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Detection of urinary metabolites of AM-2201 and UR-144, two novel synthetic cannabinoids

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Synthetic cannabinoids are the psychotropic compounds frequently identified as active components of smoking mixtures easily available via the Internet in several countries. These herbal blends have become extremely popular as a legal alternative to cannabis-based products and are difficult to detect by regular drug tests.

Here we report on an *in vitro* and *in vivo* metabolism of AM-2201, 1-[(5-fluoropentyl)-1*H*-indol-3-yl]-(naphthalen-1-yl) methanone, and UR-144 (KM-X1), (1-pentylindol-3-yl)-(2,2,3,3-tetramethylcyclopropyl)methanone isolated using preparative liquid chromatography from the smoking mixtures sold in Russia. After incubation with human liver microsomes (HLM) as well as with cytochrome isoenzymes 3A4 and 2B6, the metabolic pathways were identified by means of liquid chromatography – triple quadrupole and high resolution mass spectrometry with electrospray ionization in positive mode. It was found that the *in vitro* reactions include mono- and dihydroxylation, loss of *N*-alkyl side chain and formation of dihydrodiol metabolites in case of AM-2201. The HLM were found to be superior over the other two isoenzymes for generation of cannabinoid metabolites. Finally, forensic urine samples were analyzed to validate the *in vitro* data and it has been shown that for both cannabimimetics the recommended screening targets are the monohydroxylated metabolites. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: AM-2201; UR-144; KM-X1; synthetic cannabinoids; anti-doping analysis

Introduction

Synthetic cannabinoids are a diverse class of research chemicals that constantly appear on black market as the components of various smoking mixtures. Dozens of cannabimimetics have been identified in herbal products and fully characterized by now[1–18] and, as one may expect, many more will follow to overcome belated legislation bans. The detection of abuse of these compounds requires knowledge of their metabolism, except when blood serum[19–23] or oral fluid is used for analysis.[24] The urinary metabolites and methods of detection thereof in humans have been reported for JWH-018,[25–28] JWH-073,[29–31] JWH-081,[31] JWH-122,[31] JWH-210,[31] JWH-250,[31,32] AB-001,[33] RCS-4,[34] and AM-694.[35] An *in vitro* study was recently undertaken to identify tentative metabolites of HU-210.[36] All these publications demonstrate that synthetic cannabinoids are subject to extensive metabolism, mainly through hydroxylation and further conjugation with glucuronic acid.

Since 2010, designer cannabimimetics are classified by the World Anti-Doping Agency (WADA) as substances prohibited in sports.[37] Therefore, anti-doping laboratories should have analytical methods implemented to be able to detect these compounds. While (hypothetical) metabolites of most frequently found cannabinoids have been commercially available recently,[38] it is not always clear which ones are the most valuable for analysis because many of them were synthesized as expected, or postulated, urinary metabolites.

In smoking mixtures being sold in Russia, we have recently identified a fluorinated analog of JWH-018, termed as AM-2201,[39] and the so-called UR-144 (KM-X1), or (1-pentylindol-3-yl)-(2,2,3,3-tetramethylcyclopropyl)methanone.[40] As there have been no publications related to their metabolism in humans up to now, the aim of the present study was to identify the metabolites of these two cannabinoids using an *in vitro* assay and then compare the results obtained with the forensic urine samples to select the analytes being most valuable for doping control or toxicological analysis.

Materials and methods

Reagents

Water and methanol of gradient grade or better were purchased from Biosolve (Valkenswaard, the Netherlands) and Merck (Darmstadt, Germany), respectively. Acetonitrile of far UV / gradient grade was produced by J.T. Baker (Deventer, the Netherlands). Diethyl ether and acetone were obtained from Medkhimprom (Moscow, Russia). β-Glucuronidase from *E. Coli* K12 (solution in 50% glycerol) was purchased from Roche Diagnostics (Mannheim, Germany) and used as supplied. All other chemicals (potassium carbonate, potassium hydrocarbonate, potassium phosphate monobasic, sodium phosphate dibasic, sodium sulfate, formic acid) were obtained from Sigma-Aldrich (St Louis, MO, USA). Methyltestosterone was provided by LGC Standards (Wesel, Germany).

The CYP isoenzymes 3A4, 2B6, human liver microsomes (HLM) pooled from 150 mixed gender donors, as well as regenerating system solution A of reduced nicotinamide adenine dinucleotide phosphate (NADPH-A), regenerating system solution B of NADPH

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(NADPH-B), and phosphate buffer (0.5 M, pH 7.4), were obtained from BD Gentest (Woburn, MA, USA). Solid-phase extraction (SPE) was carried out on the columns filled with a non-polar C8 / cation exchanger sorbent (Bond Elute Certify, 130 mg × 3 ml, Varian Inc., Lake Forst, CA, USA). *N*-(5-hydroxypentyl)-JWH-018 and JWH-018-*N*-pentanoic acid were received from Cayman Chemical (Ann Arbor, MI, USA).

Smoking mixtures and urine samples

At the time of preparing this report both AM-2201 and UR-144 were legal in Russia, and respective smoking mixtures were bought via the Internet. The identity was tested by gas chromatography - mass spectrometric (GC-MS) analysis of organic extract prepared as follows: 100 mg of raw plant material was extracted with 5 ml of methanol under sonication for 10 min and centrifuged. The supernatant was diluted 1:20 with acetone and 1 μl was injected into GC-MS in full-scan mode. For *in vitro* experiments, the crude extracts obtained from smoking mixtures were additionally purified by high performance liquid chromatography (HPLC) to isolate fractions corresponding to the main component. To this end, 10 μl of the extract were mixed with 50 μl of methanol and 40 μl of water, and 90 μl were injected into the HPLC system under the conditions described below.

Urine samples were provided by local drug enforcement body and were collected from the persons detained with intoxications symptoms like agitation, mydriatic pupils ($n = 3$) or those who declared the administration of smoking mixtures in the past ($n = 8$). In total, 5 samples containing UR-144 and 6 samples containing AM-2201 metabolites were selected after preliminary analysis. As a blank urine, a certified laboratory negative control urine was used.

HPLC clean-up

Agilent 1100 HPLC system comprising a degasser unit, binary pump, autosampler, column compartment, diode array detector, and preparative scale fraction collector was employed to collect fractions. For separation a Waters SunFire C18 column (250 mm × 4.6 mm, 5 μm) protected by a Waters SunFire C18 guard column (20 mm × 4.0 mm) was used maintained at 35°C for better retention time stability. Gradient elution was applied as follows: 70% A (water) to 0% A within 20 min, then 100% B (acetonitrile) for 10 min, then 0% A to 70% A within 5 min, and equilibration at 70% A for 5 min. The eluent flow rate was 1 ml/min, detector wavelength – 254 nm. The chromatograms for UR-144 smoking mix clearly demonstrated the presence of well separated two major and two minor components, while in case of AM-2201 there was only one major component. Collected fractions: 14.2–15.9 min – unidentified analog of *N*-despentyl UR-144; 16.0–17.0 min – *N*-despentyl-UR-144; 21.4–22.3 min unidentified analog of UR-144; 22.3–23.2 min – UR-144; and 18.0–19.0 min – AM-2201.

The collected fractions were transferred to the glass 2-ml vials and subjected to the analysis by liquid chromatography coupled to tandem (LC-MS/MS) or high resolution mass spectrometry (LC-HRMS) as is. Alternatively, for an *in vitro* study, the eluent was evaporated to dryness at 50°C and reduced pressure followed by reconstitution of the residue in 50 μl of methanol.

Preparation of urine samples

Urine samples were prepared according to the protocol for non-volatile conjugated compounds used in our laboratory. Briefly, to 3 ml of urine was added 1 ml of phosphate buffer (0.8 M, pH 6.5) containing 30 μl of β-glucuronidase and 0.6 μg of methyltestosterone. After incubation at 57°C for 60 min and addition of carbonate buffer (3 M, pH 10), the samples were extracted with 5 ml of diethyl ether in the presence of Na_2SO_4. When urinary free fraction was analyzed, addition of the enzyme and incubation steps were omitted (phosphate buffer with no β-glucuronidase was used). Following the evaporation of organic layer at 70°C in a solid state heater, the residue was reconstituted in 60 μl of methanol and then 40 μl of water were added before transfer to a vial for the subsequent analysis by LC-MS/MS.

In vitro experiments

The enzyme reactions were performed in 1.7-ml Eppendorf tubes according to the protocol provided by the manufacturer. Briefly, the reaction mixture contained 20 μl of purified methanolic solution of cannabinoid, 700 μl of deionized water, 200 μl of phosphate buffer (0.5 M, pH 7.4), 50 μl of NADPH-A and 10 μl of NADPH-B. After incubation at 37°C for 5 min the samples were mixed gently prior to addition of 20 μl of either HLM or isoenzymes 3A4 or 2B6, followed by incubation at 37°C for 1 h under continuous agitation. The overall reaction was terminated by the addition of 100 μl of ice-cold acetonitrile. The reaction mixture was centrifuged at 15 000 *g* for 10 min to remove proteins. The supernatant was transferred into a fresh tube, and acetonitrile was evaporated at reduced pressure resulting in mostly aqueous specimens, which were further purified by SPE. To this end, the SPE columns were conditioned with 3 ml of methanol and 3 ml of deionized water. After sample addition, the columns were washed twice with 3 ml of deionized water. The analytes were eluted with 3 ml of methanol. The eluent was evaporated to dryness under nitrogen flow followed by reconstitution of the residue in 200 μl of water/methanol (60:40, *v:v*) and subsequent LC-MS/MS or LC-HRMS analysis.

GC-MS

The GC-MS analyses were performed in full-scan mode (50–750 amu) on the system comprising a 6890 gas chromatograph coupled to a 5973 mass spectrometer (Agilent, Palo Alto, CA, USA) with electron ionization at 70 eV. A HP-Ultra 1 column, 12 m 0.2 mm × 0.33 μm (Agilent J&W, Palo Alto, CA, USA), was used for separation. The temperature program was as follows: initial temperature 110°C for 2 min, then heating to 300°C at 15°C/min (held for 4 min). One microlitre injections were done at 300°C in the split mode (1:15) with a carrier gas flow rate set to 0.6 ml/min (helium 99.9999%). The temperature of the transfer line, ion source and quadrupole were 300, 230 and 150°C, respectively.

LC-MS/MS and LC-HRMS

In case of LC-MS/MS analyses, the system comprised an Acquity liquid chromatograph (Waters, Milford, MA, USA) coupled to a TSQ Vantage triple quadrupole mass spectrometer (ThermoFisher Scientific, San José, CA, USA). Waters Acquity BEH C18 column (100 mm × 2.1 mm, particle size 1.7 μm) maintained at 60°C and protected by a Vanguard BEH C18 column (20 mm × 2.1 mm)

was used for separation. Injection volume was 5 μl. The mobile phase flow rate was set to 0.35 ml/min. The elution program started from 0.5-min isocratic step at 95% of 0.1% formic acid in water (A) and 5% of 0.1% formic acid in methanol (B) followed by linear increase to 95% of B within 4.5 min, hold at 95% of B for 2.5 min and then re-equilibration until the end of analysis (10 min).

The heated electrospray ion (ESI) source was used for ionization. Positive ions were detected in the full-scan, MS/MS (product ion scan) and selected reaction monitoring (SRM) modes. The collision gas pressure was 0.2 Pa (or 1.5 mTorr, argon 99.9995%). Sheath and aux gas pressure (nitrogen from a nitrogen generator, 99.9% purity) was set at 55 and 35 arbitrary units, respectively.

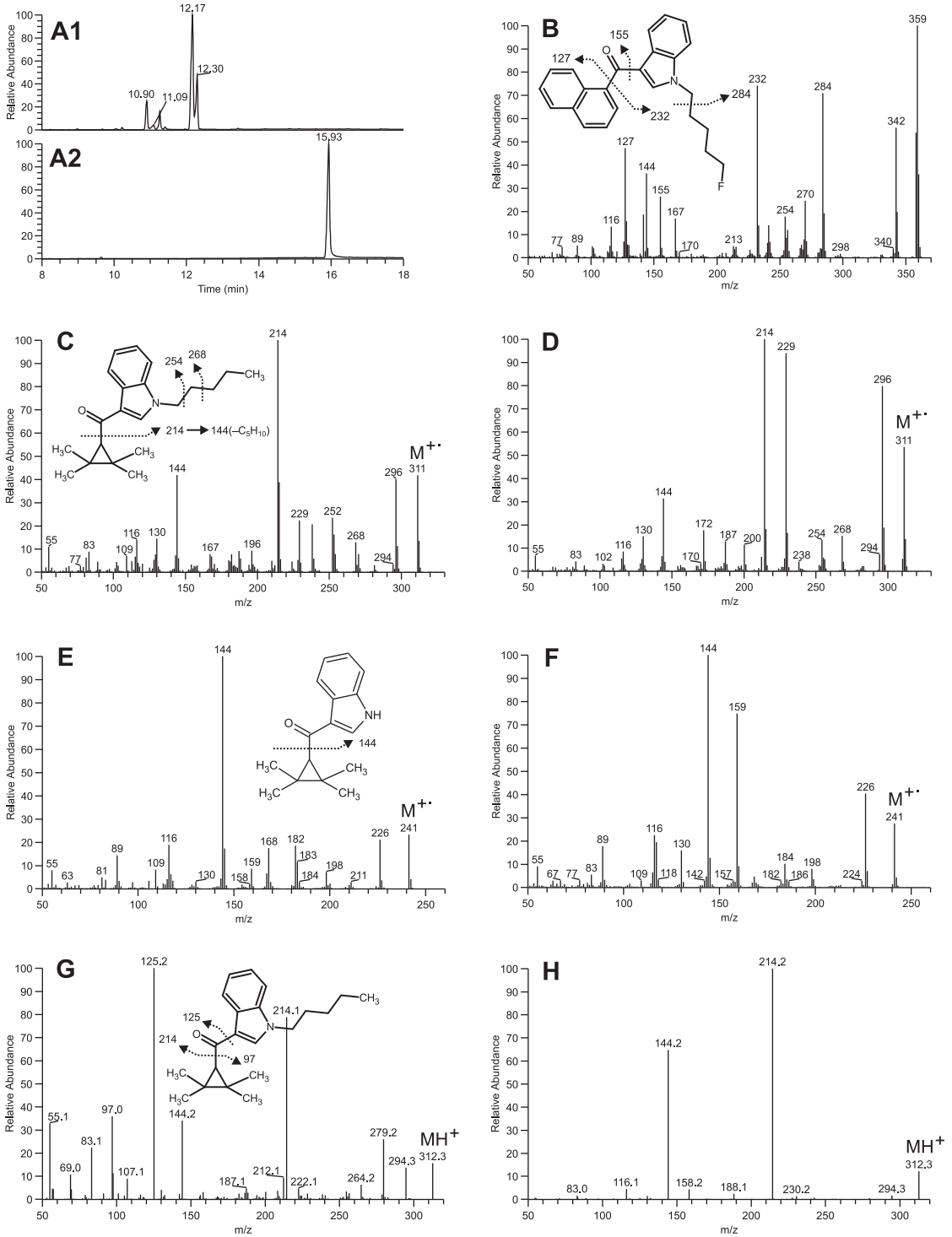

Figure 1. Total ion current chromatograms for smoking mixture Marrakesh (A1) and Nano (A2), and electron ionization mass spectra and structure of cannabinoids AM-2201 (B, corresponds to the peak with RT = 15.93 min in A2), UR-144 (C, RT = 12.17 min in A1) and its unidentified isomer (D, RT = 12.30 min in A1), N-despentyl UR-144 (E, RT = 10.90 min in A1) and its unidentified isomer (F, RT = 11.09 min in A1), as well as product ion ESI mass spectra for UR-144 (G, RT = 12.17 min in A1) and its unidentified isomer (H, RT = 12.30 min in A1).

The vaporizer and capillary temperatures were set at 370 and 300°C, respectively, with a spray voltage 4000 V. In the MS/MS experiments the collision energy (CE) was ramped from 5 to 40 eV to select the optimal value for every transition. The mass isolation widths for Q1 and Q3 were set to 0.7 and 1.0 Da (full width at half maximum, FWHM), respectively.

For an additional evaluation of *in vitro* data, the system consisting of a high-resolution mass spectrometer Exactive (Thermo Scientific, Bremen, Germany) coupled to an Accela liquid chromatograph (Thermo Scientific, San José, CA, USA) with the identical chromatographic column and gradient elution program was used. The mass spectrometer operating in positive ionization mode was calibrated using the calibration mixture containing caffeine, the tetrapeptide MRFA and Ultramark with a total of seven reference masses. The data were collected in full scan mode over the *m/z* range of 120–2000 Da at resolution of 100000 @ *m/z* 195.0882 (FWHM), or applying higher-energy collision-induced dissociation (HCD) performed at 20 and 50 V. In the latter case the resolution was set at 50 000 and the product ions were scanned over the *m/z* range 50-650 Da. All other relevant MS parameters were identical to that of TSQ Vantage.

Results and discussion

Composition of smoking mixtures bought via the Internet

Initially, the Internet websites advertising 'research chemicals' were checked to understand what compounds are currently sold and could be encountered in smoking mixtures. Therefore, the list of potential candidates was defined beforehand. It was shown by GC-MS that smoking mixture Marrakesh contained not only two isomeric compounds with the molecular weight formally corresponding to UR-144, but also two respective *N*-despentyl isomers, as tentatively identified. The total ion current (TIC) chromatogram recorded for this smoking mixture is presented in Figure 1A1. The electron ionization (EI) mass spectra of the base peak at the retention time 12.17 min (most likely UR-144), its isomeric counterpart (12.30 min), as well as *N*-despentyl-UR-144 (10.90 min) and its isomer (11.09 min) are given in Figures 1C–1F, respectively. Recently, Cayman Chemical included the reference spectrum of UR-144 in their GC-MS EI spectral library,[41] which is in good agreement with the mass spectrum shown in Figure 1C. The ions at *m/z* 144 (intact indole) and 214 (*N*-pentylindole) were earlier described as characteristic of many synthetic cannabinoids.[4,10,12,15–18,27,31,32,34,40]

Further, the ESI + mass spectra were obtained by analyzing fractions purified using HPLC. The product ion mass spectra of the component recovered in the time range 22.3–23.2 min, which is supposed to be UR-144, and its isomer (fraction 21.4–22.3 min) recorded at 25 eV are given Figures 1G and 1H, respectively. Importantly, the unidentified isomer of UR-144 which has similar EI mass spectrum (Figure 1D) with only altered relative intensities of the fragment ions, produced quite different product ion ESI + mass spectrum. As seen from Figure 1H, only fragments pertaining to 1-pentyl-1*H*-indole-3-carbaldehyde were present, while the ions at *m/z* 125 and 97 which correspond to oxo(2,2,3,3-tetramethylcyclopropyl)methylium and 2,2,3,3-tetramethylcyclopropylium were completely absent. It is suggested that this compound may have isobaric cycloalkyl or alicyclic alkyl substituent instead of 2,2,3,3-tetramethylcyclopropyl moiety. To additionally confirm the identity of UR-144, high resolution ESI + mass spectrum was recorded on an Exactive mass spectrometer in HCD mode at 20 eV. As seen from Figure 2, the accurate masses of the fragments are in a good agreement with the structure of UR-144. A higher mass error for the small fragments (*m/z* 125 and below) may be due to the fact that calibration mixture used to calibrate the mass spectrometer did not cover this mass region.

The second smoking mixture called Nano was found to contain solely AM-2201, as only one peak was detected on the TIC chromatogram (Figure 1A2). The EI mass spectrum presented in Figure 1B is identical to that reported by Nakajima[7] for AM-2201. The possibility that this mass spectrum may originate from 4-fluoropentyl isomer of AM-2201 was also evaluated. However, according to the Cayman Spectral Library[41] the EI mass spectrum of the latter cannabinoid features an abundant ion at *m/z* 339 (loss of HF), while in case of AM-2201 this ion is low.

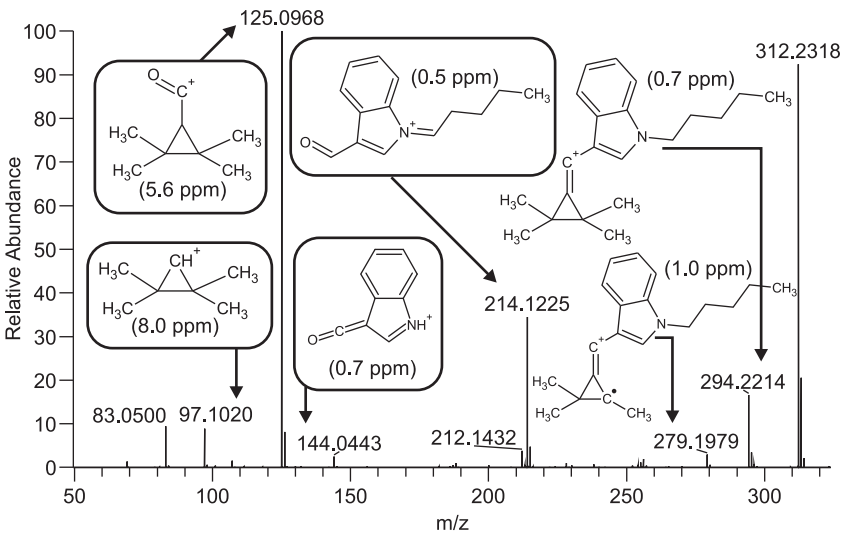

Figure 2. High resolution ESI mass spectrum of UR-144 recorded on an Exactive mass spectrometer in HCD mode at 20 eV and proposed structure of the principal fragments.

In vitro metabolism

A preliminary HPLC clean-up was necessary to isolate the main component before the *in vitro* experiments to exclude potential side reactions of plant matrix and other related cannabimimetics present in the plant material with the enzymes. It is important to emphasize that before commencing the *in vitro* reactions the purity of starting material was evaluated by the LC-MS analysis. It has been confirmed that UR-144 fraction (22.3–23.2 min) did not contain any other related cannabinoids initially found in the smoking mixture. The fraction of AM-2201 was also checked for accidental presence of JWH-018, and no trace of the latter compound was detected.

UR-144

After incubation with HLM and isoenzymes 3A4 and 2B6, *N*-despentyl (*m/z* 242), *N*-despentylhydroxy (*m/z* 258), dehydrated hydroxy (*m/z* 326), hydroxy (*m/z* 328), and dihydroxy UR-144 (*m/z* 344) were identified, with relative amount of monohydroxy metabolites being maximal amongst the others (Figure 3A). Multiple closely eluting monohydroxy metabolites were later found to be the most valuable for the analysis of urine samples. To consider the potential hydroxylation sites of UR-144, it is helpful to look at the profile of its (mono)hydroxy metabolites given in Figure 4. Taking into account the data presented in Figure 1G and Figure 4, one may see that the ion at *m/z* 125 indicates the presence of intact tetramethylcyclopropyl group, *m/z* 144 – intact indole, *m/z* 214 – intact indole and untouched *N*-pentyl side chain, *m/z* 230 – hydroxylation at indole or *N*-pentyl side chain, and finally *m/z* 160 is indicative of hydroxyindole moiety. With all this in mind, it may be concluded that the first and second hydroxy metabolites (5.22 and 5.34 min) are hydroxypentyl metabolites, while the metabolite at 5.46 min is most likely hydroxyindole. Further, the metabolites at 5.32 and 5.42 min seem to be hydroxylated at the tetramethylcyclopropyl group as they are lacking the ion at *m/z* 125.

The terminal carboxy metabolite of UR-144 (*m/z* 342) was not produced in the amount necessary to generate product ion mass spectrum and was not further evaluated. In general, the pathways of an *in vitro* transformation of UR-144 are in good agreement with the data published for JWH-018[42] and JWH-015.[43] The HLM demonstrated the highest metabolic activity towards UR-144, while isoenzyme 2B6 was the least active.

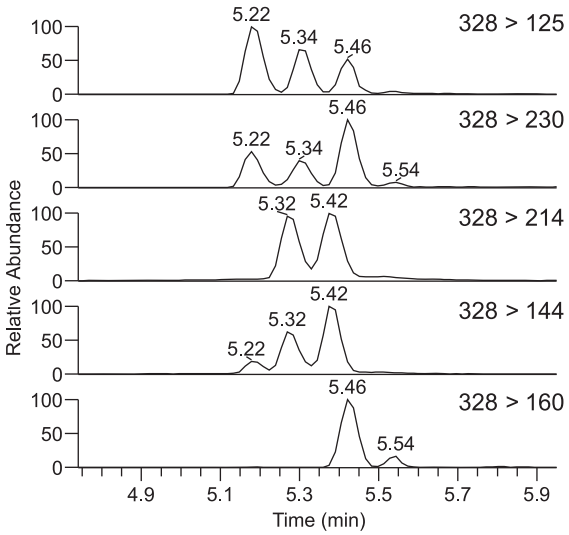

Figure 4. Profile of monohydroxy metabolites of UR-144 obtained using LC-MS/MS.

AM-2201

Incubation of AM-2201 resulted in formation of *N*-desfluoropentyl (*m/z* 272), hydroxy (*m/z* 376), dihydroxy (*m/z* 392), and dihydrodiol (*m/z* 394) metabolites. No *N*-desfluoropentyl hydroxy (*m/z* 288) metabolites were found. Again, the human liver microsomes turned out to be the most active species, and are therefore recommended to perform the metabolic reactions. In addition to the above, after the enzymatic elimination of fluorine, the non-specific metabolites common with JWH-018 were produced, namely *N*-(5-hydroxypentyl)-JWH-018 (*m/z* 358) and respective *N*-pentanoic acid (*m/z* 372), as confirmed against the synthetic reference material. The enzymatic defluorination

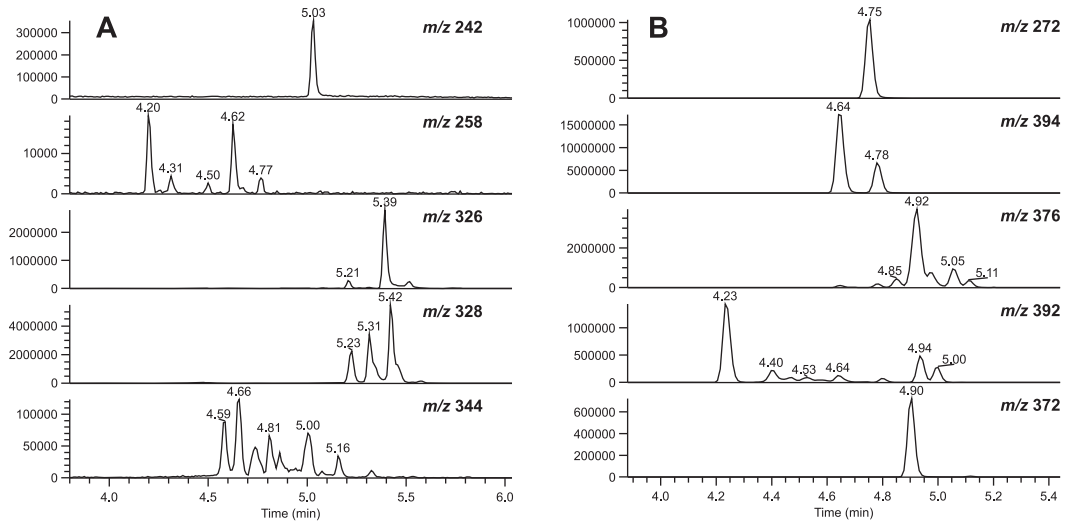

Figure 3. LC-MS ESI profile of *in vitro* synthesized metabolites of UR-144 (A) and AM-2201 (B) obtained upon incubation with human liver microsomes.

is reportedly attributed to cytochrome P450 2E1[44] and it was earlier described that organofluorine compounds such as volatile anesthetics (e.g. enflurane) undergo defluorination and liberate toxic fluoride.[45,46] Interestingly, the other defluorinated metabolites which were tentatively identified as the two isomers of *N*-(5-hydroxypentyl)-dihydrodiol-JWH-018, produced the same protonated molecular ion at *m/z* 392 as dihydroxy-AM-2201. This was quite misleading at the beginning of our study but later the accurate mass measurement allowed us to clearly differentiate between these two species. The monohydroxylated dihydrodiol of JWH-018 was first identified by Wintermeyer *et al.*[42] and the diagnostic product ions reported therein are fully compliant with our data. The TIC profile of the *in vitro* metabolites of AM-2201 is given in Figure 3B.

As a result of these *in vitro* experiments, the product ion ESI + mass spectra were obtained for the cannabinoid metabolites to establish SRM transitions necessary for their detection in urine samples (Table 1). However, in some cases it was not possible to fully characterize the metabolites due to partial or complete co-elution under the chromatographic conditions used in our study. Therefore, at this point the purpose of mass spectral characterization of the *in vitro* synthesized metabolites was not to evaluate their structure as deep as possible, but rather create a method for their determination in human urine and to identify the metabolites which are meaningful *in vivo*, i.e. abundant and easy to detect against the biological background.

Analysis of forensic urine samples and tentative structure of in vivo metabolites

Having acquired mass spectral and chromatographic data for the *in vitro* generated metabolites of UR-144 and AM-2201, post-administration urine samples collected from drug users and delivered to our laboratory were analyzed. The samples which did not contain target analytes were deselected resulting in five and six urines with UR-144 and AM-2201 metabolites, respectively. The medical examination reports were provided with these samples which allowed the (very rough) estimation of the time elapsed since the last smoking session for every person, which varied from several hours to 10 days. The samples were collected from different individuals. In addition to the cannabinoids in question these urines also contained the metabolites of JWH-018, JWH-073 and JWH-210 in varying amounts (data not shown), therefore defluorinated metabolites of AM-2201 were not evaluated. At the moment of performing this study, no metabolites of UR-144 and AM-2201 were commercially available, and therefore the identification is speculative and was solely based on the mass spectral properties of the metabolites.

We have found that the *in vitro* metabolic transformation of UR-144 correlates well with what is observed in a forensic urine sample, except that *N*-despentylhydroxy (*m/z* 242) and dehydrated hydroxy (*m/z* 326) metabolites were not detected. It was also noted that UR-144 is almost completely metabolized *in vivo*, and the metabolites are excreted as glucuronides (less than 10% unconjugated). Parent compound was detected at the low level in only one urine of the five. Based on the information from medical examination reports, we would expect that mono- and dihydroxy metabolites of UR-144 should be detectable at least 1 week after administration, but monohydroxy metabolites are preferred for screening analysis due to less interference with urinary matrix. The example chromatograms for monohydroxy metabolites whose appearance pattern, as represented by the two SRM transitions

Table 1. Metabolites of UR-144 and AM-2201 identified *in vitro*

Cannabinoid or metabolite	$[M+H]^+$ exact mass	Mass accuracy (ppm)	RT, min	Diagnostic product ions, m/z
		UR-144		
despentylhydroxy-UR-144	258.1490	1.5	4.2–4.8	116, 125, 144, 160
dihydroxy-UR-144	344.2222	0.6	4.6–5.2	125, 144, 214, 230
despentyl-UR-144	242.1538	-0.4	5.03	116, 125, 144
dehydrated hydroxy-UR-144	326.2118	0.9	5.2–5.4	116, 144, 214
				97, 125, 144, 228
hydroxy-UR-144	328.2268	-0.9	5.2–5.5	97, 125, 230
				95, 144, 214
parent compound	312.2319	-1.0	5.81	125, 144, 214
		AM-2201		
N-(5-hydroxypentyl)-dihydrodiol-JWH-018	392.1859	0.8	4.2–4.4	115, 143, 171, 189, 230
				115, 143, 171, 230
dihydroxy-AM-2201	392.1659	0.8	4.5–5.0	115, 143, 171, 187, 248
				144, 159, 187, 232
				127, 155, 176, 264
dihydrodiol-AM-2201	394.1815	0.5	4.6–4.8	115, 143, 144, 171, 189, 232
				115, 143, 144, 171, 232
hydroxy-AM-2201	376.1711	1.1	4.6–5.1	115, 143, 171, 232
				127, 144, 155, 248
despentyl-AM-2201	272.1072	0.7	4.75	116, 127, 144, 155
N-(5-hydroxypentyl)-JWH-018	358.1804	0.6	5.12	127, 155, 230
JWH-018-*N*-pentanoic acid	372.1598	1.1	4.90	127, 144, 155
parent compound	360.1754	-1.1	5.31	127, 144, 155, 232

328 > 125 and 328 > 214, is quite unique and facilitates recognition of these metabolites in urine, are given in Figure 5. The product ion mass spectra of hydroxy UR-144 isomers are presented in Figures 5C1 and 5C2.

In case of AM-2201, there was also a good agreement between the *in vitro* and *in vivo* data, with the difference that *N*-desfluoropentyl metabolite was present in forensic urine samples only in trace amounts. In the urines positive for AM-2201 we have identified abundant monohydroxy and dihydrodiol metabolites, each as two isomers excreted as glucuronides to a large extent (more than 90%). It is worth noting that the abundance of dihydroxy metabolites was low *in vivo*. The amount of parent cannabinoid was at least 100 times lower than that of the metabolites, compared by peak area. As the metabolites of JWH-018 were also present in the samples analyzed, it was impossible to draw a conclusion whether they came from defluorination of AM-2201 or from the co-administered JWH-018. The mass chromatograms of the monohydroxy and dihydrodiol metabolites in urine are given in Figure 6. Similarly to UR-144, the presence of a low-abundance ion at *m/z* 144

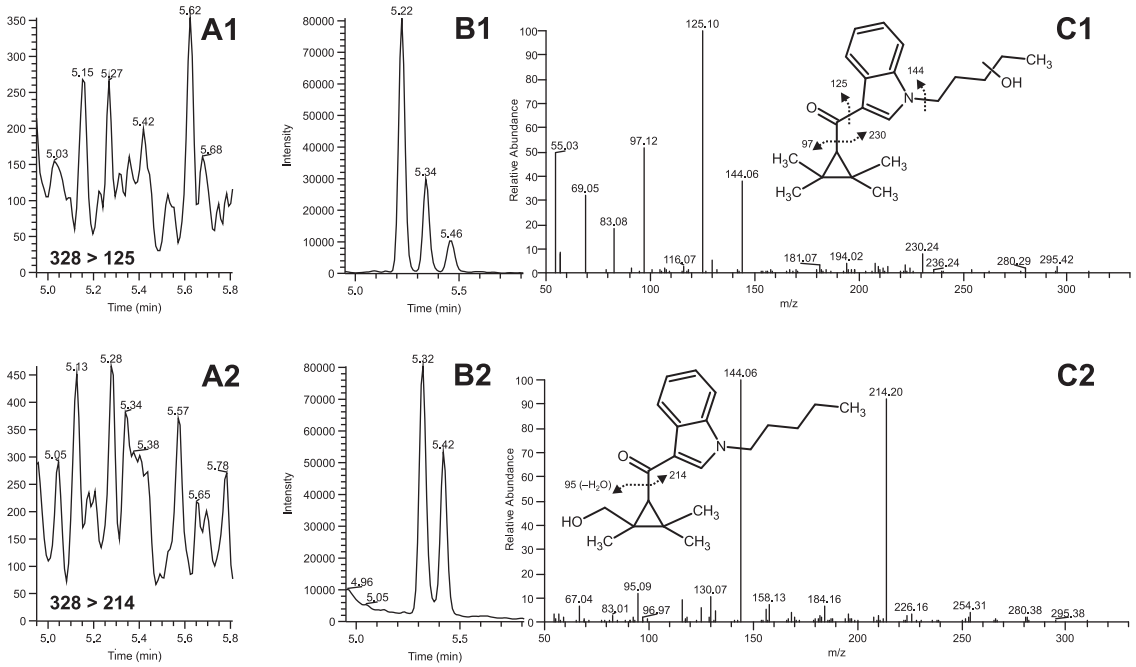

Figure 5. Mass chromatograms obtained for blank (A1, A2) and UR-144 post-administration (B1, B2) urines showing a unique pattern of monohydroxy-metabolites and their product ion ESI mass spectra (C1, RT = 5.22 min; C2, RT = 5.32 min) recorded at 25 eV. Urine (B) collected approximately 1 week after smoking session.

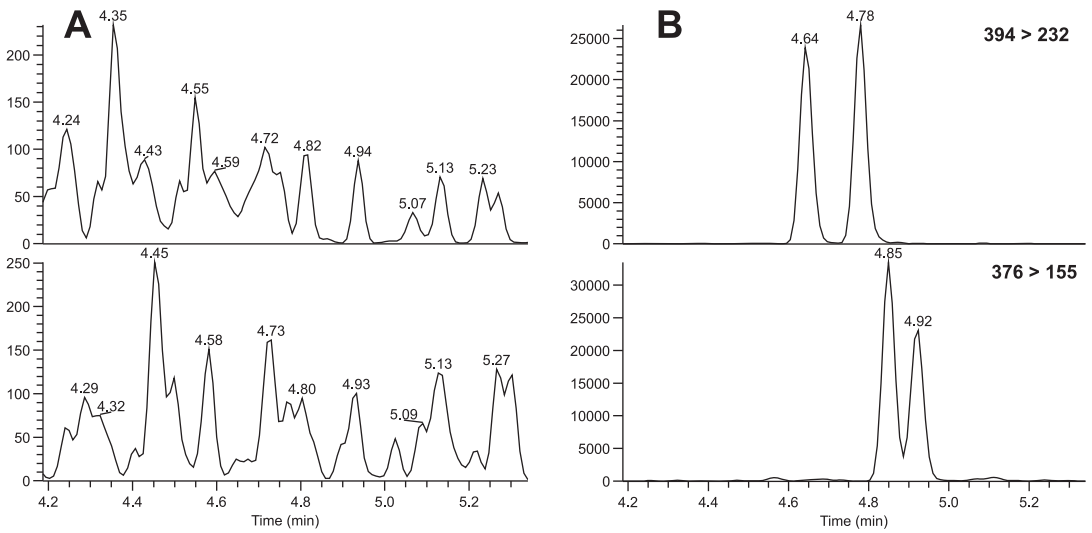

Figure 6. Mass chromatograms plotted for SRM transitions corresponding to dihydrodiol and monohydroxy metabolites of AM-2201 in blank (A) and post-administration (B) urines. Urine (B) collected approximately 1 week after smoking session.

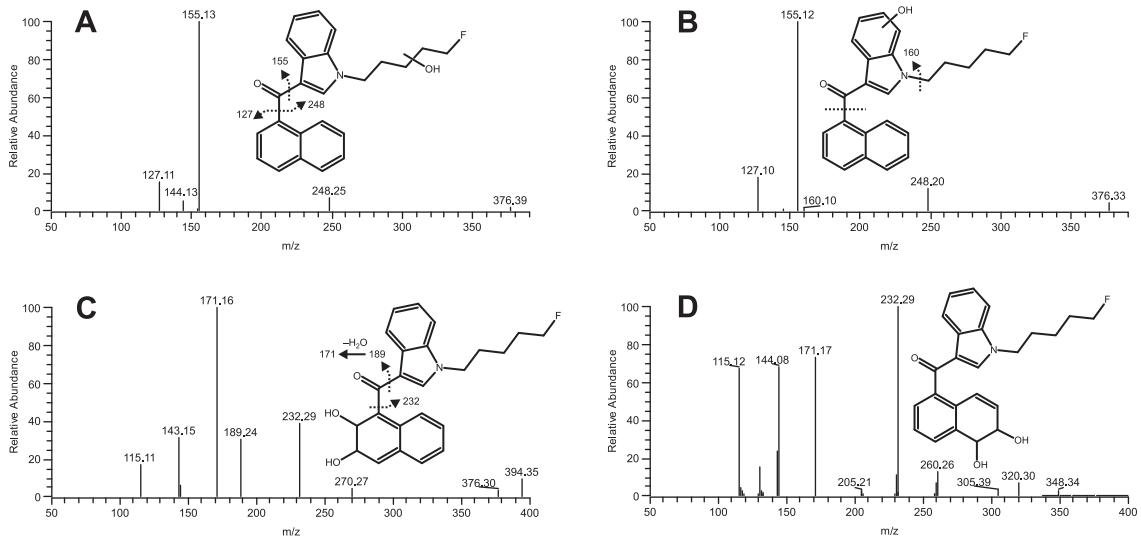

Figure 7. Product ion ESI mass spectra (recorded at 20 eV) and proposed structure of monohydroxy (A, B; RT = 4.85 min and 4.92 min), and dihydrodiol (C, D; RT = 4.64 and 4.78 min) metabolites of AM-2201.

in mass spectrum of the early eluting monohydroxy-metabolite of AM-2201 (Figure 7A) suggests that hydroxyl is located at *N*-fluoropentyl side chain, while the ion at *m/z* 160 is indicative of the hydroxyindole metabolite (Figure 7B). The ions at *m/z* 127, 155 typical of the intact naphthalene moiety[42] also support this finding.

The mass spectra of two isomeric dihydrodiol metabolites are given in Figues 7C and 7D. As one may see, the second mass spectrum is completely lacking the ion at *m/z* 189 which should correspond to (dihydroxydihydronaphthalenyl)oxomethylium, and only the ion at *m/z* 171 (loss of water from the former fragment) is detected. Possible explanation of this phenomenon is that the first metabolite could be 2,3-dihydroxy-2,3-dihydronaphthalenyl-AM-2201, whereas the second – 5,6-dihydroxy-5,6-dihydronaphthalenyl- or 7,8-dihydroxy-7,8-dihydronaphthalenyl-AM-2201. In 2,3-dihydrodiol the ion at *m/z* 189 may be stabilized by cyclization of 2-hydroxy group with carbonyl oxygen atom to give a substituted 1,2-dioxolane, while 5,6- or 7,8-dihydrodiols are more likely to eliminate water resulting in (5-oxo-5,8-dihydronaphthalen-1-yl) or (8-oxo-5,8-dihydronaphthalen-1-yl) oxomethylium ion.

Conclusion

The metabolism of two novel synthetic cannabinoids, UR-144 and AM-2201, was studied. In general, there was a good agreement between the *in vitro* and *in vivo* data, thus demonstrating the importance and applicability of simulated metabolic reactions with human liver microsomes when the new compounds need to be implemented into the existing analytical methods. To the best of our knowledge, the urinary metabolites of these cannabimimetics in human have been reported for the first time. Both compounds are subject to extensive metabolism which mainly includes hydroxylation followed by conjugation with glucuronic acid. The monohydroxy metabolites were shown to be the most valuable for the doping control or toxicological analysis due to their relatively high abundance and ease of detection in the post administration urine samples.

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