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RESEARCH PAPER

2-Methiopropamine, a thiophene analogue of methamphetamine: studies on its metabolism and detectability in the rat and human using GC-MS and LC-(HR)-MS techniques

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Abstract 2-Methiopropamine [1-(thiophen-2-yl)-2-methylaminopropane, 2-MPA], a thiophene analogue of methamphetamine, is available from online vendors selling "research chemicals." The first samples were seized by the German police in 2011. As it is a recreational stimulant, its inclusion in routine drug screening protocols should be required. The aims of this study were to identify the phase I and II metabolites of 2-MPA in rat and human urine and to identify the human cytochrome-P450 (CYP) isoenzymes involved in its phase I metabolism. In addition, the detectability of 2-MPA in urine samples using the authors' well-established gas chromatography–mass spectrometry (GC-MS) and liquid chromatography-linear ion trap-mass spectrometry (LC-MS^n) screening protocols was also evaluated. The metabolites were isolated from rat and human urine samples by solid-phase extraction without or following enzymatic cleavage of conjugates. The phase I metabolites, following acetylation, were separated and identified by GC-MS and/or liquid chromatography–high-resolution linear ion trap mass spectrometry (LC-HR-MS^n) and the phase II metabolites by LC-HR-MS^n. The following major metabolic pathways were proposed: *N*-demethylation, hydroxylation at the side chain and at the thiophene ring, and combination of these transformations followed by glucuronidation and/or sulfation. CYP1A2, CYP2C19, CYP2D6, and CYP3A4 were identified as the major phase I metabolizing enzymes. They were also involved in the *N*-demethylation of the analogue methamphetamine and CYP2C19, CYP2D6, and CYP3A4 in its ring hydroxylation. Following the administration of a typical user's dose, 2-MPA and its metabolites were identified in rat urine using the authors' GC-MS and the LC-MS^n screening approaches. Ingestion of 2-MPA could also be detected by both protocols in an authentic human urine sample.

Keywords Designer drugs · Methiopropamine · 2-MPA · Cytochrome-P450 · GC-MS · LC-MS

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Introduction

2-Methiopropamine [1-(thiophen-2-yl)-2-methylaminopropane, 2-MPA] is a methamphetamine (MA) analogue in which the benzene ring is bioisosterically exchanged with a thiophene ring. It was first synthesized in 1942 by Blicke and Burckhalter [1], and its pharmacological properties (e.g., pressor activity) were shown to be similar to that of benzene analogues although it was not developed commercially. In

2010, it first appeared on several websites selling "legal highs." In drug users' Internet forums, it was described as having stimulant effects accompanied by mild euphoria. In addition, its adverse effects such as increased heart rate, increased sweating, and chest pain were reported (http://www.bluelight.ru/vb/ and http://www.land-der-traeume.de/forum.php).

Continuing online discussions among users, recent seizures in Germany, and the occurrence in forensic case work in Switzerland underline the increasing significance of this compound as a recreational drug [2]. In Switzerland, 2-MPA became a controlled substance in 2012, but it is still legally available in Germany. Despite its widespread use, only little is known about 2-MPA. The US Drug Enforcement Administration published a profile for 2-MPA [3], but no data on its metabolism or detectability in bio-fluids are available. Therefore, the aims of this study were to identify the phase I and II metabolites of 2-MPA in rat and human urine by gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–high-resolution linear ion trap mass spectrometry (LC-HR-MS^n), and to elucidate the human cytochrome-P450 (CYP) isoenzymes involved in its main metabolic steps, as well as comparison to those enzymes involved in the *N*-demethylation and ring hydroxylation of the phenyl analogue MA. A human urine sample from a forensic case, where the ingestion of 2-MPA was suspected, was also analyzed using the GC-MS [4] and LC-MS^n [5, 6] screening protocols previously described by the authors.

Experimental

Chemicals and reagents

2-MPA was synthesized as described elsewhere [7]. Isolute C18 (500 mg, 3 mL) and HCX cartridges (130 mg, 3 mL) were obtained from Biotage (Uppsala, Sweden), MA from Lipomed (Weil am Rhein, Germany), isocitrate and isocitrate dehydrogenase 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, all other chemicals and reagents (analytical grade) from VWR, Darmstadt (Germany), and the baculovirus-infected insect cell microsomes (Supersomes) containing 1 nmol/mL of human complementary DNA-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, and pooled human liver microsomes (pHLM, 20 mg microsomal protein/mL, 400 pmol total CYP/mg protein) from BD (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 rat urine samples from male rats (Wistar, Charles River, Sulzfleck, Germany) for toxicological diagnostic reasons according to the corresponding German law (http://www.gesetze-im-internet.de/tierschg/). The compounds were administered in an aqueous suspension by gastric intubation (20 mg/kg for identification of the metabolites and 1 mg/kg body mass for toxicological analysis). 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. Blank urine samples were collected before drug administration to check whether the samples were free of interfering compounds. The samples were directly analyzed and then stored at −20 °C.

An authentic human urine sample from a road traffic case, submitted to the authors' laboratory, was also analyzed.

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

A 2.0-mL portion of urine was adjusted to pH5.2 with acetic acid (1 M, approximately 50 μL) and incubated at 56 °C for 2 h with 50 μL of a mixture of glucuronidase and arylsulfatase. The urine sample was then diluted with 1 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, and 2 mL of methanol. The basic compounds were eluted into a 1.5-mL reaction vial with 1 mL of a freshly prepared mixture of methanol/aqueous ammonia 32 % (98:2 *v*/*v*). The eluates were evaporated to dryness under a stream of nitrogen and reconstituted with 100 μL of methanol. A 50-μL aliquot was left underivatized, and another 50-μL aliquot was again evaporated to dryness and derivatized with a mixture of acetic anhydride and pyridine (3:2 *v*/*v*) using microwave irradiation (450 W, 5 min). Afterwards, the residue was reconstituted in 50 μL of methanol. A 10-μL aliquot of each extract was then injected onto the LC-MS^n and 1 μL onto the GC-MS system, respectively [8].

Sample preparation for identification of phase II metabolites by LC-HR-MS^n

A 1-mL aliquot of urine was loaded on a C18 cartridge previously conditioned with 1 mL of methanol and 3 mL of water. After passage of the sample, the cartridge was washed with 1 mL of water. Afterwards, the polar compounds were eluted with a mixture of 1 mL of methanol and 200 μL of

acetone [9]. The eluates were evaporated to dryness under a stream of nitrogen and reconstituted with 50 μL of the solvent mixture A/B (50:50, *v*/*v*; A, ammonium formate buffer (pH3); B, acetonitrile/formic acid). A 10-μL aliquot of this solution was injected onto the LC-HR-MS^n.

GC-MS apparatus for identification of the phase I metabolites

The extracts were analyzed using a ThermoFisher Scientific (TF, Dreieich, Germany) Trace GC Ultra GC coupled to a TF ISQ MS and TF Xcalibur Qual Browser software version 2.1 SP1.1160. The GC conditions were as follows: splitless injection mode; column, TF TG-1MS capillary (12 m× 0.2 mm I.D.), cross-linked methyl silicone, 330 nm film thickness; injection port temperature, 250 °C; carrier gas, helium; flow rate, 1 mL/min; and column temperature, programmed from 90 to 310 °C at 30°/min, initial time of 2 min, final time of 5 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; and capillary direct interface, heated at 280 °C.

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

The extracts were analyzed using a TF Dionex UltiMate 3000RS pump consisting of a degasser, a quaternary pump and an UltiMate 3000 RS autosampler, coupled to a TF Velos Orbitrap Pro equipped with a heated electrospray ionization (HESI) II source. Analysis was performed using a TF Hypersil Gold (150×2.1 mm, 1.9 μm) with gradient elution (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). The gradient and flow rate were programmed from 98 to 0 % A at 500 μL/min within 21 min (injection volume, 10 μL). The MS conditions for the Orbitrap were as follows: ESI, positive mode; sheath nitrogen gas flow rate, 40 AU; auxiliary gas, 20 AU; source voltage, 4 kV; source heater temperature, 400 °C; ion transfer capillary temperature, 300 °C; capillary voltage, 4 V; collision-induced dissociation MS/MS experiments were either performed in a data-dependent scan mode (*m/z* 100–800) or on the following selected precursor ions from MS^1 *m/z* 198, 184, 214, 256, 200, 242, 230, 272, 244, and 212 for phase I and 252, 334, 348, and 364 for phase II metabolites. MS^1 was performed in the full scan mode (*m/z* 100–800). Other settings were as follows: normalized collision energies, 35 %; minimum signal threshold, 100 counts; with a resolution of 30,000; isolation width, 1.5 u; activation Q, 0.25; activation time, 30 ms; and dynamic exclusion mode, repeat counts 2, repeat duration 15 s, and exclusion duration 15 s. The TF calibration mixture was used for mass calibration.

Microsomal incubations

Microsomal incubations were performed at 37 °C at a concentration of 250 μM 2-MPA or MA with the CYP isoenzymes (75 pmol/mL, each) CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, or CYP3A5 for 30 min as well as HLM (50 mg protein/mL) as positive control. Besides the enzymes and substrates, the incubation mixtures (final volume, 50 μL) contained 90 mM phosphate buffer (pH7.4), 5 mM Mg^{2+}, 5 mM isocitrate, 1.2 mM $NADP^+$, 0.5 U/mL isocitrate dehydrogenase, and 200 U/mL superoxide dismutase. For incubations with CYP2A6 or CYP2C9, phosphate buffer was replaced with 45 mM and 90 mM Tris buffer, respectively, according to the Gentest manual. Reactions were initiated by addition of the ice-cold microsomes and stopped with 50 μL of ice-cold acetonitrile. The solution was centrifuged for 2 min at 14,000×*g*; 50 μL of the supernatant phase were transferred to an autosampler vial and injected onto the LC-HR-MS/MS using the conditions described below.

LC-HR-MS/MS apparatus for analysis of the microsomal incubation metabolites

A TF Accela LC system consisting of a degasser, a quaternary pump and an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) coupled to a TF Q-Exactive system equipped with an HESI II source was used. The LC conditions were as follows: C18/cation exchange column (150× 4.6 mm, 5 μm, Grace Davis Discovery Science, Waukegan, IL) and gradient elution with 50 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. The gradient and flow rate were programmed as follows: 0–4 min 98 % A to 40 % A at 500 μL/min, 4–7 min hold 10 % A at 1,000 μL/min, and 7–10 min hold 98 % A at 750 μL/min. Injection volume was 10 μL. The MS conditions were as follows: scan experiment 1 in targeted MS^2 mode with an inclusion list containing the *m/z* for the parent compound, the hydroxylated and the *N*-demethylated metabolites of 2-MPA (*m/z* 156, 142, and 172) or MA (*m/z* 150, 136, and 166); positive scan mode (resolution 35,000 at 7 Hz) sheath gas, nitrogen at a flow rate of 12 arbitrary units (AU), auxiliary gas, nitrogen at a flow rate of 18 AU; heater temperature, 350 °C; spray voltage, 4.00 kV; ion transfer capillary temperature, 250 °C; capillary voltage, 25 V; maximum injection time, 250 ms; normalized collision energy (NCE) at 35 eV. Scan experiment 2 in positive scan mode from *m/z* 50 to 750, resolution 70,000 at 3 Hz. The instrument was mass calibrated prior to analysis infusing a Positive Mode Cal Mix (Supelco, Bellefonte, PA) at a flow rate of 5 μL/min using a syringe pump. TF Xcalibur Qual Browser software version 2.2 SP1.48 was

used for calculation of the peak areas for assessment of the relative amount of metabolites formed during incubation.

GC-MS standard urine screening protocol

The rat and human urine samples were worked-up according to published procedures [10, 11]. Briefly, the samples (5 mL) 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 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 using microwave irradiation (450 W, 5 min). Following evaporation, the residue was dissolved in 100 μL of methanol, and 2 μL were injected using a TF TriPlus autosampler onto a TF Trace GC Ultra GC coupled to a TF ISQ MS. The GC and MS conditions were the same as for the metabolism studies. For toxicological detection of 2-MPA and its metabolites, mass chromatography was used with the extracted ions at *m*/*z* 58, 86, 97, 100, and 124 for acetylated 2-MPA and its metabolites. Generation of the mass chromatograms was performed with user defined macros [10]. The identities of the peaks in the mass chromatograms were confirmed by comparison of the mass spectra underlying the peaks (after background subtraction) with reference spectra recorded during this study [4]. 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. The target library was a modified version of the Maurer/Pfleger/Weber MPW_2011 library [4]. The deconvolution parameter settings were as follows [12]: width, 32; adjacent peak subtraction, 2; resolution, high; sensitivity, very high; and shape requirements, low. The minimum match factor was set to 40.

LC-MS^n standard urine screening protocol

The rat and human urine samples (100 μL each) were precipitated by acetonitrile as already described [5]. After shaking and centrifugation, the supernatant was evaporated to dryness under a nitrogen stream and reconstituted in 50 μL of mobile phase (A/B 50:50 *v*/*v*). The worked-up 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. The LC conditions were as described for LC-HR-MS^n. Data-dependent acquisition (DDA) was conducted 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 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 were 35.0 % for MS^2 and 40.0 % for MS^3.

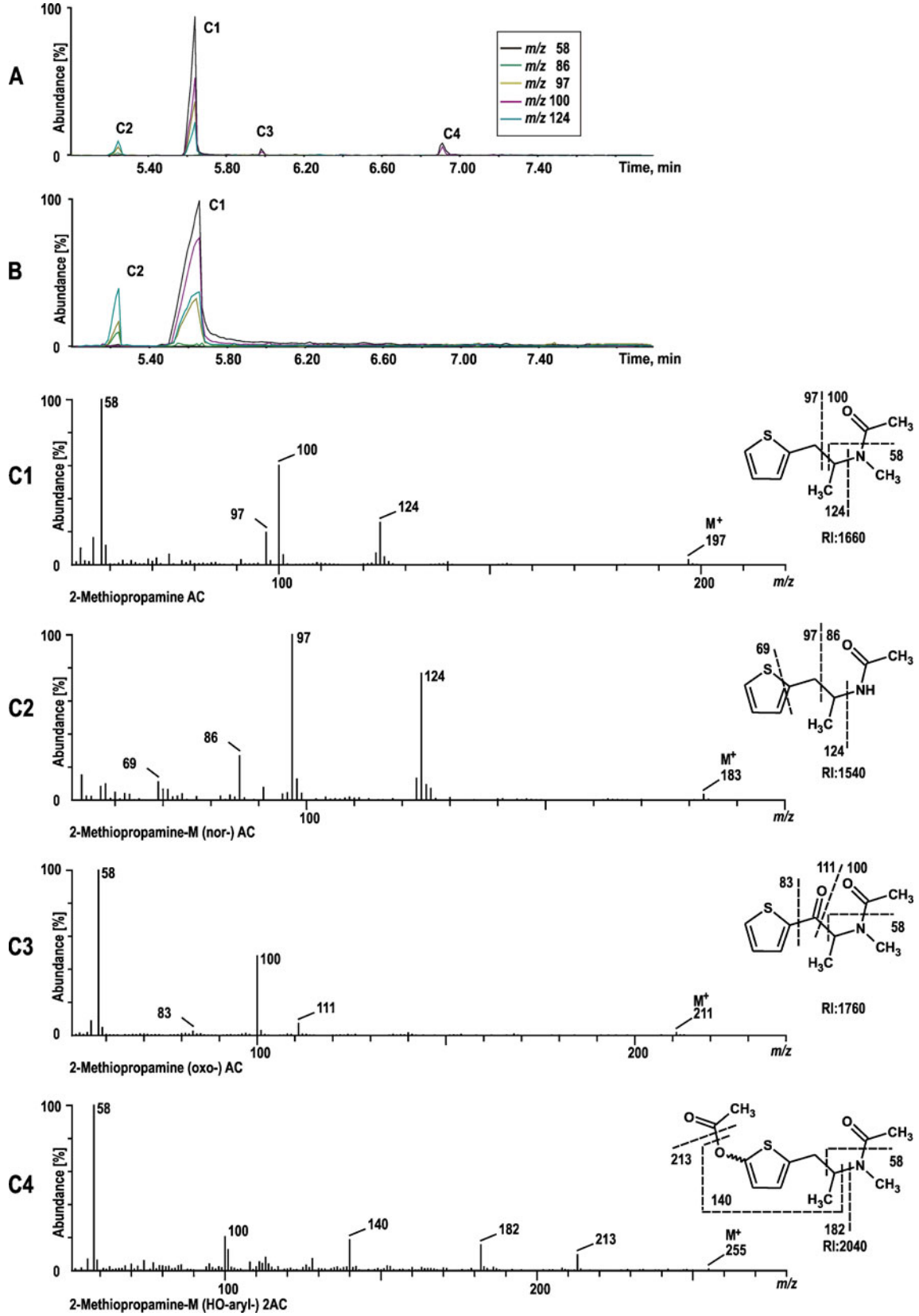
TF Xcalibur 2.1.0 software was used for data acquisition, NIST MS Search 2.0 (National Institute of Standards and Technology, Gaithersburg, MD, USA) for library generation, TF ToxID 2.1.1 for automatic target screening in the MS^2 screening mode. The settings were as follows: retention time (RT) window, 20 min; RT, 0.1 min; signal threshold, 100 counts; search index, 600; and 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.

Fig. 1 Selected mass chromatograms with the given ions of a high dose rat urine sample (20 mg/kg BW) after enzymatic cleavage, solid phase extraction and acetylation (*A*) and of a human urine sample after the STA sample preparation (*B*) indicating spectra C1-4; EI mass spectra, gas chromatographic retention indices (RI), proposed structures, and predominant fragmentation patterns of acetylated 2-MPA (*C1*), nor 2-MPA (*C2*), oxo 2-MPA (*C3*), and hydroxy-aryl 2-MPA (*C4*) arranged according to their RI ▶

Results and discussion

Identification of phase I metabolites by GC-MS

The metabolites were first identified in rat urine by full scan MS after GC separation. This was useful for the later studies on the detectability of 2-MPA as it provided the required reference spectra. The fragments in the EI spectra of the metabolites were interpreted by comparison with those of the parent compound in order to elucidate structures. The fragmentation patterns of other amphetamines and the general fragmentation rules described by Smith and Bush or McLafferty were taken into consideration [13, 14]. Figure 1 (A) shows selected mass chromatograms recorded from rat urine (enzymatic cleavage of conjugates, solid phase extraction, and acetylation) following administration of the high dose of 2-MPA. The structures, GC retention indices (RI), determined according to de Zeeuw et al. [15], and predominant fragmentation patterns of the acetylated 2-MPA and its metabolites are shown in Fig. 1 (C1–C4). In the following, important fragmentation patterns of the EI mass spectra of


A
B
C1
C2
C3
C4
m/z 58
m/z 86
m/z 97
m/z 100
m/z 124
Abundance [%]
Time, min
2-Methiopropamine AC
2-Methiopropamine-M (nor-) AC
2-Methiopropamine (oxo-) AC
2-Methiopropamine-M (HO-aryl-) 2AC
RI:1660
RI:1540
RI:1760
RI:2040

acetylated 2-MPA and its metabolites will be discussed in relation to the postulated structures depicted in Fig. 1 (C1–C4). The numbers of the corresponding mass spectra are given in brackets. The mass spectrum of acetylated 2-MPA (C1) displayed two abundant fragment ions at *m/z* 58 and 100, resulting from cleavage between positions 1 and 2 and subsequent loss of ketene as indicative of an acetyl group. These ions were also seen in the mass spectra of acetylated hydroxy-aryl 2-MPA and oxo 2-MPA (Fig. 1, C3 and C4). The thiophenylmethylium ion at *m/z* 97 in C1 is formed by an *alpha*-cleavage and the fragment ion at *m/z* 124 from loss of the *N*-acetyl-*N*-methyl part. The ion at *m/z* 111 in the mass spectrum of the oxo metabolite (C3) was identified as the thiophenyl acylium ion and the ion at *m/z* 83 as thiophenium ion. The mass spectrum of the acetylated hydroxy metabolite (C4) consisted of a base peak at *m/z* 58, fragment ions at *m/z* 140 (a shift of 16 u from *m/z* 124 in the parent drug) and at *m/z* 182 (*m/z* 140 after acetylation), indicative of the hydroxylated thiophenylpropyl part, and a fragment ion at *m/z* 213 formed by the elimination of ketene from the molecular ion. Metabolic hydroxylation of a thiophene ring has also been described for suprofen, an anti-inflammatory agent, and tienilic acid, a diuretic drug [16–18]. The acetylated nor metabolite (C2) showed a fragment ion at *m/z* 86 resulting from *N*-demethylation and derivatization by acetylation. In general, the fragment ions at *m/z* 97 and 124 were indicative of an unaltered thiophenylpropyl moiety. The observed peak at *m/z* 69 was described by Smith and Busch, resulting from the loss of methyl group from the thiophene ring [14]. In the human urine sample, only the nor metabolite could be detected beside the parent compound.

Identification of phase I metabolites by LC-HR-MS^n

LC-HR-MS^n facilitated confirmation of the metabolites identified by GC-MS and, with its higher sensitivity, revealed the presence of three further metabolites in rat urine, namely, a nor-hydroxy, a hydroxy-methoxy, and a hydroxy-alkyl-hydroxy-aryl metabolite. 2-MPA and all of its phase I metabolites (acetylated) are listed in Table 1 along with the accurate masses of their protonated molecules (PMs) recorded in MS^1, the corresponding main fragment ions in MS^2, the calculated exact masses, the corresponding elemental compositions, and the deviations of the measured accurate masses from the calculated masses, given as errors in parts per million.

The MS^2 spectrum of underivatized 2-MPA (PM at *m/z* 156.0838) contained three specific fragments, the immonium ion at *m/z* 58.0650, the thiophenylmethylium ion at *m/z* 97.0107, and the thiophenylpropylium ion at *m/z* 125.0422. As the underivatized nor 2-MPA showed only one fragment, but the acetylated metabolite some more, the HR spectra were recorded after acetylation. The spectrum of acetylated 2-MPA (PM at *m/z* 198.0942) displayed, in addition to the ions observed in the underivatized spectrum, fragment ions at *m/z* 74.0600 and 100.0758, both resulting from the acetylation of the nitrogen. The mass ion *m/z* 156.0845 was indicative of loss of ketene. The spectrum of the acetylated nor metabolite (PM at *m/z* 184.0787) contained fragment ions at *m/z* 142.0682, due to loss of ketene, at *m/z* 125.0417 from the thiophenylpropyl part, and fragment ions at *m/z* 86.0602 and 60.0444, indicative of the acetylated alkyl chain. The spectra of the acetylated hydroxy metabolites (PM at *m/z* 256.0998) displayed fragment ions at *m/z* 214.0900 indicating a *mono*-acetylated compound and at *m/z* 100.0757 for the acetylated immonium ion. The fragment ion at *m/z* 141.0370 resulted from cleavage between the nitrogen atom and the alkyl chain representing the hydroxylated thiophenylpropyl part. Another important fragment ion at *m/z* 154.0688, which was specific to the hydroxy-alkyl metabolite resulted from loss of acetic acid (60 u) from fragment ion at *m/z* 214.0900. This ion was not present in the spectrum of the hydroxy-aryl metabolite. The fragment ion at *m/z* 74.0599 in the spectrum of the hydroxy-aryl metabolite was found to be indicative of the acetylated side chain.

Two different nor-hydroxy metabolites (PM at *m/z* 242.0842, *di*-acetylated) were identified, one with the hydroxyl group on the alkyl chain and one on the thiophene ring. The spectra of both isomers contained the fragment ion at *m/z* 200.0747, formed by loss of ketene (42 u). The nor-hydroxy-alkyl metabolite produced fragment ions at *m/z* 182.0642, resulting from loss of water (18 u) from *m/z* 200.0748 and at *m/z* 140.0534 resulting from loss of the second acetyl group. The nor-hydroxy-aryl metabolite spectrum contained ions at *m/z* 158.0640, resulting from loss of the second acetyl group and at *m/z* 141.0373, which is indicative of the hydroxylated thiophenylpropyl part. The *tri*-acetylated hydroxy-alkyl-hydroxy-aryl metabolite (PM at *m/z* 314.1051) produced *m/z* 272.0956 and *m/z* 230.0849 after loss of one or two acetyl groups, followed by loss of acetic acid (60 u) forming the ion at *m/z* 170.0638. The *di*-acetylated hydroxy-methoxy metabolite (PM at *m/z* 286.1102) formed fragment ions at *m/z* 244.1010 and 202.0903, resulting from the stepwise loss of the acetyl groups. The fragment ion at *m/z* 171.0480 was representative of the thiophenylpropyl part (125 u) with one hydroxyl (16 u) and one methoxy group (30 u) on the ring. The spectrum of the acetylated oxo metabolite (PM at *m/z* 212.0738) contained the acetylated immonium ion at *m/z* 100.0760, ion at *m/z* 170.0641 resulting from loss of an acetyl group, and ion at *m/z* 128.0711 from loss of the thiophene ring.

Identification of the phase II metabolites by LC-HR-MS^n

The expected glucuronides and/or sulfates were detected using the data-dependent scan mode and identified by

Table 1 List of 2-MPA and all acetylated phase I metabolites together with the masses of their PM recorded in MS^1, the corresponding main fragment ions in MS^2, the calculated exact masses, the corresponding elemental composition, and the deviation of the measured from the calculated masses, given as errors in parts per million

Metabolites and characteristic ions, measured accurate masses (u)	Calculated exact masses (u)	Elemental composition	Error (ppm)
2-Methiopropamine			
PM at 156.0838	156.0841	C8H14NS	−2.22
Fragment ion at 125.0422	125.0419	C7H9S	1.89
Fragment ion at 58.0650	58.0651	C3H8N	−2.51
Fragment ion at 97.0107	97.0106	C5H5S	0.23
2-Methiopropamine AC			
PM at 198.0942	198.0947	C10H16ONS	−2.48
Fragment ion at 125.0422	125.0419	C7H9S	1.82
Fragment ion at 74.0600	74.0600	C3H8ON	−1.20
Fragment ion at 100.0758	100.0757	C5H10ON	0.69
Fragment ion at 156.0845	156.0841	C8H14NS	2.07
2-Methiopropamine-M (nor-) AC			
PM at 184.0787	184.0791	C9H14ONS	−1.85
Fragment ion at 125.0417	125.0419	C7H9S	−2.02
Fragment ion at 60.0444	60.0444	C2H6ON	−0.17
Fragment ion at 86.0602	86.0600	C4H8ON	−2.36
Fragment ion at 142.0682	142.0685	C7H12NS	−2.04
2-Methiopropamine-M (HO-alkyl-) 2AC			
PM at 256.0998	256.1002	C12H18O3NS	−1.68
Fragment ion at 214.0900	214.0896	C10H16O2NS	1.61
Fragment ion at 154.0688	154.0685	C8H12NS	2.10
Fragment ion at 141.0370	141.0369	C7H9OS	1.12
Fragment ion at 100.0757	100.0757	C5H10ON	0.30
2-Methiopropamine-M (HO-aryl-) 2AC			
PM at 256.0998	256.1002	C12H18O3NS	−1.56
Fragment ion at 214.0900	214.0896	C10H16O2NS	1.75
Fragment ion at 141.0370	141.0369	C7H9OS	1.12
Fragment ion at 74.0599	74.0600	C3H8ON	−1.36
Fragment ion at 100.0757	100.0760	C5H10ON	−0.01
2-Methiopropamine-M (nor-HO-alkyl-) 2AC			
PM at 242.0842	242.0845	C11H16O3NS	−1.28
Fragment ion at 200.0747	200.0740	C9H14O2NS	−3.28
Fragment ion at 182.0642	182.0634	C9H12ONS	4.37
Fragment ion at 140.0534	140.0528	C7H10NS	3.86
2-Methiopropamine-M (nor-HO-aryl-) 2AC			
PM at 242.0842	242.0845	C11H16O3NS	1.28
Fragment ion at 200.0747	200.0740	C9H14O2NS	1.36
Fragment ion at 158.0640	158.0634	C7H12ONS	3.54
Fragment ion at 141.0373	141.0369	C7H9OS	3.10
2-Methiopropamine-M (HO-alkyl-HO-aryl-) 3AC			
PM at 314.1051	314.1057	C14H20O5NS	−1.72
Fragment ion at 272.0956	272.0951	C12H18O4NS	1.64
Fragment ion at 230.0849	230.0845	C10H16O3NS	1.52
Fragment ion at 170.0638	170.0634	C8H12ONS	2.10
2-Methiopropamine-M (HO-methoxy-) 2AC			
PM at 286.1102	286.1108	C13H20O4NS	−1.94
Fragment ion at 244.1010	244.1002	C11H18O3NS	3.36

Table 1 (continued)

Metabolites and characteristic ions, measured accurate masses (u)	Calculated exact masses (u)	Elemental composition	Error (ppm)
Fragment ion at 202.0903	202.0896	C9H16O2NS	3.18
Fragment ion at 171.0480	171.0474	C8H11O2S	3.12
2-Methiopropamine-M (oxo-) AC			
PM at 212.0738	212.0740	C10H14O2NS	−0.78
Fragment ion at 170.0641	170.0634	C8H12ONS	3.99
Fragment ion at 128.0711	128.0706	C6H10O2N	4.02
Fragment ion at 100.0760	100.0757	C5H10ON	2.69

comparing their MS^2 and MS^3 with the MS^1 and MS^2 spectra of the corresponding phase I metabolites. The phase II metabolites are listed in Table 2, together with the accurate masses of their PMs recorded in MS^1, the corresponding main fragment ions in MS^2, the calculated exact masses, the corresponding elemental compositions, and the deviations of the measured from the calculated masses, given as errors in parts per million.

The mass spectrum of the hydroxy-alkyl glucuronide (PM at *m*/*z* 348.1112) contained fragment ions at *m*/*z* 172.0796, representative of its phase I metabolite and at *m*/*z* 154.0690, resulting from water loss from *m*/*z* 172.0796. In contrast, the hydroxy-aryl glucuronide (PM at *m*/*z* 348.1108) and the hydroxy-aryl sulfate (PM at *m*/*z* 252.0355) contained the fragment ion for the phase I metabolite (*m*/*z* 172.0788), and at *m*/*z* 141.0365, representative of the thiophenylpropyl group with one hydroxyl on the ring. The hydroxy-alkyl–hydroxy-aryl glucuronide (PM at *m*/*z* 364.1063) produced, through loss of glucuronic acid, fragment ions at *m*/*z* 188.0746 and at *m*/*z* 170.0641, formed by subsequent loss of water, indicating one hydroxyl group at the side chain. The *nor*-hydroxy-alkyl glucuronide (PM at *m*/*z* 334.0951) displayed a fragment ion at *m*/*z* 158.0640, indicative of the corresponding phase I metabolite and at *m*/*z* 140.0534, resulting from subsequent water loss.

In rat urine, a hydroxy-alkyl-glucuronide and a hydroxy-aryl-glucuronide (PM at *m*/*z* 348), a nor-hydroxy-alkyl-glucuronide (PM at *m*/*z* 334), a hydroxy-alkyl-hydroxy-aryl-glucuronide (PM at *m*/*z* 364), and a sulfate of the hydroxy-aryl-

Table 2 List of all phase II metabolites together with the masses of their PM recorded in MS^1, the corresponding main fragment ions in MS^2, the calculated exact masses, the corresponding elemental composition, and the deviation of the measured from the calculated masses, given as errors in parts per million

Metabolites and characteristic ions, measured accurate masses (u)	Calculated exact masses (u)	Elemental composition	Error (ppm)
2-Methiopropamine-M (HO-alkyl-glucuronide)			
PM at 348.1112	348.1111	C14H22O7NS	0.20
Fragment ion at 172.0796	172.0791	C8H14ONS	2.95
Fragment ion at 154.0690	154.0685	C8H12NS	2.94
2-Methiopropamine-M (HO-aryl-glucuronide)			
PM at 348.1108	348.1111	C14H22O7NS	0.39
Fragment ion at 172.0788	172.0791	C8H14NOS	1.87
Fragment ion at 141.0365	141.0369	C7H9OS	2.57
2-Methiopropamine-M (HO-aryl-sulfate)			
PM at 252.0355	252.0359	C8H14O4NS2	−1.65
Fragment ion at 172.0788	172.0791	C8H14ONS	−1.87
Fragment ion at 141.0365	141.0369	C7H9OS	−2.57
2-Methiopropamine-M (HO-alkyl-HO-aryl-glucuronide)			
PM at 364.1063	364.1061	C14H22O8NS	0.65
Fragment ion at 188.0746	188.0740	C8H14O2NS	3.47
Fragment ion at 170.0641	170.0634	C8H12ONS	3.99
2-Methiopropamine-M (nor-HO-alkyl-glucuronide)			
PM at 334.0951	334.0955	C13H20O7NS	−1.23
Fragment ion at 158.0640	158.0634	C8H14ONS	3.60
Fragment ion at 140.0534	140.0529	C7H10NS	3.88

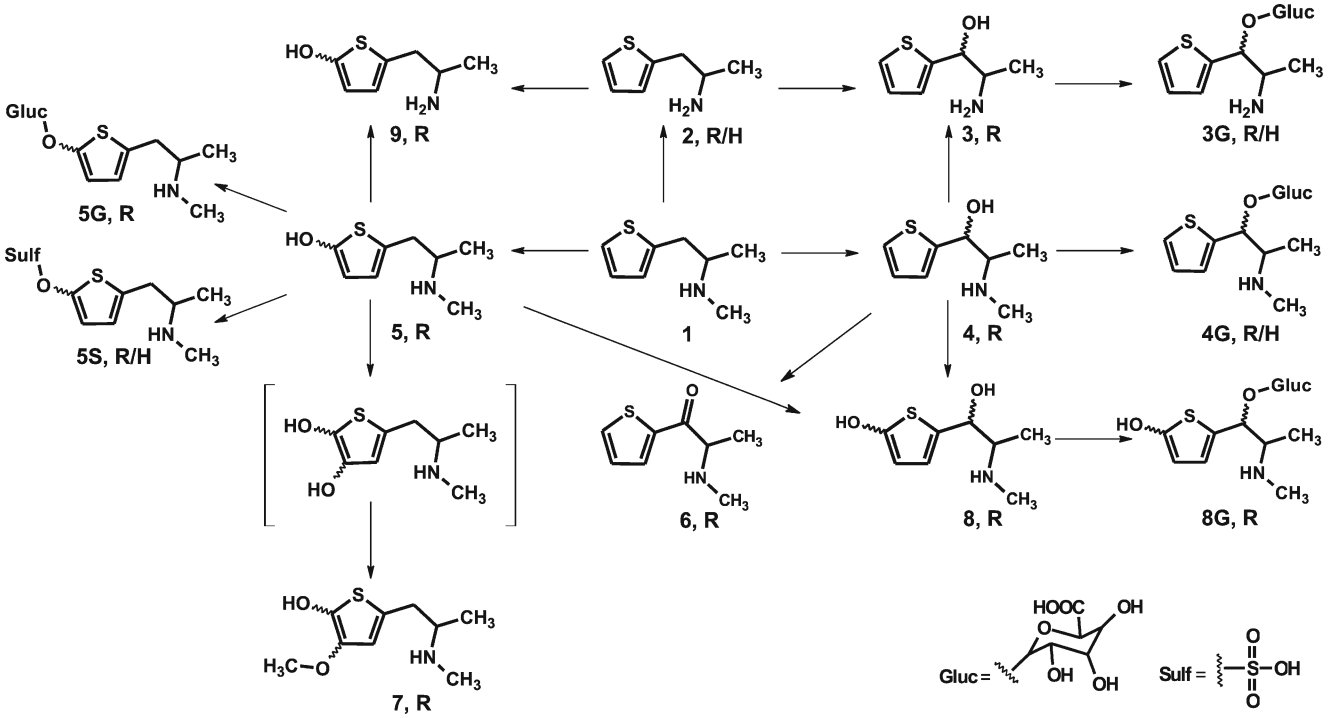


Fig. 2 Proposed metabolic pathways for 2-MPA in rats (*R*) and humans (*H*)

metabolite (PM at *m/z* 252) were detected. In human urine, the hydroxy-alkyl-glucuronide, the nor-hydroxy-alkyl-glucuronide, and the hydroxy-aryl-sulfate could be detected.

Proposed metabolic pathways

The main excretion product in rat and human urine was the unchanged drug (1 in Fig. 2). According to the mass spectral data described above, the following metabolites could be identified in rat (R in Fig. 2) or human (H) urine: nor 2-MPA (2 in Fig. 2), nor-hydroxy-alkyl 2-MPA (3), hydroxy-alkyl 2-MPA (4), hydroxy-aryl 2-MPA (5), oxo 2-MPA (6), hydroxy-methoxy-aryl 2-MPA (7), hydroxy-alkyl-hydroxy-aryl 2-MPA (8), nor-hydroxy-aryl 2-MPA (9), nor-hydroxy-alkyl 2-MPA glucuronide (3G), hydroxy-alkyl 2-MPA glucuronide (4G), hydroxy-aryl 2-MPA glucuronide (5G), hydroxy-aryl 2-MPA sulfate (5S), and hydroxy-alkyl-hydroxy-aryl 2-MPA glucuronide (8G). From these findings, the following metabolic pathways are proposed (Fig. 2): *N*-demethylation; hydroxylation at the side chain (alkyl-) or thiophene ring (aryl-), combinations of them; oxidation of the alkyl-hydroxy metabolite to the corresponding oxo metabolite; further hydroxylation of the hydroxy-aryl metabolite followed by O-methylation; and finally, glucuronidation or sulfation of the hydroxy metabolites.

CYP activity screening

The activity screening studies with the ten most abundant human hepatic CYPs were performed to identify their ability to catalyze the formation of the initial phase I metabolites in vitro. According to the supplier's advice, the incubation conditions chosen were adequate to make a statement on the general involvement of a particular CYP enzyme. The three main metabolic transformations observed in the in vitro incubations of 2-MPA with recombinant CYPs were *N*-demethylation and hydroxylation at the side chain or the thiophene ring (Table 3). This was similar to the in vivo

Table 3 General involvement of the given CYP isoenzymes in the given metabolic steps of 2-MPA and MA

Metabolic step	CYP1A2	CYP2A6	CYP2B6	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP2E1	CYP3A4	CYP3A5
2-MPA *N*-demethylation	+		+			+	+	+	+	
2-MPA ring hydroxylation	+					+	+	+	+	
2-MPA side chain hydroxylation	+					+	+		+	
MA *N*-demethylation	+		+			+	+	+	+	
MA ring hydroxylation						+	+		+	

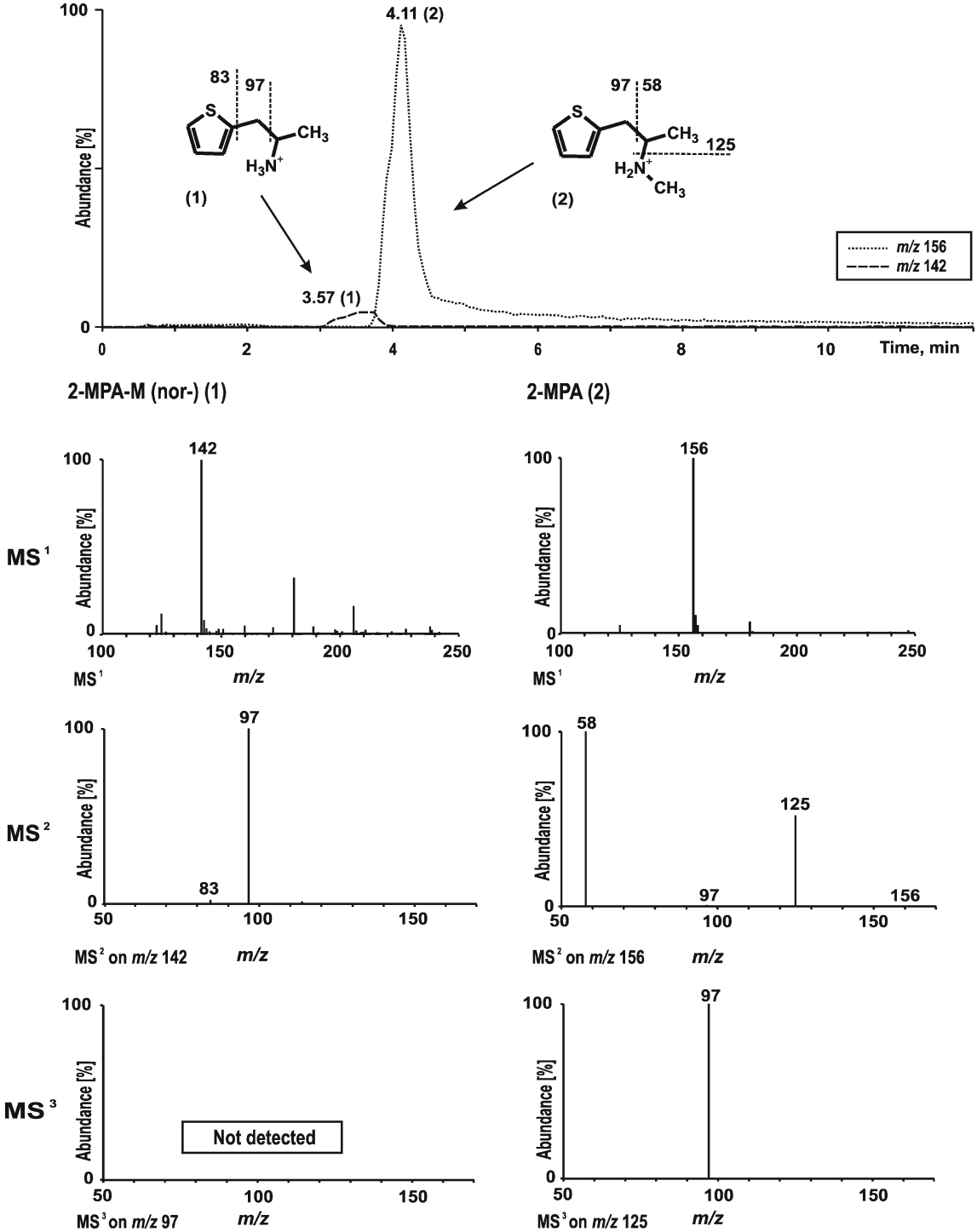


Fig. 3 Selected mass chromatograms, structures, proposed fragmentation patterns (*upper part*), and MS^1, MS^2, and MS^3 spectra of nor 2-MPA and 2-MPA after protein precipitation of an authentic human urine sample and analysis with the LC-MS^n standard screening approach (*lower part*)

results. For comparison, CYP activity screening was also performed for the phenyl analogue MA under the same conditions. *N*-Demethylation and ring hydroxylation, but no side chain hydroxylation, could be detected (Table 3). *N*-Demethylation of both analogues was generally catalyzed by the same CYPs, while there were some differences in the ring hydroxylation. This may be explained by the same chemical structure of both side chains but different ring systems. However, the actual contribution of the CYP isoenzymes in the metabolism of both drugs can only be concluded from enzyme kinetic studies, which are in progress and will be published elsewhere.

Toxicological detection of 2-MPA by GC-MS or LC-MS^n

Using the GC-MS-based systematic toxicological analysis approach described by the authors [10], an intake of 2-MPA could be monitored in rat urine after administration of 1 mg/kg BM. This dose corresponded to a human single dose of about 15 mg scaled by dose-by-factor approach according to [19]. This dose was lower than that recommended in trip reports (about 30–50 mg per single dose, http://www.bluelight.ru/vb/). Therefore, detection of the intake of 2-MPA should be possible. The possible presence of 2-MPA and its metabolites were indicated by reconstructed mass chromatography with the ions at *m/z* 58, 100, and 124 for acetylated 2-MPA; *m/z* 86, 97, and 124 for acetylated nor 2-MPA; and *m/z* 58 and 100 for acetylated oxo 2 MPA and hydroxy-aryl 2-MPA according to the corresponding reference spectra given in Fig. 1 (C1–C4). Figure 1 (B) shows the GC-MS mass chromatograms for a human urine sample collected 18 h after intake of about 200 mg (subject's statement) of 2-MPA. At the time of sampling, the subject showed no behavioural abnormities most probably due to the time lag. In agreement to the mass chromatograms recorded in rat urine after the high 2-MPA dose (Fig. 1, A), the parent compound and its nor metabolite were identified and thus considered to be suitable candidates for routine toxicological screening. In cases of severe overdose and/or genetic variability, other metabolites may also occur in human urine and should therefore be included in the mass chromatograms. The identity of the peaks indicated by the selected mass chromatogram was confirmed by computerized comparison of the underlying full scan mass spectrum with reference spectra recorded during this study [4]. Additionally, the full scan data files acquired by the GC-MS system were evaluated by AMDIS allowing the detection of 2-MPA in the prepared urine samples using the previously described procedure [12].

Using the LC-MS^n screening approach described previously [6], in rat urine after the 1 mg/kg BW dose, both the parent compound and its nor metabolite were also detected. As indicated by the mass chromatograms depicted in Fig. 3 (upper part), in the human urine (after a 200 mg dose), the nor metabolite could also be detected. The underlying spectra, the proposed fragmentation patterns, and the structures are given in Fig. 3 for identification of the indicated analytes. For 2-MPA, the main fragment ions were at *m/z* 58 from the immonium ion and at *m/z* 125 from the thiophenylpropyl part. In addition, a small fragment ion at *m/z* 97 could be detected representing the thiophenylmethyl part, resulting from an alpha-cleavage between positions 1 and 2. This fragment ion is as well the only one present in the MS^3 spectra of 2-MPA. For nor 2-MPA, the MS^2 spectrum shows solely one fragment ion at *m/z* 97, the same as described before. For this metabolite no MS^3 spectra could be obtained.

Conclusions

This study shows that the MA analogue, 2-MPA, was metabolized in rats and humans only to a minor extent. Various CYPs were involved in the main metabolic steps. Both screening approaches tested should be suitable for drugs of abuse testing in urine focusing on the detection of the parent drug and its nor metabolite.

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