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Tetrodotoxin poisoning evidenced by solid-phase extraction combining with liquid chromatography–tandem mass spectrometry

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ABSTRACT

The toxicity and toxin component of gastropod *Niotha clathrata* implicated to a food paralytic poisoning incident in Kaohsiung, Taiwan in November 2006 were studied. The highest scores of average toxicity in the digestive gland and other portions from collected gastropods were 62 ± 24 (mean ± S.D.) and 32 ± 16 μg/g according to tetrodotoxin (TTX) bioassay, respectively. The toxin from these gastropods was large amount and easily identified as tetrodotoxin by traditional method of HPLC-FLD. The toxin of patient's blood serum was trace amount and analyzed by a new developed method LC–MS/MS. LC–MS/MS was contracted by the LC system interfaced with the MS/MS system with a turbo ion spray interface. Positive ion detection and multiple reaction monitoring mode were used for TTX of patient serum. It was found that linearity in serum was observed within concentration ranged of 1–100 ng/ml and limit of detection was 0.1 ng/ml. The LOQ was reproducible at 1 ng/ml in serum. The blood serum showed to contain TTX of 3.30 ± 0.08 ng/ml. It indicated that LC–MS/MS was more lower detectable and believable method for TTX determination than LC–MS reported previously. Furthermore, the causative agent of gastropod food poisoning was identified as TTX.

1. Introduction

The food poisoning incident due to ingesting a small gastropod *Niotha clathrata* occurred in Kaohsiung, Taiwan, in November 2006. The 5 years daughter ate about 10–20 unknown spiral shells in dinner with her parents was reported. Onset of symptoms began approximately 1–2 h after ingestion, the victims presented symptoms of paresthesia of lips, tongue, abdominal pain and vomiting. All patients were discharged uneventfully from the hospital after 1 day of management, and now are healthy.

Recently, several poisoning cases due to ingestion of toxic puffers and gastropods have occurred in Taiwan, Japan, China and Southeast Asia [1,2]. Especially small gastropods in China have caused many poisoning incidents for a long time, resulting in many deaths and severe problems on public health [3].

A number of human fatalities stemming from the ingestion of gastropod associated poisoning have also been reported in Taiwan [1,4]. The causative agents are commonly reported as tetrodotoxin (TTX) and/or paralytic shellfish poisons (PSP) [1,5]. Both toxins are the most public health concern worldwide, but TTX is distributed mainly in Asia, especially Japan, Taiwan, and the southeast coast of China [6–10].

TTX and PSP are nervous toxins that block voltage-gated sodium channels, resulting in respiratory paralysis and often death in human [11,12]. TTX has been detected in some Taiwanese gastropods including *N. clathrata*, *Nassarius conidalis* [6], *Na. castus*, *Na. papillosus* [13,14], *Na. glans* [15], *Oliva miniacea*, *O. mustelina*, *O. hirasei* [16], *Polinices didyma*, *Zeuxis scalaris* [17], and *Z. sufflatus* [18]. Among them, most of toxins were contained in the digestive gland of gastropods except *O. miniacea*, *O. mustelina*, and *O. nirasei*. On the other hand, some gastropods, including *Tectus fenestratus*, *T. niloticus*, *T. pyramis*, *T. hanleyanus* and *Turbo argyrostomus*, contained PSP only [19]. But several gastropods including *Na. papillosus* [14], *N. clathrata* [20], and *Natica lineata* [21] contained TTX and PSP.

The gastropod *N. clathrata* is a species of carnivorous gastropod Nassariidae. In the previous reports, the gastropod *N. clathrata* contained mainly toxin TTX, with the minor GTX [3,4,20]. Furthermore, several gastropod species of Nassariidae [17] collected from

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Table 1
Toxicity in specimens of *Niotha clathrata* collected from Kaohsiung in November 2006

Specimen no.	Body weight (g)	Body length (cm)	Digestive gland		Muscle		Total toxicity (μg/specimen)
			Weight (g)	Toxicity[a] (μg/g)	Weight (g)	Toxicity (μg/g)	
1	2.86	2.48	0.42	110	0.68	40	73
2	2.91	2.50	0.37	104	0.72	70	88
3	2.28	1.98	0.12	42	0.26	17	9
4	2.25	2.02	0.16	29	0.33	13	9
5	2.45	2.22	0.23	49	0.52	33	28
6	2.47	2.28	0.28	54	0.42	22	24
7	2.83	2.65	0.35	65	0.62	24	38
8	2.55	2.23	0.22	57	0.42	46	32
9	2.58	2.61	0.21	40	0.52	20	18
10	2.86	2.65	0.33	72	0.64	43	52
11	2.77	2.65	0.25	66	0.60	27	33
12	2.54	2.31	0.18	52	0.48	34	25
Mean ± S.D.	2.61 ± 0.23	2.38 ± 0.24	0.26 ± 0.09	63 ± 24	0.52 ± 0.14	32 ± 16	36 ± 24

[a] Toxicity was examined by tetrodotoxin bioassay and is expressed as μg/g of TTX in the digestive gland and muscle.

southern Taiwan also contained TTX and PSP. Hwang et al. [17] reported that the gastropod *N. clathrata* collected from southern Taiwan showed regional and seasonal variations.

In this paper, we report three patients with tetrodotoxication due to the gastropod *N. clathrata.* The uneaten specimens of *N. clathrata* from the same meal, were collected to detect the toxicity and the causative agents were identified in the digestive gland and other portions of the gastropods and blood of the daughter.

So far, several analytical methods for trace amount of TTX in urine and blood samples of poisoned patients have been reported. Kurono et al. [22] has developed a GC-MS for human blood sample. Although the limit of quantification (LOQ) of TTX in this method was low, but required a complex extraction procedure and time-consuming. The immunoaffinity chromatography method showed high sensitivity to detect TTX from urine, but using the monoclonal antibody was expensive [23]. Recent liquid chromatography with mass spectrometry (LC–MS–ESI) has also been developed [5,24].

In this study, the toxin of gastropod was large and was confirmed to contain TTX by using traditional method of HPLC-FLD. However, the toxin in the blood sample was trace amount and the new LC–MS/MS method was developed to confirm the presence of TTX. This study showed that LC–MS/MS combining with solid phase extraction (SPE) cartridges was more lower detectable and believable for determining TTX in the blood samples than LC–MS reported previously [24].

2. Materials and methods

2.1. Materials

Twelve specimens of causative gastropod *N. clathrata* remaining from the food poisoning case in November 2006 were collected and frozen at −20 °C until the assay was carried out. The edible parts of these specimens were removed from the shells and separated into the digestive gland and edible portions. Blood sample (6 ml) was collected from the daughter after 12 h of digestion and frozen at −20 °C for use. Authentic TTX, 4-*epi* TTX and anhydrotetrodotoxin (anh-TTX) were obtained from Wako Pure Chemical Industry (Tokyo, Japan). Authentic PSP components GTX1-4 isolated and purified from the purple clam *Soletellina diphos* in Taiwan were used [25]. Authentic saxitoxin (STX) and neoSTX obtained from the crab *Zosimus aeneus* in Japan (Daigo, Noguchi, Miwa, Kawai, and Hashimoto, 1988) were kindly provided by Professor O. Arakawa, Nagasaki University, Japan. These authentic toxins have been checked their identity and purity according to the method of Sullivan and Iwaoka [26]. The calibration curve of those toxins have been checked by NRC CRM-STXdiAc agent (National Research Council Canada, Nova Scotia, Canada). The toxins were separately kept in several small black tube as stock solutions (180 μg/ml), freeze-dried and stored at −70 °C. Among them, one stock toxin was dissolved in 1 ml of 0.03 M acetic acid and kept at −20 °C until use.

2.2. Bioassay method of toxicity

Toxic muscle and digestive gland were separately homogenized for 5 min with 3 volumes of 1% acetic acid in methanol and centrifuged (20,000 × *g*, 20 min). The operation was repeated twice. The supernatants were combined, concentrated under reduced pressure at 45 °C and examined for the toxicity by the mouse assay for TTX. Lethal potency was expressed in mouse units (MU). One MU is equal to 0.178 μg TTX, defined here as the amount of toxin required to kill 20 g of ICR strain male mice in 30 min after intraperitoneal injection [27].

2.3. Sample preparation

2.3.1. Extraction and purification of toxin from gastropod

The remaining extracts from the toxic specimens of each species were mixed, partially purified by using a C18 Sep-Pack cartridge column, and then ultrafiltered as previously described [5]. It is meant that the retained supernatant was passed through a cartridge column (C18 Sep-Pack cartridges, Millipore, Waters, MA, USA), previously regenerated with 10 ml methanol and equilibrated with 10 ml water. Toxin was absorbed in column and eluted using

Table 2
Patient symptoms and tetrodotoxin concentration in the serum

Case no.	Age (years)	Sex	Serum concentration (ng/ml)	Estimated amount of ingested gastropods (specimens)	Clinical effects
1	5	F	3.30[a]	20	Paresthesia of lips, tongue, abdominal pain and vomiting
2	37	F	–	8	Paresthesia of lips, tongue
3	40	M	–	12	Paresthesia of lips, tongue and slight abdominal pain

[a] Serum sample collected in E-DA hospital after of ingestion approximate 12 h. The limit of detection was 0.1 ng/ml. The LOQ was reproducible at 1 ng/ml in serum.

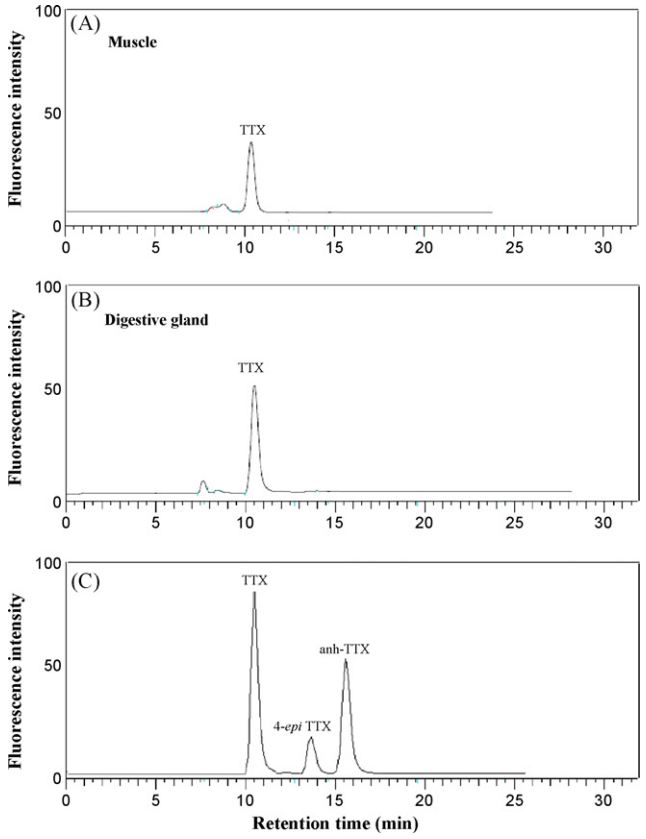

Fig. 1. HPLC chromatograms of TTX toxin from the (A) muscle (118 μg/ml) and (B) digestive gland (135 μg/ml) of *N. clathrata* specimens and (C) authentic TTX, 4-*epi* TTX and anh-TTX (250 μg/ml) by using HPLC-FLD method. The injection volume was generally 20 μl on column. The retention time for TTX was 10.5 min.

10 ml methanol, resulting in the pigments being retained in the column. The elute was freeze-dried, dissolved in 2 ml of 0.5% acetic acid and filtered through a 3000 MW cut-off Ultrafree microcentrifuge filters (Micron YM-3, Millipore, Waters, MA, USA). The filtrate was dried down, made the volume to 1 ml and submitted to subsequent HPLC-FLD [5,28].

2.3.2. Extraction of toxin from the blood serum

The sample cleanup procedure used was based on the procedure developed by O'Leary et al. [28] for TTX in serum and urine of poisoned patients. Each 1.0 ml of the blood sample was thawed and mixed with 500 μl of 0.5 M acetic acid to keep it acidic. The sample was immediately centrifuged at 10,000 × *g* for 3 min. The blood serum was loaded onto the cartridge (Oasis MCX cartridges, Millipore, Waters, MA, USA), previously regenerated with 1 ml methanol and equilibrated with 1 ml water. The TTX was then eluted with two portions of 0.2 M HCl/20% methanol. The eluate was freeze-dried, dissolved in 0.5 ml of 0.5% acetic acid and filtered through a 3000 MW cut-off Ultrafree microcentrifuge filters. The combined eluates were analyzed by LC–MS/MS assay [24].

2.4. LC– MS/MS analysis of TTX

The tested solutions were separated using a liquid chromatograph HP 1100 (Agilent Technologies, Waldbronn, Germany) consisting of a quaternary pump for the mobile phase and Waters Cosmosil Hilic 4.6 × 150 mm column (Waters Corporation, USA).

Mobile phase A consisted of 0.1% formic acid in water, while mobile phase B consisting of methanol. The gradient started within 10% B for 5 min and then linearly increased to 90% B within 15 min. The mobile phase was kept at isocratic conditions 90% B for 10 min and then equilibrated for 10 min. The total analysis time was 15 min and the flow rate was 700 μl/min. All LC–MS/MS experiments were recorded on a 4000Q TRAP mass spectrometer (ABI-Sciex, Toronto, Canada) equipped with an electrospray ion (ESI) source with data system in the positive-ion mode. Optimum ion source parameters were as follows: curtain gas = 10 psi; ion spray voltage = 5500 V; temperature at 550 °C; ion source gas 1 = 50 psi; ion source gas 2 = 50 psi for MS/MS. The collision gas was set to medium mode and the interface heater to on mode. Tuning parameters were optimized in order to get the best signal to noise for TTX. The mass spectrometer was operated in MS/MS mode using multiple reaction monitor (MRM) to detect specific precursor ion to product ion transitions for each analyte. The collision full scan (Q1) spectra were collected in the mass range *m*/*z* 100–330. The mass spectral Q1/Q3 transitions, monitored for TTX, were 320/302, 320/256 and 320/162, respectively [29,30].

2.5. Determination of standard curve, LOD and recovery by using LC–MS/MS

Stock solution of TTX was prepared in 0.03 M acetic acid and stored at −20°C. Standard solutions of TTX (1, 5, 10, 25, 50, 100 ng/ml) were dilution of the stock solutions with 0.03 M acetic acid. An internal standard working solution (10 ng/ml TTX) was similarly prepared. TTX calibration curve was linear within the range 1–100 ng/ml ($Y = 408.0\ X + 81.8$, $r = 0.9996$) and the limit of detection was 0.1 ng/ml. Spiked samples were prepared by dissolving approximate 1, 5, 10, 25 and 30 ng of TTX in normal blood serum (1.0 ml). The samples were mixed with 500 μl of 0.5 M acetic acid, centrifuged, passed through the cartridge column, and extracted methods as described above. The LOD of assay method for TTX was the quantity in blood serum after sample clean-up corresponding to three times the baseline noise (S/N > 3). The lowest limit of quantification (LLOQ) was defined as the concentration, which was quantified with less than 20% variation in precision. The LOQ was reproducible at 1 ng/ml in blood serum.

2.6. HPLC-FLD analysis for TTX and PSP

All chemicals and solvents used were HPLC or analytical grade. Reversed phase HPLC (L-2100, Hitachi Ltd., Tokyo, Japan) was performed on a reversed-phase column (Merck Lichromsper 100 RP-18, 4 mm I.D. × 20 cm; E. Merck, Darmstadt, Germany) with a fluorescence detector (F-1000, Hitachi Ltd., Tokyo, Japan). The mobile phase for TTX analysis was sodium 1-heptane sulfonate (2 mM) in methanol (1%)-potassium phosphate buffer (0.05 M, pH 7.0). The TTX was detected by mixing the eluate with 3N NaOH at a ratio of 1:1, followed by heating at 99 °C for 0.4 min, and monitoring the fluorescence at 505 nm with 381 nm excitation [31]. For PSP analysis, the mobile phase consisted of sodium1-heptanesulfonate (2 mM) as an ion-pairing reagent, with 10 mM ammonium phosphate (pH 7.1) for GTXs group and sodium1-heptanesulfonate (2 mM) in 30 mM phosphoric acid (pH 7.1) containing 5% of acetonitrile for STXs group, pumped at a flow rate of 0.6 ml/min. In all cases the eluate from the column was continuously oxidized with periodic acid (7 mM) in 50 mM potassium phosphate buffer (pH 9.0), during passing through a teflon tubing followed by heating at 90 °C for 0.5 min and then mixed with an equal volume of acidifying reagent (0.5 M acetic acid) before entering a fluorescence detector. The intensity of fluorescence was measured at 330 nm with 390 nm excitation [32]. Toxin concentrations in the gastropod extracts were determined by comparing the peak area of each of the individual toxin.

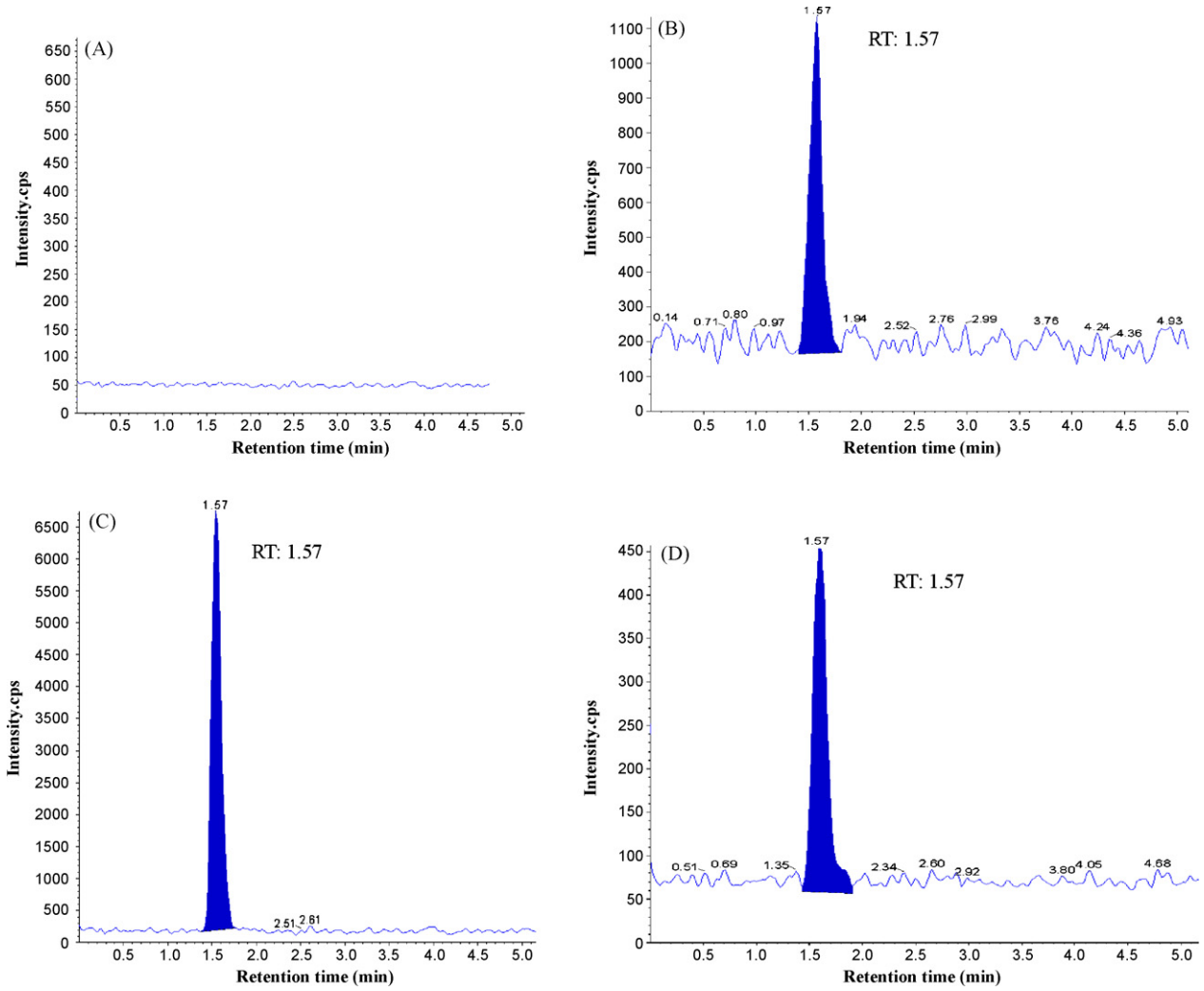

Fig. 2. Full scan TIC chromatography for ion spray LC–MS/MS analysis of (A) blank blood serum, (B) blood serum spiked with TTX (10 ng/ml, limit of quantification level), (C) *N. clathrata* specimens (25.9 ng/ml) and (D) blood serum sample of patient (3.3 ng/ml).

3. Result

3.1. Matrix

The SPE optimization proved to be robust as matrix effects did not affect significantly the accuracy of the method, as evidenced by analyzing tissue and serum sample. It was possible that the extent of the matrix effect can vary by varying the source of a given biological matrix. In this case, use of a C18 cartridge as a preliminary clean-up step is essential to remove material from the tissue samples. The recovery of spiked TTX in sample was 90%. Moreover, using the Oasis MCX cartridge as cation exchange resins enables to improve sensitivity by eliminating matrix effect from the blood serum samples. The average recovery of TTX in the blood serum of human was more than 95%, indicating that the clearing procedure was adequate for purifying.

3.2. Toxin and toxicity identification for HPLC-FLD

Twelve specimens of *N. clathrata* were analyzed for toxicity by the TTX bioassay. The toxicity of the *N. clathrata* specimens collected from the food poisoning incident showed in Table 1. All of the specimens detected were toxic. The mean value of toxicity was 36 ± 24 μg/specimen (mean ± S.D.), 62 ± 24 μg/g digestive gland and 32 ± 16 μg/g other portions. Toxicity of individual specimen was variable, ranging 9–88 μg/specimen. The bioassay method cannot distinguish between TTX and PSP, lethal potencies are expressed in TTX.

The main toxin of gastropod specimen was confirmed by HPLC-FLD analysis. The chromatography profiles of standard TTX and its analogues are shown in Fig. 1. The detection limit of TTX was 1 μg/ml and the calibration curve was linear in the range of 1–500 μg/ml ($Y = 112.42X + 27.75$, $r = 0.993$). The retention time of one peak almost coincided with of TTX (10.5 min). The major toxic compound was identified as TTX. The concentration in the digestive gland and other portions was 65 μg/g (ranging 32–82 μg/g) and 25 μg/g (11–35 μg/g), respectively. The paralytic shellfish toxins were not found by HPLC-FLD (Table 2).

3.3. Mass spectrometry

The presence of TTX was also confirmed by LC–MS/MS analysis, exhibiting a molecular mass of 320 Da, assignable to TTX + H ($C_{11}H_{17}N_3O_3 = 320$). For monitoring TTX at m/z 320.3 Da in the total ion current (TIC) mode, the calibration curve was linear within the range 1–100 ng/ml ($Y = 408.0\ X + 81.8$, $r = 0.9996$) and the limit of detection was 0.1 ng/ml. The ion acquisition along time in the range of 100–330 TIC mode. Typical chromatography is shown in Fig. 2.

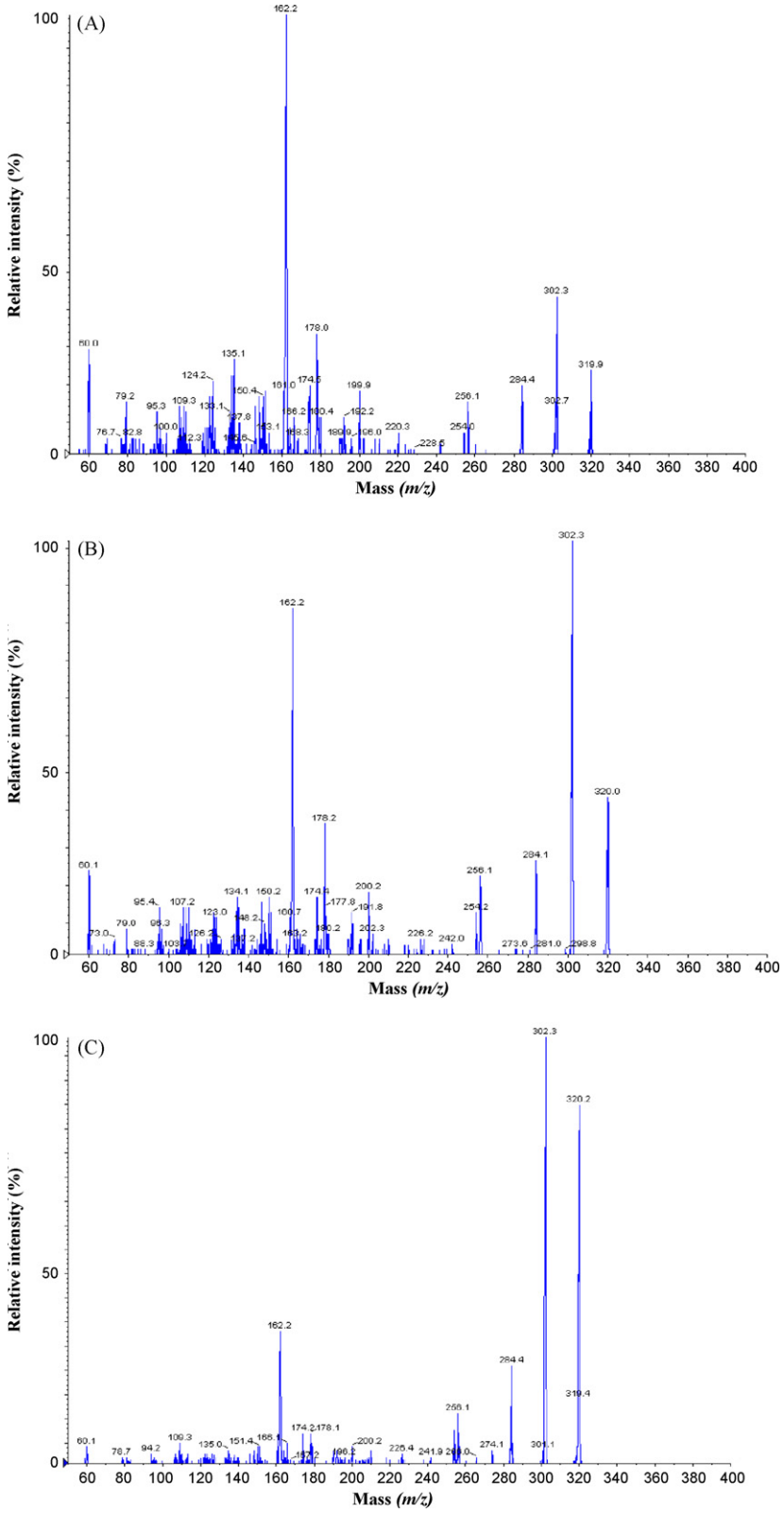

Fig. 3. Fragmentation ion profile in LC–MS/MS system of (A) blood serum sample of patient, (B) *N. clathrata* specimens, and (C) authentic TTX.

In order to obtain TTX analysis and sensitivity, positive ESI mass spectra of TTX are shown in Fig. 3. Multiple reaction monitoring (MRM) was performed at unit resolution using mass transition ion pairs m/z 320 → m/z 302 (declustering potential (DP) 89 eV; collision energy 35 eV), m/z 320 → m/z 256 (DP 89 eV; CE 40 eV), m/z 320 → m/z 162 (DP 89 eV; CE 40 eV) for TTX. The gastropod sample containing of TTX was applied to the LC–MS/MS in the ESI mode detecting at m/z 320–162 for TTX. The presence of TTX in *N. clathrata*

was also confirmed by LC–MS/MS analysis. Searching specific selective ions m/z 162, 256, 302 and 320 Da, corresponding to the ions of TTX fragmentation. The ion fragment at m/z 162 can be interpreted as 2-aminohydroquinazoline [29]. The product ion m/z 162 was monitored because it had the most abundant and stable ion for tissue and the blood serum analysis.

3.4. Assay of blood serum

Therefore, spiked samples were prepared by dissolving 1, 5, 10, 25 and 30 ng/ml of TTX in normal blood serum. The standard TTX showed fragmentation pattern as shown in Fig. 3, this pattern is applicable to the detection of TTX. The blood serum of poisoned daughter has fragmented ions similar to those of TTX and the amount of toxin was 3.30 ± 0.08 ng/ml. In LC–MS/MS analysis. Judging from these data, the causative agent of the food poisoning incident due to ingesting gastropod *N. clathrata* was identified as TTX.

4. Discussion

In this study, the amount of TTX in these gastropods ranges from 9 to 88 μg/specimen by TTX bioassay. The toxicity of gastropod specimens showed distinct individual variation like other TTX containing animals. Accordingly, TTX in TTX bearing organisms is found to come from concentration through several steps of the food web, starting with TTX production by bacteria [33,34]. It is not clear at present whether TTX is biosynthesized in toxic animals or is accumulated through the food web. However, Hwang et al. [35] demonstrated that more toxic snails were more attracted by TTX so that they could accumulate more TTX toxin from the food chain. Furthermore, poisonous starfish and flatworm may also contribute to the accumulation of TTX [36].

In food poisoning incident, the clinical characteristics of patients from E-DA hospital including paresthesia of lips, tongue, abdominal pain and vomiting. The estimated ingested amount of gastropods was an approximate number (8–20 specimens) according to the statements of the victims. Therefore, the victims have ingested 1000–5000 MU (0.2–0.9 mg) of TTX in this food poisoning incident. Based on the minimal toxic dose of 1000 MU TTX to human (equivalent to 0.2 mg of TTX), the symptoms of victims will appear. The mortal dose of TTX is 2 mg of TTX (10,000 MU) for human, so there is no death in the case because they all ate less than 5000 MU of TTX [37].

There are some reports that TTX concentrations in poisoning patients have been determined. O'Leary et al. [28] reported that the minimum quantifiable concentrations of TTX in the urine and blood serum samples from TTX-poisoned patients were 5 and 20 ng/ml, respectively. Akaki and Hatano [38] analyzed 30 samples of puffer from wholesale markets, and seven serum and five urine samples of poisoned humans after consuming puffer by using LC–MS/MS. TTX was detected in serum and urine samples at the levels of 0.9–1.8 ng/ml and 15–150 ng/ml, respectively. In this study, the amount of TTX in the serum was 3.30 ng/ml. Oda et al. [39] demonstrated that TTX elimination from the body took about 5 days. Hence, it is necessary to collect urine and blood from patients as soon as possible after falling ill. Until now, the TTX-associated food poisonings were usually identified judging from the toxin of causative food, and the approach for examining TTX in the urine and blood by using LC–MS/MS was also important, because it could confirm the toxin.

5. Conclusion

TTX poisoning due to consumption of the gastropod *N. clathrata* has been identified by LC–MS/MS method. Herein, a rapid and reliable LC–MS/MS method has been described for detecting TTX in the patient blood serum.

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