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PAPER IN FOREFRONT

Structure characterisation of urinary metabolites of the cannabimimetic JWH-018 using chemically synthesised reference material for the support of LC-MS/MS-based drug testing

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Abstract As recently reported, the synthetic cannabinoid JWH-018 is the subject of extensive phase I and II metabolic reactions in vivo. Since these studies were based on LC-MS/MS and/or GC-MS identification and characterisation of analytes, the explicit structural assignment of the metabolites was only of preliminary nature, if possible at all. Here, we report the chemical synthesis of five potential in vivo metabolites of JWH-018 derivatives featuring an alkylcarboxy (M1), a terminal alkylhydroxy (M2), a 5-indolehydroxy (M3), an *N*-dealkylated 5-indolehydroxy (M4) and a 2′-naphthylhydroxy (5) analogue, respectively, and their characterisation by nuclear magnetic resonance spectroscopy. The collision-induced dissociation (CID) patterns of the protonated compounds were studied by high-resolution/high-accuracy tandem mass spectrometry (MS^n) applying an LTQ Orbitrap with direct infusion and electrospray ionisation of target analytes. An unusual dissociation behaviour including a reversible ion–molecule reaction between a naphthalene cation (*m/z* 127) and water in the gas phase of the MS was shown to be responsible for nominal neutral losses of 10 u in the course of the CID pathway. LC-MS/MS-supported comparison of synthesised reference standards with an authentic urine sample using an API 4000 QTrap mass spectrometer identified the synthetic JWH-018 analogues M1–M4 as true in vivo metabolites, presuming a chromatographic separation of potentially present regioisomeric analogues. Existing doping control methods were expanded and validated according to international guidelines in order to allow for the detection of the carboxy and the alkylhydroxy metabolites, respectively, as urinary markers for the illegal intake of the synthetic cannabinoid JWH-018. Both metabolites were quantified in authentic doping control urine samples that had been suspicious of JWH-018 abuse after routine screening procedures, and a stable isotope-labelled $^{13}C_8$-^{15}N-carboxy metabolite was synthesised for future analytical applications.

Keywords Spice · Synthetic cannabinoids · JWH-018 · Metabolite structure · CID · Ion–molecule reaction

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Introduction

The synthetic cannabinoid JWH-018 (naphthalen-1-yl-(1-pentyl-1*H*-indol-3-yl)methanone) was first identified in 2008 as an active ingredient in different herbal blends referred to as ‘spice’ [1, 2]. Later, many European countries took legal action to ban or control this substance

and some of its analogues [3], and also the World Anti-Doping Agency prohibited JWH-018 in elite sports during competition [4]. Therefore, analytical methods had to be developed in forensic and doping control laboratories to enable the detection of an illegal intake of this psychoactive substance. Two quantitative LC-MS/MS methods targeting the intact drug in serum are described [5, 6], but as a fast drop of the JWH-018 concentration in serum was observed [6], only short detection windows are expected. Moreover, in doping control analysis, the availability of urine is much higher than for blood or plasma samples, requiring the establishment of an analytical procedure for the detection of JWH-018 abuse in urinary matrix. For this purpose, the metabolic pathways of JWH-018 in the human body were elucidated by an in vitro assay [7] as well as two in vivo studies in post-administration samples [8, 9], yielding a multitude of mainly hydroxylated and *N*-dealkylated metabolites, whilst the intact parent compound was not detected in urine. However, as to the best of our knowledge, the definite structures of the postulated metabolites could not yet be affirmed and no certified reference material for the confirmation of JWH-018 abuse is available so far. Therefore, we report in the present study the synthesis of five JWH-018 analogues, belonging to a list of potential in vivo metabolites, featuring an alkylcarboxy, a terminal alkylhydroxy, a 5-indolehydroxy, a 2′-naphthalenehydroxy or an *N*-dealkylated 5-indolehydroxy analogue, respectively. After their characterisation by nuclear magnetic resonance (NMR) spectroscopy as well as high-resolution/high-accuracy tandem mass spectrometry, the synthesised standards were compared to authentic JWH-018 urine specimens. For the first time, this allows for an explicit structure confirmation of major JWH-018 urinary metabolites as well as their quantification by means of LC-MS/MS.

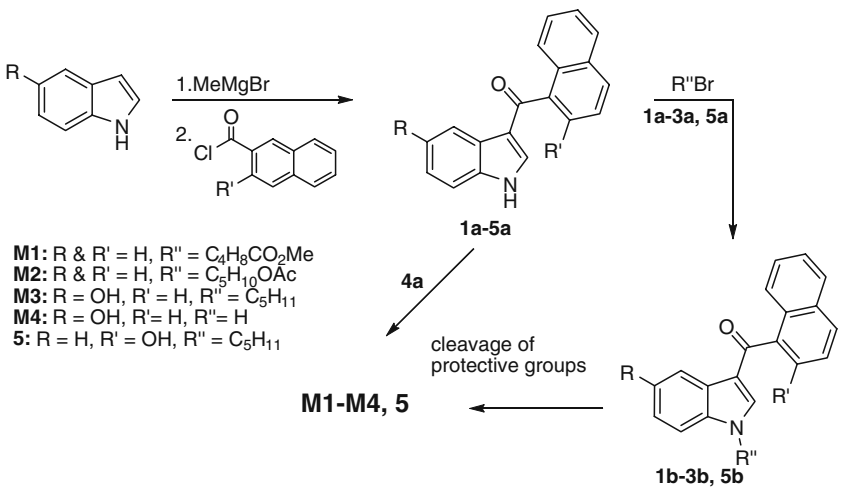
Experimental

Chemicals and reagents

All reagents, chemicals and JWH-015 were purchased from Sigma Aldrich (Deisendorf, Germany) or Alfa Aesar (Karlsruhe, Germany), and solvents (all analytical grade) were obtained from Merck (Darmstadt, Germany). $^{13}C_8$-^{15}N-indole (96–99% isotopic enrichment) was obtained from Cambridge Isotopes (Andover, MA, USA) and β-glucuronidase (*Escherichia coli*) from Roche Diagnostics (Mannheim, Germany).

Procedures for the preparation of compounds M1–M4 and 5

In accordance with Makriyannis and Deng [10], the indole was activated by treatment with 3 equivalents (eq.) of methylmagnesium bromide and subsequently coupled with 1 eq. of naphthoylchloride to yield the 3-acyl-1*H*-indoles **1a**–**5a** after aqueous workup (Scheme 1). The amines **1a**–**3a** and **5a** were deprotonated with 3 eq. of sodium hydride and further alkylated by reaction with the respective alkylbromide (methyl 5-bromovalerate for **1a**, 5-bromopentyl acetate for **2a**, 1-bromopentane for **3** and **5**) to yield the 1-alkyl-3-acyl-1*H*-indoles **1b**–**3b** and **5b**, respectively, which were purified by silica gel column chromatography using ethyl acetate/*n*-heptane 1:1 (*v/v*). The methyl ester and acyl protective groups of **1b** and **2b**, respectively, were removed by hydrolysis in a 1:1 (*v/v*) mixture of methanol and 2 M aqueous sodium hydroxide solution for 1 h at room temperature. Crude **M1** hydrolysis product was worked up by silica gel column chromatography (ethyl acetate/methanol 6:1, *v/v*). The product was dissolved in methanol, diluted with *n*-heptane and extracted with 2 M aqueous NaOH solution. The aqueous phase was



Scheme 1 Synthetic route for the preparation of the JWH-018 metabolites **M1**–**M4** and **5**. The molecular structures of the target compounds are included in the product ion spectra in Fig. 1

adjusted to pH 2, the resulting white precipitate filtered off, and washed with water and *n*-heptane to yield **M1** as a white solid. The **M2** hydrolysis product was purified by column chromatography (ethyl acetate/*n*-heptane 3:1, *v/v*) to afford **M2** as colourless oil. The removal of the methoxy and ethoxy protective groups of compounds **4a**, **5b** and **3b**, respectively, was achieved by the following general procedure: To 1 equivalent of acyl indole in dry dichloromethane, 5 eq. of boron tribromide (1 M solution in dichloromethane) was added at 0 °C under argon atmosphere and the mixture stirred for 3 h at room temperature. The product solution was quenched by the addition of saturated aqueous $NaHCO_3$ solution and extracted with ethyl acetate. The resulting product from the concentrated combined organic phases was purified by silica gel column chromatography (ethyl acetate/*n*-heptane 1:1, *v/v*) to yield compounds **M3**, **M4** and **5**, respectively, as pale yellow solids. The stable isotope-labelled analogue **$^{13}C_8$-^{15}N-M1** was prepared in accordance to **M1**, but starting from $^{13}C_8$-^{15}N-1*H*-indole as educt.

All molecular structures were confirmed by NMR spectroscopy with ^{1}H, H,H-COSY, H,C-HMQC, H,C-HMBC, ^{13}C DEPT and ^{15}N experiments employing Bruker AV 600/AV 400 instruments (Bruker, Karlsruhe, Germany) equipped with a 5-mm inverse probe head (z-gradient coil). All spectra were recorded at room temperature from solutions of approximately 10 mg/mL analyte concentration. The spectra were calibrated using the solvent residual peak as reference signal.

M1: ^{1}H NMR ($CDCl_3$, 600 MHz): δ (ppm)=8.45–8.49 (m, 1H, H-4), 8.17 (d, 1H, H-8′), 7.95 (d, $^3J_{4',5'}$=8.3 Hz, 1H, H-4′), 7.89 (d, $^3J_{5',4'}$=8.3 Hz, 1H, H-5′), 7.64 (dd, 1H, H-2′), 7.48–7.54 (m, 2H, H-3′, H-6′), 7.43–7.47 (m, 1H, H-7′), 7.33–7.39 (m, 3H, H-5, H-6, H-7), 7.35 (s, 1H, H-2), 4.08 (t, 2H, H-9), 2.31 (t, 2H, H-12), 1.85 (m, 2H, H-10), 1.59 (m, 2H, H-11). **^{13}C NMR** ($CDCl_3$, 150 MHz): δ (ppm)=192.8 (C-8), 178.1 (C-13), 139.1 (C-1′), 138.0 (C-2), 133.8 (C-4′a), 130.8 (C-8′a), 130.1 (C-4′), 128.2 (C-5′), 127.1 (C-3a), 126.8 (C-7′), 126.3 (C-6′), 126.0 (C-8′), 125.9 (C-2′), 124.6 (C-3′), 123.8 (C-6), 123.0 (C-4, C-5), 117.8 (C-3), 109.9 (C-7), 46.8 (C-9), 33.1 (C-12), 29.1 (C-10), 21.8 (C-11).

M2: ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm)=8.51-8.44 (m, 1H, H-4), 8.18 (m, 1H, H-8′), 7.96 (m, 1H, H-4′), 7.90 (m, 1H, H-5′), 7.65 (m, 1H, H-2′), 7.54–7.43 (m, 3H, H-3′, H-6′, H-7′), 7.46 (m, 1H, H-7′), 7.41–7.33 (M, 3H, H-5, H-6, H-7), 7.35 (s, 1H, H-2), 4.09 (t, *J*=7.3 Hz, 2H, H-9), 3.56 (t, *J*=6.4 Hz, 3H, H-13), 1.83 (m, 2H, H-10), 1.56–1.48 (m, 2H, H-12), 1.40–1.31 (m, 2H, H-11). **^{13}C NMR** ($CDCl_3$, 100 MHz): δ (ppm)=192.2 (C-8), 139.3 (C-1′), 138.1 (C-2); 137.2 (C-7a), 133.9 (C-4′a),130.9 (C-8′a), 130.2 (C-4′), 128.3 (C-5′), 127.2 (C-3a), 126.9 (C-7′), 126.5 (C-6′), 126.2 (C-8′), 125.9 (C-2′), 124.7 (C-3′, C-6), 123.1 (C-4, C-5), 117.2 (C-3), 110.1 (C-7), 62.6 (C-13), 47.3 (C-9), 32.2 (C-12), 29.8 (C-10), 23.3 (C-11).

M3: 1H NMR (CDCl3, 600 MHz): δ (ppm)=8.68 (d, *J*= 2.4 Hz, 1H, H-4), 8.28 (s, 1H, 5-OH), 8.19 (d, 1H, H-8′), 7.97 (d, *J*=8.3 Hz, 1H, H-4′), 7.91 (d, *J*=8.3 Hz, 1H, H-5′), 7.68 (dd, 1H, H-2′), 7.55 (m, 1H, H-3′), 7.48 (m, 1H, H-6’), 7.46 (m, 1H, H-7′), 7.21 (s, 1H, H-2), 7.20 (d, *J*=8.6 Hz, 1H, H-7), 6.88 (dd, *J*=8.6/2.4 Hz, 1H, H-6), 3.97 (t, *J*= 7.3 Hz, 2H, H-9), 1.76 (p, *J*=7.3 Hz, 2H, H-10), 1.30–1.24 (m, 2H, H-12), 1.24–1.17 (m, 2H, H-11), 0.83 (t, 3H, H-13). **^{13}C NMR** ($CDCl_3$, 150 MHz): δ (ppm)=193.1 (C-8), 154.6 (C-5), 139.3 (C-2), 139.0 (C-1), 133.9 (C-4′a), 131.8 (C-7a), 130.9 (C-8′a), 130.2 (C-4′), 128.4 (C-5′), 128.2 (C-3a), 126.9 (C-7′), 126.5 (C-6′), 126.2 (C-8′), 126.0 (C-2′), 124.9 (C-3′), 116.9 (C-3), 113.9 (C-6), 111.0 (C-7), 107.8 (C-4), 47.6 (C-9), 29.6 (C-10), 29.0 (C-11), 22.3 (C-12), 14.0 (C-13).

M4: ^{1}H NMR (CD_3OD, 400 MHz): δ (ppm)=8.02–7.97 (m, 2H, H-4′, H-8′), 7.92 (m, 1H, H-5’), 7.74 (dd, *J*= 2.5 Hz, *J*=0.5 Hz, 1H, H-4), 7.60 (dd, 1H, H-2′), 7.54–7.50 (m, 1H, H-3′), 7.50–7.42 (m, 2H, H-6′, H-7′), 7.40 (s, 1H, H-2), 7.28 (dd, *J*=8.7/0.5 Hz, 1H, H-7), 6.80 (dd, *J*=8.7/ 2.5 Hz, 1H, H-6). **^{13}C NMR** (CD_3OD, 100 MHz): δ (ppm)= 195.1 (C-8), 154.9 (C-5), 140.2 (C-1′), 138.5 (C-2), 135.2 (C-4′a), 133.2 (C-7a), 132.0 (C-8′a), 131.0 (C-4′), 129.4 (C-5′), 128.6 (C-3a), 127.8 (C-7′), 127.4 (C-6′), 126.8 (C-2′), 126.6 (C-8′), 125.8 (C-3′), 118.8 (C-3), 114.5 (C-6), 113.7 (C-7), 107.5 (C-4).

5: ^{1}H NMR ($CDCl_3$, 600 MHz): δ (ppm)=9.86 (s, 1H, 2′-OH), 8.36-8.32 (m, 1H, H-4), 7.94 (d, 1 H, H-8′), 7.88 (d, *J*=8.0 Hz, 1H, H-4’), 7.70 (d, *J*=8.0 Hz, 1H, H-5′), 7.40–7.37 (m, 1H, H-7), 7.36–7.32 (m, 2H, H-5, H-6), 7.31–7.27 (m, 1H, H-6′), 7.30 (s, 1H, H-2), 7.25 (m, 1H, H-3′), 7.23–7.19 (m, 1H, H-7′), 4.01 (t, *J*=7.1 Hz, 2H, H-9), 1.75 (p, *J*=7.1 Hz, 2H, H-10), 1.31–1.24 (m, 2H, H-12), 1.24–1.17 (m, 2H, H-11), 0.85 (m, 3H, H-13). **^{13}C NMR** ($CDCl_3$, 150 MHz): δ (ppm)=192.3 (C-8), 157.5 (C-2′), 138.4 (C-2), 136.9 (C-7a), 133.6 (C-4′), 132.7 (C-8′a), 128.8 (C-4′a), 128.4 (C-5′), 127.0 (C-3a), 126.3 (C-7′, C-8’), 123.8 (C-6), 123.6 (C-6a′), 123.2 (C-5), 122.8 (C-4), 119.1 (C-3′), 117.9 (C-1′), 117.5 (C-3), 47.4 (C-9), 29.6 (C-10), 28.9 (C-11), 22.4 (C-12), 14.0 (C-13).

^{15}N-$^{13}C_8$-M1: ^{1}H NMR ($CDCl_3$, 600 MHz): δ (ppm)= 8.46–8.53 (m, 1H, H-4), 8.18 (d, 1H, H-8′), 7.97 (d, *J*= 8.3 Hz, 1H, H-4′), 7.90 (d, *J*=8.3 Hz, 1H, H-5′), 7.65 (m, 1H, H-2′), 7.49–7.55 (m, 2H, H-3′, H-6′), 7.44–7.49 (m, 1H, H-7′), 7.33–7.41 (m, 3H, H-5, H-6, H-7), 7.33–7,41 (m, 1H, H-2), 4.10 (t, *J*=7.2 Hz, 2H, H-9), 2.32 (t, *J*= 7.2 Hz, 2H, H-12), 1.85 (m, 2H, H-10), 1.59 (m, 2H, H-11). **^{13}C NMR** ($CDCl_3$, 150 MHz): δ (ppm)=192.3 (C-8), 178.6 (C-13), 137.4 (m, ^{13}C-2), 136.4 (m, ^{13}C-7a), 133.8 (C-4′a), 130.8 (C-8′a), 130.2 (C-4′), 128.3 (C-5′), 126.3–127.6 (m, ^{13}C-3a), 126.1 (C-8′), 126.0 (C-2′), 124.7 (C-3′), 122.42–124.59 (m, ^{13}C-4, ^{13}C-5, ^{13}C-6), 117.6 (m, ^{13}C-3), 109.5–

110.5 (m, ^{13}C-7), 46.9 (C-9), 33.3 (C-12), 29.2 (C-10), 21.9 (C-11). **^{15}N NMR** ($CDCl_3$, 60 MHz): δ (ppm)=148.6 (^{15}N).

High-resolution/high-accuracy mass spectrometry

High-resolution/high-accuracy tandem mass spectrometry ($HRMS^n$) of the synthesised compounds was realised by the direct infusion of analyte solutions (5 μg/mL in acetonitrile/0.5% formic acid solution, 1:1, *v/v*) into an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) at a flow rate of 5 μL/min and electrospray ionisation in positive mode. The ionisation voltage was set to 5,000 V and the capillary temperature to 275 °C; for MS^n experiments, protonated precursor ions were isolated with a width of 1.5 Da prior to collision-induced dissociation. Damping and collision gas in the linear ion trap was helium (purity grade 5.0), whilst nitrogen was used for the curved linear ion trap. Prior to the acquisition, calibration was achieved with the manufacturer's calibration mixture (containing caffeine, MRFA and ultramark). Spectra were evaluated using Xcalibur software version 2.0 (Thermo).

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

For comparison of the synthesised standards with urine specimens containing JWH-018 metabolites, multiple reaction monitoring experiments (MRM) were performed using an Agilent 1100 Series LC (Waldbronn, Germany) coupled to an Applied Biosystems API 4000 QTrap mass spectrometer (Darmstadt, Germany) with electrospray ionisation (ESI) in positive mode. The LC was equipped with a Kinetex C18 column (2.1×100 mm, particle size 2.6 μm) obtained from Phenomenex (Aschaffenburg, Germany) and 5 mM ammonium acetate containing 0.1% acetic acid (A) and acetonitrile (B) as eluents. At a flow rate of 200 μL/min, the following gradient was used: from 0 to 15 min, linear gradient from 80% A to 55% A; in further 5 min to 50% A; holding there for 5 min before linear decrease to 0% A up to 30 min; after a hold for 2 min, re-equilibration at starting conditions for 8 min. For some of the in vivo metabolites, a chromatographic procedure including an extended isocratic elution step was performed on an Agilent Zorbax Eclipse XDB-C18 column (4.6× 150 mm, particle size 5 μm) with 800 μL/min flow rate: 13 min isocratic elution at 50% A followed by a 1-min linear gradient to 0% A and a hold for 1 min before re-equilibration for 5 min. For validation purposes, the same setup was used, but with a Kinetex C18 column of only 50 mm length. The flow rate was set to 250 μL/min and the gradient ran from 90% A to 0% A within 8 min, maintaining there for 1 min followed by re-equilibration for 5 min. The injection volume was set to 5 μL and the characteristic product ions detected using MRM mode (Table 1).

Urine sample preparation

Sample preparation of authentic doping control samples was performed according to established screening procedures as described elsewhere [9, 11]. Briefly, 1 mL of urine, fortified with 10 ng of JWH-015 as internal standard (ISTD), was subjected to enzymatic hydrolysis followed by liquid–liquid extraction at pH 9.6. For methylation of carboxyl, phenolic hydroxyl and amino groups by means of iodomethane in the presence of a base [12], 2 mL of urine was prepared as described in detail in an earlier article [9].

Assay validation

Validation for quantitative purposes was performed for **M1** and **M2** according to guidelines of the International Conference on Harmonisation [13]. The employed ion transitions as well as the mass spectrometric parameters are listed in Table 1.

Table 1 Ion transitions and mass spectrometric parameters for validation purposes

Compound	Declustering potential (V)	Ion transition (*m/z*)	Collision offset voltage (V)	Dwell time (ms)
M1	81	372–155	37	40
	81	372–144	55	40
	81	372–127	81	40
M2	91	358–155	40	40
	91	358–144	51	40
	91	358–127	80	40
ISTD	85	328–155	35	40
	85	328–127	60	40

Specificity Ten different blank urine samples from five healthy male and female volunteers, respectively, were prepared and analysed as described above in order to check for interfering peaks at the respective retention times of **M1** and **M2**. For evaluating possible interferences from high concentrations of other cannabimimetic substances, JWH-018, JWH-073, JWH-200, JWH-250, HU-210 as well as the C_8-homologue of CP47,497 were added to blank urine samples (100 ng/mL each) fortified with **M1** and **M2** at LOQ level and compared to blank urine specimens fortified with the target analytes only.

Recovery The recovery of **M1** and **M2** was calculated by the comparison of mean peak area ratios of eight urine samples fortified with 1 ng/mL of each metabolite prior to analysis and eight urine samples in which the analytes were added after preparation into the final ether extract.

Limit of detection and limit of quantification Ten urine samples were fortified with 0.5 ng/mL of each standard and another ten samples were spiked with the ISTD only. The limit of detection (LOD) as well as the limit of quantification (LOQ) were both estimated by the evaluation of the signal-to-noise ratio (S/N≥3 for LOD and S/N≥10 for LOQ) for the respective compounds.

Linearity The linearity of the method was determined for both analytes with fortified samples (n=12) at concentrations between 0 and 100 ng/mL.

Intraday and interday precision Intra- and interday precisions were calculated from ten urine samples of low (0.5 ng/mL=LOQ), medium (10 ng/mL) and high (100 ng/mL) concentrations of both metabolites, respectively, which were prepared and analysed on three consecutive days (n=10+10+10).

Bias The bias of the method was tested by the fivefold determination of fortified urine samples at three concentration levels (0.5, 10, 50 ng/mL). The content of **M1** and **M2** was quantified by a linear regression with external calibration curves ranging from 0 to 75 ng/mL for medium and high concentrations and from 0 to 2.5 ng/mL for the low concentration, respectively. As for the whole validation, JWH-015 was used as the internal standard in all samples. For the calculation of the bias, the ratio of the determined concentrations to the theoretical values was formed.

Stability In order to check the stability of the analytes in urinary matrix under regular storage conditions of doping control specimens, urine fortified with 10 ng/mL of **M1** and **M2**, respectively, was kept at room temperature as well as at 4 °C over a period of 32 days. After 1, 4, 8, 11, 15, 20, 25 and 32 days, an aliquot was taken and stored at −20 °C until the end of the study, when all samples were prepared and analysed at once.

Ion suppression/enhancement effects The influence of matrix effects on the ESI process were tested by analysing three male and three female blank urine samples as well as solvent only with continuous co-infusion of **M1** and **M2** (solution concentration 100 ng/mL for each analyte, flow rate 5 μL/min) using a post column T-connector [14, 15].

Results and discussion

Characterisation of target analytes by NMR and MS^n analysis

During NMR analysis of compounds **M1**–**M4** and **5**, the explicit assignment of ^1H and ^{13}C NMR signals to the correspondent nuclei, supported by heteronuclear 2D NMR experiments, substantiated the molecular structures of the analytes, as shown in Fig. 1. Also in Fig. 1, the ion trap product ion spectra of protonated analytes **M1**–**M4** and **5**, as well as the stable isotope-labelled **M1** (see below for discussion) are shown. The corresponding high-resolution/high-accuracy MS^n data are presented in Table 2 and further substantiate the shown molecular structures by the confirmation of the elemental compositions. The mass spectrometric collision-induced dissociation (CID) behaviour of all target analytes is well in accordance with the earlier reported CID pathway of JWH-018 parent compound as well as its postulated metabolites. Despite minor variation in product ion intensities, which are due to differences in instrumental setup, ESI-MS^n analysis of the JWH-018 derivatives combined with high-resolution/high-accuracy mass measurements support the recently suggested pathways of product ion formation as well as the conclusions on the correspondent metabolic transformations derived thereof [7, 9].

Most strikingly, however, tandem mass spectrometry (MS^n) measurement of the compounds yielded novel information on the most dominant CID pathway leading the product ions at *m/z* 155, 145 and 127 of **M1**–**M4**, respectively. The ion at *m/z* 155 was earlier proposed to be formed by the elimination of the alkylindole moiety, leading to a naphthalene-1-acylium cation structure (Scheme 2) [7]. This ion further releases carbon monoxide, yielding the naphthalene cation at *m/z* 127. Selection and subsequent excitation of *m/z* 155 in MS^3 analysis, however, yielded the observation that the ion at *m/z* 145 also constitutes a product ion of *m/z* 155, corresponding to a neutral loss of 10 u (Table 2). Such unusual neutral losses

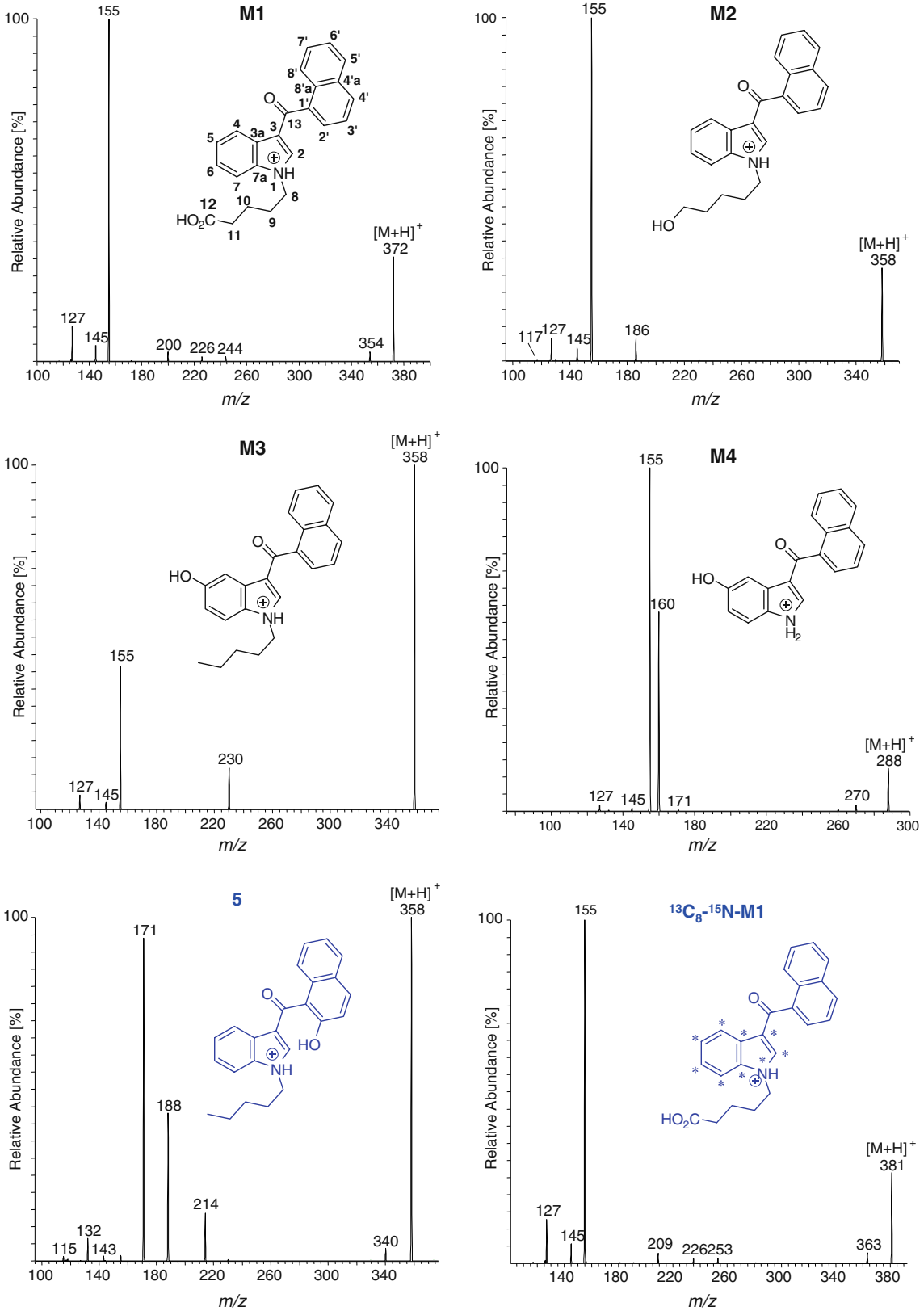


Fig. 1 ESI-MS/MS product ion spectra of verified JWH-018 metabolites **M1–M4** (*black structures*), JWH-018 derivative **5** and **$^{13}C_8$-^{15}N-M1** (both *blue*), generated by direct infusion of analyte solutions (5 µg/mL in acetonitrile/0.5% formic acid solution, 1:1, *v/v*) and collision-induced dissociation of protonated precursor ions (collision energy 17 au) in the LTQ ion trap

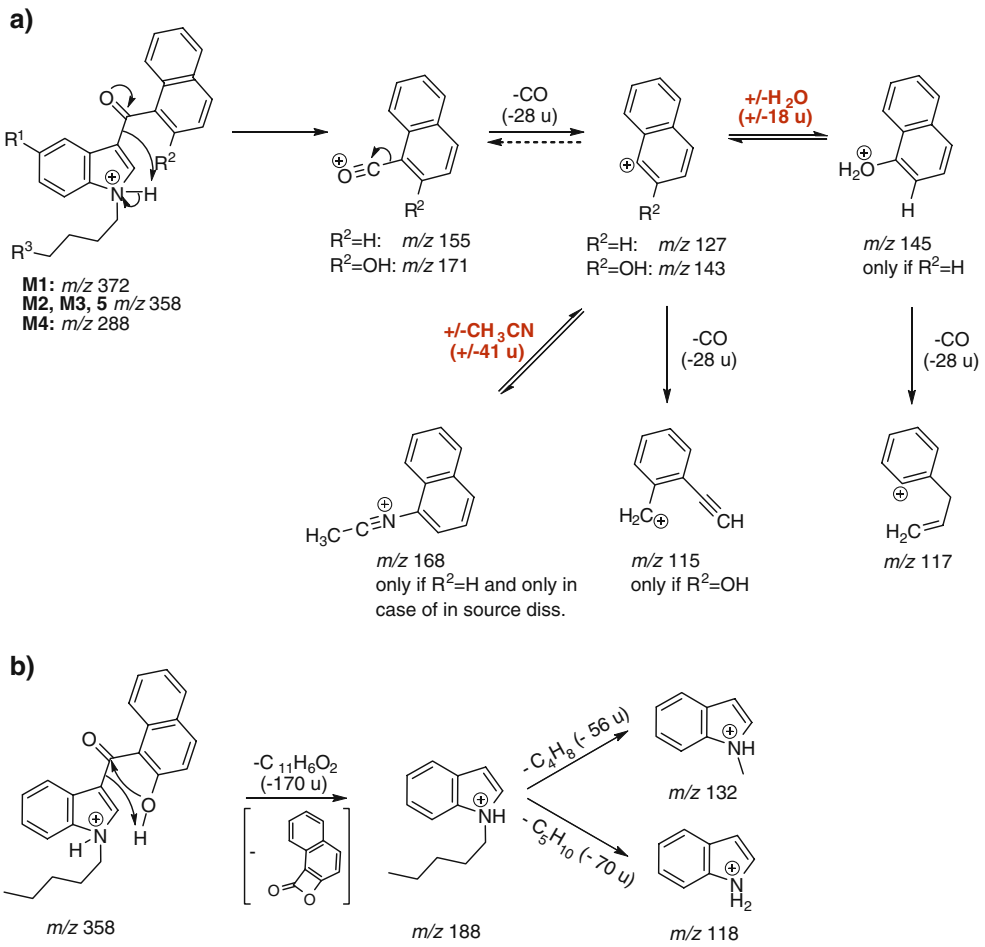
Table 2 High-resolution/high-accuracy LTQ Orbitrap MS^n data of analytes shown in Fig. 1

Compound	MS^2	MS^3	MS^4	Elemental comp.	Error (ppm)	Product ions	Elemental comp.	Error (ppm)	Cleaved species
M1	372.1601			$C_{24}H_{22}O_3N$	1.8	354.1494	$C_{24}H_{20}O_2N$	1.7	$-H_2O$
						272.1073	$C_{19}H_{14}O_1N$	1.2	$-C_4H_7CO_2H$
						244.0972	$C_{14}H_{14}O_3N$	1.5	$-C_{10}H_8$
						226.0864	$C_{14}H_{12}O_2N$	0.8	$-H_2O$, $-C_{10}H_8$
						200.1072	$C_{13}H_{14}ON$	0.9	$-C_{10}H_8$, $-CO_2$
						182.0964	$C_{13}H_{12}N$	0.1	$-C_{10}H_8$, $-CO_2$, $-H_2O$
						172.1121	$C_{12}H_{14}N$	0.4	$-C_{10}H_8$, $-CO_2$, $-CO$
						155.0492	$C_{11}H_7O$	0.1	$-C_{13}H_{15}O_2N$
						145.0648	$C_{10}H_9O$	0.2	$-C_{13}H_{15}O_2N$, $-CO$, $+H_2O$,
						127.0542	$C_{10}H_7$	−0.2	$-C_{13}H_{15}O_2N$, $-CO$
						117.0698	C_9H_9	−0.3	$-C_{13}H_{15}O_2N$, $-CO$, $+H_2O$, $-CO$
		354.1493		$C_{24}H_{20}O_2N$	1.3	226.0864	$C_{14}H_{12}O_2N$	0.8	$-C_{10}H_8$
						155.0491	$C_{11}H_7O$	−0.1	$C_{13}H_{13}ON$
		244.0971		$C_{14}H_{14}O_3N$	1.2	144.0444	C_9H_6ON	−0.0	$-C_4H_7CO_2H$
		226.0864		$C_{14}H_{12}O_2N$	0.8	170.0964	$C_{12}H_{12}N$	−0.4	$-CO$, $-CO$
		200.1070		$C_{13}H_{14}ON$	0.1	182.0964	$C_{13}H_{12}N$	−0.0	$-H_2O$
						172.1120	$C_{12}H_{14}N$	−0.2	$-CO$
						156.0807	$C_{11}H_{10}N$	−0.3	$-C_2H_4O$
		155.0491		$C_{11}H_7O$	−0.4	145.0647	$C_{10}H_9O$	−0.6	$-CO$,
						127.0541	$C_{10}H_7$	−0.9	$-CO$
						117.0697	C_9H_9	−1.1	$-CO$, $+H_2O$, $-CO$
			127.0541	$C_{10}H_7$	−0.8	145.0647	$C_{10}H_9O$	−0.6	$+H_2O$
						117.0697	C_9H_9	−1.1	$-CO$, $+H_2O$
M2	358.1806			$C_{24}H_{24}O_2N$	1.3	230.1177	$C_{14}H_{16}O_2N$	0.6	$-C_{10}H_8$
						186.1278	$C_{13}H_{16}N$	0.6	$-C_{11}H_8O$
						155.0491	$C_{11}H_7O_1$	0.0	$-C_{13}H_{17}ON$
						145.0648	$C_{10}H_9O$	0.1	$-C_{13}H_{17}ON$, $-CO$, $+H_2O$,
						127.0542	$C_{10}H_7$	−0.2	$-C_{13}H_{17}ON$, $-CO$
		186.1278		$C_{13}H_{16}N$	0.2	130.0650	C_9H_8N	−0.7	$-C_4H_8$
		155.0491		$C_{11}H_7O_1$	−0.2	145.0647	$C_{10}H_9O$	−0.5	$-CO$, $+H_2O$
						127.0541	$C_{10}H_7$	−0.8	$-CO$
						117.0697	C_9H_9	−1.1	$-CO$, $+H_2O$, $-CO$
			127.0541	$C_{10}H_7$	−0.8	145.0647	$C_{10}H_9O$	−0.5	$+H_2O$
						117.0697	C_9H_9	−1.1	$-CO$
M3	358.1807			$C_{24}H_{24}O_2N$	1.5	230.1179	$C_{14}H_{16}O_2N$	1.4	$-C_{10}H_8$
						155.0492	$C_{11}H_7O_1$	0.3	$-C_{13}H_{17}ON$
						145.0648	$C_{10}H_9O$	0.3	$-C_{13}H_{17}ON$, -CO, $+H_2O$
						127.0542	$C_{10}H_7$	−0.2	$-C_{13}H_{17}ON$, $-CO$
		230.1176		$C_{14}H_{16}O_2N$	0.4	174.0549	$C_{10}H_8O_2N$	−0.3	$-C_4H_8$
						160.0392	$C_9H_6O_2N$	−0.6	$-C_5H_{10}$
		155.0490		$C_{11}H_7O_1$	−0.7	145.0647	$C_{10}H_9O$	−0.6	$-CO$, $+H_2O$
						127.0541	$C_{10}H_7$	−0.9	$-CO$
						117.0698	C_9H_9	−1.1	$-CO$, $+H_2O$, $-CO$
			127.0541	$C_{10}H_7$	−0.9	145.0647	$C_{10}H_9O$	−0.6	$+H_2O$
						117.0697	C_9H_9	−1.3	$+H_2O$, $-CO$
M3 in source diss.		168.0807		$C_{12}H_{10}N$	−0.5	145.0647	$C_{10}H_9O$	−0.7	$-CH_3CN$
						127.0541	$C_{10}H_7$	−1.0	$-CH_3CN$, $-CO$

Table 2 (continued)

Compound	MS^2	MS^3	MS^4	Elemental comp.	Error (ppm)	Product ions	Elemental comp.	Error (ppm)	Cleaved species
						117.0697	C_9H_9	−1.3	$-CH_3CN$, $-CO$, $+H_2O$, $-CO$
M4	288.1023			$C_{19}H_{14}O_2N$	1.3	270.0918	$C_{19}H_{12}ON$	1.5	H_2O
						160.0393	$C_9H_6O_2N$	−0.1	$-C_{10}H_8$
						155.0492	$C_{11}H_7O$	0.1	$-C_8H_7ON$
						145.0648	$C_{10}H_9O$	0.1	$-C_8H_7ON$, $-CO$, $+H_2O$
						127.0543	$C_{10}H_7$	0.3	$-C_8H_7ON$, $-CO$
		160.0392		$C_9H_6O_2N$	−0.7	132.0443	C_8H_6ON	−1.0	$-CO$
		155.0491		$C_{11}H_7O$	−0.5	145.0647	$C_{10}H_9O$	−0.7	$-CO$, $+H_2O$
						127.0541	$C_{10}H_7$	−0.9	$-CO$
						117.0697	C_9H_9	−1.2	$-CO$, $+H_2O$, $-CO$
			127.0541	$C_{10}H_7$	−0.8	145.0647	$C_{10}H_9O$	−0.6	$+H_2O$
						117.0697	C_9H_9	−1.2	$+H_2O$, $-CO$
5	358.1807			$C_{24}H_{24}O_2N$	1.4	340.1702	$C_{24}H_{22}ON$	1.8	$-H_2O$
						214.1229	$C_{14}H_{16}ON$	1.2	$-C_{10}H_8$
						188.1435	$C_{13}H_{18}N$	0.8	$-C_{11}H_6O_2$
						171.0442	$C_{11}H_7O_2$	0.6	$-C_{13}H_{17}N$
						143.0492	$C_{10}H_7O$	0.3	$-C_{13}H_{17}N$, $-CO$
						132.0808	$C_9H_{10}N$	0.0	$-C_{11}H_6O_2$, $-C_4H_8$
						118.0651	C_8H_8N	−0.1	$-C_{11}H_6O_2$, $-C_5H_{10}$
						115.0542	C_9H_7	−0.5	$-C_{13}H_{17}N$, $-CO$, $-CO$
		214.1227		$C_{14}H_{16}ON$	0.1	158.0600	$C_{10}H_8ON$	−0.5	$-C_4H_8$
						144.0443	C_9H_6ON	−0.8	$-C_5H_{10}$
		188.1434		$C_{13}H_{18}N$	−0.1	132.0807	$C_9H_{10}N$	−0.9	$-C_4H_8$
						118.0650	C_8H_8N	−1.2	$-C_5H_{10}$
		171.0440		$C_{11}H_7O_2$	−0.2	143.0491	$C_{10}H_7O$	−0.6	$-CO$
						115.0541	C_9H_7	−1.3	$-CO$, $-CO$
		143.0491		$C_{10}H_7O$	−0.5	115.0541	C_9H_7	−1.3	$-CO$
$^{13}C_8$-^{15}N-M1	381.1835			$C_{16}{}^{*}C_8H_{22}O_3{}^{*}N$	2.0	363.1735	$C_{16}{}^{*}C_8H_{20}O_2{}^{*}N$	2.2	$-H_2O$
						281.1315	$C_{11}{}^{*}C_8H_{14}O{}^{*}N$	2.4	$-C_4H_7CO_2H$
						253.1211	$C_6{}^{*}C_8H_{14}O_3{}^{*}N$	1.8	$-C_{10}H_8$
						235.1105	$C_6{}^{*}C_8H_{12}O_2{}^{*}N$	1.7	$-H_2O$, $-C_{10}H_8$
						209.1312	$C_5{}^{*}C_8H_{14}O{}^{*}N$	1.4	$-C_{10}H_8$, $-CO_2$
						191.1204	$C_5{}^{*}C_8H_{12}{}^{*}N$	0.8	$-C_{10}H_8$, $-CO_2$, $-H_2O$
						181.1361	$C_4{}^{*}C_8H_{14}{}^{*}N$	0.9	$-C_{10}H_8$, $-CO_2$, $-CO$
						155.0492	$C_{11}H_7O_2$	0.5	$-{}^{*}C_8H_6{}^{*}NC_5H_9O_2$
						145.0649	$C_{10}H_9O$	0.5	$-{}^{*}C_8H_6{}^{*}NC_5H_9O_2$, $-CO$, $+H_2O$,
						127.0543	$C_{10}H_7$	0.3	$-{}^{*}C_8H_6{}^{*}NC_5H_9O_2$, $-CO$
						117.0699	C_9H_9	0.0	$-{}^{*}C_8H_6{}^{*}NC_5H_{11}O_2$, $-CO$, $+H_2O$, $-CO$

have earlier been reported and shown to be caused by ion-neutral complex formation in the gas phase [16]. Here, the abnormal observation can be explained by a spontaneous ion–molecule reaction between the naphthalene cation (*m/z* 127) and residual water (18 u) in the gas phase of the ion trap to form the water adduct product ion at *m/z* 145 (Scheme 2). In fact, the MS^3 spectra of *m/z* 127 display the same product ion at *m/z* 145 ($+H_2O$) as observed in MS^3 experiments with *m/z* 155 (Table 2). This water adduct formation is highly reversible and allows for the conduction of MS^n 'ping-pong' experiments [17], switching back and forth between the ions at *m/z* 127 and *m/z* 145 (not shown). Finally, elimination of carbon monoxide (−28 u) from the ion–molecule reaction product ion at *m/z* 145 yields the



Scheme 2 **a** Suggested mechanism of most dominant product ion formation after collision-induced dissociation of protonated precursor ions of **M1**–**M4** and derivative **5**. The neutral reactants highlighted in *red* take part in reversible ion–molecule reactions. The *dotted arrow* indicates a minor reaction pathway. **b** Proposed mechanism of the dissociation pathway exclusively observed for compound **5**

fragment ion at *m/z* 117, which is also observable in the MS^3 spectrum of *m/z* 127 (constituting another formal neutral loss of 10 u, Scheme 2). In accordance with recently published observations on the mass spectrometric behaviour of protonated 1- and 2-naphthol [18], the strong tendency of the naphthalene cation to form ion-neutral complexes with Lewis basic compounds in general is further substantiated by the observation of small amounts of carbon monoxide adduct (*m/z* 155) in the ion trap product ion spectra of *m/z* 127 (not shown). Moreover, an abundant ion-neutral complex of *m/z* 127 with acetonitrile (CH_3CN, 41 u) can be observed (*m/z* 168, Scheme 2) if the naphthalene cation is generated by a nozzle skimmer induced in source dissociation (shown for **M3** in Table 2). Whether the neutral reactants in fact establish a covalent bond to the naphthalene moiety as shown in Scheme 2 or are bound by complexation cannot be fully elucidated at this point.

Interestingly, the ion–molecule reactions cannot be observed in the case of **5**, which comprises a hydroxyl function in the 2-position of the naphthalene ring. Compared to the acylium product ions of **M1**–**M4** at *m/z* 155, the hydroxylation leads to a mass shift of 16 u, giving rise to the analogous 2-hydroxy-naphthalene-1-acylium ion at *m/z* 171 (Fig. 1, compound **5**; see also Scheme 2). This ion also eliminates carbon monoxide under the formation of the product ion at *m/z* 143, for which no ion-neutral complex with water can be observed. Instead, a neutral loss of carbon monoxide (−28 u) yields the product ion at *m/z* 115 (Scheme 2).

Moreover, considering the MS/MS spectrum of **5**, the exclusive formation of the product ion at *m/z* 188 ($C_{13}H_{18}N$, Table 2) stands out from the usual dissociation pattern. The mechanism is proposed to include a nucleophilic attack of the 2′-hydroxyl-oxygen at the C13 carbonyl group and transfer of the 2′-hydroxyl-hydrogen to the C3 position concerted with the cleavage of the C3–C13 linkage, finally releasing the protonated *N*-pentylindolium product ion with *m/z* 188 and a neutral naphthalene fragment presumably comprising a *ß*-propiolactone function (Scheme 2b). Taking into account these mechanistic suggestions, an observation of the ion at *m/z* 188 requires a hydroxylation precisely at the 2′-position of the naphthalene moiety since lactone formation might not occur if the

hydroxyl group was located at another site of the aromatic system. The relevance of this will be discussed in the course of the following chapter.

Comparison of synthesised standards and urinary JWH-018 metabolites

In order to check whether the synthesised JWH-018 derivatives in fact constitute true in vivo metabolites, a doping control urine sample, which appeared to be suspicious in the course of the routine screening procedure for JWH-018 abuse, was reanalysed by LC-MS/MS in positive MRM mode. It was then compared to a mixture of all synthesised standards in aqueous solution (all 10 ng/mL) regarding retention times as well as the relative abundances of peak areas obtained from diagnostic ion transitions of the respective compounds. Different elution conditions were necessary for some analytes in order to ensure sufficient separation of the variety of JWH-018 metabolites excreted into the urine. As can be retraced in Fig. 2, the analytes **M1**–**M4** (e.g. the alkylcarboxy-, the terminal alkylhydroxy-, the 5-indolehydroxy- and the *N*-dealkyl-5-indolehydroxy-JWH-018, respectively) could be verified as urinary in vivo metabolites of JWH-018, according to the rules established by WADA [19]. It is to be said that this conclusion can only be drawn on the presumption that all regioisomeric hydroxy

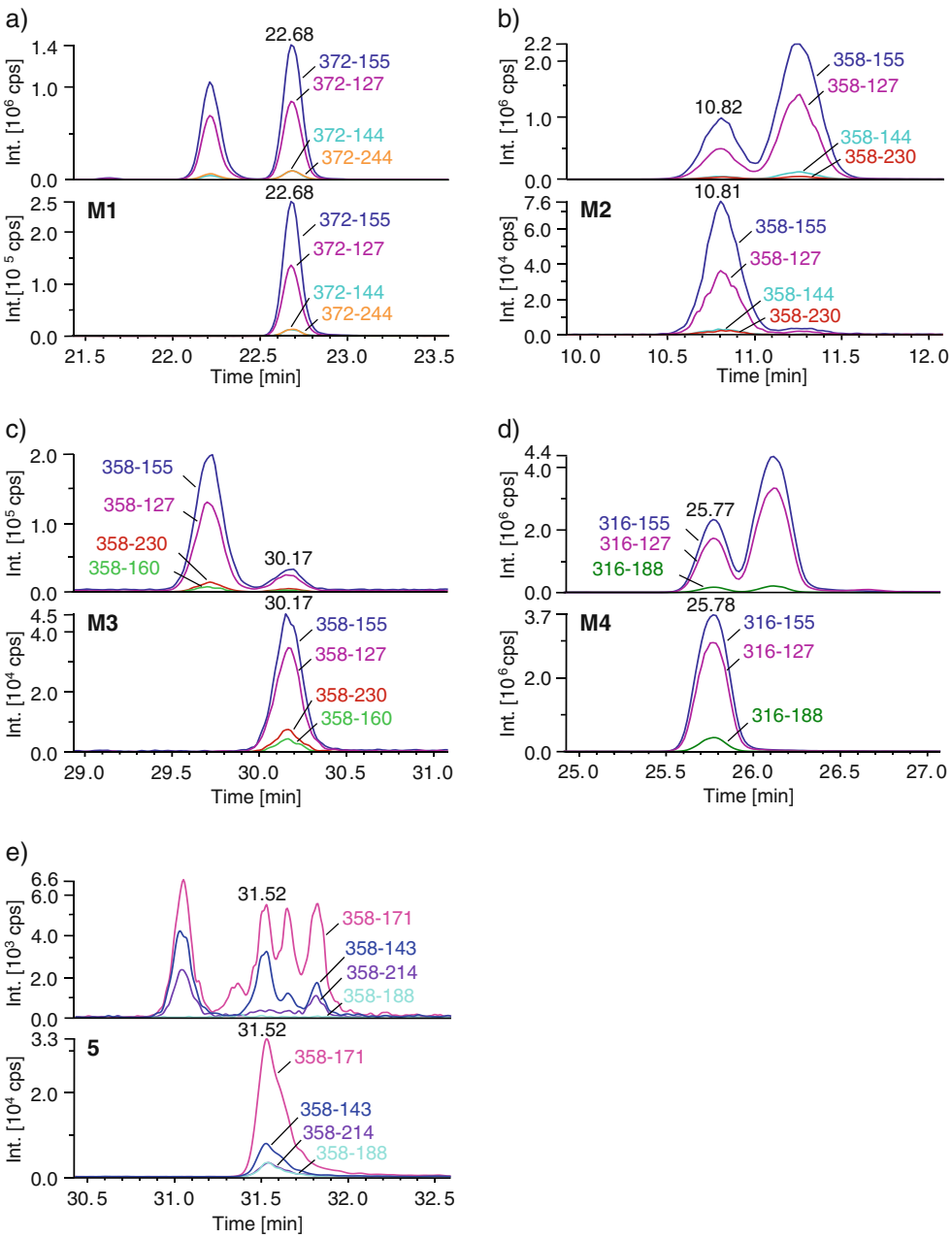


Fig. 2 LC-MS/MS analyses for comparison of the synthesised standards (*lower chromatogram*) with urine specimens containing JWH-018 metabolites (*top chromatogram*) recorded on an API 4000 QTrap mass spectrometer using MRM mode and gradient elution conditions unless otherwise specified: **M1** (**a**), **M2** using isocratic elution (**b**), **M3** (**c**), **M4** after methylation of the hydroxyl- as well as the amino function (**e**), compound **5** (**d**)

Table 3 Summary of assay validation results of **M1** and **M2**

	LOD (ng/mL)	LOQ (ng/mL)	Recovery (%) at 100 ng/mL		Precision			Bias (n=5+5+5)		
					Conc. (ng/mL)					
					0.5	10	50	0.5	10	50
M1	0.1	0.5	44	Intraday (n=30)	9.8%	10.2%	5.8%	112.6%	98.0%	91.0%
				Interday (n=90)	13.4%	11.6%	9.4%			
M2	0.1	0.5	77	Intraday (n=30)	9.3%	5.8%	6.3%	89.3%	95.1%	107.0%
				Interday (n=90)	12.4%	6.8%	7.0%			

compounds potentially present in the excretion urine (e.g. all five theoretically possible hydroxyindoles) have distinct chromatographic retention properties. For the confirmation of **M4** as metabolite (Fig. 2d), a further derivatisation step with iodomethane for site-specific methylation was necessary as endogenous compound(s) from the urine matrix interfered with most of the characteristic ion transitions [9]. Interestingly, the optimised chromatographic conditions allowed for the revelation of evidence that **M2**, **M3** and **M4** are not detected as the most abundant metabolites in their respective ion traces and that other regioisomers of the respective hydroxylation sites are also present in the urine (Figs. 2b–d). Figure 2e shows extracted ion chromatograms of compound **5** in the urine sample as well as the standard, respectively. The considerable number of peaks detected using the most abundant ion transitions 358–171, 358–214 and 358–143 (all indicative of naphthalene hydroxylations) at similar retention times as the standard indicate that several naphthalene-hydroxylated regioisomers are present in the sample. In contrast to the aforementioned findings, however, compound **5** could not be verified as a urinary metabolite because no signal is observed in the extracted ion chromatogram of 358–188 in the urine sample, which serves as a selective diagnostic tool for the presence of a hydroxylation in the 2′-position of the naphthalene ring (see previous chapter).

Taken together, these results suggest using **M1** as a well-defined, chromatographically separated and mainly unconjugated urinary metabolite [9] for confirmation analysis of suspicious urine samples. For screening purposes, however, **M2** is proposed as the most efficient target since it is detected as the most abundant peak in co-elution with other isobaric alkylhydroxy metabolite species.

Assay validation

The assay was validated for the quantitative determination of **M1** as target analyte for the confirmation analysis of JWH-018 abuse. Although not recommended for confirmation purposes due to the chromatographic overlap of urinary metabolites, validation was also performed for **M2** in order to test for suitability as a screening tool. The main validation results are summarised in Table 3. The method demonstrated specificity as no interfering peaks were observed at the expected retention times of the metabolites and the ISTD using extracted ion chromatograms of diagnostic ion transitions (Table 1) in all blank urine specimens. Moreover, the presence of (structurally) analogous cannabimimetics in 200-fold excess did not interfere with the determination of the target analytes.

The test for stability of the analytes in urinary matrix showed no significant decrease over a period of 4 weeks under storage at room temperature as well as 4 °C, and ion suppression/enhancement effects, studied by a continuous infusion of the analytes, accounted for <10% at the expected retention times of **M1** and **M2**, respectively. Both analytes were found linear according to Mandel [20] over a concentration range of 0–100 ng/mL with a slope of 0.069 and 0.052, an intercept of 0.064 and 0.082, as well as correlation coefficient of r=0.999 and 0.997 for **M1** and **M2**, respectively. The LOD was estimated at 0.1 ng/mL by a signal-to-noise ratio≥3 and the LOQ at 0.5 ng/mL (S/N≥10) for both metabolites. The relative standard deviations were calculated below 10% for the precision at LOQ level for both metabolites. A recovery of 77% was determined for **M2** at 1 ng/mL, whereas the recovery for **M1** was only

Table 4 Quantification of **M1** and **M2** in authentic doping control samples

	M1 (ng/mL)	M2[a] (ng/mL)
Sample 1 (0.1 mL[b])	44	710
Sample 2 (0.5 mL[b])	6	101
Sample 3 (0.5 mL[b])	18	139
Sample 4 (0.5 mL[b])	12	147

[a] Sum of **M2** and another metabolite monohydroxylated at the alkyl side chain which chromatographically overlap under the employed gradient elution conditions

[b] Urine aliquot of the doping control specimen, filled up to a volume of 1 mL with blank urine

moderate under the routinely employed screening conditions (44%), but improved considerably to 79% using a neutral extraction medium. The calculated relative standard deviations for intra- and interday precisions were, in accordance with the FDA guidelines [21], all below 15% (20% for LOQ, respectively), and a mean bias of 101% and 97% for **M1** and **M2**, respectively, was determined with relative standard deviations of <10%.

Authentic doping control samples-quantification

Using this validated method, the concentration of **M1** and **M2** in four doping control samples, suspicious for JWH-018 intake according to the routine screening procedure, was determined (Table 4). Unfortunately, for **M2**, a complete overlap with another peak, corresponding to a hydroxylation at another position of the alkyl side chain, was observed under these chromatographic conditions. But owing to the fact that the MS/MS spectra obtained from both peaks are quite similar and a good peak shape was observed (not shown), the sum of both metabolite peaks was quantified against a standard curve of **M2**. Additionally, **M2** was selectively quantified using isocratic elution conditions. It accounts for about 30% of the sum of both metabolites (not shown). For **M1**, considerably lower concentrations were observed in the four samples compared to the summed response of **M2**.

Finally, in order to facilitate the quantification of the carboxy metabolite **M1** in the future, a stable isotope-labelled analogue of **M1** was synthesised in accordance with the synthetic route described above, but starting from $^{13}C_8$-^{15}N-indole as educt. The incorporation of stable isotope labels into the molecule of **$^{13}C_8$-^{15}N-M1** at the specified positions is confirmed by ^{13}C and ^{15}N NMR analysis, whilst ^{1}H NMR results are in accordance to those of **M1** (see "Experimental"). Moreover, determination of the elemental composition was achieved by HRMS analysis (Table 2).

Conclusion

With the help of the synthesised reference standards, an unequivocal structural assignment of four urinary JWH-018 metabolites was achieved for the first time. The CID behaviour of all metabolites includes an ion–molecule reaction with water in the gas phase, resulting in formal neutral losses of 10 u in the fragmentation pathway. LC-MS/MS analysis of doping control urine samples that appeared to be suspicious of JWH-018 intake in the course of routine screening procedures revealed that the most abundant metabolite peak in the chromatogram corresponds to a co-eluting mixture of at least two alkylhydroxylated metabolites, including the terminal alkylhydroxy analogue **M2**. Screening for this mixture of regioisomers in urinary drug testing is therefore likely to yield the most sensitive results. For confirmatory analysis, however, the chromatographically separated and mass spectrometrically well-defined carboxy metabolite **M1** is recommended, which can be determined with an LOD of 0.1 ng/mL even under alkaline extraction conditions. Since this metabolite proved to be the most robust target analyte, an isotope-labelled analogue of **M1** was synthesised and characterised in order to serve as ideal internal standard for future quantitative LC-MS applications.

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