Forensic Toxicol
DOI 10.1007/s11419-015-0274-9

ORIGINAL ARTICLE

Urinary excretion and metabolism of the α-pyrrolidinophenone designer drug 1-phenyl-2-(pyrrolidin-1-yl)octan-1-one (PV9) in humans

Noriaki Shima[1] · Hidenao Kakehashi[1] · Shuntaro Matsuta[1] · Hiroe Kamata[1] · Shihoko Nakano[1] · Keiko Sasaki[1] · Tooru Kamata[1] · Hiroshi Nishioka[1] · Kei Zaitsu[2] · Takako Sato[3] · Akihiro Miki[1] · Munehiro Katagi[1] · Hitoshi Tsuchihashi[3]

Received: 30 January 2015 / Accepted: 20 March 2015

Abstract 1-Phenyl-2-(pyrrolidin-1-yl)octan-1-one (PV9) and 16 metabolites, including diastereomers and conjugates, were identified or tentatively detected in human urine by gas chromatography–mass spectrometry and liquid chromatography–high-resolution tandem mass spectrometry. These urinary metabolites indicated that the metabolic pathways of PV9 include: (1) the reduction of ketone groups to their corresponding alcohols; (2) oxidation of the pyrrolidine ring to the corresponding pyrrolidone; (3) aliphatic oxidation of the terminal carbon atom to the corresponding carboxylate form, possibly through an alcohol intermediate (not detected); and (4) hydroxylation at the penultimate carbon atom to the corresponding alcohols followed by further oxidation to ketones, and combinations of these steps. In addition, results from the quantitative analyses of five phase-I metabolites using newly synthesized authentic standards suggested that the main metabolic pathway includes the aliphatic oxidation of terminal and/or penultimate carbons. Human metabolism of PV9 differed significantly from those of α-pyrrolidinovalerophenone and α-pyrrolidinobutiophenone, suggesting that the main metabolic pathways of α-pyrrolidinophenones significantly change depending on the alkyl chain length of the parent molecule.

✉ Noriaki Shima
shima6499@hotmail.co.jp

[1] Forensic Science Laboratory, Osaka Prefectural Police Headquarters, 1-3-18 Hommachi, Chuo-ku, Osaka 541-0053, Japan

[2] Department of Legal Medicine and Bioethics, Nagoya University Graduate School of Medicine, Nagoya, Japan

[3] Division of Preventive and Social Medicine, Department of Legal Medicine, Osaka Medical College, Takatsuki, Japan

Keywords PV9 · Cathinone derivative · Metabolism · Urine · α-POP · LC–MS-MS

Introduction

Various cathinone-derived designer drugs have recently appeared on the drug market in many countries, including Japan [1–5]. In particular, α-pyrrolidinophenones, including 3,4-methylenedioxypyrovalerone (MDPV) [6, 7], α-pyrrolidinovalerophenone (α-PVP) [8–13], α-pyrrolidinobutiophenone (α-PBP) [13–15], 4-methyl-α-pyrrolidinohexanophenone (MPHP) [16, 17], and 3,4-methylenedioxy-α-pyrrolidinobutiophenone (MDPBP) [18], have rapidly become a popular class of designer drugs. 1-Phenyl-2-(pyrrolidin-1-yl)octan-1-one (PV9) is an α-PVP analog in which the alkyl chain is elongated up to eight carbons, also known as α-pyrrolidinooctanophenone (α-POP). PV9 recently appeared as one of the alternatives to various controlled cathinones such as α-PVP and MDPV, and a fatal case of acute intoxication with this substance has recently been reported [19, 20].

Synthetic cathinones can be obtained through Internet websites and local dealers, and are often sold as "fragrance powder," "aroma liquid," or "herbal incense." They are most commonly taken by oral ingestion, intravenous injection, or smoking. A limited understanding of the mechanisms of drug action has been derived, mostly from in vitro studies and animal models [21–26]. MDPV and α-PVP are selective inhibitors of presynaptic norepinephrine and dopamine reuptake transporters, and show little effect on serotonin transporters, resulting in overall stimulation of the central nervous system [23, 24]. The pharmacological effects include increased energy, empathy, openness, and libido. These factors contribute to a serious drug addiction,

Published online: 08 April 2015

and can lead to crimes including murder, robbery, suicide, and traffic accidents. To regulate the abuse of these substances and/or determine the cause of death or poisoning in overdose cases, information about the metabolic pathways and metabolite analyses are indispensable for forensic science and toxicology. However, there is little data on long-alkyl-chained α-pyrrolidinophenones such as PV9 [19], although several reports have explored the metabolism of various cathinones and detection of the resulting metabolites in humans and rats [7–11, 22, 27–29].

In the present study, PV9 and its urinary metabolites were deduced from the mass spectra and relative retention times utilizing gas chromatography–electron ionization-mass spectrometry (GC–EI-MS) and liquid chromatography–high-resolution tandem mass spectrometry (LC–HR-MS-MS). The key metabolites for estimating metabolic pathways of PV9 were identified and quantified using authentic standards synthesized in our laboratory. Based on these results, the metabolism of PV9 in humans is discussed and compared to the metabolic pathways of α-PVP and α-PBP. In addition, the effectiveness of the hydrolysis of conjugate metabolites was verified. These analytical studies are of great importance in both forensic toxicological evaluations and drug control enforcement.

Materials and methods

Reagents

Ten compounds (M1–M10), as shown in Fig. 1, were tested as phase I metabolite candidates in this study. PV9 and the eight metabolites except M5 and M7 were synthesized in our laboratory, according to the methods described below. M1, M2, M4, M6, M8 and M9 were confirmed by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and liquid chromatography–high-resolution mass spectrometry (LC–HR-MS). M3 and M10 were confirmed only by LC–HR-MS, because they were simply synthesized by reduction reactions from M2 and M9, respectively. PV9, M1, M4, M6 and M9 utilized for quantitative analyses were ensured as > 98 % pure, based on LC–MS analysis by the direct flow injection method and ^{1}H NMR spectroscopy. Purifications by the flash column of the synthesized standards were performed on Flash Purification System Isolera™ Prime (Biotage, Uppsala, Sweden) attached by a silica gel cartridge, with ethyl acetate/*n*-hexane mixture as eluent. Dibenzylamine (DBA), used as an internal standard (IS), was obtained from Wako Pure Chemical (Osaka, Japan). Standard stock solutions of all compounds were prepared in methanol and adjusted to appropriate concentrations with distilled water immediately prior to use. β-Glucuronidase/sulfatase (*Helix pomatia*, Type H-1) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents of analytical grade were obtained from Wako Pure Chemical or Tokyo Chemical Industry (Tokyo, Japan). Distilled water and LC/MS-grade methanol were used throughout the experiments.

Chemical syntheses

α-Pyrrolidinovalerophenone (PV9)

To a solution of benzonitrile in anhydrous tetrahydrofuran (THF), ethyl magnesium bromide in THF was added dropwise with stirring and cooling in an external ice bath over 5 min. The mixture was stirred for an additional 1 h at room temperature. The reaction was quenched by the addition of saturated ammonium chloride solution, and the mixture was extracted with ethyl acetate. The organic extract was washed with brine, dried over anhydrous sodium sulfate (Na_2SO_4), and evaporated to obtain crude 1-phenyloctan-1-ol. To a solution of 1-phenyloctan-1-ol in a solvent mixture of *n*-hexane/diethyl ether (1:1, v/v), activated manganese dioxide (MnO_2) was added portionwise, and the mixture was stirred at room temperature for 36 h. The solids were removed by filtration, and the organic extract was concentrated under vacuum to give octanophenone. To a solution of octanophenone in dichloromethane, one drop of bromine in dichloromethane was added. This solution was stirred for 5 min for the reaction to initiate. An equimolar amount of bromine solution was added over a period of a further 10 min. The solvent was removed under vacuum to yield 2-bromooctanophenone. Pyrrolidine in THF was added dropwise to a solution of 2-bromooctanophenone in THF. The mixture was stirred at room temperature overnight. The reaction mixture was treated with 10 % HCl aqueous solution to make it acidic and was then washed with diethyl ether. The aqueous layer was made basic with 10 % sodium carbonate and extracted with ethyl acetate. The organic extract was washed with brine, dried over anhydrous Na_2SO_4, and evaporated to give crude PV9 as pale yellow oil. Finally, 10 % HCl methanol solution was added dropwise to the oil. After removal of the solvent, the crude hydrochloride salt was purified by recrystallization from diethyl ether/2-propanol.

1-Phenyl-2-(pyrrolidin-1-yl)octan-1-ol (M1)

To a solution of PV9 in ethanol at 60 °C, sodium tetrahydroborate ($NaBH_4$) was added, and the mixture was stirred at 60 °C for 30 min. It was evaporated under vacuum, and the residue was treated with 10 % HCl aqueous solution to make it acidic before it was washed

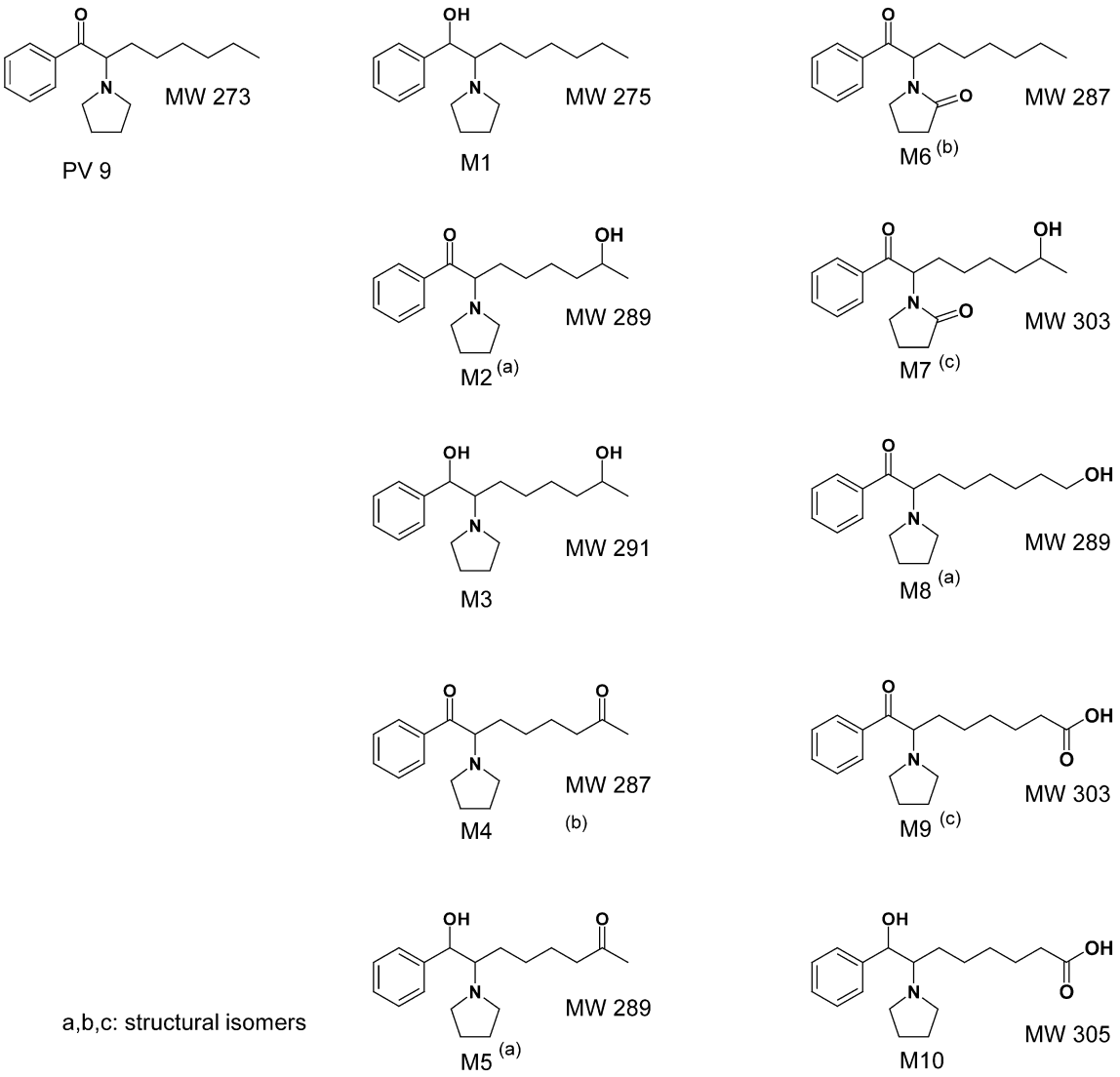


Fig. 1 PV9 and putative phase I metabolites. *MW* molecular weight

with diethyl ether. The aqueous layer was then made basic with 10 % sodium carbonate and extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and evaporated to give diastereomeric M1 [referred to M1-diastreomer1 (M1-D1) and M1-diastreomer2 (M1-D2), as defined below] as pale yellow oil. The product ratio of the diastereomers was determined by NMR spectroscopy.

^{1}H NMR (400 MHz, $CDCl_3$; the compound existed as a mixture of diastereomers, M1-D1 denoted by § and M1-D2 denoted by *): δ 7.39–7.29 (m, 4H*, 4H§), 7.29–7.20 (m, 1H*, 1H§), 5.02 (d, J = 4.0 Hz, 1H*), 4.18 (d, J = 9.6 Hz, 1H§), 2.84–2.65 (m, 4H*, 5H§), 2.56 (m, 1H*), 1.84–1.76 (m, 4H*, 4H§), 1.52–0.95 (m, 10H*, 10H§), 0.81 (t, J = 7.0 Hz, 3H*), 0.80 (t, J = 7.0 Hz, 3H§); HR-MS: cacld for $[M+H]^+$ 276.2322, found 276.2321 (M1-D1) and 276.2322 (M1-D2).

7-Hydroxy-1-phenyl-2-(pyrrolidin-1-yl)octan-1-one (M2)

To a solution of 6-bromo-1-hexanol in dimethylformamide (DMF) stirred at room temperature, *tert*-butyldimethylchlorosilane (TBSCl) was added, followed by an addition of imidazole. The mixture was stirred at room temperature for 3 h. The reaction was quenched by the addition of saturated ammonium chloride aqueous solution, and the mixture was extracted with ethyl acetate. The organic extract was washed with water and brine, dried over anhydrous Na_2SO_4, and evaporated to remove solvent. The residue was purified by the above flash column to yield

6-bromohexyloxytrimethylsilane. To a solution of 6-bromohexyloxytrimethylsilane in anhydrous THF, an equimolar amount of magnesium turnings was added. The mixture was refluxed for 1 h, and then cooled to room temperature and an equimolar amount of benzonitrile was added dropwise over 5 min. The mixture was refluxed for an additional 2 h. The reaction was quenched by the addition of pre-cooled saturated ammonium chloride aqueous solution, and the mixture was extracted with ethyl acetate. The organic extract was washed with water and brine, dried over anhydrous Na_2SO_4, and evaporated to remove solvent. The residue was purified by the flash column to yield 1-phenyl-7-trimethylsilyloxyheptan-1-one. To a solution of 1-phenyl-7-trimethylsilyloxyheptan-1-one in dichloromethane, one drop of bromine in dichloromethane was added for initiation of the reaction. After this mixture was stirred for 5 min, an equimolar amount of bromine solution was added over 10 min. The solvent was removed under vacuum to yield 2-bromo-1-phenyl-7-trimethylsilyloxyheptan-1-one. To a solution of 2-bromo-1-phenyl-7-trimethylsilyloxyheptan-1-one in THF, pyrrolidine was added dropwise. The mixture was stirred at room temperature overnight. The reaction mixture was treated with 10 % HCl aqueous solution to make it acidic, and was washed with diethyl ether. The aqueous layer was then made basic with 10 % sodium carbonate and extracted with ethyl acetate. The organic extract was washed with brine, dried over anhydrous Na_2SO_4, and evaporated to remove solvent. The residue was purified by the flash column to yield 7-hydroxy-1-phenyl-2-(pyrrolidin-1-yl)heptan-1-one.

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.10 (d, $J = 7.4$ Hz, 2H), 7.56 (t, $J = 7.4$ Hz, 1H), 7.46 (t, $J = 7.4$ Hz, 2H), 3.93 (dd, $J = 4.1$, 9.8 Hz, 1H), 3.57 (t, $J = 6.4$ Hz, 2H), 2.67–2.57 (m, 4H), 1.99–1.89 (m, 1H), 1.76 (m, 6H), 1.53–1.46 (m, 2H), 1.36–1.22 (m, 4H).

To a solution of 7-hydroxy-1-phenyl-2-(pyrrolidin-1-yl)heptan-1-one in dichloromethane, Dess-Martin periodinane (DMP) was added. The mixture was stirred at room temperature for 1 h. The reaction mixture was treated with saturated sodium carbonate aqueous solution and saturated sodium thiosulfate aqueous solution. The organic extract was washed with brine, dried over anhydrous Na_2SO_4, and evaporated to remove solvent. The residue was purified by the flash column to yield 7-oxo-7-phenyl-6-(pyrrolidin-1-yl)heptanal.

^{1}H NMR (400 MHz, $CDCl_3$): δ 9.71 (s, 1H), 8.09 (d, $J = 7.4$, 2H), 7.56 (t, $J = 7.4$, 1H), 7.46 (t, $J = 7.4$, 2H), 3.93 (dd, $J = 4.1$, 8.7 Hz, 1H), 2.67–2.56 (m, 4H), 2.38 (t, $J = 7.3$ Hz, 2H), 2.00–1.90 (m, 1H), 1.76 (br, 5H), 1.63–1.54 (m, 2H), 1.32–1.24 (pent, $J = 7.8$, 2H).

To a solution of 7-oxo-7-phenyl-6-(pyrrolidin-1-yl)heptanal in THF at 0 °C, methyl magnesium bromide in THF was added dropwise. The mixture was stirred at 0 °C for 5 min. The reaction mixture was warmed up to room temperature and treated with 10 % HCl aqueous solution to make it acidic, and was then washed with diethyl ether. The aqueous layer was then made basic with 10 % sodium carbonate and extracted with ethyl acetate. The organic extract was washed with brine, dried over anhydrous Na_2SO_4, and evaporated to remove solvent. The residue was purified by the flash column to yield 7-hydroxy-1-phenyl-2-(pyrrolidin-1-yl)octan-1-one (M2) as pale yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.09–8.01 (m, 2H), 7.52 (t, $J = 7.3$, 1H), 7.48–7.41 (m, 2H), 3.89–3.69 (m, 2H), 2.62–2.55 (m, 4H), 2.01–1.82 (br, OH), 1.72 (m, 6H), 1.69–1.36 (m, 4H), 1.29–1.11 (m, 5H); HR-MS: cacld for $[M+H]^+$ 290.2115, found 290.2118.

1-Phenyl-2-(pyrrolidin-1-yl)octan-1,7-diol (M3)

To a solution of M2 in ethanol at 60 °C, $NaBH_4$ was added, and the mixture was stirred at 60 °C for 30 min. The reaction mixture was evaporated under vacuum, and the residue was treated with 10 % HCl aqueous solution to make it acidic before it was washed with diethyl ether. The aqueous layer was then made basic with 10 % sodium carbonate and extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and evaporated to give diastereomeric M3 (M3-D1 through -D4) as pale yellow oil.

HR-MS: cacld for $[M+H]^+$ 292.2271, found 292.2275 (M3-D1 through -D4).

1-Phenyl-2-(pyrrolidin-1-yl)octan-1,7-dione (M4)

To a solution of M2 in dichloromethane, DMP was added. The mixture was stirred at room temperature for 1 h. The reaction mixture was treated with saturated sodium carbonate aqueous solution and saturated sodium thiosulfate aqueous solution. The organic extract was washed with brine, dried over anhydrous Na_2SO_4, and evaporated to remove solvent. The residue was purified by the flash column to yield M4 as pale yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.10 (d, $J = 7.3$ Hz, 2H), 7.57 (t, $J = 7.3$ Hz, 1H), 7.47 (t, $J = 7.3$ Hz, 2H), 4.00 (dd, $J = 4.6$, 8.7 Hz, 1H), 2.67–2.56 (m, 4H), 2.37 (t, $J = 7.3$ Hz, 2H), 2.09 (s, 3H), 1.95–1.93 (m, 1H), 1.78 (m, 5H), 1.57–1.50 (m, 2H), 1.29–1.24 (m, 2H); HR-MS: cacld for $[M+H]^+$ 288.1958, found 288.1960.

1-Phenyl-2-(2-oxopyrrolidin-1-yl)octan-1-one (M6)

To a solution of 2-bromooctanophenone, an intermediate for the synthesis of PV9, in THF at 0 °C, a suspension of 2-pyrrolidinone and sodium hydride (60 %, dispersion in paraffin liquid) in THF was added dropwise. The mixture

was stirred at 0 °C for 1 h. The reaction mixture was added to water and extracted with ethyl acetate. The organic extract was washed with brine, dried over anhydrous Na_2SO_4, and evaporated under vacuum. The resultant residue was purified by the flash column to give M6 as pale yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.05 (d, $J = 7.6$ Hz, 2H), 7.58 (t, $J = 7.6$ Hz, 1H), 7.47 (t, $J = 7.6$ Hz, 2H), 5.64 (dd, $J = 5.6$, 9.6 Hz, 1H), 3.36 (ddd, $J = 6.0$, 8.0, 9.6 Hz, 1H), 3.26 (ddd, $J = 5.2$, 8.0, 9.6 Hz, 1H), 2.43 (ddd, $J = 7.6$, 9.2, 17 Hz, 1H), 2.33 (ddd, $J = 5.4$, 9.2, 17 Hz, 1H), 2.05–1.84 (m, 3H), 1.79–1.68 (m, 1H), 1.38–1.21 (m, 8H), 0.87 (t, $J = 6.6$ Hz, 3H); HR-MS: cacld for $[M+H]^+$ 288.1958, found 288.1960.

8-Hydroxy-1-phenyl-2-(pyrrolidin-1-yl)octan-1-one (M8)

A solution of 7-bromo-1-heptanol in DMF was stirred at room temperature, and mixed with TBSCl and then with imidazole. The mixture was stirred at room temperature for 3 h. The reaction was quenched by the addition of saturated ammonium chloride aqueous solution, and the mixture was extracted with ethyl acetate. The organic extract was washed with water and brine, dried over anhydrous Na_2SO_4, and evaporated to remove solvent. The residue was purified by the flash column to yield 7-bromoheptanoxytrimethylsilane. To a solution of 7-bromoheptanoxytrimethylsilane in anhydrous THF, an equimolar amount of magnesium turnings was added. The mixture was refluxed for 2 h, and then cooled to room temperature and an equimolar amount of benzonitrile was added dropwise over 5 min. The mixture was refluxed for an additional 2 h. The reaction was quenched by the addition of pre-cooled saturated ammonium chloride aqueous solution, and the mixture was extracted with ethyl acetate. The organic extract was washed with water and brine, dried over anhydrous Na_2SO_4, and evaporated to remove solvent. The residue was purified by the flash column to yield 1-phenyl-8-trimethylsilyloxyoctan-1-one. To a solution of 1-phenyl-8-trimethylsilyloxyoctan-1-one in dichloromethane, one drop of bromine in dichloromethane was added because of initiation of the reaction. After this mixture was stirred for 5 min, an equimolar amount of bromine solution was added over 10 min. The solvent was removed under vacuum to yield 2-bromo-1-phenyl-8-trimethylsilyloxyoctan-1-one. To a solution of 2-bromo-1-phenyl-8-trimethylsilyloxyoctan-1-one in THF, pyrrolidine was added dropwise. The mixture was stirred at room temperature overnight. The reaction mixture was treated with 10 % HCl aqueous solution to make it acidic and then washed with diethyl ether. The aqueous layer was then made basic with 10 % sodium carbonate and extracted with ethyl acetate. The organic extract was washed with brine, dried over anhydrous Na_2SO_4, and evaporated to remove solvent. The residue was purified by the flash column to yield M8 as pale yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.13–8.08 (m, 2H), 7.59–7.53 (m, 1H), 7.48–7.43 (m, 2H), 3.93 (dd, $J = 4.4$, 8.8 Hz, 1H), 3.58 (t, $J = 8.6$ Hz, 2H), 2.73–2.64 (m, 2H), 2.64–2.55 (m, 2H), 1.98–1.81 (m, 2H and br, OH), 1.81–1.71 (m, 4H) 1.54–1.44 (m, 2H), 1.32–1.20 (m, 6H); HR-MS: cacld for $[M+H]^+$ 290.2115, found 290.2115.

8-Oxo-8-phenyl-7-(pyrrolidin-1-yl)octanoic acid (M9)

To a solution of M8 in acetone at 0 °C, chromium(VI) oxide in 20 % sulfuric acid aqueous solution was added, and the mixture was stirred at room temparature for 1 h. The reaction mixture was mixed with water and extracted with ethyl acetate. The organic extract was washed with 5 % sodium bicarbonate and brine, respectively, dried over anhydrous Na_2SO_4, and evaporated under vacuum. The resultant residue was purified by preparative thin-layer chromatography on silicagel plates with chloroform–methanol mixture to give M9 as pale yellow oil.

^{1}H NMR (400 MHz, CD_3OD): δ 8.07 (d, $J = 8.0$ Hz, 2H), 7.72 (t, $J = 8.0$ Hz, 1H), 7.59 (t, $J = 8.0$ Hz, 2H), 4.98–4.90 (m, 1H), 3.29–3.11 (m, 4H), 2.11 (t, $J = 7.2$, 2H), 2.09–1.94 (m, 6H), 1.49 (quin, $J = 7.2$ Hz, 2H), 1.34–1.21 (m, 4H); HR-MS: cacld for $[M+H]^+$ 304.1907, found 304.1910.

8-Hydroxy-8-phenyl-7-(pyrrolidin-1-yl)octanoic acid (M10)

To a solution of M9 in ethanol at 60 °C, $NaBH_4$ was added, and the mixture was stirred at 60 °C for 1 h. The reaction mixture was mixed with saturated ammonium chloride aqueous solution and extracted with ethyl acetate. The organic extract was washed with 5 % sodium bicarbonate and brine respectively, dried over anhydrous Na_2SO_4, and evaporated under vacuum to give diastereomic M10 (M10-D1 and M10-D2).

HR-MS: cacld for $[M+H]^+$ 306.2064, found 306.2066 (M10-D1) and 306.2065 (M10-D2).

Urine specimens

Urine specimens obtained from PV9 users were submitted to our laboratory for forensic analysis. The specimens were collected from two abusers, and were stored at − 20 °C until analysis. Drug-free urine specimens used for validation experiments were obtained from healthy volunteers.

Table 1 Targeted compounds and their selected reaction monitoring parameters for quatitative analysis

Compound	Q1 (*m/z*)	Q3 (*m/z*)	Time (ms)	DP (V)	EP (V)	CEP (V)	CE (eV)	CXP (V)
PV9	274.2	91.1	30	42	6	15	34	3
M1-D1 and D2	276.2	258.2	30	35	6	15	20	3
M4	288.2	182.2	30	42	6	15	35	3
M6	288.2	91.1	30	42	6	15	35	3
M9	304.2	91.1	30	42	6	15	35	3
IS (Dibenzylamine)	198.1	91.1	30	31	7	12	27	4

Q1 quadrupole 1, *Q3* quadrupole 3, *DP* declustering potential, *EP* entrance potential, *CEP* collision cell entrance potential, *CE* collision energy, *CXP* collision cell exit potential

Sample preparation for GC–MS

A 100-μl aliquot of a urine sample was adjusted to pH 4 or 9 with a diluted hydrochloric acid (0.6 M HCl) or carbonate buffer (1 M, pH 9.5), respectively. To the solution, 200 μl of a chloroform/2-propanol (3:1, v/v) was added and the mixture was vortex-mixed for 1 min. After centrifuging at 7000 *g* for 10 min, the organic layer was transferred to a stoppered glass test tube. After addition of 50 μl of acetic acid, it was evaporated to dryness under a nitrogen stream at 40 °C. The residue (extracted at pH 9) was reconstituted in 100 μl of chloroform/2-propanol (3:1, v/v), and a 1-μl aliquot of the solution was injected into the GC–MS system. Another residue (extracted at pH 4) was trimethylsilylated at 70 °C for 30 min after reconstitution in 100 μl of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), and a 1-μl aliquot of the reaction mixture was injected into the GC–MS system.

Sample preparation for LC–MS-MS

A 1-ml aliquot of urine was adjusted to pH 5 with 1 M acetic acid and incubated at 37 °C for 6 h with *β*–glucuronidase/sulfatase (15,000/750 U/ml urine, respectively). To aliquots of pre-hydrolysis urine and post-hydrolysis urine (100 μl each), 100 μl of DBA aqueous solution (0.2 μg/ml) was added as IS. After mixing briefly, 600 μl of methanol was added to each solution, and each mixture was vortex-mixed for 1 min, and centrifuged at 7000 *g* for 10 min. Each supernatant fraction was transferred to a stoppered glass test tube and evaporated to dryness under a nitrogen stream at 50 °C. The residue was dissolved in 100 μl of distilled water. After centrifuging at 7000 *g* for 10 min, a 5-μl aliquot of the supernatant was used for the LC–MS-MS analysis.

NMR spectroscopy

All NMR spectra of the synthetic compounds were acquired on a JNM-ECS 400 FT NMR system (JEOL Resonance, Akishima, Japan) in $CDCl_3$ using tetramethylsilane as the IS.

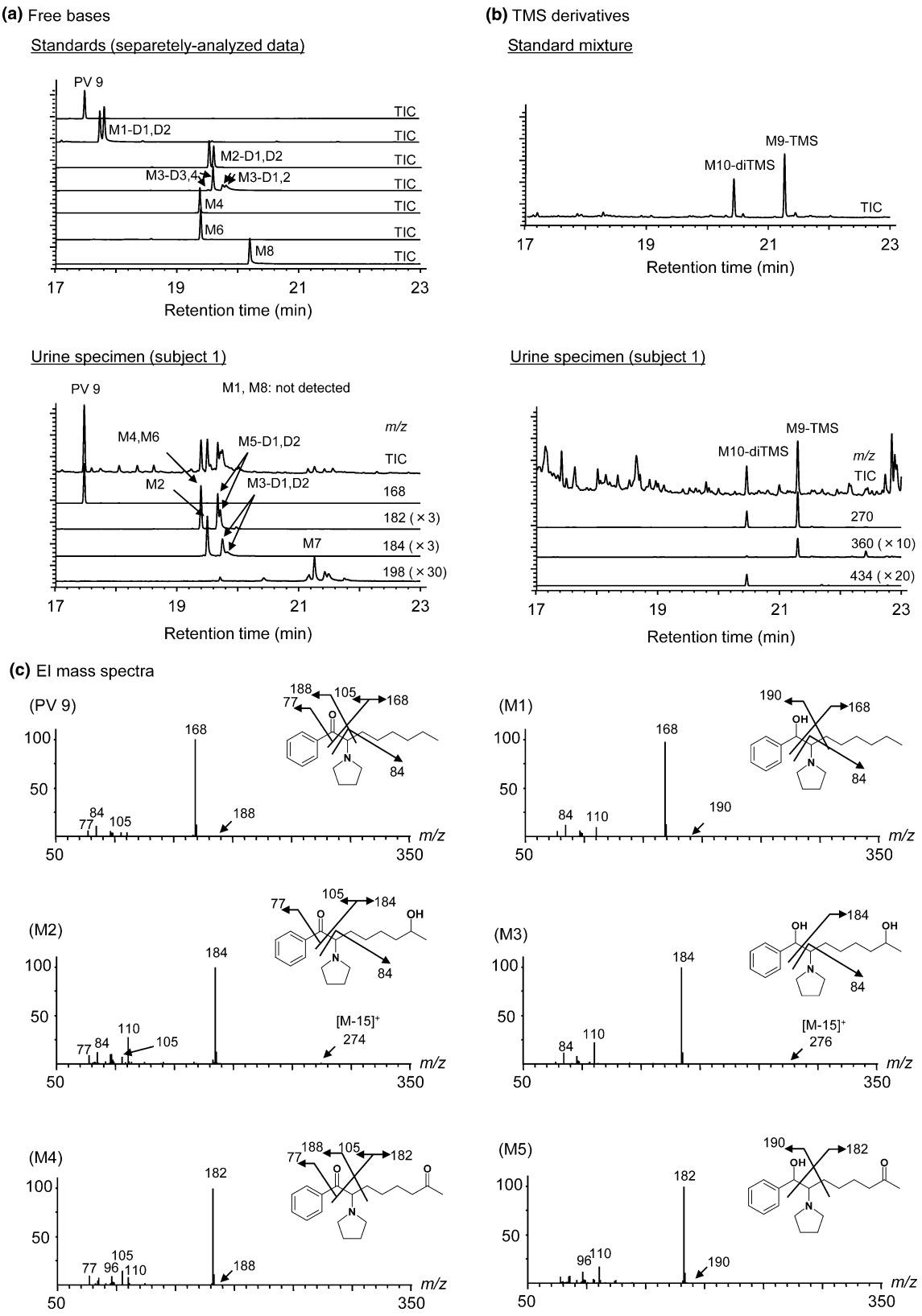
Fig. 2 Total ion chromatograms (TICs) and extracted ion chromatograms obtained from an authentic standard mixture and a PV9 user's urine specimen (subject 1), without derivatization (**a**) and with trimethylsilyl (TMS) derivatization (**b**), and electron ionization (EI) mass spectra of PV9 and its metabolites with/without TMS derivatization (**c**) obtained by gas chromatography–mass spectrometry. The *numbers in parentheses* represent magnification ratios ▶

GC–MS conditions

A GCMS-QP2010 Ultra gas chromatograph-mass spectrometer (Shimadzu, Kyoto, Japan) was operated in the EI mode using a CP-Sil 8CB-MS capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm; Agilent, Santa Clara, CA, USA). Injections were made automatically in the splitless mode at an injection port temperature of 250 °C. The carrier gas was high-purity helium at a flow rate of 3.0 ml/min. The column temperature was initially held at 80 °C for 1 min, and increased to 300 °C at 10 °C/min. The ionization energy and interface temperature were set at 70 eV and 250 °C, respectively.

LC–MS-MS conditions

LC–HR-MS-MS analysis was conducted on a Prominence Series UFLC system (Shimadzu) linked to a Triple TOF 5600 hybrid quadrupole time-of-flight tandem mass spectrometer (AB Sciex, Concord, ON, Canada) equipped with an electrospray ionization (ESI) interface. The MS conditions were as follows: ion spray voltage, 5.5 kV; turbo spray temperature, 500 °C; declustering potential (DP), 80 V; collision energy (CE), 35 eV. Nitrogen was used as a nebulizer and collision gas. A full scan was run in the positive mode with a mass range from *m/z* 100 to 1000 and with a 250-ms accumulation time. For the information-dependent acquisition criteria, the 20 most intense ions that exceeded 100 cps counts were selected to do a product ion scan, and the ion scan ranged from *m/z* 50 to 1000 with a


(a) Free bases
Standards (separetely-analyzed data)
PV 9
M1-D1,D2
M2-D1,D2
M3-D3,4
M3-D1,2
M4
M6
M8
TIC
17
19
21
23
Retention time (min)
(b) TMS derivatives
Standard mixture
M9-TMS
M10-diTMS
TIC
Retention time (min)
Urine specimen (subject 1)
PV 9
M1, M8: not detected
M4,M6
M5-D1,D2
M2
M3-D1,D2
M7
m/z
TIC
168
182 (×3)
184 (×3)
198 (×30)
Retention time (min)
Urine specimen (subject 1)
M9-TMS
M10-diTMS
m/z
TIC
270
360 (×10)
434 (×20)
Retention time (min)
(c) EI mass spectra
(PV 9)
(M1)
(M2)
(M3)
(M4)
(M5)
[M-15]+
274
276
m/z
50
350
100

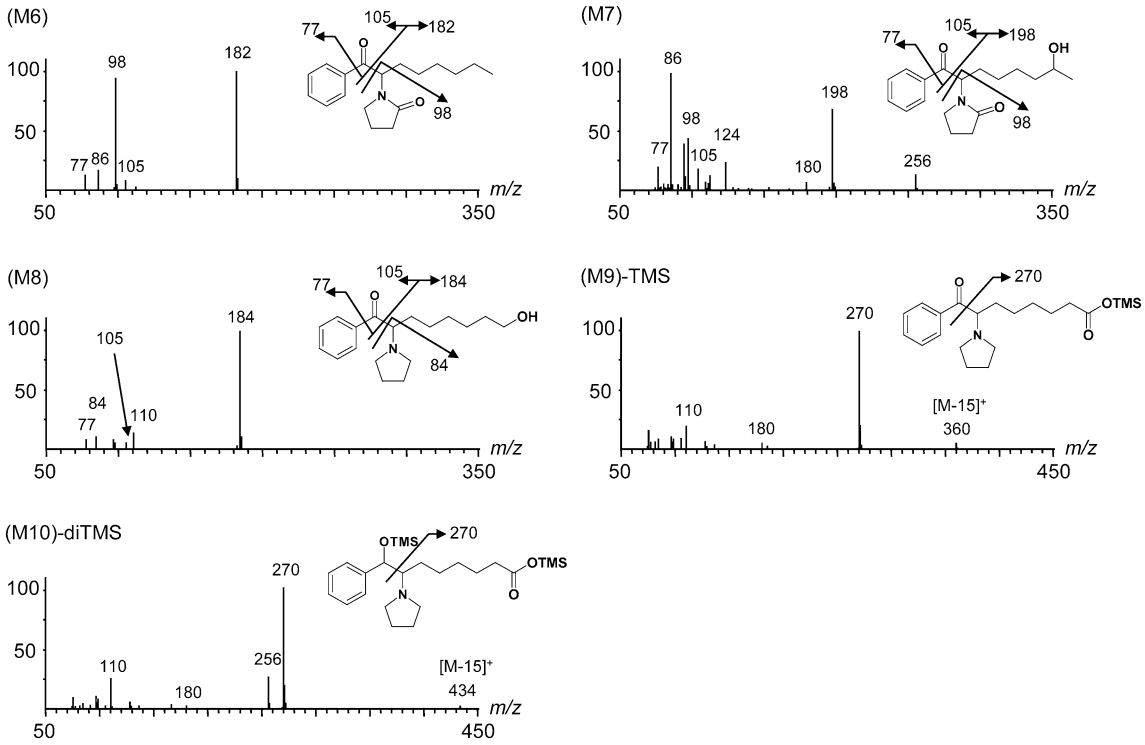


Fig. 2 continued

50-ms accumulation time. The instrument was mass-calibrated prior to analysis, infusing a Positive Calibration Solution (AB Sciex) at a flow rate of 100 µl/min on an automated calibration delivery system. Quantitative analysis was performed on a Prominence Series UFLC system linked to an API 3200 QTRAP hybrid triple quadrupole linear ion-trap mass spectrometer (AB Sciex) equipped with an ESI interface in the selected reaction monitoring (SRM) mode. Nitrogen was used as a nebulizer and collision gas, and the SRM parameters for each compound are shown in Table 1.

Liquid chromatography separation was carried out using L-column2 ODS semi-micro column (150 mm × 1.5 mm i.d., 5 µm particles; Chemicals Evaluation and Research Institute, Tokyo, Japan). The analytes were chromatographed by linear gradient elution with (A) 10 mM ammonium acetate buffer (pH 5); and (B) methanol at a flow rate of 0.1 ml/min and at a column temperature of 40 °C. A gradient was applied starting from 95 % A/5 % B, and linearly increased to 5 % A/95 % B over the 15 min, and held for 10 min.

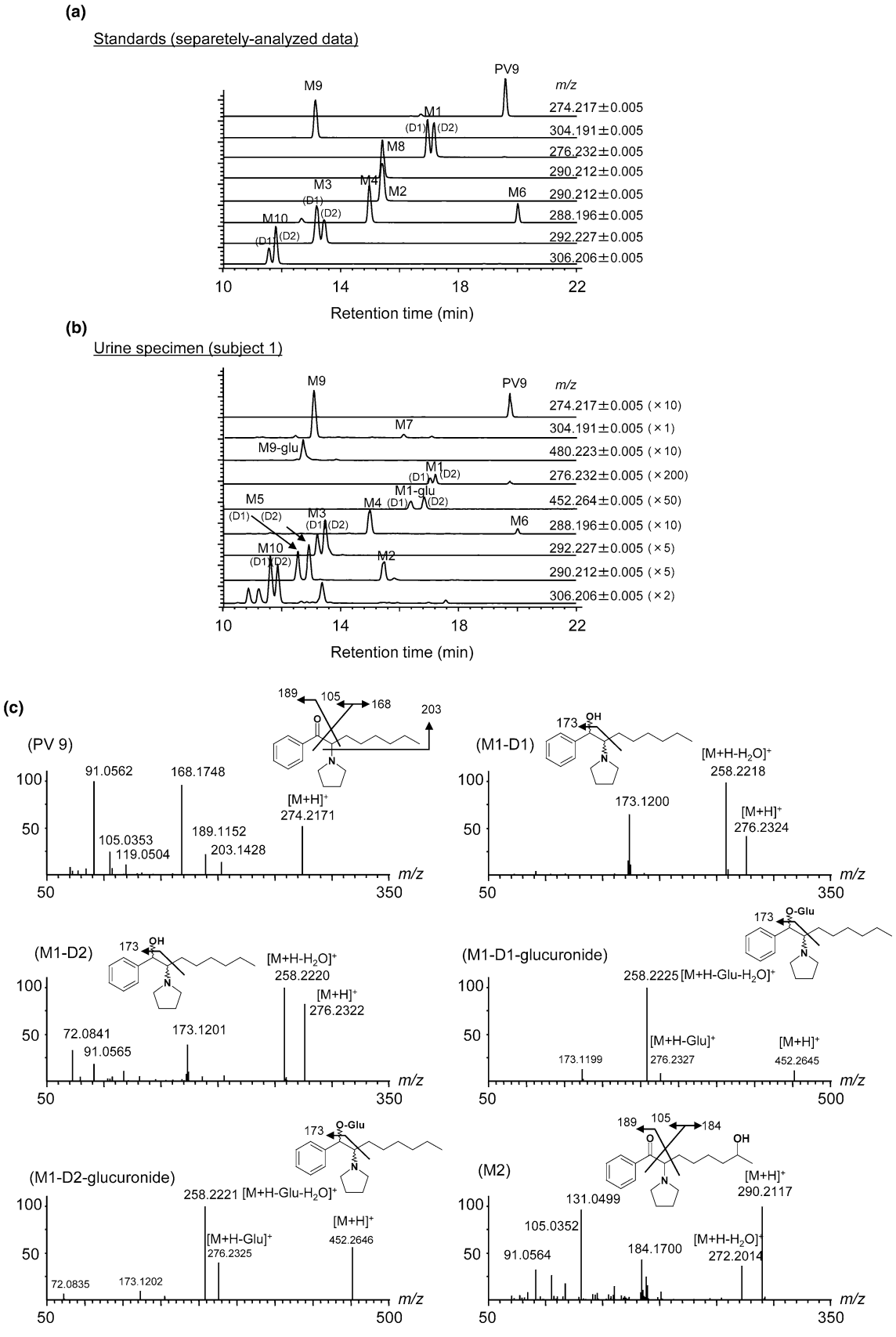
Results and discussion

PV9 and putative metabolites

Previous reports have detailed the human metabolism of α-PVP and α-PBP [8, 14], which are structurally similar to PV9. The studies showed that the primary metabolic pathways include the reduction of the keto groups to their corresponding alcohols, and oxidation at the 2″ position of pyrrolidine rings to form the corresponding ketones. Other reports [9, 10, 13] have also described metabolites utilizing these pathways. Recently, Hasegawa et al. [19, 20] reported a fatal case of PV9 poisoning in which a PV9 metabolite (M1, via reduction of keto group) was identified based on LC–MS-MS data. Minakata et al. [30] also reported detections of M1 and an oxidized metabolite of PV9 in human blood.

Fig. 3 Extracted ion chromatograms obtained from an authentic ▶ standard mixture (**a**) and a PV9 user's urine specimen (**b**), and product ion mass spectra of PV9 and its metabolites (**c**) obtained by liquid chromatography–tandem mass spectrometry (LC–MS-MS). The *numbers in parentheses* represent magnification ratios. Each protonated molecule was selected as a precursor ion

Results from our preliminary experiments using GC–MS and LC–HR-MS-MS suggested that the metabolic pathways of PV9 include not only the reductions and oxidations described above, but also the aliphatic oxidation at terminal (omega) and/or penultimate (omega-1) carbon atoms. The nine compounds (PV9, M1–M4, M6, M8–M10) needed for the metabolic studies were chemically synthesized, and five of them (PV9, M1, M4, M6, M9) were used for quantitative analyses. It was expected that metabolites containing two or three chiral centers (M1, M2, M3, M10) would be


(a)
Standards (separetely-analyzed data)
M9
PV9
M1
(D1)
(D2)
M8
M3
M4
M2
M6
(D1)
(D2)
M10
(D1)
(D2)
m/z
274.217±0.005
304.191±0.005
276.232±0.005
290.212±0.005
290.212±0.005
288.196±0.005
292.227±0.005
306.206±0.005
10
14
18
22
Retention time (min)
(b)
Urine specimen (subject 1)
M9
PV9
M7
M9-glu
M1
(D1)
(D2)
M1-glu
M5
(D1)
(D2)
M4
(D1)
(D2)
M3
(D1)
(D2)
M6
M10
(D1)
(D2)
M2
m/z
274.217±0.005 (×10)
304.191±0.005 (×1)
480.223±0.005 (×10)
276.232±0.005 (×200)
452.264±0.005 (×50)
288.196±0.005 (×10)
292.227±0.005 (×5)
290.212±0.005 (×5)
306.206±0.005 (×2)
10
14
18
22
Retention time (min)
(c)
(PV 9)
189
105
168
203
91.0562
168.1748
[M+H]+
274.2171
105.0353
119.0504
189.1152
203.1428
100
50
50
350
m/z
(M1-D1)
173
OH
[M+H-H2O]+
258.2218
173.1200
[M+H]+
276.2324
100
50
50
350
m/z
(M1-D2)
173
OH
[M+H-H2O]+
258.2220
[M+H]+
276.2322
72.0841
91.0565
173.1201
100
50
50
350
m/z
(M1-D1-glucuronide)
173
O-Glu
258.2225 [M+H-Glu-H2O]+
[M+H-Glu]+
276.2327
[M+H]+
452.2645
173.1199
100
50
50
500
m/z
(M1-D2-glucuronide)
173
O-Glu
258.2221 [M+H-Glu-H2O]+
[M+H-Glu]+
276.2325
[M+H]+
452.2646
72.0835
173.1202
100
50
50
500
m/z
(M2)
189
105
184
OH
[M+H]+
290.2117
131.0499
105.0352
91.0564
184.1700
[M+H-H2O]+
272.2014
100
50
50
350
m/z

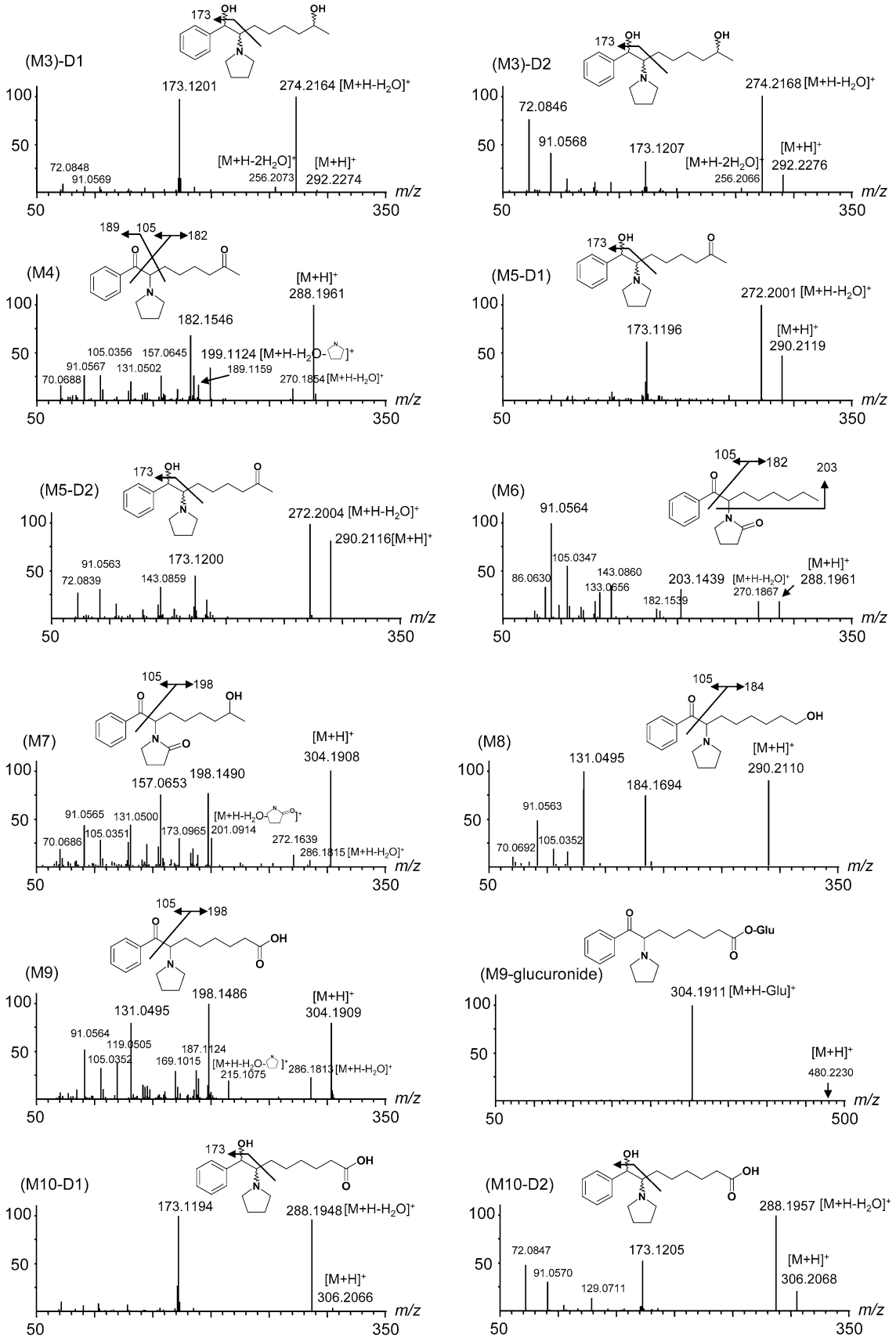


Fig. 3 continued

excreted in urine as diastereomers. Their syntheses were therefore designed to result in the appropriate diastereomers, as described in the "Materials and methods" section. In particular, the diastereomic ratio (M1-D1/M1-D2) of synthesized M1 was 1.0 based on NMR analyses [8, 31].

Identification of PV9 and its phase I metabolites in urine by GC–MS

Figure 2 shows the total ion chromatogram, extracted ion chromatograms, and EI mass spectra obtained from a PV9 user's urine specimen and authentic standards obtained by GC–MS. To achieve high sensitivity of M9 and M10, trimethylsilyl (TMS) derivatization with MSTFA was adopted as pretreatment for GC–MS, and the other metabolites were analyzed as free bases. Unchanged PV9 and six phase I metabolites, M2, M3, M4, M6, M9-TMS, and M10-diTMS, were identified in the PV9 user's urine, whereas M1 and M8 were not detected. In addition, putative metabolites M5 (D1 and D2) and M7 were detected at retention times of 19.7 and 21.3 min, respectively, based on the fragment ions (Fig. 2) and molecular weights obtained by GC–MS analysis with the chemical ionization (data not shown), although the reference standards of M5 and M7 were not available.

The GC conditions used in the present study provided baseline separation of peaks corresponding to structural isomers M2 (diastereomers) and M8, although no significant differences were observed in their mass spectra, except for an extremely weak fragment ion at *m/z* 274 (Fig. 2c). In contrast, another pair of structural isomers, M4 and M6, was not adequately separated under several sets of GC conditions, including Agilent DB1-MS, DB5-MS, and DB17-MS columns (data not shown). However, background subtraction techniques based on slight differences in retention time allowed the acquisition of mass spectra, which in turn allowed for the identification of M4 and M6 (Fig. 2c).

Two diastereomic metabolites, M2 and M3, were detected in PV9 abusers' urine as only one diastereomer and two diastereomers, respectively, although the diastereomic standards of M2 and M3 produced two and four peaks (Fig. 2a), respectively. This finding suggested that PV9 would be enantioselectively hydroxylated at penultimate carbon (omega-1 position) to only one diastereomer (M2), followed by the reductions of the keto group to two diastereomers (M3): hydroxylation at omega-1 position would not produce the diastereomers, while the reductions of the keto group would result in production of the diastereomers (Fig. 2a). This hypothesis was supported by the detection of diastereomic M5. The column separation of diastereomic standard M10 was not achieved under GC conditions in this study, but it was easily separated by LC as described below.

Analyses of PV9 and its metabolites in urine by LC–HR-MS-MS

To simultaneously detect PV9 and its metabolites, a sensitive and reliable LC–HR-MS-MS was performed. A C18 semi-micro column with a gradient elution program optimized in the present study provided clear detection of PV9 and 16 metabolites, including diastereomers and conjugates, as shown in Fig. 3. Table 2 summarizes measured mass numbers of the protonated molecules and their predominant product ions with proposed molecular formulae. These data were used for structural elucidation prior to the synthesis of authentic standards. Of the 16 detected metabolites, ten were identified using the synthesized standards, and six other metabolites, such as M1-glucuronide-D1, M1-glucuronide-D2, M5-D1, M5-D2, M7, and M9-glucuronide, were deduced from the proposed formulae of product ions and relative retention times. Although M1 and M10 were either not detected in urine or were not stereoisomerically separated by GC–MS (Fig. 2a, b), both metabolites were detected as diastereomers by LC–HR-MS-MS (Fig. 3b). Besides these two metabolites, M1-glucuronides, M3, and M5 were found to be excreted in urine as diastereomers. On the other hand, the presence of M8 in urine could not be confirmed under this LC–MS-MS condition (Fig. 3). D2 of each diastereomic metabolite produced the product ions at *m/z* 72 and 91 more abundantly by collision-induced dissociation (CID) than D1 did (Fig. 3c), corresponding to data on the urinary metabolites of α-PVP and α-PBP [8, 14].

Table 3 summarizes the relative ion intensities of protonated metabolites obtained in the single full-scan mode of LC–HR-MS. These intensities suggest that M9 and M10 (metabolites via omega oxidation), and M2 through M5 (via omega-1 oxidation), are the main metabolites of PV9, whereas M1 and M6 (via reduction of keto groups and oxidation of pyrrolidine rings) are minor metabolites.

Urinary concentrations of PV9 and its metabolites

To obtain more reliable quantitative results, simultaneous determinations of PV9 and its metabolites were further performed on a triple quadrupole mass spectrometer system (API 3200 QTRAP), as described above. To investigate the main metabolic pathways, unchanged PV9 and its five metabolites, including M9, estimated to be a main metabolite in the previous section, were quantified by using the validated LC–MS-MS method in the SRM mode. Table 4 summarizes the validation data obtained by analyzing various concentrations of analytes spiked into drug-free urine. The pretreatment of urine specimens with methanol (a protein precipitation method) provided high recoveries of greater than 95 %. Calibration curves were

Table 2 Accurate mass numbers of the protonated molecular and predominant product ions, and their proposed molecular formulae measured by liquid chromatography–high-resolution-tandem mass spectrometry (LC–HR-MS-MS)

	Measured accurate mass	Molecular formula	Error (ppm)
PV 9	274.2171	$C_{18}H_{28}NO$	2.0
	203.1428	$C_{14}H_{19}O$	−1.2
	189.1152	$C_{12}H_{15}NO$	2.0
	168.1748	$C_{11}H_{22}N$	0.7
M1	276.2322	$C_{18}H_{3}ONO$	0.03
	258.2220	$C_{18}H_{28}N$	1.4
	173.1201	$C_{12}H_{15}N$	1.2
M1-glucuronide	452.2646	$C_{24}H_{38}NO_{7}$	0.8
	276.2325	$C_{18}H_{30}NO$	1.1
	258.2221	$C_{18}H_{28}N$	1.8
	173.1202	$C_{12}H_{15}N$	1.7
M2	290.2117	$C_{18}H_{28}NO_{2}$	0.8
	272.2014	$C_{18}H_{26}NO$	1.9
	189.1143	$C_{12}H_{15}NO$	−2.7
	188.1067	$C_{12}H_{14}NO$	−1.5
	184.1700	$C_{11}H_{22}NO$	2.2
	131.0499	$C_{9}H_{7}O$	5.8
M3	292.2276	$C_{18}H_{30}NO_{2}$	1.7
	274.2168	$C_{18}H_{28}NO$	0.9
	256.2066	$C_{18}H_{26}N$	2.4
	173.1207	$C_{12}H_{15}N$	4.6
M4	288.1961	$C_{18}H_{26}NO_{2}$	1.0
	270.1854	$C_{18}H_{24}NO$	0.7
	199.1124	$C_{14}H_{15}O$	3.3
	189.1159	$C_{12}H_{15}NO$	5.8
	182.1546	$C_{11}H_{20}NO$	3.6
	157.0645	$C_{11}H_{9}O$	−1.9
	131.0502	$C_{9}H_{7}O$	8.1
M5	290.2116	$C_{18}H_{28}NO_{2}$	0.5
	272.2004	$C_{18}H_{26}NO$	−1.8
	173.1200	$C_{12}H_{15}N$	0.6
	143.0859	$C_{11}H_{11}$	2.6
M6	288.1961	$C_{18}H_{26}NO_{2}$	1.0
	270.1867	$C_{18}H_{24}NO$	5.4
	203.1439	$C_{14}H_{19}O$	4.2
	182.1539	$C_{11}H_{20}NO$	−0.2
	143.0860	$C_{11}H_{11}$	3.3
	133.0656	$C_{9}H_{9}O$	6.1
M7	304.1908	$C_{18}H_{26}NO_{3}$	0.3
	286.1815	$C_{18}H_{24}NO_{2}$	4.7
	272.1639	$C_{17}H_{22}NO_{2}$	−2.2
	201.0914	$C_{13}H_{13}O_{2}$	1.9
	198.1490	$C_{11}H_{20}NO_{2}$	0.7
	173.0965	$C_{12}H_{13}O$	2.4
	157.0653	$C_{11}H_{9}O$	3.2
	131.0500	$C_{9}H_{7}O$	6.6
M8	290.2110	$C_{18}H_{28}NO_{2}$	−1.6
	184.1694	$C_{11}H_{22}NO$	−1.0
	131.0495	$C_{9}H_{7}O$	2.7
M9	304.1909	$C_{18}H_{26}NO_{3}$	0.6
	286.1813	$C_{18}H_{24}NO_{2}$	4.0
	215.1075	$C_{14}H_{15}O_{2}$	3.9
	198.1486	$C_{11}H_{20}NO_{2}$	−1.3
	187.1124	$C_{13}H_{15}O$	3.5
	169.1015	$C_{13}H_{13}$	1.9
	131.0495	$C_{9}H_{7}O$	2.7
M9-glucuronide	480.2230	$C_{24}H_{34}NO_{9}$	0.5
	304.1911	$C_{18}H_{26}NO_{3}$	1.2
M10	306.2068	$C_{18}H_{28}NO_{3}$	1.4
	288.1957	$C_{18}H_{26}NO_{2}$	−0.4
	173.1205	$C_{12}H_{15}N$	3.5
	129.0711	$C_{10}H_{9}$	9.5

Automatic calibrations of scans by time-of-flight (TOF) mass spectrometry and TOF tandem mass spectrometry were performed using an external calibrant delivery system that infuses calibration solution

Table 3 Relative ion intensities of protonated molecules in the single full-scan mode [ratio (%) to PV9] obtained by LC–HR-MS

Compound	Subject 1	Subject 2
PV 9	100	100
M1-D1	2	10
M1-D1-glucuronide	6.3	Trace
M1-D2	3.4	6.8
M1-D2-glucuronide	12	Trace
M2	140	110
M3-D1	110	39
M3-D2	230	61
M4	92	67
M5-D1	200	94
M5-D2	330	160
M6	7.0	5.5
M7	1.7	n.d.
M8	n.d.	n.d.
M9	1100	2000
M9-glucuronide	94	250
M10-D1	560	380
M10-D2	402	200

n.d. not detected

linear over the concentration range of 1–1000 ng/ml, with a correlation coefficients greater than 0.99. At urinary concentrations above this range, samples were diluted to appropriate concentrations with drug-free urine and re-analyzed. The accuracy bias and precision of this technique were less than 15 % for all of the compounds at all of the concentrations examined.

Table 5 summarizes the urinary concentrations obtained from urine specimens of two actual PV9 users. M9 was the

Table 4 Validation data for the LC–MS-MS procedure established in the present study

Item for validation	PV9	M1-D1	M1-D2	M4	M6	M9
Recovery (%) (500 ng/ml) [a]	98	99	95	101	98	98
Correlation coefficient (r)[b]	0.996	0.997	0.998	0.997	0.995	0.995
Intraday accuracy relative error (%)[c]						
(50 ng/ml)	1.2	−3.7	−2.0	−13.7	3.5	−9.1
(500 ng/ml)	0.9	−6.1	3.3	−8.9	11.9	−11.5
Pecision RSD (%)[c]						
Intraday (50 ng/ml)	3.0	6.9	3.8	2.7	10.4	6.9
(500 ng/ml)	2.2	4.0	3.8	5.3	7.9	2.1
Interday(50 ng/ml)	7.4	8.1	4.9	6.3	9.1	6.0
(500 ng/ml)	5.1	8.8	5.2	8.2	6.2	4.4

The instrument used was an LC–triple quadrupole mass spectrometer (API 3200 QTRAP)

RSD relative standard deviation

[a] The recoveries were calculated by comparing peak areas of the analytes extracted from spiked samples with those spiked in urinary extract ($n = 5$)

[b] Linearity ranges were tested with seven different concentrations (1–1000 ng/ml) of analytes spiked into drug-free urine

[c] Accuracy and precision data were obtained from five spiked samples for each concentration

Table 5 Urinary concentrations of PV9 and its metabolites in urine specimens of PV9 users

	Concentration (ng/ml)					
	PV 9	M1-D1	M1-D2	M4	M6	M9
Subject 1	2300	50 (650)[a]	42 (90)[a]	3000	890	50,000 (54,000)[a]
Subject 2	67	2.3 (6.5)[a]	1.2 (1.4)[a]	51	17	2700 (2800)[a]

[a] The values after hydrolysis are shown in parentheses

most abundant metabolite in both subjects, while M1 was the lowest concentration metabolite, even when both diastereomers were combined. M4 (produced via omega-1 oxidation) was more abundant in both samples than its structural isomer M6 (produced via oxidation of the pyrrolidine ring). These results suggest that M9 is the main metabolite of PV9, and that the main metabolic pathway of PV9 includes aliphatic oxidation at terminal and/or penultimate carbon atoms.

With regard to diastereoselectivity, the concentration ratios of M1-D1 to M1-D2 ([M1-D1]/[M1-D2]) were 1.2 for subject 1, and 1.9 for subject 2. These values are much lower than those of reductive diastereomic metabolites of α-PBP (average ± standard deviation (SD) = 173.5 ± 57.2, $n = 9$) [14] and α-PVP (average ± SD = 41.0 ± 11.8, $n = 16$) [8]. This decreasing ratio, associated with extension of the aliphatic side chain, may be caused by a lowering of the stereoselectivity of carbonyl reduction.

Our previous studies and those of several other researchers [8, 14, 28, 31–34] have shown that hydrolysis of urine samples is a partially effective pretreatment for GC–MS and LC–MS analyses. Presently, the detections of conjugated metabolites M9-glucuronide and M1-glucuronide imply that the concentrations of their respective aglycones increase after acidic/enzymatic hydrolysis.

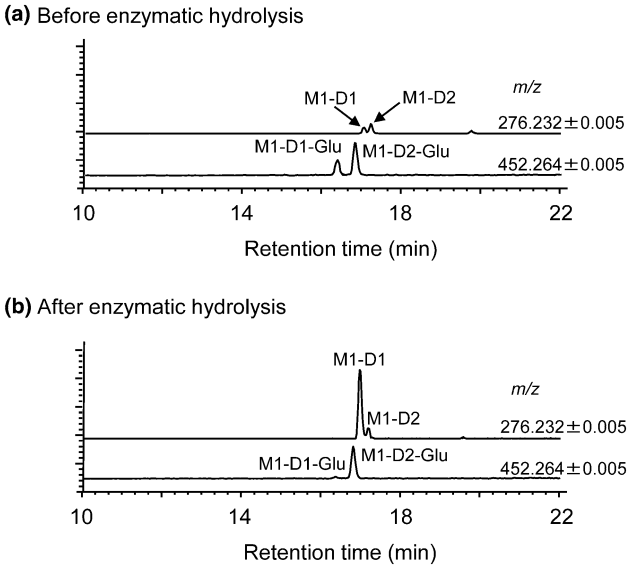


Fig. 4 Extracted ion chromatograms obtained from a PV9 user's urine specimen (subject 1) before (**a**) and after (**b**) the enzymatic hydrolysis obtained by LC–MS-MS

Indeed, urinary concentrations of M9, M1-D1, and M1-D2 increased by about 8, 1200, and 110 % for subject 1, and by 4, 180, and 17 % for subject 2, respectively, after

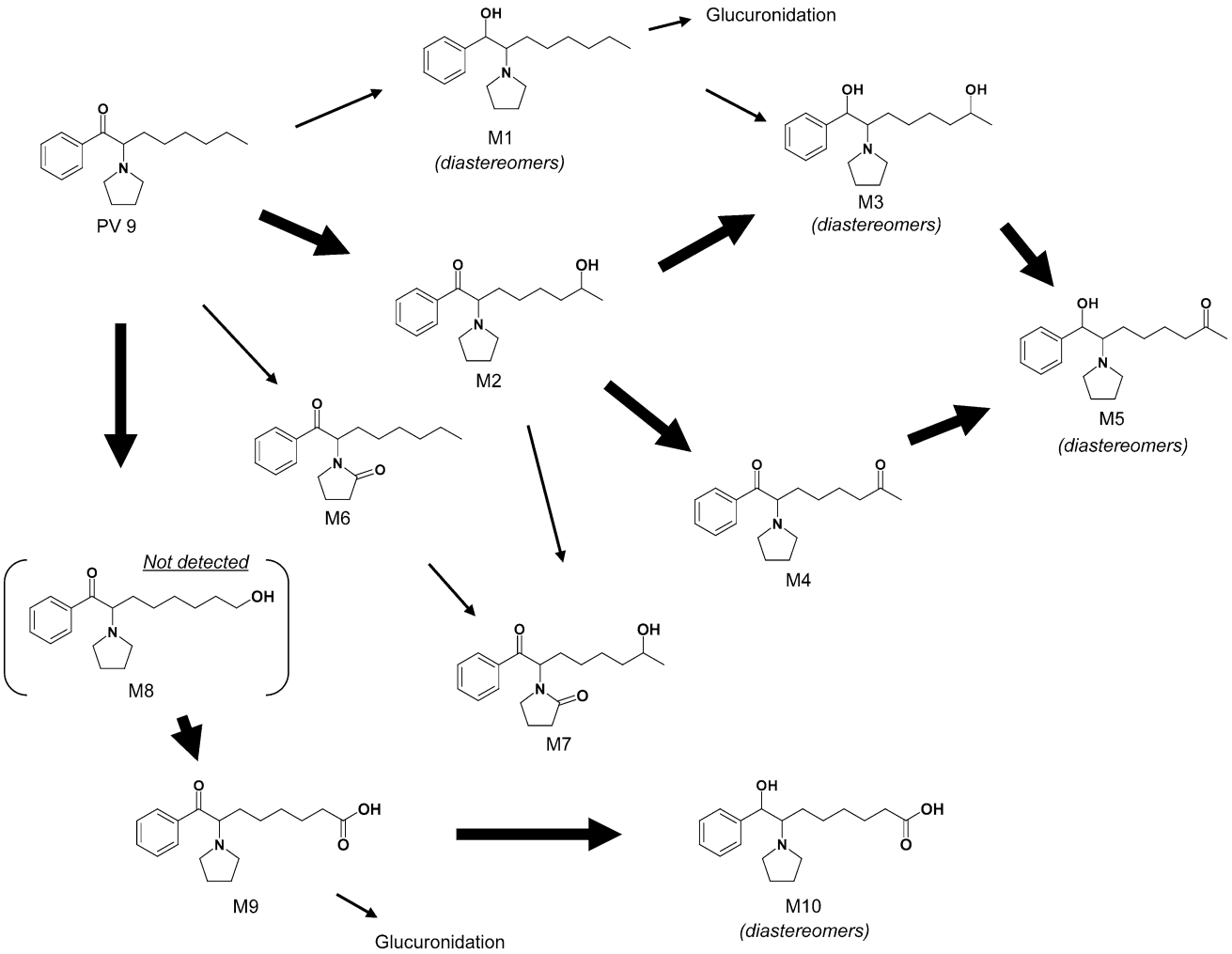


Fig. 5 Proposed metabolic pathways for PV9 in humans

enzymatic hydrolysis (Table 5). Interestingly, the amount of M1-D1 after hydrolysis increased at a higher rate than that of M1-D2, although M1-D2-glucuronide before hydrolysis was more abundant than M1-D1-glucuronide (Fig. 4a). This effect can be probably attributed to the incomplete digestion of M1-D2-glucuronide by glucuronidase (Fig. 4b).

Proposed metabolic pathways

The urinary metabolites detected in the present study suggest the metabolic pathways of PV9 shown in Fig. 5. These include: (1) reduction of the ketone group to M1 (diastereomers); (2) oxidation of the pyrrolidine ring to M6; (3) aliphatic oxidation of the omega carbon atom to M9 perhaps via M8 (not detected); and (4) oxidations at the omega-1 carbon atom to M2, followed by further oxidation to M4. Various combinations of these steps can result in the formation of M3 (diastereomers), M5 (diastereomers), M7, and M10 (diastereomers). In addition, M1 and M9 were hypothesized to be partially conjugated to mainly glucuronides. Based on the urinary concentrations and detected intensities of these metabolites, the main metabolite was M9, and the main metabolic pathway included aliphatic oxidation at the omega and omega-1 carbon atoms. Thus, PV9 metabolism would differ significantly from that of α-PVP or α-PBP, which contain shorter alkyl chains [8, 14]. This suggests that the alkyl chain length of α-pyrrolidinophenones influences the main metabolic pathways.

Conclusions

In the present study, PV9 and its 16 metabolites, including diastereomers, were detected in PV9 users' urine using newly synthesized authentic standards and/or based on their mass spectra and relative retention times obtained

from GC–MS and LC–HR-MS-MS. The proposed metabolic pathway of PV9 in humans includes the reduction of keto groups to alcohols, the oxidation of pyrrolidine rings to pyrrolidones, the aliphatic oxidation of omega and/or omega-1 carbon atoms to form the corresponding carboxylic acids, alcohols, and ketones, and combinations of these steps. Interestingly, it was suggested that the main metabolic pathways of α-pyrrolidinophenones depend on alkyl chain length. These findings will contribute to the establishment of a reliable analytical procedure for proving the intake of newly encountered designer drugs, as well as in the prediction of their metabolic pathways.

Conflict of interest There are no financial or other relations that could lead to a conflict of interest.

Ethical approval Informed consent was obtained from all healthy individuals included in the study, who supplied several 10 ml each of urine as blank matrix.

References

1. Valente MJ, Guedes de Pinho P, de Lourdes Bastos M, Carvalho F, Carvalho M (2014) Khat and synthetic cathinones: a review. Arch Toxicol 88:15–45
2. Paillet-Loilier M, Cesbron A, Le Boisselier R, Bourgine J, Debruyne D (2014) Emerging drugs of abuse: current perspectives on substituted cathinones. Subst Abuse Rehabil 5:37–52
3. Cottencin O, Rolland B, Karila L (2014) New designer drugs (synthetic cannabinoids and synthetic cathinones): review of literature. Curr Pharm Des 20:4106–4111
4. Uchiyama N, Shimokawa Y, Kawamura M, Kikura-Hanajiri R, Hakamatsuka T (2014) Chemical analysis of a benzofuran derivative, 2-(2-ethylaminopropyl)benzofuran (2-EAPB), eight synthetic cannabinoids, five cathinone derivatives, and five other designer drugs newly detected in illegal products. Forensic Toxicol 32:266–281
5. Jane MP, Lewis SN (2012) The toxicology of bath salts: a review of synthetic cathinones. J Med Toxicol 8:33–42
6. Wyman JF, Lavins ES, Engelhart D, Armstrong EJ, Snell KD, Boggs PD, Taylor SM, Norris RN, Miller FP (2013) Postmortem tissue distribution of MDPV following lethal intoxication by "bath salts". J Anal Toxicol 37:182–185
7. Meyer MR, Du P, Schuster F, Maurer HH (2010) Studies on the metabolism of the α-pyrrolidinophenone designer drug methylenedioxy-pyrovalerone (MDPV) in rat and human urine and human liver microsomes using GC–MS and LC–high resolution MS and its detectability in urine by GC–MS. J Mass Spectrom 45:1426–1442
8. Shima N, Katagi M, Kamata H, Matsuta S, Sasaki K, Kamata T, Nishioka H, Miki A, Tatsuno M, Zaitsu K, Ishii A, Sato T, Tsuchihashi H, Suzuki K (2014) Metabolism of the newly encountered designer drug α-pyrrolidinovalerophenone in humans: identification and quantitation of urinary metabolites. Forensic Toxicol 32:59–67
9. Tyrkkö E, Pelander A, Ketola R, Ojanperä I (2013) In silico and in vitro metabolism studies support identification of designer drugs in human urine by liquid chromatography/quadrupole-time-of-flight mass spectrometry. Anal Bioanal Chem 405:6697–6709
10. Hasegawa K, Suzuki O, Wurita A, Minakata K, Yamagishi I, Nozawa H, Gonmori K, Watanabe K (2014) Postmortem distribution of α-pyrrolidinovalerophenone and its metabolite in body fluids and solid tissues in a fatal poisoning case measured by LC–MS-MS with the standard addition method. Forensic Toxicol 32:225–234
11. Sauer S, Peters FT, Haas C, Meyer MR, Fritschi G, Maurer HH (2009) New designer drug α-pyrrolidinovalerophenone (PVP): studies on its metabolism and toxicological detection in rat urine using gas chromatographic/mass spectrometric techniques. J Mass Spectrom 44:952–964
12. Saito T, Namera A, Osawa M, Aoki H, Inokuchi S (2013) SPME–GC–MS analysis of α-pyrrolidinovalerophenone in blood in a fatal poisoning case. Forensic Toxicol 31:328–332
13. Namera A, Konuma K, Kawamura M, Saito T, Nakamoto A, Yahata M, Ohta S, Miyazaki S, Shiraishi H, Nagao M (2014) Time-course profile of urinary excretion of intravenously administered α-pyrrolidinovalerophenone and α-pyrrolidinobutiophenone in a human. Forensic Toxicol 32:68–74
14. Matsuta S, Shima N, Kamata H, Kakehashi H, Nakano S, Sasaki K, Kamata T, Nishioka H, Miki A, Katagi M, Zaitsu K, Sato T, Tsuchihashi H, Suzuki K (2015) Metabolism of the designer drug α-pyrrolidinobutiophenone (α-PBP) in humans: identification and quantification of the phase I metabolites in urine. Forensic Sci Int 249:181–188
15. Namera A, Urabe S, Saito T, Torikoshi-Hatano A, Shiraishi H, Arima Y, Nagao M (2013) A fatal case of 3,4-methylenedioxypyrovalerone poisoning: coexistence of α-pyrrolidinobutiophenone and α-pyrrolidinovalerophenone in blood and/or hair. Forensic Toxicol 31:338–343
16. Sauer C, Hoffmann K, Schimmel U, Peters FT (2011) Acute poisoning involving the pyrrolidinophenone-type designer drug 4′-methyl-alpha-pyrrolidinohexanophenone (MPHP). Forensic Sci Int 208:20–25
17. Westphal F, Junge T, Rösner P, Fritschi G, Klein B, Girreser U (2007) Mass spectral and NMR spectral data of two new designer drugs with an alpha-aminophenone structure: 4′-methyl-alpha-pyrrolidinohexanophenone and 4′-methyl-alpha-pyrrolidinobutyrophenone. Forensic Sci Int 169:32–42
18. Meyer MR, Mauer S, Meyer GM, Dinger J, Klein B, Westphal F, Maurer HH (2014) The in vivo and in vitro metabolism and the detectability in urine of 3′,4′-methylenedioxy-alpha-pyrrolidinobutyrophenone (MDPBP), a new pyrrolidinophenone-type designer drug, studied by GC–MS and LC–MS(n.). Drug Test Anal 6:746–756
19. Hasegawa K, Wurita A, Minakata K, Gonmori K, Nozawa H, Yamagishi I, Suzuki O, Watanabe K (2014) Identification and quantitation of a new cathinone designer drug PV9 in an "aroma liquid" product, antemortem whole blood and urine specimens, and a postmortem whole blood specimen in a fatal poisoning case. Forensic Toxicol 32:243–250
20. Hasegawa K, Wurita A, Minakata K, Gonmori K, Nozawa H, Yamagishi I, Watanabe K, Suzuki O (2015) Postmortem distribution of PV9, a new cathinone derivative, in human solid tissues in a fatal poisoning case. Forensic Toxicol 33:141–147
21. Iversen L, White M, Treble R (2014) Designer psychostimulants: pharmacology and differences. Neuropharmacology 87:59–65
22. Zaitsu K, Katagi M, Tsuchihashi H, Ishii A (2014) Recently abused synthetic cathinones, α-pyrrolidinophenone derivatives: a review of their pharmacology, acute toxicity, and metabolism. Forensic Toxicol 32:1–8
23. Marusich JA, Antonazzo KR, Wiley JL, Blough BE, Partilla JS, Baumann MH (2014) Pharmacology of novel synthetic stimulants structurally related to the "bath salts" constituent 3,4-methylenedioxypyrovalerone (MDPV). Neuropharmacology 87:206–213
24. Kaizaki A, Tanaka S, Numazawa S (2014) New recreational drug 1-phenyl-2-(1-pyrrolidinyl)-1-pentanone (alpha-PVP) activates

central nervous system via dopaminergic neuron. J Toxicol Sci 39:1–6
25. Simmler LD, Buser TA, Donzelli M, Schramm Y, Dieu LH, Huwyler J, Chaboz S, Hoener MC, Liechti ME (2013) Pharmacological characterization of designer cathinones in vitro. Br J Pharmacol 168:458–470
26. Baumann MH, Partilla JS, Lehner KR, Thorndike EB, Hoffman AF, Holy M, Rothman RB, Goldberg SR, Lupica CR, Sitte HH, Brandt SD, Tella SR, Cozzi NV, Schindler CW (2013) Powerful cocaine-like actions of 3,4-methylenedioxypyrovalerone (MDPV), a principal constituent of psychoactive 'bath salts' products. Neuropsychopharmacology 38:552–562
27. Strano-Rossi S, Cadwallader AB, de la Torre X, Botrè F (2010) Toxicological determination and in vitro metabolism of the designer drug methylenedioxypyrovalerone (MDPV) by gas chromatography/mass spectrometry and liquid chromatography/quadrupole time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 24:2706–2714
28. Springer D, Fritschi G, Maurer HH (2003) Metabolism of the new designer drug α-pyrrolidinopropiophenone (PPP) and the toxicological detection of PPP and 4-methyl-α-pyrrolidinopropiophenone (MPPP) studied in rat urine using gas chromatography mass spectrometry. J Chromatogr B 796:253–266
29. Shin H-S, Shin Y-SO, Lee S, Park B-B (1996) Detection and identification of pyrovalerone and its hydroxylated metabolite in the rat. J Anal Toxicol 20:568–572
30. Minakata K, Yamagishi I, Nozawa H, Hasegawa K, Wurita A, Gonmori K, Suzuki M, Watanabe K, Suzuki O (2015) Determination of new pyrrolidino cathinone derivatives, PVT, F-PVP, MPHP, PV8, PV9 and F-PV9, in human blood by MALDI-Q-TOF mass spectrometry. Forensic Toxicol 33:148–154
31. Shima N, Katagi M, Kamata H, Matsuta S, Nakanishi K, Zaitsu K, Kamata T, Nishioka H, Miki A, Tatsuno M, Sato T, Tsuchihashi H, Suzuki K (2013) Urinary excretion and metabolism of the newly encountered designer drug 3,4-dimethylmethcathinone in humans. Forensic Toxicol 31:101–112
32. Zaitsu K, Katagi M, Kamata HT, Kamata T, Shima N, Miki A, Tsuchihashi H, Mori Y (2009) Determination of the metabolites of the new designer drugs bk-MBDB and bk-MDEA in human urine. Forensic Sci Int 188:131–139
33. Kamata HT, Shima N, Zaitsu K, Kamata T, Miki A, Nishikawa M, Katagi M, Tsuchihashi H (2006) Metabolism of the recently encountered designer drug, methylone, in humans and rats. Xenobiotica 36:709–723
34. Meyer MR, Wilhelm J, Peters FT, Maurer HH (2010) Beta-keto amphetamines: studies on the metabolism of the designer drug mephedrone and toxicological detection of mephedrone, butylone, and methylone in urine using gas chromatography-mass spectrometry. Anal Bioanal Chem 397:1225–1233