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SHORT COMMUNICATION

Determination of new pyrrolidino cathinone derivatives, PVT, F-PVP, MPHP, PV8, PV9 and F-PV9, in human blood by MALDI-Q-TOF mass spectrometry

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Abstract A rapid and sensitive detection method using matrix-assisted laser desorption ionization (MALDI)–quadrupole time-of-flight–mass spectrometry (Q-TOF–MS) was developed for the analysis of six pyrrolidino cathinone derivatives, α-pyrrolidinopentiothiophenone (PVT), 4′-fluoro-α-pyrrolidinopentiophenone (F-PVP), 4′-methyl-α-pyrrolidinohexanophenone (MPHP), α-pyrrolidinoheptanophenone (PV8), α-pyrrolidinooctanophenone (PV9) and 4′-fluoro-α-pyrrolidinooctanophenone (F-PV9), that were newly designated as illegal drugs in Japan. In this method, α-cyano-4-hydroxycinnamic acid was used as the matrix to assist the ionization of cathinones. MS spectra of these cathinones showed a protonated molecule $[M+H]^+$ and iminium, respectively, and hence the main ions of MALDI–MS were a mixture of the main ions detected by liquid chromatography–electrospray ionization–mass spectrometry and that by gas chromatography–electron ionization–mass spectrometry. The quantification of these cathinones was performed using α-pyrrolidinopentiophenone as the internal standard. The limit of detection was 1 ng/ml and the quantification range was 2–100 ng/ml for these cathinones using 20 μl of blood. We encountered a fatal poisoning case where PV9 was abused. The PV9 levels in postmortem blood samples from the right heart, left heart and femoral vein were 198, 209 and 163 ng/ml, respectively. The metabolites of PV9 were detected, and the peak height of an oxidized metabolite was higher than those of the protonated molecule and the other metabolites.

Keywords α-Pyrrolidinooctanophenone (PV9) · Pyrrolidino cathinone derivatives · MALDI–Q-TOF mass spectrometry · α-Cyano-4-hydroxy cinnamic acid · Blood

Introduction

Application of matrix-assisted laser desorption ionization (MALDI)–mass spectrometry (MS) for the determination of four pyrrolidino catinone derivatives was reported in 2013 [1], but soon after, new pyrrolidino cathinone derivatives, α-pyrrolidinopentiothiophenone (PVT) [2], 4′-fluoro-α-pyrrolidinopentiophenone (F-PVP) [3], 4′-methyl-α-pyrrolidinohexanophenone (MPHP) [3–5], α-pyrrolidinoheptanophenone (PV8) [3] and α-pyrrolidinooctanophenone (PV9) [3, 6], were detected in illegal drug market in Japan. Therefore, in the present work, these newly reported cathinones were examined by MALDI–MS, although detections of these catinones by gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS) have been recently reported [2–6]. In the present work, 4′-fluoro-α-pyrrolidinooctanophenone (F-PV9) was also included as an analyte, since it has a structure similar to those of the above five newly reported cathinones [2–6]. These six cathinones were designated as illegal drugs in 2013–2014 in Japan. The present method was successfully applied to samples obtained from a victim intoxicated with PV9. In the present detection system, the delayed extraction voltage was set at 20 V to detect not only cathinones, but also the metabolites of PV9. In addition to the β-oxygen–

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reduced metabolite mentioned previously on PV9 [6] and α-pyrrolidinopentiophenone (PVP) [7, 8], oxidized metabolites were also detected by MALDI–MS.

Materials and methods

Materials

PVP, β-oxygen–reduced metabolite of PVP (OH-α-PVP), PVT, F-PVP, MPHP, PV8, PV9 and F-PV9 were obtained from Cayman Chemicals, Ann Arbor, MI, USA; α-cyano-4-hydroxycinnamic acid (CHCA) from Sigma-Aldrich, St. Louis, MO, USA; acetonitrile (ACN) and methanol that were suitable for LC–MS and other chemicals of analytical grade from Wako Pure Chemicals, Osaka, Japan. Pure water with a specific resistance of 18 MΩ cm was used (Millipore, Bedford, MA, USA). Blood samples from normal subjects under permission were used as blank samples, and those spiked with several amounts of cathinones were used as positive samples. The postmortem blood samples of a victim intoxicated with PV9 were obtained at autopsy performed in our laboratory [6].

Standard solutions

Individual stock solutions of cathinones were prepared separately by dissolving an appropriate amount of each compound in ethanol at 1 mg/ml, respectively, and stored at –20 °C. Working calibration solutions and quality control solutions were prepared daily by diluting the stock solutions with blank blood at 1–100 ng/ml. PVP at 100 ng/ml in blood was used as internal standard (IS) for the determination of six cathinones.

Blood preparation for the assay

One microliter of IS and 59 μl of 0.14 M K_2CO_3 were added to 20 μl of blood placed in a tube (Eppendorf AG, Hamburg, Germany) using a MICROMAN M10 (Gilson S.A.S., Villiers-le-Bel, France), and they were mixed and centrifuged at 10,000 *g* for 2 min. The supernatant (72 μl) was placed in a new tube, 220 μl of 1-chlorobutane (CB) were added, and the contents of the tube were vortex-mixed for 1 min and centrifuged at 10,000 *g* for 2 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, i.e., 0.7 mg CHCA/ml in an ACN-ethanol-aqueous solution (ACN/ethanol/H_2O: 90/5/5, v/v/v). Two microliters of the solution were loaded on a stainless steel MALDI sample target plate (V700666, AB SCIEX, Framingham, MA, USA) and allowed to dry.

Fig. 1 Matrix-assisted laser desorption ionization—mass spectrometry (MALDI–MS) spectrum in each *upper panel* and product ion mass spectrum derived from the protonated molecule with collision voltage at 30 V in each *lower panel* for PVT, F-PVP, MPHP, PV8, PVP and F-PV9 at 20 μg/ml each with the range of spectrum at *m/z* 50–300. The peaks marked with *asterisk* corresponded to the matrix-related ions of α-cyano-4-hydroxycinnamic acid solution

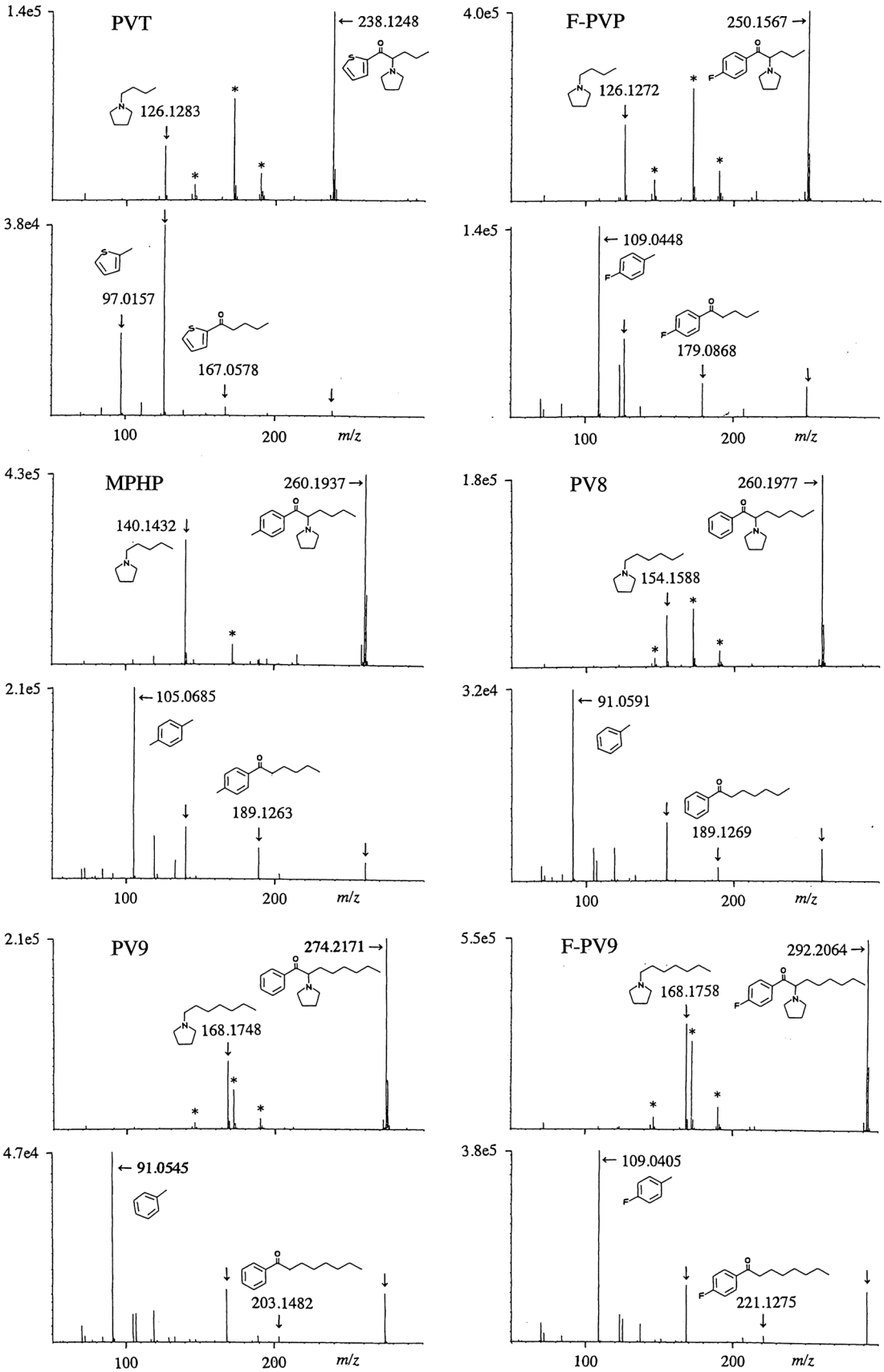
Instrument

A reflector type Q-TOF mass spectrometer, QSTAR Elite Hybrid (AB SCIEX), was used in the positive ion mode. The 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 a maximum of 100 samples on it was inserted into the machine. Mass spectra were obtained using 355 nm radiation from an Nd:YAG laser system (AB SCIEX). The pulse energy of the excitation was set at 3.4 μJ with the delayed extraction voltage at 20 V. In MS–MS detection, the collision voltage was set at 30 V. The instrument was equipped with a video camera for displaying the sample image on a monitor and permitting the laser to be focused on a specific well within the area of the target. Spectra were obtained with 20 cycles at 100 Hz for the detection of a cathinone derivative at 1–100 ng/ml. Only a part of a sample in a 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.

Results and discussion

Instrumental optimizations in MALDI–MS detection of cathinones and their metabolites

Cathinone and its metabolites in blood were extracted and detected together. In the present detection, the laser energy for excitation was set at 3.4 μJ, with a delayed extraction voltage at 20 V to detect the metabolites of cathinones, because the detection of metabolites of cathinones was quite difficult under our previous instrumental condition, i.e., laser energy of 8.2 μJ with a delayed extraction voltage of 60 V [1]. In ionizations such as electrospray ionization (ESI), electron ionization (EI) and chemical ionization used in LC–MS or GC–MS, the initial speeds of various ions in a sample were the same. On the contrary, the initial speeds of various ions in a sample were different in the laser desorption ionization, and hence the delayed extraction (voltage) was used to adjust them [9]. In the present work on PV9 and its metabolites, when the delayed extraction voltage was changed from 20 V to 30 V , the peak height of [M+H] became 0.8 times that of the original, and the peak heights of the three metabolites [M+H_3], [M+H+O] and [M+H_3+O] became 0.3 times


1.4e5
PVT
← 238.1248
126.1283
3.8e4
97.0157
167.0578
100
200
m/z
4.0e5
F-PVP
250.1567 →
126.1272
1.4e5
← 109.0448
179.0868
100
200
m/z
4.3e5
MPHP
260.1937 →
140.1432
2.1e5
← 105.0685
189.1263
100
200
m/z
1.8e5
PV8
260.1977 →
154.1588
3.2e4
← 91.0591
189.1269
100
200
m/z
2.1e5
PV9
274.2171 →
168.1748
4.7e4
← 91.0545
203.1482
100
200
m/z
5.5e5
F-PV9
292.2064 →
168.1758
3.8e5
← 109.0405
221.1275
100
200
m/z

those of the originals, whereas the peak height of [M+H+O–H_2O] became twice the original. Because 20 V was most suitable for the detection of the protonated molecule [M+H] as well as the three metabolites [M+H_3], [M+H+O] and [M+H_3+O], the voltage was adopted in the following work. This drastic decrease (0.3 times the original) observed in the three metabolites according to the increase of the voltage from 20 to 30 V was also observed in the standard sample of OH-α-PVP obtained from Cayman Chemicals, and these facts suggested some structural similarity amongst these four metabolites. Although the increase of the energy level from 3.4 to 8.2 μJ enhanced the signals, it also enhanced noises, and hence an energy level of 3.4 μJ was adopted in the following work.

Single mass spectra and product ion mass spectra obtained by MALDI

Two microliters of each CHCA solution containing cathinones at 20 μg/ml were placed in target wells and the spectrum was detected by MALDI–MS, as shown in the upper panel of each compound in Fig. 1, where two kinds of ions, i.e., a protonated molecule and an iminium, were observed respectively. Therefore, MPHP and PV8 could be identified from the respective iminiums by MALDI–MS, even though their protonated molecules showed the same *m*/*z* values. The main ions of the cathinones in LC–ESI–MS were the protonated molecules [2, 3, 6], whereas those in GC–EI–MS were iminium ions [2–4, 6], and hence the main ions in MALDI–MS were a mixture of the main ion in LC–ESI–MS and that in GC–EI–MS.

The protonated molecules in MALDI–MS were selected as the precursor ions, and the spectra of MALDI–MS–MS at a collision voltage of 30 V were shown in lower panels in Fig. 1, where *m*/*z* values and schematic structures were described for the two ions that were not described in upper panel spectra. In the present work, schematic structures mean that some structures should contain a proton in the figure where the *m*/*z* value increased by 1, and/or some single or double bond in the figure should be a double or a triple bond, respectively, where the *m*/*z* value decreased by 1.

Reliability of the method

A calibration curve was prepared by spiking a cathinone at 0, 2, 10 or 100 ng/ml to the blank blood containing IS at 100 ng/ml ($n = 6$) as described in Table 1, where x was the concentration of the cathinone in ng/ml and y was the relative value of the peak height of the cathinone to that of IS. Precision and accuracy were assessed by analyzing blood spiked with the cathinone at 2, 10 or 100 ng/ml three times a day, as well as on three different days, and values are listed in Table 2. The coefficient of variation was < 16.1 % and the accuracy was 85.3–121 % for intraday and interday variations. Therefore, 2–100 ng/ml could be considered as the quantification range of the six cathinones. Figure 2 indicated the spectra of six cathinones at 1 ng/ml each in blood in the upper panels and the blank spectra in lower panels, respectively, where only a narrow range, i.e., width of 1 Da was indicated there, because the line width of signal was ca. 0.026 Da for each of these cathinones, respectively. The signal-to-noise ratio was three for PVT, F-PVP and F-PV9 and about ten for MPHP, PV8 and PV9, respectively.

Table 1 Regression equations for six cathinones

	Equation	Correlation coefficient
PVT	$y = 0.008174\ x + 0.000798$	0.995
F-PVP	$y = 0.013624\ x + 0.005176$	0.994
MPHP	$y = 0.030190\ x + 0.003602$	0.997
PV8	$y = 0.017607\ x + 0.001752$	0.994
PV9	$y = 0.033724\ x + 0.004023$	0.995
F-PV9	$y = 0.016312\ x + 0.001274$	0.992

Table 2 Intraday (3 times) and interday (3 days) accuracy/precision of cathinones spiked into blood

Spiked (ng/ml)	Intraday		Interday	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
PVT				
100	99.8	11.9	100	1.2
10	100	3.6	98.8	4.2
2	93.0	6.1	103	10.2
F-PVP				
100	97.8	11.3	93.0	5.8
10	100	7.1	104	4.5
2	101	8.2	121	14.3
MPHP				
100	98.5	1.5	102	8.6
10	98.5	3.8	98.1	2.4
2	96.6	4.5	97.4	7.0
PV8				
100	108	5.4	92.5	4.7
10	102	6.0	95.0	8.2
2	106	16.1	101	12.3
PV9				
100	107	5.6	93.5	5.8
10	102	13.6	89.7	10.3
2	85.3	4.3	86.1	6.3
F-PV9				
100	94.8	9.2	105	9.5
10	98.7	5.6	99.6	2.9
2	96.3	8.5	96.5	3.9

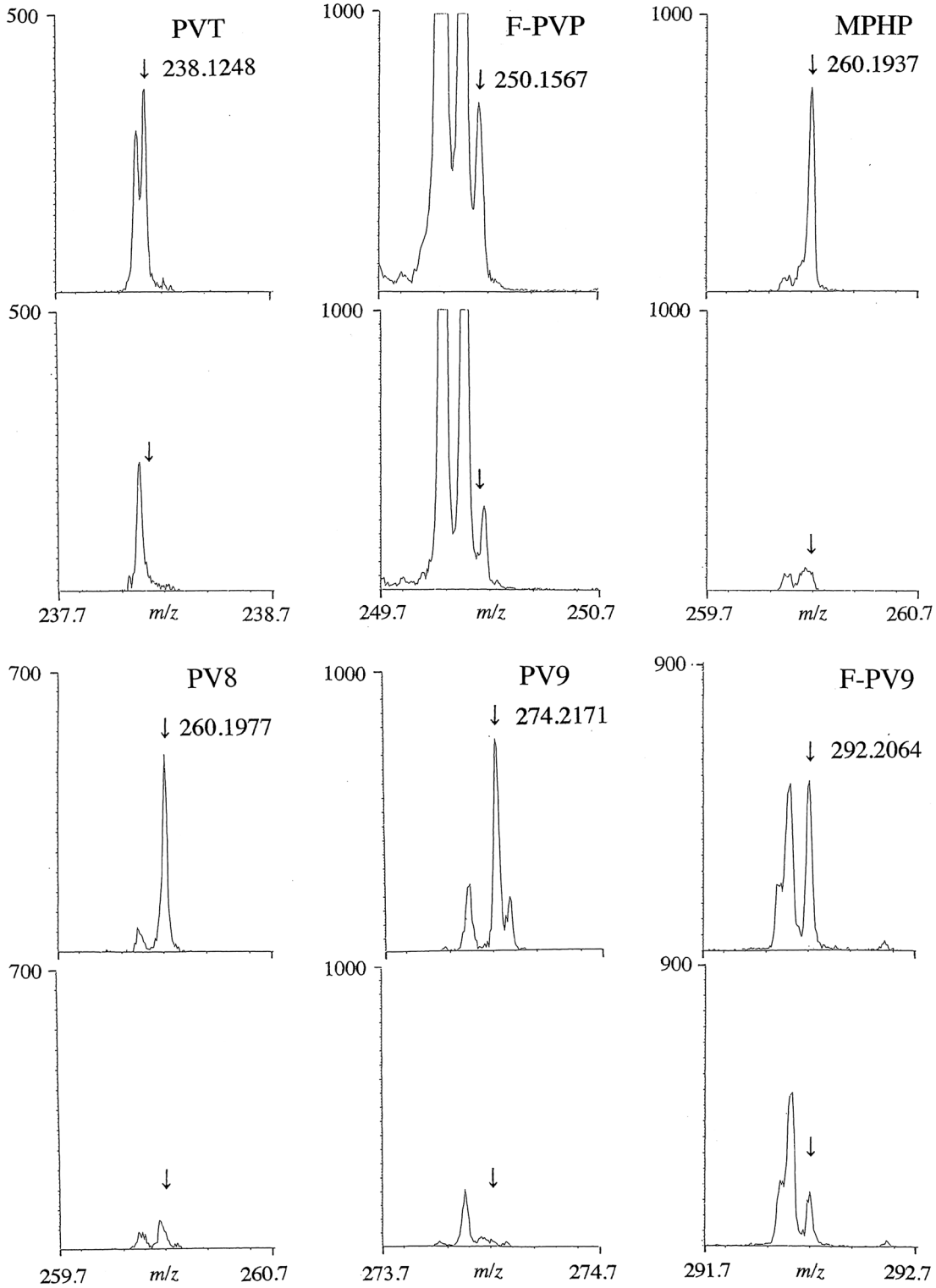


Fig. 2 MALDI–MS spectra of six cathinones at 1 ng/ml each. Blood spiked with each cathinone at 1 ng/ml was shown in each *upper panel* spectrum and blank blood was shown in each *lower panel* spectrum. The ranges of spectra were *m/z* 237.7–238.7 for PVT, *m/z* 249.7–250.7 for F-PVP, *m/z* 259.7–260.7 for MPHP, and *m/z* 259.7–260.7 for PV8, *m/z* 273.7–274.7 for PV9, and *m/z* 291.7–292.7 for F-PV9

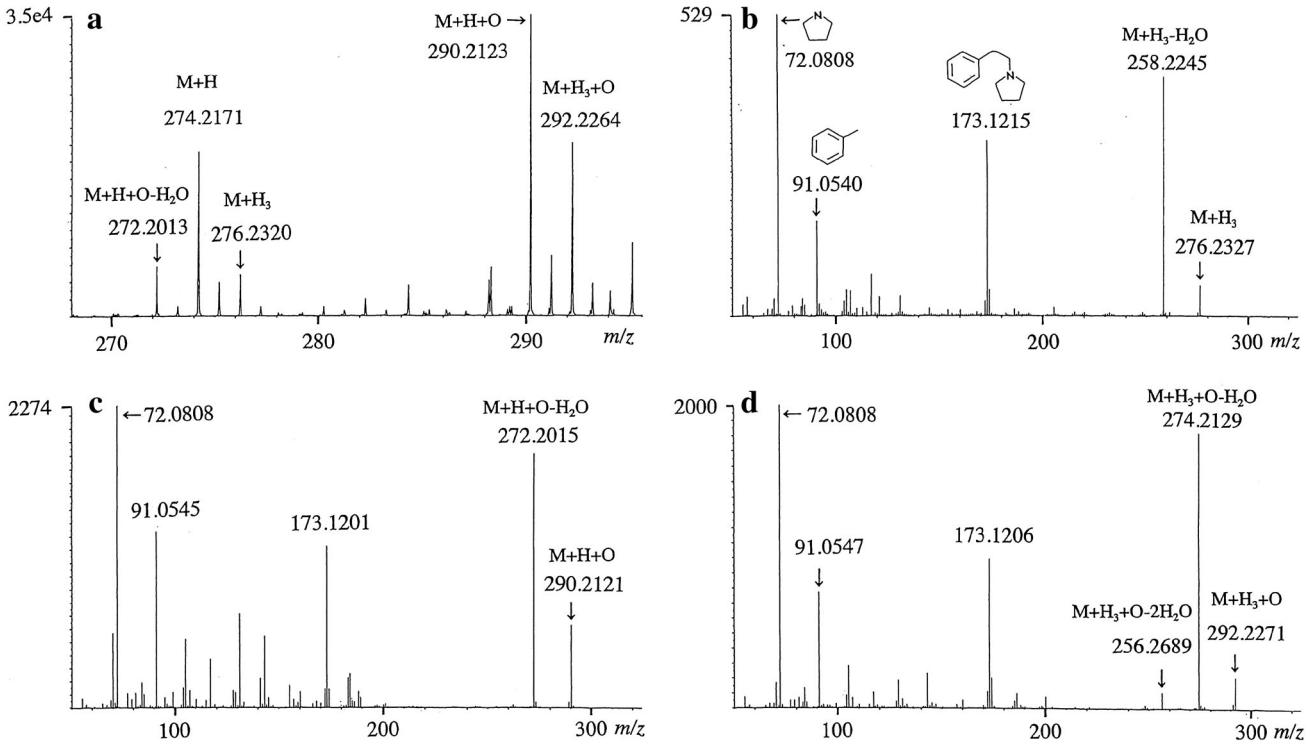


Fig. 3 MALDI–MS spectrum of the blood in the right heart of a victim intoxicated with PV9 (**a**), as well as the product ion spectrum from $[M+H_3]$ at *m/z* 276.2327 (**b**), that from $[M+H+O]$ at *m/z* 290.2121 (**c**) and that from $[M+H_3+O]$ at *m/z* 292.2271 (**d**), obtained from the same blood sample as **a**. Here, M stands for PV9

The recovery was calculated by comparing the peak height obtained from the extract of spiked blood with the peak height obtained from the standard cathinone dissolved in the CHCA solution. The recoveries of PVT, F-PVP and MPHP were 80–100 %, whereas those of PV8, PV9 and F-PV9 were 70–90 %, respectively, at 2–100 ng/ml.

Practical application

The PV9 levels of blood samples in the right heart, left heart and femoral vein were determined ($n = 4$) to be 198 ± 15, 209 ± 5 and 163 ± 7 ng/ml, respectively. The MALDI–MS spectra of blood in the right heart of a victim shown in Fig. 3a indicated not only the protonated molecule, but also four ions related to PV9. The sum of the peak heights of the four ions was more than three times that of the protonated molecule. Most metabolites are more hydrophilic than the original molecule, and hence the ionization efficiency of metabolites is estimated to be lower than that of the original molecule. Therefore, the original concentration of PV9 in blood may be higher than four times that of protonated molecule. The observed *m/z* values and (calculated ones) of five ions related to PV9 were as follows; $[M+H+O–H_2O]$ at *m/z* 272.2013 (272.2014), $[M+H]$ at *m/z* 274.2171 (274.2170), $[M+H_3]$ at *m/z* 276.2320 (276.2327), $[M+H+O]$ at *m/z* 290.2123 (290.2120) and $[M+H_3+O]$ at *m/z* 292.2264 (292.2271). The maximum difference between the observed *m/z* value and calculated one was 0.0007.

The MALDI–MS–MS spectra of the three metabolites with the collision voltage at 30 V are shown in Fig. 3b–d by selecting the ions at 276.2327, 290.2121 and 292.2271 as the precursor ions, respectively. The main fragment ions derived from the β-oxygen reduced metabolite ion $[M+H_3]$ were its dehydrated ion $[M+H_3–H_2O]$ at *m/z* 258.2245 [6] as well as ions at *m/z* 173.1215, 91.0540 and 72.0808.

$[M+H+O]$ at *m/z* 290.2123 and $[M+H_3+O]$ at *m/z* 292.2264 were the oxidized and oxidized-reduced metabolites of PV9, respectively. Figure 3c indicates that the observed ion at *m/z* 272.2013 in Fig. 3a was the dehydrated product ion derived from $[M+H+O]$. When PV9 is oxidized, there are roughly three positions that oxygen attaches to; that is, either a benzene ring, pyrrolidine ring or alkyl branch. When benzene ring was oxidized, the fragment ions at *m/z* 173.1215 and 91.0540 should be oxidized. When pyrroridine ring was oxidized, the fragment ions at *m/z* 173.1215 and 72.0808 should be oxidized. Because the main fragment ions from $[M+H+O]$ in Fig. 3c and those from $[M+H_3+O]$ in Fig. 3d were observed at *m/z*

173.1215, 91.0540 and 72.0808, oxygen may had been attached to the alkyl branch in [M+H+O] and [M+H_3+O], respectively.

Conclusions

There have not been many researches on low mass molecules by MALDI–MS, although high mass molecules have been well studied by MALDI–MS. Therefore, the suitable conditions for the detection of small molecules by MALDI–MS have yet to be thoroughly examined. In the present work, we found that the delayed extraction voltage, which is only used in laser desorption ionization, is an important factor for the sensitive detection of small molecules by MALDI–MS.

The identification of metabolites is a difficult task because standard samples of various metabolites are difficult to obtain. The present work shows that MALDI–TOF–MS is one of the suitable methods for the identification of metabolites, because it can give precise molecular masses to identify them without the use of standard samples. The maximum difference between the observed molecular mass and the calculated one for the three metabolites of PV9 was only 0.0007 Da.

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Conflict of interest The authors state that they have no financial or other relations that could lead to a conflict interest.

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