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LETTER TO THE EDITOR

Diphenidine and its metabolites in blood and urine analyzed by MALDI-Q-TOF mass spectrometry

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Dear Editor

New psychoactive substances that are different from synthetic cannabinoids or cathinone derivatives have appeared very recently on illicit drug markets. In 2014, three reports were published on diphenidine distribution in Japan. The first study characterized diphenidine and 1-benzylpiperidine in a powdered product called "fragrance powder" [1], while the second determined diphenidine and 5-fluoro-AB-PINACA in a dubious herbal product [2]. The third study dealt with a fatal case in which a victim smoked a herbal product containing diphenidine together with a herbal product containing AB-CHMINACA and 5-fluoro-AMB [3]. The postmortem distributions of these substances determined by gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–tandem mass spectrometry (LC–MS-MS) indicated that the main cause of death was diphenidine poisoning [3]. To date, the toxicity and metabolism of diphenidine have not been studied in any scientific context because it is a research chemical [4] with very little history of use in humans [5]. To tentatively identify unreported molecules, such as metabolites, their exact masses with sensitivities better than 0.001 Da are necessary [6]. Matrix-assisted laser desorption ionization (MALDI) can achieve a softer ionization than electrospray ionization under some conditions, and hence is suitable for the detection of intact molecules. Therefore, in the present work we examined diphenidine and its metabolites by MALDI quadrupole time-of-flight mass spectrometry (Q-TOF-MS) with a resolution better than 0.001 Da.

Diphenidine [1-(1,2-diphenyl-ethyl)piperidine]-HCl, acetonitrile (ACN), and ethanol that were suitable for LC–MS, and other chemicals of analytical grade were obtained from Wako Pure Chemicals (Osaka, Japan). α-Cyano-4-hydroxycinnamic acid (CHCA) was obtained from Sigma-Aldrich (St. Louis, MO, USA), and amitriptyline, to be used as internal standard (IS) for diphenidine quantitation, was obtained from Astellas Pharma (Tokyo, Japan). Pure water with a specific resistance of 18 MΩ cm was used (Millipore, Bedford, MA, USA). Blood samples from healthy volunteers under permission were used as blank samples, and those spiked with several amounts of diphenidine were used as positive samples. The postmortem blood and urine samples of a victim intoxicated mainly with diphenidine were obtained at autopsy performed in our laboratory [3].

Individual stock solutions of diphenidine and amitriptyline were prepared separately by dissolving the appropriate amount of each compound in ethanol at 1 mg/ml. The solutions were stored at −20 °C. Working calibration solutions and quality control solutions were prepared daily by diluting the stock solution of diphenidine with blank blood at 1–100 ng/ml. Amitriptyline at 50 ng/ml in blood was used as IS for the determination.

Diphenidine and its metabolites in a sample were extracted and detected together. One microliter of IS and 59 μl of water were added to 20 μl of blood or urine placed in a tube (Eppendorf AG, Hamburg, Germany) using a MICROMAN M10 pipette (Gilson S.A.S., Villiers le Bel, France), and the mixture was centrifuged at 10,000 *g* for 3 min. The supernatant (72 μl) was placed in a new tube,

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and 4 µl of 2 M K_2CO_3 and 220 µl of 1-chlorobutane (CB) were added. The mixture was vortex-mixed for 1 min and centrifuged at 10,000 *g* for 3 min. The upper 198 µl of the CB layer was transferred to another tube and evaporated to dryness at room temperature using a centrifugal dryer (Savant Speed Vac Plus SC110A; Savant, Holbrook, NY, USA). The residue was mixed with 16 µl of prepared CHCA solution (1.0 mg CHCA/ml in ACN–ethanol–water; 80:10:10, v/v/v). Two microliters of the solution was loaded on a stainless steel MALDI sample target plate (V700666, AB SCIEX, Framingham, MA, USA) and allowed to dry.

A reflector type Q-TOF mass spectrometer, QSTAR Elite Hybrid (AB SCIEX), was used in the positive ion mode. MS scan was performed at 7 kHz. Mass resolution was 15,000 and mass accuracy was 5 ppm. A stainless steel MALDI target plate with maximum 100 samples was inserted into the machine. Mass spectra were obtained using 355-nm radiation from the Nd:YAG laser system (AB SCIEX). The pulse energy of the excitation was set at 3.9 µJ with the delayed extraction voltage at 20 V. In MS-MS detection, the collision voltage was set at 20 V. The instrument was equipped with a video camera for displaying the sample image on a monitor and to allow the laser to be focused on a specific well within the target area. Spectra were obtained with 20 cycles at 200 Hz. Only part of a sample each well was consumed in the detection. Mass calibration was conducted using ions at *m/z* 172.0393 and *m/z* 379.0925 produced from CHCA.

Two microliters of CHCA solution containing diphenidine at 10 µg/ml was placed on a target well and was detected by MALDI-MS (see Fig. 1a). The protonated molecule of diphenidine $[M+H]^+$ at *m/z* 266.1910 was selected as the precursor ion, and the product ion spectrum at a collision voltage of 20 V is shown in Fig. 1b in which *m/z* values and schematic structures are described for fragment ions. In the present work, schematic structures were estimated when the *m/z* value increased by 1. Similarly, the conversion of single or double bonds to double or triple bonds, respectively, was also considered when the *m/z* value decreased by 1. In the spectrocopic analysis that followed, fragment ions observed at *m/z* 86.0964 $[C_5H_{12}N_1]^+$, 103.0543 $[C_8H_7]^+$, and 181.1016 $[C_{14}H_{13}]^+$ were denoted as ions (A), (B), and (C), respectively. The ion at *m/z* 166.0776 was produced from the precursor ion (C) by collision-induced dissociation, and assigned as $C_{13}H_{10}$ from its calculated value of 166.0777. As shown in Fig. 1b, the two phenyl rings became equivalent after the deletion of piperidine from diphenizine, although they were nonequivalent in the intact diphenidine.

The signal of diphenidine $[M+H]^+$ at *m/z* 266.1910 and that of IS, amitriptyline, at *m/z* 278.1914 were used for quantitation. A calibration curve was prepared by spiking diphenidine at 0, 3, 10, or 100 ng/ml ($n = 6$) into blank blood containing IS at 50 ng/ml. The calibration equation was $y = 0.01095x + 0.001191$, with a correlation coefficient of 0.997, where x is the concentration of diphenidine (ng/ml) and y is the relative ratio of the peak height of the diphenidine to that of IS. Figure 1c shows the mass spectra where the concentrations of diphenidine spiked into blood were 0, 1, and 3 ng/ml. The signal-to-noise ratio was 3 when the blood concentration was 1 ng/ml, as shown in Fig. 1c. Precision and accuracy were assessed by analyzing blood spiked with diphenidine at 3, 10, or 100 ng/ml three times a day as well as on three different days. The coefficient of variation was <15.4 % and the accuracy was 87.9–116.0 % for intraday and interday variations. Therefore, the quantitation range of diphenidine in blood was considered to be 3–100 ng/ml.

Recovery was in the range of 80–100 % at 3–100 ng/ml, as calculated by comparing the peak height obtained from the extract of spiked blood with the peak height obtained from the standard diphenidine dissolved in CHCA solution. The diphenidine levels in blood from the right atrium and in urine of the victim were determined ($n = 5$) as 726 ± 32 and 304 ± 19 ng/ml, respectively.

The MALDI–MS spectra at *m/z* 260–305 for the right atrium blood and urine of the victim are shown in Figs. 2a and 3a, respectively. They indicated not only the ions observed in Fig. 1a, but also a mono-oxidized metabolite $[M+H+O]^+$ at *m/z* 282.1855, a dioxidized metabolite $[M+H+O_2]^+$ at *m/z* 298.1798, and a dioxidized dehydrogenated metabolite $[M–H+O_2]^+$ at *m/z* 296.1642. The relative ratios of the peaks at *m/z* 266.1910, 282.1855, 298.1798, and 296.1642 in urine were 100:68:29:28, whereas those in blood were 100:19:1.5:1.0, respectively, indicating that the relative amounts of metabolites were higher in urine than those in blood.

The product ion spectrum from the protonated molecule $[M+H]^+$ at *m/z* 266.1910 extracted from blood is shown in Fig. 2b and is almost the same as that shown in Fig. 1b for the diphenidine standard solution. The product ion spectrum from the metabolite at *m/z* 282.1855 extracted from blood and the product ion spectra from the metabolites at *m/z* 298.1798 and 296.1642 extracted from urine are shown in Figs. 2c, 3b, and 3c, respectively. The product ion spectra from the precursor ions at *m/z* 266.1098 and 282.1852 extracted from urine were almost the same as those extracted from blood; similarly, the product ion spectra from the precursor ions at *m/z* 298.1798 and 296.1642 extracted from blood were almost the same as those extracted from urine, and hence are not shown.

The product ion derived from mono-oxidized metabolite $[M+H+O]^+$ is shown in Fig. 2c. There are two possibilities as to the position of mono-oxidation of diphenidine:

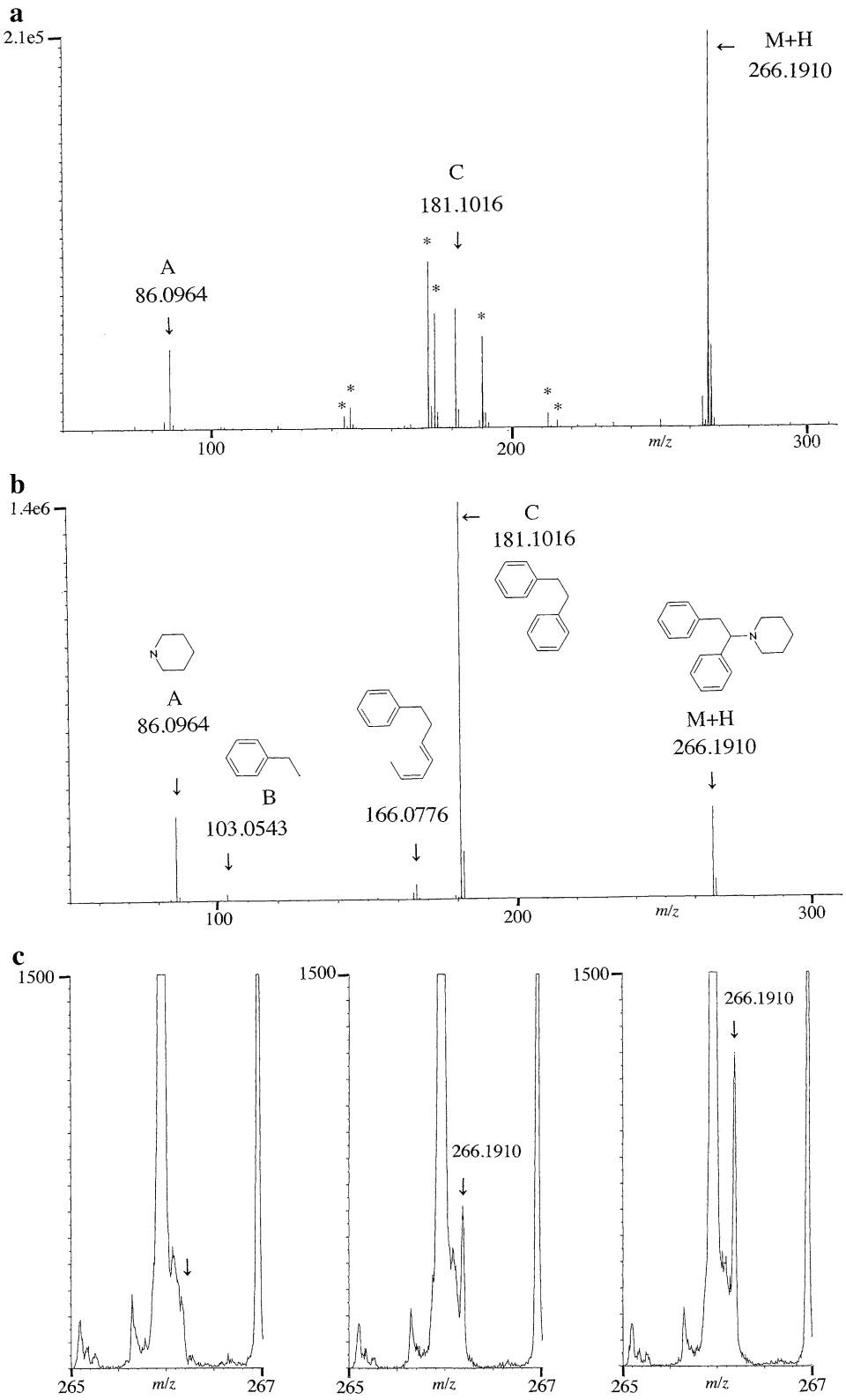

Fig. 1 Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) spectrum for *m/z* 50–310 of diphenidine at 10 μg/ml in α-cyano-4-hydroxycinnamic acid (CHCA) solution (**a**), its product ion spectrum for *m/z* 50–310 derived from the precursor ion $[M+H]^+$ at *m/z* 266.1908 with collision voltage of 20 V (**b**), and MALDI-MS spectra at *m/z* 265–267 of three blood samples that were spiked with diphenidine at 0, 1, or 3 ng/ml (**c**). The matrix-related ions of CHCA solution are marked with *asterisk*

either on piperidine (regioisomer *M1*) or on the phenyl ring (regioisomer *M2*). The position was assigned based on the product ion spectrum, because the MS-MS reaction dissociated diphenidine to the two fragments of piperidine (A) and the remainder (C), and then (C) decomposed to (B) as shown in Figs. 1b and 2b. The ions at *m/z* 102.0916

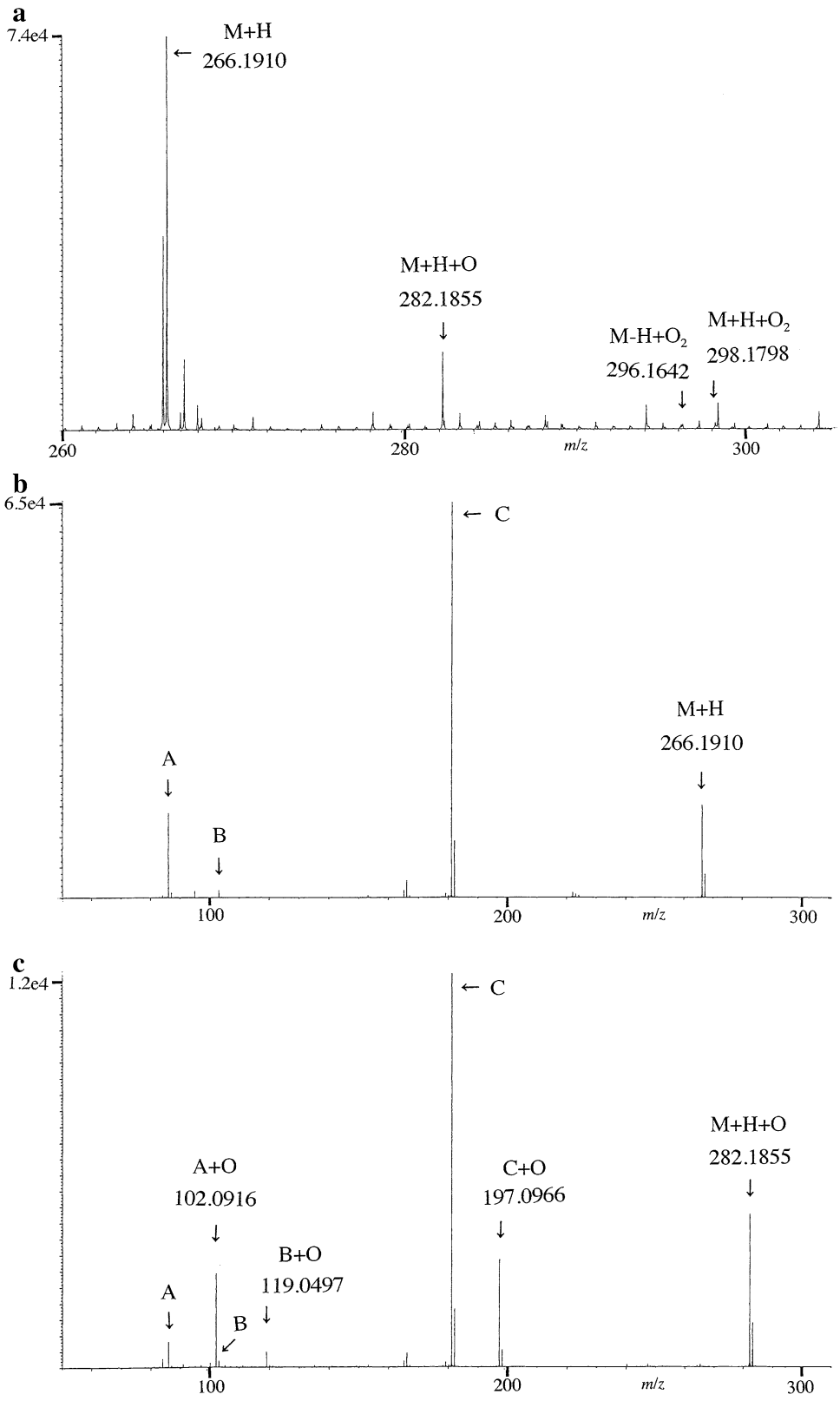

Fig. 2 MALDI-MS spectrum at *m/z* 260–305 of the extract from victim's blood (**a**), its product ion spectrum at *m/z* 50–310 from the precursor ion $[M+H]^+$ at *m/z* 266.1910 with collision voltage of 20 V (**b**), and its product ion spectrum at *m/z* 50–310 from the precursor ion $[M+H+O]^+$ at *m/z* 282.1855 with collision voltage of 20 V (**c**)

(A+O), 103.0543 (B), and 181.1016 (C) are considered to come from the regioisomer *M1* and those at *m/z* 86.0964 (A), 119.0497 (B+O), 103.0543 (B), and 197.0966 (C+O) from regioisomer *M2*. The observed and calculated masses of metabolites and their product ions are listed in Table 1. The signal height of (A+O) was 3.7 times that of (A) and

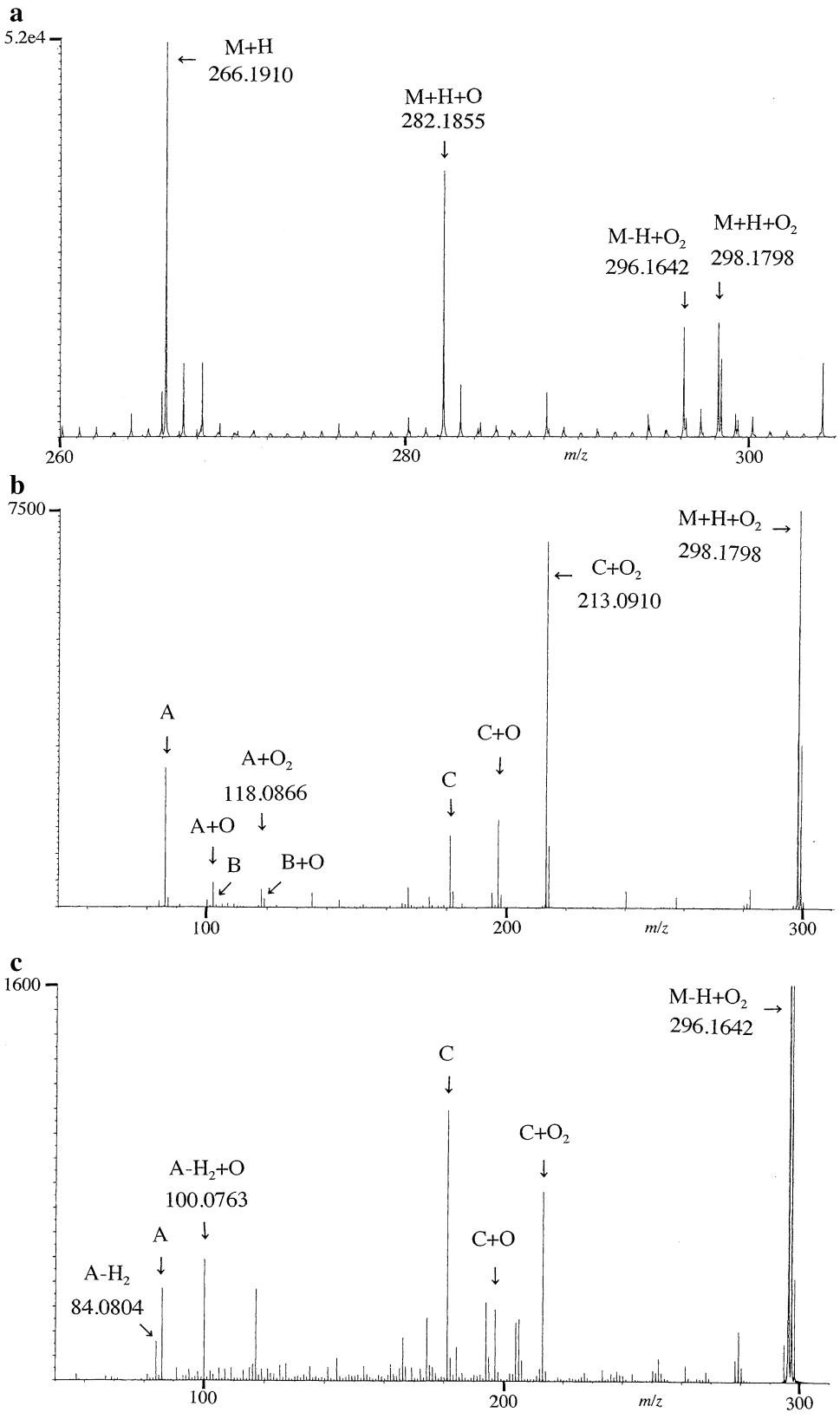

Fig. 3 MALDI-MS spectrum at *m/z* 260–305 of the extract from victim's urine (**a**), its product ion spectrum at *m/z* 50–310 derived from the precursor ion $[M+H+O_2]^+$ at *m/z* 298.1798 with collision voltage of 20 V (**b**), and its product ion spectrum at *m/z* 50–310 derived from the precursor ion $[M-H+O_2]^+$ at *m/z* 296.1642 with collision voltage of 20 V (**c**)

Table 1 Characterization of the metabolites of diphenidine in blood and urine

Metabolite ion	Fragment ion	Observed (*m/z*)	Calculated (*m/z*)	Difference (*m/z*)
1. M+H+O ($C_{19}H_{24}NO$)		282.1855	282.1852	0.003
Regioisomer *M1*	A+O	102.0916	102.0913	0.003
	B (C_8H_7)	103.0543	103.0542	0.001
	C ($C_{14}H_{13}$)	181.1016	181.1011	0.005
Regioisomer *M2*	A ($C_5H_{12}N$)	86.0964	86.0964	0.0
	B+O	119.0497	119.0491	0.006
	B	103.0543	103.0542	0.001
	C+O	197.0966	197.0960	0.006
2. M+H+O_2 ($C_{19}H_{24}NO_2$)		298.1798	298.1801	0.003
Regioisomer *M3*	A+O_2	118.0866	118.0862	0.004
	B	103.0543	103.0542	0.001
	C	181.1017	181.1011	0.006
Regioisomer *M4*	A+O	102.0911	102.0913	0.002
	B+O	119.0497	119.0491	0.006
	B	103.0543	103.0542	0.001
	C+O	197.0966	197.0960	0.006
Regioisomer *M5*	A	86.0964	86.0964	0.0
	B	103.0543	103.0542	0.001
	C+O_2	213.0910	213.0910	0.0
3. M−H+O_2 ($C_{19}H_{22}NO_2$)		296.1642	296.1645	0.003
	A+O−H_2	100.0763	100.0756	0.007
	A	86.0964	86.0964	0.0
	A−H_2	84.0804	84.0807	0.003
	C+O_2	213.0914	213.0910	0.004
	C+O	197.0957	197.0960	0.003
	C	181.1013	181.1011	0.002

For the structural characterization of the fragments A, B, and C, see Fig. 1

M metabolite

that of (C) was 3.7 times that of (C+O), indicating that the oxidation on piperidine occurred 3.7 times more frequently than on the phenyl ring. The mono-oxidation probably means the formation of one hydroxyl group.

The product ions derived from dioxidized metabolite $[M+H+O_2]^+$ are shown in Fig. 3b. There are three possibilities for the dioxidation of diphenidine: dioxidation on piperidine (regioisomer *M3*), mono-oxidation on piperidine and on a phenyl ring (regioisomer *M4*), or dioxidation on phenyl rings (regioisomer *M5*). The ions at *m/z* 118.0866 (A+O_2), 103.0543 (B), and 181.1017 (C) are considered to come from regioisomer *M3*, those at *m/z* 102.0911 (A+O), 119.0497 (B+O), 103.0543 (B), and 197.0966 (C+O) from regioisomer *M4*, and those at *m/z* 86.0964 (A), 103.0543 (B), and 213.0910 (C+O_2) from regioisomer *M5* (Table 1). The calculation of the relative ratio of *M4*:*M5*:*M6* was not possible.

The product ions derived from dioxidized dehydrogenated metabolite $[M–H+O_2]^+$ are shown in Fig. 3c. Although the molecular masses agreed with the calculated values (Table 1), assignment of the metabolites was not possible. However, we concluded that the dehydrogenation occurred mainly on piperidine because ions at *m/z* 84.0804 (A−H_2) and 100.0763 (A−H_2+O) were observed clearly, whereas (C−H_2) and (C−H_2+O_2) were not observed. The maximum difference between the observed mass and calculated mass of metabolites of diphenidine and their product ions was only 0.0007 Da, as listed in Table 1.

The present work indicated that oxidation occurred more abundantly on piperidine than on phenyl ring, and a similar tendency was also observed on the oxidized metabolites of pyrrolidinophenone derivatives having a pyrrolidine ring and a phenyl ring [7–9], although piperidine is a six-membered ring whereas pyrrolidine is a five-membered ring. In the case of α-pyrrolidinopropiophenone (PPP), 2″-oxo-PPP (position 2 on pyrrolidine ring) was mainly detected, and 4′-hydroxy-PPP (position 4 on phenyl ring) made up only 10 % of the total metabolites that were extracted from rat urine by solid-phase extraction (SPE) and detected by GC–MS [7]. In the case of oxidized metabolites of α-pyrrolidinovalerophenone (PVP), only 2″-oxo-PVP and 2″-hydroxy-PVP (including 2″-glucuronide) were

extracted from human urine by SPE and detected by LC–MS-MS [8]. In another work on α-pyrrolidinobutiophenone (PBP) and α-PVP, only 2″-oxo-PBP and 2″-oxo-PVP were extracted from human urine by MonoSpin C_{18} treatment and detected by GC–MS [9].

The metabolism of diphenidine has not been reported to date. For confirmation of drugs that are difficult to detect in the unchanged form, knowledge of probable metabolites is prerequisite for proof drug intake. The present work clarified that the oxidation of diphenidine occurred firstly on piperidine ring and secondly on phenyl ring, while monooxidation was observed to occur together with dioxidation. The maximum difference between the observed mass and calculated mass for diphenidine, its metabolites, and their product ions was only 0.0007 Da. Therefore, it is possible to tentatively identify the metabolites with high certainty without standard compounds, although the position of oxidization on each ring cannot be determined.

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

Ethical approval Informed consent was obtained from all individuals included in the study, who supplied about 1 ml each of whole blood for use as blank matrix.

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