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Lefetamine-derived designer drugs *N*-ethyl-1,2-diphenylethylamine (NEDPA) and *N-iso*-propyl-1,2-diphenylethylamine (NPDPA): Metabolism and detectability in rat urine using GC-MS, LC-MS^n and LC-HR-MS/MS

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***N*-Ethyl-1,2-diphenylethylamine (NEDPA) and *N-iso*-propyl-1,2-diphenylethylamine (NPDPA) are two designer drugs, which were confiscated in Germany in 2008. Lefetamine (*N,N*-dimethyl-1,2-diphenylethylamine, also named L-SPA), the pharmaceutical lead of these designer drugs, is a controlled substance in many countries. The aim of the present work was to study the phase I and phase II metabolism of these drugs in rats and to check for their detectability in urine using the authors' standard urine screening approaches (SUSA). For the elucidation of the metabolism, rat urine samples were worked up with and without enzymatic cleavage, separated and analyzed by gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-high resolution-tandem mass spectrometry (LC-HR-MS/MS). According to the identified metabolites, the following metabolic pathways for NEDPA and NPDPA could be proposed: *N*-dealkylation, mono- and bis-hydroxylation of the benzyl ring followed by methylation of one of the two hydroxy groups, combinations of these steps, hydroxylation of the phenyl ring after *N*-dealkylation, glucuronidation and sulfation of all hydroxylated metabolites. Application of a 0.3 mg/kg BW dose of NEDPA or NPDPA, corresponding to a common lefetamine single dose, could be monitored in rat urine using the authors' GC-MS and LC-MS^n SUSA. However, only the metabolites could be detected, namely *N*-deethyl-NEDPA, *N*-deethyl-hydroxy-NEDPA, hydroxy-NEDPA, and hydroxy-methoxy-NEDPA or *N*-de-*iso*-propyl-NPDPA, *N*-de-*iso*-propyl-hydroxy-NPDPA, and hydroxy-NPDPA. Assuming similar kinetics, an intake of these drugs should also be detectable in human urine.** **Copyright © 2014 John Wiley & Sons, Ltd.**

Keywords: designer drugs; lefetamine derivatives; metabolism; GC-MS; LC-HR-MS/MS

Introduction

Lefetamine (*N,N*-dimethyl-1,2-diphenylethylamine, also named santenol, L-SPA) was marketed as a central analgesic with opioid effects.[1,2] Due to its abuse, it was scheduled in many countries.[3] In 2008, two lefetamine derivatives, *N*-ethyl-1,2-diphenylethylamine (NEDPA) and *N-iso*-propyl-1,2-diphenylethylamine (NPDPA), were confiscated by the German police.[4] These derivatives were already described as drugs with amphetamine-like effects in the early 1940s.[5] Berger *et al*. showed that the respective primary amine 1,2-diphenylethylamine (the potential *N*-dealkyl metabolite of lefetamine, NEDPA, and NPDPA) acts as an N-Methyl-D-aspartate (NMDA) channel blocker similar to phencyclidine and ketamine.[6] Abuse or poison cases of the derivatives have not yet been described, but potential use is discussed in online forums.[7–9] Metabolism data are not yet published, but GC-MS and GC-MS/MS data of the parent compounds lefetamine, NEDPA, and NPDPA were conducted by Westphal *et al*.[4] This is helpful in identifying confiscated material, but in the case of abuse or intoxication, these data are not sufficient for drug testing in urine because many of such drugs are completely metabolized. Therefore, the aim of the present study was to elucidate the phase I and II metabolism of NEDPA and NPDPA in rat urine to define the targets for urine screening. 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).[10,11]

Experimental

Chemicals and reagents

NEDPA and NPDPA were provided by the Landeskriminalamt Baden-Wuerttemberg (Stuttgart, Germany) for research purposes. Isolute HCX cartridges (130 mg, 3 mL) were obtained from Biotage (Uppsala, Sweden), all other chemicals (analytical grade),

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reagents, a mixture (100 000 Fishman units/mL) of glucuronidase (EC No. 3.2.1.31) and arylsulfatase (EC No. 3.1.6.1) from Helix Pomatia L. from VWR (Darmstadt, Germany).

Urine samples

The investigations were performed using urine of male Wistar rats (Charles River, Sulzfeld, Germany) for toxicological diagnostic reasons according to the corresponding German law.[12] They were administered a single 20 mg/kg body mass dose (for metabolism studies) or 0.3 mg/kg (for detectability studies) of NEDPA or NPDPA in aqueous suspension by gastric intubation as described elsewhere.[13] The rats were housed in metabolism cages for 24 h, having water ad libitum. Urine was collected separated from faeces over a 24 h period and stabilized with sodium fluoride. All samples were directly analyzed and then stored at -20°C. Blank urine samples were collected before drug administration to check whether the samples were free of interfering compounds.

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

As described previously,[14] 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 30 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 an 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,

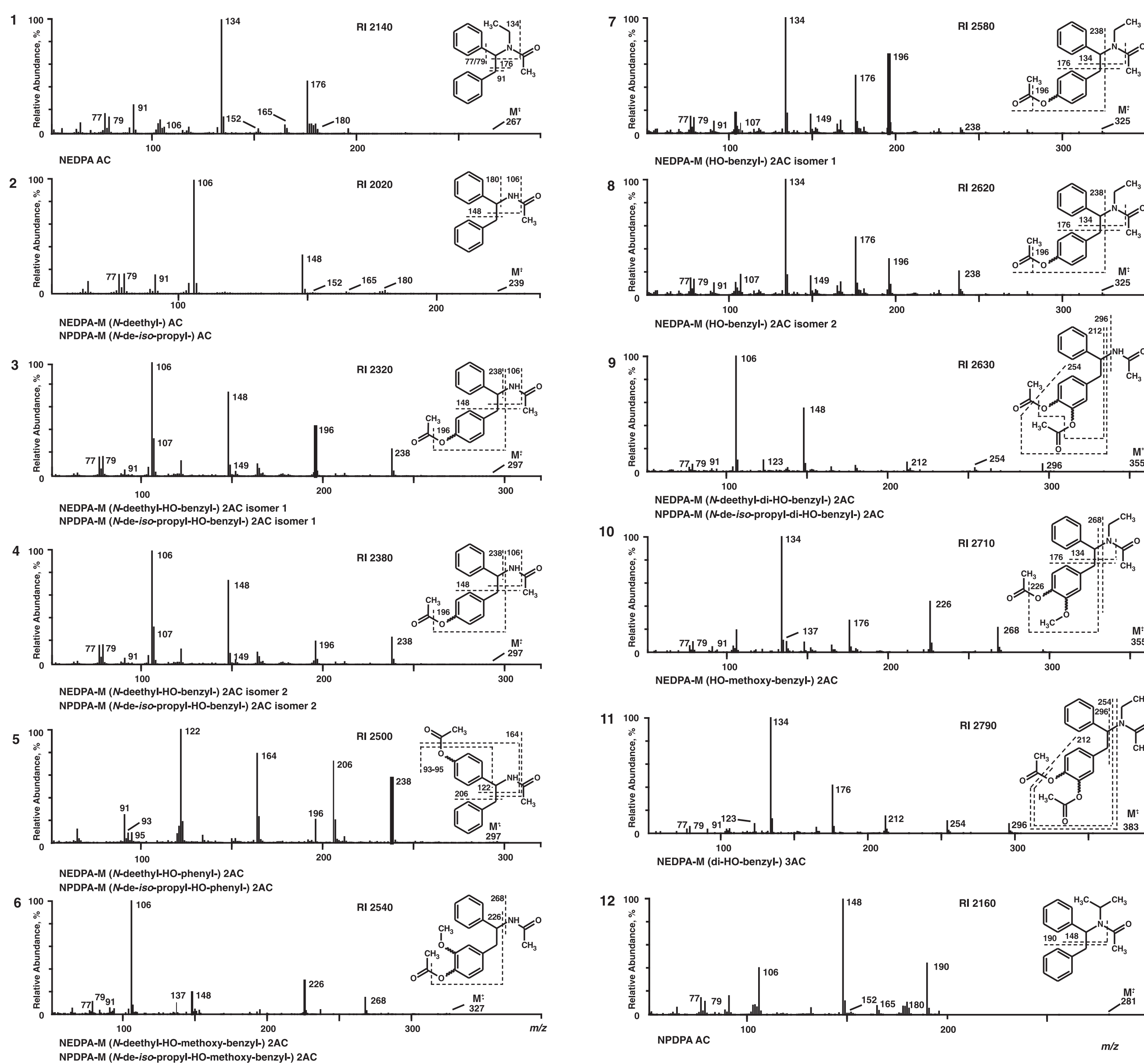

Figure 1. EI mass spectra, gas chromatographic retention indices (RI), proposed structures (unclear hydroxy positions are indicated by tildes), and predominant fragmentation patterns of NEPDA, NPDPA and their metabolites arranged according to the RIs, first for NEDPA and its metabolites and second for NPDPA and its unique metabolites.

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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 liquid chromatography-high resolution-tandem mass spectrometry (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 a gas chromatography-mass spectrometry (GC-MS) system.

Sample preparation for identification of phase II metabolites 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.[11,15] 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.

GC-MS apparatus for identification of the phase I metabolites

The extracts were analyzed 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

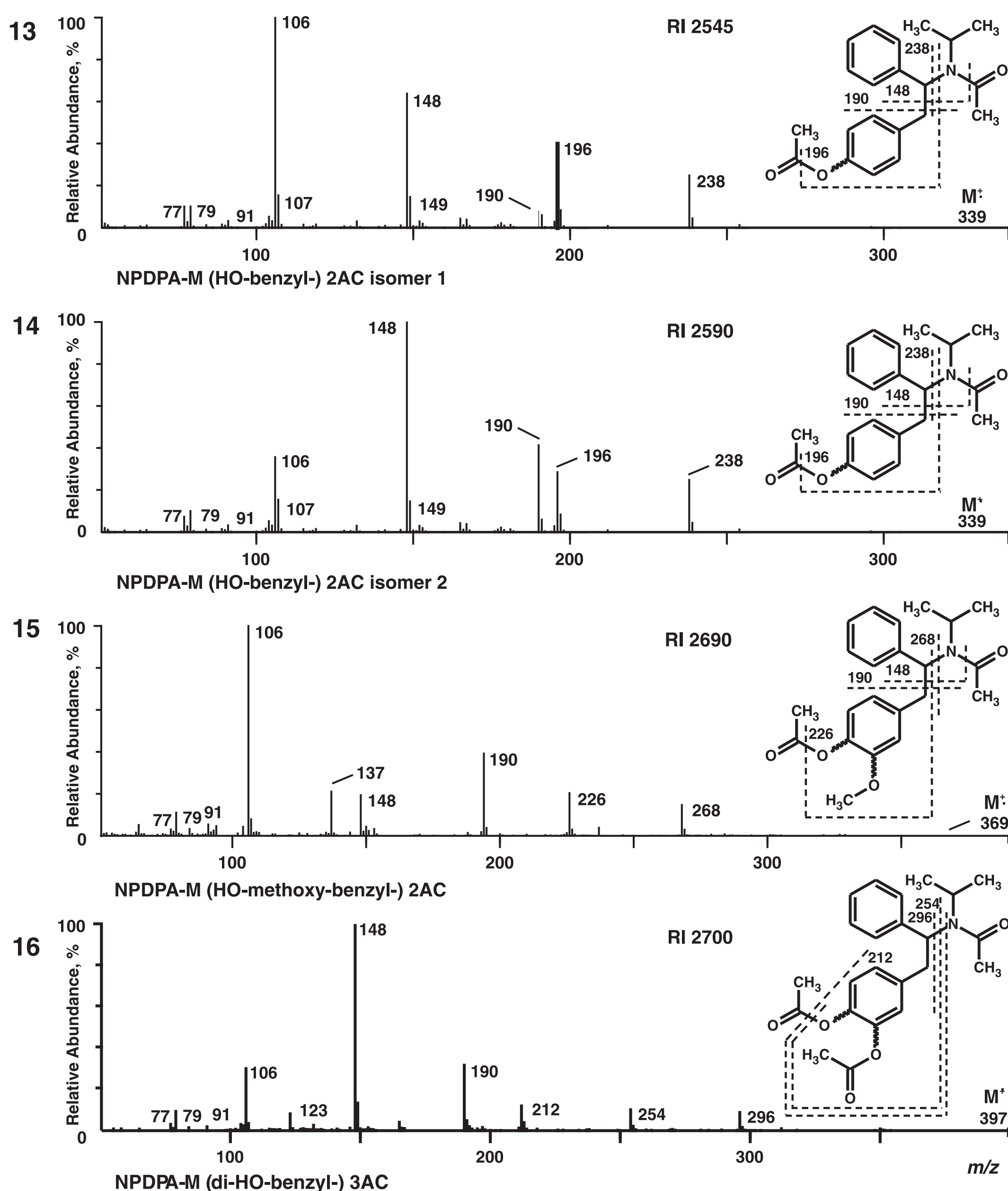

Figure 1. (Continued)

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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 12 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

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, USA) 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: 0–8.5 min 2% B to 100% B, 8.5–12 min hold 100% B, and 12–15 min hold 98% A, constantly at 500 μL/min.[14]

Two methods were created, one for phase I and one for phase II metabolites with the following MS conditions: electrospray ionization (ESI), positive full scan mode, *m/z* 50–500, followed by targeted MS^2 modes on the masses of interest (MS^1 and MS^2, resolution 17.500 at 12 Hz) sheath gas, nitrogen at a flow rate of 60 arbitrary units (AU); auxiliary gas, nitrogen at a flow rate of 10 AU; heater temperature, 320°C; spray voltage, 3.00 kV; ion transfer capillary temperature, 320°C; S-lens RF, level 50; maximum injection time, 250 ms, normalized collision energy (NCE), 30% in the high energy collisional dissociation cell (HCD); automatic gain control, 2e5.

Standard urine screening approach (SUSA) using GC-MS

For work-up (hydrolysis, liquid-liquid extraction, and microwave-assisted acetylation), already published procedures were used.[10] 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 90–310°C at 30°/min, final time 14 min.

For toxicological detection of NEDPA, NPDPA and their metabolites, mass chromatography was used with the extracted ions at *m/z* 106, 107, 123, 134, 148, 176, 190, and 196. Generation of the mass chromatograms was performed with user defined macros.[10] 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)[16] in simple mode.[17] 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.[18]

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

In accordance to Wissenbach *et al.*,[11,15] 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 an 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.[19]

The MS^2 and MS^3 reference spectra were recorded in the high dose urine after the above-mentioned workup and analysis. 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 phase I metabolites were identified by full-scan EI-MS after GC separation. According to the general fragmentation rules described by, for example McLafferty and Turecek and Smith and Busch,[20,21] metabolites are postulated from shifts of the parent

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compound fragments. The GC retention indices (RI) were determined in correlation with the Kovats' indices as described elsewhere.[10] The EI spectra, structures (unclear hydroxy positions are indicated by tildes), and proposed fragmentation patterns of acetylated NEDPA, NPDPA, and their metabolites are shown in Figure 1. They are arranged according to the RIs, first for NEDPA and its metabolites and second for NPDPA and its unique metabolites. The EI spectra of acetylated NEDPA and NPDPA (spectra nos. 1 and 12) were identical to those published by Westphal *et al.*[4]

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

NEDPA, NPDPA and their unique (non-dealkylated) metabolites were degraded by alpha-cleavage to the ions at *m/z* 176 or 190 followed by the loss of the acetyl residue to the ions at *m/z* 134 or 148 (Figure 1, spectra nos. 1, 7, 8, 10–16). Fragment ion at *m/z* 106 could be formed either by elimination of the corresponding *N*-alkyl residues of the fragments at *m/z* 134 or 148 (spectra nos. 1, 7, 8, 10–16) or by alpha-cleavage of the *N*-dealkyl metabolites after loss of the acetyl residue (spectra nos. 2–4, 6, 9). Cleavage of the phenyl residue at C1 led to the common fragments at *m/z* 77 and 79 for all compounds with the exception of the hydroxy-phenyl metabolite that led to ions at *m/z* 93 and 95 (spectrum no. 5). All compounds with unchanged benzyl residue (defined as phenyl at position C2, spectra nos. 1, 2, 12) formed the common fragment ion at *m/z* 91. NEDPA, NPDPA, and their *N*-dealkyl metabolites formed fragment ions for diphenylethane at *m/z* 152, 165, and a cluster of 178–181 by loss of the amino part.

All mono-hydroxy benzyl metabolites formed the fragment at *m/z* 149 and after loss of the corresponding acetyl residue to the ion at *m/z* 107 (spectra nos. 3, 4, 7, 8, 13, 14), all di-hydroxy benzyl metabolites the ion at *m/z* 123 (spectra nos. 9, 11, 16), and all hydroxy-methoxy benzyl metabolites the ion at *m/z* 137 (spectra nos. 6, 10, 15). Furthermore, the mono-hydroxy benzyl metabolites formed the fragments at *m/z* 238 and after loss of the corresponding acetyl residue to the ion at *m/z* 196 (spectra nos. 3, 4, 7, 8, 13, 14), the di-hydroxy benzyl metabolites to the ion at *m/z* 296 and after loss of one acetyl residue *m/z* 254 and of two acetyl residues *m/z* 212 (spectra nos. 9, 11, 16), and the hydroxy-methoxy benzyl metabolites the ion at *m/z* 268 after loss of the acetyl residue *m/z* 226 (spectra nos. 6, 10, 15). As the methylation after bis-hydroxylation should be catalyzed by the catechol-*O*-methyl-transferase (COMT) with preference of the meta-position,[22,23] the remaining hydroxy group should be in para-position. Otherwise, the exact position of the hydroxy group could not be determined by mass spectrometry. For ^{1}H NMR analysis, the concentrations of the metabolites were too low.

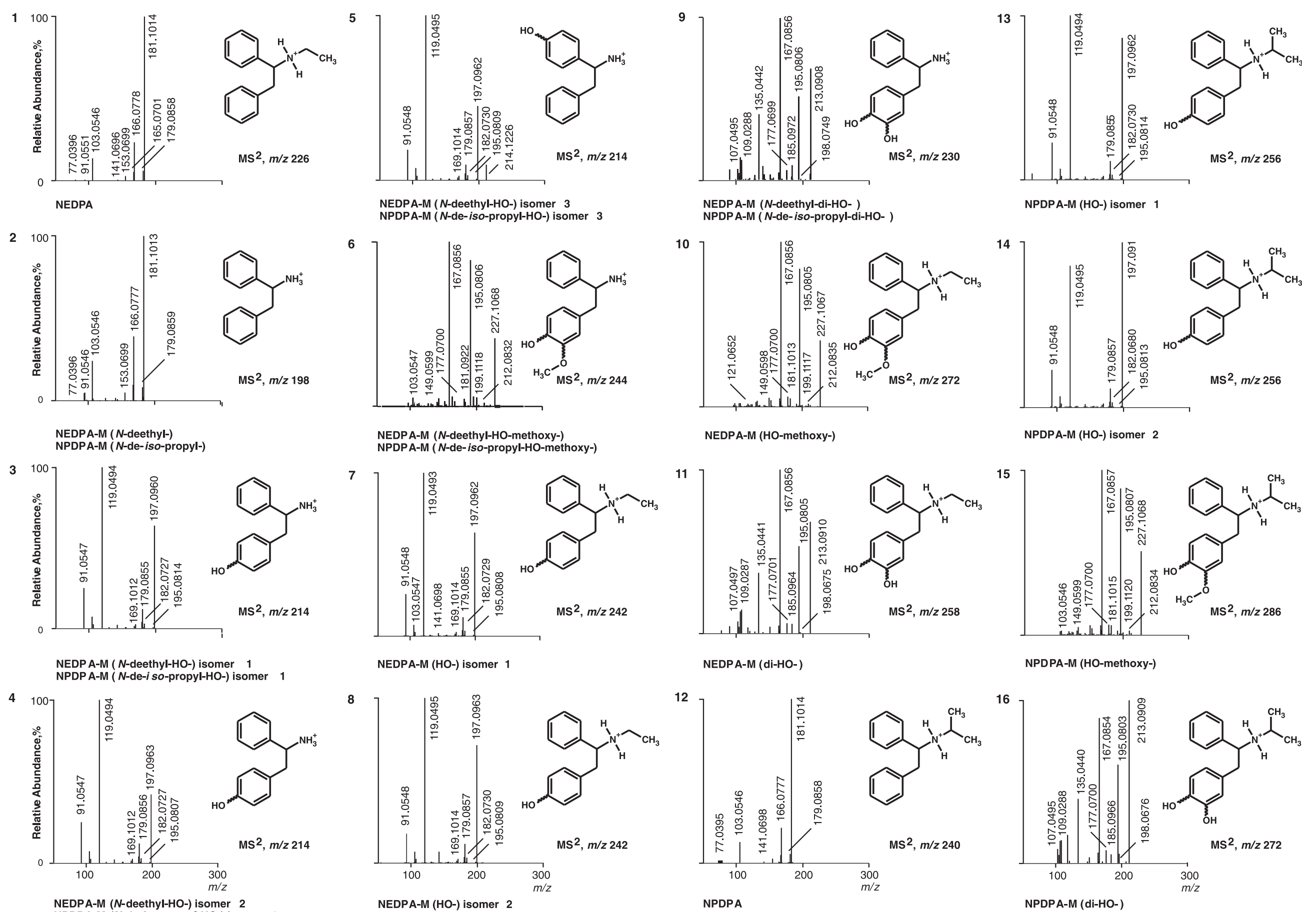

Figure 2. ESI positive mode MS/MS spectra with measured accurate masses (for reasons of visibility, the common *N*-dealkylated compounds are only represented by those of NEDPA), the selected ions for targeted MS^{2}, and proposed structures (unclear hydroxy positions are indicated by tildes) for NEDPA, NPDPA, and their metabolites arranged according to the numbering used in Figure 1.

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

The ESI positive mode MS/MS spectra, the selected ions for targeted MS^2, and proposed structures (unclear hydroxy positions are indicated by tildes) for NEDPA, NPDPA, and their metabolites are shown in Figure 2 in order of the numbering used in Figure 1. Their exact (theoretical) and accurate (measured) masses, the corresponding delta parts per million (ppm) values (rounded to two decimals), the calculated elemental compositions, and the retention times are listed in Table 1 according to the numbering of Figure 1. The hydroxy metabolites, which

Table 1. Compound numbers according to Figure 1, exact masses, accurate masses (ESI, positive mode), delta ppm values (rounded to two decimals), and retention times (RT) of the phase I and II metabolites of NEDPA and NPDPA.

No.	Compounds	Exact Mass (*m/z*)	Accurate Mass (*m/z*)	delta (ppm)	Proposed elemental composition	RT (min)
1	NEDPA	226.1590	226.1591	0.26	$C_{16}H_{20}N$	5.7
2	NEDPA-M (*N*-deethyl-)	198.1277	198.1279	1.07	$C_{14}H_{16}N$	4.6
2	NPDPA-M (*N*-de-*iso*-propyl-)	198.1277	198.1278	0.43	$C_{14}H_{16}N$	4.6
3	NEDPA-M (*N*-deethyl-hydroxy-) isomer 1	214.1226	214.1229	1.37	$C_{14}H_{16}NO$	3.1
3	NPDPA-M (*N*-de-*iso*-propyl-hydroxy-) isomer 1	214.1226	214.1227	0.38	$C_{14}H_{16}NO$	3.1
3G	NEDPA-M (*N*-deethyl-hydroxy-) glucuronide	390.1547	390.1556	0.38	$C_{20}H_{24}NO_7$	2.8
3G	NPDPA-M (*N*-de-*iso*-propyl-hydroxy-) glucuronide isomer 1	390.1547	390.1550	0.77	$C_{20}H_{24}NO_7$	2.8
3S	NEDPA-M (*N*-deethyl-hydroxy-) sulfate isomer 1	294.0794	294.0796	0.46	$C_{14}H_{16}NO_4S$	3.6
3S	NPDPA-M (*N*-de-*iso*-propyl-hydroxy-) sulfate isomer 1	294.0794	294.08	1.81	$C_{14}H_{16}NO_4S$	3.6
4	NEDPA-M (*N*-deethyl-hydroxy-) isomer 2	214.1226	214.1227	0.38	$C_{14}H_{16}NO$	3.6
4	NPDPA-M (*N*-de-*iso*-propyl- hydroxy-) isomer 2	214.1226	214.1227	0.46	$C_{14}H_{16}NO$	3.6
4S	NEDPA-M (*N*-deethyl-hydroxy-) sulfate isomer 2	294.0794	294.0791	-0.46	$C_{14}H_{16}NO_4S$	3.9
4S	NPDPA-M (*N*-de-*iso*-propyl-hydroxy-) sulfate isomer 2	294.0794	294.0799	1.50	$C_{14}H_{16}NO_4S$	3.9
5	NEDPA-M (*N*-deethyl-hydroxy-) isomer 3	214.1226	214.1228	0.71	$C_{14}H_{16}NO$	4.2
5	NPDPA-M (*N*-de-*iso*-propyl- hydroxy-) isomer 3	214.1226	214.1227	0.30	$C_{14}H_{16}NO$	4.2
5S	NEDPA-M (*N*-deethyl-hydroxy-) sulfate isomer 3	294.0794	294.0796	0.46	$C_{14}H_{16}NO_4S$	4.2
5S	NPDPA-M (*N*-de-*iso*-propyl-hydroxy-) sulfate isomer 3	294.0794	294.0797	0.67	$C_{14}H_{16}NO_4S$	4.2
6	NEDPA-M (*N*-deethyl-hydroxy-methoxy-)	244.1332	244.1329	-1.11	$C_{15}H_{18}NO_2$	3.5
6	NPDPA-M (*N*-de-*iso*-propyl-hydroxy-methoxy-)	244.1332	244.1333	0.25	$C_{15}H_{18}NO_2$	3.5
6G	NEDPA-M (*N*-deethyl-hydroxy-methoxy-) glucuronide	420.1652	420.1655	0.41	$C_{21}H_{26}NO_8$	3.0
6G	NPDPA-M (*N*-de *iso*-propyl-hydroxy-methoxy-) glucuronide	420.1652	420.1654	0.19	$C_{21}H_{26}NO_8$	3.0
6S	NEDPA-M (*N*-deethyl-hydroxy-methoxy-) sulfate	324.0900	324.0904	1.19	$C_{15}H_{18}NO_5S$	3.7
6S	NPDPA-M (*N*-de-*iso*-propyl-hydroxy-methoxy-) sulfate	324.0900	324.0901	0.30	$C_{15}H_{18}NO_5S$	3.7
7	NEDPA-M (hydroxy-) isomer 1	242.1539	242.1540	0.25	$C_{16}H_{20}NO$	3.6
7G	NEDPA-M (hydroxy-) glucuronide	418.1860	418.1862	0.53	$C_{22}H_{28}NO_7$	3.0
7S	NEDPA-M (hydroxy-) sulfate	322.1107	322.1115	2.44	$C_{16}H_{20}NO_4S$	4.0
8	NEDPA-M (hydroxy-) isomer 2	242.1539	242.1541	0.84	$C_{16}H_{20}NO$	4.1
9	NEDPA-M (*N*-deethyl-di-hydroxy-)	230.1175	230.1182	2.59	$C_{14}H_{16}NO_2$	3.0
9	NPDPA-M (*N*-de-*iso*-propyl-di-hydroxy-)	230.1175	230.1182	2.61	$C_{14}H_{16}NO_2$	3.0
9G	NEDPA-M (*N*-deethyl-di-hydroxy-) glucuronide	406.1496	406.1489	-1.84	$C_{20}H_{24}NO_8$	2.8
9G	NPDPA-M (*N*-de-*iso*-propyl-di-hydroxy-) glucuronide	406.1496	406.1493	-0.79	$C_{20}H_{24}NO_8$	2.8
9S	NEDPA-M (*N*-deethyl-di-hydroxy-) sulfate	310.0743	310.0750	1.89	$C_{14}H_{16}NO_5S$	3.8
9S	NPDPA-M (*N*-de-*iso*-propyl-di-hydroxy-) sulfate	310.0743	310.0748	1.34	$C_{14}H_{16}NO_5S$	3.8
10	NEDPA-M (hydroxy-methoxy-)	272.1645	272.1647	0.69	$C_{17}H_{22}NO_2$	4.0
10G	NEDPA-M (hydroxy-methoxy-) glucuronide	448.1965	448.1969	0.58	$C_{23}H_{30}NO_8$	3.1
10S	NEDPA-M (hydroxy-methoxy-) sulfate	352.1213	352.1212	-0.28	$C_{17}H_{22}NO_5S$	3.9
11	NEDPA-M (di-hydroxy-)	258.1488	258.1492	1.35	$C_{16}H_{20}NO_2$	3.1
11G	NEDPA-M (di-hydroxy-) glucuronide	434.1809	434.1810	0.20	$C_{22}H_{28}NO_8$	3.0
11S	NEPDA-M (di-hydroxy-) sulfate	338.1056	338.1061	1.21	$C_{16}H_{20}NO_5S$	4.2
12	NPDPA	240.1746	240.1750	1.42	$C_{17}H_{22}N$	6.2
13	NPDPA-M (hydroxy-) isomer 1	256.1695	256.1697	0.57	$C_{17}H_{22}NO$	4.2
13G	NPDPA-M (hydroxy-) glucuronide	432.2016	432.2017	0.01	$C_{23}H_{30}NO_7$	3.3
13S	NPDPA-M (hydroxy-) sulfate	336.1264	336.1250	-4.18	$C_{17}H_{22}NO_4S$	4.3
14	NPDPA-M (hydroxy-) isomer 2	256.1695	256.1697	0.52	$C_{17}H_{22}NO$	4.5
15	NPDPA-M (hydroxy-methoxy-)	286.1801	286.1805	1.06	$C_{18}H_{24}NO_2$	4.5
15G	NPDPA-M (hydroxy-methoxy-) glucuronide	462.2122	462.2121	-0.38	$C_{24}H_{32}NO_8$	3.4
16	NPDPA-M (di-hydroxy-)	272.1645	272.1650	1.73	$C_{17}H_{22}NO_2$	3.8
16G	NPDPA-M (di-hydroxy-) glucuronide	448.1965	448.1969	0.68	$C_{23}H_{30}NO_8$	3.2
16S	NPDPA-M (di-hydroxy-) sulfate	352.1213	352.1220	1.87	$C_{17}H_{22}NO_5S$	4.6

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could not be differentiated by LC-MS, are arranged according to their LC retention times. The elemental composition of all metabolites of NEDPA and NPDPA proposed by GC-MS could be verified using LC-HR-MS/MS. In contrast to EI, the alpha-cleavage between carbon 1 and 2 leading to immonium ions did not occur. The initial fragmentation step was the loss of the amino part, represented by a loss of 45.0578 u for all *N*-ethyl, 59.0734 u for all *N*-*iso*-propyl, and 17.0265 u for all *N*-dealkyl compounds. The fragmentation of the common *N*-dealkylated metabolite of NEDPA and NPDPA (Figure 2, spectrum no. 2) led to the same ions as those of the parent drugs because the diphenylethane structure was not changed. However, differentiation of the spectra was possible via their precursor masses and RT. The following common fragment ions did not allow further distinguishing of the location of metabolic modifications at the side chain or ring systems: ions at *m/z* 181.1011 for compounds with unchanged ring systems, 197.0960 for the hydroxy, 213.0910 for the di-hydroxy, or 227.1066 for the hydroxy-methoxy compounds. Loss of two hydrogen atoms led to diphenylethene (*m/z* 179.0855) according to the description of Grützmacher *et al*.[24] in the spectra of NEDPA, NPDPA and their common dealkylated metabolite (spectra nos. 1, 2, 12). Rearrangements are postulated in accordance to Weibel and Hesse[25] as a result of forming alternating double bonds for increased stability, represented by losses of CH_3 (*m/z* 166.0777) as a radical, CH_4 (*m/z* 165.0698), C_2H_4 (*m/z* 153.0698), C_3H_4 (*m/z* 141.0698), and C_5H_6 (*m/z* 115.0542). Fragmentation between a phenyl and the ethane bridge to an ion at *m/z* 77.0385 and *m/z* 103.0542.

The metabolic introduction of a hydroxy group into the diphenylethane part led to a shift of 15.9949 u from the ion at *m/z* 181.1011 (spectra nos. 1, 2, 12) to *m/z* 197.0960 (spectra nos. 3-5, 7, 8, 13, 14), from *m/z* 166.0777 to *m/z* 182.0726, and from *m/z* 103.0542 to *m/z* 119.0491. Again, the loss of hydrogen is detectable at ion *m/z* 195.0804. The ion at *m/z* 169.1011, absent in the spectrum of the parent compounds, resulted by loss of CO. Due to high resolution, the ion at *m/z* 169.0647 could be detected apart from *m/z* 169.1011, standing for another shift of 15.9949 u from the ion at *m/z* 153.0698, but with very low abundance. A loss of 18.0106 u from fragment ion at *m/z* 197.0960 to the diphenylethene ion at *m/z* 179.0855 (spectra nos. 3–5, 7, 8, 13, 14) resulted from a loss of water, which would be expected if the hydroxy group was aliphatically bond. Such a loss of water is quite untypical for phenols, but should be possible similar to the description of Bourcier and Hoppilliard.[26] After this loss of water, CO was deduced from this phenolic fragment resulting in the ion at *m/z* 169.1011. This CO loss is a well-known fragmentation step for phenolic compounds.[21,26] In addition, fragment ion at *m/z* 91.0542 was also recorded, due to the symmetry of the deaminated molecules or as a loss of CO from the shifted *m/z* 119.0491.

For the bis-hydroxylated metabolites of both compounds, another shift of 15.9949 u from fragment ion at *m/z* 197.0960 to *m/z* 213.0910, from *m/z* 182.0726 to *m/z* 198.0675, and from *m/z* 119.0491 to *m/z* 135.0440 was detected (spectra nos. 9, 11, 16). From the latter fragment, again the loss of CO is detected at *m/z* 107.0491. As already seen for the mono-hydroxylated metabolites, a loss of water occurred from the ion at *m/z* 213.0910 to *m/z* 195.0804, followed by another loss of water to *m/z* 177.0698. But also a direct loss of CO from the ion at *m/z* 213.0910 to *m/z* 185.0960, followed by a loss of water to *m/z* 167.0855 was detectable. The fragment ion at *m/z* 109.0284 referred to the phenylic ion (*m/z* 77.0385) shifted by 31.9898 u from the metabolic introduction of two hydroxy groups at one aromatic ring. Introduction of a methyl group to one of the hydroxy groups resulting in hydroxy-methoxy metabolites, which could be a product of COMT as described above, were detectable (spectra nos. 6, 10, and 15). The shifts were from fragment ion at *m/z* 213.0910 to *m/z* 227.1066, from *m/z* 198.0675 to *m/z* 212.0831, and from *m/z*

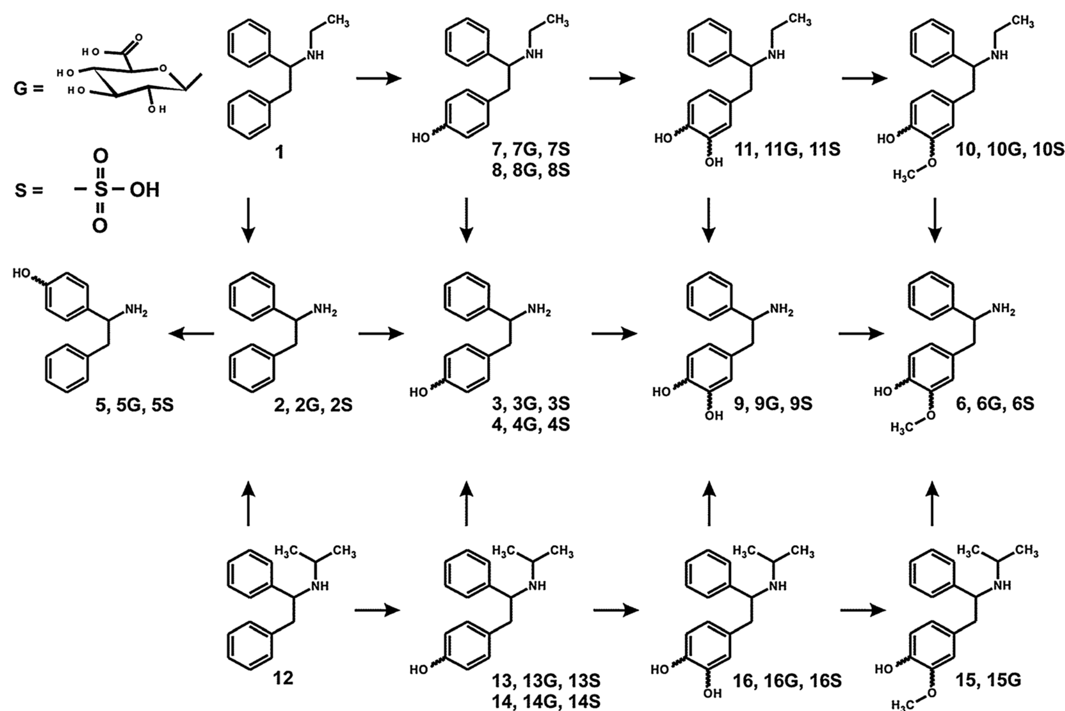

Figure 3. Metabolic pathways of NEDPA and NPDPA (numbering according to Figure 1, unclear hydroxy positions indicated by tildes, glucuronides or sulfates by G or S).

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Table 2. Compound numbers according to Figure 1, precursor ions, characteristic MS^2 ions, and the two most abundant ions selected for the following MS^3 spectra (ESI, positive mode, n.d. = not detected).

No.	Compounds	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 (%)
1	NEDPA	226	181 (100)	181: 103 (90), 115 (10), 141 (10), 153 (100), 165 (10), 179 (10), 181 (2)
2	NEDPA-M (*N*-deethyl-)	198	103 (10), 141 (10), 153 (20), 165 (10), 166 (100), 179 (2), 181 (10)	153: n.d. 166: n.d.
2	NPDPA-M (*N*-de-*iso*-propyl-)	198	103 (10), 141 (10), 153 (20), 165 (10), 166 (100), 179 (2), 181 (10)	153: n.d. 166: n.d.
3	NEDPA-M (*N*-deethyl-hydroxy-) isomer 1	214	91 (10), 103 (2), 119 (100), 141 (2), 169 (2), 179 (10), 197 (2)	91: n.d. 119: 91 (100)
3	NPDPA-M (*N*-de-*iso*-propyl-hydroxy-) isomer 1	214	91 (10), 103 (2), 119 (100), 141 (2), 169 (2), 179 (10), 197 (2)	91: n.d. 119: 91 (100)
3G	NEDPA-M (*N*-deethyl-hydroxy-) glucuronide	390	119 (10), 141 (2), 167 (2), 179 (2), 197 (100), 214 (10)	119: n.d. 197: 79 (2), 91 (10), 103 (2), 119 (100), 141 (2), 153 (2), 167 (2), 169 (2), 197 (2)
3G	NPDPA-M (*N*-de-*iso*-propyl-hydroxy-) glucuronide isomer 1	390	119 (10), 141 (2), 167 (2), 179 (2), 197 (100), 214 (10)	119: n.d. 197: 79 (2), 91 (10), 103 (2), 119 (100), 141 (2), 153 (2), 167 (2), 169 (2), 197 (2)
6	NEDPA-M (*N*-deethyl-hydroxy-methoxy-)	244	121 (2), 149 (20), 167 (60), 177 (10), 181 (20), 195 (100), 199 (10), 212 (2), 227 (2)	167: n.d. 195: 153 (10), 167 (100)
6	NPDPA-M (*N*-de-*iso*-propyl-hydroxy-methoxy-)	244	121 (2), 149 (20), 167 (60), 177 (10), 181 (20), 195 (100), 199 (10), 212 (2), 227 (2)	167: n.d. 195: 153 (10), 167 (100)
6G	NEDPA-M (*N*-deethyl-hydroxy-methoxy-) glucuronide	420	197 (100), 227 (70), 272 (2)	197: 79 (2), 91 (10), 103 (2), 119 (100), 141 (2), 153 (2), 167 (2), 169 (2), 197 (2) 227: 91 (2), 105 (2), 121 (2), 131 (20), 149 (20), 167 (50), 177 (10), 181 (20), 191 (20), 195 (100), 227 (10)
7	NEDPA-M (hydroxy-) isomer 1	242	119 (2), 197 (100)	119: n.d. 197: 91 (10), 103 (2), 119 (100), 169 (2), 197 (2)
7G	NEDPA-M (hydroxy-) glucuronide	418	119 (10), 141 (2), 167 (2), 179 (2), 197 (100), 242 (10)	119: 91 (100) 197: 79 (2), 91 (10), 103 (2), 119 (100), 141 (2), 153 (2), 167 (2), 169 (2), 197 (2)
8	NEDPA-M (hydroxy-) isomer 2	242	197 (100)	197: 79 (2), 91 (10), 103 (2), 119 (100), 141 (2), 153 (2), 167 (2), 169 (2)
9	NEDPA-M (*N*-deethyl-di-hydroxy-)	230	103 (5), 105 (10), 107 (20), 109 (10), 135 (100), 167 (90), 177 (10), 185 (40), 195 (80), 213 (2)	135: 107 (100) 167: n.d.
9	NPDPA-M (*N*-de-*iso*-propyl-di-hydroxy-)	230	103 (5), 105 (10), 107 (20), 109 (10), 135 (100), 167 (90), 177 (10), 185 (40), 195 (80), 213 (2)	135: 107 (100) 167: n.d.
9G	NPDPA-M (*N*-de-*iso*-propyl-di-hydroxy-) glucuronide	406	167 (2), 213 (100)	167: n.d. 213: 79 (2), 91 (2), 103 (10), 105 (10), 107 (20), 109 (10), 121 (2), 135 (90), 141 (2), 153 (2), 167 (2), 177 (10), 185 (20), 195 (2)

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10	NEDPA-M (hydroxy-methoxy-)	272	195 (2), 227 (100)	227: 91 (2), 103 (2), 105 (2), 107 (2), 121 (2), 131 (10), 149 (10), 153 (2), 167 (60), 177 (10), 181 (20), 191 (10), 195 (100), 199 (10)
10G	NEDPA-M (hydroxy-methoxy-) glucuronide	448	227 (100), 272 (20)	227: 91 (2), 105 (2), 121 (2), 131 (10), 149 (10), 167 (60), 177 (10), 181 (20), 191 (10), 195 (100), 199 (10) 272: 227 (100)
11	NEDPA-M (di-hydroxy-)	258	197 (2), 213 (100)	197: 91 (100), 119 (50) 213: 79 (2), 91 (2), 103 (10), 105 (10), 107 (20), 109 (10), 121 (2), 135 (90), 141 (2), 153 (2), 167 (2), 177 (10), 185 (20), 195 (2)
11G	NEDPA-M (di-hydroxy-) glucuronide	434	135 (10), 167 (10), 195 (10), 213 (100), 258 (2)	167: n.d. 213: 79 (2), 91 (2), 103 (10), 105 (10), 107 (20), 109 (10), 121 (2), 135 (90), 141 (2), 153 (2), 167 (2), 177 (10), 185 (20), 195 (2)
12	NPDPA	240	179 (2), 181 (100)	181: 103 (70), 115 (10), 141 (2), 153 (100), 181 (10)
13	NPDPA-M (hydroxy-) isomer 1	256	119 (2), 197 (100)	119: n.d. 197: 91 (10), 103 (2), 119 (100), 169 (2), 197 (2)
13G	NPDPA-M (hydroxy-) glucuronide	432	119 (100), 197 (100), 256 (10)	119: 91 (100) 197: 79 (2), 91 (10), 103 (2), 119 (100), 141 (2), 153 (2), 167 (2), 169 (2), 197 (2)
14	NPDPA-M (hydroxy-) isomer 2	256	119 (10), 197 (100)	119: n.d. 197: 79 (2), 91 (10), 103 (2), 119 (100), 141 (2), 153 (2), 167 (2), 169 (2), 197 (2)
15	NPDPA-M (hydroxy-methoxy-)	286	227 (100)	227: 91 (2), 103 (2), 105 (2), 107 (2), 121 (2), 131 (10), 149 (10), 153 (2), 167 (60), 177 (10), 181 (20), 191 (10), 195 (100), 199 (10)
15G	NPDPA-M (hydroxy-methoxy-) glucuronide	462	167 (10), 177 (2), 195 (10), 213 (2), 227 (100), 286 (10)	167: n.d. 227: 91 (2), 103 (2), 105 (2), 107 (2), 121 (2), 131 (10), 149 (10), 153 (2), 167 (60), 177 (10), 181 (20), 191 (10), 195 (100), 199 (10)
16	NPDPA-M (di-hydroxy-)	272	197 (2), 213 (100)	197: 169 (, 119, 91 213: 79 (2), 91 (2), 103 (10), 105 (10), 107 (20), 109 (10), 121 (2), 135 (90), 141 (2), 153 (2), 167 (2), 177 (10), 185 (20), 195 (2)
16G	NPDPA-M (di-hydroxy-) glucuronide	448	135 (10), 167 (10), 195 (10), 213 (100), 258 (2)	167: n.d. 213: 79 (2), 91 (2), 103 (10), 105 (10), 107 (20), 109 (10), 121 (2), 135 (90), 141 (2), 153 (2), 167 (2), 177 (10), 185 (20), 195 (2)

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135.0440 to *m/z* 149.0597. The fragment ion at *m/z* 212.0831 could also be seen as a loss of a methyl radical of the methoxy group from the ion at *m/z* 227.1066. This moiety was most abundant lost as methanol, resulting in the fragment ion at *m/z* 195.0804, followed by the loss of water to the ion at *m/z* 177.0698 or the loss of CO to *m/z* 167.0855 as described before. But also the loss of CO followed by the loss of water occurred (*m/z* 227.1066 to *m/z* 199.1117 to *m/z* 181.1011). This fragmentation line was also detected for the fragment ion at *m/z* 149.0597 (to *m/z* 121.0647 to *m/z* 103.0542), which could also be a moiety from the unchanged phenyl ring.

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. The exact (theoretical) and accurate (measured) masses, the corresponding delta ppm values, the calculated elemental compositions, and the retention times (RT) of the phase II metabolites are also listed in Table 1 according to the numbering of Figure 1. For confirmation of the conjugate structure, the targeted MS^2 spectra after HCD fragmentation were compared to those of the underlying phase I metabolites. In case of glucuronidation, the recorded MS^2 spectra contained the characteristic fragment ions of the phase I metabolites, in case of sulfation the initial loss of the amino moiety as described above was also observed.

Proposed metabolic pathways

According to the identified metabolites of NEDPA and NPDPA, hydroxylation, di-hydroxylation, followed by methylation of one of the hydroxy groups, *N*-dealkylation, and combinations of them as well as glucuronidation and sulfation of hydroxy metabolites could be proposed. The pathways are depicted in Figure 3 and the metabolites numbered in accordance to Figure 1. As can be seen, *N*-dealkylation of NEDPA and NPDPA led to the same metabolites (nos. 2–5, 9).

Detectability of NEDPA and NPDPA by GC-MS or LC-MS^n

For the detectability studies, the drugs were administered in a dose corresponding to a common lefetamine single dose scaled by dose-by-factor approach from man to rat according to Sharma and McNeill[27] as described above. Because so far no dosages of abuse are known, the low dose chosen corresponded to a single therapeutic dose (50 mg tablet for an adult) of lefetamine.

Using the authors' GC-MS SUSA,[10,17] the parent drugs could not be detected, but the following metabolites: *N*-deethyl-NEDPA (no. 2), *N*-deethyl-hydroxy-NEDPA (nos. 3, 4), hydroxy-NEDPA (nos. 7, 8), hydroxy-methoxy-NEDPA (no. 10), and di-hydroxy-NEDPA (no. 11) or *N*-de-*iso*-propyl-NPDPA (no. 2), *N*-de-*iso*-propyl-hydroxy-NPDPA (nos. 3, 4), *N*-de-*iso*-propyl-hydroxy-phenyl-NPDPA (no. 5) and hydroxy-NPDPA (nos. 13, 14). The identity could be confirmed by comparison of the underlying spectra with the reference spectra depicted in Figure 1.

Using the authors' LC-MS^n SUSA,[11,15,19,28] NEDPA administration could be monitored by detection of hydroxy-NEDPA (nos. 7), di-hydroxy-NEDPA (no. 11), *N*-deethyl-NEDPA (no. 2), and the glucuronides of its *N*-deethyl-hydroxy (no. 3G), hydroxy (nos. 7G), di-hydroxy (no. 11G), and hydroxy-methoxy metabolites (no. 10G) and NPDPA by detection of its *N*-de-*iso*-propyl-hydroxy-NPDPA (no. 3), hydroxy-NPDPA (no. 13), di-hydroxy-NPDPA (no. 16), and hydroxy-NPDPA glucuronide (13G). For differentiation of an intake of NEDPA or NPDPA, the unique, not dealkylated, metabolites could be used.

The identity could be confirmed by comparison of the underlying spectra with the reference MS^2 and MS^3 spectra. The corresponding precursor ions and the mass/abundance list of the MS^2 and MS^3 spectra are given in Table 2. The above-mentioned sulfates were not detectable because of different chromatography, apparatus, and MS data acquisition. These reference spectra were recorded with the SUSA approach, but in high dose urine extracts to gain also the reference data of the minor metabolites.

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 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 drugs NEDPA and NPDPA were extensively metabolized and that an intake of these drugs of abuse should be detectable using the authors' general screening protocols. The targets were the *N*-deethyl, *N*-deethyl-hydroxy, hydroxy, and hydroxy-methoxy metabolites of NEDPA or the *N*-de-*iso*-propyl, *N*-de-*iso*-propyl-hydroxy, and hydroxy metabolites of NPDPA. Assuming similar kinetics, an intake of these drugs should also be detectable in human urine.

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