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Screening for the synthetic cannabinoid JWH-018 and its major metabolites in human doping controls

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Referred to as 'spice', several new drugs, advertised as herbal blends, have appeared on the market in the last few years, in which the synthetic cannabinoids JWH-018 and a C_8 homologue of CP 47,497 were identified as major active ingredients. Due to their reported cannabis-like effects, many European countries have banned these substances. The World Anti-Doping Agency has also explicitly prohibited synthetic cannabinoids in elite sport in-competition. Since urine specimens have been the preferred doping control samples, the elucidation of the metabolic pathways of these substances is of particular importance to implement them in sports drug testing programmes. In a recent report, an *in vitro* phase-I metabolism study of JWH-018 was presented yielding mainly hydroxylated and *N*-dealkylated metabolites. Due to these findings, a urine sample of a healthy man declaring to have smoked a 'spice' product was screened for potential phase-I and -II metabolites by high-resolution/high-accuracy mass spectrometry in the present report. The majority of the phase-I metabolites observed in earlier *in vitro* studies of JWH-018 were detected in this urine specimen and furthermore most of their respective monoglucuronides. As no intact JWH-018 was detectable, the monohydroxylated metabolite being the most abundant one was chosen as a target analyte for sports drug testing purposes; a detection method was subsequently developed and validated in accordance to conventional screening protocols based on enzymatic hydrolysis, liquid-liquid extraction, and liquid chromatography/electrospray tandem mass spectrometry analysis. The method was applied to approximately 7500 urine doping control samples yielding two JWH-018 findings and demonstrated its capability for a sensitive and selective identification of JWH-018 and its metabolites in human urine.

Keywords: cannabimimetics; aminoalkylindole; designer drugs; exactive; sport

Introduction

In the last few years, several different herbal blends have appeared on the market referred to as 'spice'. Although declared as exotic incense and not for human consumption, they were smoked by some consumers and cannabis-like effects were described.[1] As most of the labeled potentially psychoactive herbal ingredients are unverifiable in these products, the psychotropic effects could hardly be traced back to the plants.[2] Therefore it was assumed that added synthetic cannabinoids, not reported on the label, were responsible for these biological and psychological effects. In 2008, a C_8 homologue of the synthetic cannabinoid CP 47,497[3,4] as well as the new psychoactive substance JWH-018[5] were identified as active ingredients. Hence, many European countries took legal action to ban or control these substances and some of their related compounds in 2009.[2]

Several non-traditional cannabinoids (e.g. 3-arylcyclohexanols and aminoalkylindoles) have been discovered in the past few decades which, despite no obvious structural similarity to classic cannabinoids, possess binding capacity to the cannabinoid receptors CB_1 and CB_2 and cause cannabinomimetic responses in mice.[6–8] JWH-018 (1-pentyl-3-(1-naphthoyl)indole) belongs to the aminoalkylindole family having approximately a four-fold increased affinity to the CB_1 and about a ten-fold affinity to the CB_2 receptor compared with Δ^9-tetrahydrocannabinol (THC).[3]

Cannabinoids have been classified as doping substances by the World Anti-Doping Agency (WADA) under paragraph S8[9] because reducing stress and anxiety as well as potential misjudgment during competition is considered to be beneficial or hazardous, respectively, in some sports.[10] Since synthetic cannabinoids such as JWH-018, JWH-073, JWH-200, JWH-250, the C_8 homologue of CP-47,497 and HU-210 (Figure 1) have also shown high binding affinity to the cannabinoid receptors,[6,11–14] they are consequently regarded as relevant to doping controls.

However, for the implementation of these substances into doping control screening procedures, the knowledge of their respective metabolism is essential as urine specimens are the most frequently provided doping control samples and the substances undergo metabolization in the body before being excreted.

In an *in vivo* rat metabolism study of JWH-018 Krämer *et al*. reported hydroxylated *N*-dealkylated species being the main metabolites whereas the parent compound as well as the *N*-dealkylated metabolite could only be detected in small

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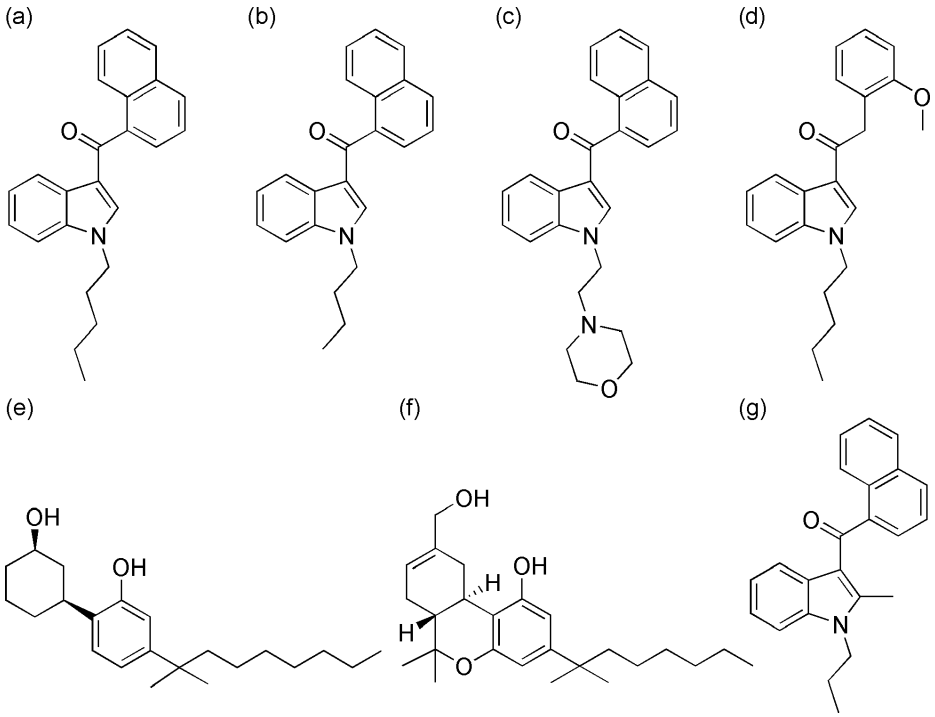


Figure 1. Structures of some synthetic cannabinoids: (a) JWH-018; (b) JWH-073; (c) JWH-200; (d) JWH-250; (e) C_8 homologue of CP 49,497; (f) HU-210; and the utilized internal standard JWH-015 (g).

amounts.[15] An *in vitro* metabolism experiment[16] using human liver microsomal fraction identified analogously to the *in vitro* metabolism of JWH-015[17] hydroxylated compounds as main metabolites and also *N*-dealkylated species to a moderate extend. Furthermore, a carboxy metabolite of JWH-018 was also observed. In a very recent report, monohydroxylated and predominantly glucuronidated metabolites of JWH-018 were described as the main metabolic products found in post-administration human urine samples.[18] In the present report, a urine specimen of a 'spice' user was screened for potential phase-I and phase-II metabolites using high-resolution/high-accuracy mass spectrometry; routine doping control screening protocols were subsequently extended to enable the detection of JWH-018 and its major metabolites. The method was validated for JWH-018 and the performance estimated for the monohydroxylated metabolite, which has served as a target analyte in a routine initial testing procedure.

Experimental

Chemicals and reference substance

Sodium phosphate dibasic (Na_2HPO_4), potassium phosphate monobasic trihydrate (KH_2PO_4), potassium carbonate (K_2CO_3), potassium hydrogen carbonate ($KHCO_3$), ammonium acetate, iodomethane and acetic acid were purchased from Merck (Darmstadt, Germany). Methanol and acetonitrile (both high performance liquid chromatography (HPLC) grade) were obtained from VWR (Leuven, Belgium) and Honeywell (Seelze, Germany) respectively; *tert.*-butyl methyl ether from AppliChem (Darmstadt, Germany); and β-glucuronidase (*E. coli*) from Roche Diagnostics (Mannheim, Germany). OASIS HLB solid-phase extraction (SPE) cartridges (60 mg, 3 mL) were purchased from Waters (Eschborn, Germany). The reference substance JWH-018 was supplied by LGC Standards (Wesel, Germany) and the internal standard JWH-015 by Sigma-Aldrich (Steinheim, Germany). All chemicals were used in the highest quality available and de-ionized water used for aqueous buffers and solutions was of Milli-Q grade.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The urine specimens were analyzed using a Thermo Accela LC coupled to a Thermo Exactive mass spectrometer (Bremen, Germany). The LC was equipped with a Thermo Scientific Hypersil GOLD column (2.1 × 50 mm, particle size 1.9 µm) and 5 mM ammonium acetate containing 0.1% acetic acid (A) and methanol (B) as solvents. The gradient at a flow rate of 200 µL/min decreased from 90% A to 20% A within 12 min and then to 0% A in a further 3 min. After maintaining 0% A for another 3 min, the column was re-equilibrated for 6 min at starting conditions. The mass spectrometer, operated in positive ionization mode, was calibrated using the manufacturer's calibration mixture (seven reference masses) and mass accuracies <5 ppm were accomplished for the period of analysis. The ionization voltage was set to $+3.5$ kV and the capillary was heated to 275 °C. Consistently, three MS settings were used: a) full scan MS from *m/z* 100–800 at a resolution of 50 000 (full width at half maximum), b) full scan MS (*m/z* 100–800 at a resolution of 25 000) with higher-energy collision-induced dissociation (HCD) set to 20 V, c) full scan MS (*m/z* 100–800, resolution of 25 000) with HCD set to 50 V. Nitrogen for the curved linear ion trap was obtained from a CMC nitrogen generator (CMC Instruments, Eschborn, Germany).

Further characterization of JWH-018 and its main metabolites by means of MS/MS- and MS^3-experiments was performed using an Agilent 1100 Series LC (Waldbronn, Germany) coupled to an Applied Biosystems API4000 Qtrap mass spectrometer (Darmstadt, Germany) with electrospray ionization (ESI) and identical LC

conditions. The ion source was operated in the positive mode at 450 °C using a spray voltage of 5000 V and nitrogen, obtained from a nitrogen generator (CMC Instruments, Eschborn, Germany), was employed as curtain and collision gas (5×10^{-3} Pa).

Validation of the routine doping control procedure was conducted using the aforementioned LC-MS system consisting of an Agilent 1100 Series LC and an API4000 Qtrap and a Kinetex C18 column (2.1 × 100 mm, particle size 2.6 µm) with a flow rate of 200 µL/min. The gradient started at 90% A, decreased firstly to 20% A in 12 min, then in a further 3 min to 0% A and maintained there for another 3 min followed by re-equilibration for 6 min. The injection volume was set to 2 µL and the characteristic product ions of JWH-018 and JWH-015 (ISTD) were detected using the multiple reaction monitoring mode (MRM). The respective ion transitions are summarized in Table 1.

Urine sample preparation

The sample preparation was performed according to established doping control screening procedures to allow a rapid implementation of the new target analyte into existing protocols.[19] Briefly, 1 mL of urine, fortified with 20 ng of JWH-015 as internal standard (20 µL of a 1 ng/µL solution), was buffered to pH 7.0 (0.8 M phosphate buffer) and hydrolyzed using β-glucuronidase. After incubation at 50 °C for 1 h and adjusting the pH to 9.6 with an aqueous solution containing potassium carbonate and potassium hydrogen carbonate (20%, 1 : 1, *w/w*), the solution was extracted with 4 mL of *tert.*-butyl methyl ether. The organic layer was transferred into a fresh glass tube and evaporated to dryness. The dry residue was reconstituted in 100 µL of water/acetonitrile (4 : 1, *v/v*), transferred to HPLC vials and a total of 5 µL was injected into the LC-MS/MS system.

In order to obtain information on potentially formed phase-II metabolites, 2 mL of urine and 20 ng of the internal standard were loaded on an OASIS HLB SPE cartridge preconditioned with 2 mL of methanol and water respectively. After washing with 2 mL of water, the analytes were eluted with 1.5 mL of methanol into a polypropylene tube and the solvent was evaporated in a vacuum centrifuge. Reconstitution is performed as aforementioned.

To further confirm the locations of modifications especially hydroxylations carboxyl, phenolic hydroxyl, and amino groups were selectively methylated by means of iodomethane in the presence of a base[20] whereas aliphatic hydroxyl groups remained unchanged. Here, 2 mL of urine and 20 ng of the internal standard were enzymatically hydrolyzed, liquid-liquid extracted and evaporated to dryness as described above. The dry residue was reconstituted in 180 µL of acetonitrile and 20 µL of iodomethane as well as a spatula tip of potassium carbonate were added. The mixture was heated at 50 °C under constant stirring and after 3 h, the solution was evaporated to dryness and the residue resuspended in 50 µL of acetonitrile for LC-MS/MS analysis.

Stock and working solutions

The stock solutions of JWH-018 and the internal standard were prepared at 1 mg/mL in methanol and stored at +4 °C. Over a period of 4 weeks, no degradation of the analytes was observed. The working solutions for validation purposes were freshly prepared prior to use at concentrations between 0.1 and 10.0 µg/mL.

Assay validation

Validation for qualitative purposes was performed considering the parameters specificity, recovery, limit of detection, linearity, intra-day and inter-day precision according to guidelines of the International Conference on Harmonisation (ICH).[21]

Table 1. Elemental compositions of JWH-018 and its urinary metabolites using high resolution/high-accuracy mass spectrometry. The relationship between precursor and diagnostic product ions was determined by MS/MS experiments on an API4000 Qtrap mass spectrometer, ion transitions used for validation purposes are written in bold text

Cmp.	Precursor ion (*m/z*)	Elemental comp. (exp.)	Error (ppm)	Observed characteristic product ions (*m/z*)
JWH-018	342.1852	$C_{24}H_{24}NO$	−0.0	272, **214, 155, 144, 127**
M1	358.1802	$C_{24}H_{24}NO_2$	0.1	340, 272, **230, 155, 144, 127**
M2	358.1802	$C_{24}H_{24}NO_2$	0.2	214, 171, 144, 143
M3	358.1802	$C_{24}H_{24}NO_2$	−0.3	288, 230, 160, 155, 127
M4	374.1750	$C_{24}H_{24}NO_3$	−0.1	246, 155, 144, 127
M5	374.1751	$C_{24}H_{24}NO_3$	0.1	288, 246, 160, 155, 127
M6	374.1749	$C_{24}H_{24}NO_3$	−0.5	288, 230, 171, 144, 143
M7	374.1752	$C_{24}H_{24}NO_3$	0.3	230, 171, 160, 143
M8	390.1700	$C_{24}H_{24}NO_4$	0.0	304, 288, 262, 246, 230, 187, 176, 171, 160, 155, 144, 143, 127
M9	376.1907	$C_{24}H_{26}NO_3$	0.0	358, 214, 189, 171, 144, 143
M10	392.1856	$C_{24}H_{26}NO_4$	0.0	374, 230, 189, 171, 144, 143
M11	408.1806	$C_{24}H_{26}NO_5$	0.0	390, 306, 246, 230, 189, 171, 160, 144, 143
M12	356.1646	$C_{24}H_{22}NO_2$	0.4	228, 155, 144, 127
M13	372.1594	$C_{24}H_{22}NO_3$	0.0	288, 171, 144, 143
M14	372.1595	$C_{24}H_{22}NO_3$	0.2	288, 244, 160, 155, 127
M15	372.1595	$C_{24}H_{22}NO_3$	0.3	272, 244, 155, 144, 127
M16	372.1594	$C_{24}H_{22}NO_3$	0.2	244, 155, 144, 127
M17	288.1019	$C_{19}H_{14}NO_2$	−0.1	160, 155, 127
M18	288.1019	$C_{19}H_{14}NO_2$	−0.1	171, 144, 143
M19	306.1124	$C_{19}H_{16}NO_3$	−0.2	288, 189, 171, 144, 143

As the monohydroxylated target analyte is, to our knowledge, not commercially available, the method was validated for the parent compound as a representative of 1-alkyl-3-(1-naphthoyl)indole-based compounds allowing the estimation of the assay performance for structurally related substances such as the target metabolite.

Specificity

Ten different blank urine samples obtained from five healthy male and female volunteers, respectively, were prepared as described above and analyzed with LC-MS in order to check for interfering peaks at the respective retention times of JWH-018 and its most abundant metabolite used as target analyte as well as the ISTD. The employed ion transitions are listed in Table 1.

Recovery

The recovery of JWH-018 was calculated by comparison of mean peak area ratios of analyte and ISTD of a total of 20 urine samples at a concentration of 100 ng/mL. Ten specimens were fortified with JWH-018 before sample preparation whereas at the other ten specimens, the analyte was added into the final *tert.*-butyl methyl ether extract.

Lower limit of detection (LLOD)

The LLOD is defined as the 'lowest content that can be measured with reasonable statistical certainty'[22] at a signal-to-noise ratio $\geq$3. Ten urine specimens were fortified with 1 ng/mL of JWH-018 and another ten samples were spiked with the ISTD only. All specimens were prepared and analyzed as described above in order to provide the data necessary to estimate the LLOD. The injection volume was set to 5 µL.

Linearity

In order to cover a wide concentration range, the linearity of the method was determined with fortified samples (n = 6) at concentrations of 5, 25, 50, 100, 150, and 250 ng/mL.

Intra-day and inter-day precision

The intra-day and inter-day precisions were calculated from six urine samples of low (20 ng/mL), medium (100 ng/mL) and high (200 ng/mL) concentrations of JWH-018, respectively, which were prepared and analyzed on three consecutive days (n = 18 + 18 + 18). Further to these, the post-administration urine specimen was prepared and analyzed on three consecutive days using six replicates each (n = 6 + 6 + 6).

Test for ion suppression/enhancement effects

In order to probe for a possible influence of matrix effects on JWH-018, remarkable especially when employing ESI, five different blank urine samples and solvent only were analyzed with continuous co-infusion of JWH-018 (solution concentration 0.5 mg/L, flow rate 5 µL/min) using a post-column T-connector.[23,24]

Authentic urine specimens

Proof-of-concept for the applicability of the validated assay was obtained by analyzing a spot urine sample obtained from a healthy male individual declaring to have smoked a 'spice'-enriched cigarette 12 h prior to urine collection. Written consent was obtained and the sample analyzed using the above-reported approach. Finally, the established method was applied to routine doping controls and approximately 7500 urine specimens were tested for the presence of JWH-018 and its metabolic products.

Results and discussion

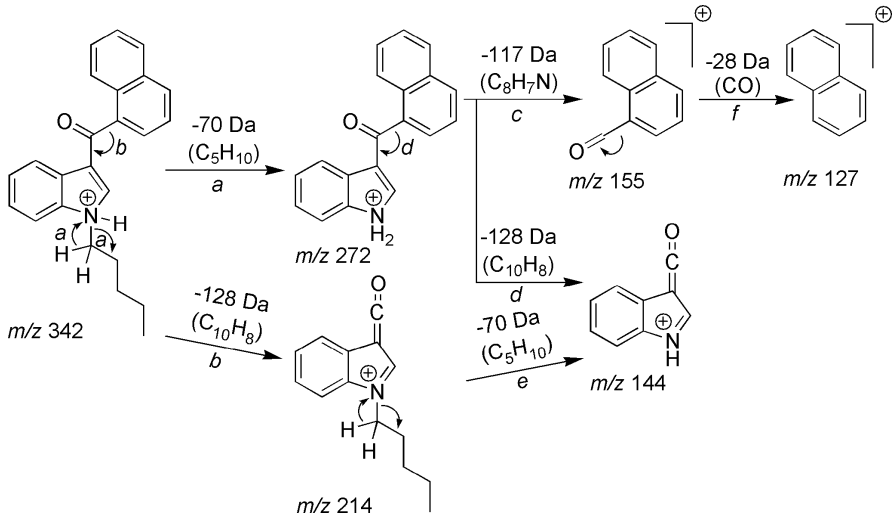
Mass spectrometry

The mass spectrometric behaviour of JWH-018 under positive ESI and collision-induced dissociation (CID) conditions was shown by Auwärter *et al.*[25] and yielded characteristic product ions at *m/z* 214, 155, 144, and 127, which were further confirmed in the present study using high-resolution/high-accuracy (tandem) mass spectrometry. The origin of *m/z* 272 (−70 Da, Figure 2a) was attributed to the loss of the *N*-alkyl side chain releasing pent-1-ene (Scheme 1, route *a*) as supported by its measured elemental composition (see Supplemental Material). The fragment ion is of low abundance and assumed to be rapidly cleaved at either side of the alpha carbon of the carbonyl group resulting in the product ions at *m/z* 144 and 155 by the elimination of naphthalene and indole, respectively. The latter ion represents the most abundant signal under the chosen CID conditions and was shown to generate the product ion at *m/z* 127 by the loss of carbon monoxide in MS^3 experiments (Scheme 1, routes *c*, *d*, *f*). The initial elimination of naphthalene (128 Da) from the protonated molecule gives rise to the product ion at *m/z* 214 (Scheme 1, route *b*) yielding, after the loss of the *N*-alkyl side chain, also the fragment at *m/z* 144 (Scheme 1, route *e*). These characteristic product ions were employed for screening *in vitro* metabolism and urine samples for the presence of metabolites and identifying the locations of modifications such as hydroxylations, oxidations, etc.

In a recent *in vitro* metabolism study of JWH-018 with human liver microsomal fractions, various hydroxylated derivatives of JWH-018 were reported as main metabolites.[16] Several mono- (*m/z* 358), bis- (*m/z* 374) and trishydroxylations (*m/z* 390) were detected as well as arene oxidation leading to two dihydrodiols (*m/z* 376) and the respective mono- (*m/z* 392) and bishydroxylated dihydrodiols (*m/z* 408) with the monohydroxylated species being the most abundant metabolites. Furthermore dehydrogenation (*m/z* 340), dehydrogenation and monohydroxylation (*m/z* 356) as well as bishydroxylation (*m/z* 372) were observed. A carboxy metabolite (*m/z* 372) was detected as well as the *N*-dealkylated JWH-018 (*m/z* 272), which could also be monohydroxylated at different positions (*m/z* 288) and oxidized at the arene residue leading to two *N*-dealkylated dihydrodiols (*m/z* 306).

Some of these *in vitro*-derived metabolites were identified in human urine samples after administration of a 'spice' smoking mixture.[18] Two monohydroxylations (*m/z* 358) were reported as main metabolites and furthermore a carboxy metabolite (*m/z* 372), bishydroxylations (*m/z* 374), dihydrodiol formations (*m/z* 376) also in combination with monohydroxylation (*m/z* 392), as well as *N*-dealkylation and monohydroxylation (*m/z* 288).

Due to the relevance of cannabinoids for in-competition doping controls, currently established methods should be expanded to these newly identified and investigated compounds. Therefore, a



Scheme 1. Proposed dissociation pathways of JWH-018 after positive ESI and CID.

urine specimen of a healthy male person declaring to have smoked 'spice' presumably containing JWH-018 was analyzed and tested for target analytes that are potentially useful for sports drug testing purposes. On the basis of the determination of accurate masses of the intact molecules as well as their corresponding diagnostic product ions, the presence of all phase-I metabolites identified in earlier *in vitro* metabolism studies[16] (except for the dehydrogenated and the *N*-dealkylated metabolite) were confirmed in this urine specimen (Figure 3) with the monohydroxylation (M1) being the most abundant metabolite. However, in contrast to *in vitro* metabolism experiments[16] that yielded various different monohydroxylated species, only one hydroxylated metabolite was identified in the human urine sample in appreciable intensity (Figure 4b top chromatogram, and Figure 5b). Recently reported data obtained from administration studies[18] also described one major assigned to monohydroxylated JWH-018 bearing the hydroxyl group at the indole residue. Although the obtained product ion spectra are very similar, the results of the present study suggest the alkyl side chain as position of hydroxylation for this abundant metabolite (M1): Comparing the product ion mass spectra of JWH-018 (Figure 2a) and the hydroxylated analog (Figure 2b), only selected characteristic product ions are incremented by 16 mass units. While the ion at *m/z* 155, which represents the naphthalene moiety, remains unchanged, the ion at *m/z* 230 (214 + 16) indicates the hydroxylation of either the indole residue or the alkyl side chain. The observed dehydratization reaction of the protonated molecule (*m/z* 358) leading to *m/z* 340 as well as the presence of *m/z* 272, which corresponds to the unmodified combined ring systems (Scheme 1), indicate hydroxylation of the alkyl side chain. This assumption is further supported by the observed fragment ion at *m/z* 284 (Figure 2b), which is attributed to the loss of butanol from the protonated molecule of M1 as corroborated by its measured elemental composition ($C_{20}H_{14}NO$: exact mass: 284.1070, determined mass: 284.1071, error: 0.3 ppm). The retention of the hydroxyl function in *m/z* 284 as suggested in an earlier report[18] is consequently not very likely. Additional evidence for the introduction of the hydroxyl group in the alkyl side chain was obtained by site-specific methylation reactions. These allow methylation of phenolic hydroxyl, carboxyl and amino groups in contrast to aliphatic hydroxyl groups, and M1 remained unchanged under the employed conditions substantiating suggested location of hydroxylation.

At least seven additional monohydroxylations (*m/z* 358) were observed but were of considerably lower intensity. A more precise determination of the location of the hydroxylation (naphthalene moiety, indole residue or alkyl side chain) is enabled by monitoring which characteristic product ions for the respective parts of the molecule are incremented by 16 mass units and site-specific methylation experiments. In this way, three monohydroxylations at the alkyl side chain (M1) and the naphthalene moiety (M2) respectively as well as two monohydroxylations at the indole residue (M3) were identified. Analogously, other metabolites were studied but with an increasing number of modifications the characterization of single metabolites becomes considerably more complex. Bishydroxylation (*m/z* 374) was observed with at least four metabolites comprising two hydroxyl functions at the alkyl side chain (M4), one hydroxyl moiety each at the alkyl side chain and the indole residue (M5), the alkyl side chain and the naphthalene moiety (M6) or the indole residue and the naphthalene moiety (M7) respectively. Furthermore, numerous trishydroxylations (M8, *m/z* 390) with hydroxylations at different positions of the molecule were observed but the individual metabolites were not clearly differentiable due to their highly similar LC behaviour and resulting insufficient separation.

Comparable to the *in vitro*[16] as well as the post-administration urine studies[18] two peaks for dihydrodiols (M9, *m/z* 376) and also monohydroxylated dihydrodiol metabolites (M10, *m/z* 392) were detected. For the latter a minimum of four peaks was found, all of which were also observed unchanged after methylation. In combination with detected characteristic product ions (*m/z* 230 – monohydroxylation at the indole residue or the alkyl side chain, *m/z* 144 – no modification at the indole residue, Table 1) the alkyl side chain was concluded as hydroxylation position for these metabolites in contrast to earlier published results.[18] In analogy to the *in vitro* experiments[16] but not yet reported for post-administration urine samples,[18] the bishydroxylated dihydrodiol of JWH-018 (M11, *m/z* 408) and at least three peaks for M12 (*m/z* 356, dehydrogenation and monohydroxylation both at the alkyl side chain) were observed as well as seven peaks representing dehydrogenation and bishydroxylation (*m/z* 372) of JWH-018. While the double bond is located at the alkyl side chain in all

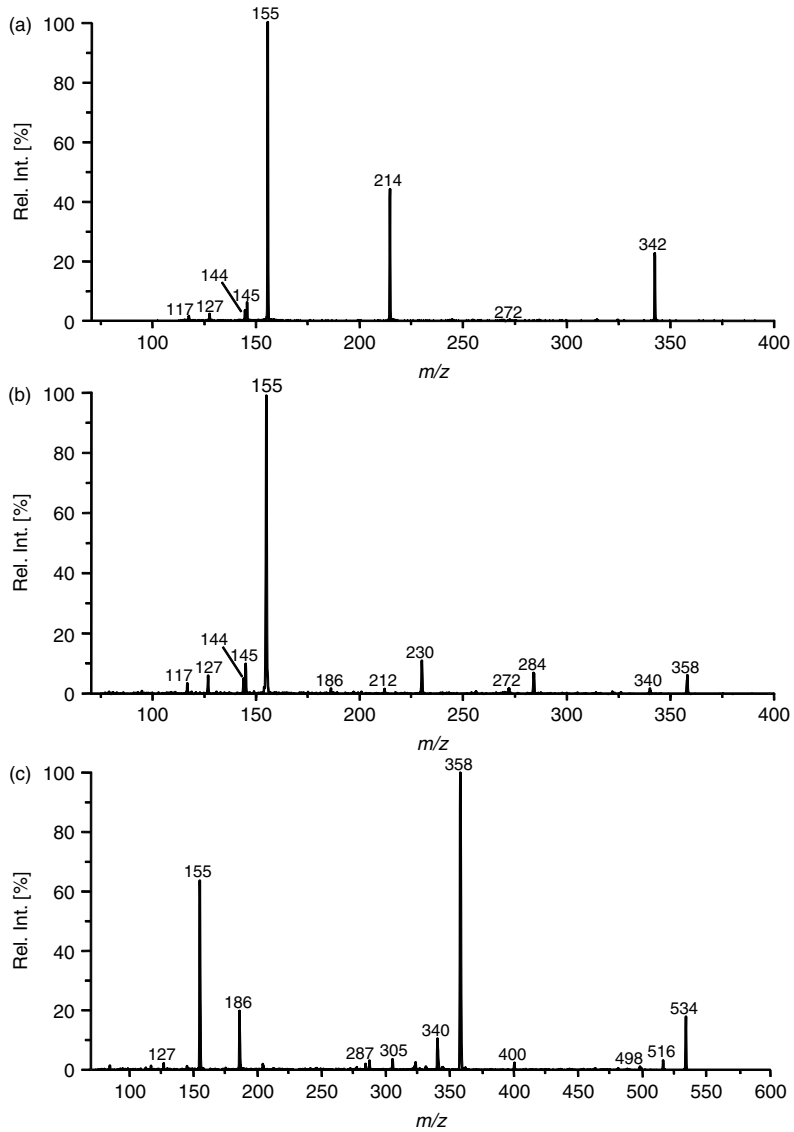


Figure 2. ESI product ion spectra of $[M+H]^+$ ions of (a) JWH-018; (b) monohydroxylated phase-I metabolite (M1); and (c) monoglucuronide of M1, recorded on an API4000 Qtrap mass spectrometer.

cases, four metabolites are hydroxylated once each at the alkyl side chain and the naphthalene moiety (M13), one at the alkyl side chain and the indole residue (M14) and two metabolites (M15) twice at the alkyl side chain (Table 1). Furthermore the carboxy metabolite (M16) was also detected as previously described.[16,18] Monohydroxylations after the loss of the *N*-alkyl side chain (*m/z* 288) were difficult to detect as endogenous compound(s) from urine interfered with the respective metabolites on most of the meaningful ion transitions. Only the ion transitions 288-155 and 288-127 (corresponding to an unchanged naphthalene moiety) were not affected so that the two observed peaks were attributed to monohydroxylations at the indole residue (M17) which was also in agreement with results obtained from methylation experiments. Due to the loss of the *N*-alkyl side chain all hydroxylation reactions lead to phenolic hydroxyl groups and thus two further metabolites hydroxylated at the naphthalene moiety (M18) were detected by means of the methylation reaction, additionally to earlier published results from urine samples.[18] However, it has to be taken into consideration that these molecules have, after the loss of the *N*-alkyl side chain, an amino function that is also methylated under the chosen reaction conditions so that an additional mass shift by 14 is observed. This methylation of the amino group was also detected for the *N*-dealkylated dihydrodiol (M19, *m/z* 306) which has not yet been reported in urine samples either[18] yielding the characteristic product ion at *m/z* 158 (144 + 14). Comparable to M9, two peaks were observed corresponding (in analogy to the proposed metabolic pathway of JWH-015[17]) to the dihydrodiol at the upper (R) or lower ring (R′) of the naphthalene residue (Figure 3), respectively.

Moreover, the urine specimen, prepared without hydrolysis, was screened for potential phase-II metabolites. While no sulfates were observed, the monoglucuronides (Gluc.) of most of the identified phase-I metabolites were detected by means of their MS/MS spectra as well as exact masses (Table 2). As already reported for some metabolites,[18] the detected phase-I metabolites are excreted into urine as glucuronides to a large extent (Figure 4a). Except for the carboxy metabolite M16, which can be detected also in the sample prepared without hydrolysis (Figure 4a; ion trace

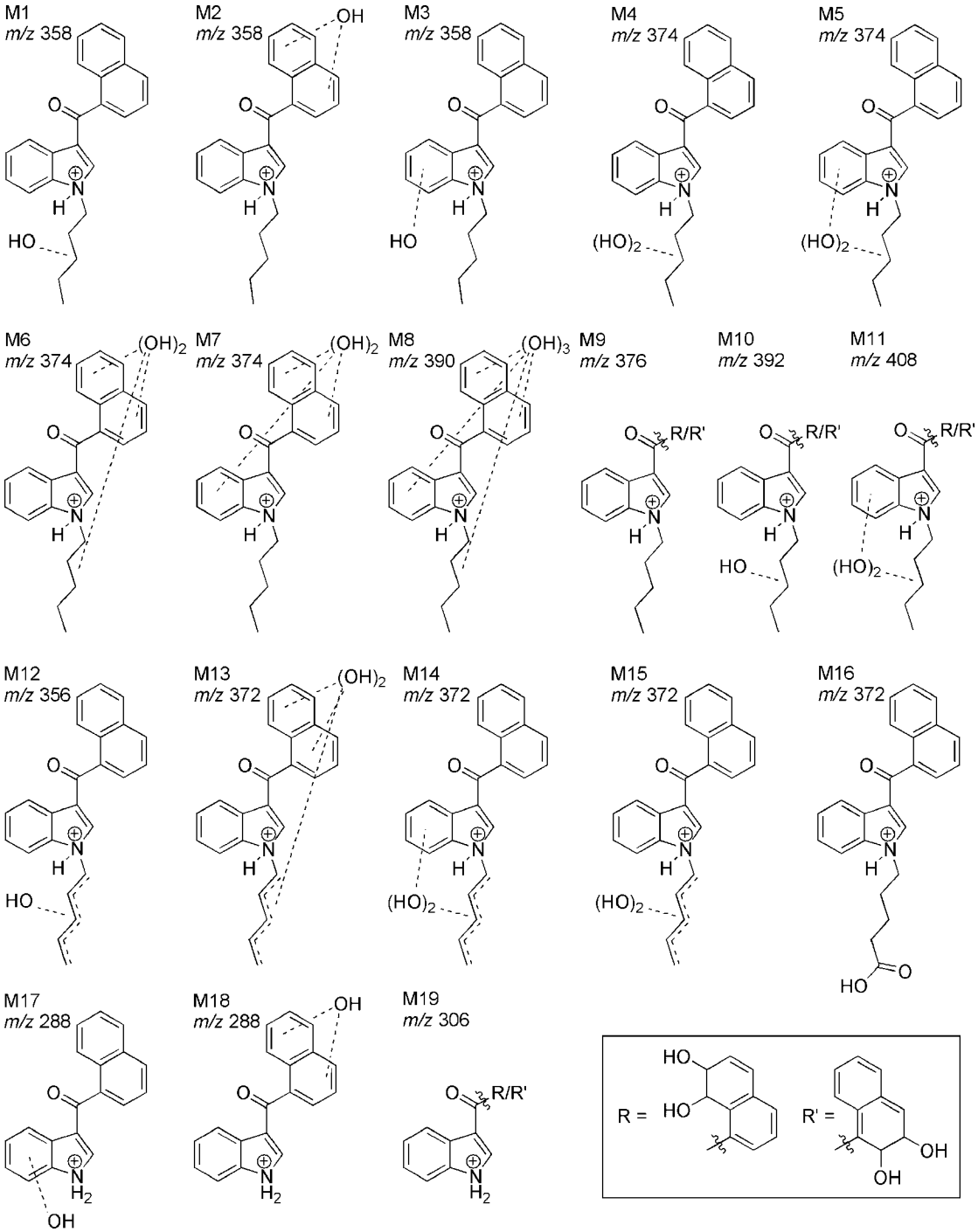


Figure 3. Structures of the major JWH-018 metabolites M1-M19 in analogy to the earlier proposed metabolic pathway of JWH-015.[17] The dihydrodiol in M9-M11 and M19 can be located at the upper (R) or lower ring (R′) of the naphthalene residue, respectively.

372, peak at 14.5 min), hardly any unconjugated metabolites were observed in the non-hydrolyzed urine sample. The glucuronides were completely converted into the respective aglycons by enzymatic hydrolysis with β-glucuronidase as illustrated in Figure 4b.

As an example, the product ion mass spectrum of the glucuronic acid conjugate of the most abundant metabolite M1 is shown in Figure 2c. Besides two dehydratization reactions forming to the product ions at *m/z* 516 and 498, the fragment at *m/z* 358 corresponding to the aglycon (M1) is observed as main product ion. In addition, the ions at *m/z* 340, 155 and 127 further substantiate the attributed structure of the glucuronide of M1 in accordance to the above-described results.

Additionally to the less abundant glucuronides of M1 two further but less abundant were detected as well as one glucuronide of M2 and M3, respectively. For the bishydroxylated product, four monoglucuronides of M4, one of M5 and further two of M6 (*m/z* 550) were identified whereas the different glucuronides of the

Table 2. Elemental compositions of urinary JWH-018 phase-II metabolites using high-resolution/high-accuracy MS. The relationship between precursor and diagnostic product ions was determined by MS/MS experiments on an API4000 Qtrap mass spectrometer and the accurate masses were confirmed using an Exactive mass spectrometer

Cmp.	Precursor ion (*m/z*)	Elemental comp. (exp.)	Error (ppm)	Product ion* (*m/z*)	Elemental comp. (exp.)	Error (ppm)	Cleaved species
Gluc. M1	534.2124	$C_{30}H_{32}NO_8$	0.2	516 (516.2011)	$C_{30}H_{30}NO_7$	−1.1	H_2O
				358 (358.1802)	$C_{24}H_{24}NO_2$	0.1	$C_6H_8O_6$
				340 (340.1695)	$C_{24}H_{22}NO$	−0.2	$C_6H_{10}O_7$
				272 (272.1068)	$C_{19}H_{14}NO$	−0.7	$C_{11}H_{18}O_7$
				230 (230.1173)	$C_{14}H_{16}NO_2$	−0.9	$C_{16}H_{16}O_6$
				155 (155.0491)	$C_{11}H_7O$	0.0	$C_{19}H_{25}NO_7$
				144 (144.0443)	C_9H_6NO	−0.3	$C_{21}H_{26}O_7$
				127 (127.0546)	$C_{10}H_7$	2.9	$C_{20}H_{25}NO_8$
Gluc. M2	534.2122	$C_{30}H_{32}NO_8$	−0.1	358 (358.1803)	$C_{24}H_{24}NO_2$	0.3	$C_6H_8O_6$
				288 (288.1020)	$C_{19}H_{14}NO_2$	0.2	$C_{11}H_{18}O_6$
				214 (214.1230)	$C_{14}H_{16}NO$	1.8	$C_{16}H_{16}O_7$
				171 (171.0442)	$C_{11}H_7O_2$	0.7	$C_{19}H_{25}NO_6$
				144 (144.0445)	C_9H_6NO	0.4	$C_{21}H_{26}O_7$
				143 (143.0493)	$C_{10}H_7O$	1.3	$C_{20}H_{25}NO_7$
Gluc. M3	534.2124	$C_{30}H_{32}NO_8$	0.2	516 (516.2023)	$C_{30}H_{30}NO_7$	1.1	H_2O
				358 (358.1803)	$C_{24}H_{24}NO_2$	0.4	$C_6H_8O_6$
				288 (288.1019)	$C_{19}H_{14}NO_2$	0.2	$C_{11}H_{18}O_6$
				230 (230.1176)	$C_{14}H_{16}NO_2$	0.1	$C_{16}H_{16}O_6$
				160 (160.0394)	$C_9H_6NO_2$	0.4	$C_{21}H_{26}O_6$
				155 (155.0492)	$C_{11}H_7O$	0.1	$C_{19}H_{25}NO_7$
				127 (127.0543)	$C_{10}H_7$	0.5	$C_{20}H_{25}NO_8$
Gluc. M4	550.2072	$C_{30}H_{32}NO_9$	0.1	374 (374.1753)	$C_{24}H_{24}NO_3$	0.7	$C_6H_8O_6$
				272 (272.1067)	$C_{19}H_{14}NO$	−1.0	$C_{11}H_{18}O_8$
				155 (155.0491)	$C_{11}H_7O$	0.0	$C_{19}H_{25}NO_8$
				144 (144.0444)	C_9H_6NO	0.1	$C_{21}H_{26}O_8$
				127 (127.0545)	$C_{10}H_7$	1.8	$C_{20}H_{25}NO_9$
Gluc. M5	550.2072	$C_{30}H_{32}NO_9$	0.0	374 (374.1749)	$C_{24}H_{24}NO_3$	−0.4	$C_6H_8O_6$
				246 (246.1122)	$C_{14}H_{16}NO_3$	−1.1	$C_{16}H_{16}O_6$
				155 (155.0491)	$C_{11}H_7O$	−0.6	$C_{19}H_{25}NO_8$
				127 (127.0544)	$C_{10}H_7$	1.5	$C_{20}H_{25}NO_9$
Gluc. M6	550.2074	$C_{30}H_{32}NO_9$	0.4	532 (532.1967)	$C_{30}H_{30}NO_8$	0.2	H_2O
				374 (374.1750)	$C_{24}H_{24}NO_3$	−0.3	$C_6H_8O_6$
				230 (230.1178)	$C_{14}H_{16}NO_2$	1.0	$C_{16}H_{16}O_7$
				171 (171.0441)	$C_{11}H_7O_2$	0.2	$C_{19}H_{25}NO_7$
				144 (144.0442)	C_9H_6NO	−1.0	$C_{21}H_{26}O_8$
				143 (143.0490)	$C_{10}H_7O$	−1.1	$C_{20}H_{25}NO_8$
Gluc. M8	566.2021	$C_{30}H_{32}NO_{10}$	0.1	390 (390.1702)	$C_{24}H_{24}NO_4$	0.4	$C_6H_8O_6$
				246 (246.1132)	$C_{14}H_{16}NO_3$	3.1	$C_{16}H_{16}O_7$
				187 (187.0391)	$C_{11}H_7O_3$	0.5	$C_{19}H_{25}NO_7$
				171 (171.0441)	$C_{11}H_7O_2$	0.2	$C_{19}H_{25}NO_8$
				155 (155.0492)	$C_{11}H_7O$	0.4	$C_{19}H_{25}NO_9$
				144 (144.0445)	C_9H_6NO	0.5	$C_{21}H_{26}O_9$
				143 (143.0492)	$C_{10}H_7O$	0.7	$C_{20}H_{25}NO_9$
				127 (127.0546)	$C_{10}H_7$	3.0	$C_{20}H_{25}NO_{10}$
Gluc. M9	552.2229	$C_{30}H_{34}NO_9$	0.2	516 (516.2016)	$C_{30}H_{30}NO_7$	−0.2	2x H_2O
				376 (376.1907)	$C_{24}H_{26}NO_3$	0.0	$C_6H_8O_6$
				358 (358.1803)	$C_{24}H_{24}NO_2$	0.4	$C_6H_{10}O_7$
				214 (214.1226)	$C_{14}H_{16}NO$	−0.1	$C_{16}H_{18}O_8$
				189 (189.0543)	$C_{11}H_9O_3$	−1.9	$C_{19}H_{25}NO_6$
				171 (171.0440)	$C_{11}H_7O_2$	−0.2	$C_{19}H_{27}NO_7$
				144 (144.0445)	C_9H_6NO	0.6	$C_{21}H_{28}O_8$
				143 (143.0493)	$C_{10}H_7O$	1.3	$C_{20}H_{27}NO_8$
Gluc. M10	568.2180	$C_{30}H_{34}NO_{10}$	0.5	392 (392.1855)	$C_{24}H_{26}NO_4$	−0.3	$C_6H_8O_6$
				374 (374.1749)	$C_{24}H_{24}NO_3$	−0.4	$C_6H_{10}O_7$
				230 (230.1172)	$C_{14}H_{16}NO_2$	−2.6	$C_{16}H_{18}O_8$
				189 (189.0546)	$C_{11}H_9O_3$	0.0	$C_{19}H_{25}NO_7$

Table 2. (*Continued*)

Cmp.	Precursor ion (*m/z*)	Elemental comp. (exp.)	Error (ppm)	Product ion* (*m/z*)	Elemental comp. (exp.)	Error (ppm)	Cleaved species
				171 (171.0439)	$C_{11}H_7O_2$	−0.6	$C_{19}H_{27}NO_8$
				144 (144.0445)	C_9H_6NO	0.6	$C_{21}H_{28}O_9$
				143 (143.0492)	$C_{10}H_7O$	0.3	$C_{20}H_{27}NO_9$
Gluc. M12	532.1968	$C_{30}H_{30}NO_8$	0.4	356 (356.1646)	$C_{24}H_{22}NO_2$	0.3	$C_6H_8O_6$
				272 (272.1063)	$C_{19}H_{14}NO$	−2.6	$C_{11}H_{16}O_7$
				228 (228.1024)	$C_{14}H_{14}NO_2$	2.1	$C_{16}H_{16}O_6$
				155 (155.0492)	$C_{11}H_7O$	0.1	$C_{19}H_{23}NO_7$
				144 (144.0443)	C_9H_6NO	−0.9	$C_{21}H_{24}O_7$
				127 (127.0545)	$C_{10}H_7$	2.4	$C_{20}H_{23}NO_8$
Gluc. M13	548.1913	$C_{30}H_{30}NO_9$	−0.3	530 (530.1806)	$C_{30}H_{28}NO_8$	−0.6	H_2O
				372 (372.1594)	$C_{24}H_{22}NO_3$	−0.1	$C_6H_8O_6$
				288 (288.1019)	$C_{19}H_{14}NO_2$	0.1	$C_{11}H_{16}O_7$
				228 (228.1023)	$C_{14}H_{14}NO_2$	1.8	$C_{16}H_{16}O_7$
				171 (171.0439)	$C_{11}H_7O_2$	−0.7	$C_{19}H_{23}NO_7$
				144 (144.0445)	C_9H_6NO	0.5	$C_{21}H_{24}O_8$
				143 (143.0493)	$C_{10}H_7O$	1.4	$C_{20}H_{23}NO_8$
Gluc. M14	548.1916	$C_{30}H_{30}NO_9$	0.2	530 (530.1813)	$C_{30}H_{28}NO_8$	0.6	H_2O
				372 (372.1598)	$C_{24}H_{22}NO_3$	1.1	$C_6H_8O_6$
				288 (288.1018)	$C_{19}H_{14}NO_2$	−0.2	$C_{11}H_{16}O_7$
				244 (244.0969)	$C_{14}H_{14}NO_3$	0.5	$C_{16}H_{16}O_6$
				155 (155.0491)	$C_{11}H_7O$	−0.1	$C_{19}H_{23}NO_8$
				127 (127.0546)	$C_{10}H_7$	2.7	$C_{20}H_{23}NO_9$
Gluc. M15	548.1915	$C_{30}H_{30}NO_9$	0.0	530 (530.1809)	$C_{30}H_{28}NO_8$	−0.2	H_2O
				372 (372.1598)	$C_{24}H_{22}NO_3$	1.1	$C_6H_8O_6$
				272 (272.1071)	$C_{19}H_{14}NO$	0.4	$C_{11}H_{16}O_8$
				244 (244.0967)	$C_{14}H_{14}NO_3$	−0.3	$C_{16}H_{16}O_6$
				155 (155.0491)	$C_{11}H_7O$	−0.1	$C_{19}H_{23}NO_8$
				144 (144.0444)	C_9H_6NO	0.0	$C_{21}H_{24}O_8$
				127 (127.0546)	$C_{10}H_7$	3.1	$C_{20}H_{23}NO_9$
Gluc. M17	464.1341	$C_{25}H_{22}NO_8$	0.2	288 (288.1019)	$C_{19}H_{14}NO_2$	0.0	$C_6H_8O_6$
				160 (160.0392)	$C_9H_6NO_2$	−0.6	$C_{16}H_{16}O_6$
				155 (155.0492)	$C_{11}H_7O$	0.1	$C_{14}H_{15}NO_7$
				127 (127.0545)	$C_{10}H_7$	2.2	$C_{15}H_{15}NO_8$
Gluc. M18	464.1342	$C_{25}H_{22}NO_8$	0.4	288 (288.1021)	$C_{19}H_{14}NO_2$	0.6	$C_6H_8O_6$
				171 (171.0440)	$C_{11}H_7O_2$	−0.2	$C_{14}H_{15}NO_6$
				144 (144.0444)	C_9H_6NO	0.2	$C_{16}H_{16}O_7$
				143 (143.0492)	$C_{10}H_7O$	0.6	$C_{15}H_{15}NO_7$
Gluc. M19	482.1447	$C_{25}H_{24}NO_9$	0.3	306 (306.1124)	$C_{19}H_{16}NO_3$	−0.1	$C_6H_8O_6$
				288 (288.1019)	$C_{19}H_{14}NO_2$	−0.1	$C_6H_{10}O_7$
				189 (189.0546)	$C_{11}H_9O_3$	−0.1	$C_{14}H_{15}NO_6$
				171 (171.0441)	$C_{11}H_7O_2$	0.0	$C_{14}H_{17}NO_7$
				144 (144.0445)	C_9H_6NO	1.0	$C_{16}H_{18}O_8$
				143 (143.0494)	$C_{10}H_7O$	1.8	$C_{15}H_{17}NO_8$

* Nominal mass recorded on an API4000 Qtrap (accurate mass, determined by an Exactive mass spectrometer)

trishydroxlations (M8) at *m/z* 566 were not clearly distinguishable (Table 2). At least three glucuronic acid conjugates of M9 (*m/z* 552), five of M10 (*m/z* 568) and two of M12 (*m/z* 532) were observed while no glucuronides of M11 were detected probably due to its low intensity. Besides the monoglucuronides of M13 (*m/z* 548, two peaks) and M14, two double peaks for M15 were observed with the two peaks probably corresponding to the two detected phase-I metabolites dehydrogenated and bishydroxylated at the alkyl side chain while the splitting in double peaks may be due to the glucuronidation of one of the two hydroxyl groups respectively or the formation of diastereomeric glucuronides. For the *N*-dealkylated and monohydroxylated species, two metabolites glucuronidated at a hydroxyl group at the indole residue (M17) as well as at least one peak for the glucuronide of M18 (*m/z* 464) were observed and also a minimum of two peaks corresponding to the monoglucuronide of M19 at *m/z* 482 (Table 2).

As the active compound JWH-018 was not detected in either the herein presented authentic urine specimen or in three post-administration urine samples,[18] information on the human metabolic pathway of JWH-018 is of particular importance to enable a mass spectrometric detection of JWH-018 abuse in urine samples. Hence, the monohydroxylated metabolite (M1) as most

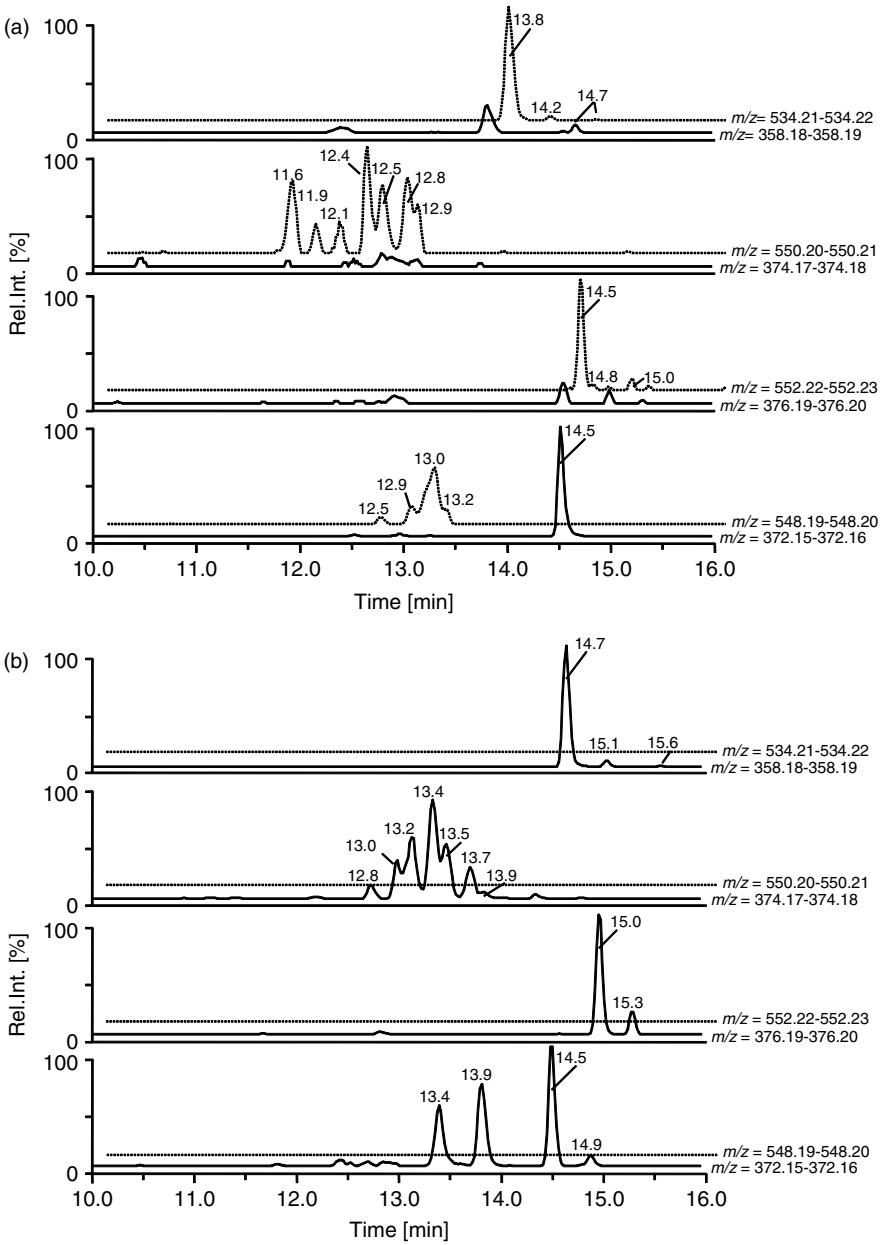


Figure 4. Extracted ion chromatograms recorded on a Thermo Exactive mass spectrometer using the high-resolution/high-accuracy MS data of the following phase-I metabolites (solid lines) with *m/z* 358.18–358.19 (corresponding to M1–3), 374.17–374.18 (M4–7), 376.19–376.20 (M9) and 372.15–372.16 (M13–16) as well as their respective monoglucuronides (dotted lines) with *m/z* 534.21–534.22, 550.20–550.21, 552.22–552.23 and 548.19–548.20 after analysis of the authentic urine sample of a 'spice' user without (a) and with (b) enzymatic hydrolysis.

abundant urinary metabolite was chosen and implemented as target analyte into routine screening procedures that include an enzymatic hydrolysis step.

Assay validation

Based on the mass spectrometric data, a method to detect JWH-018 and its metabolites in urine samples was established and validated using positive ESI and MRM enabling a sensitive determination of these analytes. As the most abundant urinary metabolite (M1) serving as a target analyte for doping control purposes is not commercially available, assay validation was performed with the active compound JWH-018, largely mimicking the physicochemical properties of 1-alkyl-3-(1-naphthoyl)indole substances and serving as a representative of this class of compounds. However, the validation data presented for JWH-018 should be considered as estimates for M1 only (Table 3).

Specificity, LLOD, linearity, and recovery

The specificity of the method was demonstrated as no interfering peaks were detected at expected retention times of JWH-018, M1 and the ISTD using extracted ion chromatograms of diagnostic ion transitions (Table 1) in all blank urine specimens. The analyte JWH-018 was found linear according to Mandel[26] over a concentration range of 5–250 ng/mL (slope: 0.016; intercept: 0.137; correlation coefficient r = 0.998) and a recovery of 88% at 100 ng/mL was determined. The LLOD was estimated at 0.1 ng/mL by a signal-to-

Table 3. Summary of assay validation results of JWH-018

	LLOD (ng/mL)	Recovery (%) at 100 ng/mL	Intra-day precision (n = 18)		Inter-day precision (n = 18 + 18 + 18)	
			Concentration (ng/mL)	CV (%)	Concentration (ng/mL)	CV (%)
JWH-018	0.1	88	20	7.4	20	9.0
			100	6.3	100	14.0
			200	3.5	200	13.7

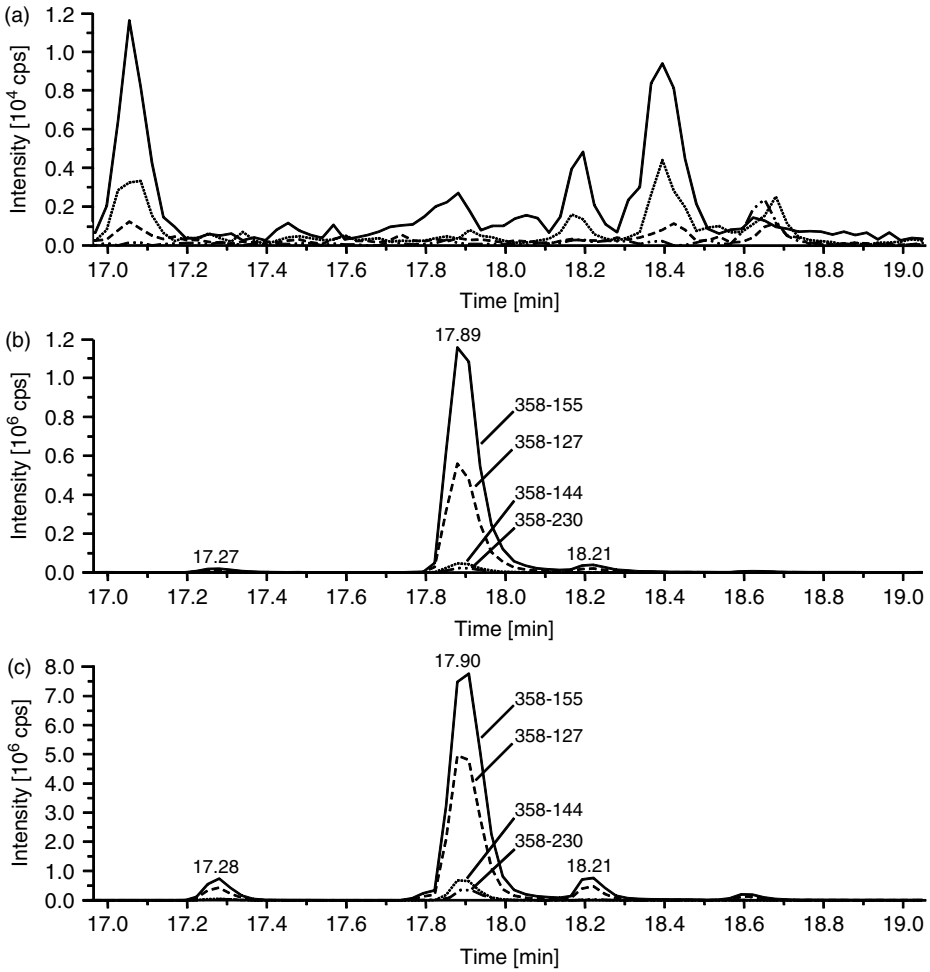


Figure 5. LC-MS/MS analyses of authentic urine samples recorded on an API4000 Qtrap mass spectrometer using MRM mode: (a) blank urine specimen; (b) urine sample of a 'spice' user; (c) doping control sample containing JWH-018. The peak at 17.9 min corresponds to the monohydroxylated and most abundant metabolite M1.

noise ratio ≥3. The relative standard deviation of the peak area ratios at 1 ng/mL was 21.8% (n = 10).

Intra-day and inter-day precision

Inter-day precisions at three concentration levels (20, 100 and 200 ng/mL) were determined with relative standard deviations between 9.0% and 14.0%. Intra-day precisions at these concentration levels ranged from 6.7% to 8.4%, 4.4% to 8.5% and 2.5% to 8.9% for low, medium, and high concentrations, respectively. The inter-day precision of the most abundant urinary JWH-018 metabolite (M1) determined by repeated preparation and analysis of the authentic urine sample was 10.8% with intra-day precisions between 7.0% and 11.3%.

Ion suppression/enhancement effects

Five different urine samples from three male and two female donors were prepared and measured as described. Ion suppression/enhancement effects, studied by continuous infusion of JWH-018, were observed less than 10% at expected retention times of the analyte and its metabolites.

Authentic doping control samples

The validated procedure was established into an existing screening assay as described elsewhere.[19,27,28] Since September 2009, approximately 7500 urine specimens were measured yielding two atypical findings in routine screening LC-MS/MS measurements.

The presence of the monohydroxylated analog of JWH-018 (M1) was unambiguously confirmed using four diagnostic ion transitions as illustrated in Figure 5 showing a blank urine specimen (a), a urine sample of a 'spice' user (b) and a doping control sample containing JWH-018 (c). Retention times and relative abundances of peaks obtained from selected ion transitions were all within the limits established by WADA.[29]

Further to the target analyte (M1), which represents the most abundant metabolite in urine, all additional metabolites discussed above were also found in these two routine doping control samples as well as most of their respective monoglucuronides. The active drug JWH-018 was, as in the urine specimen of the 'spice' user, not detectable and is therefore unsuitable for the implementation in screening procedures. Hence, the investigated metabolites serve as target analytes for the detection of abuse of JWH-018 in elite sports.

But although the JWH-018 metabolites were clearly identified in these two doping control samples, they were not categorized as adverse analytical findings due to out-of-competition testing since synthetic cannabinoids have been prohibited only in-competition.

Conclusion

JWH-018 was reported as one active ingredient in 'spice' blends and cannabis-like effects were described after smoking these products. As the administered drug is not detectable in urine samples, the elucidation of its major metabolic pathways, for which high-resolution/high-accuracy mass spectrometry has demonstrated great utility, is of particular importance to doping controls. After confirmation of most of the *in vitro* identified metabolites in a urine specimen of a 'spice' user, the most abundant urinary JWH-018 phase-I metabolite could successfully be implemented in a routine doping control assay which can be expanded to further structural analogs subsequent to studies of their respective metabolism reactions. However, the structures of the metabolites were proposed based solely on chemical reactivity, chromatographic, and mass spectrometric analyses as well as in analogy to earlier published results; hence, further confirmation by means of synthesis of the respective metabolites is the aim of future projects.

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