RESEARCH PAPER

Qualitative metabolism assessment and toxicological detection of xylazine, a veterinary tranquilizer and drug of abuse, in rat and human urine using GC–MS, LC–MS^n, and LC–HR-MS^n

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Abstract Xylazine is used in veterinary medicine for sedation, anesthesia, and analgesia. It has also been reported to be misused as a horse doping agent, a drug of abuse, a drug for attempted sexual assault, and as source of accidental or intended poisonings. So far, no data concerning human metabolism have been described. Such data are necessary for the development of toxicological detection methods for monitoring drug abuse, as in most cases the metabolites are the analytical targets. Therefore, the metabolism of xylazine was investigated in rat and human urine after several sample workup procedures. The metabolites were identified using gas chromatography (GC)–mass spectrometry (MS) and liquid chromatography (LC) coupled with linear ion trap high-resolution multistage MS (MS^n). Xylazine was N-dealkylated and S-dealkylated, oxidized, and/or hydroxylated to 12 phase I metabolites. The phenolic metabolites were partly excreted as glucuronides or sulfates. All phase I and phase II metabolites identified in rat urine were also detected in human urine. In rat urine after a low dose as well as in human urine after an overdose, mainly the hydroxy metabolites were detected using the authors' standard urine screening approaches by GC–MS and LC–MS^n. Thus, it should be possible to monitor application of xylazine assuming similar toxicokinetics in humans.

Keywords Xylazine · Metabolism · Human · Detectability · Gas chromatography–mass spectrometry · Liquid chromatography–multistage mass spectrometry · Liquid chromatography–high-resolution multistage mass spectrometry

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Introduction

Xylazine is used in veterinary medicine for sedation, anesthesia, and analgesia. It has also been reported to be misused as a horse doping agent, a drug of abuse, a drug for attempted sexual assault, and as source of accidental or intended poisonings [1–6]. In the case of an overdose, sedation, respiratory depression, bradycardia, severe hypotension, and hyperglycemia were observed in humans [1, 3, 7]. During the last decade, it has gained popularity as a recreational drug [2, 8–11]. For all these reasons, xylazine should be monitored not only in horse doping [4], but also in clinical and forensic toxicology. Before possible quantification in blood, e.g., by gas chromatography (GC)–mass spectrometry (MS) [12], drugs and poisons have to be detected typically with general screening approaches [13]. Urine screening is the most comprehensive approach, covering a broad range of (also low-dosed) drugs for a longer time. However, most drugs are excreted in urine, mainly or exclusively as metabolites. Thus, the metabolism and the detectability of new drugs in urine must be studied.

So far, no data concerning metabolism or detectability in humans have been described, only data from rats and/or horses [4, 14–16]. In a current poisoning case [1], more metabolites were detected in human urine samples than were described in the rat or horse studies. Thus, the metabolism of xylazine in rats should be reinvestigated using the authors' standard study design. These studies should allow assessment of whether the human metabolites, so far not described in animals, were formed only by humans or also by rats, but not yet identified. The question of whether the application of a common user's dose can be monitored by common screening approaches should also be answered. Therefore, the aim of the study presented was the identification of the phase I and phase II metabolites of xylazine in rat and human urine with and without gentle enzymatic cleavage of conjugates and solid-phase

extraction (SPE) using GC–MS and liquid chromatography (LC)–linear ion trap high-resolution (HR) multistage MS (MS^n). In addition, the detectability of xylazine and/or its metabolites was assessed using the authors' standard urine screening approaches by GC–MS after acid hydrolysis, liquid–liquid extraction, and acetylation as well as using LC–MS^n after protein precipitation [17–20].

Experimental

Chemicals and reagents

Xylazine was obtained from Sigma-Aldrich (Steinheim, Germany), Isolute Confirm HCX (130 mg, 3 mL) and Isolute Confirm C_{18} (500 mg, 3 mL) SPE cartridges were obtained from Biotage (Grenzach-Wyhlen, Germany), N-nitroso-N-methylurea was obtained from Jenachem (Jena, Germany), and acetonitrile (LC–MS grade), ammonium formate (analytical grade), formic acid (for MS), methanol (LC–MS grade), and all other chemicals and biochemicals were obtained from VWR (Darmstadt, Germany).

Rat urine samples

Metabolism studies were performed using male Wistar rats (Charles River, Sulzfeld, Germany). They were given a single 1 or 15 mg/kg body mass dose in aqueous suspension by gastric intubation of xylazine for toxicological diagnostic reasons according to the corresponding German law (http://www.gesetze-im-internet.de/tierschg/). 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. All urine samples were analyzed directly or stored at -20 °C until further analysis.

Human urine samples

Authentic urine samples of a healthy 14-year-old Caucasian male were submitted to the authors' laboratory for toxicologic diagnostic reasons. They were handled according to the institutional protocol and regulations. Urine was directly analyzed or stored at -20 °C until further analysis.

Sample preparation of urine samples for metabolism studies using GC–MS

For identification of phase I metabolites by GC–MS, 1.0 mL of urine (rat urine 15 mg/kg drug administration or patients' urine) was processed by enzymatic cleavage of conjugates and SPE (HCX) [21]. Briefly, urine was loaded on an Isolute Confirm HCX cartridge previously conditioned with methanol and water. After passage of the sample, the cartridge was washed with

0.1 M hydrochloric acid and purified water. Elution was performed with a mixture of methanol and ammonia (98:2 v/v). The evaporated extracts were acetylated after addition of 100 μL of an acetic anhydride–pyridine mixture (3:2; v/v) and incubation for 5 min under microwave irradiation at about 400 W. After evaporation of the derivatization mixture (60 °C, reduced pressure), the residue was dissolved in 100 μL methanol.

Sample preparation of urine samples for metabolism studies using LC–HR-MS^n

For identification of phase I metabolites, 1.0 mL of urine was enzymatically hydrolyzed and extracted as described for GC–MS. However, the evaporated underivatized extract was reconstituted in 50 μL of mobile phase (A/B 1:1 v/v).

For identification of phase II metabolites, 1.0 mL of urine (rat urine 15 mg/kg drug administration or patients' urine) was processed by SPE (C_{18}) [22]. Briefly, urine was diluted with 2.0 mL purified water before the sample was loaded on a previously conditioned (1.0 mL methanol, 1.0 mL water) Isolute Confirm C_{18} cartridge. After passage of the sample, the cartridge was washed with 2.0 mL of purified water. Elution was performed using 1.5 mL of methanol and 0.2 mL acetone. The extracts were gently evaporated to dryness and reconstituted in 50 μL of mobile phase (A/B 1:1 v/v).

For protein precipitation, 100 μL of urine was precipitated by 500 μL acetonitrile [23]. After shaking and centrifugation, the supernatant was gently evaporated to dryness and reconstituted in 50 μL of mobile phase (A/B 1:1 v/v).

Methylation with diazomethane for GC–MS and LC–HR-MS^n analysis

For further structure elucidation, the HCX urine extracts were methylated using diazomethane. This was freshly prepared as follows: N-nitroso-N-methylurea was dissolved in aqueous potassium hydroxide solution (40 %) and diethyl ether, and the reaction vessel was slightly capped and stored for 30 min at 4 °C. Afterwards, the organic layer was removed and dried over potassium hydroxide. The solution was stored at -20 °C and could be used for several days. For derivatization, the evaporated HCX extract were reconstituted in 50 μL methanol, and 100 μL of the methylation reagent was added and the resulting mixture was incubated for 30 min at room temperature in a closed vial. After evaporation of reagent, the residue was reconstituted in 50 μL of methanol for GC–MS or mobile phase (A/B 1:1 v/v) for LC–HR-MS^n.

GC–MS apparatus for identification of the phase I metabolites

The settings were the same as described by Meyer et al. [24]. Briefly, an HP 5890 series II gas chromatograph (Agilent, Waldbronn, Germany) combined with an HP 5972 MSD mass

spectrometer and HP MS ChemStation (DOS series, version C.03.00) was used. The gas chromatograph was programmed from 100 to 310 °C at 30 °C/min (initial time 3 min, final time 8 min). The mass spectrometer was operated in the electron ionization full-scan mode (m/z 50–550).

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

The settings were the same as described by Meyer et al. [19] and Welter et al. [20]. Briefly, a Thermo Fisher Scientific (Dreieich, Germany) Dionex LC system was coupled with a Thermo Fisher Scientific LTQ Orbitrap equipped with a HESI-II source. Separation was performed by a Thermo Fisher Scientific Hypersil Gold column (15 mm×2.1 mm, 1.9 μm) and gradient elution. The mass spectrometer was operated in positive electrospray ionization mode. Collision-induced dissociation MS/MS experiments were performed by using information-dependent acquisition (IDA): MS^1 was performed in the full-scan mode (m/z 150–800) with a resolution of 15,000. MS^2 and MS^3 were performed in the IDA mode: four IDA MS^2 experiments were chosen to provide MS^2 data on the four most intense signals from MS^1 and additionally, eight MS^3 experiments were chosen to record MS^3 data on the most and second most intense signals from MS^2. MS^2 spectra were collected with a higher priority than MS^3 spectra. The normalized wideband collision energies were 35 % for MS^2 and 40 % for MS^3. Other settings were as follows: for MS^2, minimum signal threshold, 100 counts, isolation width, 1.5 u; for MS3, minimum signal threshold, 50 counts, isolation width, 2.0 u; for both stages, activation Q, 0.25, activation time, 30 ms, dynamic exclusion mode, repeat counts, 2, repeat duration, 15 s, exclusion list, 50, exclusion duration, 15 s, average full-scan to full-scan cycle time, 4 s. To obtain more structure information, samples were measured without wideband activation additionally. Thermo Fisher Scientific Xcalibur version 2.2 was used for data acquisition.

GC–MS standard urine screening protocol

The rat and human urine samples were worked up according to published procedures [17, 25]. Briefly, the samples (5 mL) were divided into two aliquots and one part was submitted to acid hydrolysis. Thereafter, the sample pH was adjusted to 8-9 and the other aliquot of untreated urine was added. This mixture was extracted with a dichloromethane–2-propanol–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 was injected into the GC–MS system as described above.

For toxicological detection of xylazine and its metabolites, mass chromatography was used with the extracted ions at m/z 121, 137, 146, 162, 179, 221, 250, and 278. Generation of the mass chromatograms was performed with user-defined macros [17]. 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 (Maurer HH, Pfleger K, Weber AA, Mass spectral library of drugs, poisons, pesticides, pollutants and their metabolites. 5th edition, Wiley-VCH, Weinheim, in preparation). In addition, the full-scan data files acquired by GC–MS were evaluated by the Automated Mass Spectral Dconvolution and Identification System (http://chemdata.nist.gov/mass-spc/amdis/) in simple mode. The deconvolution parameter settings were as follows [26]: width, 32, adjacent peak subtraction, 2; resolution, high; sensitivity, very high; and shape requirements, low. The minimum match factor was set to 50.

LC–MS^n standard urine screening protocol

The rat and human urine samples (100 μL each) were precipitated by acetonitrile as already described for the metabolism studies. The worked up samples were separated and analyzed using a Thermo Fisher Scientific LXQ linear ion trap MS equipped with a HESI-II source and coupled to a Thermo Fisher Scientific Accela LC system consisting of a degasser, a quaternary pump, and an autosampler. A Thermo Fisher Scientific Hypersil Gold column (10 mm×2.1 mm, 1.9 μm) was used, and the applied gradient and MS settings are described elsewhere [20, 23]. Thermo Fisher Scientific Xcalibur version 2.2 was used for data acquisition, NIST MS Search 2.0 (National Institute of Standards and Technology, Gaithersburg, MD, USA) was used for library generation, and Thermo Fisher Scientific ToxID 2.1.1 was used for automatic target screening in the MS^2 screening mode. The settings were as follows: retention time window, 20 min; retention time, 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 the precursor tolerance option and retention time locking. Further settings were as follows: score threshold, 0.1; minimum number of peak matches, 0. ToxID and SmileMS were run automatically after file acquisition using an Xcalibur processing method starting both software tools.

Results and discussion

Identification of phase I metabolites by GC–MS

In the upper part of Fig. 1, reconstructed mass chromatograms are depicted of the ions at m/z 121, 179, 146, 162, and 221,

indicating the underlying marked metabolite spectra b–j. In the lower part of Fig. 1, GC–MS spectra with the chemical structure, retention index, and proposed fragmentation (unclear hydroxy positions are indicated by tildes) of xylazine and its metabolites are shown. The mass spectrum of xylazine

(Fig. 1, spectrum a) is characterized by the base peak at m/z 205 resulting from radical loss of a methyl group of the 2,6-dimethylaniline ring. Further radical fragmentations of the thiazine ring could explain the fragment ions at m/z 177 (loss of C_3H_7, 43 u), m/z 145 (loss of C_3H_7S, 75 u), and m/z 130
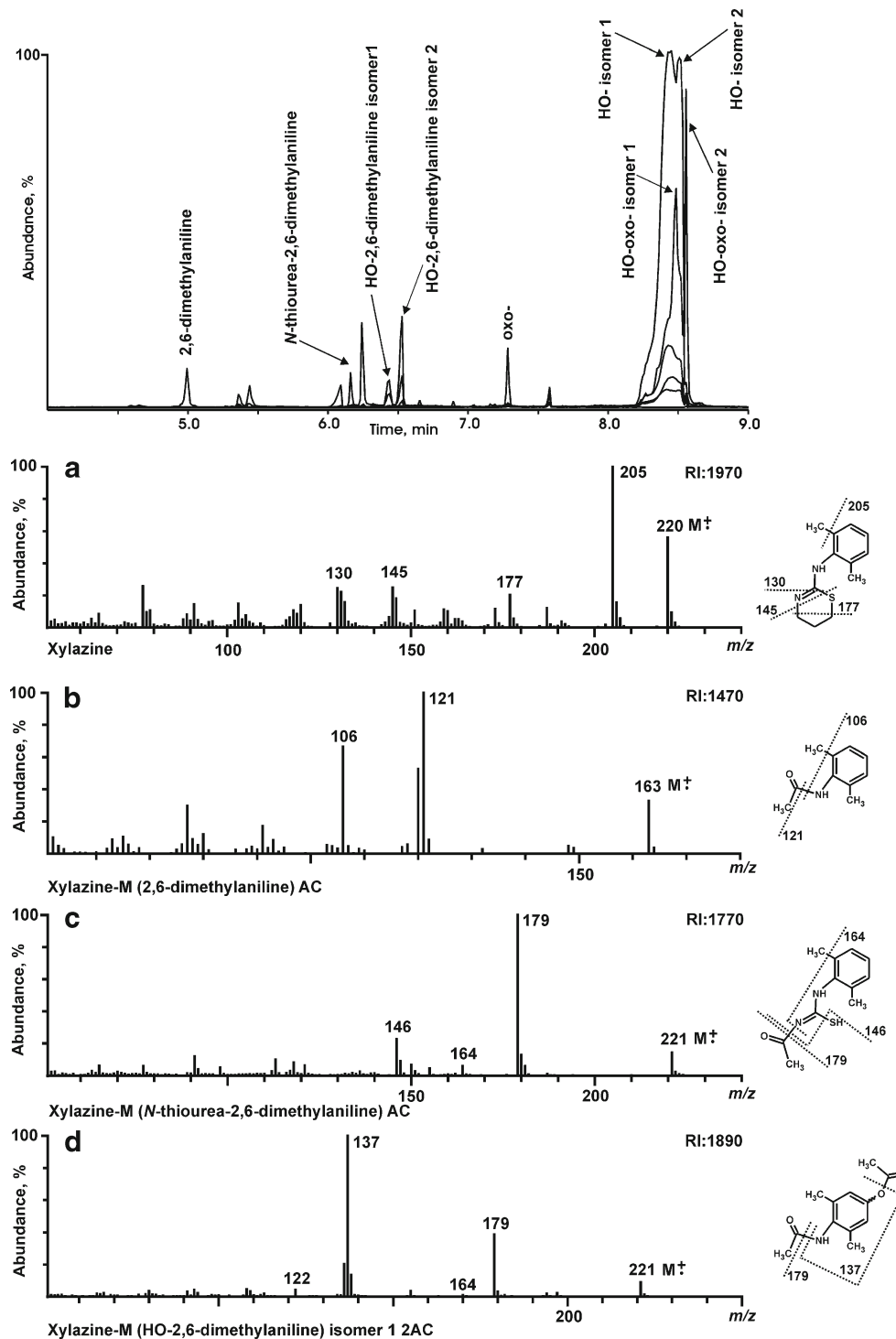

Fig. 1 Reconstructed mass chromatograms (*upper part*) of the ions at m/z 121, 179, 146, 162, and 221 indicating the underlying marked metabolite spectra b–j. Gas chromatography–mass spectrometry spectra (*lower part*) of xylazine and its phase I metabolites with the chemical structure, retention index (*RI*), and proposed fragmentation. Unclear hydroxy positions are indicated by *serrated lines*. *M* metabolite, *HO* hydroxy
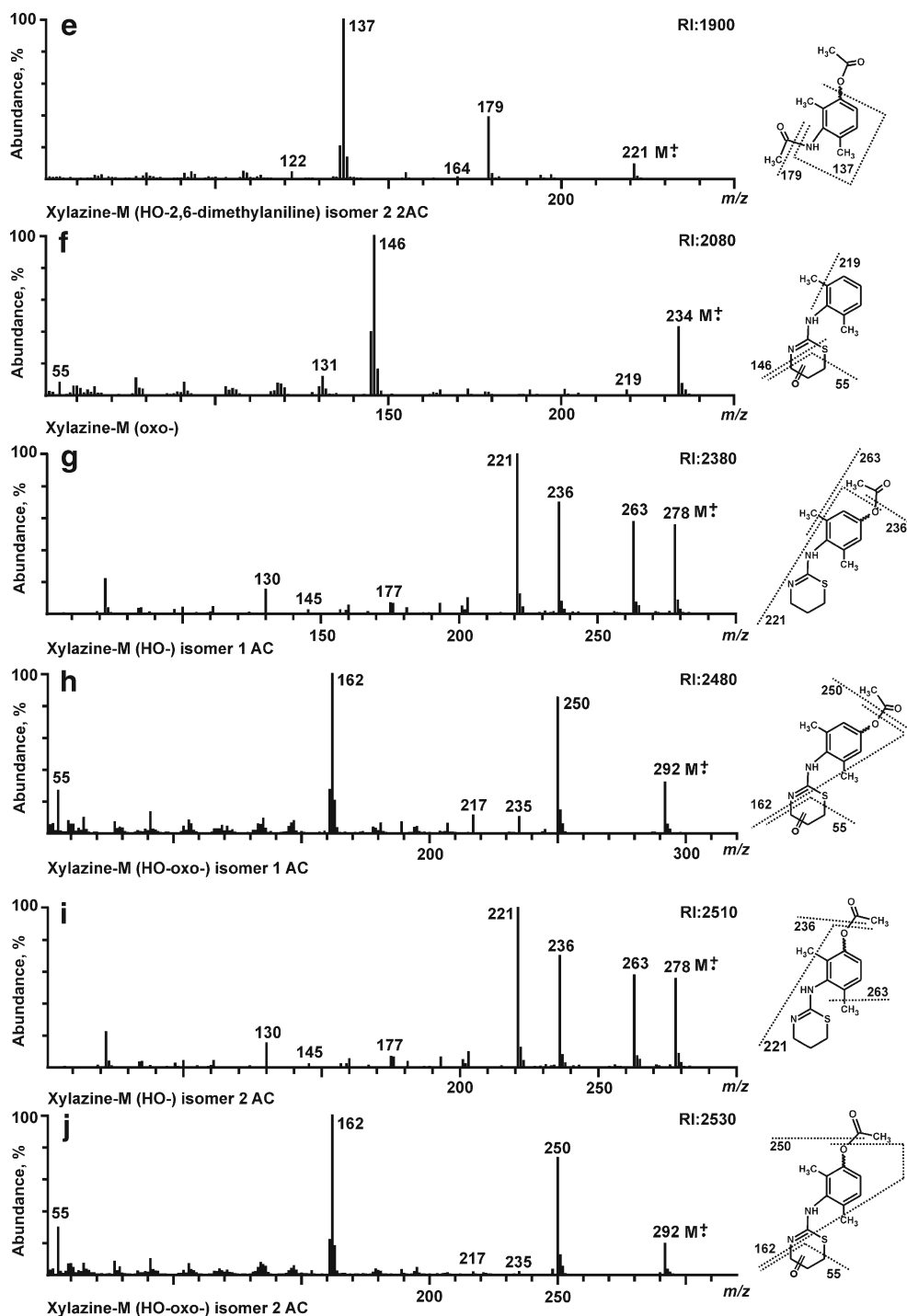
e RI:1900

137

179

122 164 221 M⁺

200 m/z

Xylazine-M (HO-2,6-dimethylaniline) isomer 2 2AC

f RI:2080

146

234 M⁺

55 131 219

150 200 m/z

Xylazine-M (oxo-)

g RI:2380

221

236

263 278 M⁺

130 145 177

150 200 250 m/z

Xylazine-M (HO-) isomer 1 AC

h RI:2480

162

250

292 M⁺

55 217 235

200 300 m/z

Xylazine-M (HO-oxo-) isomer 1 AC

i RI:2510

221

236

263 278 M⁺

130 145 177

200 250 m/z

Xylazine-M (HO-) isomer 2 AC

j RI:2530

162

250

292 M⁺

55 217 235

200 250 m/z

Xylazine-M (HO-oxo-) isomer 2 AC

Fig. 1 (continued)

(loss of C_3H_8NS, 130 u). For identification of metabolites, molecular ions and fragment ions, calculated according to the expected molecule modifications, including derivatization, were extracted from the total ion chromatogram. In the case of the acetylated hydroxyxylazine isomers (Fig. 1, spectra g, I), the molecular ion should be at m/z 278 (220+16+42). Typical fragmentations of these metabolites were primary loss of 42 u (C_2H_2O, acetyl) or 15 u (CH_3, methyl radical),

resulting in fragment ions at m/z 263 (278 - 15), m/z 236 (278 - 42), and m/z 221 (236 - 15). As the fragment ions characteristic of the thiazine ring (m/z 177, 145, and 130) were not changed in the spectra of the hydroxy metabolites, it could be concluded that hydroxylation occurred at the aromatic ring. The exact position of hydroxylation could not be determined, and thus the hydroxy group was connected with a tilde in the structure. The oxo-xylazine metabolite (Fig. 1,
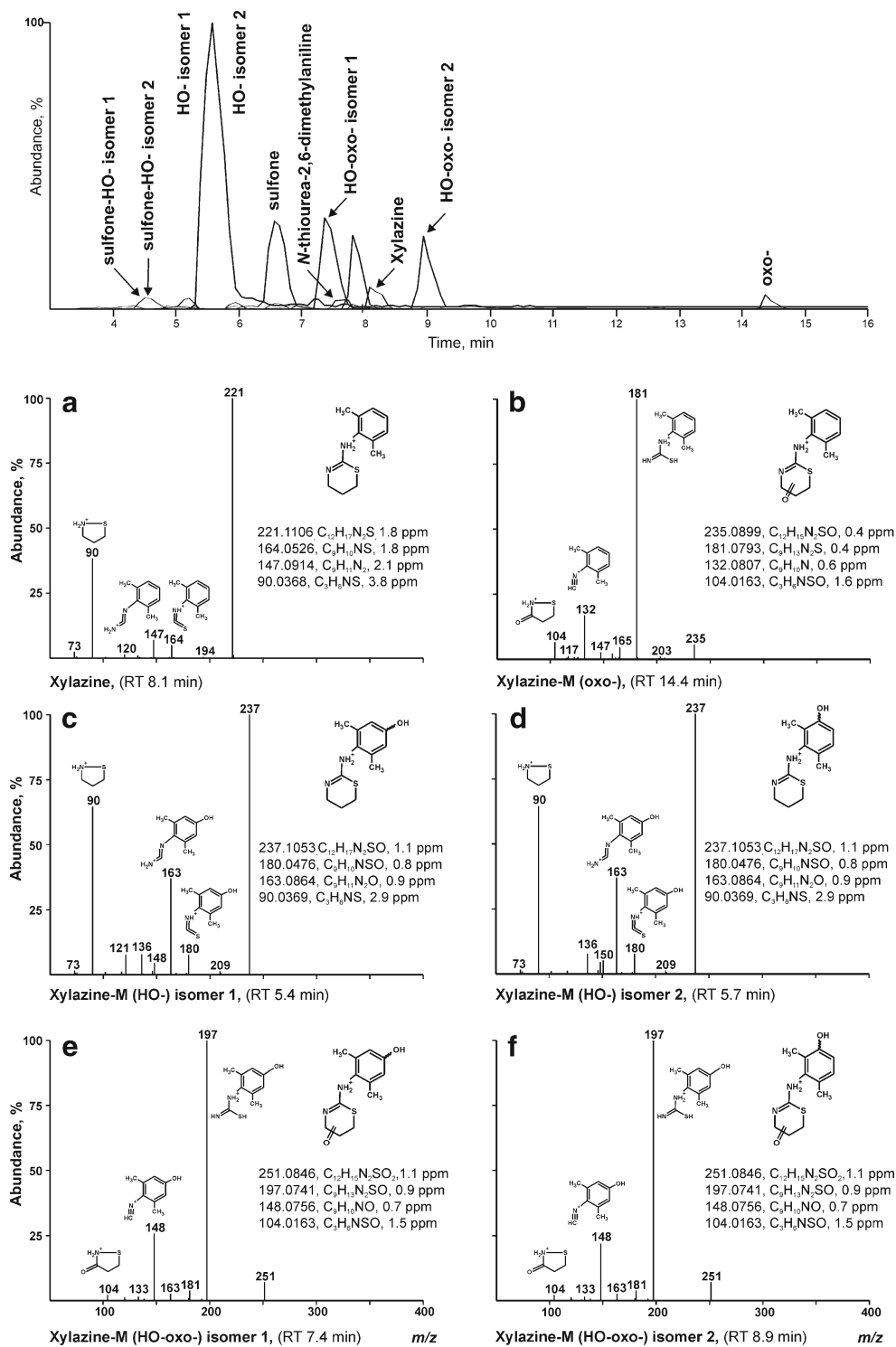
Fig. 2 Reconstructed mass chromatograms (*upper part*) of the ions at *m/z* 181.0795, 221.1106, 235.0899, 237.1053, 251.0846, 253.1005, and 269.0956 indicating the underlying marked metabolite spectra *a–j*. Liquid chromatography–high-resolution tandem mass spectrometry spectra (*lower part*) of xylazine and its phase I metabolites (for better readability, only the nominal masses are depicted in the spectra). Partial structures, measured accurate masses with error values, calculated elemental compositions, and retention times (*RT*) are given if relevant for interpretation. Unclear hydroxy positions indicated by *serrated lines*. M metabolite, HO hydroxy

spectrum f) could be detected in same matter: owing to radical loss of a methyl group from the molecular ion at *m/z* 234 (220+14), the fragment ion at *m/z* 219 occurred. As marked in Fig. 1 (spectrum f), cleavage of the thiazine ring led to the fragment ion at *m/z* 146 (base peak) and a characteristic fragment ion at *m/z* 55 (C₃H₃O) indicating the oxidation of

g Xylazine-M (*N*-thiourea-2,6-dimethylaniline), (RT 7.6 min)

181.0795, $C_9H_{13}N_2S$, 0.58 ppm
122.0964, $C_9H_{12}N$, 0.36 ppm

h Xylazine-M (sulfone), (RT 6.6 min)

253.1005, $C_{12}H_{17}N_2SO_2$, 1.4 ppm
181.0792, $C_9H_{13}N_2S$, 1.0 ppm
164.0527, $C_9H_{10}NS$, 1.1 ppm
147.0914, $C_9H_{11}N_2$, 0.1 ppm

i Xylazine-M (sulfone-HO-) isomer 1, (RT 4.5 min)   *m/z*

269.0956, $C_{12}H_{17}N_2SO_3$, 0.5 ppm
197.0741, $C_9H_{13}N_2SO$, 0.2 ppm
163.0865, $C_9H_{11}N_2O$, 0.1 ppm
146.0600, C_9H_8NO, 0.1 ppm

j Xylazine-M (sulfone-HO-) isomer 2, (RT 4.7 min)   *m/z*

269.0956, $C_{12}H_{17}N_2SO_3$, 0.5 ppm
197.0741, $C_9H_{13}N_2SO$, 0.6 ppm
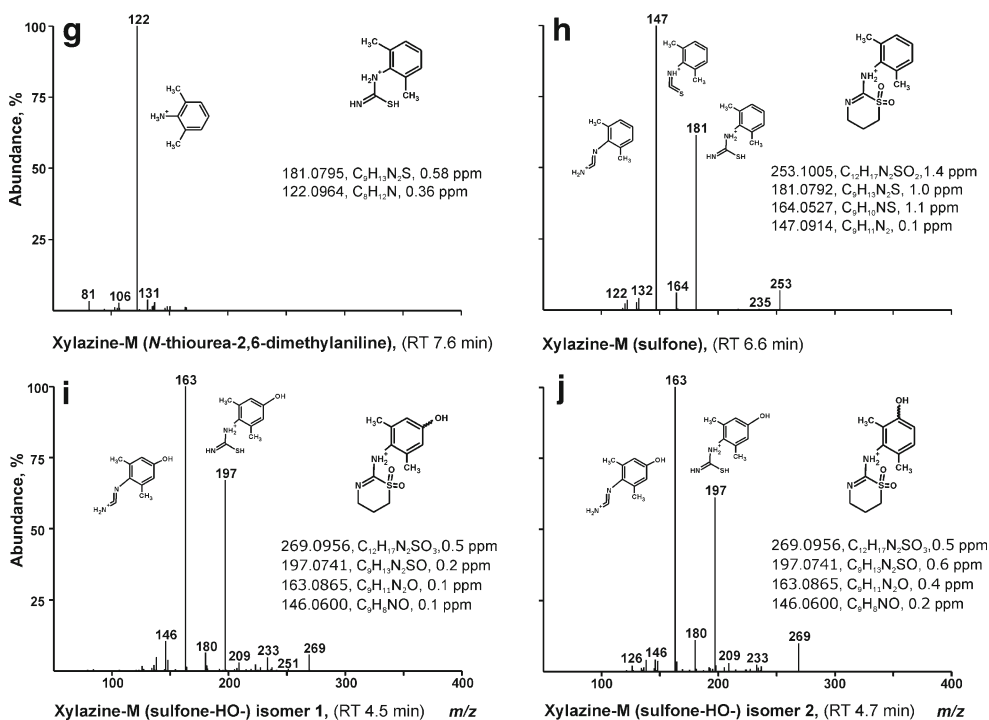163.0865, $C_9H_{11}N_2O$, 0.4 ppm
146.0600, C_9H_8NO, 0.2 ppm

Fig. 2 (continued)

the thiazine ring. The hydroxy-oxo-xylazine isomers (Fig. 1, spectra h, j) also showed a fragment ion at m/z 55 and a fragment ion at m/z 162 (146+16). The molecular ion was at m/z 292 (220+16+42+14) and loss of the acetyl groups and a methyl radical resulted in fragment ions at m/z 250 (292 - 42) and m/z 235 (250 - 15), respectively. The acetylated thiourea metabolite (Fig. 1, spectrum c) exhibited analogous fragmentation patterns: loss of acetyl groups led to a fragment ion at m/z 179 (221 – 42), followed by radical loss of a methyl group, leading to a fragment ion at m/z 164 (179 – 15), or cleavage of thiourea, leading to a fragment ion at m/z 146. By loss of the acetyl group, the acetylated metabolite 2,6-dimethylaniline (Fig. 1, spectrum b) led to a fragment ion at m/z 121 (163 – 42), followed by radical loss of a methyl group, leading to a fragment ion at m/z 106 (121 – 15). By loss of the two acetyl groups, the diacetylated hydroxy-2,6-dimethylaniline metabolite isomers (Fig. 1, spectra d, e) led to fragment ions at m/z 179 and 137, followed by radical loss of a methyl group, leading to fragment ions at m/z 164 and 122.

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

In the upper part of Fig. 2, reconstructed mass chromatograms are depicted of the ions at m/z 181.0795, 221.1106, 235.0899, 237.1053, 251.0846, 253.1005, and 269.0956 indicating the underlying marked metabolite spectra a–j. LC–HR-MS/MS spectra of xylazine and its phase I metabolites are shown in the lower part of Fig. 2. Partial structures, measured accurate

masses with calculated elemental compositions (error values in parts per million), and retention times are given if relevant for interpretation. Unclear hydroxy positions are indicated by tildes. The spectra were recorded in HR, providing the best structural information, but for better readability, only data for important ions are given. For most metabolites, no MS^3 spectra could be recorded because the molecules were too small for further fragmentation. The MS^2 spectrum of xylazine (Fig. 2, spectrum a) contains besides the protonated molecular ion at m/z 221.1106, three characteristic fragment ions at m/z 90.0368, 147.0914, and 164.0526 resulting from different cleavages of the thiazine ring. These fragment ions as well as the protonated molecular ion were changed according to the corresponding modifications of the molecule caused by the metabolizing enzymes (cytochromes P450, alcohol dehydrogenases, glucuronyltransferases, sulfotransferases, etc.). The spectrum with the protonated molecular ion of the proposed oxo metabolite (Fig. 2, spectrum b) at m/z 235.0899 (221.1106+15.9949 - 2.0156) shows the shifted fragment ion at m/z 104.0163 (90.0369 +15.9949 - 2.0156) and a less abundant unchanged fragment ion at m/z 147.0914. The xylazine fragment ion at m/z 164.0526 could not be observed, but additional fragment ions at m/z 132.0807 and 181.0793 were observed. This might be the result of a different fragmentation behavior of the changed thiazine ring. The spectra of the hydroxy metabolites (Fig. 2, spectra c, d) showed a protonated molecular ion at m/z 237.1053 (221.1106+ 15.9949) and an unchanged fragment ion at m/z 90.0369. The other fragment ions were changed to fragment ions at
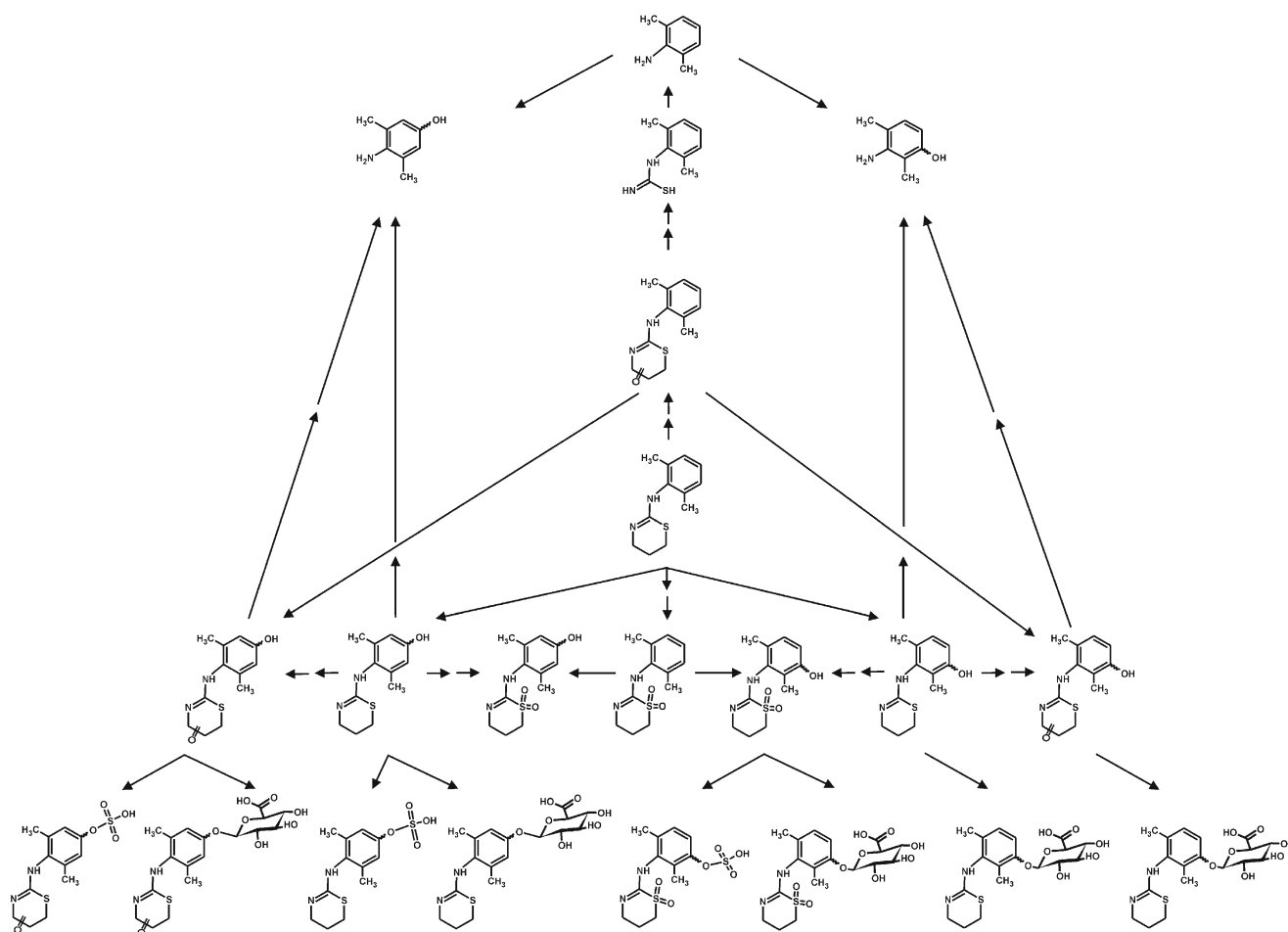
Fig. 3 Proposed metabolic pathways of xylazine in rats and humans (unclear hydroxy positions are indicated by *serrated lines*)

m/z 163.0864 (147.0914+15.9949) and m/z 180.0476 (164.0526+15.9949). The unchanged fragment ion at m/z 90.0369 indicated that hydroxylation occurred at the 2,6-dimethylaniline ring. The lack of loss of water from the protonated molecular ion (sample measured without wideband activation) further confirmed that neither the nitrogen nor the thiazine ring was oxidized. The oxohydroxy metabolite isomers (Fig. 2, spectra e, f) with a protonated molecular ion at m/z 251.0846 (221.1106+15.9949 - 2.0156+15.9949) formed a fragment ion at m/z 104.0163 (90.0369+15.9949 - 2.0156), indicating thiazine ring oxidation, as well as a fragment ions at m/z 148.0756 (132.0807+15.9949) and 197.0741 (181.0793+15.9949), indicating hydroxylation at the 2,6-dimethylaniline ring.

The N-dealkylated metabolites (2,6-dimethylaniline and the two hydroxy-2,6-dimethylaniline isomers) detected by GC–MS could not be detected by LC–HR-MS^n, most probably because of the small molecular size. The spectrum of N-thiourea-2,6-dimethylaniline (Fig. 2, spectrum g) with the precursor ion at m/z 181.0795 showed a fragment ion at m/z 122.0965 corresponding to 2,6-dimethylaniline. In addition to the metabolites detected by GC–MS, a further metabolite

(Fig. 2, spectrum h) with a protonated molecular ion at m/z 253.1005 (221.1106+15.9949 +15.9949) was detected, indicating a dihydroxy, sulfone, or, after opening of the thiazine ring, carboxy metabolite. Hydroxylation of the aromatic ring could be excluded owing to existence of fragment ions at m/z 147.0914 and 164.0526, markers of the unchanged 2,6-dimethylaniline ring, and at m/z 181.0793, indicating changes at the thiazine ring. Dihydroxylation at the thiazine ring could be excluded owing to the lack of water elimination. Oxidation to a carboxy compound could be excluded owing to the lack of a methyl ester after derivatization of the urine extracts by diazomethane. Consequently, the sulfone structure was proposed. In the same manner, two hydroxysulfone isomers could be identified (Fig. 2, spectra i, j) with a protonated molecular ion at m/z 269.0956 (221.1106+15.9949+15.9949+15.9949), indicating hydroxylation of the sulfone metabolite. Hereby, fragment ions at m/z 163.0865, 180.0474, and 197.0741 indicated hydroxylation of the 2,6-dimethylaniline ring in comparison with the sulfone metabolite (fragment ions at m/z 147.0914, 164.0527, and 181.0792, each + 15.9949).

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

The phase II metabolites were isolated using C_{18} SPE because the mixed-mode HCX SPE could not retain sufficiently the acidic glucuronides. They were identified by comparison of the MS^3 spectra of conjugated metabolites with the MS^2 spectra of corresponding phase I metabolites. Glucuronides of the two hydroxy isomers with a protonated molecular ion at m/z 413.1371, of the two oxohydroxy isomers with a protonated molecular ion at m/z 427.1170, and of one of the hydroxysulfone isomers with a protonated molecular ion at m/z 445.1265 were detected. The possible glucuronide of the other isomer was most probably not sufficiently abundant for detection. This might also be the reason why only one sulfate could be detected for the hydroxy isomers with a protonated molecular ion at m/z 317.062, the oxohydroxy isomers with a protonated molecular ion at m/z 331.0418, and the hydroxysulfone isomers with a protonated molecular ion at m/z 349.0522. Another reason could be that the isomeric conjugates could not be separated under the applied conditions LC and the variations of them tested.

Metabolic pathways in rats and humans

All metabolites identified in rat urine could also be detected in human urine with similar relative abundances. On the basis of these metabolite data, the following metabolic pathways are proposed (Fig. 3): N-dealkylation to 2,6-dimethylaniline, hydroxylation followed by dehydration to the oxothiazine metabolites, N,S-di-dealkylation to 2,6-dimethylphenylthiourea, S-oxidation to a sulfone, hydroxylation of the aromatic ring to two isomeric metabolites, followed by glucuronidation or sulfation. In addition, various combinations of these steps could occur.

These pathways, firstly described in this study for humans, are similar to those deduced from studies with rat urine [16], rat liver microsomes [14, 15], or horses urine [4, 14], with some differences. These could be caused by different species, body samples, or analytical techniques. In addition to the known pathways, the following were described for the first time: hydroxylation followed by dehydration to the oxothiazine metabolites in combination with hydroxylation of the aromatic ring to two isomeric metabolites, glucuronidation or sulfation of the phenolic metabolites, and S-oxidation to a sulfone. To exclude that the latter was formed artificially under the aerobic storage and workup conditions used, blank urine spiked with xylazine (0.2 mg/mL), left at ambient temperature for 12 h, was analyzed. As no sulfone could be detected, metabolic formation could be assumed. The N-oxide detected by Lavoie et al. [15] in rat liver microsomes could not be detected in rat and human urine. N-Acetyl conjugates, typical for aniline derivatives, could also not be detected either because of too low concentrations or because of steric hindrance of their formation by the vicinal methyl groups.

Table 1 Detectability of xylazine and its phase I metabolites in rat and human urine by gas chromatography–mass spectrometry (*GC–MS*) or liquid chromatography–multistage mass spectrometry (*LC–MS^n*) standard urine screening approaches

Species	Rat urine (low dose)		Human urine (overdose)	
	GC–MS	LC–MS^n	GC–MS	LC–MS^n
Xylazine	−	+	−	+
Xylazine-M (2,6-dimethylaniline)	+	−	+	−
Xylazine-M (N-thiourea-2,6-dimethylaniline)	+	+	+	+
Xylazine-M (HO-2,6-dimethylaniline isomer 1)	+	−	+	−
Xylazine-M (HO-2,6-dimethylaniline isomer 2)	+	−	+	−
Xylazine-M (oxo-)	+	+	+	+
Xylazine-M (HO-) isomer 1	++	++	++	++
Xylazine-M (HO-) isomer 1 glucuronide	−	++	−	++
Xylazine-M (HO-) isomer 2	++	++	++	++
Xylazine-M (HO-) isomer 2 glucuronide	−	++	−	++
Xylazine-M (HO-oxo-) isomer 1	+	+	+	+
Xylazine-M (HO-oxo-) isomer 1 glucuronide	−	+	−	+
Xylazine-M (HO-oxo-) isomer 2	+	+	+	+
Xylazine-M (HO-oxo-) isomer 2 glucuronide	−	+	−	+
Xylazine-M (sulfone)	−	++	−	++
Xylazine-M (sulfone-HO-) isomer 1	−	+	−	+
Xylazine-M (sulfone-HO-) isomer 2	−	+	−	+

M metabolite, *HO* hydroxy, − not detected, + detected, ++ main target

Toxicological detection by GC–MS and LC–MS^n

In contrast to the metabolism studies, routine screening approaches are based on a fast sample workup, namely, acid hydrolysis, in contrast to time-consuming but gentle enzymatic hydrolysis or liquid–liquid extration or protein precipitation versus SPE. The results of the detectability testing are summarized in Table 1. With use of the authors' GC–MS standard urine screening approaches, an intake of xylazine could be detected in rat urine at least after a 1 mg/kg body mass dose. This dose corresponded to that described in a case report in which the patient showed disorientation [27]. This extrapolation was necessary because the human urine samples tested came from cases in which the applied dose was not documented. Toxicological detection should focus on the acetylated hydroxy isomers being the most abundant metabolites the rat and human urine. 2,6-Dimethylaniline and the corresponding hydroxy isomers are also metabolites of lidocaine and therefore are not selective for monitoring xylazine administration [17, 28]. With use of the authors' LC–MS^n standard urine screening approaches, administration of a 1 mg/kg body mass dose could also be monitored. The main targets in rat and human urine were also the hydroxy isomers and in addition their glucuronides and the sulfone metabolite. Low-resolution LC–MS^n was sufficient for the screening approach because it produced the same fragments as the HR device, so the reference spectra recorded during the metabolism study could be used for the screening and confirmation process.

Conclusions

The study presented has shown that xylazine was extensively metabolized by hydroxylation, dealkylation, C-oxidation, S-oxidation, and their combinations. All 20 phase I and phase II metabolites could be identified in rat and human urine. This helped us study the detectability of xylazine administration because the metabolites were the main targets in urine.

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