ORIGINAL ARTICLE



Toxicological analysis of satratoxins, the main toxins in the mushroom *Trichoderma cornu-damae*, in human serum and mushroom samples by liquid chromatography–tandem mass spectrometry

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Abstract

Purpose Many poisoning cases involving the deadly toxic mushroom *Trichoderma cornu-damae* have been reported, but there are very few reports on toxicological analysis of the poisoning. In this study, a simple and sensitive method was developed for detecting and quantifying satratoxins, which are the main toxins found in *T. cornu-damae*, in human serum and mushroom samples.

Methods The four main toxins, namely, satratoxin H and its 12'-acetate, 13'-acetate and 12',13'-diacetate, were isolated from *T. cornu-damae* and used as analytical standards. These standards were spiked into human serum and effective methods were developed for extraction and detection/quantification using liquid chromatography–tandem mass spectrometry (LC–MS/ MS). Quantification of satratoxins in *T. cornu-damae* samples was performed by the standard addition method.

Results Although satratoxins, which have neutral terpene structures, showed very low sensitivities in conventional LC–MS/ MS analysis, they could be detected with enough sensitivity by our developed method. In human serum, the limit of detection was 0.1 ng/mL and the limit of quantification was 1 ng/mL for all four satratoxins. The recovery rate ranged from 70.5 to 86.6%, and the coefficients of determination for calibration curves were > 0.999. Satratoxins in *T. cornu-damae* samples were also well detected and quantified with coefficients of determination for calibration for calibration curves of > 0.997 and intraday/interday precision (relative standard deviation) ranging from 2.98 to 10.3%.

Conclusions To our knowledge, this is the first report of toxicological analysis of satratoxins using analytical standards.

Keywords *Trichoderma cornu-damae* poisoning · Satratoxins · Liquid chromatography-tandem mass spectrometry · Toxicological analysis · Standard addition method

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Introduction

Trichoderma cornu-damae (Hypocreaceae, formerly known as *Podostroma cornu-damae* or *Hypocrea cornu-damae*, Fig. 1), also called "poison fire coral" or "kaen-take" (in Japanese), is a deadly toxic mushroom native to East Asia, Southeast Asia and Oceania [1–3]. Poisoning cases involving *T. cornu-damae* have been reported [4–11] including fatal cases [4–8, 11], and the main toxins in *T. cornu-damae* have been identified as trichothecene macrolides, specifically satratoxin H and its 12'-acetate, 13'-acetate and 12',13'-diacetate (Fig. 2) [12]. These compounds are deadly poisons and their lethal doses in mice are less than 0.5 mg per capita per day [12]. The intraperitoneal LD₅₀ of satratoxin H in rats is reported as 1 mg/kg [13].



Fig. 1 *Trichoderma cornu-damae*. **a** Found in Ohtsu City, Japan, on 8 September 2013, **b** found in Ohtsu City on 7 September 2014 and collected for analysis, **c** found in Kyoto City, Japan, on 14 September 2014 and collected for analysis, **d** found in Ohtsu City on 14 September 2019

Fig. 2 Chemical structure of the four satratoxins and internal standard



Several mechanisms are known for satratoxin poisoning [14–18]. Satratoxins contain a trichothecene moiety and are classified as type-D trichothecenes, and act as trichothecene-type mycotoxins [14–18]. The macrolide moieties in the satratoxins may simply affect their toxicity by altering their hydrophobicity [12]. Trichothecenes act on serotonin-mediated neurons to induce severe vomiting, diarrhea, dehydration and hypotension as acute symptoms that usually appear within about 30 min [4, 6, 8, 9, 17]. They also strongly inhibit the activity of peptidyl transferase, which is an integral part of the 60S subunit of the ribosome, resulting in inhibition of protein synthesis, destruction of bone marrow, and dysfunction of the gastrointestinal mucosal epithelium and skin, where cell division is active [14, 15]. Trichothecenes also stimulate the production of inflammatory cytokines [18]. Consequently, satratoxins can cause cytokine storm or endotoxic shock-like symptoms within a few days of ingestion, such as circulatory failure [4, 6, 8], multiple organ failure [4–6, 8], renal failure [4–6, 8], organ necrosis [4, 6, 8], and progressive severe respiratory distress from lung necrosis [4, 5, 8]. The severity of these endotoxic shock-like symptoms depends on the amount of *T. cornu-damae* ingested. If only a small amount is ingested, these symptoms are mild [7, 9, 10], but if a lethal amount is

ingested, these symptoms are severe and can result in death [4–8, 11]. Ingestion of 1 g of *T. cornu-damae* can be fatal for an adult male [4]. Within a week of *T. cornu-damae* ingestion, protein synthesis inhibition and skin capillary disorders cause desquamation of the palms, sole, face or chest, and hair loss [4, 7, 9, 10] and *T. cornu-damae* is known as the only mushroom that causes contact dermatitis. These are considered to be characteristic symptoms of *T. cornu-damae* poisoning in both non-fatal and fatal cases. Destruction of bone marrow causes hematopoietic dysfunction, leukocytopenia and thrombocytopenia [4, 8].

Accidental poisoning with T. cornu-damae has occurred in several ways. First, the adult fruiting body of T. cornudamae is quite similar to that of the edible mushrooms Clavulinopsis laeticolor (coral mushroom, "kaben-take" in Japanese), and Clavulinopsis miyabeana ("naginata-take" in Japanese), and many poisoning cases have resulted from confusion between these mushrooms [4-6, 8] even when victims have checked the identity of collected mushrooms using mushroom guides [4]. There has also been a fatal case of ingestion of sake (Japanese rice wine) infused with dried T. cornu-damae, because it was mistaken for a medicinal mushroom [4]. Furthermore, the young fruiting body of T. cornu-damae is similar to that of the rare edible mushrooms Ganoderma lucidum ("ling zhi" in Chinese or "reishi" in Japanese) [7, 10] and Cordyceps sobolifera (caterpillar fungus, "chong cao" in Chinese or "semi-take" in Japanese) [4, 9], which are important in traditional Chinese medicine. Consequently, there have been many cases of accidental ingestion of T. cornu-damae in decoctions or brewed drinks [4, 7, 9, 10], which are common preparations in traditional Chinese medicine.

Satratoxin H is the main mycotoxin in Stachybotrys species, and there are many reports on the environmental analysis of satratoxin H including direct ionization-electron ionization mass spectrometry [19], gas chromatography-mass spectrometry with trimethylsilyl derivatization [20], liquid chromatography (LC) with ultraviolet detection [21, 22] and LC-tandem mass spectrometry (LC-MS/MS) [23-26]. Yet, despite many reports of poisoning cases involving T. cornudamae, including fatal cases, there is only one report on the toxicological analysis of T. cornu-damae and satratoxins and no analytical standards for satratoxins were used [11]. In addition, although T. cornu-damae has been previously thought of as an uncommon mushroom, in 2015, it suddenly began growing naturally in urban areas throughout Japan, and now it is common and well-known deadly poisonous mushroom readily obtained in Japan and easily identified because of its characteristic appearance. Consequently, the forensic toxicologists have to prepare for both accidental poisonings and cases of suicide/homicide (including attempted cases), injury, or food tampering using T. cornu-damae. The aim of this study was to develop a simple and precise method for detecting and quantifying satratoxins in human serum using LC–MS/MS and analytical standards of satratoxins. In addition, a standard addition method for satratoxins in *T. cornu-damae* was also investigated.

Materials and methods

Chemicals and reagents

Satratoxin H, its 12'- and 13'-acetates and its 12',13'-diacetate were isolated from T. cornu-damae samples collected in Kyoto City, Japan, on 14 September 2014 and Ohtsu City, Japan, on 7 September 2014 (Fig. 1) by the method reported by Saikawa et al. [12]. The isolated satratoxins were used as analytical standards after their nuclear magnetic resonance spectra (Fig. S1) were confirmed to coincide with previously reported spectra [12, 27]. The purities of satratoxin H, its 12'-acetate, 13'-acetate and 12',13'-diacetate used to prepare stock solutions were 84.5%, 98.2%, 99.1% and 78.9%, respectively (Fig. S2). Diacetoxyscirpenol (Fig. 2) was purchased from Fuji Film Wako Pure Chemical Corporation (Osaka, Japan). Stock solutions of the above compounds were prepared as 100 µg/mL acetonitrile solutions and stored at -20 °C in the dark. Human serum was purchased from Sigma-Aldrich (St. Louis, MO, USA) 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 commercially available and of analytical grade.

Instrumentation

The LC-MS/MS system consisted of an Eksigent ekspert ultra 100-XL system (Sciex, Framingham, MA, USA) and a QTrap6500 mass spectrometer (Sciex). For detection and quantification, MS measurements were performed in multiple reaction monitoring (MRM) mode. For structural elucidation, MS measurements were performed in enhanced product ion (EPI) mode with collision energy (CE) of 20, 35, and 50 V and in collision energy spread (CES) mode with CE ramped from 20 to 50 V. The optimized parameters for the MRM transitions used are shown in Table 1. The chromatographic separation was performed on an Ascentis C18 column (150×2.1 mm i.d., particle size 3 µm, Sigma-Aldrich) in combination with an Ascentis C18 Guard Cartridge (20 mm × 2.1 mm i.d., particle size 3 µm, Sigma-Aldrich) as a guard column. The column temperature was maintained at 40 °C. The buffer (pH 3) for the LC mobile phase was prepared by adding 0.1% formic acid to 4 mm ammonium acetate (A). Methanol was the organic mobile phase (B). The gradient was started at 30% B, ramped linearly to 90% B over 30 min, and then held at 30% B until Table 1Retention times andMRM transitions of satratoxinsand the internal standard

Compound	Retention time (min)	Transition	Precursor Ion/Q1	Monitoring Ion/Q3	DP (V)	EP (V)	CE (V)	CXP (V)
Satratoxin H	19.0	1 (quantifier ion)	529.24	231.14	131	10	25	16
		2 (qualifier ion)	529.24	201.08	131	10	35	20
Satratoxin H 12'-Acetate	23.9	1 (quantifier ion)	571.25	245.08	111	10	23	16
		2 (qualifier ion)	571.25	231.14	111	10	23	16
Satratoxin H 13'-Acetate	22.6	1 (quantifier ion)	571.25	245.08	111	10	23	16
		2 (qualifier ion)	571.25	231.14	111	10	23	16
Satratoxin H 12',13'-Diacetate	26.4	1 (quantifier ion)	613.26	245.08	126	10	23	16
		2 (qualifier ion)	613.26	231.14	126	10	23	14
Diacetoxyscirpenol (internal standard)	14.2	1 (quantifier ion)	384.20	307.15	35	10	14	17

MRM multiple reaction monitoring, *DP* declustering potential, *EP* entrance potential, *CE* collision energy, *CXP* collision cell exit potential

40 min for equilibration. The mobile phase flow rate was 0.2 mL/min, and the injection volume was 3μ L.

Serum sample cleanup procedure

A serum sample (1.0 mL) was loaded into an Oasis HLB cartridge (1 mL, 30 mg) (Waters Japan, Tokyo, Japan) that had been pre-conditioned with 1 mL of methanol followed by 1 mL of water. The cartridge was washed with 1 mL of water, followed by 1 mL of 30% aqueous methanol, and then the target compounds were eluted with 1 mL of methanol. After evaporation of the eluate, the residue was carefully redissolved in 30 μ L methanol and further diluted with 70 μ L of 4 mm ammonium acetate with 0.1% formic acid buffer (pH 3). The solution was transferred into a 250- μ L deactivated glass insert (Agilent 5181-8872, Agilent Technologies Japan, Ltd., Tokyo, Japan). Aliquots (3 μ L) of the prepared samples were injected into the LC–MS/MS system.

Validation procedures for serum samples

Calibration standards were prepared by mixing blank serum with a standard solution containing the four satratoxins at 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, and 50 ng/mL. Diacetoxyscirpenol was added to the serum samples at 10 ng/mL as an internal standard (IS) before extraction. The samples were cleaned up by the method described above and analyzed by LC-MS/MS. The LOD was defined as the lowest concentration giving a signal-to-noise (S/N) ratio higher than 3. The LOQ was defined as the lowest concentration giving a relative standard deviation (RSD) of quantification values within 20%. Calibration curves were constructed by plotting the peak area ratios of analytes to IS (y-axis) versus the concentrations of the calibration standards (x-axis). Each calibration curve was fitted with a linear equation by leastsquares approximation. Extraction recovery was determined at concentrations of 1, 5, and 20 ng/mL for the four target compounds. For each concentration, five replicate aliquots (1.0 mL) of the serum sample were spiked with the appropriate amount of analytes (pre-spiked samples). An additional five replicate aliquots (1.0 mL) had no analytes added (postspiked samples). After extraction of the post-spiked samples, the eluate from the Oasis HLB cartridge was spiked with the same amount of analytes as the pre-spiked samples. IS (100 ng/mL) was added to the eluates of all samples. Then, all the spiked eluates were evaporated to dryness and redissolved in a final volume of 100 µL with the initial mobile phase for LC-MS/MS as described above. The recoveries were calculated by comparing the peak area ratios of analytes to IS for the pre-spiked samples with those for the postspiked samples. The results for each set of five replicates were averaged. Quality control samples were independently prepared at 1, 5, and 20 ng/mL. The samples were quantified using the above calibration curve. The accuracy (% relative error) is expressed as the difference between the concentration at which the sample was spiked and the measured concentration. Intraday precision was evaluated using the RSD of the calculated concentrations for five replicates spiked at three levels which were subjected to extraction and analysis on the same day. Interday precision was assessed using the RSD of the mean concentrations measured on three different days at three levels (three replicates at each level on each day). Matrix effects of serum on the sensitivities for the analytes were also determined. Five blank serum samples (1 mL) were extracted by the same method as used for the post-spiked samples. To each eluate from the Oasis HLB column, 100 µL of 10 µg/mL mixed standard solution of the four analytes was added. The solution was evaporated to dryness and re-dissolved in a final volume of 100 µL with the initial mobile phase for LC-MS/MS. Matrix effects were calculated by comparing the peak areas of analytes in serum with those in standard aqueous solutions. The results for each set of five replicates were averaged.

Stabilities of satratoxins in serum samples

Satratoxin-spiked serum samples were independently prepared at 1 and 20 ng/mL as described above, and 1 mL of 20 ng/mL solutions were stored and putrefied at room temperature (rt) in the dark for 1 week (n=5) to study degradation of satratoxins. Further sets of serum samples were stored at -20 °C for 1 week and thawed at rt (n=5), stored at -20 °C in the dark for 1 week and thawed at 4 °C (n = 5), and stored at -20 °C in the dark for 3 weeks (n = 5) and that the true of 1 ng/mL sample was stored at -20 °C for 1 week and thawed at rt (n = 5). After storage and thawing, 100 µL of 1 µg/mL IS solution was added to each sample before solid-phase extraction as described in the "Serum sample cleanup procedure" section. Freshly prepared serum samples spiked with satratoxins at 1 and 20 ng/mL (1 mL, n = 5) were also added 100 μ L of 1 μ g/mL IS solution and submitted to solid-phase extraction in the same manner as the stored samples. Satratoxins in the freshly prepared and stored samples were quantified by LC-MS/MS as described in the "Validation procedures for serum sample" section. The recoveries of the satratoxins after storage were calculated by comparing the peak area ratios of the analytes to the IS for the stored samples with those for the freshly prepared samples.

Mushroom sample preparation

A 1-g mushroom sample was finely chopped (< 0.5 mmcube) and mixed well. Then, 50-mg samples were accurately weighed, transferred to 50-mL polyethylene capsules, and stored at - 20 °C in the dark until qualitative/ quantitative analysis and method validation experiments. These 50-mg samples were frozen in liquid nitrogen for 5 min, and then pulverized using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) at 3000 rpm for 10 s. Then, they were allowed to warm to rt. Next, 1 mL of methanol was added to the obtained powders in the capsules, and the mixtures were again homogenized using the Multi-Beads Shocker at 3000 rpm for 10 s at rt. The homogenates were transferred into 50-mL centrifuge tubes, and the capsules were washed with 1 mL of methanol three times. The washings were also transferred to the centrifuge tubes. The extracts were ultrasonicated for 30 min, and centrifuged at $3000 \times g$ for 30 min at 4 °C. The supernatants were collected, and the total volumes were adjusted with methanol to give 10.0-mg mushroom/mL methanol extract solutions. These solutions were used as 10-mg mushroom/ mL stock solutions of the mushroom sample extracts, stored at - 80 °C in the dark, and analyzed by LC-MS/ MS as soon as possible.

Qualitative analysis of the satratoxins

The LC–MS/MS samples of spiked serum samples (1, 5, and 20 ng/mL) were analyzed in MRM mode. The 10-mg mushroom/mL stock solutions of the mushroom sample extracts were diluted to give 10-µg mushroom/mL LC–MS/MS samples as described above and analyzed in MRM mode. The ratio of the peak area of transition 1 (quantifier ion, Table 1) to transition 2 (qualifier ion, Table 1) was required to be within the tolerance values specified in European Union guidelines for qualitative analysis [28]. For qualitative analysis of the relatively concentrated samples (5 and 20 ng/mL serum samples and 100-µg mushroom/mL LC–MS/MS sample of mushroom extract), appropriate EPI/CES spectra were also compared.

Quantitative analysis of satratoxins in mushroom samples by the standard addition method

Calibration reference standards for the absolute calibration curves of the four satratoxins were prepared at concentrations of 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, and 100 ng/mL from 100 µg/mL standard solution by dilution with LC mobile phase [buffer-methanol (70:30, v/v)]. IS was added at 100 ng/mL to each calibration reference standard as an IS. Absolute calibration curves were constructed by plotting the peak area ratio of the analytes to the IS (y-axis) versus the concentration of the calibration standard (x-axis). Each calibration curve was fitted with a linear equation by leastsquares approximation. For quantification of satratoxins in the mushroom samples, 10-µg mushroom/mL LC-MS/MS samples were first quantified using the above absolute calibration curves to determine an approximate concentration of each satratoxins in the LC-MS/MS sample (x ng/mL). Then, LC–MS/MS samples spiked with satratoxins of x, $2 \times$ and $3 \times ng/mL$ were prepared and used to construct four-point calibration curves, in which the peak area ratio of the analytes to the IS (y-axis) was plotted against the concentration of the calibration standards (x-axis). Each calibration curve was fitted with a linear equation by least-squares approximation. The concentrations of satratoxins in the LC-MS/ MS samples were determined as the absolute values of the x-intercepts, and the initial concentrations of satratoxins in the mushroom samples were re-calculated. This quantification procedure was performed for each analyte separately.

Validation procedures for mushroom samples

Validation was performed for the quantification of satratoxins in *T. cornu-damae* samples collected at Kyoto City on 14 September 2014 and Ohtsu City on 7 September 2014. The stored 50-mg subdivided samples were pulverized, extracted to give 10 mg/mL stock solutions of the mushroom sample as described in the "Mushroom sample preparation" section, and the stock solutions were subjected to LC–MS/MS analysis as described in the "Quantitative analysis of satratoxins in mushroom samples by the standard addition method" section. Intraday precision was evaluated using the RSD of the calculated concentrations for five replicates subjected to extraction and analysis on the same day. Interday precision was assessed using the RSD of the mean concentrations measured on three different days (three replicates on each day). Matrix effects on the quantification of satratoxins in mushroom samples were calculated by comparing peak areas between 10- μ g mushroom/mL LC–MS/MS samples and the aqueous solutions of the analytical standard satratoxins at the calculated concentrations of LC–MS/MS samples by the standard addition method.

Stabilities of satratoxins in methanol extract of mushroom sample

For stability testing, 1 mL of 10-mg mushroom/mL methanol extract stock solution of the *T. cornu-damae* sample collected in Kyoto City on 14 September 2014 prepared as described in the "Mushroom sample preparation" section were stored under the following conditions: at rt in ambient light indoors for 1 week (n=5), at rt in the dark for 1 week (n=5), at 4 °C in the dark for 1 week (n=5) and at – 20 °C in the dark for 1 week (n=5). After storage, each sample was diluted to 10-µg mushroom/mL for LC–MS/MS samples, to which IS was added at 100 ng/mL. Freshly prepared 1 mL of 10-mg mushroom/mL methanol extracts of the *T. cornu-damae* sample collected in Kyoto City on 14 September 2014 (n=5) was also diluted to give 10 µg mushroom/mL LC–MS/MS and IS was added to each sample at 100 ng/mL.

Then, satratoxins in the freshly prepared and stored samples were quantified by LC–MS/MS as described in the "Quantitative analysis of satratoxins in mushroom samples by the standard addition method" section. The recoveries after storage were calculated by comparing the peak area ratios of the analytes to the IS for the stored samples with those for the freshly prepared samples.

Results

MS detection

The EPI spectra (CE: 20 V) of four satratoxins are shown in Fig. 3, and the other EPI spectra (CE: 20, 35 and 50 V) and



Fig. 3 Enhanced product ion (EPI) spectra of the four satratoxins with collision energy (CE) at 20 V. a Satratoxin H, b satratoxin H 12'-acetate, c satratoxin H 13'-acetate, d satratoxin H 12',13'-diacetate

CES spectra are shown in Figs. S3–S6. In all the spectra, the fragmentation series of m/z 249 => 231 and m/z 263 => 245 were observed as major ions at relatively low CE (Fig. 3, Figs. S3–S6). These ions were used as the monitoring ions in MRM mode after optimization. Typical MRM chromatograms of four satratoxins are shown in Fig. 4. The peaks of all the compounds (including the IS) were well separated with good peak shapes.

Validation of the analytical method for serum samples

Before constructing the calibration curves, LODs and LOQs were determined for the four satratoxins in human serum. All the satratoxins have neutral terpene structures and were detected with extremely low sensitivity by MS in both positive and negative mode, especially satratoxin H. The QTrap6500 instrument could detect the protonated molecular ions of all the satratoxins with practical sensitivities. The LODs of the satratoxins were 0.1 ng/mL in serum (Table S1; Fig. 5b), and both the quantifier and qualifier ions were detected with an S/N ratio of > 3 at this concentration. LOO is usually defined as an appropriate S/N ratio. However, the ionization of satratoxins, which are neutral terpene compounds, is likely to be influenced by unexpected matrix effects or heterogeneity of the samples. Therefore, LOQ was defined as RSD within 20% [29] and determined to be 1 ng/mL for each compound (Table S1) during the following validation process. For each compound, the linearity of the calibration curve was examined over the concentration range from the LOQ of the compound up to 50 ng/mL. The serum samples were finally concentrated from 1 mL to 100 µL; thus, a 50 ng/mL spiked sample had a final concentration of 500 ng/ mL and this point was set as the upper limit. For the four satratoxins, the calibration curves showed satisfactory linearity with coefficients of determination of > 0.999 (Table S1). Matrix effects ranged from 88 to 114% (Table S1), which could be a negilible level in actual analysis.

Recovery experiments for satratoxins in serum were performed with spiked samples at 1, 5, and 20 ng/mL. The results are shown in Table S2. The average recovery yield was between 70.5 and 86.6% for all analytes at all the spiked levels tested.

The repeatability of quantification for the four satratoxins was assessed using intraday precision (n=5) at three concentrations (1, 5 and 20 ng/mL), and the results are shown in Table S3. At the spiked level of 1 ng/mL, the intraday RSDs were in the range 3.2–18.4% (Table S3). In this spiked level, the quantification results started to be influenced irregularly by unexpected matrix effects or sample heterogeneity but the *S/N* ratio was sufficient (Fig. 5a). Thus, the LOQ was determined to be 1 ng/mL (Table S1, S3). At spiked levels of 5 and 20 ng/mL, the intraday RSDs were in the range of 5.3–7.8% (Table S3). The interday RSDs at spiked levels of 1, 5, and 20 ng/mL were in the range 3.4-7.6% (Table S3). There were no interference peaks in blank serum (Fig. 5c), even though the samples were concentrated tenfold.

Stabilities of satratoxins in serum samples

The stabilities of the satratoxins in serum under various storage conditions were evaluated using recovery rates (Table S4). In all the serum samples cryopreserved at -20 °C, the concentrations of all four satratoxins showed no obvious decreases regardless of the spiked level, storage period, or thawing temperature (Table S4). In the putrefied serum samples stored at rt for a week, a slight decrease in the concentration of satratoxin H 12',13'-diacetate and

Fig. 4 Multiple reaction monitoring (MRM) chromatograms of a satratoxin H 12',13'-diacetate, b satratoxin H 12'-acetate and 13'-acetate, c satratoxin H, d internal standard (each 100 ng/mL)



Fig. 5 MRM chromatograms of human serum spiked with the satratoxins. a Limit of quantification (LOQ) level (1 ng/mL), b limit of detection (LOD) level (0.1 ng/mL), c blank serum



slight increases in the concentrations of satratoxin H and its monoacetates were observed (Table S4). The sum of the recoveries of the four satratoxins was almost 400% under all the storage conditions (Table S4).

Validation of the standard addition method for mushroom samples

The MRM chromatograms of the $10-\mu g$ mushroom/mL extracts of the two *T. cornu-damae* samples are shown in Fig. 6. For the four satratoxins, quantifier and qualifier ions were successfully detected with sufficient sensitivity. For both samples, in the transitions for satratoxin H, an unknown peak was observed at 20.5 min (Fig. 6a, b) and this was thought to be 12'-epi-satratoxin H [30] or isosatratoxin H [31] since these are the only currently known isomers of satratoxin H. However, we ultimately

could not identify this peak because of the limited amount of mushroom samples. The standard addition calibration equations for satratoxins in mushroom samples are shown in Table S5. Matrix effects calculated by comparing the peak areas of analytes in quantified methanol extract samples (10 μ g mushroom/mL) with those in standard aqueous solutions of the same concentration are also shown in Table S5. Table S6 shows the intraday and interday precision quantification of satratoxins in mushroom samples. The intraday and interday repeatability for satratoxins ranged from 5.05 to 10.3% and 2.98 to 10.3%, respectively, and in terms of matrix effects, each compound suf-

fered moderate ion suppression (54.1-80.5%, Table S5).

Concentrations of the four satratoxins in the mushroom

samples collected in Kyoto City and Ohtsu City were very

similar (Table S6), probably because Kyoto City and Ohtsu

Fig. 6 MRM chromatograms of *T. cornu-damae* sample extracts (each 10 μg mushroom/mL). **a** Collected in Kyoto City, Japan, on 14 September 2014, **b** collected in Ohtsu City, Japan, on 7 September 2014



City are close together (Fig. 1).

Stabilities of satratoxins in methanol extracts of mushroom sample

The stabilities of the satratoxins in 10-mg mushroom/mL methanol extracts of mushroom sample that were stored under various conditions were evaluated using recovery rates (Table S7). Under – 20 °C storage for 1 week, the concentrations of four satratoxins did not show any obvious increase or decrease (Table S7), as in the case of the serum samples. However, as the storage temperature increased (4 °C to rt in the dark to rt in ambient light), the concentrations of satratoxin H 12',13'-diacetate and 12'-acetate decreased, and those of satratoxin H and its 13'-diacetate increased (Table S7). The effect of changing from storage in the dark to in ambient light was minimal or within the experimental error (Table S7).

Discussion

MS detection

The major fragmentation series of m/z 249 => 231 observed in all the spectra at relatively low CE (Fig. 3, Figs. S3–S6) which was used for MRM detection could be due to the trichothecene verrucarol moiety (Fig. 7a, b) and also could be due to fragment ions common to trichothecene-type mycotoxins, including the IS diacetoxyscirpenol. The dehydration process producing m/z 231 could occur at the 15-OH group of the verrucarol moiety, but could also occur at the epoxide (Fig. 7a) [20]. In the EPI and CES spectra, the fragmentation series of m/z 263 => 245 was also observed as major fragment ions at relatively low CE (Fig. 3, Figs. S3–S6) and could be due to the macrocyclic moiety (Fig. 7b).



Fig. 7 Proposed structures of the product ions. a Fragment ions from the trichothecene vertucarol moiety, b fragment ions from the macrocyclic moiety

Validation of the analytical method for serum samples

For quantification of satratoxins in serum samples, high linearity (>0.999) and weak matrix effects (88-114%) were demonstrated in this experiment, and these could be attributed to the strong adsorpsion of the satratoxins onto the Oasis HLB cartridges. This enabled thorough washing of the cartridges and efficient cleanup of the serum samples, even though the process includes concentrating the samples tenfold. The average recovery, which ranged between 70.5 and 86.6% for all analytes at all the spiked levels tested, can be considered a satisfactory result. The low LOD (0.1 ng/ mL), LOQ (1 ng/mL) and detection range (0.1–50 ng/mL) for all the analytes in serum can enable detection/quantification with satisfactory sensitivity. The intraday RSDs (5.3-7.8%) at spiked levels of 5 and 20 ng/mL and the interday RSDs (3.4-7.6%) at spiked levels of 1, 5, and 20 ng/mL indicate that satratoxins can be quantified with satisfactory repeatability.

The satratoxins showed unexpected stabilities in serum. In all the serum samples cryopreserved at -20 °C, the concentrations of all four satratoxins showed no obvious decreases, indicating that there is no risk of satratoxin degradation for serum samples stored at this temperature. The slight decrease of the concentration of satratoxin H 12',13'-diacetate and slight increases of the those of satratoxin H and its monoacetates in the putrefied serum samples stored at rt for a week could be due to non-enzymic or enzymic chain hydrolysis (Fig. 8) [32]. The sum of the recoveries of the four satratoxins was almost 400% under all the storage conditions, meaning that only interchange between the four satratoxins occurred, with no other degradation processes such as hydrolysis of the lactone ring or reaction of the epoxide group. Importantly, only minimal degradation of satratoxins occurred in the putrefied human serum samples stored at rt for a week. This suggests that satratoxins could remain in blood in fatal T. cornu-damae poisoning cases and could be detectable for a while after death.



Fig. 8 Proposed hydrolysis/methanolysis pathways of satratoxins in human serum at room temperature and in methanol solutions

Validation of the standard addition method for mushroom samples

In contrast to serum samples, "blank" T. cornu-damae samples are not available. Thus, to overcome matrix effects in the quantification of satratoxins in T. cornu-damae samples, we employed a four-point standard addition method. The coefficients of determination showed satisfactory linearity (0.9967–0.9999) for quantification of satratoxins in actual T. cornu-damae samples. The intraday RSDs (5.05–10.1%) and interday RSDs (2.98-10.3%) showed satisfactory repeatability and would be precise enough for quantification of satratoxins in actual T. cornu-damae samples. In terms of matrix effects, each compound suffered moderate ion suppression (54.1-80.5%), because no clean-up was performed for mushroom samples and only high dilution was used in this study. As a consequence, mushroom constituents remained in the extract and could suppress the ionization of satratoxins.

Stabilities of satratoxins in methanol extracts of mushroom sample

Information on stability in actual T. cornu-damae samples during storage is important, but we did not have a sufficient amount of T. cornu-damae samples, so we performed stability experiment using satratoxins in T. cornu-damae methanol extracts instead. Under - 20 °C storage, no obvious methanolysis of satratoxins were observed. However, unlike the frozen serum samples, the methanol solutions do not freeze under the storage temperatures used in this study, which meant that molecular motions did not stop and slight methanolysis was observed under 4 °C or rt storage. In addition, the concentrations of satratoxin H 12',13'-diacetate and 12'-acetate decreased, and those of satratoxin H and its 13'-diacetate increased. This could have occurred because the 12'-acetoxy group is readily methanolyzed but the 13'-acetoxy group is resistant to methanolysis (Fig. 8). The reason for this difference in susceptibility to methanolysis is currently unknown. The effect of changing from storage in the dark to storage in ambient light was minimal or within the experimental error (Table S7). In summary, satratoxins in methanol stock solutions were stable at -20 °C for at least a week.

Conclusions

Poisoning cases involving the deadly poisonous mushroom *Trichoderma cornu-damae* ("poison fire coral") have been reported, including fatal cases, but there have been very few reports on toxicological analysis of *T. cornu-damae*

poisoning. Here, we developed a simple analytical method using LC-MS/MS detection and quantification of the deadly mycotoxins satratoxin H and its 12'-acetate, 13'-acetate and 12',13'-diacetate in human serum at the parts per billion to parts per trillion level with satisfactory precision. To our knowledge, this is the first report of toxicological analysis of these four known satratoxins in human serum using analytical standards. This means that absolutely no data on the blood levels of satratoxins in poisoning cases have been reported to date. In one fatal poisoning case, unchanged satratoxins were detected in blood samples by LC–MS/MS in full scan mode [11] indicating that unchanged satratoxins were present in blood at nanogram per milliliter levels. Therefore, our method, which enables detection of satratoxins at picogram per milliliter levels, could be a practical method for toxicological analysis of T. cornu-damae poisoning. We also performed detection and quantification of satratoxins in T. cornu-damae samples. Quantification by the standard addition method successfully overcame the matrix effects from mushroom constituents. The four satratoxins were successfully detected in mushroom samples and quantified with satisfactory repeatability and reproducibility. Satratoxins were unexpectedly stable in human serum and methanol extracts when stored at -20 °C for at least a week, with only minimal degradation occurring in putrefied human serum after storage at rt for a week. This method is expected to be useful in T. cornu-damae poisoning cases for analyzing both patient samples and residual cooked mushroom samples because of its simple pre-treatment and highly sensitive detection and quantification.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent There was no requirement for informed consent in this study.

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