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CASE REPORT

Analysis of toxic *Veratrum* alkaloids in plant samples from an accidental poisoning case

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Abstract
Purpose Four individuals (aged 50–60 years) at a dinner ate a wild plant that had been collected in a forest and looked similar to the edible wild plant *Hosta montana* (plantain lily). About 30–40 min after eating the plant, two of these individuals suffered from severe vomiting and a rapid decrease in blood pressure, and were hospitalized. Another individual initially showed mild symptoms, but was then hospitalized as symptoms increased in severity. The last person did not show any symptoms. The plant was found to be the very toxic *Veratrum* species.
Methods Although biological specimens were not available in this case, the remaining uncooked *Veratrum* sample was analyzed to determine what compounds contributed to the poisoning symptoms by liquid chromatography—tandem mass spectrometry.
Results Two toxic alkaloids, protoveratrines A and B, were identified and quantified at 146 and 1302 μg/g, respectively. Reproducible experiments showed that the cooked samples contained 400-600 μg/g levels of protoveratrine B. Protoveratrine A and B are the most toxic compounds in *Veratrum* alkaloids. Other toxic *Veratrum* alkaloids were also present in trace amounts or not detected. Thus, the patients' symptoms were assigned to protoveratrine B poisoning. To prevent further poisoning, wild *Veratrum* species were collected from two other colonies over years and analyzed. The composition of *Veratrum* alkaloids of the poisoning sample and that of the young *Veratrum* sprout samples of two other colonies were very similar, and had not changed largely over the years when they were young sprouts, showing that all the young sprouts of *Veratrum* species are dangerous to eat.
Conclusions This is a rare report on the direct correlation of protoveratrine B ingestion with poisoning symptoms.

Keywords *Veratrum* alkaloid poisoning · Vomiting and hypotension · LC–MS/MS · Protoveratrines A and B · Standard addition method

Introduction

Veratrum species are native to the Northern Hemisphere, and most of them produce various kinds of toxic C-nor-D-homo *Veratrum* alkaloids (VAs) (Fig. 1; Table 1) [1–13]. The main symptoms of VA ingestion are immediate vomiting, oral paraesthesia, bradycardia (decreased to 35/min), and a large decrease in blood pressure (decreased to 50 mm Hg), which is the most characteristic symptom for *Veratrum* poisoning [14–18]. Some cases of VAs ingestion are fatal for humans [12].

In Japan, two variants of *Veratrum album* L. (false hellebore), *V. album* L. subsp. *oxysepalum* Hulten and *V. stamineum* Maxim., are the main native species [19]. The young sprouts of these plants are very similar to those of the edible plant *Hosta montana* F. Maekawa (plantain lily). Consequently, there have been many accidental *Veratrum*

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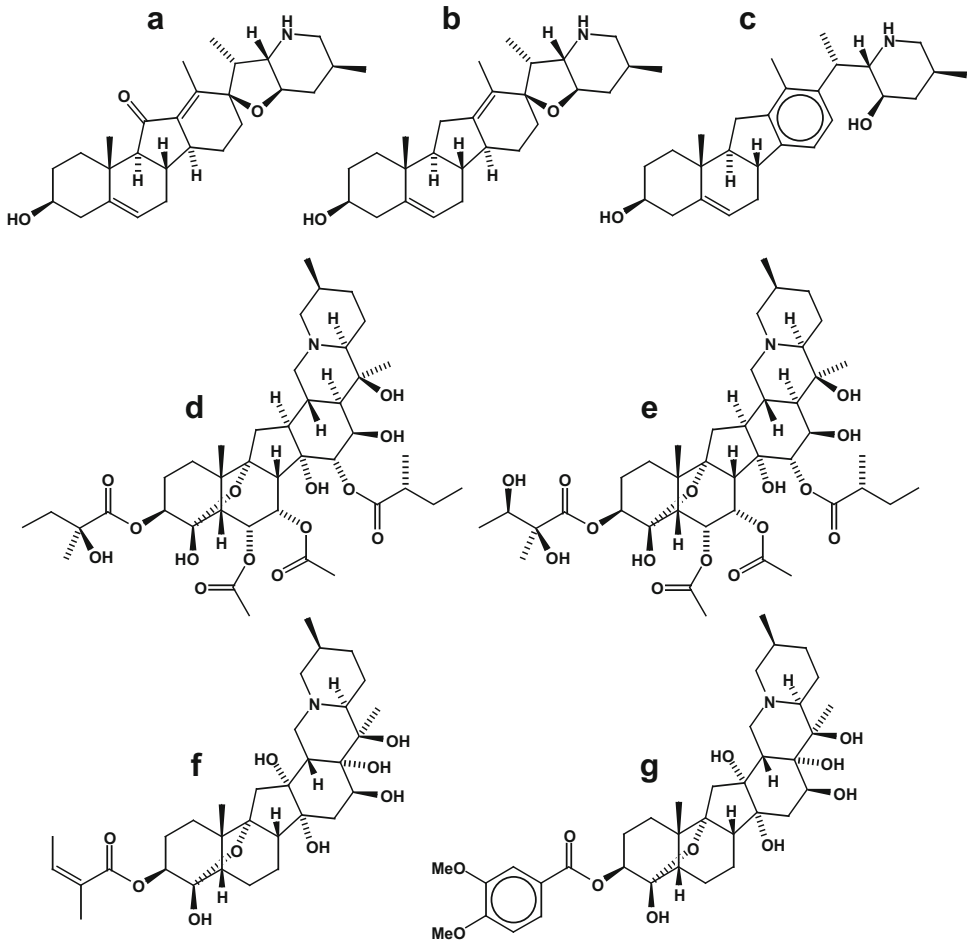

Fig. 1 Structures of toxic *Veratrum* alkaloids (VAs). **a** Jervine, **b** cyclopamine, **c** veretramine, **d** protoveratrine A (PV-A), **e** protoveratrine B (PV-B), **f** cevadine, and **g** veratridine

Table 1 LD_{50} values of the toxic *Veratrum* alkaloids reported in the literature

Compound	LD_{50} in mice (mg/kg)				LD_{50} in rats (mg/kg)		
	i. v.	s. c.	i. p.	p. o.	s. c.	i. p.	p. o.
Jervine		29 [1]		260 [2]			240 [3]
Cyclopamine	43.5 [4]						
Veratramine	3.1 [5]	4.5 [1]					
Protoveratrine A		0.29 [1]	0.2 [6]		0.48 [7]		
Protoveratrine B	0.065 [7]	0.21 [1]			0.92 [7]		
Cevadine	1.0 [8]	4.9 [1]	3.5 [9]				
Veratridine	0.42 [10]	6.3 [1]	1.35 [9]			3.5 [11]	

LD_{50} median lethal dose, *i. v.* intravenous, *s. c.* subcutaneous, *i. p.* intraperitoneal, *p.o.* per os

poisoning cases in Japan, and most of these have occurred during spring because *Veratrum* and *Hosta* species look quite similar in this season [19].

This case report is of an accidental poisoning after ingestion of toxic *Veratrum* plant taken in Gujo, Japan. Four individuals ate cooked young sprouts of the *Veratrum* plant at dinner, and three of them showed typical symptoms of *Veratrum* poisoning [14–18]. To identify the compounds responsible for the poisoning symptoms, the residual uncooked plant sample was analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) in our laboratory. In addition, for a risk assessment and to prevent further accidental poisoning, an inspection was conducted for other colonies of *Veratrum* species in the area. Two more colonies were found in Gifu Prefecture. Samples were taken from these colonies in multiple years and analyzed by LC–MS/MS to investigate changes in the VA composition over time.

Fig. 2 Sample collection points for the *Veratrum* species in Gifu Prefecture, Japan. **a** Poisoning sample (*Veratrum* sp., Gujo), **b** colony 1 (*V. album* subsp. *oxysepalum*, Yamanaka Pass, Takayama), **c** colony 2 (*V. stamineum* var. *micranthum*, Nenoue Highland, Nakatsugawa)

Case history

In April 2010, four individuals (ages 50–60 years) at dinner ingested a wild plant that was collected in the forest in Gujo, which is a city in Gifu Prefecture, Japan (Fig. 2a). The residual raw plant (Fig. 3) looked very similar to young sprouts of the edible plant *H. montana*. However, it was found to be the very toxic *Veratrum* species, but more detailed classification was impossible. The plant was served boiled and dressed with vinegar and miso ("sumiso" style), and as tempura. The exact quantity eaten by each person was unclear. About 30–40 min after eating the plant, two individuals (patient 1, male and patient 2, female) suffered from severe vomiting with a rapid decrease in blood pressure, and were hospitalized that day. Three hours after the dinner, a third individual (patient 3, female) showed mild symptoms. After these symptoms worsened the next day, patient 3 was also hospitalized. The fourth person showed no symptoms, probably, because the ingested amount was less than the toxic dose. None of the cooked plant sample remained. Instead, about 2 g of the aerial part of the remaining uncooked plant sample was submitted to our laboratory for analysis. Human specimens were not available in this case.

Materials and methods

Sampling of *Veratrum* species in Gifu Prefecture

For a risk assessment and to prevent further poisoning cases, an inspection for other colonies of *Veratrum* species

Fig. 3 Aerial part of the residual uncooked plant sample. A sample of this (2 g) was submitted to our laboratory for liquid chromatography–tandem mass spectrometry analysis

was conducted in Gifu Prefecture, and two more colonies were found. At Yamanaka Pass in Takayama, a colony of *V. album* subsp. *oxysepalum* (colony 1, Fig. 2b) was found. Then, at Nenoue Highland in Nakatsugawa, a colony of *V. stamineum* var. *micranthum* (colony 2, Fig. 2c) was also found. Samples were collected from the Yamanaka Pass colony in April 2012 and May 2013, and similar samples were collected from the Nenoue Highland colony in April 2012 and April 2014 as whole bodies of plants. All the samples were still young sprouts; thus the aerial parts of all the samples were mostly leaves, and stems were not observed. These samples were stored at −80 °C in the dark until required for analysis. The samples were divided into aerial parts and rhizomes, and analyses were performed for both.

Chemicals and reagents

Yohimbine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All the VAs used in this study were isolated as free bases in our laboratory. Jervine, cyclopamine and veratramine were isolated from rhizomes of *V. stamineum* collected in Fukushima Prefecture, Japan. Protoveratrine A (PV-A) and protoveratrine B (PV-B) were isolated from rhizomes of *V. stamineum* collected in Tottori Prefecture, Japan. Generally, homogenization of rhizomes and extraction of VAs were performed in the same manner as described in the below "Sample preparation" section. Because cevadine and veratridine were not found in the collected Japanese *Veratrum* samples, they were isolated from a crude veratrine hydrochloride mixture (Sigma-Aldrich, St. Louis, MO, USA). Purification of the VAs were performed by using preparative thin-layer chromatography (p-TLC) using silicagel F_{254} plates (20 × 20 cm, 1 mm thickness, Merck Millipore, Billerica, MA, USA). Cyclohexane/diethylamine (mainly 7:3, v/v),

ethyl acetate/methanol (mainly 1:1, v/v) and ethyl acetate containing 1% ammonia water were used as the main mobile phases. The acidic mobile phase was not used, because it may cause isomerization or decomposition at the hemiacetal or allylfuran moiety of VAs. During the isolation processes, ultraviolet absorption at 254 nm and fluorescence at 366 nm on the developed p-TLC plates, and coloring on the subdivided p-TLC plates by Dragendorff reagent were used for monitoring of VAs. The purified VAs were used as analytical reference standards after their nuclear magnetic resonance spectra were confirmed to coincide with previously reported spectra [20–22]. Stock solutions of the above compounds were prepared as 100 μg/mL acetonitrile solutions and stored at −20 °C in the dark. Ultrapure water was prepared using a Milli-Q Gradient A10 system (Merck Millipore, Billerica, MA, USA). All other chemicals were of analytical grade commercially available.

Sample preparation

The plant samples were chopped and mixed well, and 1-g samples were accurately weighed. These samples were put in 50-mL polyethylene capsules, frozen on liquid nitrogen for 5 min, and then pulverized using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) at 3000 rpm for 10 min. Then, they were allowed to warm to room temperature. Next, 5 mL of methanol was added to the powders in the capsules, and the mixtures were again homogenized using the Multi-Beads Shocker at 3000 rpm for 10 min at room temperature. The homogenates were transferred into 50-mL centrifuge tubes, and the capsules were washed with 3 mL of methanol. The washings were also transferred to the centrifuge tubes. The extracts were ultrasonicated for 30 min, and centrifuged at 3000×*g* for 30 min at 4 °C. The supernatants were transferred to 10-mL volumetric flasks, and the volumes were adjusted to 10 mL with methanol. These solutions were used as 100 mg/mL stock solutions of the plant samples.

Instrumentation

The LC–MS/MS system consisted of an Acquity UPLC system (Waters, Milford, MA, USA) and a Xevo TQ-S mass spectrometer (Waters). The chromatographic separation was performed on an Ascentis Express C18 column (100 × 2.1 mm i.d., particle size 2.7 μm, Sigma-Aldrich) in combination with an Ascentis Express C18 Guard Cartridge (5 × 2.1 mm i.d., particle size 2.7 μm, Sigma-Aldrich) as a guard column. The MS measurements were performed in the multiple reaction monitoring (MRM) mode, and the optimized parameters for the MRM transitions used are shown in Table 2. The ion source temperature was 150 °C, the desolvation temperature 350 °C, and the column temperature maintained at 40 °C. The LC mobile phase consisted of 4 mM ammonium acetate with 0.1% formic acid in water (pH 3) (A) and acetonitrile (B). The gradient started at 20% B, ramped to 43% B over 30 min, and then held at 20% B until 40 min for equilibration. The mobile phase flow rate was 0.2 mL/min, and the injection volume was 5 μL.

Qualitative analysis of the samples

The 100-mg/mL stock solutions of the plant samples were diluted 10, 100, and 1000 times with methanol to give 10, 1, and 0.1 mg/mL sample extracts. LC–MS/MS samples with concentrations of 10, 1, 0.1, and 0.01 mg/mL were prepared by adding 100 ng/mL yohimbine in methanol (100 μL) and 4 mM ammonium acetate–0.1% formic acid (pH 3) buffer solution (800 μL) to 100 μL aliquots of the 100, 10, 1, and 0.1 mg/mL extracts, respectively. The LC–MS/MS samples were used for identification of the VAs. The ratio of the peak area of detection transition 1 to confirmation transition 2 (Table 2) was required to be within the tolerance specified in European Union guidelines [23].

Quantitative analysis of the extracts of the samples by the standard addition method

Calibration reference standards for the absolute calibration curves for seven VAs were prepared at concentrations of 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, and 100 ng/mL from 100 ng/mL standard solution by dilution with LC mobile phase (mixture of 4 mM ammonium acetate with 0.1% formic acid and acetonitrile (80:20, v/v)). Yohimbine was added at 10 ng/mL to each calibration reference standard as internal standard. Absolute calibration curves were plotted using the peak area ratio of analytes to internal standard (*y* axis) and the concentrations of the calibration standard (*x* axis), and fitted with a linear equation by least-squares approximation. The limit of detection (LOD) was defined as the lowest concentration giving a signal-to-noise (S/N) ratio higher than 3. Similarly, the limit of quantification (LOQ) was defined as the lowest concentration giving an S/N ratio higher than 10.

For quantification of each detected VA, an appropriate LC–MS/MS sample (10, 1, 0.1, or 0.01 mg/mL) was selected so that the concentration of the target VA was within the calibration range. Using the above absolute calibration curves, an approximate concentration of each detected VA in the selected LC–MS/MS sample (*x* μg/g) was determined. If *x* was less than 1, further quantification was not required, because it would not cause the poisoning

Table 2 Multiple reaction monitoring conditions used for the *Veratrum* alkaloids and the internal standard

Compound	Precursor ion (*m/z*)	Cone voltage (V)	Transition 1 (detection/quantification)		Transition 2 (confirmation)	
			Product ion (*m/z*)	Collision energy (eV)	Product ion (*m/z*)	Collision energy (eV)
Jervine	426.30	48	114.09	32	109.10	34
Cyclopamine	412.32	46	109.10	34	114.09	28
Veratramine	410.31	42	295.21	28	84.08	28
Protoveratrine A	794.43	58	776.42	40	658.36	52
Protoveratrine B	810.43	58	792.42	40	658.36	52
Cevadine	538.34	62	520.33	52	456.28	48
Veratridine	674.35	66	83.05	58	456.28	50
Yohimbine	355.20	36	144.08	28		

symptoms. Then, LC–MS/MS samples spiked with the detected VA at 0, x, $2x$ and $3x$ μg/g were prepared and used for the construction of four-point calibration curves, which were plotted using the peak area ratio of analytes to internal standard (y axis) and the concentrations of the calibration standards (x axis), and fitted with a linear equation by least-squares approximation. The concentration of VA was determined as the absolute value of the intersection point on the x axis. This quantification procedure was performed for each detected VA separately.

Preparation of cooked samples

In this case, cooked samples were not available. However, during the cooking process, VAs may thermally decompose or dissolve out into hot water or hot oil. Thus, to estimate the VA concentrations in the actual poisoning cooked samples, it was necessary to prepare the tempura and sumiso of *Veratrum* plant. The amounts of the poisoning sample, Yamanaka sample and Nenoue samples were very limited, and there was almost no residue. Therefore, to prepare the cooked samples, we collected new *Veratrum* samples at Yamanaka Pass in Takayama in July 2017 again. The collected *Veratrum* plants had already grown and had stems; the leaves were collected and used for preparation of the cooking samples. One leaf was cut at median line into two pieces, one of which was cooked and the other of which was used as non-cooked reference sample. For the preparation of the tempura sample, tempura batter was prepared from 30 g of flour, 7.5 g of starch, 1.25 g of salt, 1/4 of egg and 32.5 mL of water. Then 2.35 g of the half-cut *Veratrum* leaf was coated with the tempura batter and fried in the oil at 180 °C for 1 min. Then the batter of the tempura was completely removed and weighed. The weight of the leaf decreased by 50.2%; 0.498 g (equivalent to 1 g of raw leaf) of the sample was used for analysis. VAs in the tempura samples were extracted in mostly the same manner as described in the "Sample preparation" section, but the methanol extract was finally defatted with *n*-hexane three times. For the preparation of the sumiso sample, sumiso sauce was prepared from 2 teaspoons of vinegar, 3 teaspoons of miso and 2 teaspoons of sugar. Then 3.7 g of the half-cut *Veratrum* leaf was boiled for 1 min. After cooling down, the boiled leaf was cut into bite-sized pieces and coated with sumiso sauce. The weight of the leaf increased by 59.5%; 1.60 g (equivalent to 1 g of raw leaf) of the sample was used for analysis. Before analysis, the sumiso sauce was washed away. VAs were extracted in the same manner as described in the "Sample preparation" section. For the preparation of the sautéed sample, 8 g of the half-cut *Veratrum* leaf was further cut in 5-cm widths and sautéed with 1 teaspoon of oil, 0.4 g of salt and 1 mL of soy sauce for 1 min. The weight of the leaf decreased by 8.7%; 0.913 g (equivalent to 1 g of raw leaf) of the sample was analyzed in the same manner as the tempura sample.

Results and discussion

Validation of the analytical method

The standard addition calibration equations for VAs in plant samples, in which VAs were quantifiable, are also shown in Table 3. The matrix effects were calculated by comparing the peak areas of analytes in quantified methanol extract samples (PV-A/B: 0.1 mg plant/mL, Jervine: 10 mg plant/mL) with those for the same concentraion of reference standard aqueous solutions (neat samples), and are also shown in Table 3. The coefficient of correlation varied between 0.9966 and 0.9999 (Table 3); this seemed to be satisfactory for quantification. The LODs of jervine,

Table 3 Standard addition calibration equations for *Veratrum* alkaloids in plant samples, in which *Veratrum* alkaloids were quantifiable

Sample	*Veratrum* alkaloid	Plant sample extract used	Equation	Coefficient of correlation (r)	Matrix effect (%)
Poisoning sample					
Leaf[a]	Jervine	10 mg/mL	$y = 9.049 \times 10^{-2}x + 0.2443$	0.9988	87.6
	Protoveratrine A[b]	0.1 mg/mL	$y = 1.206 \times 10^{-3}x + 0.1758$	0.9969	116
	Protoveratrine B	0.1 mg/mL	$y = 1.258 \times 10^{-3}x + 1.6384$	0.9992	92.5
Samples of Yamanaka Pass, Takayama					
April 2012					
Leaf[a]	Protoveratrine A[b]	0.1 mg/mL	$y = 6.708 \times 10^{-4}x + 0.0664$	0.9966	132
	Protoveratrine B	0.1 mg/mL	$y = 8.285 \times 10^{-4}x + 0.7655$	0.9986	88.9
Rhizome	Protoveratrine A[b]	0.1 mg/mL	$y = 1.151 \times 10^{-3}x + 0.2636$	0.9973	124
	Protoveratrine B	0.1 mg/mL	$y = 1.142 \times 10^{-3}x + 3.2311$	0.9992	96.2
May 2013					
Leaf[a]	Jervine	10 mg/mL	$y = 8.054 \times 10^{-2}x + 0.2208$	0.9995	78.3
	Protoveratrine A[b]	0.1 mg/mL	$y = 1.119 \times 10^{-3}x + 0.1332$	0.9981	138
	Protoveratrine B	0.1 mg/mL	$y = 1.028 \times 10^{-3}x + 1.1292$	0.9999	91.4
Rhizome	Jervine	10 mg/mL	$y = 8.524 \times 10^{-2}x + 0.1501$	0.9995	84.5
	Protoveratrine A[b]	0.1 mg/mL	$y = 1.070 \times 10^{-3}x + 0.2758$	0.9992	109
	Protoveratrine B	0.1 mg/mL	$y = 1.165 \times 10^{-3}x + 2.9122$	0.9989	95.7
Samples of Nenoue Highland, Nakatsugawa					
April 2012					
Leaf[a]	Jervine	10 mg/mL	$y = 9.353 \times 10^{-2}x + 0.3200$	0.9976	80.7
	Protoveratrine A[b]	0.1 mg/mL	$y = 9.872 \times 10^{-4}x + 0.0116$	0.9969	124
	Protoveratrine B	0.1 mg/mL	$y = 1.096 \times 10^{-3}x + 0.0595$	0.9998	86.3
Rhizome	Jervine	10 mg/mL	$y = 1.075 \times 10^{-1}x + 0.1755$	0.9991	93.5
	Protoveratrine A[b]	0.1 mg/mL	$y = 1.018 \times 10^{-4}x + 0.0855$	0.9978	144
	Protoveratrine B	0.1 mg/mL	$y = 1.208 \times 10^{-3}x + 0.4479$	0.9975	92.3
April 2014					
Leaf[a]	Jervine	10 mg/mL	$y = 8.852 \times 10^{-2}x + 2.0469$	0.9990	85.3
	Protoveratrine A[b]	0.1 mg/mL	$y = 1.157 \times 10^{-4}x + 0.0031$	0.9971	109
	Protoveratrine B	0.1 mg/mL	$y = 1.150 \times 10^{-3}x + 0.0171$	0.9997	94.1
Rhizome	Jervine	10 mg/mL	$y = 9.423 \times 10^{-2}x + 0.3223$	0.9987	91.7
	Protoveratrine A[b]	0.1 mg/mL	$y = 2.321 \times 10^{-4}x + 0.0137$	0.9983	134
	Protoveratrine B	0.1 mg/mL	$y = 1.175 \times 10^{-3}x + 0.3771$	0.9994	90.9

[a] From March to early May, the leaves are the main aerial part of the plant. Stem growth occurs after June

[b] In all the samples, the peak for protoveratrine A was accompanied by a peak for an unknown isomer

[c] The concentration of *Veratrum* alkaloid was determined as the absolute value of the intersection point on the x axis

PV-A and PV-B were about 1, 20 and 20 ng/g, and the LOQs of jervine, PV-A and PV-B were about 5, 100 and 100 ng/g, respectively. Concerning the matrix effect, PV-A showed irregular ionization enhancement for all the samples (Table 3). The reason was unknown, but there is a need to be careful for quantification of PV-A by the absolute calibration curve method, because the irregular enhancement may cause error. Therefore, PV-A in plant samples should be quantified by the standard addition method. For PV-B and jervine, moderate ionization supressions were observed (Table 3).

Because the accuracy and precision data cannot be presented from the standard addition method, we instead presented data on intraday and interday repeatability. The amounts of the samples were very limited; thus analytical repeatability of the standard addition method also could not be determined. Thus, the leaves of the Yamanaka adult sample collected in July 2017 was also used for measurements of intraday ($n = 5$) and interday ($n = 3$) repeatability of PV-A and PV-B. The results are shown in Table 4. The repeatability was expressed as relative standard deviation (RSD) of the measured concentrations of analytes.

Table 4 Examples of intraday and interday repeatability for determination of protoveratrine A and B in *Veratrum* album subsp. *oxysepalum* collected in July 2017 at Yamanaka Pass, Takayama, Japan

Veratrum alkaloid	Intraday ($n = 5$)		Interday ($n = 3$)	
	Measured concentration (μg/g)[a]	Repeatability (RSD, %)[b]	Measured concentration (μg/g)[a]	Repeatability (RSD, %)[b]
Protoveratrine A[c]	11.8 ± 1.85	15.7	10.4 ± 1.28	12.3
Protoveratrine B	133 ± 6.52	4.9	128 ± 9.22	7.2

[a] Mean ± standard deviation

[b] *RSD* relative standard deviation

[c] In all the samples, the peak for protoveratrine A was accompanied by a peak for an unknown isomer

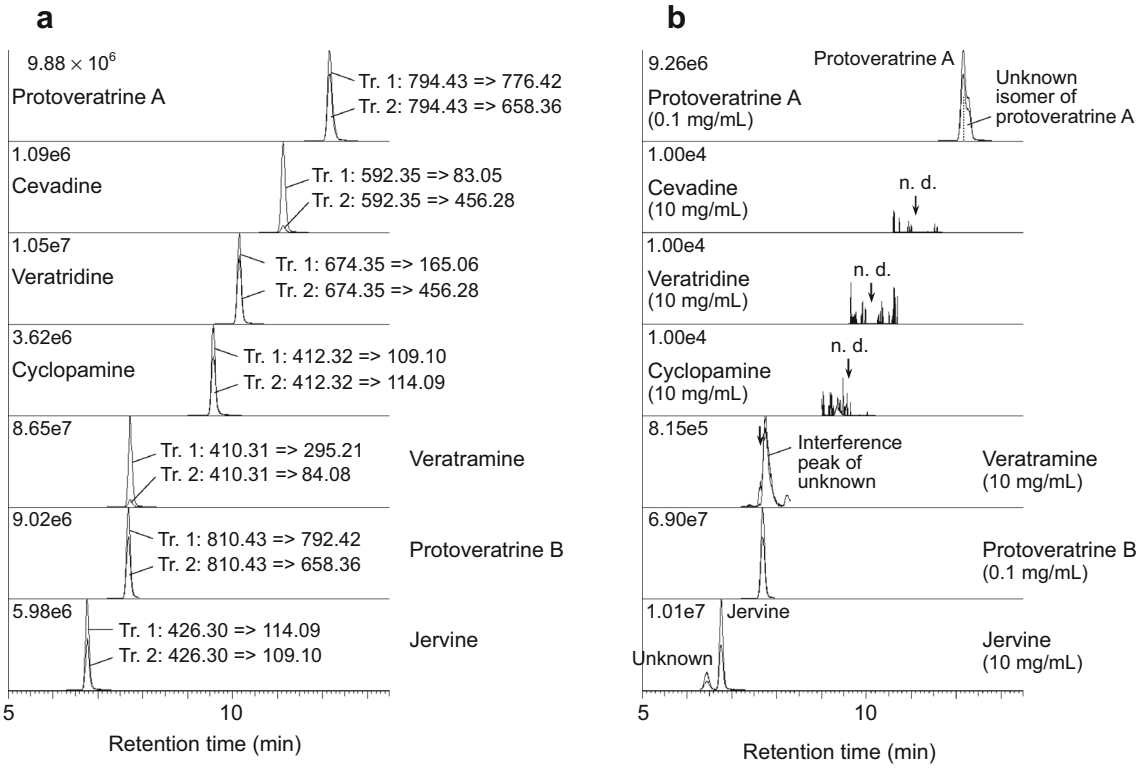

Fig. 4 Multiple reaction monitoring chromatograms of **a** seven reference standard VAs (50 pg each) and **b** the poisoning sample. *Tr* transition, *n. d.* not detected

The repeatability for PV-B was less than 7.2%. However, PV-A showed a higher RSD (but less than 15.7%), probably due to irregular ionization enhancement shown in Table 3.

LC–MS/MS analysis of VAs

The MRM chromatograms of the reference standard VAs (50 pg each) and the extract of the poisoning sample are shown in Fig. 4a, b, respectively. All seven reference standard VAs were detected as symmetric peaks (Fig. 4a). In the poisoning sample, PV-A and B, and jervine were detected (Fig. 4b). Veratramine could have been present, but this was difficult to judge and could have been an interference. Even if it was present, the peak area indicated the concentration would be much less than 1 μg/g. Other toxic VAs, especially cevadine and veratridine, which are major toxic components in the European and American species [12, 13], were not detected. The quantitative analysis data for all the samples are shown in Table 5. In all the samples, PV-A and B were detected as the main toxic compounds, and a small quantity of jervine was also detected.

Table 5 Results for the detection and quantitative analysis of *Veratrum* alkaloids in the samples

Skeleton	Compound	Concentration (μg/g)								
		Poisoning sample	Sample collected area							
			Yamanaka Pass, Takayama				Nenoue Highland, Nakatsugawa			
			April 2012		May 2013		April 2012		April 2014	
		Leaf[a]	Leaf[a]	Rhizome	Leaf[a]	Rhizome	Leaf[a]	Rhizome	Leaf[a]	Rhizome
Jervanine	Jervine	2.70	<1	<1	2.74	1.76	3.42	1.63	23.1	3.42
	Cyclopamine	–	–	–	–	–	–	–	–	–
Veratranine	Veratramine	<1[b]	–	–	–	–	–	–	–	–
Protoverine	Protoveratrine A[c]	146	75.0	229	119	258	11.8	84	2.68	5.9
	Protoveratrine B	1302	924	2831	1099	2501	54.3	371	14.9	321
Cevanine	Cevadine	–	–	–	–	–	–	–	–	–
	Veratridine	–	–	–	–	–	–	–	–	–

– Not detected

[a] From March to early May, the leaves are the main aerial part of the plant. Stem growth occurs after June

[b] Including interference peak

[c] In all the samples, the peak for protoveratrine A was accompanied by a peak for an unknown isomer

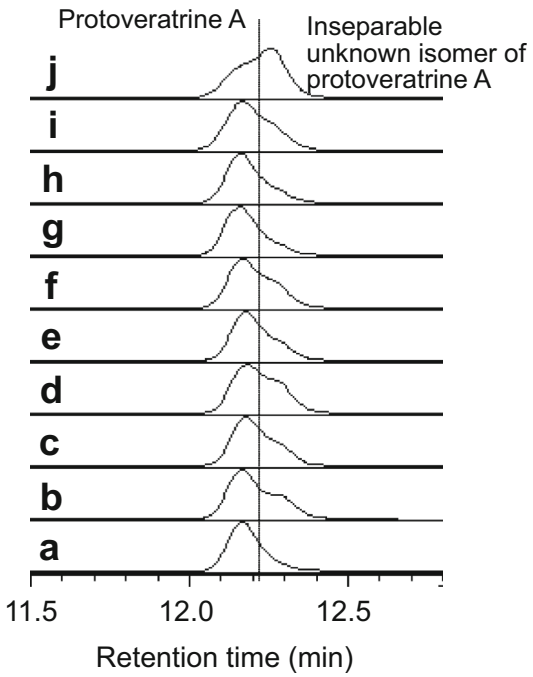

Fig. 5 Multiple reaction monitoring chromatograms of **a** reference standard PV-A (50 pg), **b** PV-A in the poisoning sample, **c** leaf and **d** rhizome of *V. album* subsp. *oxysepalum* from Yamanaka Pass (April 2012), **e** leaf and **f** rhizome of *V. album* subsp. *oxysepalum* from Yamanaka Pass (May 2013), **g** leaf and **h** rhizome of *V. stamineum* var. *micranthum* from Nenoue Highland (April 2012), and **i** leaf and **j** rhizome of *V. stamineum* var. *micranthum* from Nenoue Highland (April 2014)

The VA composition was quite similar among all the samples, even though the locations of some of the sampling sites were separated by more than 60 km (Fig. 2a–c) or 80 km (Fig. 2b, c). In addition, for both colonies 1 and 2, there were no time courses on the VA composition in each sample in the span of 1 or 2 years when they are young sprouts. In general, PV-A/B contents in rhizomes were higher than in leaves. The poisoning leaf and Yamanaka Pass leaf contained 1000 μg/g (0.1%) levels of PV-B (Table 5), which is the most toxic alkaloid among the VAs [1, 7]. This means that 100 g of young sprouts collected in April or May could contain 100 mg levels of PV-B, which could be a fatal dose [1, 7, 12]. In general, the concentrations of VAs were higher in the rhizomes than in the aerial parts. In this case, the amount of PV-B in the poisoning sample was about ten times that of PV-A. Trace amount of jervine, which is a much weaker poison than either of the PVs [1–10], was also detected in all the samples, but it would not contribute to the symptoms. Thus, the symptoms of the patients could be assigned to PV-B poisoning. This case is the first report of a *Veratrum* poisoning case with the symptoms caused by a single PV-B.

Unknown isomer of PV-A

An unknown inseparable peak was detected just after PV-A in all of the samples (Fig. 5b–j). The product ion spectrum of the reference standard PV-A (Fig. 6a) coincided with that of the unknown inseparable peak (Fig. 6b). This strongly suggests the unknown peak has the same planar structure as PV-A, and is an unknown epimer of PV-A.

Because of the limited quantities of the samples, isolation and structural analysis of the isomer were quite difficult. However, based on the detection of the 2′-epimer of

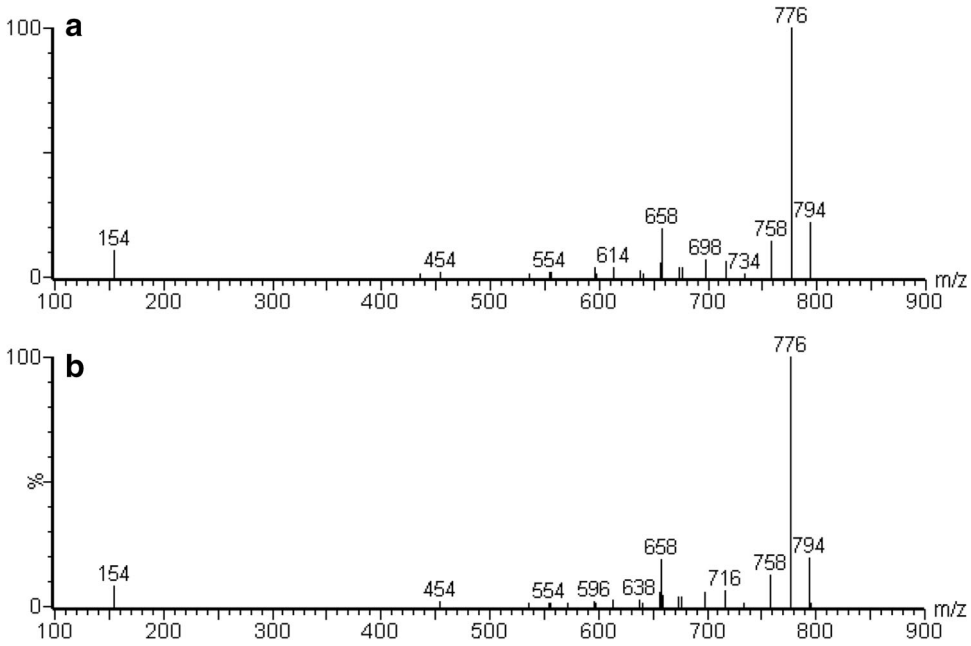

Fig. 6 Product ion spectra of **a** reference standard PV-A and **b** unknown isomer of PV-A (collision energy, 40 eV each)

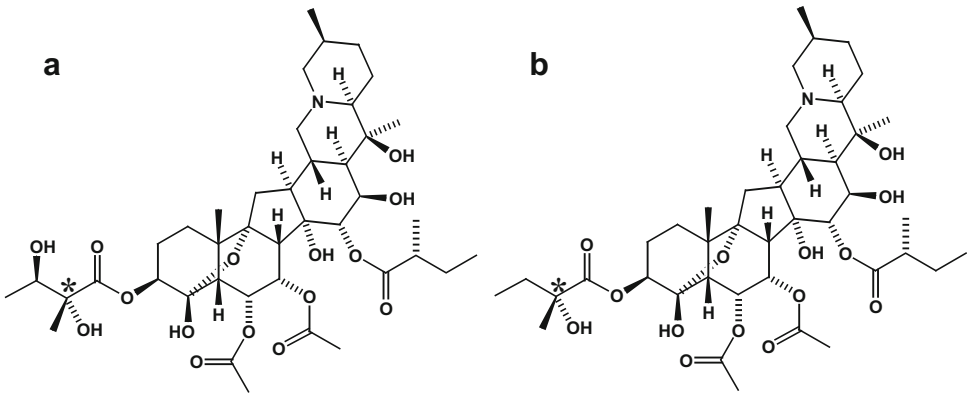

Fig. 7 Structures of **a** the known isomer of PV-B, PV-C (2′-epi-PV-B), and **b** the possible structure of the unknown isomer of PV-A (2′-epi-PV-A). *2′-position

PV-B, protoveratrine C (PV-C, Fig. 7a) in 1982 [24], there is a possibility that the unknown isomer is the 2′-epimer of PV-A (Fig. 7b). In the quantitative analysis, the peak of the unknown isomer was omitted.

Decrease of VAs during cooking

The decreases of VAs during cooking are shown in Table 6. First, VA concentrations in the reference Yamanaka adult sample collected in July 2017 were much lower than those given in Table 5, but contained enough amounts of PV-A and PV-B for the experiments. Individual differences of VA concentrations were larger than those in young sprouts in Table 5. Because the concentration of jervine was less than 1 μg/g for all the samples, its amount was not measured in the standard addition experiments. As shown in Table 6, in the tempura sample, 63.9% of PV-B remained; in contrast, most of PV-A was lost. In the sautéed sample, the loss of PV-A and B were limited (not more than 20.5%); the loss of PV-A/B in the tempura sample may mainly be due to dissolving out into hot oil. Because PV-A is more lipophilic than PV-B, PV-A seemed to be dissolved out into the oil preferentially. In contrast to the tempura, most of PV-A remained in the sumiso sample; more than a half of PV-B was lost. PV-B is more hydrophilic than PV-A; it seemed to be dissolved out into the hot water during boiling. For both tempura and sumiso, from 42.6 to 63.9% of PV-B remained during the cooking process. The raw poisoning sample contained more than 0.1% PV-B (Table 5), and thus the cooked *Veratrum* plants that the victims ate were estimated to contain more than 400–600 μg/g levels of PV-B. This would be still a dangerous level for humans [1, 7]; finally it was confirmed that the cause of this poisoning case was PV-B.

Table 6 Decrease of *Veratrum* alkaloid during cooking

Skeleton	Compound	Concentration (μg/g)					
		Yamanaka Pass, Takayama, July 2017					
		Tempura		Sumiso		Sautéed	
		Cooked leaf[a]	Reference leaf[a]	Cooked leaf[a]	Reference leaf[a]	Cooked leaf[a]	Reference leaf[a]
Jervanine	Jervine	<1	<1	<1	<1	<1	<1
Protoverine	Protoveratrine A[b]	0.130	4.61	0.424	0.562	8.18	10.3
	Protoveratrine B	160	250	86	201	140	160
Decrease (%)	Protoveratrine A[b]	−97.2		−24.4		−20.5	
	Protoveratrine B	−36.1		−57.4		−12.5	

[a] In July, the aerial part of the plant consisted of stem and leaves. The leaves are taken and used for the experiments

[b] In all the samples, the peak for protoveratrine A was accompanied by a peak for an unknown isomer

Conclusions

Many poisoning cases with *Veratrum* species occur worldwide every year, but there have been few reports on the toxicological analysis of *Veratrum* poisoning. We performed LC–MS/MS analysis of the residual leaves of the ingested plant in a *Veratrum* poisoning case. The main toxic compound in the poisoning sample was PV-B (about 0.1%), which is most toxic among the VAs. Other toxic compounds detected were PV-A (about 1/10th of the PV-B concentration) and jervine (about 1/500th of the PV-B concentration) as minor components. After cooked as tempura or sumiso, the poisoning sample was estimated to contain more than 400-600 μg/g levels of PV-B. Therefore, the severe vomiting and rapid decrease in blood pressure observed in the patients were considered to be primarily caused by PV-B. This is a rare report on the direct correlation of PV-B ingestion with poisoning symptoms. If the patient ingested 100 g of the cooked leaves, this would provide more than 40–60 mg levels of PV-B, which might be fatal. However, severe vomiting may relieve the symptoms and increase the survival rate. For a risk assessment and to prevent further *Veratrum* poisoning, an inspection for other *Veratrum* species was conducted, and two more colonies were found in Gifu Prefecture. The VA composition of the poisoning sample was compared with those of other colonies, and all were found to be rich in PV-B, with PV-A and jervine as minor components when they are young sprouts. Over time (1–2 years), there were no major changes in the VA composition in young sprouts of Yamanaka Pass and Nenoue Highland colonies. This means that *Veratrum* species in Gifu Prefecture would be very toxic when they are young sprouts in every year. In addition, all the samples contained an unknown isomer of PV-A. These characteristics appeared to be specific to the *Veratrum* species in the Gifu Prefecture area.

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Compliance with ethical standards

Conflict of interest We declare that there are no conflicts of interest.

Ethical approval Informed consent was obtained from all participants included in the study.

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