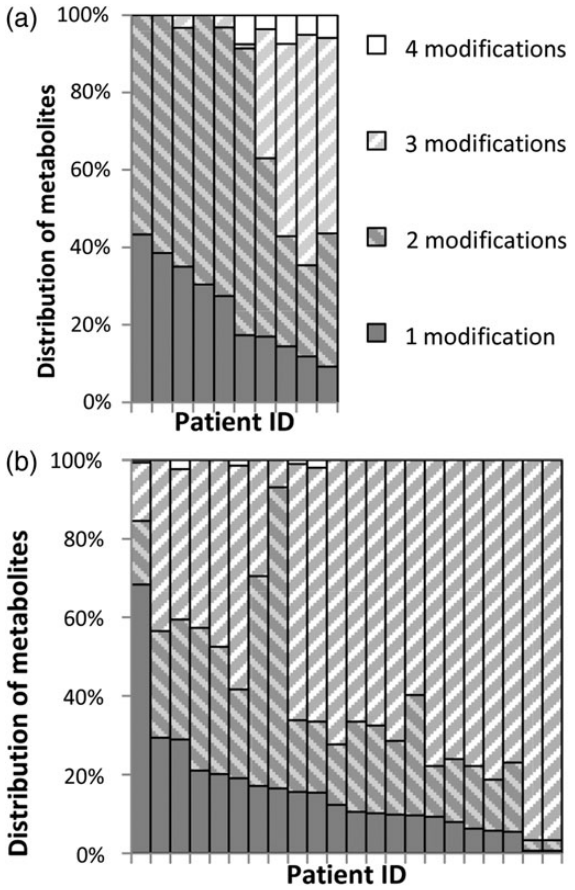

Figure 4. Distribution of metabolites in case samples based on the number of functions added (hydroxyl- and keto-groups) for cases with metabolite patterns indicating an intake of AKB-48 (a) and 5F-AKB-48 (b).

The parent compounds of AKB-48 and 5F-AKB-48 could unfortunately not be determined due to strong retention in the chromatographic system used. However, by analyzing solutions of AKB-48, 5F-AKB-48 and HLM incubations using an extended gradient we concluded that no metabolites eluted after our data collection were stopped (data not shown). Holm *et al.* (16) showed that the majority of 5F-AKB-48 metabolites in their urine sample was present as glucuronides. In this study, case samples were analyzed in positive ionization mode and after β-glucuronidase treatment most of the glucuronide would be detected as their hydrolysis products. Although glucuronides of AKB-48 and 5F-AKB-48 were not commercially available, other glucuronides showed almost 100% hydrolysis efficiency in this set-up (data not shown).

HLMs as a tool in assay development

Due to the high similarity between AKB-48 and 5F-AKB-48, it is difficult to determine which cannabinoid a metabolite is produced from (with the exception of the halogenated metabolites) by simply examining the structure. Instead HLMs were used to generate and compare metabolites from both analogs.

A majority of the metabolites identified in the case samples and the possibility to divide the case samples into three distinct groups based on metabolite findings (Table II and above) indicate

that HLMs were a suitable model system for identifying potential metabolites of these cannabinoids in case samples.

However, in this study it was not possible to predict which metabolites that would be most abundant in the case samples based on the HLM incubations. This indicates, as expected, that pharmacokinetic and individual metabolic pathways are important for the metabolism of AKB-48 and 5F-AKB-48 in specific cases.

Holm *et al.* (16) also used HLMs for their work on 5F-AKB-48 while Gandhi *et al.* (25) used cryopreserved human hepatocytes. They correctly point out that the hepatocytes have a more complete metabolic system making their metabolism more representative of that *in vivo*. It is, however, difficult to determine how well their findings correlate with those *in vivo* as they did not report metabolites from any case samples.

Conclusions

In this study, we have identified metabolites suitable as abundant and unique markers of AKB-48 and 5F-AKB-48 intake for urine screening in forensic cases. These compounds have few metabolites in common even though the dehalogenated metabolites of 5F-AKB-48 account for a majority of the combined area in case samples. Mono-, di- and trihydroxylations are common, sometimes in combination with keto-group formation and/or N-dealkylations.

As used in this study, the HLMs might prove to be a valuable and suitable tool for the production and identification of probable metabolites from synthetic cannabinoids.

Supplementary data

Supplementary data are available at *Journal of Analytical Toxicology* online.

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