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Elucidation of the metabolites of the novel psychoactive substance 4-methyl-*N*-ethyl-cathinone (4-MEC) in human urine and pooled liver microsomes by GC-MS and LC-HR-MS/MS techniques and of its detectability by GC-MS or LC-MS^n standard screening approaches

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4-methyl-*N*-ethcathinone (4-MEC), the *N*-ethyl homologue of mephedrone, is a novel psychoactive substance of the beta-keto amphetamine (cathinone) group. The aim of the present work was to study the phase I and phase II metabolism of 4-MEC in human urine as well as in pooled human liver microsome (pHLM) incubations. The urine samples were worked up with and without enzymatic cleavage, the pHLM incubations by simple deproteinization. The metabolites were separated and identified by gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-high resolution-tandem mass spectrometry (LC-HR-MS/MS). Based on the metabolites identified in urine and/or pHLM, the following metabolic pathways could be proposed: reduction of the keto group, *N*-deethylation, hydroxylation of the 4-methyl group followed by further oxidation to the corresponding 4-carboxy metabolite, and combinations of these steps. Glucuronidation could only be observed for the hydroxy metabolite. These pathways were similar to those described for the *N*-methyl homologue mephedrone and other related drugs. In pHLM, all phase I metabolites with the exception of the *N*-deethyl-dihydro isomers and the 4-carboxy-dihydro metabolite could be confirmed. Glucuronides could not be formed under the applied conditions. Although the taken dose was not clear, an intake of 4-MEC should be detectable in urine by the GC-MS and LC-MS^n standard urine screening approaches at least after overdose. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: 4-MEC; designer drugs; urine; human liver microsomes; GC-MS; LC-HR-MS/MS

Introduction

For several years, the use of recreational drugs, also called legal highs, designer drugs, or novel psychoactive substances (NPS), has been increasing particularly due to easy access via the Internet. These new synthetic drugs are often phenethylamine derivatives. Besides the well-known ecstasy compounds, the cathinone derivatives (beta-keto amphetamines) are more and more consumed.[1–7] Cathinone, a natural product from Khat (*Catha edulis*), is the chemical lead and mephedrone (4-methyl-methcathinone) was one of the first derivatives. Overdose of such psychostimulant cathinones lead to elevated hyperthermia, sweating, convulsions, but also to hallucinations and paranoia.[8,9]

Simmler *et al.* described the interaction of cathinones with the monoamine transporters and receptors.[10] In some countries, the whole cathinone group was scheduled, but in other countries, only particular derivatives were.

Besides other cathinones, 4-methyl-ethcathinone (4-MEC) appeared on the market and since then several overdose cases have been reported, including some analytical data.[11–13] The symptoms were similar to that described above. 4-MEC was described as inhibitor of all monoamine transporters and as serotonin releaser.[10] As mentioned in the case reports, the *in vivo* metabolism of 4-MEC was not yet elucidated in contrast to that of other cathinones.[1,2,14–18]

Therefore, the aim of the present study was to elucidate the phase I and II metabolites of 4-MEC in human urine and to con-

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firm them in pooled human liver microsome (pHLM) incubations using gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-high resolution-tandem mass spectrometry (LC-HR-MS/MS). In addition, it should be tested whether an intake could be monitored by the authors' GC-MS and LC-MS^n standard urine screening approaches (SUSA).[19,20]

Experimental

Chemicals and reagents

4-MEC was obtained from LGC Standards (Wesel, Germany), isocitrate and isocitrate dehydrogenase from Sigma (Taufkirchen, Germany), $NADP^+$ from Biomol (Hamburg, Germany), acetonitrile (LC-MS grade), ammonium formate (analytical grade), formic acid, methanol (both LC-MS grade), a mixture (10,000 Fishman units/mL) of glucuronidase (EC No. 3.2.1.31) and arylsulfatase (EC No. 3.1.6.1) from Helix Pomatia L., and all other chemicals (analytical grade) from VWR (Darmstadt, Germany). pHLM (20 mg microsomal protein/mL, 400 pmol total CYP/mg protein) were obtained from BD Biosciences (Heidelberg, Germany). After delivery, the microsomes were thawed at 37 °C, aliquoted, snap-frozen in liquid nitrogen, and stored at -80 °C until use.

Urine samples

The investigations were performed using urine samples submitted to the authors' laboratories for toxicological diagnostics: one from an overdose case[13] and one from a routine drug testing case.

Sample preparation for identification of phase I metabolites in urine by GC-MS and LC-HR-MS/MS

As described previously,[14,21] 2 mL of urine were adjusted to pH 5.2 with acetic acid (1 M, approximately 50 μL) and incubated at 56 °C for 1.5 h with 50 μL of a mixture (100 000 Fishman units/mL) of glucuronidase and arylsulfatase from *Helix Pomatia L*. The urine sample was then diluted with 2 mL of water and loaded on a HCX cartridge, previously conditioned with 1 mL of methanol and 1 mL of water. After passage of the sample, the cartridge was washed with 1 mL of water, 1 mL of 0.01 M hydrochloric acid, and again with 1 mL of water. The retained non-basic compounds were first eluted into a 1.5 mL reaction vial with 1 mL of methanol (fraction 1), whereas the basic compounds were eluted in a second step into a different vial with 1 mL of a freshly prepared mixture of methanol/aqueous ammonia 32% (98:2, *v/v*, fraction 2). The eluates were divided into two vials and gently evaporated to dryness under a stream of nitrogen at 56 °C. One part was reconstituted in 50 μL of a mixture of 10 mM ammonium formate buffer and acetonitrile (1:1, *v/v*) and a 10 μL aliquot injected onto the LC-HR-MS/MS system. The other part was derivatized by acetylation according to the published procedure (acetanhydride/pyridine)[14] and 1 μL injected onto the GC-MS system.

Sample preparation for identification of phase II metabolites in urine by LC-HR-MS/MS

Also similar to published methods, a 100 μL aliquot of urine was mixed with 500 μL of acetonitrile for protein precipitation.[20–22] After shaking and centrifugation, the supernatant was gently evaporated to dryness and reconstituted in 50 μL of a mixture of 10 mM ammonium formate buffer and acetonitrile (1:1, *v/v*) and 10 μL injected onto the LC-HR-MS/MS system.

Pooled human liver microsomes incubation and work-up

Microsomal incubations were performed under conditions published before.[23] Briefly, 4-MEC (50 μM) was incubated with pHLM (50 mg protein/mL) for 30 and 120 min. After incubation, the solution was centrifuged, the supernatant transferred to an autosampler vial, and 10 μL injected onto the LC-HR-MS/MS.

GC-MS apparatus for identification of the phase I metabolites in urine

The extracts were analyzed as previously described[21] using a Hewlett Packard (HP, Agilent, Waldbronn, Germany) 5890 Series II gas chromatograph combined with an HP 5972A MSD mass spectrometer and an HP MS ChemStation (DOS series) with HP G1034C software version C03.00. The GC conditions were as follows: splitless injection mode; column, HP-1 capillary (12 m x 0.2 mm I.D.), cross linked methyl silicone, 330 nm film thickness; injection port temperature, 280 °C; carrier gas, helium; flow-rate, 0.8 mL/min; column temperature, programmed from 90–230 °C at 30°/min, hold for 3 min, then 230-310 °C at 30°/min, initial time 2 min, final time 2 min. The MS conditions were as follows: full scan mode, mass range *m/z* 50–550; electron ionization (EI) mode; ionization energy, 70 eV; ion source temperature, 220 °C; capillary direct interface, heated at 280 °C.

LC-HR-MS/MS apparatus for confirmation and identification of phase I and II metabolites in urine and pHLM

The extracts were analyzed using a ThermoFisher Scientific (TF, Dreieich, Germany) Accela LC system consisting of a degasser, a quaternary pump and an HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), coupled to a TF Q-Exactive system equipped with heated electrospray ionization (HESI) II source. The instrument was mass calibrated prior to analysis by infusion of a Positive Mode Cal Mix provided by Supelco (Bellefonte, PA) at a flow rate of 5 μL/min using a syringe pump. The LC conditions were as follows: TF Accucore PhenylHexyl (100 mm x 2.1 mm, 2.6 μm); gradient elution with Millipore water containing formic acid (0.1%, *v/v*) as mobile phase A and acetonitrile containing formic acid (0.1%, *v/v*) as mobile phase B.

The gradient and flow rate were programmed as follows: 0–4 min 2% B to 60% B, 4–7 min hold 90% B, and 7–10 min hold 2% B, constantly at 500 μL/min.[14] The HESI-II source conditions were as follows: heater temperature, 320 °C; ion transfer capillary temperature, 320 °C; sheath gas, 60 arbitrary units (AU); auxiliary gas, 10 AU; spray voltage, 3.00 kV, and S-lens RF level, 50.0. Mass spectrometry was performed in positive polarity mode using full scan data and a subsequent data dependent MS^2 (dd-MS^2) mode with an inclusion list on the masses of interest. The settings for full scan data acquisition were as follows: resolution, 35 000; automatic gain control (AGC) target, 1e6; maximum injection time (IT), 120 ms; scan range, *m/z* 50 to 750. The settings for the (dd-MS^2) mode with an inclusion list were as follows: resolution, 17,500; AGC target, 2e5; maximum IT, 250 ms; high collision dissociation (HCD) cell with stepped normalized collision energy (NCE), 35 +/- 50 %.

Standard urine screening approach (SUSA) using GC-MS

For work-up (hydrolysis, liquid-liquid extraction, and microwave-assisted acetylation), already published procedures were used.[19,21] Briefly, 5 mL of urine were divided into two aliquots and one part was submitted to acid hydrolysis. Thereafter, the sample was adjusted to pH 8-9 and the other aliquot of untreated urine was added. This mixture was extracted with 5 mL of a dichloromethane-isopropanol-ethyl acetate mixture (1:1:3, *v/v/v*) and the organic layer was evaporated to dryness. The residue was acetylated with an acetic anhydride-pyridine mixture (3:2, *v/v*) under microwave irradiation. After evaporation of the derivatization mixture, the residue was dissolved in 100 μL of methanol and 1 μL were injected onto the GC-MS system.

The GC conditions were similar to those for the metabolism studies with the oven programmed from 90 to 310 °C at 30°/min, final time 2 min.

For toxicological detection of 4-MEC and its metabolites, mass chromatography was used with the extracted ions at *m/z* 72, 86, 114, 119, 121, and 129. Generation of the mass chromatograms was performed with user defined macros.[19] The identity of the peaks in the mass chromatograms was confirmed by comparison of the mass spectra underlying the peaks (after background subtraction) with reference spectra recorded during this study. In addition, the full scan data files acquired by GC-MS were evaluated by the automated mass spectral deconvolution and identification system (AMDIS) (http://chemdata.nist.gov/mass-spc/amdis/) in simple mode.[24] The deconvolution parameter settings were as follows: width 32; adjacent peak subtraction two; resolution high; sensitivity very high; and shape requirements low. The minimum match factor was set to 40. The target library was a modified version of the Maurer/Pfleger/Weber MPW_2011 library.[25]

Standard urine screening approach (SUSA) using LC-MS^n

In accordance with Wissenbach *et al.*,[20,22] the urine samples (100 μL) were worked up by protein precipitation as described above for identification of phase II metabolites. The samples were separated and analyzed using a TF LXQ linear ion trap MS equipped with a HESI II source and coupled to a TF Accela LC system consisting of a degasser, a quaternary pump, and an autosampler. Gradient elution was performed using a TF Hypersil Gold (150 x 2.1 mm, 1.9 μm) and 10 mM aqueous ammonium formate buffer containing formic acid (0.1%, *v/v*) as mobile phase A and acetonitrile containing formic acid (0.1%, *v/v*) as mobile phase B. The gradient and flow rate were programmed from 98% to 0% A at 500 μL/min within 21 min (injection volume 10 μL). Data-dependent acquisition (DDA) was conducted on precursor ions selected from MS^1. MS^1 was performed in full scan mode (*m/z* 100-800). MS^2 and MS^3 were performed in DDA mode: four DDA MS^2 scan filters were chosen to provide MS^2 on the four most intense signals from MS^1 and additionally, eight MS^3 scan filters were chosen to record MS^3 on the most and second most intense signals from the MS^2. MS^2 spectra were collected with a higher priority than MS^3 spectra. Normalized wideband collision energies with collision induced dissociation (CID) were 35% for MS^2 and 40% for MS^3.

TF ToxID 2.1.1 was used for automatic target screening in the MS^2 screening mode. The settings were as follows: retention time (RT) window, 20 min; RT, 0.1 min; signal threshold, 100 counts; search index, 600; reverse search index, 700. SmileMS version 1.1 (GeneBio, Geneva, Switzerland) was used for automatic target screening using the precursor tolerance option and for automatic untargeted screening without precursor tolerance option and RT locking. Further settings were as follows: score threshold, 0.1; minimum number peak matches, 0. ToxID and SmileMS were run automatically after file acquisition using an Xcalibur processing method starting both software tools.[26]

The MS^2 and MS^3 reference spectra were recorded in urine after the above-mentioned workup and analysis (mass list given in Table 2). They were confirmed by comparison with the corresponding LC-HR-MS/MS spectra.

Results and discussion

Identification of the phase I metabolites by GC-MS

The urinary metabolites of 4-MEC were identified by full-scan EI MS after GC separation. The postulated structures of the metabolites were deduced from the fragments detected in the EI mode, which were interpreted in correlation to those of the parent compound according to the fragmentation pattern of mephedrone[2] and the general fragmentation rules described by McLafferty and Turecek and Smith and Busch, for example.[27–29] The gas chromatographic retention indices (RI) were determined according to de Zeeuw *et al.*[30] Mass spectra, RIs, structures and predominant fragmentation patterns of 4-MEC and its metabolites are shown in Figure 1.

Proposed fragmentation patterns for identification of the phase I metabolites by GC-MS

In the following, important fragmentation patterns of the EI mass spectra of 4-MEC and its derivatized metabolites will be discussed. The numbers of the corresponding mass spectra in Figure 1 are given in brackets.

Alpha cleavage between position 1 and 2 led to the fragment ions at *m/z* 119 and/or *m/z* 114 (nos. I, II, V, VI, and VII) as a main fragmentation step.[31] Further fragmentation of the fragment ion at *m/z* 114 led to the imonium ion at *m/z* 72 indicating a conserved ethyl part (nos. I, V, VI, and VII). In contrast, the iminium ion at *m/z* 86 formed via alpha cleavage indicated a primary amine due to metabolic *N*-deethylation (II, III, and IV). The unchanged methylbenzyl part was represented by the fragment ion at *m/z* 91 (I, II, III, IV, and V). Again, in accordance with Zuba,[31] a further loss of acetylene (C_2H_2) from the fragment ion at *m/z* 91 resulted in the fragment at *m/z* 65 (I and II). Acetylation of the dihydro metabolites in beta position (nos. III, IV, and V) was characterized by a shift of *m/z* 59 (*m/z* 249 to 190 and *m/z* 277 to 218) because of the loss of acetate. The fragment ion at *m/z* 121 corresponded to the fragment ion at *m/z* 119 shifted by two hydrogens (nos. III, and IV). The fragment ion at *m/z* 129 being characteristic for the dihydro metabolites was a result of multiple fragmentation steps and should have been a 1-(acetylamino)-2-(formyloxy)ethylium-2-yl ion.

Oxidation of the tolyl part to the carboxylic acid and artificial methylation by methanol in the injection port[19] (no. VI) was indicated by the fragment ion at *m/z* 163 (alpha cleavage between position 1 and 2) and the fragment ion at *m/z* 246 (loss of methanol). Hydroxylation and acetylation of the tolyl part (no. VII) led to the fragment ion at *m/z* 177 based also on this alpha cleavage.

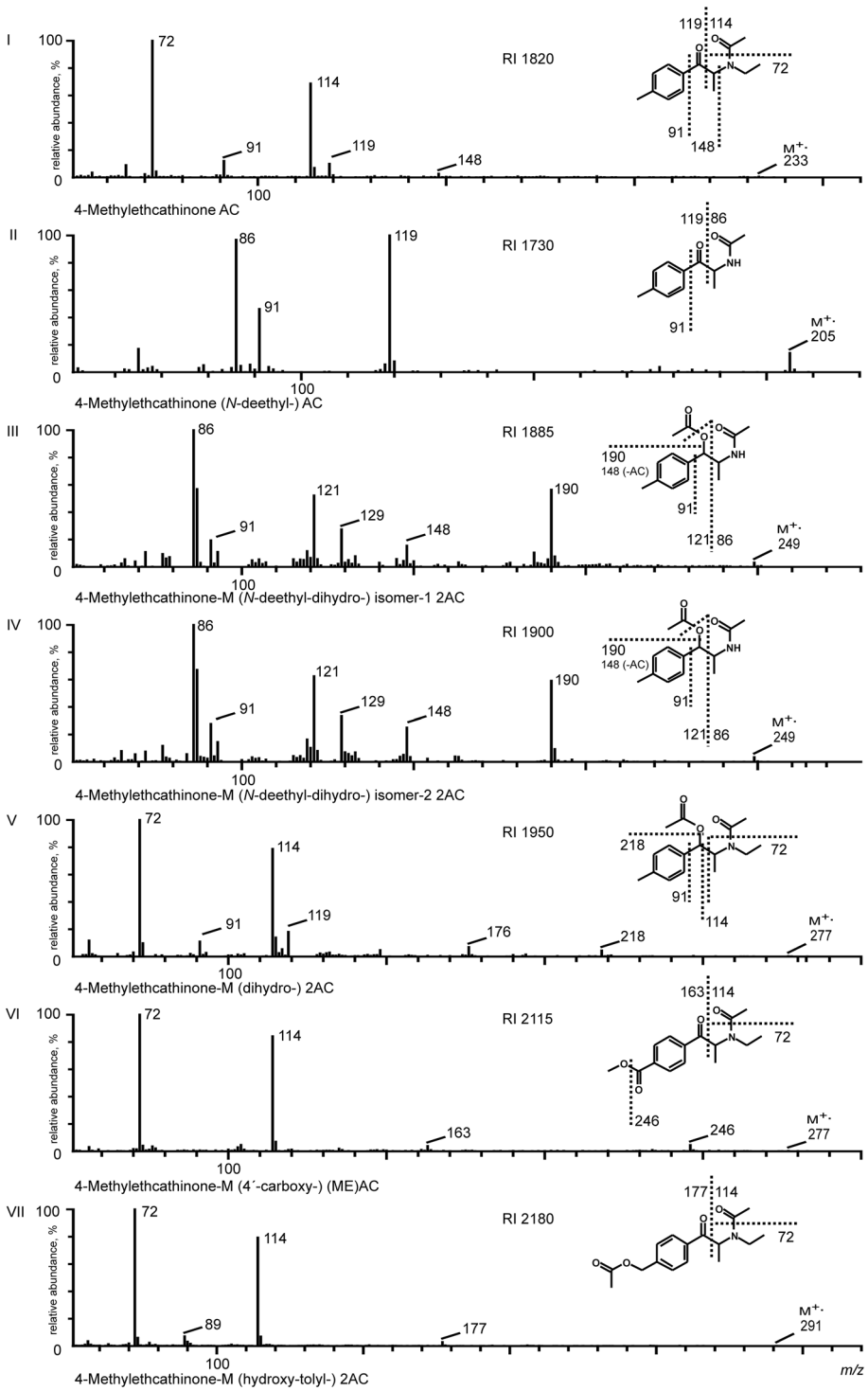

Figure 1. EI mass spectra, gas chromatographic retention indices (RI), proposed structures, and predominant fragmentation patterns of 4-MEC and its metabolites (all after acetylation) arranged according to ascending mass of the metabolites and RIs

Proposed fragmentation patterns for confirmation of the phase I metabolites by LC-HR-MS/MS

The phase I metabolites indicated by GC-MS could be confirmed by LC-HR-MS/MS and the 4-carboxy-dihydro-metabolite could additionally be detected. In Table 1, their exact masses, accurate masses, delta ppm values, retention times (RT), precursor ions, and characteristic MS^2 ions of the phase I and II metabolites of 4-MEC are summarized. The initial fragmentation step of 4-MEC was the loss of water (18.0105 u) in accordance to Zuba.[31] This initial step was also seen for the dihydro, *N*-deethyl, *N*-deethyl-dihydro isomers, 4-carboxy, 4-carboxy-dihydro, and hydroxy-tolyl metabolites. Furthermore, a loss of CH_3 as a radical at *m/z* 159.1043 occurred. A characteristic α-cleavage of the amine led to the fragments at *m/z* 146.0964 (not shown in Table 1) and at *m/z* 145.0887. A rearrangement by spontaneous formation

Table 1. 4-MEC and its metabolites (numbers correspond to Figure 1 and Table 2), protonated molecular ions (PM), characteristic fragment ions (FI) with measured accurate masses (ESI, positive mode), corresponding calculated exact masses, delta ppm values (rounded to two decimals), proposed elemental composition, and retention times (RT) recorded by LC-HR-MS/MS (in order of ascending mass of the metabolites)

No.	4-MEC and its metabolites, characteristic ions with measured accurate masses *(m/z)*		Exact Mass *(m/z)*	delta (ppm)	Proposed elemental composition	RT (min)
I	**4-MEC**					
	MS^1	PM *at m/z* 192.1385	192.1382	1.56	$C_{12}H_{18}NO$	2.4
	MS^2	FI at *m/z* 174.1279	174.1282	1.72	$C_{12}H_{16}N$	
		FI at *m/z* 159.1043	159.1047	2.51	$C_{11}H_{13}N$	
		FI at *m/z* 145.0887	145.0891	2.76	$C_{10}H_{11}N$	
		FI at *m/z* 131.0730	131.0734	3.05	C_9H_9N	
		FI at *m/z* 119.0858	119.0860	1.68	C_9H_{11}	
		FI at *m/z* 91.0547	91.0547	0	C_7H_7	
II	**4-MEC-M (*N*-deethyl-)**					
	MS^1	PM at *m/z* 164.1071	164.1069	1.22	$C_{10}H_{14}NO$	2.2
	MS^2	FI at *m/z* 146.0965	146.0969	2.74	$C_{10}H_{12}N$	
		FI at *m/z* 131.0731	131.0734	2.29	C_9H_9N	
		FI at *m/z* 119.0858	119.0860	1.68	C_9H_{11}	
		FI at *m/z* 91.0547	91.0547	0	C_7H_7	
III	**4-MEC-M (*N*-deethyl-dihydro-) isomer 1**					
	MS^1	PM at *m/z* 166.1227	166.1226	0.6	$C_{10}H_{16}NO$	1.9
	MS^2	FI at *m/z* 148.1121	148.1126	3.38	$C_{10}H_{14}N$	
		FI at *m/z* 131.0857	131.0860	2.89	$C_{10}H_{11}$	
		FI at *m/z* 116.0624	116.0626	1.72	C_9H_8	
		FI at *m/z* 91.0547	91.0547	0	C_7H_7	
IV	**4-MEC-M (*N*-deethyl-dihydro-) isomer 2**					
	MS^1	PM at *m/z* 166.1225	166.1226	0.6	$C_{10}H_{16}NO$	2.1
	MS^2	FI at *m/z* 148.1121	148.1126	3.38	$C_{10}H_{14}N$	
		FI at *m/z* 131.0855	131.0860	3.81	$C_{10}H_{11}$	
		FI at *m/z* 116.0621	116.0626	4.3	C_9H_8	
		FI at *m/z* 91.0546	91.0547	1.10	C_7H_7	
V	**4-MEC-M (dihydro-)**					
	MS^1	PM at *m/z* 194.1536	194.1544	4.12	$C_{12}H_{20}NO$	2.3
	MS^2	FI at *m/z* 176.1438	176.1439	0.57	$C_{12}H_{18}N$	
		FI at *m/z* 161.1204	161.1204	0	$C_{11}H_{15}N$	
		FI at *m/z* 147.1049	147.1047	1.36	$C_{10}H_{13}N$	
		FI at *m/z* 131.0857	131.0860	2.29	$C_{10}H_{11}$	
		FI at *m/z* 116.0623	116.0626	2.58	C_9H_8	
		FI at *m/z* 91.0547	91.0547	0	C_7H_7	
VI	**4-MEC-M (4-carboxy-)**					
	MS^1	PM at *m/z* 222.1127	222.1124	1.35	$C_{12}H_{16}NO_3$	1.6
	MS^2	FI at *m/z* 204.1020	204.1024	1.96	$C_{12}H_{14}NO_2$	
		FI at *m/z* 186.0915	186.0918	1.61	$C_{12}H_{12}NO$	
		FI at *m/z* 160.1122	160.1126	2.50	$C_{11}H_{14}N$	
		FI at *m/z* 132.0810	132.0813	2.27	$C_9H_{10}N$	
		FI at *m/z* 105.0704	105.0704	0	C_8H_9	
VII	**4-MEC-M (hydroxy-tolyl-)**					
	MS^1	PM at *m/z* 208.1332	208.1337	2.4	$C_{12}H_{18}NO_2$	1.7
	MS^2	FI at *m/z* 190.1228	190.1231	1.58	$C_{12}H_{16}NO$	
		FI at *m/z* 172.1122	172.1126	2.32	$C_{12}H_{14}N$	
		FI at *m/z* 160.1122	160.1122	0	$C_{11}H_{14}N$	
		FI at *m/z* 132.0810	132.0813	2.27	$C_9H_{10}N$	
VIII	**4-MEC-M (4-carboxy-dihydro-)**					
	MS^1	PM at *m/z* at *m/z* 224.1279	224.1281	0.89	$C_{12}H_{18}NO_3$	1.5
	MS^2	FI at *m/z* 206.1176	206.1181	2.43	$C_{12}H_{16}NO_2$	
		FI at *m/z* 191.0940	191.0946	3.14	$C_{11}H_{13}NO_2$	

(Continues)

Table 1. (Continued)

No.	4-MEC and its metabolites, characteristic ions with measured accurate masses (*m/z*)	Exact Mass (*m/z*)	delta (ppm)	Proposed elemental composition	RT (min)
	FI at *m/z* 178.0863	178.0868	2.81	$C_{10}H_{12}NO_2$	
	FI at *m/z* 161.0596	161.0602	3.73	$C_{10}H_9O_2$	
	FI at *m/z* 117.0701	117.0704	2.56	C_9H_9	
IX	**4-MEC-M (hydroxy-tolyl- glucuronide)**				
	MS^1 PM at *m/z* 384.1651	384.1652	0.26	$C_{18}H_{26}NO_8$	1.4
	MS^2 FI at *m/z* 208.1333	208.1332	0.48	$C_{12}H_{18}NO_2$	
	FI at *m/z* 190.1227	190.1231	2.1	$C_{12}H_{16}NO$	
	FI at *m/z* 172.1120	172.1126	3.49	$C_{12}H_{14}N$	
	FI at *m/z* 160.1120	160.1120	0	$C_{11}H_{14}N$	
	FI at *m/z* 132.0810	132.0813	2.27	$C_9H_{10}N$	

of a more stable indole system can be postulated in accordance to Pedersen *et al*.[18] The radical was supposed to lose a hydrogen atom to form an even electron fragment (*m/z* 144.0808, not shown in Table 1).[32] A further loss of methyl as a radical resulted in the ion at *m/z* 131.0730. The ion at *m/z* 119.0858 resulted from a neutral loss containing a nitrogen after dissociation of the C-N bond being abundant in cathinones with tertiary amines in combination with a rearrangement reaction[31]. Loss of C_2H_4 formed a tropylium ion at *m/z* 91.0547.

For *N*-deethyl 4-MEC (no. II, protonated mass, PM at *m/z* 164.1071), the same fragment ions as observed for the parent drug could be detected because of the unchanged 4-methylphenalkylamine structure. However, in contrast to the parent drug, only the more stable fragment ion at *m/z* 146.0965 was formed by loss of CH_3 as a radical. The fragmentation of the *N*-deethyl-dihydro 4-MEC isomers (nos. III and IV, PM at *m/z* 166.1227 and 166.1225) was similar that of dihydro 4-MEC. Only the fragment at *m/z* 148.1121 was shifted to *m/z* 147.1049 for the dihydro metabolite. The *N*-deethylation and reduction of the keto moiety was supposed to favor the conversion to the more stable postulated indole form.[18] Differentiation of the isomers was possible because of different RTs. These findings were in accordance to previous studies.[2]

The metabolic reduction of the keto moiety to the respective alcohol (no. V in Table 1, PM at *m/z* 194.1536) led to a shift of 2.0156 u from the ion at *m/z* 174.1279 to 176.1438, from ion at *m/z* 159.1043 to 161.1204 and from ion at *m/z* 145.0887 to 147.1049 (see Table 1). In contrast to the parent drug, the dihydro part was fragmented to the ion at *m/z* 131.0857 due to neutral loss of NH_3 and at *m/z* 116.0623 after loss of CH_3 as a radical. The tropylium ion at *m/z* 91.0547 was detected in accordance to the parent drug. All the other fragment ions were equivalent to the parent compound.

The metabolic introduction of a hydroxy group at the tolyl part (no. VII, PM at *m/z* 208.1332) led after the initial loss of water (*m/z* 190.1228) to a second loss of water to fragment ion at *m/z* 172.1122. This water elimination was followed by a loss of a carbon atom indicated by a fragment ion at *m/z* 160.1122 and a further loss of C_2H_4 to *m/z* 132.0810. Consequently, for the hydroxy-tolyl metabolite, the 4-methyl-phenalkylamine structure was shown to be stable.

Metabolic oxidation of the hydroxy-tolyl metabolite to the corresponding carboxylic acid (no. VI, PM at *m/z* 222.1127) led in addition to the initial loss of water (*m/z* 204.1020) to a loss of CO_2 (*m/z* 204.1020 to 160.1122) illustrating that the initial water loss resulted from the carbonyl part. In analogy to the parent drug fragmentation patterns, ion at *m/z* 186.0915 was formed by loss of water of ion at *m/z* 204.1020. Therefore, it could be postulated, that after loss of CO_2 and water, a phenylalkylamine residue, stabilized by mesomery, was formed for 4-carboxy 4-MEC. As described above, the ion at *m/z* 132.0810 occurred from fragment at *m/z* 160.1122 by alpha cleavage of the amine with a loss of C_2H_4. Dissociation of the C-N bond and loss of the methyl part resulted in a phenethyl residue at *m/z* 105.0704.

The metabolic oxidation of the hydroxy-tolyl metabolite in combination with the reduction of the keto moiety of 4-carboxy-dihydro 4-MEC (no. VIII, PM at *m/z* 224.1279) led to an initial loss of water at *m/z* 206.1175. In contrast to the carboxy metabolite, no elimination of CO_2 or water could be observed as a second or third fragmentation step. This might be caused by lack of mesomery stabilization of the phenethylamine residue. Furthermore, the initial loss of water was unlikely to result from the carbonyl part. Again, a loss of CH_3 at *m/z* 191.0940 and C_2H_4 at *m/z* 178.0862 and C-N bond dissociation at *m/z* 178.0862 to 161.0596 was observed. A characteristic shift of 43.9895 u from the ion at *m/z* 161.0596 to 117.0701 indicated the loss of CO_2 as last fragmentation step. This illustrated different fragmentation patterns for the 4-carboxy-dihydro metabolite in contrast to the non-reduced metabolite.

Identification of the phase II metabolites by LC-HR-MS/MS

For detection of the phase II metabolites, ion chromatograms of the calculated exact masses of the protonated conjugates of the identified phase I metabolites were reconstructed in the full scan mode and are included in Table 1. For confirmation of the conjugate structure, the MS^2 spectra after HCD fragmentation were compared to those of the underlying phase I metabolites. Using this approach, the glucuronide of hydroxy-tolyl 4-MEC could be detected (no. IX in Table 1, PM at *m/z* 384.1651). The MS^2 spectrum contained all characteristic fragment ions of the phase I metabolite (Table 1). No sulphates and no carboxy glucuronides could be detected.

Proposed metabolic pathways

As depicted in Figure 2, the following metabolic pathways of 4-MEC could be proposed: reduction of the keto group, *N*-deethylation,

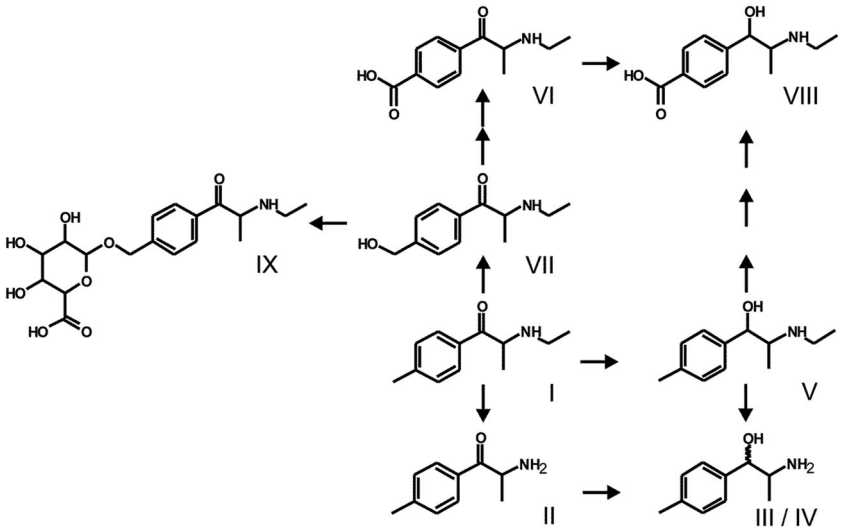

Figure 2. Metabolic pathways of 4-MEC (numbering according to Figure 1 and Tables 1 and 2).

hydroxylation of the 4-methyl group followed by further oxidation to the corresponding 4-carboxy metabolite, and combinations of these steps. Glucuronidation could only be observed for the hydroxy-tolyl metabolite. These pathways were similar to those described for the *N*-methyl homologue mephedrone and other related drugs.[1,2,14,16,18,33] In pHLM, all phase I metabolites with the exception of the *N*-deethyl-dihydro isomers and the 4-carboxy-dihydro metabolite could be confirmed. Glucuronides could not be formed under the applied conditions. The incubation for 30 or 120 min resulted in the same metabolites.

According to previous studies on mephedrone,[2] reduction of the keto group should lead to diastereomers. Only primary amines formed by *N*-demethylation were reduced to both diastereomeric alcohols. These diastereomers were also detected after *N*-deethylation and reduction of 4-MEC. In accordance to Meyer *et al.*,[2] these diastereomers were supposed to be formed enzymatically. In contrast to *N*-deethyl-dihydro 4-MEC, only one diastereomer was detectable for dihydro 4-MEC. This confirmed the previously published hypothesis that the *N*-dealkyl group could sterically hinder the enzymatic reaction to one of the isomers.[2] However, it should be kept in mind that 4-MEC was also reduced under storage conditions at room temperature.[34]

Detectability of 4-MEC and its metabolites by GC-MS or LC-MS^n

An intake of 4-MEC could be monitored by both SUSAs in the overdose case as well as the routine drug testing case by detection of 4-MEC and its metabolites. The identity could be confirmed by comparison of the underlying GC-MS spectra (Figure 1 and Maurer/Pfleger/Weber MPW_2015 library in preparation) or LC-MS^n spectra (Table 2 and Maurer *et al.*[26]).

If an intake of these drugs should be monitored by other screening approaches, the corresponding metabolite and/or mass spectral data should be transformed according to the applied technique, for example ions selected from the GC-MS or LC-MS reference spectra presented here. In case of species differences, genetic variations, and/or severe overdose, the minor metabolites may also occur in human urine and should therefore

Table 2. 4-MEC and its metabolites (numbers correspond to Figure 1 and Table 1), precursor ions, characteristic MS^2 fragment ions, and up to two most abundant ions selected for the following MS^3 spectra (ESI, positive mode, n.d. = not detected) recorded for the LC-MS^n SUSA (in order of ascending mass of the metabolites)

No.	4-MEC and its metabolites	Precursor Ions (*m/z*)	MS^2 spectra with the given ions (*m/z*) and their relative abundance (%)	MS^3 spectra of the ions in bold (*m/z*) and their relative abundance (%)	RT
I	4-MEC	192	146 (100), 147 (50), 145 (30), 174 (10), 159 (5), 119 (3)	**146:** 119 (100), 146 (1)	6.8
II	4-MEC-M (*N*-deethyl-)	164	119 (100), 146 (21), 131 (16), 129 (16)	n.d.	5.9
III	4-MEC-M (*N*-deethyl-dihydro-) isomer 1	166	131 (100), 148 (4)	n.d.	5.5
IV	4-MEC-M (*N*-deethyl-dihydro-) isomer 2	166	131 (100), 148 (4)	n.d.	5.8
V	4-MEC-M (dihydro-)	194	176 (100), 148 (60), 131 (50), 105 (45), 161 (30), 119 (30)	n.d.	7.0
VI	4-MEC-M (4-carboxy-)	222	160 (100), 186 (40), 132 (30)	**160:** 132 (100), 160 (10) **186:** 158 (100), 186 (5)	2.6
VIII	4-MEC-M (4-carboxy-dihydro-)	224	178 (100), 206 (60), 117 (21), 191 (15), 160 (11)	n.d.	2.9

be included in the SUSA. However, for assessing the detection window, urine samples from a controlled drug administration study would be necessary.

Conclusions

The presented study showed that the new designer drug 4-MEC was transformed to various metabolites and that an intake of 4-MEC could be monitored by the authors' GC-MS and LC-MS^n SUSAs approaches.

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