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Metabolic fate, mass spectral fragmentation, detectability, and differentiation in urine of the benzofuran designer drugs 6-APB and 6-MAPB in comparison to their 5-isomers using GC-MS and LC-(HR)-MS^n techniques

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Abstract The number of so-called new psychoactive substances (NPS) is still increasing by modification of the chemical structure of known (scheduled) drugs. As analogues of amphetamines, 2-aminopropyl-benzofurans were sold. They were consumed because of their euphoric and empathogenic effects. After the 5-(2-aminopropyl)benzofurans, the 6-(2-aminopropyl)benzofuran isomers appeared. Thus, the question arose whether the metabolic fate, the mass spectral fragmentation, and the detectability in urine are comparable or different and how an intake can be differentiated. In the present study, 6-APB (6-(2-aminopropyl)benzofuran) and its *N*-methyl derivative 6-MAPB (*N*-methyl-6-(2-aminopropyl)benzofuran) were investigated to answer these questions. The metabolites of both drugs were identified in rat urine and human liver preparations using GC-MS and/or LC-HR-MS^n. Besides the parent drug, the main metabolite of 6-APB was 4-carboxymethyl-3-hydroxy amphetamine and the main metabolites of 6-MAPB were 6-APB (*N*-demethyl metabolite) and 4-carboxymethyl-3-hydroxy methamphetamine. The cytochrome P450 (CYP) isoenzymes involved in the 6-MAPB *N*-demethylation were CYP1A2, CYP2D6, and CYP3A4. An intake of a common users' dose of 6-APB or 6-MAPB could be confirmed in rat urine using the authors' GC-MS and the LC-MS^n standard urine screening approaches with the corresponding parent drugs as major target allowing their differentiation. Furthermore, a differentiation of 6-APB and 6-MAPB in urine from their positional isomers 5-APB and 5-MAPB was successfully performed after solid phase extraction and heptafluorobutyrylation by GC-MS via their retention times.

Keywords: designer drugs; 6-APB; 6-MAPB, metabolism; GC-MS; LC-(HR)-MS^n

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Introduction

The number of so-called novel psychoactive substances (NPS) is still increasing by modification of the chemical structure of known (scheduled) drugs [1]. As analogues of amphetamines, 2-aminopropyl-benzofurans were sold since 2010. After the 5-(2-aminopropyl)benzofurans, the 6-(2-aminopropyl)benzofuran isomers appeared. As already discussed [2], the benzofurans can also be seen as MDA (3,4-methylenedioxy-amphetamine) and MDMA (3,4-methylendioxy-methamphetamine) analogues regarding a bioisosteric substitution of one oxygen (-O-) in the furan ring by methine (-CH=). The MDA- and MDMA-like effects of these compounds have been discussed on several drug users forum on the Internet (e.g. www.bluelight.org; www.land-der-traeume.de) and they were used as stimulants or entactogens because of their euphoric and empathogenic effects. Described negative effects were tachycardia, jaw tensions, insomnia, and severe paranoia [3]. Acute psychosis together with agitation was also described in a case report where 6-APB was consumed together with cannabis and the synthetic cannabinoid JWH-122 [4]. Due to the multiple substance ingestion, it is not clear if the acute psychosis comes from 6-APB alone or the mixture of the ingested drugs. Nevertheless, Iversen et al. showed that 6-APB has inhibitory effects on the monoamine reuptake transporters with higher affinity on the dopamine (DA) and noradrenalin (NA) transporter and lower affinity on the serotonin (5-HT) transporter [5]. In addition, they showed that 6-APB acted as a full agonist at the 5-HT_{2B} receptor (calcium mobilization assay) which may be relevant when considering potential vasoconstrictive effects and cardiotoxicity. Unfortunately, clinical studies are not available for 6-MAPB but experience reports available on the Internet suggest that there may be similarities. To date, only few analytical data are available for 6-APB and 6-MAPB. Stanczuk et al. and Casale et al. differentiated between the several positional isomers in purchased products but no data are available on the differentiation between the positional isomers in

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urine [6, 7]. Welter et al. investigated the metabolism and the detectability of the positional isomers 5-APB and 5-MAPB [2].

Thus, the question arose whether the metabolic fate, the mass spectral fragmentation, and the detectability in urine is comparable or different with the varied position of the alkyl chain or the oxygen in the ring. Another question was how an intake can be differentiated for the case that once the legal status might be different or if the particular drug used for homicide by poisoning can be found at the suspect. In the present study, 6-APB (6-(2-aminopropyl)benzofuran) and its *N*-methyl derivative 6-MAPB (*N*-methyl-6-(2-aminopropyl)benzofuran) were investigated to answer these questions.

Experimental

Chemicals and reagents

6-APB, 5-APB, and 5-MAPB were synthesized [6] and provided by the Department of Pharmacology and Therapeutics, Trinity Centre for Health Sciences, St. James's Hospital (Dublin, Ireland), before 5-APB and 6-APB were scheduled. 6-MAPB was synthesized and provided by the School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University (Liverpool, UK). Isolute C18 (500 mg, 3 mL) and HCX cartridges (130 mg, 3 mL) were obtained from Biotage (Uppsala, Sweden), isocitrate and isocitrate dehydrogenase, heptafluorobutyric anhydride (HFBA), sodium phosphate from Sigma (Taufkirchen, Germany), $NADP^+$ from Biomol (Hamburg, Germany), acetonitrile (LC-MS grade), ammonium formate (analytical grade), formic acid (LC-MS grade), methanol (LC-MS grade), 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, and all other chemicals and reagents (analytical grade)

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from VWR (Darmstadt, Germany). The baculovirus-infected insect cell microsomes (Supersomes) containing 1 nmol/mL of human cDNA-expressed CYP 1A2, CYP 2A6, CYP 2B6, CYP 2C8, CYP 2C9, CYP 2C19, CYP 2D6, CYP 2E1 (2 nmol/mL), CYP 3A4, or CYP 3A5 (2 nmol/mL), and pooled human liver microsomes (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

Urine samples were used from male Wistar rats (Charles River, Sulzfeld, Germany) after administration of the compounds by gastric intubation using an aqueous suspension for toxicological diagnostic reasons according to the corresponding German law (http://www.gesetze-im-internet.de/tierschg/). For identification of the metabolites a single 20 mg/kg body mass (BM) dose for 6-APB and 10 mg/kg body mass dose for 6-MAPB and for toxicological analysis, a single 3 mg/kg body mass dose for 6-APB or 1 mg/kg body mass dose for 6-MAPB were administered once. The rats were housed in metabolism cages for 24 h, having water *ad libitum*. Urine was collected separately from the feces over a 24 h period. To check if the samples were free of interfering peaks, blank urine samples were collected before drug administration. The samples were directly analyzed and then stored at -20°C.

Sample preparation for identification of phase I and II metabolites

Urine samples were prepared as described previously [2, 8]. Briefly, urine samples were extracted after conjugate cleavage with a mixture of glucuronidase and arylsulfatase by solid phase extraction (SPE) with HCX cartridges. After elution, evaporation, and reconstitution in

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100 µL of methanol, 50 µL were left underivatized and another 50 µL were again gently evaporated to dryness and derivatized with a mixture of acetic anhydride and pyridine (3:2 v/v) under microwave irradiation (450 W, 5 min). Afterwards, the residue was reconstituted in 50 µL of methanol. A 3-µL aliquot of the extract was injected onto the GC-MS or 5 µL onto the LC-HR-MS^n.

For the phase II metabolism studies, 1 mL of urine was extracted with SPE (C18) cartridges. Analytes were eluted, evaporated, and reconstituted with 50 µL of the solvent mixture A/B (50:50, v/v; A: ammonia formate buffer, pH 3; B: acetonitrile/formic acid). A 5-µL aliquot of the extract was then injected onto the LC-HR-MS^n.

GC-MS apparatus for identification of the phase I metabolites

For analysis of the extracts a combination of a Hewlett Packard (HP, Agilent, Waldbronn, Germany) 6890 Series gas chromatograph with an HP 6890 MSD mass spectrometer and an HP MS ChemStation (DOS series) with HP G1701AA software version A03.00 was used. The GC conditions were as follows: splitless injection mode; column, Optima 5 MS capillary (12 m x 0.2 mm I.D.), cross linked methyl silicone, 0.35 µm film thickness (Macherey-Nagel, Düren, Germany); injection port temperature, 280°C; carrier gas, helium; flow-rate, 0.5 ml/min; column temperature, programmed from 85-310°C at 30°/min; initial time, 2 min; final time, 7 min; total time, 16 min. The MS conditions were as follows: full-scan mode, *m/z* 50-550 u; electron ionization (EI) mode; ionization energy, 70 eV; ion source temperature, 220°C; capillary direct interface, heated at 280°C.

LC-HR-MS^n apparatus for identification of phase I and II metabolites

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As described previously [2, 8], a Thermo Fisher Scientific (TF) Dionex UltiMate 3000 RS pump consisting of a degasser, a quaternary pump and an UltiMate 3000 RS autosampler, coupled to a TF Orbitrap Velos Pro equipped with a heated electrospray ionization (HESI) II source was used for analysis of the prepared extracts. For details of the LC and MS conditions see ref. [8]. Briefly, a TF Hypersil Gold (C18) column (150 x 2.1 mm, 1.9 µm) with gradient elution with 10 mM aqueous ammonium formate buffer containing 0.1 % (v/v) formic acid as mobile phase A and acetonitrile containing 0.1 % (v/v) formic acid as mobile phase B was applied. The Orbitrap was run in the positive mode and the collision-induced dissociation (CID)-MS/MS experiments (normalized collision energies, 35%) were either performed in a data-dependent acquisition (DDA) mode (*m/z* 100-800) or on the following selected precursor ions from MS^1 at *m/z* 218, 232, 234, 276, 278, 290, 308, 322, 336, 380, and 394 for the acetylated parent compounds and phase I metabolites as well as 368, 372, 386, 388, and 400 for phase II metabolites.

Microsomal incubations for initial CYP activity screening studies

According to published protocols [2, 8], each isomer was incubated. Briefly, incubation with the CYP isoenzymes (50 pmol/mL, each) CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5 or HLM (1 mg protein/mL) was performed at 150 µmol/L substrate concentration. After 30 min, the reaction was stopped with ice-cold acetonitrile, centrifuged and the supernatant transferred to an autosampler vial, afterwards 5 µL were injected onto the LC-HR-MS^n.

LC-HR-MS^n apparatus for analysis of the microsomal incubation

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For the analysis of the incubations the same system was used as described for the metabolism studies with the following modifications: CID-MS/MS experiments were performed either in the DDA mode or on the precursor ions from MS^1 for 6-APB, hydroxy, hydroxy-dihydro, 4-carboxymethyl-3-hydroxy amphetamine (*m/z* 176.1069, 192.1019, 194.1175, 210.1124) or 6-MAPB, *N*-demethyl, hydroxy, hydroxy-dihydro, and 4-carboxymethyl-3-hydroxy methamphetamine (*m/z* 176.10169, 190.1226, 206.1175, 208.1332, 224.1281). For calculation of the relative amount of metabolites formed during incubations the peak areas were used determined with TF Xcalibur Qual Browser software version 2.2 SP1.48.

GC-MS standard urine screening approach (SUSA)

As published before [2, 9, 10], the sample preparation procedure consisted of acid hydrolysis for fast conjugate cleavage and extraction with a dichloromethane-isopropanol-ethyl acetate mixture (1:1:3 v/v/v). After evaporation, the residue was acetylated with an acetic anhydride-pyridine mixture under microwave irradiation (450 W, 5 min), again evaporated and reconstituted in 100 µL of methanol. The GC and MS conditions for analysis of the extracts were the same as described above for the metabolism studies.

For toxicological detection, mass chromatography was used with the extracted ions at *m/z* 58, 86, 100, 131, 147, 160, 163, 174, 190, 206, 218, and 220 for the acetylated 6-APB, 6-MAPB, and their metabolites. Confirmation of the peak identity in the mass chromatograms was performed by computerized comparison of the mass spectra underlying the peaks (after background subtraction) with reference spectra recorded during this study. The automated mass spectral deconvolution and identification system (AMDIS) (http://chemdata.nist.gov/mass-spc/amdis/) was also used for evaluation of the full scan data files acquired by the GC-MS system, as described previously [11].

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LC-MS^n standard urine screening approach

The urine samples (100 µL) were diluted with acetonitrile, shaken, centrifuged, evaporated to dryness, and reconstituted in mobile phase A/B (v/v; 50:50) as described elsewhere [2, 12]. The samples were analyzed using a TF LXQ linear ion trap MS equipped with an HESI II source and coupled to a TF Accela LC system. The LC conditions were as described for LC-HR-MS^n and the MS settings are described elsewhere [2, 12]. In brief, data-dependent acquisition (DDA) was performed on precursor ions selected from MS^1: MS^1 was performed in the full scan mode (*m/z* 100-800). MS^2 and MS^3 were performed in the DDA mode: four DDA MS^2 scan filters and eight MS^3 scan filters were chosen to provide MS^2 on the four most intense signals from MS^1 and MS^3 on the most and second most intense signals from the MS^2.

Sample preparation and apparatus for differentiation of the isomers by GC-MS

Blank urine samples were spiked with each of the isomers (5-APB, 6-APB, 5-MAPB and 6-MAPB) in a final concentration of 100 µg/L. The spiked urine samples or the rat urines after administration of the low doses of 5-APB, 6-APB, 5-MAPB, or 6-MAPB were worked up by conjugates cleavage followed by solid phase extraction with an HCX cartridge as described above but with HFBA derivatization according to Peters et al. [13]. Afterwards, 3 µL of the extracts were analyzed with the GC-MS apparatus described above but with the following modifications: column, Optima 5 MS capillary (25 m x 0.2 mm I.D.), 0.35 µm film thickness (Macherey-Nagel, Düren, Germany); column temperature, programmed from 80-200°C at 5°C/min, 2 min hold, 200-310°C at 35°C/min; initial time, 2 min; final time, 3 min; total time, 34 min; carrier gas, helium; flow-rate, 0.8 ml/min.

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Results and discussion

The metabolites were identified in rat urine samples after administration of doses that corresponded to the doses reported for multiple and/or higher dosing scaled by dose-by-factor approach according to Sharma et al. [14]. The different workup procedures were used as for the 5-isomers. Preliminary tests with the parent drugs showed that their efficiencies were comparable (data not shown).

GC-MS identification of phase I metabolites

Metabolite structures were elucidated by interpreting the fragments in the EI spectra of the metabolites in correlation to that of the parent compound. The fragmentation patterns of other amphetamines and furan or benzofuran containing compounds [2, 6, 15-22] as well as the general fragmentation rules described by e.g. Smith and Bush or McLafferty were taken into consideration [23, 24]. Furthermore, published data on the metabolism of 5-APB and 5-MAPB were considered [2]. Figure 1 shows the EI mass spectra, structures, predominant fragmentation patterns, and GC retention indices (RI) of the acetylated parent compounds and their acetylated metabolites (1–10). These reference data are essential for identification of these drugs in any GC-MS laboratory.

EI-MS fragmentation of the phase I metabolites

Below, important fragmentation patterns of the EI mass spectra of acetylated 6-APB and 6-MAPB and their metabolites will be discussed in depth allowing postulation of the structures depicted in Fig. 1.1 – 1.10. The numbers of the corresponding mass spectra are given in brackets. 6-APB and most of its metabolites were also the corresponding *N*-demethyl

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metabolites of 6-MAPB, this is in accordance to published data about 5-APB and 5-MAPB [2]. The EI fragmentation of the 6-isomer metabolites was often the same as described for the corresponding 5-isomer metabolites (e.g. 1-5), therefore, only the spectra with differences were described in depth. The spectrum of twofold acetylated hydroxy 6-MAPB (6) showed the fragment ions at *m/z* 58 and 100 typical for methamphetamine derivatives and resulting from a cleavage between position 1 and 2 in the propylamine side chain. The fragment ions at *m/z* 174 and 216 resulted from the cleavage of *N*-methyl-acetamide (73 u) and a following loss of the second acetyl group (42 u). The spectra of three fold acetylated 4-hydroxyethyl-3-hydroxy amphetamine/*N*-demethyl-4-hydroxyethyl-3-hydroxy methamphetamine (7) and 4-hydroxyethyl-3-hydroxy methamphetamine (9) showed fragment ions either at *m/z* 86 or 58 and 100 for the primary amine or the secondary amine, respectively. Cleavage of acetamide (59 u) or *N*-methyl-acetamide from the precursor ions, respectively, lead to fragment ion at *m/z* 262, a following loss of acetic acid (60 u) then lead to fragment ion at *m/z* 202, and a subsequent loss of another acetyl group lead to fragment ion at *m/z* 160. Spectrum no. 10, threefold acetylated 4-dihydroxy-3-hydroxy amphetamine/*N*-demethyl-4-dihydroxy-3-hydroxy methamphetamine $-H_2O$ (10), was an artifact most likely formed in the GC injection port. A loss of water is often described when a hydroxy group is located at a side chain, therefore this artifact derived most probably from 4-dihydroxy-3-hydroxy amphetamine/*N*-demethyl-4-dihydroxy-3-hydroxy methamphetamine. The spectrum showed fragment ions at 260 after the loss of acetamide, a following stepwise loss of two acetyl groups lead to fragment ions at *m/z* 218 and 176, respectively. As can be seen in Figure 1, for both compounds, ring opened metabolites could be observed as already described for 5-APB, 5-MAPB and other benzofuran containing compounds. The corresponding mechanism has been described by Kobayashi et al. and Connelly et al. [17, 18]. They could show that the ring cleavage occurs via an unsaturated aldehyde and leads to either an alcohol or the carboxylic acid by reduction or oxidation of the intermediate. In summary, there are only small

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differences in the fragmentation of the 6-isomers compared to the 5-isomers and by comparing only the spectra of the metabolites mass spectral differentiation was not possible.

LC-HR-MS^n fragmentation and identification of phase I metabolites

The fragmentation of 6-APB, 6-MAPB and their metabolites detected by LC-HR-MS^n will be explained in detail according to the fragments given in Table 1. All metabolites identified by GC-MS could be confirmed with the exception of hydroxy-deamino-dihydro 6-APB/hydroxy-deamino-dihydro 6-MAPB most probably because of poor ionization with the chosen ionization mode due to the loss of the nitrogen moiety. Three additional metabolites could be detected, namely dihydro 6-MAPB (11), hydroxy-dihydro 6-APB/*N*-demethyl-hydroxy-dihydro 6-MAPB (12), and 4-dihydroxyethyl-3-hydroxy methamphetamine (13) most likely due to the higher sensitivity of the LC-HR-MS^n system. As already seen for 5-APB and other amines [2, 8], underivatized 6-APB formed only few fragments. Therefore, all HR spectra were recorded in urine extracts after acetylation resulting in more specific MS^2 and MS^3 spectra. In Table 1, all identified acetylated phase I metabolites of 6-APB and 6-MAPB are listed together with the accurate and the calculated exact masses, the corresponding two main fragment ions in MS^2 and the two most abundant fragment ion in the corresponding MS^3 spectra, if available, the elemental compositions, the deviations of the measured accurate masses from the calculated masses, given as errors in ppm (all within 5 ppm deviation), and the retention times.

The fragmentation of 6-APB, 6-MAPB and their metabolites will be explained in detail according to the fragments given in Table 1. The MS^2 spectrum of underivatized 6-APB (1a; protonated molecular mass, PM, at *m/z* 176.1064) showed two fragment ions, the benzofuranyl-methylium ion at *m/z* 131.0492 resulting from an alpha cleavage and the fragment ion at *m/z* 117.0699 resulting from loss of oxygen most likely followed by a

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rearrangement of the ring. Acetylation resulted in more specific spectra due to the formation of more fragment ions. After derivatization the MS^2 spectrum of 6-APB (1b; PM at *m/z* 218.1167) showed fragment ions at *m/z* 159.0805 and 176.1072, resulting from the loss of the acetyl group (42.0105 u) and a following loss of ammonia (17.0265 u). Detected fragments in the MS^3 spectra were at *m/z* 131.0492 and 117.0699, which could also be observed in the spectrum of underivatized 6-APB. Underivatized 6-MAPB (2a, PM at *m/z* 190.1220) formed more fragments; nevertheless the acetylated extracts were also used. Acetylated 6-MAPB (2b; PM at *m/z* 232.1321) showed fragment ions at *m/z* 190.1228 after loss of the acetyl group and 159.0805 for the benzofuranyl-propylium ion. The MS^2 of two fold acetylated hydroxy 6-APB/*N*-demethyl-hydroxy 6-MAPB (4; PM at *m/z* 276.1223) and of two fold acetylated hydroxy 6-MAPB (6; PM at *m/z* 290.1376) showed fragment ions at *m/z* 217.0860 after loss of acetamide (59.0371 u) and *N*-methyl-acetamide (73.0527 u), respectively or fragment ions at *m/z* 234.1126 or 248.1282 after an alternative loss of only the acetyl group. The MS^3 spectra showed fragment ions at *m/z* 175.0755, resulting from the loss of the second acetyl group from ion at *m/z* 217 and representing the hydroxy-benzofuranyl-propylium ion (shift of 16 u from *m/z* 159). The acetoxy-benzofuranyl-methylium ion at *m/z* 189.0548 resulted from alpha cleavage. The MS^2 spectrum of the methylester of two fold acetylated 4-carboxymethyl-3-hydroxy amphetamine/*N*-demethyl-4-carboxymethyl-3-hydroxy methamphetamine (5; PM at *m/z* 308.1480) showed fragment ions at *m/z* 266.1385 (loss of one acetyl group) and 234.1127 (following loss of methanol *m/z* 32.0262 from the ester). In the MS^3 spectra, the fragment ion at *m/z* 224.1286 was present, resulting from the loss of the second acetyl group and representing the methylester of 4-carboxymethyl-3-hydroxy amphetamine. Fragment ions at *m/z* 192.1014 and 175.0755 resulted from cleavage of one acetyl group from fragment at *m/z* 234.1125 and subsequent loss of ammonia. The spectrum of 4-carboxymethyl-3-hydroxy methamphetamine (8; PM at *m/z* 322.1637) showed fragment ions at *m/z* 280.1543 and 238.1441 resulting from the consecutive loss of two acetyl groups and leading to the

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methylester of 4-carboxymethyl-3-hydroxy methamphetamine. In the MS^3 of 280, fragment ions were observed at *m/z* 238.1437 and 207.1016 resulting from either the loss of one acetyl group or of *N*-methyl-acetamide. The MS^2 spectrum of three fold acetylated 4-hydroxyethyl-3-hydroxy amphetamine/*N*-demethyl-4-hydroxyethyl-3-hydroxy methamphetamine (7; PM at *m/z* 322.1639) showed fragment ions at *m/z* 280.1543 resulting from loss of one acetyl group and fragment ion at *m/z* 220.1335 indicating the hydroxy group at the alkyl chain because of the loss of acetic acid (60.0211 u) from *m/z* 280.1543. The most abundant fragment ions in the MS^3 spectra were generated by loss of one acetyl group followed by loss of water from fragment at *m/z* 280.1543 resulting in fragment ions at *m/z* 238.1438 and 220.1333. Fragment ions at *m/z* 178.1227 and 161.0961 represent either the 4-hydroxy-3-vinylphenyl-propanaminium or the 4-hydroxy-3-vinylphenyl-propylium ion resulting from consecutive loss of one acetyl group and ammonia. The MS^2 spectrum of three fold acetylated 4-hydroxyethyl-3-hydroxy methamphetamine (9; PM at *m/z* 336.1794) showed fragment ions at *m/z* 294.1699 and 252.1594, resulting from consecutive loss of two acetyl groups. The corresponding MS^3 spectra showed fragment ions at *m/z* 179.1065, resulting from loss of *N*-methyl-acetamide and after loss of ammonia fragment ion at *m/z* 161.0960. Four fold acetylated 4-dihydroxyethyl-3-hydroxy amphetamine/*N*-demethyl-4-dihydroxyethyl-3-hydroxy methamphetamine (10; PM at *m/z* 380.1691) showed in the MS^2 spectrum as most abundant fragment ions those at *m/z* 320.1491 resulting from loss of acetic acid indicating a hydroxy group in the side chain and at *m/z* 278.1378, from a subsequent loss of one acetyl group. The MS^3 spectrum of fragment ion at *m/z* 320.1491 showed ions at *m/z* 278.1385 and 236.1281 resulting from the loss of two acetyl groups. The most abundant fragments in the MS^2 spectrum of acetylated dihydro 6-MAPB (11; PM at *m/z* 234.1484) were at *m/z* 161.0960 resulting from the loss of *N*-methyl-acetamide and at *m/z* 133.0648 after a following loss of ethylene (28.0313 u), representing either the dihydro-benzofuranyl-propylium or the dihydro-benzofuranyl-methylium ion. Fragment ions at *m/z* 236.1280 and 218.1174 were present in

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the MS^2 spectrum of two fold acetylated hydroxy-dihydro 6-APB/ *N*-demethyl-hydroxy-dihydro 6-MAPB (12; PM at *m/z* 278.1378), resulting from the loss of one acetyl group followed by the loss of water (18.1015 u). Fragments at *m/z* 177.0909 and 159.0803 were present in the MS^3 spectrum of fragment ion at *m/z* 236 representing the hydroxy-dihydro-benzofuranyl-propylium ion and after loss of water the corresponding benzofuranyl-propylium ion. Four fold acetylated hydroxy-dihydro 6-MAPB (13; PM at *m/z* 394.1846) showed fragment ion at *m/z* 334.1645, resulting from loss of acetic acid and at *m/z* 292.1540 after a subsequent loss of one acetyl group. The MS^3 spectra of *m/z* 334 showed fragment ions at *m/z* 292.1539 and 261.1119, resulting from loss of one acetyl group and a subsequent loss of methanamine (31.0421 u).

The fragmentation patterns were similar to those described for 5-APB and 5-MAPB, but those of the ring-opened metabolites showed some differences, maybe due to the position of the oxygen and the alkyl chain after ring opening. The meta-position of the oxygen and the side chain for the 6-isomers might lead to a different fragmentation pattern than the para-position, due to different mesomeric and inductive effects.

LC-HR-MS^n fragmentation and identification of the phase II metabolites

For the identification of glucuronides and/or sulfates, two different modes were used, either data-dependent scan mode with a parent mass list containing the precursor ions calculated from those of the corresponding phase I metabolites or by searching for a specific neutral loss of 176.0320 u for glucuronides and/or 79.9568 u for sulfates. For identification, the MS^3 spectra of the detected glucuronides were, if possible, compared with the MS^2 spectra of the corresponding phase I metabolites. Unfortunately no MS^3 spectra could be detected most probably due to small formation rates. In Table 2, all identified phase II metabolites are listed together with the accurate and the calculated exact masses, the corresponding two main

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fragment ions in MS^2 spectra, the corresponding elemental compositions, the deviations of the measured accurate masses from the calculated masses, given as errors in ppm (all within 5 ppm deviation), and the retention times.

In accordance to the 5-isomers, no sulfates could be observed, but the following glucuronides: 4-carboxymethyl-3-hydroxy amphetamine glucuronide (5G), 4-hydroxyethyl-3-hydroxy amphetamine glucuronide (7G), and 4-dihydroxyethyl-3-hydroxy amphetamine glucuronide (10G). For 6-MAPB, *N*-demethyl-4-carboxymethyl-3-hydroxy methamphetamine glucuronide (5G), 4-carboxymethyl-3-hydroxy methamphetamine glucuronide (8G), *N*-demethyl-4-hydroxyethyl-3-hydroxy methamphetamine glucuronide (7G), 4-hydroxyethyl-3-hydroxy methamphetamine glucuronide (9G), and *N*-demethyl-4-dihydroxy-3-hydroxy methamphetamine glucuronide. In addition, hydroxy-aryl 6-APB glucuronide (4G) could be detected.

In summary, most MS^2 spectra recorded after a neutral loss of 176 u showed the accurate mass of the corresponding phase I metabolites and the accurate mass after loss of ammonia or methanamine. For example, the hydroxy-aryl 6-APB glucuronide (4G; PM at *m/z* 368.1348) showed in MS^2 most abundant fragment ions at *m/z* 192.1014 and *m/z* 175.0749, representing the phase I metabolite and after loss of ammonia or methanamine the hydroxy-benzofuranyl-propylium ion. For 4-carboxymethyl-3-hydroxy amphetamine/*N*-demethyl-4-carboxymethyl-3-hydroxy methamphetamine glucuronide (5G; PM at *m/z* 386.1430) and 4-hydroxyethyl-3-hydroxy amphetamine/*N*-demethyl 4-hydroxyethyl-3-hydroxy methamphetamine glucuronide (7G; PM at *m/z* 372.1636) the previously described fragmentation pattern was observed. 4-Hydroxyethyl-3-hydroxy methamphetamine (9G; PM at *m/z* 386.1787) showed most abundant fragment ions in MS^2 at *m/z* 210.1484 representing the corresponding phase I metabolite and at *m/z* 179.1062 resulting from the loss of methanamine. 4-Carboxymethyl-3-hydroxy methamphetamine glucuronide (8G; PM at *m/z* 400.1595) showed a different fragment pattern by ions at *m/z* 224.1276, after loss of the conjugate, and at *m/z* 206.1171

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after loss of water from the carboxy group. This loss of water has been described before [25]. The spectrum of 4-dihydroxyethyl-3-hydroxy amphetamine/*N*-demethyl-4-dihydroxyethyl-3-hydroxy methamphetamine glucuronide (10G; PM at *m/z* 388.1602) was the only one where the fragment for the corresponding phase I metabolite was not one of the most abundant ones. The most abundant fragments present in this spectrum were at *m/z* 195.1010 and 177.0905. Fragment at *m/z* 195.1010 resulted from loss of the conjugate and a subsequent loss of ammonia and a following loss of water then lead to fragment ion at *m/z* 177.0905.

Proposed metabolic pathways

The main metabolic pathways for 6-APB and 6-MAPB proposed according to the identified metabolites are summarized in Fig. 2. They were in accordance to those of 5-APB and 5-MAPB as well as of furan and benzofuran [2, 17, 18, 21]. Hydroxylation of 6-APB (1) at the furan ring (4) was the initial step. The following ring cleavage and reduction of the resulting unsaturated aldehyde lead to the corresponding aldehyde, which was either oxidized to the corresponding carboxylic acid (5) or reduced to the alcohol (7). The alcohol was further hydroxylated (10) and this one as well as the carboxylic acid and alcohol, were glucuronidated (5G, 7G, 10G). Hydrogenation of the hydroxy metabolite could also be observed (12), as well as deamination followed by reduction (3). For 6-MAPB (2), the predominant step was *N*-demethylation to 6-APB (1), which underwent the same pathways as described for 6-APB. 6-MAPB itself also underwent most of the described pathways, namely benzofuran hydroxylation (6) followed by enzymatic ring cleavage to either an alcohol (9) by reduction or carboxylic acid (8) by oxidation of the intermediate aldehyde. The alcohol was further hydroxylated (13) and both carboxylic acid and alcohol were glucuronidated (8G, 9G). A further pathway was hydrogenation (11) of 6-MAPB. In summary, *N*-demethylation was the predominant step due to the corresponding relative GC-MS and LC-MS peak areas for 6-

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MAPB. Enzymatic cleavage of the benzofuran ring was another step and the most important step for 6-APB, finally leading to the corresponding carboxy or hydroxy metabolite by oxidation or reduction.

All together there were not much differences between the metabolites formed for 6-APB, 6-MAPB compared to the corresponding 5-isomers. Slight differences could be observed in the formation of dihydroxy metabolites, which could be detected only for the 5-isomers. Additional metabolites detected only for the 6-isomers were dihydro 6-MAPB (11) and the glucuronide of 4-dihydroxyethyl-3-hydroxy amphetamine/*N*-demethyl-4-dihydroxyethyl-3-hydroxy methamphetamine (10G). These differences might be caused by lower formation rates due to different affinity of the meta-position of the oxygen in the 6-isomers to the active center of the involved enzymes in contrast to the para-position of the 5-isomers and/or by different elimination rates.

HLM incubations and initial CYP activity screening

The drugs were incubated with HLM to see whether the humans form in principle the same metabolites as the rat. Unfortunately, for 6-APB no metabolites could be detected in HLM incubations and for 6-MAPB only *N*-demethyl 6-MAPB (1) could be found. This is more or less in accordance to the low dose rat urine results, where the *N*-demethyl 6-MAPB was the only metabolite detected with the GC-MS approach and the case report of Chan et al. [4], where only 6-APB could be detected. Again, the difference to the 5-isomers, for which several metabolites could be detected [2], might be caused by different affinity to the catalyzing enzymes.

The initial CYP activity screening [2] was not possible for 6-APB due to the very low metabolite formation rate maybe caused by the CYP inhibition potential of such benzofuran designer drugs [26]. Only the *N*-demethylation of 6-MAPB could be monitored. The enzymes

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involved in this step were CYP1A2, CYP2D6, and CYP3A4. CYP enzyme kinetics could not be performed due to the low metabolite formation rates.

Toxicological detection of 6-APB and 6-MAPB by GC-MS or LC-MS^n

Application of the compounds could be monitored in rat urine after administration of 3 mg/kg BM 6-APB or 1 mg/kg BM 6-MAPB, respectively, using the GC-MS-based SUSA described by the authors [8, 9]. The used low doses corresponded to reported human single doses of about 30 mg for 6-APB or 10 mg for 6-MAPB scaled by dose-by-factor approach according to Sharma et al. [14]. Therefore, detection of the intake of these compounds should be possible also in human urine. The possible presence of 6-APB or 6-MAPB and their metabolites, within the GC-MS SUSA, were indicated by reconstructed mass chromatography with the ions at *m/z* 86, 100, 131, 147, 190, and 206 for the acetylated parent compounds and their metabolites according to the corresponding reference spectra given in Fig. 1. Figure 3 shows reconstructed ion chromatograms after a low dose of 6-APB (Fig. 3A) and 6-MAPB (3B). As can be seen, the main excretion products were 6-APB and 6-MAPB themselves, whereas for 6-MAPB also the *N*-demethyl metabolite could be detected with the GC-MS SUSA. Furthermore, the microsomal incubations with HLM showed that *N*-demethyl 6-MAPB was the only metabolite detectable, which was in accordance to the *in vivo* results from the rat studies. This supports the assumption that the approach should be suitable for drug testing even in human urine. In addition, Welter et al. [2] showed that *N*-demethyl 5-MAPB was the main metabolite in rat and in human urine, indicating that this might also be true for 6-MAPB. The other identified metabolites may also occur in human urine in case of severe overdoses and/or genetic variations of metabolisms (as well as species differences,) and should therefore be included in the mass ion chromatograms. Confirmation of the peak identity indicated by the selected mass chromatogram was performed by computerized

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comparison of the underlying full scan mass spectrum with reference spectra recorded during this study. Additionally, evaluation of the full scan data files acquired by GC-MS was done by AMDIS allowing the detection of 6-APB and 6-MAPB in the prepared urine samples using the previously described procedure [11].

With the LC-MS^n SUSA [27], the main excretion products detected in low dose rat urine were again 6-APB and 6-MAPB. As can be seen in Fig. 3, it was also possible to detect 4-carboxymethy-3-hydroxy amphetamine in the low dose 6-APB rat urine (Fig. 3C) as well as *N*-demethyl 6-MAPB, 4-carboxymethyl-3-hydroxy amphetamine, and *N*-demethyl-4-carboxymethy-3-hydroxy methamphetamine in the low dose 6-MAPB rat urine (3D). As indicated by Table 1, the respective parent compounds did not show characteristic fragmentations without prior acetylation. Nevertheless, it was possible to confirm the intake of 6-APB and 6-MAPB via the spectra and retention times of the unchanged drugs and the detected metabolites. Table 3 summarizes the detected metabolites together with their PMs and the corresponding two main fragments in MS^2 and MS^3, if available.

Analytical differentiation of 5-APB, 6-APB, 5-MAPB, and 6-MAPB by GC-MS

For the case, that the isomers must be differentiated, both SUSAs were not suitable due to very similar chromatographic properties. Thus, another sample work-up was tested according to Peters et al. [13]. Figure 4A shows a reconstructed ion chromatogram of a mixture of the positional isomers 5-APB, 6-APB, 5-MAPB, and 6-MAPB in a pure compound solution (concentration 100 µg/L, each) without extraction but analyzed with the GC-MS method described for differentiation of isomers. The observed elution order for the HFB derivatized compounds was as follows: 5-APB < 6-APB <5-MAPB < 6-MAPB. This elution order for APBs in pure compound solutions after heptafluorobutyrylation has already been described by Stanczuk et al. [6]. Figure 4B shows reconstructed ion chromatograms of a mixture of

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prepared low dose rat urine samples. Each isomer could be detected at the expected retention time even in matrix. The spectra of the HFB derivatives are shown in Figure 4C. As can be seen, the spectra for the respective positional isomers are very similar, and therefore also not suitable for differentiation.

Conclusions

6-APB and 6-MAPB were metabolized only to a minor extent in rats. CYP1A2, CYP2D6, and CYP3A4 mainly catalyze the *N*-demethylation of 6-MAPB. An intake of the compounds in urine could be proved by both SUSAs tested, focusing on the detection of the parent drugs and the *N*-demethyl 6-MAPB. Intake of the 6-isomers from that of the 5-isomers in urine could be differentiated by GC-MS after heptafluorobutyrylation providing different retention times.

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Table 1 List of 6-APB, 6-MAPB, and all acetylated phase I metabolites together with the accurate and the exact masses of their PM recorded in MS^1, the corresponding main fragment ions in MS^2 and MS^3, the corresponding elemental compositions, the deviations of the measured accurate masses from the calculated masses, given as errors in ppm, and the retention times

No.	Metabolites and characteristic ions Measured accurate masses [u]		Calculated exact masses [u]	Elemental composition	Error [ppm]	RT [min]
1a	**6-APB** **6-MAPB-M (*N*-demethyl-)**					
	MS^1	PM at *m/z* 176.1064	176.1069	C11H14NO	-3.09	5.1
	MS^2	fragment ion at *m/z* 131.0492	131.0491	C9H7O	0.10	
		fragment ion at *m/z* 117.0699	117.0698	C9H9	0.20	
	MS^3	n.d.				
1b	**6-APB AC** **6-MAPB-M (*N*-demethyl-) AC**					
	MS^1	PM at *m/z* 218.1167	218.1175	C13H16NO2	-4.15	
	MS^2	fragment ion at *m/z* 159.0805	159.0804	C11H11O	0.62	
		fragment ion at *m/z* 176.1072	176.1069	C11H14NO	1.08	10.8
	MS^3 on 159	fragment ion at *m/z* 131.0491	131.0491	C9H7O	0.52	
		fragment ion at *m/z* 117.0700	117.0698	C9H9	0.65	
	MS^3 on 176	fragment ion at *m/z* 131.0491	131.0491	C9H7O	0.05	
		fragment ion at *m/z* 117.0699	117.0698	C9H9	0.20	
2a	**6-MAPB**					
	MS^1	PM at *m/z* 190.1220	190.1226	C12H16NO	-3.37	
	MS^2	fragment ion at *m/z* 159.0805	159.0804	C11H11O	0.40	5.8
		fragment ion at *m/z* 131.0493	131.0491	C9H7O	0.81	
	MS^3 on 159	fragment ion at *m/z* 131.0491	131.0491	C9H7O	-0.06	
		fragment ion at *m/z* 117.0699	117.0698	C9H9	0.19	
2b	**6-MAPB AC**					
	MS^1	PM at *m/z* 232.1321	232.1332	C14H18NO2	-4.76	
	MS^2	fragment ion at *m/z* 159.0805	159.0804	C11H11O	0.24	
		fragment ion at *m/z* 190.1228	190.1226	C12H16NO	0.88	13.0
	MS^3 on 159	fragment ion at *m/z* 131.0491	131.0491	C9H7O	-0.06	
		fragment ion at *m/z* 117.0699	117.0698	C9H9	0.07	
	MS^3 on 190	fragment ion at *m/z* 159.0803	159.0804	C11H11O	-0.65	
		fragment ion at *m/z* 131.0488	131.0491	C9H7O	-2.85	
4	**6-APB-M (hydroxy-) 2AC** **6-MAPB-M (*N*-demethyl-hydroxy-) 2AC**					
	MS^1	PM at *m/z* 276.1223	276.1230	C15H18NO4	-2.51	
	MS^2	fragment ion at *m/z* 217.0860	217.0859	C13H13O3	0.15	
		fragment ion at *m/z* 234.1126	234.1124	C13H16NO3	0.40	10.0
	MS^3 on 217	fragment ion at *m/z* 189.0548	189.0546	C11H9O3	1.16	
		fragment ion at *m/z* 175.0755	175.0753	C11H11O2	0.99	

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	MS^3 on 234	fragment ion at *m/z* 189.0548 fragment ion at *m/z* 175.0754	189.0546 175.0753	C11H9O3 C11H11O2	0.75 0.30	
5	**6-APB-M (4-carboxymethyl-3-hydroxy amphetamine) ME2AC** **6-MAPB-M (*N*-demethyl-4-carboxymethyl-3-hydroxy methamphetamine) ME2AC**					
	MS^1	PM at *m/z* 308.1480	308.1492	C16H22NO5	-4.02	
	MS^2	fragment ion at *m/z* 266.1385 fragment ion at *m/z* 234.1125	266.1386 234.1124	C14H20NO4 C13H16NO3	-0.50 0.32	9.6
	MS^3 on 266	fragment ion at *m/z* 234.1127 fragment ion at *m/z* 224.1286	234.1124 224.1281	C13H16NO3 C12H18NO3	1.05 2.21	
	MS^3 on 234	fragment ion at *m/z* 192.1014 fragment ion at *m/z* 175.0755	192.1019 175.0753	C11H14NO2 C11H11O2	-2.81 1.08	
6	**6-MAPB-M (hydroxy-) 2AC**					
	MS^1	PM at *m/z* 290.1376	290.1386	C16H20NO4	-3.63	
	MS^2	fragment ion at *m/z* 248.1282 fragment ion at *m/z* 217.0860	248.1281 217.0859	C14H18NO3 C13H13O3	0.31 0.39	12.7
	MS^3 on 248	fragment ion at *m/z* 217.0858 fragment ion at *m/z* 175.0754	217.0859 175.0753	C13H13O3 C11H11O2	-0.55 0.04	
	MS^3 on 217	fragment ion at *m/z* 175.0754 fragment ion at *m/z* 189.0547	175.0753 189.0546	C11H11O2 C11H9O3	0.21 0.19	
7	**6-APB-M (4-hydroxyethyl-3-hydroxy amphetamine) 3AC** **6-MAPB-M (*N*-demethyl-4-hydroxyethyl-3-hydroxy methamphetamine) 3AC**					
	MS^1	PM at *m/z* 322.1639	322.1648	C17H24NO5	-2.97	
	MS^2	fragment ion at *m/z* 280.1543 fragment ion at *m/z* 220.1335	280.1543 220.1332	C15H22NO4 C13H18NO2	-0.25 1.20	11.3
	MS^3 on 280	fragment ion at *m/z* 220.1333 fragment ion at *m/z* 238.1438	220.1332 238.1437	C13H18NO2 C13H20NO3	0.57 0.24	
	MS^3 on 220	fragment ion at *m/z* 161.0961 fragment ion at *m/z* 178.1227	161.0960 178.1226	C11H13O C11H16NO	0.05 0.05	
8	**6-MAPB-M (4-carboxymethyl-3-hydroxy methamphetamine) ME2AC**					
	MS^1	PM at *m/z* 322.1637	322.1648	C17H24NO5	-3.81	
	MS^2	fragment ion at *m/z* 280.1543 fragment ion at *m/z* 238.1441	280.1543 238.1437	C15H22NO4 C13H20NO3	-0.05 1.40	11.6
	MS^3 on 280	fragment ion at *m/z* 238.1437 fragment ion at *m/z* 207.1016	238.1437 207.1015	C13H20NO3 C12H15O3	-0.26 0.10	
	MS^3 on 238	fragment ion at *m/z* 207.1015	207.1015	C12H15O3	-0.55	
9	**6-MAPB-M (4-hydroxyethyl-3-hydroxy methamphetamine) 3AC**					
	MS^1	PM at *m/z* 336.1794	336.1805	C18H26NO5	-3.58	
	MS^2	fragment ion at *m/z* 294.1699 fragment ion at *m/z* 252.1594	294.1699 252.1594	C16H24NO4 C14H22NO3	-0.31 -0.13	12.9
	MS^3 on 294	fragment ion at *m/z* 252.1593 fragment ion at *m/z* 161.0960	252.1594 161.0960	C14H22NO3 C11H13O	-0.28 -0.13	
	MS^3 on 252	fragment ion at *m/z* 179.1065 fragment ion at *m/z* 161.0959	179.1066 161.0960	C11H15O2 C11H13O	-0.66 -0.89	
10	**6-APB-M (4-dihydroxyethyl-3-hydroxy amphetamine) 4AC** **6-MAPB-M (*N*-demethyl-4-dihydroxyethyl-3-hydroxy methamphetamine) 4AC**					
	MS^1	PM at *m/z* 380.1691	380.1703	C19H26NO7	-3.36	
	MS^2	fragment ion at *m/z* 320.1491 fragment ion at *m/z* 278.1378	320.1492 278.1386	C17H22NO5 C15H20NO4	-0.49 -3.01	11.2
	MS^3 on 320	fragment ion at *m/z* 278.1385 fragment ion at *m/z* 236.1281	278.1386 236.1281	C15H20NO4 C13H18NO3	-0.59 0.09	
11	**6-MAPB-M (dihydro-) AC**					

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	MS^1	PM at *m/z* 234.1484	234.1488	C14H20NO2	-1.98	11.7
	MS^2	fragment ion at *m/z* 161.0960 fragment ion at *m/z* 133.0648	161.0960 133.0647	C11H13O C9H9O	-0.07 0.18	
	MS^3	n.d.				
12	**6-APB-M (hydroxy-dihydro-) 2AC** **6-MAPB-M (*N*-demethyl-hydroxy-dihydro-) 2AC**					
	MS^1	PM at *m/z* 278.1378	278.1386	C15H20NO4	-3.21	11.3
	MS^2	fragment ion at *m/z* 236.1280 fragment ion at *m/z* 218.1174	236.1281 218.1175	C13H18NO3 C13H16NO2	-0.69 -0.60	
	MS^3 **on 236**	fragment ion at *m/z* 159.0803 fragment ion at *m/z* 177.0909	159.0804 177.0910	C11H11O C11H13O2	-0.74 -0.70	
13	**6-MAPB-M (4-dihydroxyethyl-3-hydroxy methamphetamine) 4AC**					
	MS^1	PM at *m/z* 394.1846	394.1860	C20H28NO7	-3.62	12.9
	MS^2	fragment ion at *m/z* 334.1645 fragment ion at *m/z* 292.1540	334.1648 292.1543	C18H24NO5 C16H22NO4	-1.16 -0.98	
	MS^3 **on 334**	fragment ion at *m/z* 292.1539 fragment ion at *m/z* 261.1119	292.1543 261.1121	C16H22NO4 C15H17O4	-1.38 -0.87	

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Table 2 List of all detected phase II metabolites for 6-APB and 6-MAPB together with the masses of their PM recorded in MS^1, the corresponding main fragment ions in MS^2, the exact masses, the corresponding elemental composition, and the deviation of the measured from the calculated masses, given as errors in ppm

No.	Metabolites and characteristic ions Measured accurate masses [u]		Calculated exact masses [u]	Elemental composition	Error [ppm]	RT [min]
4G	**6-APB-M (hydroxy-aryl glucuronide)**					
	MS^1	PM at *m/z* 368.1348	368.1339	C17H22NO8	2.19	3.9
	MS^2	fragment ion at *m/z* 175.0749	175.0753	C11H11O2	-2.66	
		fragment ion at *m/z* 192.1014	192.1019	C11H14NO2	-2.49	
5G	**6-APB-M (4-carboxymethyl-3-hydroxy amphetamine glucuronide)** **6-MAPB-M (*N*-demethyl-4-carboxymethyl-3-hydroxy amphetamine glucuronide)**					
	MS^1	PM at *m/z* 386.1430	386.1445	C17H24NO9	-4.11	3.2
	MS^2	fragment ion at *m/z* 210.1120	210.1124	C11H16NO3	-2.37	
		fragment ion at *m/z* 193.0854	193.0859	C11H13O3	-2.54	
7G	**6-APB-M (4-hydroxyethyl-3-hydroxy amphetamine glucuronide)** **6-MAPB-M (*N*-demethyl-4-hydroxyethyl-3-hydroxy methamphetamine glucuronide)**					
	MS^1	PM at *m/z* 372.1636	372.1652	C17H26NO8	-4.28	2.8
	MS^2	fragment ion at *m/z* 196.1327	196.1332	C11H18NO2	-2.39	
		fragment ion at *m/z* 179.1062	179.1066	C11H15O2	-2.62	
8G	**6-MAPB-M (4-carboxymethyl-3-hydroxy methamphetamine glucuronide)**					
	MS^1	PM at *m/z* 400.1595	400.1602	C18H26NO9	-1.71	2.7
	MS^2	fragment ion at *m/z* 224.1276	224.1281	C12H18NO3	-2.90	
		fragment ion at *m/z* 206.1171	206.1175	C12H16NO2	-2.06	
9G	**6-MAPB-M (4-hydroxyethyl-3-hydroxy methamphetamine glucuronide)**					
	MS^1	PM at *m/z* 386.1793	386.1809	C18H28NO8	-4.12	3.8
	MS^2	fragment ion at *m/z* 210.1484	210.1488	C12H20NO2	-2.26	
		fragment ion at *m/z* 179.1062	179.1066	C11H15O2	-2.54	
10G	**6-APB-M (4-dihydroxyethyl-3-hydroxy amphetamine glucuronide)** **6-MAPB-M (*N*-demethyl-4-dihydroxyethyl-3-hydroxy methamphetamine glucuronide)**					
	MS^1	PM at *m/z* 388.1602	388.1602	C17H26NO9	0.02	4.7
	MS^2	fragment ion at *m/z* 177.0905	177.0910	C11H13O2	-2.94	
		fragment ion at *m/z* 195.1010	195.1015	C11H15O3	-2.78	

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Table 3 List of all detected phase metabolites for 6-APB and 6-MAPB in the low dose rat urine with the LC-MS^n SUSA together with the masses of their precursor ions, the corresponding main fragment ions in MS^2 and MS^3

No.	Compounds	Precursor ions (*m/z*)	MS^2 spectra ions (*m/z*) and their relative abundances (%)	MS^3 spectra ions in bold (*m/z*) and their relative abundance (%)
1	6-APB *N*-demethyl 6-MAPB	176	131 (100), 159 (1), 117 (1)	**131:** n.d. **159:** n.d.
2	6-MAPB	190	159 (100), 131 (2)	**159:** 131 (100), 117 (2) **131:** n.d.
5	6-APB-M (4-carboxymethyl-3-hydroxy amphetamine) 6-MAPB-M (*N*-demethyl-4-carboxymethyl-3-hydroxy methamphetamine)	210	165 (100), 147 (45), 175 (20), 193 (3)	**165:** 121 (100), 77 (5) **147:** 119 (100), 91 (20)
8	6-MAPB-M (4-carboxymethyl-3-hydroxy methamphetamine)	224	193 (100), 165 (20), 147 (10), 175 (5)	**193:** 165 (100), 147 (50) **165:** 121 (100)

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Legends to Figures

Fig. 1 EI mass spectra, gas chromatographic retention indices (RI), proposed structures, and predominant fragmentation patterns of acetylated 6-APB (1) and 6-MAPB (2) and their metabolites (3 - 10) arranged according to their RI

Fig. 2 Proposed metabolic pathways for 6-APB and 6-MAPB in rat

Fig. 3 Reconstructed ion chromatograms with the given ions from rat urines after a low dose of 6-APB (A) and 6-MAPB (B) after acid hydrolysis, liquid-liquid extraction and acetylation and analysis with the GC-MS standard urine screening approach SUSA and after protein precipitation of those urines (C-D) and analysis with the LC-MS^n SUSA

Fig. 4 Reconstructed ion chromatograms with the given ions of a reference compound mixture of 5-APB, 6-APB, 5-MAPB, and 6-MAPB after heptafluorobutyrylation (A), or of a mixture of low dose rat urine samples after conjugates cleavage, solid phase extraction, and heptafluorobutyrylation (B), structures, proposed fragmentation patterns, and corresponding EI mass spectra (C)

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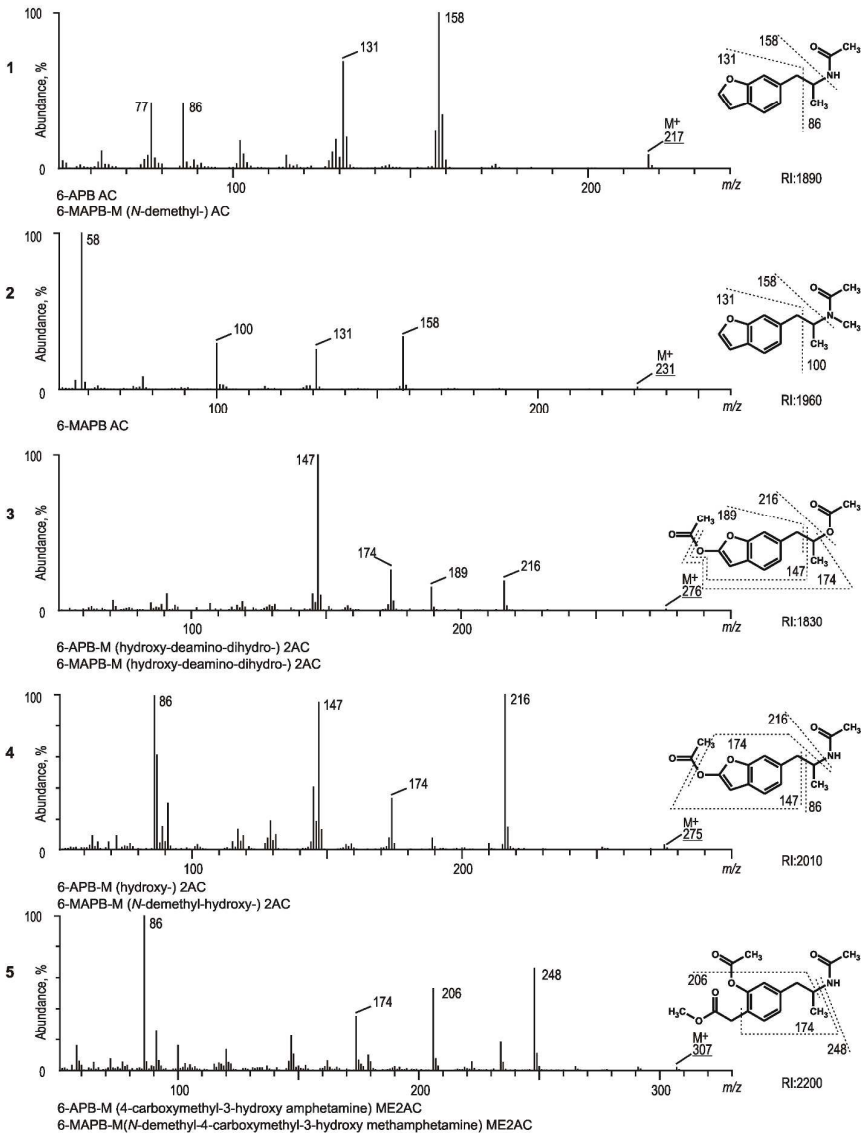

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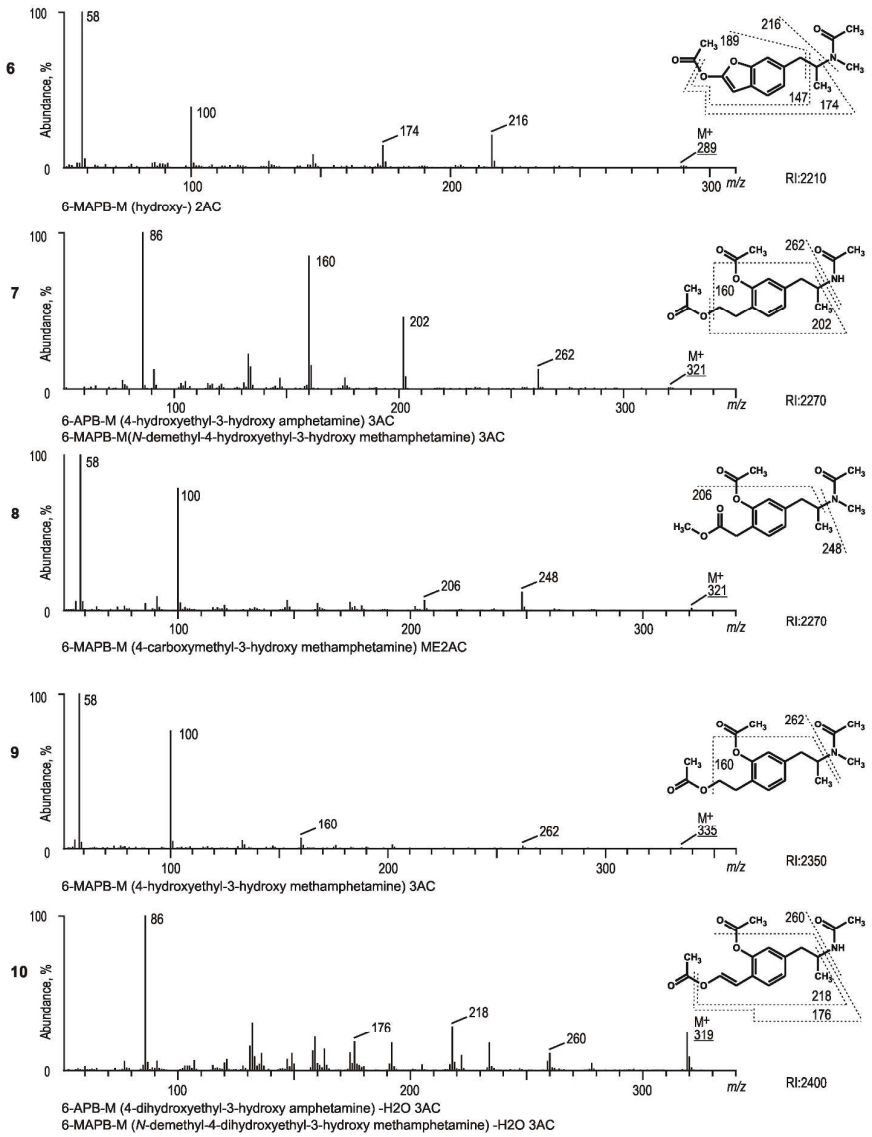
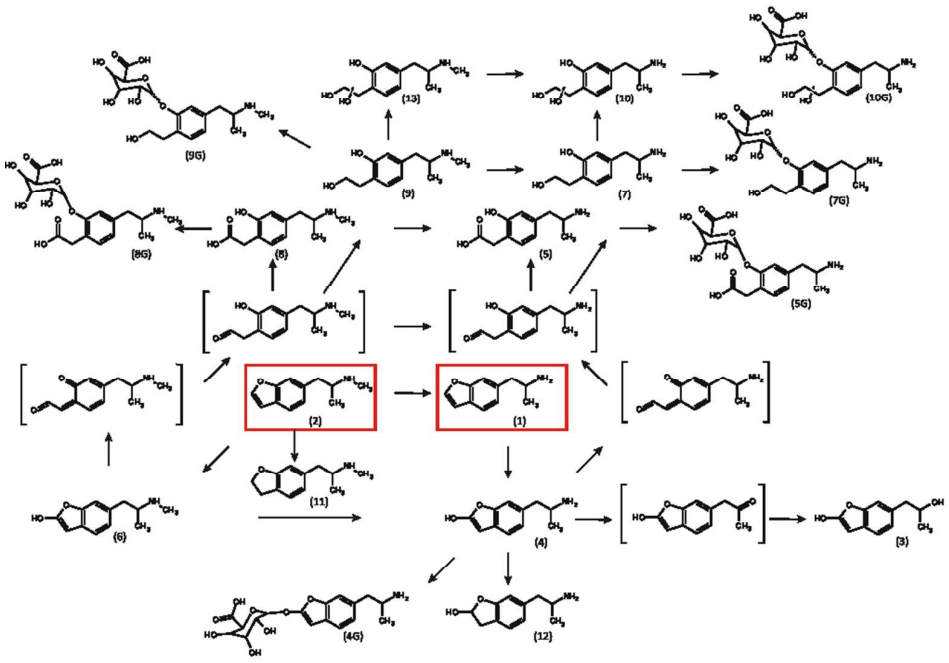


Fig. 1 continued



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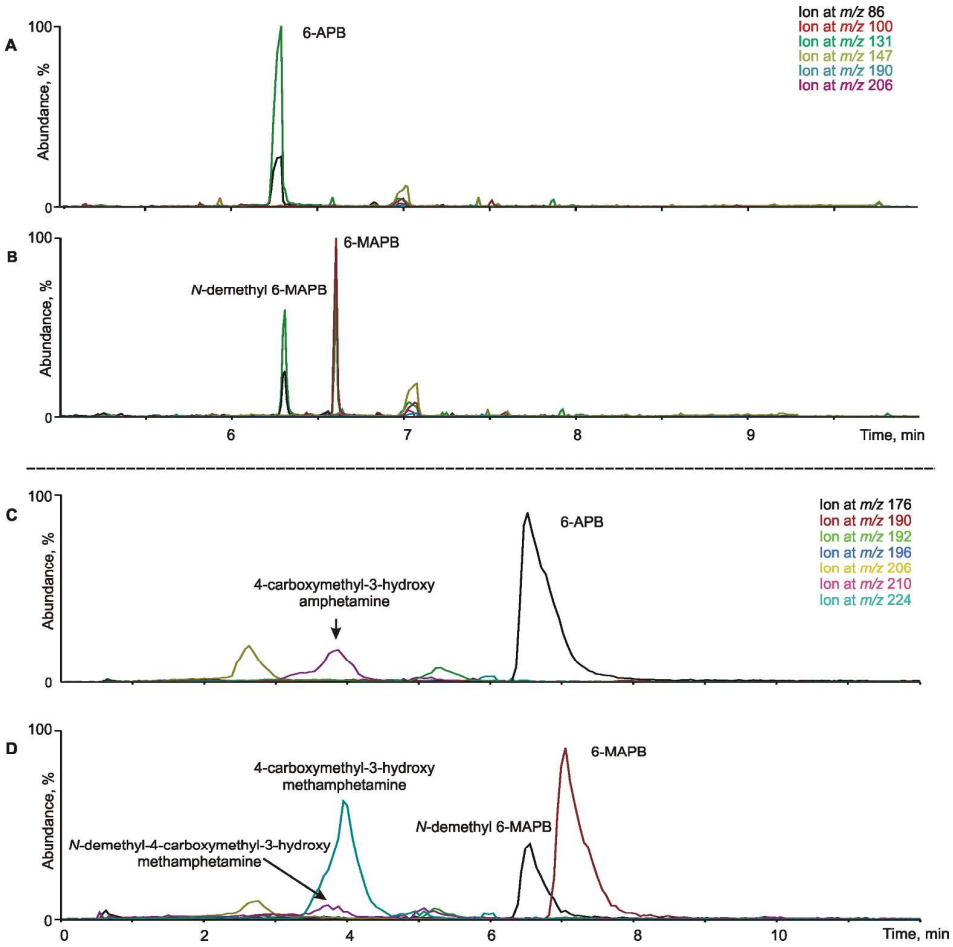

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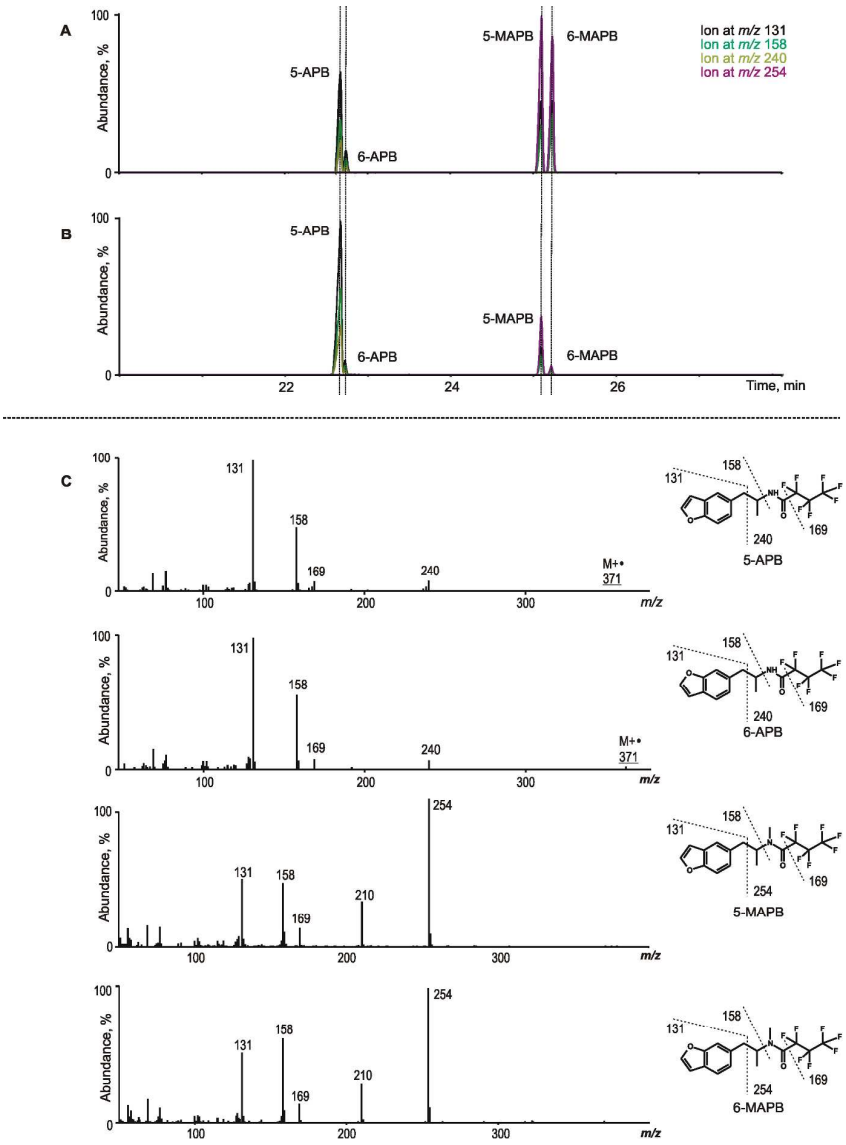

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