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journal or publication title	Forensic Toxicology
volume	32
number	1
page range	169-175
year	2014-01
URL	http://hdl.handle.net/10271/2912

doi: 10.1007/s11419-013-0212-7

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Abstract A rapid and sensitive detection method using matrix-assisted laser desorption ionization (MALDI) – time-of-flight (TOF) – mass spectrometry (MS) was developed for the analysis of four pyrrolidino cathinones, such as α -pyrrolidinopropiophenone (PPP), 4'-methyl- α -pyrrolidinopropiophenone (MPPP), α -pyrrolidinobutiophenone (PBP), and α -pyrrolidinovalerophenone (PVP). In this method, α -cyano-4-hydroxy cinnamic acid was used as the matrix to assist the ionization of cathinones. Each MALDI-TOF-MS spectrum of these cathinones showed not only protonated molecular ion $[M + H]^+$, but also several fragment ions having comparable intensities to that of $[M + H]^+$. Hence MPPP and PBP could be clearly discriminated by the mass spectra alone, although they have almost the same mass numbers in their protonated molecular ions. The quantification of MPPP, PBP or PVP was performed using PPP as internal standard, respectively, and that of PPP was performed using PBP as internal standard. The limit of detection was 1 ng/ml, and the quantification range was 2 – 200 ng/ml for the four cathinones using 20 μ l of blood. In a fatal poisoning case in which PVP was abused, the PVP levels in whole blood samples obtained from the right heart, left heart and femoral vein were 0.597, 0.635 and 0.580 μ g/ml, respectively. We recommend the MALDI-TOF-MS method without any chromatography for both identification and quantification of the pyrrolidino cathinones in various matrices in forensic toxicological analysis, because of the simplicity, rapidness and reliability of the method.

Keywords α -Pyrrolidinovalerophenone (α -PVP) • Pyrrolidino cathinones • Designer drugs • MALDI-TOF-mass spectrometry • α -Cyano-4-hydroxy cinnamic acid • Blood

Introduction

Cathinone is a beta-ketone amphetamine analogue contained naturally in the shoots and leaves of the *Catha edulis Forsk* [1], and its various derivatives have recently been synthesized and widely distributed on illegal drug markets. Because these drugs induce psychoactive, central nervous stimulation like amphetamines, governments of most countries have controlled the use, supply and possession of them, but new drugs appear to circumvent the current controls. In 2009 the most abused derivative of cathinone was 4-methylmethacathinone in Japan [2], and afterwards derivatives of cathinone having pyrrolidine ring also became to be abused. For the detection of these cathinones, high-performance liquid chromatography (HPLC) [3], gas chromatography (GC)-mass spectrometry (MS) [4-9] and liquid chromatography (LC)-MS (-MS) [10-15] were used currently. Fragment ions were mainly observed in GC-electron ionization (EI)-MS, whereas protonated molecular ions $[M+H]^+$ were mainly observed in GC-chemical ionization (CI)-MS and LC-electrospray ionization (ESI)-MS. In GC-EI-MS, different molecules having the same branch structure sometimes give the same signal of the branch, and hence they were identified after the chromatographic separation [8]. In some LC-ESI-MS(-MS) analyses the tandem MS method was applied to determine the structure of a molecule more precisely as well as to increase the sensitivity of its detection [11,12,14,15].

Matrix assisted laser desorption ionization (MALDI) is one of the ionization methods that use the energy of light for the ionization where the energy is absorbed by matrix substances firstly and then the energy is transferred to the analytes to be excited. MALDI-MS has usually applied to the analysis of high-mass biomolecules [16].

Matrices themselves show strong signals at around m/z 100-300, and hence these signals of matrices were assumed to interfere with the analysis of low-mass molecules having signals at m/z 100-300. We noticed, however, that the interferences from matrices could be avoided by MALDI-time-of-flight (TOF)-MS more often than being assumed [17]. Two signals differing only 0.08 Da could be discriminated by MALDI-TOF-MS, because the line width of signals was less than 0.03 Da [17]. Therefore, in the present work, we tried the application of MALDI-TOF-MS method for the detection of four pyrrolidino cathinones, such as α -pyrrolidinopropiophenone (PPP), 4'-methyl- α -pyrrolidinopropiophenone (MPPP), α -pyrrolidinobutiophenone (PBP) and α -pyrrolidinovalerophenone (PVP), because the abuse by this group of cathinones is now widespread worldwide and causing serious social problems.

Liquid-liquid extraction (LLE) [3, 4, 10-12, 15] and solid phase extraction (SPE) [5,6,8,9,13,14] were performed to eliminate large amounts of interfering substances contained in biological samples. Comparing the results after LLE with those after SPE, they were equivalent in terms of recovery, linearity and accuracy. In the present work, LLE was adopted for the purification of cathinones in human fluids, because of its rapidness, low cost and applicability to small amounts of specimen.

Case history

A man in his 40s acted violently in a rented room, and hence was reported to the police by the owner of the room. The police arrived there but the man continued acting violently as well as tried to fall down from his room on the third floor. Firemen and

paramedical men also arrived there. After about two hours, his physical condition was suddenly deteriorated, and his cardiopulmonary arrest was observed. He was brought to a hospital, but was pronounced dead at the hospital. Autopsy was performed in our laboratory about two days after his death.

Materials and methods

Materials

PPP, MPPP, PBP and PVP were obtained from Cayman Chemicals, Ann Arbor, MI, USA; α -cyanono-4-hydroxy cinnamic acid (CHCA), trifluoroacetic acid and angiotensin I from Sigma-Aldrich, St. Louis, MO, USA; acetonitrile (ACN) and methanol that were suitable for LC-MS(-MS), and other chemicals of analytical grade were obtained from Wako Pure Chemicals, Osaka, Japan. Pure water with a specific resistance of $18\text{ M}\Omega \cdot \text{cm}$ was used (Millipore, Bedford, MA, USA). Blood samples from healthy subjects under their permission with informed consent were used as blank samples, and those spiked with several amounts of cathinones were used as quality control samples.

Standard solutions

Individual stock solutions of cathinones were prepared separately by dissolving an appropriate amount of each compound in ethanol at 1 mg/ml and stored at $-20\text{ }^{\circ}\text{C}$, respectively. Working calibration solutions and quality control solutions were prepared

daily by diluting the stock solutions with blank blood at 1 – 200 ng/ml. PPP at 100 ng/ml in blood was used as internal standard (IS) for the determination of MPPP, PBP or PVP, and PBP at 100 ng/ml in blood was used as IS for the determination of PPP.

Extraction procedure

To 20 µl of human whole blood with or without addition of a target cathinone in a tube (Eppendorf AG, Hamburg, Germany), 1 µl of IS at 2 µg/ml, 51 µl of water, and 8 µl of 1 M K₂CO₃ were added. A 200 µl aliquot of 1-chlorobutane (CB) was added to the solution and vortex-mixed for 1 min, centrifuged at 10,000 g for 3 min, and upper 160 µl of the CB layer was transferred to another tube and evaporated to dryness (it took less than 8 min) at room temperature using a centrifugal dryer (Savant Speed Vac Plus SC110A; Savant, Holbrook, NY, USA). The residue was reconstituted with 16 µl of CHCA-solution prepared, i.e., 0.7 mg CHCA/ml in an ACN-ethanol-aqueous solution (ACN/ethanol/H₂O, 85:5:10, 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.

Instrument and its conditions

A reflector type QTOF 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 5ppm. A stainless steel MALDI target plate with maximum 100 samples on it was inserted into the machine. Mass spectra were obtained using 355-nm

radiation from Nd: YAG laser system (AB SCIEX). 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 at 2 – 200 ng/ml and 100 cycles at 100 Hz and 1 ng/ml, i.e., the limit of detection (LOD), respectively. Only a part of a sample on a well was consumed in the detection. Mass calibration was conducted using ions at m/z 172.0393 and m/z 1296.6848 produced from CHCA and angiotensin I, respectively.

Results and discussion

Optimizations for MALDI-TOF-MS analysis of cathinones

The concentrations of CHCA were varied at 0, 0.1, 0.2, 0.3, 0.4, 0.8, 1, 1.5 and 2 mg/ml in 50 – 90 % ACN solution. The 0.4 – 1.0 mg CHCA/ml in 85 % ACN solution gave the highest signals of cathinones. When ethanol was added at 5 % and H₂O was reduced to 10 %, a deposit of sample evaporated on target plate became more homogeneous and the signals of cathinones increased by 10 %. Therefore, 0.7 mg CHCA/ml in the solution of ACN/ethanol/H₂O (85:5:10, v/v/v) (named CHCA solution here after) was used in the present work. When CHCA was absent, the signal of cathinones was not observed at all, and hence it could be said that CHCA contributed greatly to the ionization of cathinones. Although trifluoroacetic acid often enhanced signals in MALDI detections, it did not work in the present case, and was not used. 2,5-Dihydroxybenzoic acid, a commonly used matrix substance in MALDI, was not used in the present assay, because the signals of cathinones with the matrix were < 5 % of those with CHCA.

MALDI-single TOF and MALDI-tandem quadrupole (Q)-TOF mass spectra of cathinones

Two microliters of CHCA solution containing the cathinones at 50 µg/ml each were placed on a target well and detected by MALDI-single stage TOF-MS as shown in Fig. 1 (a in each panel). The accurate m/z values of major ions observed were shown in the figure. The protonated molecular ions in the spectra were selected as the precursor ions, and the results of MALDI-tandem quadrupole (Q)-TOF mass spectra (product ion mass spectra) at collision voltage of 30 V are also shown in Fig. 1 (b in each panel); the mass numbers of fragment peaks appearing in the tandem mass spectra were the same as those found in the single-stage mass spectra. The estimated structures of major ions were described in the panels of the tandem mass spectra. Each structure should contain a proton; the m/z value increased by 1 should be observed, and/or a single or double bond shown in the figure should be a double or a triple bond, where the m/z value decreased by 1.

We also tested ESI-tandem Q mass spectra for cathinones using a different type of an instrument. The mass numbers of the fragment peaks were essentially the same as those found in the present study (unpublished observation).

Extraction and detection of cathinones in whole blood

The extraction efficiencies were compared by changing the amounts of alkalizing agents such as K_2CO_3 and NH_4OH as well as the amounts of extractors such as methyl isobutyl ketone, butyl ether and CB, respectively. The detection was made by MALDI-TOF-MS. The condition, 52 µl of water, 8 µl of 1 M K_2CO_3 and 200 µl of CB, was found to be most suitable for 20 µl of blood. When we tried to collect the CB layer completely, aqueous layer was partially collected together with CB layer in some occasions, and hence 160 µl of CB out of 200 µl of CB was collected (80 % of total amount collected). When the CB extract itself was mixed with the CHCA solution, they could not make

uniform deposit on the MALDI target due to hydrophobicity of CB. Therefore, the CB was eliminated by evaporation. Then the residue was dissolved with 16 μ l of CHCA solution to adjust the concentration of cathinone to its original concentration in blood, because 80 % of the CB extract was collected as residue. The recovery was calculated by comparing the peak height obtained from the extract of spiked blood with the peak height obtained from the standard cathinones dissolved in the CHCA solution. The recoveries of cathinones at 2 – 200 ng/ml blood were in the range of 85 – 100 %. No interfering signals from impurities in blood were observed for the cathinone analysis.

Reliability of MALDI-TOF-MS method

Each calibration curve was prepared by spiking cathinones at 2, 20 or 200 ng/ml to blank blood containing IS at 100 ng/ml ($n = 6$) as described in Table 1, where x was the concentration of cathinone in ng/ml and y was the relative value of the peak height of the cathinone to the peak height of IS. Precision and accuracy were assessed by analyzing blood spiked with cathinones at 2, 20 or 200 ng/ml three times a day as well as on three different days, and they were listed in Table 3. The coefficient of variation was <19 % and the accuracy was 84 – 107 % for intraday and interday variations. Therefore 2 – 200 ng/ml could be considered as the quantification range, and the limit of quantification (LOQ) was 2 ng/ml in blood. The LOD ($S/N = 3$) was 1 ng/ml for four cathinones as shown in Fig. 2. Only a narrow range, i.e., width of 1 Da was indicated there, because the line width of signal was ca. 0.024 Da for four cathinones, respectively. Figure 2 also indicated that the signals of matrix at m/z 100 – 300 do not interfere with the detection of four cathinones even at 1 ng/ml due to high mass resolution of MALDI-TOF-MS, although some signals of matrix appeared very strongly at m/z 100 – 300.

Comparison of LODs and LOQs in several works

Several works have been performed not only for detection of derivatives of cathinones and their metabolites but also for quantification of them in biological samples. The LOD was 39 ng/ml using 0.5 ml of plasma (the absolute amount of the cathinone required for the detection: 20 ng) in an HPLC work [3]. The LODs were 100 ng/ml using 0.5 ml of urine (absolute amount: 50 ng) [5], and 1 ng/ml using 0.5 ml of blood (absolute amount: 0.5 ng) [13] in two GC-MS works. The LOD was 0.5 ng/ml using 0.05 ml of blood (absolute amount: 0.025 ng) in an SPME-GC-MS work [8]. The LODs were 0.01–0.1 ng/ml using 1 ml of serum (absolute amounts: 0.01–0.1 ng) in an LC-MS-MS work [14]. Some works described only LOQs; they were 100 ng/ml using 0.05 ml of urine (absolute amount: 5 ng) [10], 10 ng/ml using 0.1 ml of urine (absolute amount: 1 ng) [12] and 5 ng/ml using 1 ml of blood (absolute amount: 5 ng) [15] in three LC-MS-MS works. In summary, the absolute amounts of cathinones required at LODs and LOQs in the above previous works were 0.01–50 ng and 1–5 ng, respectively. The present MALDI-TOF-MS method can be considered as one of the most sensitive ones, because the absolute amounts of cathinones required at LOD and LOQ in the present study were 0.02 ng (1 ng/ml using 0.02 ml of blood) and 0.04 ng (2 ng/ml using 0.02 ml of blood), respectively.

Practical application

The above established method was applied to actual whole blood samples obtained from a fatal poisoning case. For the quantification of PVP in whole blood samples, PPP was added as IS to each sample to normalize the intensities of signals. Blood samples obtained from the right ventricle, left ventricle and femoral vein were diluted 10-fold with water before quantification. As results, the PVP levels were determined to be 0.597,

0.635 and 0.580 µg/ml in the poisoning case, respectively.

Conclusions

The mass spectral profiles obtained by MALDI-TOF-MS for each of four pyrrolidino cathinones were unique. They looked like that the spectrum of ESI-MS and that of ESI-MS-MS were combined; the protonated molecular ion as well as some fragment ions could be observed with similar intensities, and hence MPPP and PBP having almost the same molecular mass could be identified on the basis of their fragment ions. MALDI is moderately tolerant to salts, and hence the sample preparation required in the present work was only liquid-liquid extraction of four pyrrolidino cathinones that have been recently disclosed in Japan [2]. Therefore, the MALDI-TOF-MS method is recommendable for both identification and quantification of pyrrolidino cathinones in various matrices in forensic toxicology, because of its simplicity, rapidness and reliability. This is the first trial to identify and quantify cathinones in human samples by MALDI-TOF-MS to our knowledge

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

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Figure captions

Fig. 1 Matrix assisted laser desorption ionization (MALDI)-single stage time-of-flight (TOF) mass spectra (**a**) and MALDI-tandem quadrupole-TOF mass spectra with collision voltage of 30 V (**b**) for four pyrrolidino cathinones in whole blood at 50 μ g/ml

each. The mass numbers of peaks observed in the tandem mass spectra (**b**) are the same as those described in the upper panels (**a**). *PPP* α -pyrrolidinopropiophenone; *MPPP* 4'-methyl- α -pyrrolidinopropiophenone; *PBP* α -pyrrolidinobutiophenone; *PVP* α -pyrrolidinovalerophenone

Fig. 2 MALDI-MS spectra at the limit of detection. The ranges of spectra were m/z 203.65 – 204.65 for PPP (**a**), m/z 217.65 – 218.65 for MPPP (**b**), m/z 217.65 – 218.65 for PBP (**c**) and m/z 231.65 – 232.65 for PVP (**d**). Whole blood spiked with each cathinone at 1 ng/ml (the limit of detection) is shown in each *upper* panel spectrum and blank whole blood was shown in *lower* panel spectrum.

Table 1 Regression equations for four cathinones in whole blood for the present method

	Regression equation	Correlation coefficient
PPP	$y = 0.018458 x + 0.007313$	0.999
MPPP	$y = 0.013874 x + 0.001050$	0.997
PBP	$y = 0.005418 x + 0.001122$	0.996
PVP	$y = 0.005039 x + 0.001872$	0.994

Each regression equation was obtained as a mean of six determinations with four plots of a cathinone at different concentrations. *PPP* α -pyrrolidinopropiophenone, *MPPP* 4'-methyl- α -pyrrolidinopropiophenone, *PBP* α -pyrrolidinobutiophenone, *PVP* α -pyrrolidinovalerophenone

Table 2 Intraday and interday accuracy and precision for
measurments of cathinones spiked into blank whole blood for the present method

Spiked (ng/ml)	Intraday ($n = 3$)		Interday ($n = 3$)	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
PPP				
2	94.1	3.6	97.1	7.5
20	107.0	8.4	95.4	7.2
200	98.8	3.1	101.3	1.3
MPPP				
2	100.4	5.0	103.5	10.2
20	103.9	5.6	97.4	10.4
200	95.7	4.7	105.1	4.9
PBP				
2	83.8	7.4	105.5	15.9
20	104.8	18.6	94.7	11.3
200	103.2	4.3	96.2	9.5
PVP				
2	98.7	9.5	105.3	12.4
20	99.8	3.0	99.3	4.8
200	102.8	10.6	99.5	2.7

PPP

MPPP

PBP

PVP