CASE REPORT

A fatal case involved in pyrethroid insecticide ingestion: quantification of tetramethrin and resmethrin in body fluids of a deceased by LC–MS/MS

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Abstract

Purpose The quantification of parent molecules of pyrethroids tetramethrin and resmethrin in human specimens by a mass spectrometry (MS) technique has not been reported yet. A woman in her 60s was found dead in a wasteland. At the scene, an empty beer can and a spray for insecticides containing tetramethrin and resmethrin were found. Therefore, the concentrations of tetramethrin and resmethrin in postmortem specimens and the methanol solution used for rinsing the inside of the beer can were determined using liquid chromatography (LC)–tandem mass spectrometry (MS/MS).

Methods The quantification method by LC–MS/MS for intact parent molecules of tetramethrin and resmethrin in whole blood and urine has been devised and validated in this work. The method was applied to the quantification of tetramethrin and resmethrin in whole blood, urine and stomach contents obtained from a cadaver at autopsy.

Results The limits of detection of tetramethrin and resmethrin were 0.06 and 0.03 ng/mL; limits of quantification were 0.2 and 0.1 ng/mL in blood and urine, respectively. The concentrations of tetramethrin of the deceased were 11.1 ± 1.2 and 0.425 ± 0.017 ng/mL for stomach contents and urine, respectively; the concentration of resmethrin in stomach contents was 1.77 ± 0.18 ng/mL. The tetramethrin and resmethrin were unstable in blood and urine at room temperature; they should be kept at not higher than 4 ℃.

Conclusions To our knowledge, this is the first report for quantification of unchanged tetramethrin and resmethrin in human specimens obtained in a fatal case.

Keywords Tetramethrin · Resmethrin · Pyrethroid insecticides · Authentic human specimens · Fatal case · LC–MS/MS

Introduction

The pyrethroid insecticides are about 2300 times more toxic to insects as compared to mammals, making them preferred and approved insecticides for household use [1, 2]. They are considered to be very safe insecticides for humans; the fatal cases due to pyrethroid exposure are very rare [3]. The symptoms after pyrethroid ingestion in humans are usually nausea, vomiting, abdominal pain and others [1, 2]. There are many kinds of pyrethroids such as tetramethrin (TM), resmethrin (RM), deltamethrin, permethrin, fenpropathrin and cypermethrin after the modification of the basic structure of natural pyrethrins. The acceptable daily intake for humans varies between 0.01 and 0.05 mg/kg body weight per day for deltamethrin and permethrin, respectively [2].

A gas chromatography (GC)–mass spectrometry (MS) detection method after liquid-liquid (L-L) extraction for 13 pyrethroids in human blood was reported in 2004 [4], and it was applied to 45 real blood specimens obtained from the population exposed continuously to pyrethroids, but all blood specimens showed the peaks below the limit of quantification (LOQ) and could not be quantified. In 2018, GC–tandem mass spectrometry (MS/MS) method with ultrasound-assisted dispersive L-L microextraction was developed for 9 pyrethroids in blood [5]. The method was

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applied to two blood specimens obtained from two subjects who were rescued without significant harm; they showed 82 and 91 ng/mL of fenpropathrin, respectively.

The metabolites of pyrethroids were used for the quantification of pyrethroids in human urine specimens because intact pyrethroids are hydrolyzed rapidly [2, 6]. GC–MS was used for the determination of the metabolites such as *trans*-chrysanthemumdicarboxylic acid (CDCA), 3-phenoxybenzoic acid (3-PBA) and others, that were esterified with hexafluoroisopropanol [2, 6]. The maximum concentrations of CDCA and 3-PBA were 54 and 26 ng/mL, respectively, in urine specimens from 30 persons who had used commercially available vaporizers or sprays containing pyrethrum [2], and the maximum concentration of 3-PBA was 3.0 ng/mL in urine specimens obtained from 132 persons who were not exposed to pesticides occupationally [6].

In the above works, however, the parent molecule could not be specified. That is, not parent ions but product ions were used in the detection by GC–MS with electron ionization mode. The metabolites cannot specify parent molecules; CDCA was produced from TM, RM and others, and 3-PBA, from deltamethrin, permethrin, cypermethrin and others [2, 6].

Liquid chromatography (LC)–MS/MS is also known as a suitable method to detect intact parent molecules [7, 8]. Cypermethrin in plasma specimens from 396 persons was tried to quantify in 2011 by online solid-phase extraction coupled with LC–MS/MS, and showed the maximum level at 4.8 ng/mL, and their major exposure pathways were attributed to dietary intakes of vegetables and fruits [7]. However, none of these people were appreciably poisoned. The detection method of seven pyrethroids in rat plasma was reported in 2016, in which the oral administration of cypermethrin in rats was examined, and the maximum concentration in plasma was detected after 1 h [8].

In the present study, a sensitive quantification method for TM and RM (shown in Fig. 1) in whole blood and urine was devised using LC–MS/MS, and was applied to the determination of TM and RM in authentic human specimens obtained from a cadaver. Concerning the detection of TM and RM by LC–MS/MS, only detection method models were reported for TM and RM spiked into animal muscle, liver

and egg [9, 10]. In these works, the LOQs were 11.7 and 2.0 ng/g for RM in bovine muscle [9] and for TM in egg [10], respectively. Of course, there have been reports neither on the fatal case due to TM and/or RM exposure nor on actual quantifications of TM and RM in human specimens.

Case history

A woman in her 60s was found dead in a wasteland near her house. Her family saw her lastly 16 h before the discovery of her body. A spray can to be used against wide range of insects and an empty beer can (Fig. 2) were found together with a kitchen knife and her suicide note at the scene. The body was stored in a refrigerated morgue at 3 ℃, and after six days the autopsy was started at our department. The empty beer can was enclosed in a plastic bag and also stored at 3 ℃ for 6 days.

The external examinations showed several incised wounds at her neck and wrist, but they were all slight injuries. Around her nostril and mouth, there was vomit debris attached. The petechiae were observed at the conjunctiva palpebrarum. The internal examinations showed congestion for several organs, especially in both lungs. The heart blood was fluid and dark. The trachea was filled with stomach contents, which reached the tracheal bifurcation. The stomach was almost empty and only 1 mL of viscous fluid could be collected.

The alcohol concentrations in right heart blood, left heart blood and urine were almost undetectable, not higher than 0.1 mg/mL analyzed by GC. The immunological drug screening with the Triage DOA kit for urine (Alere, Waltham, MA, USA) showed a weak positive line for benzodiazepine drugs. The NAGINATA GC–MS screening test [11] concerning conventional drugs and toxic compounds was negative for blood, but it detected only α-hydroxytriazolam at 3.2 μg/mL in glucuronidase hydrolyzed urine.

Above all results suggested a possibility that the victim sprayed the contents of the spray can into the beer can and ingested the fluid. The new spray can was shown to contain 0.675 g of TM, 0.090 g of RM, 33.8 mL of kerosene and

Fig. 1 Structures of tetramethrin and resmethrin

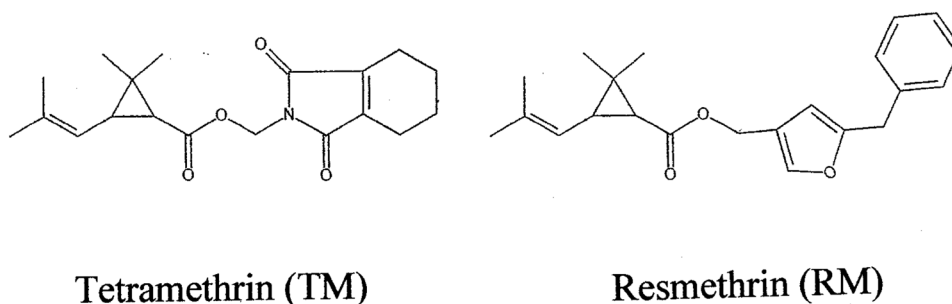Tetramethrin (TM)   Resmethrin (RM)

Fig. 2 The spray can for insecticides and the empty beer can found at the scene

416.2 mL of LP gas in 450 mL of its volume if not used, and the remaining volume in the used spray can was 260 mL. However, it is difficult to estimate the amount ingested by the victim because there is a possibility that the spray had already been used to some extent previously. Vomiting is a common clinical effect of not only kerosene [12] but also pyrethroids [13]. Therefore, present findings of the victim were consistent with asphyxia as the direct cause of death after aspiration of stomach contents into the trachea and bifurcation; the indirect cause appeared to be pyrethroid plus kerosene poisoning.

The examined specimens for TM/RM analysis in the present study were blood, urine, stomach contents and the beer can sample.

Materials and methods

Materials

Methanol, acetonitrile and 2-propanol suitable for LC–MS, 1-chlorobutane (CB) for amino acid analysis, TM, RM and other chemicals of analytical grade were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan); 1-(5-fluoropentyl)-*N*-(naphthalen-1-yl)-1*H*-indole-3-carboxamide (5F-NNEI) from Cayman Chemical (Ann Arbor, MI, USA); pure water with a specific resistance of 18 MΩ cm was used (Millipore, Bedford, MA, USA). Blood and urine samples obtained from healthy subjects were used as blank samples, and those spiked with several amounts of drugs were used as quality control samples.

The authentic specimens obtained from a cadaver were stored at – 80 ℃ until analyses. The inside of the empty beer can was rinsed with 4.7 mL of methanol, and the methanol solution was stored at – 30 ℃ until analysis.

Standard solutions

Stock solutions of TM and RM were prepared at 1 mg/mL in *n*-octanol; and the stock solution of 5F-NNEI at 1 mg/mL in acetonitrile, and all stored at − 30 ℃. 5F-NNEI is selected as the internal standard (IS) because all the pyrethroids are relatively unstable, whereas 5F-NNEI is very stable and lipophilic, and has a molecular weight relatively similar to the pyrethroid insecticides. The stock solutions were diluted with acetonitrile as required. The IS was spiked to the victim's blood, urine and stomach contents at 1 ng/mL, respectively.

Stability tests

Pyrethroids are known to be unstable against UV light, hydrolysis, oxidation and heat [14]. Therefore, to find out a suitable L-L extraction method for TM and RM, stability tests were performed using TM and RM spiked into blood, urine, water or four kinds of organic solvents (methanol, acetonitrile, 2-propanol or CB) placed in a plastic tube with cap. We confirmed that 5F-NNEI in any one of the seven solutions mentioned above was stable at 22 ℃ for 6 days under LED room light and also stable after 10 cycles of freeze/thaw treatments, and hence it was used as IS for the quantification of TM and RM.

TM and RM at 10 ng/mL each in seven solutions mentioned above were placed in the tube with cap and examined concerning the stabilities against heat, freeze/thaw treatment and LED room-light irradiation as follows ($n = 3$ in each condition).

The temperature stability was examined at four temperatures as follows. TM and RM in blood, urine or water were preserved at 37 or 22 ℃ for 7 h or 1 day; those in each of four organic solvents at 22 ℃ for 4 days; those in blood and urine at 4 ℃ for 2–8 days; those in seven solutions at – 30 ℃ for 30 days.

The freeze/thaw stability test was performed using TM and RM in blood or urine. They were frozen at – 80 ℃ and

thawed at 4 ℃. This treatment was repeated in either 4 or 10 cycles.

The LED room-light stability was tested at 22 ℃ by placing the samples on a desk (lighted) or in a drawer (unlighted), where transparent solutions such as water and four organic solvents were examined. TM and RM in water and four organic solvents were treated for 1 or 4 days.

After these treatments, the amounts of TM and RM were compared with those of the freshly prepared samples corresponded.

Extraction of tetramethrin and resmethrin from urine

Samples and reagents were cooled in a container filled with ice. To 100 μL of urine in a tube, 100 μL of IS (at 1 ng/mL in acetonitrile) and 900 μL of CB were added, vortexed for 1 min, and centrifuged at $10,000 \times g$ for 1 min. The 900 μL of the upper layer was transferred to a new tube and evaporated to ca. 5 μL at room temperature using the centrifugal dryer (miVac Duo LV; Genevac Ltd, Ipswich, England). The residue was reconstituted in 90 μL of acetonitrile and centrifuged at $10,000 \times g$ for 1 min. The supernatant (5 μL) was used for LC–MS/MS analysis.

Extraction of tetramethrin and resmethrin from blood and stomach contents

Samples and reagents were cooled in a container filled with ice. Two stainless beads (3 mm diameter), 50 μL of blood or stomach contents and 50 μL of water were placed in a tube and mixed. To the sample, 100 μL of IS (at 1 ng/mL in acetonitrile) and 100 μL of acetonitrile were added, vortexed for 1 min, and centrifuged at $10,000 \times g$ for 2 min. The 270 μL of upper layer was transferred to a new tube and 900 μL of CB was added, vortexed for 1 min, and centrifuged at $10,000 \times g$ for 1 min. The 1000 μL of upper layer was transferred to a new tube and evaporated to ca. 5 μL at room temperature using the centrifugal dryer. The residue was reconstituted in 80 μL of acetonitrile and centrifuged at $10,000 \times g$ for 1 min. The supernatant (5 μL) was used for LC–MS/MS analysis.

Instrumental conditions

LC–MS/MS was performed on an Acquity LC instrument (Waters, Milford, MA, USA) connected with an electrospray ionization QTRAP 4000 MS/MS system (AB SCIEX, Framingham, MA, USA) in the positive ion mode. A filter named SUMIPAX Filter PG-ODS (Sumika Chemical Analysis Service, Osaka, Japan) was attached to the precolumn before LC separation. The LC column for the chromatographic separation was TSK-GEL ODS-100 V

(150×2.0 mm i.d., particle size 5 μm; Tosoh, Tokyo, Japan). The mobile phase consisting of 35% B (i.e., 65% A) was set at a flow rate of 200 μL/min, and then the gradient elution was started from 35 to 100% B over 9 min, switched to 100% B, held for 3 min, and returned to initial conditions over 8 min, where solvent A was pure water containing 0.1% formic acid and 10 mM ammonium acetate, and solvent B, 100% methanol. The MS/MS conditions were: ion source temperature, 700 ℃; spray needle voltage, + 5.5 kV; sheath gas pressures, 30 units for gas 1 and 50 units for gas 2; curtain gas flow, 50 units. For sensitive detection of product ions, the selected reaction monitoring (SRM) mode was used. Tandem MS ion transitions with their suitable collision voltages were m/z 332.2→164.1 with 31 V (quantifier: QT), m/z 332.2→135.0 with 25 V (qualifier: QL1) and m/z 332.2→79.1 with 71 V (QL2) for TM; m/z 339.2→171.1 with 23 V (QT), m/z 339.2→143.1 with 39 V (QL1) and m/z 339.2→128.1 with 53 V (QL2) for RM; and m/z 375.2→232.1 with 31 V for 5F-NNEI (IS), respectively.

High-resolution LC–MS/MS was performed on an UltiMate 3000 coupled to a Thermo Scientific Q Exactive (quadrupole-Orbitrap) mass spectrometer (Thermo Scientific, Waltham, MA, USA) operated in positive ionization mode. Chromatographic separation was achieved with the same column under the same solvent conditions described above for the LC–MS/MS performed on a 4000 QTRAP MS/MS system. The MS or MS/MS conditions were: spray voltage, 3.5 kV; capillary temperature, 250 ℃; heater temperature, 350 ℃; sheath gas flow rate, 50 units and auxiliary gas flow rate, 15 units. Nitrogen was used for the collision-induced dissociation experiment. The instrument was calibrated every 24 h. The full MS resolution was 70,000 with scan range of m/z 220–2000 and MS/MS resolution was 17,500 with scan range of m/z 50–2000.

Results

Selected reaction monitoring chromatograms and product ion spectra of tetramethrin and resmethrin

The empty beer can found at the scene was expected to contain a small amount of insecticides, and hence the inside of the can was rinsed with methanol (the amount was 4.7 mL totally). This methanol solution was named hereafter as the "beer can solution" and was also examined.

The SRM chromatograms by LC–MS/MS are shown for the detection of TM at the transition m/z 332.2→164.1 and RM at the transition m/z 339.2→171.1 in Fig. 3, where the reference standard at 10 ng/mL in methanol, the beer can solution, the extract from stomach contents and urine (concentrated threefold) were shown from the top to the bottom.
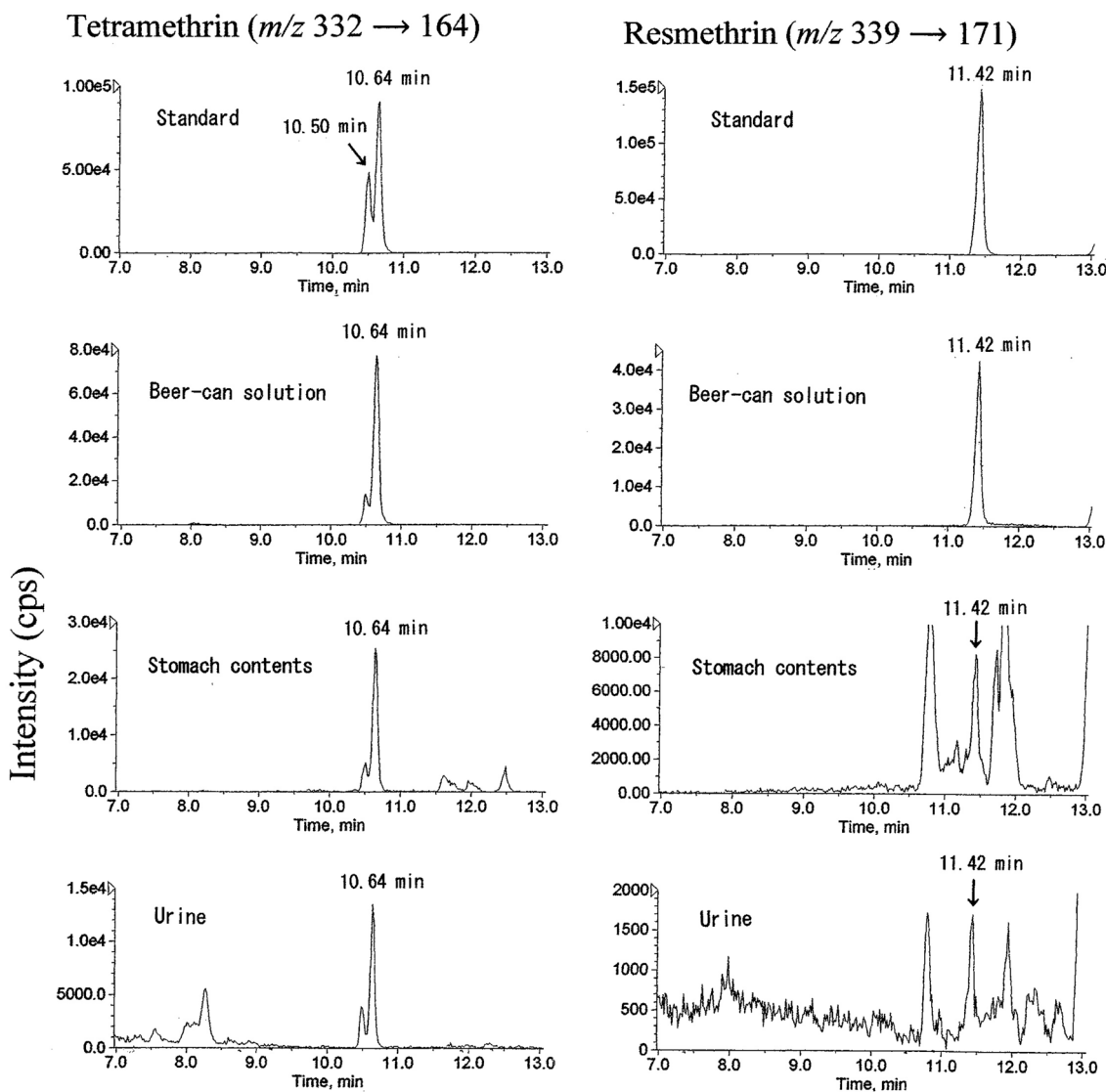
Fig. 3 Selected reaction monitoring chromatograms by liquid chromatography (LC)–tandem mass spectrometry (MS/MS) for the detection of tetramethrin and resmethrin, where the standard in methanol (10 ng/mL), the methanol solution used for rinsing of the empty beer can, the extract from stomach contents and urine (concentrated threefold) are shown from the top to the bottom

The extract from blank blood and blank urine are not shown here because they did not show any peaks that interfered with the detection of the analytes. Here TM shows two peaks at 10.50 min and 10. 64 min due to the isomers. The relative intensities of the two peaks of the beer can solution, the extracts from stomach contents and urine were nearly the same, but they were different from that of the reference standard. This may be due to the difference between the two production companies; Fumakilla Limited (Tokyo, Japan) produced a spray of insecticides, and FUJIFILM Wako Pure Chemical Corporation produced the reference standards.

Figure 4 shows the product ion spectra for TM (a) and RM (b) detected by LC–Orbitrap-MS/MS. The reference standard at 200 ng/mL in methanol is shown in the upper panel and the extract from the stomach contents (concentrated twofold), in the lower panel. Here the collision voltages were selected at 55 V for TM and 45 V for RM to show five product ions, respectively. The *m/z* values and their relative ratios of five product ions in the stomach contents agreed quite well with those in the reference standards, respectively. Furthermore, the maximum error between the *m/z* values of the product ions in stomach contents and those of the theoretical values calculated from the estimated structures was only 8 ppm as listed in Table 1.

The relative peak height ratios of three principal product ions (QT, QL1 and QL2) produced under their suitable collision voltages in the reference standard solution and those in the beer can solution, stomach contents and urine were
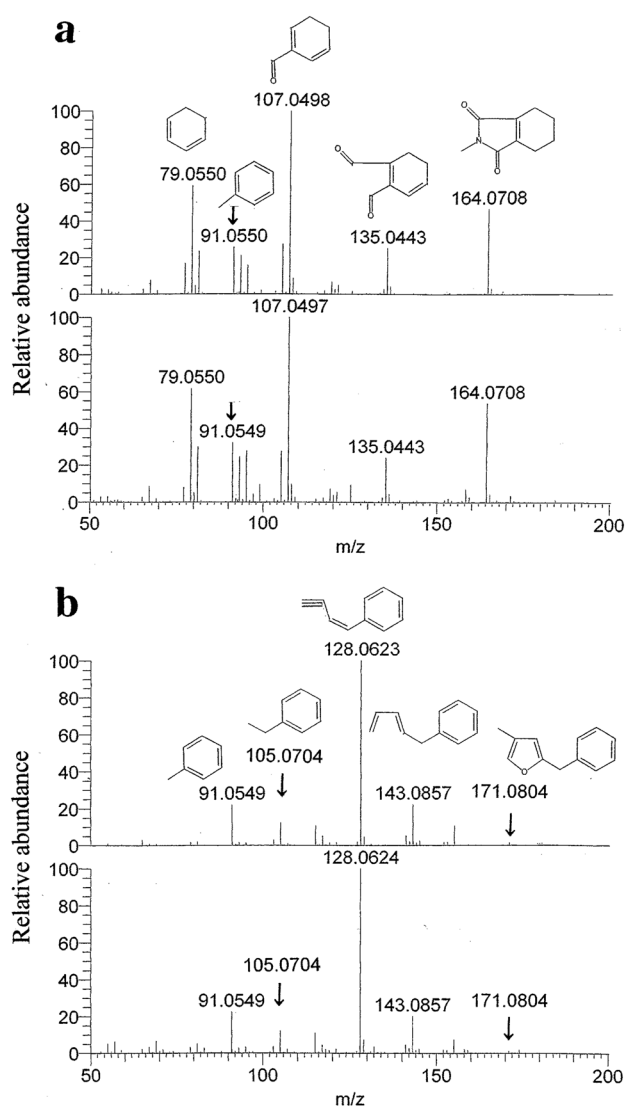
Fig. 4 Product ion spectra detected by LC–Orbitrap-MS/MS and structures of product ions for tetramethrin (**a**) and resmethrin (**b**), where the reference standard at 200 ng/mL in methanol is shown in the upper panel and the extract from stomach contents (concentrated to twofold), in the lower panel, respectively

detected by LC–MS/MS, and are listed in Table 2 by taking the highest product ions to be 100. The retention times of the three product ions were the same within the error of 0.01 min. The relative ratios of the product ions of reference standards and those of specimens agreed well with each other.

The observed retention times of the peaks in the three specimens in Fig. 3, the product ion spectra of the stomach contents in Fig. 4 and the relative peak height ratios in the three specimens in Table 2 agreed well with those of the reference standards, TM and RM, respectively, confirming that the peaks from the authentic specimens were due to TM and RM.

Reliability of the quantification method of tetramethrin or resmethrin in blood and urine

The concentration linearity of TM or RM using the present method was examined by spiking the compound to blank blood at 0, 0.2, 0.6, 2.0, 6.0 and 20 ng/mL and urine samples at 0, 0.1, 0.3, 1.0, 3.0 and 10 ng/mL ($n=6$ at each concentration), respectively. The regression equations for the calibration curves are listed in Table 3, where the correlation coefficients were 0.996–0.999. The limits of detection (LODs) (signal-to-noise ratio = 3) of both TM and RM were 0.06 and 0.03 ng/mL for blood and urine, respectively. The precisions and the accuracies were assessed by analyzing samples spiked with TM or RM at 0.2, 2.0 and 20 ng/mL in blood and 0.1, 1.0 and 10 ng/mL in urine, respectively, three times a day as well as on three different days. The accuracy data were 90.8–121% and the precision data were not greater than 16.1% for intraday and interday measurements as listed in Table 4. The recoveries in the quantification ranges were 77.4–92.6% ($n=3$ at each concentration) and the matrix effects were 85.4–106% ($n=3$ at each concentration). These validation data could be considered to be within the acceptable range for the quantification.

Table 1 Accurate mass data showing elemental compositions, retention times, precursor ions and diagnostic product ions with their mass errors in parentheses (ppm) for tetramethrin and resmethrin detected from stomach contents by liquid chromatography (LC)–Orbitrap-tandem mass spectrometry (MS/MS)

Analyte	Elemental composition	Retention time (min)	Precursor ion in m/z observed (mass error, ppm)	Collision voltage (V)	Diagnostic product ions in m/z observed (mass error, ppm)
Tetramethrin	$C_{19}H_{25}NO_4$	10.64	332.1856 (0)	55	164.0708(+1), 135.0443(+1), 107.0497(+6), 91.0549(+7), 79.0550(+8)
Resmethrin	$C_{22}H_{26}O_3$	11.42	339.1955(0)	45	171.0804(0), 143.0857(+1), 128.0624(+2), 105.0704(+5), 91.0549(+7)

Table 2 Relative peak height intensities of the product ions of tetramethrin and resmethrin in the standard solution, the beer can solution, stomach contents and urine from the victim taking the intensity of the highest ion to be 100 detected by LC–MS/MS

Analyte	Protonated molecular ion (*m/z*)	Product ion (*m/z*) with collision voltage		
Specimen		Percent product ion intensity		
Tetramethrin	(332)	(164) 31 V (QT)	(135) 25 V (QL1)	(79) 71 V (QL2)
Standard		100	65.2 ± 1.5	19.4 ± 0.4
Beer can solution		100	62.8 ± 2.8	24.4 ± 3.6
Stomach contents		100	_a	29.5 ± 1.6
Urine		100	62.4 ± 4.3	21.0 ± 1.7
Resmethrin	(339)	(171) 23 V (QT)	(143) 39 V (QL1)	(128) 53 V (QL2)
Standard		100	41.2 ± 1.9	46.3 ± 1.4
Beer can solution		100	39.7 ± 2.1	47.7 ± 4.7
Stomach contents		100	40.0 ± 0.8	45.7 ± 0.7
Urine		100	42.1 ± 1.7	50.0 ± 2.5

QT quantifier, *QL* qualifier

[a] Product ion that could not be quantified due to the interference by impurities

Table 3 Regression equations, correlation coefficients and limits of detection of tetramethrin and resmethrin spiked into blank blood and urine detected by LC–MS/MS

Compound	Range	Equation	Correlation coefficient	Limit of detection (ng/mL)
Tetramethrin in blood	0.2–20 ng/mL	$y = 0.1311\,x + 0.0014$	0.998	0.06
Resmethrin in blood	0.2–20 ng/mL	$y = 0.2954\,x + 0.0008$	0.997	0.06
Tetramethrin in urine	0.1–10 ng/m	$y = 0.1259\,x + 0.0012$	0.999	0.03
Resmethrin in urine	0.1–10 ng/mL	$y = 0.3192\,x + 0.0004$	0.996	0.03

Table 4 Intraday and interday accuracy/precision, recovery and matrix effect data of tetramethrin and resmethrin in blank blood and urine

Analyte	Conc. Spiked (ng/mL)	Intraday		Interday		Recovery (%)	Matrix effect (%)
		Accuracy %	Precision (% RSD)	Accuracy (%)	Precision (% RSD)		
Blood tetramethrin	0.2	102	13.7	107	9.7	86.7	86.5
	2	109	8.1	108	10.1	92.6	106
	20	96.2	2.2	93.7	3.3	88.9	102
Blood resmethrin	0.2	121	15.3	116	16.1	77.4	85.4
	2	102	8.9	106	5.8	88.1	93.9
	20	97.8	8.1	90.8	13.4	86.5	97.2
Urine tetramethrin	0.1	114	7.3	107	11.1	84.9	98.1
	1	113	7.1	106	6.5	86.2	104
	10	99.4	3.4	92.5	5.8	86.1	91.1
Urine resmethrin	0.1	96.4	9.1	101	5.9	79.5	97.7
	1	95.1	9.2	96.2	7.7	84.1	98.6
	10	93.6	3.8	94.0	3.2	77.9	93.5

All values were obtained from triplicate determinations

RSD relative standard deviation

Stability tests

The results of stability tests against temperature, freeze/thaw treatment and light irradiation of TM and RM in blood, urine, water or four organic solvents (methanol, acetonitrile, 2-propanol and CB) are listed in Table 5 and Tables S1 and S2. At 37 or 22 ℃, TM and RM in blood, urine and water were unstable (Table 5). On the contrary, those in four organic solvents were completely stable at 22 ℃ even after 4 days. The remaining TM and RM in blood or urine

Table 5 Stabilities of tetramethrin and resmethrin (10 ng/mL) in blank blood, urine and water at –30, 4, 22 and 37 ℃

Analyte	Temperature	Duration	Blood (Remaining %)	Urine (Remaining %)	Water (Remaining %)
Tetramethrin	– 30 ℃	30 days	98 ± 7	97 ± 10	99 ± 11
	4 ℃	2 days	95 ± 7	96 ± 8	–
		4 days	91 ± 6	96 ± 7	–
		6 days	86 ± 6	88 ± 5	–
		8 days	84 ± 7	84 ± 11	–
	22 ℃	7 h	78 ± 5	84 ± 7	88 ± 15
		1 day	63 ± 4	69 ± 5	80 ± 7
		4 days	–	–	41 ± 6
	37 ℃	7 h	51 ± 5	36 ± 6	54 ± 7
		1 day	35 ± 3	18 ± 3	25 ± 1
Resmethrin	– 30 ℃	30 days	99 ± 5	95 ± 7	96 ± 8
	4 ℃	2 days	93 ± 6	91 ± 6	–
		4 days	89 ± 7	80 ± 3	–
		6 days	86 ± 2	67 ± 6	–
		8 days	77 ± 2	58 ± 2	–
	22 ℃	7 h	78 ± 4	48 ± 9	48 ± 4
		1 day	55 ± 5	22 ± 5	32 ± 2
		4 days	–	–	6 ± 5
	37 ℃	7 h	49 ± 3	15 ± 2	13 ± 1
		1 day	41 ± 4	12 ± 1	12 ± 1

All values were obtained from triplicate determinations

– Not examined

were > 90% at 4 ℃ after 2 days, and those in blood, urine, water or four organic solvents were also > 90% at – 30 ℃ after 30 days (Tables 5, S1). Therefore, the above results suggested that aqueous samples such as blood, urine and stomach contents should be treated at low temperatures, and TM and RM in aqueous samples should be transferred into organic solvents as soon as possible. Accordingly, samples and reagents were cooled in a container filled with ice before the extraction of TM and RM from samples.

According to the above results, freeze/thaw treatments were performed between – 80 and 4 ℃. The remaining TM and RM in blood or urine were > 90% after 4 or 10 cycles of the treatments (Table S2).

The remaining TM and RM under the LED room light at 22 ℃ after 1 or 4 days in water and four organic solvents were the same as those under no light, respectively (Table S2).

The recoveries of TM and RM decreased when the organic solvents had dried up in the extraction, although TM and RM were stable when they were dissolved in four organic solvents at 22 ℃ for 4 days. Therefore, about 5 μL of the organic layer was left at the last evaporation step in the extraction of TM and RM from samples.

Quantification of tetramethrin and resmethrin in authentic specimens collected at autopsy and the methanol solution used for rinsing the inside of the empty beer can

The levels of TM were 11.1 ± 1.2 and 0.425 ± 0.017 ng/mL for stomach contents and urine, respectively. The level of RM was 1.77 ± 0.18 ng/mL for stomach contents. However, TM and RM in blood and RM in urine were below LOQ levels and thus could not be quantified. The total amounts detected in the empty beer can could be calculated to be 136 ± 10 ng and 27.0 ± 1.2 ng for TM and RM, respectively.

Discussion

The total amount of the analyte (i.e., concentration x sample volume) can be used for the indication of the sensitivity of the method. In this section, the limit of detection (LOD) and the sample volume were compared, because some of the methods reported previously did not describe the LOQs. In the present detection, LOD of 0.03 ng/mL

using 0.1 mL of urine and LOD of 0.06 ng/mL using 0.05 mL of blood were achieved. The LODs of the previous GC–MS and LC–MS/MS detections were as follows: LOD of 0.1 ng of pyrethroid metabolites/mL using 3 mL of urine [2]; LODs of 0.05–2 ng of pyrethroids/mL using 5 mL of blood [4]; LODs of 0.01–0.1 ng of pyrethroids/mL using 1 mL of blood [5]; LODs of 0.05–0.1 ng of metabolites/mL using 3 mL of urine [6]; LOD of 0.05 ng of cypermethrin/mL using 0.3 mL of plasma [7]; LODs of 0.2–7.8 ng of pyrethroids/mL using 0.3 mL of plasma [8]; LOD of 3.5 ng of RM/g using 10 g of bovine muscle and LOD of 5.9 ng of RM/g using 10 g of bovine liver [9] and LOD of 0.2 ng of TM/g using 5 g of egg [10]. The above results indicated that the LODs in the present work are comparable to those of previous works, but the present sample volumes are much smaller than those of the previous works.

Although urine is a suitable specimen due to its noninvasiveness for humans, and the metabolites of pyrethroids in urine were quantified [2, 6], the quantification of the parent molecules in authentic urine specimens was a difficult task. The reasons may be as follows: the pyrethroids are lipophilic and hence intact pyrethroids were not excreted appreciably into urine; the ester bond of pyrethroids was hydrolyzed easily by several esterases in human body. Usually, the drugs spiked into urine are more stable than those spiked into blood because the activities of esterases and oxidants in urine are much weaker than those in blood. In the present work, however, RM spiked into urine or water was less stable than that spiked into the blood at high temperature suggesting other factors such as evaporation may also be involved for its instability. Therefore, the extraction of pyrethroids should be performed at low temperature. The present sensitive quantification could be achieved according to the special attention paid to sample treatment where the temperatures of samples were maintained to be low, because TM and RM in blood, urine and water were unstable at 22 and 37 ℃ (Table 5).

The quantification of pyrethroids in real human specimens in toxic and fatal cases is quite limited. Some of such cases were reported previously in 1989–1998 [13, 15], in which the quantifications of the pyrethroids were performed by GC. Nowadays, however, the quantifications by GC–MS/MS and LC–MS/MS are considered to give the most trustworthy data, and hence only those cases detected by GC–MS are introduced as follows. In bifenthrin intoxication cases, the concentrations detected by GC–MS were 20 ng/mL serum in one living subject reported in 2001 [16] and 500 ng/mL plasma in another living subject reported in 2013 [17]. Another GC–MS work in 2017 reported 2.46 and 0.41 µg/mL of α-cypermethrin in blood and urine, respectively, and 2.4 and 0.46 µg/mL of deltamethrin in blood and urine, respectively, in a fatal case [18]. In fenpropathrin

intoxication cases, the concentrations detected by GC–MS/MS were 82 and 91 ng/mL blood in two patients, respectively, reported in 2018 [5].

LC–MS/MS can determine parent molecules of pyrethroids and does not require the derivatization of the molecules that is required sometimes by GC–MS. However, the quantification of pyrethroids by LC–MS/MS in human toxic/fatal cases has not been reported yet to our knowledge. Therefore, careful sample treatments and a quick sensitive detection method are described in this article.

Precise metabolism of TM and RM in humans has not been reported yet and, we hope to investigate the quantification/qualification of the metabolites in the near future using human postmortem samples. Metabolism of TM in rats and that of RM in cows were studied using ^{14}C labeled analytes more than 20 years ago [19, 20], but the metabolisms in animals are not exactly the same as those of humans due to different CYP enzymes; it is also a problem that in animal model experiments, the administration doses as per body weights to gain metabolites are usually much higher than those in the authentic human cases. This makes it more difficult to regard the results obtained from animals as good models of human results.

Conclusions

A sensitive method was established for the quantification of TM and RM in whole blood and urine using LC–MS/MS. The LOQs of both TM and RM were 0.1 ng/mL in 0.1 mL of urine and 0.2 ng/mL in 0.05 mL of whole blood, respectively. To our knowledge, this is the first report to quantify TM and RM in authentic human specimens obtained from a cadaver.

Declarations

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