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APPLICATION OF LC-MS/MS IN IDENTIFICATION OF TOXIN IN THE CAUSATIVE GASTROPOD AND VICTIM SAMPLES

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Key words: food poisoning, gastropod, TTX, Nassariidae, LC-MS/MS.

ABSTRACT

Food paralytic poisoning incident was detected in the gastropod Nassariidae *Niotha clathrata* and *Zeuxis scalaris* occurred in December 2012 in Kaohsiung, Taiwan. The toxin was purified from each remain of cooked gastropod and victim tissues by ultrafiltration using a YM-3 membrane, followed by C18 cartridge column purification. Based on analyses by LC-MS/MS, the causative agent of gastropod food poisoning was identified as tetrodotoxin. The toxicity of remain of cooked sample was 245 MU/g (1MU = 0.178 μg TTX) for *N. clathrata* and 203 MU/g for *Z. scalaris.* The blood and excrements of victim was shown to contain TTX of 0.13 and 0.18 μg/g, respectively. There was positive between results from LC-MS/MS and mouse bioassay in the causative gastropod. The LC-MS/MS method is suitable for rapid screening of TTX.

I. INTRODUCTION

Food poisoning incident caused by Nassariidae (*Niotha clathrata*, *Zeuxis scalaris*) involving one victim occurred in December 2012 in Kaohsiung, Taiwan. The victim was a 45 years old male, who had diabete without medical control. The symptoms of the victim included dizziness, vertigo, nausea, vomiting, paresthesia of lips, tongue and symmetric fingers numbness, and gait disturbance. On the next day, the numbness ascended up to forearms and also presented in toes. After three days, the victim recovered and now is healthy. These symptoms exhibited from food poisoning were similar to those of tetrodotoxin (TTX) [7].

Paralytic poisoning incidents continue to occur in Taiwan. Among them, gastropod poisoning incidents have occasionally occurred in Taiwan and Mainland China, as well as in Japan and Cuba [7, 18]. In Taiwan and Mainland China, gastropods were a delicious traditional food in fishing villages, because gastropods are cheap and high in nutrients value, but now gastropods became one kind of toxic food. From 1994 to 2012, there were a total of 13 cases of gastropod poisoning incidents in Taiwan (Table 1).

In Taiwan, toxic gastropods have been reported to contain marine toxin TTX and sometimes with minor PSP. The detecting methods for paralytic toxins TTX and/or PSP include bioassay, liquid chromatography-fluorescence detection, thin-layer chromatography, immunoassay, gas chromatography-mass spectrometry, liquid chromatography-mass spectrometry (LC-MS), and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [5, 7, 13, 15].

Among them, mouse bioassay is a simple, convenient and convenient without distinguish TTX from PSP but it can't give any information on toxin composition [2]. In addition, all over the world are strongly to oppose to bioassay using even mice. With HPLC method in which TTX components were separated by silica gel C-18 chromatography and the toxin eluate is then mixed with NaOH [7]. TTX compounds were spectro fluorometrically detected by means of TTX derivative C_9-base [2, 25]. However, such data can not directly to identify TTX derivatives. Recently, LC-MS and LC-MS/MS methods have been explored for both qualitative and quantitative analyses of TTX and PSP by researchers [12, 23]. These analyses provided direct evidence to prove TTX. Furthermore, LC-MS/MS has been used to directly identify TTX and PSP compounds. Therefore, this study applied LC-MS/MS method to detect and confirm the level and distribution of toxin in the tissue of gastropod and the victim.

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Table 1. Gastropod poisoning incidents in Taiwan during 1994-2012.

Date	Causative gastropod	Toxin	Total toxicity (MU/ specimen)	Patients (Death)	Place	Reference
May, 1994	*Niotha clathrata*	TTX, minor PSP	345 ± 192	26 (0)	Pingtung	(9)
	Zeuxis scalaris	TTX, minor PSP	98 ± 46	—	—	
May, 1995	*Nassarius castus*	TTX	—	1 (0)	—	(1)
	N. conidalis	TTX	—	—	—	
Jul, 2000	*Polinices didyma*	TTX	118 ± 105	1 (0)	Chiayi	(21)
Apr. 2001	*Zeuxis sufflatus*	TTX	730 ± 337	5 (0)	Taipei	(14)
	Niotha clathrata	TTX	409 ± 147	—	—	
Feb, 2002	*Olive miniacea*	TTX	149 ± 91	1 (0)	Pingtung	(9)
	O. mustelina	TTX	35 ± 14	—	—	
	O. hirasei	TTX	58 ± 31	—	—	
May, 2002	*Nassarius papillosus*	TTX	320	2 (1)	Kaohsiung	(5)
	N. gruneri	TTX	386	—	—	
Apr, 2004	*Nassarius glans*	TTX	5188 ± 1959	6 (2)	Tungsa island	(16, 25)
Oct, 2005	*Nassarius papillosus*	TTX, minor PSP	210 ± 104	1 (0)	Liuchiu Island	(13)
Nov, 2006	*Niotha clathrata*	TTX	345 ± 115	3 (0)	Kaohsiung	(5)
Sep, 2007	*Nassarius papillosus*	TTX	1044 ± 706	3 (1)	Penghu	No reported
May, 2009	Unknown gastropod	TTX	—	8 (0)	Kaohsiung	No reported
Dec, 2012	*Niotha clathrata*	TTX	—	1 (0)	Kaohsiung	This study
	Zeuxis scalaris	TTX				

—: No information.

II. MATERIALS AND METHODS

1. Materials

Urine and excrements were immediately collected from 45-years-old victim when he had eaten gastropods for 1 hr and was admitted to a hospital December, 2012. The remain of cooked gastropods and victim samples were frozen at −20°C until the assay was carried out. In the causative gastropods, the muscle was eaten and the digestive gland was retained.

The ICR (Institute of Cancer Research, Charles River, USA) strain mouse was purchased from National Laboratory Animal Breeding and Research Center, Taipei, R.O.C. Healthy mice weighing between 18 and 20 g were used. Authentic TTX and derives *epi*-TTX and anh-TTX, obtained from Wako Pure Chemical Industries (Tokyo, Japan), were used as the reference standards.

2. Assay Method for Toxicity

The digestive gland of causative gastropod was weighed, homogenized with 5 ml of 1% acetic acid solution and centrifuged (20,000 × *g*, 20 min). The operation was repeated twice. The supernatants were combined, concentrated under pressure at 45°C and examined for toxicity by the TTX mouse bioassay [5].

According to Hwang and Jeng's table for dose-death time relationship and toxicity determined by bioassay was expressed in mouse units. One mouse unit (MU) equal to 0.178 μg of TTX, is defined here as the amount of toxin required to kill a 20 g ICR strain male mouse in 30 min via ip injection.

3. Purification of TTX for Gastropod and Victim's Clinical Sample

The purification method of TTX for causative gastropod and victim's clinical samples is briefly explained as follows [23]. Each (1.0 ml or 1.0 g) of the samples was thawed. The samples were homogenized with 5 ml of 1% acetic acid solution and centrifuged at 20,000 × *g* for 20 min. The operation was repeated twice. The supernatant was freeze-dried, dissolved with 5 ml distilled H_2O and passed through an extraction column (C18 Sep-Pack cartridges, Millopore, Water, MA), previously regenerated with 10 ml methanol and 10 ml water. Toxin was absorbed in the column and eluted with 10 ml 0.3% acetic acid. The eluant was freeze-dried, dissolved in 2 ml 0.3% acetic acid and filtered through a 3,000 MW cut-off Ultrafree microcentrifuge filter (Micron YM-3, Millipore, Waters). The filtrate was dehydrated, dissolved with water to 1 ml, and submitted for subsequent analysis.

4. Qualitative aand Quantitative Range of LC-MS/MS

LC-MS/MS experiments were obtained by a 4000Q TRAP mass spectrometer (ABI-Sciex, Toronto, Canada) equipped with an electrospray ion (ESI) source, with data system in the positive-ion mode. The tested solution was separated using a liquid chromatography HP/100 (Agilent Technologies, Waldbronn, Germany) consisting of a quaternary pump for the

Table 2. Anatomical distribution of toxicity in specimens of remained cooked gastropods from Kaohsiung in December, 2012 by using mouse bioassay.

Specimen No.	Body length of gastropod (cm)	shell weight of gastropod (g)	Weight of digestive gland (g)	Toxicity of digestive gland (MU/g)	TTX concentration in digestive gland (μg/g)		A: B (%)
					Calculated from mouse assay (A)	LC-MS/MS* (B)	
Niotha clathrata							
1	2.8	2.05	0.56	107	10.68	9.36	114
2	2.7	1.62	0.25	133	5.87	6.68	88
3	2.3	1.96	0.45	303	24.39	26.09	93
4	2.0	1.97	0.30	330	17.62	16.78	105
5	1.8	1.92	0.27	304	14.60	15.65	93
6	2.0	2.05	0.25	292	13.00	14.28	91
Mean ± S.D.	2.3 ± 0.4	1.93 ±0.16	0.35 ± 0.13	245 ± 98	14.36 ± 6.30	14.81 ± 6.75	97.33 ± 10.01
Zeuxis scalaris							
1	3.1	1.71	0.63	108	12.10	14.30	85
2	3.0	1.96	0.67	76	9.08	8.62	105
3	1.7	0.77	0.55	297	29.01	30.06	97
4	2.5	1.1	0.61	287	31.15	32.28	96
5	2.2	1.16	0.60	320	34.18	34.14	100
6	2.8	1.82	0.60	130	13.88	12.78	109
Mean ± S.D.	2.6 ± 0.5	1.42 ± 0.48	0.61 ± 0.04	203 ± 110	21.57 ± 11.05	22.03 ± 11.33	98.67 ± 8.31
Victim sample							
Urine						0.13	
Excrements						0.18	

*Toxicity was calculated from TTX concentration of TTX by using LC-MS/MS (1 MU = 0.178 μg of TTX) (Hwang and Jeng, 1988).

mobile phase and Waters Cosmosil Hilic 4.6 × 150 mm column (Waters Corporation, Milford, USA). Mobile phase A consisted of 0.1% formic acid in water, while mobile phase B consisted of methanol. Optimum ion source parameters were as follows: curtain gas, 10 psi; ion spray voltage, 5,500 V; temperature at 550°C; ion source gas 1, 50 psi; ion source gas 2, 50 psi. These parameters were optimized using the best signal noise for TTX standard. The mass spectrometer was operated in MS/MS mode using a multiple reaction monitor (MRM) to detect a specific precursor ion to product ion transitions for each analysis. For TTX analysis, 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 *m/z* 320/302, *m/z* 320/256 and *m/z* 320/162, respectively [13].

III. RESULTS

1. Toxicity of TTX in the Gastropods by Using Mouse Bioassay and LC-MS/MS

The toxicities of the remain of sample *N. clathrata* and *Z. scalaris* are shown in Table 2. The mice exhibited symptoms of neurotoxin exposure after implicating the extract of remained cook gastropods. The average toxicity of digestive gland in each specimen was 245 ± 98 MU/g (mean ± S.D.) for *N. clathrata*, and 203 ± 110 MU/g for *Z. scalaris*. The calculated TTX concentration of digestive gland of gastropod was 14.36 ± 6.30 μg/g for *N. clathrata* and 21.57 ± 11.05 μg/g for *Z. scalaris*. The data of TTX concentration in the gastropod determined by LC-MS/MS were also shown in Table 2. These calculated TTX concentration was almost same as those determined by LC-MS/MS.

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). The ion acquisition timeframe showed in the range of a 100-330 TIC mode. The TTX standard curve was linear within the range of 1 to 300 ng/ml (y = 817 x + 2190, r = 0.9996). For each analyte, the combined ion current profile for all transitions was extracted from the LC–MRM dataset, and the plot of ratio between the peak area of the TTX and the internal standards versus injected amount or concentration was obtained by measuring the resulting peak areas. The contents of TTX in the remain of gastropods and victim's sample urine and excrements were 14.81 μg/g, 22.03 μg/g, 0.13 μg/g and 0.18 μg/g (Table 2). The data in the remain of gastropod from mouse bioassay were similarity these of LC-MS/MS method. It indicated that LC-MS/MS is useful for detecting the concentration of TTX. Typical chromatography is shown in Fig. 1. Multiple reaction monitoring (MRM) was performed at unit resolution using a mass transition ion pair *m/z* 320 → *m/z* 302 (declustering potential (DP) 89 eV; collision energy 35 eV), *m/z* 320 → *m/z* 256 (DP 89 eV;

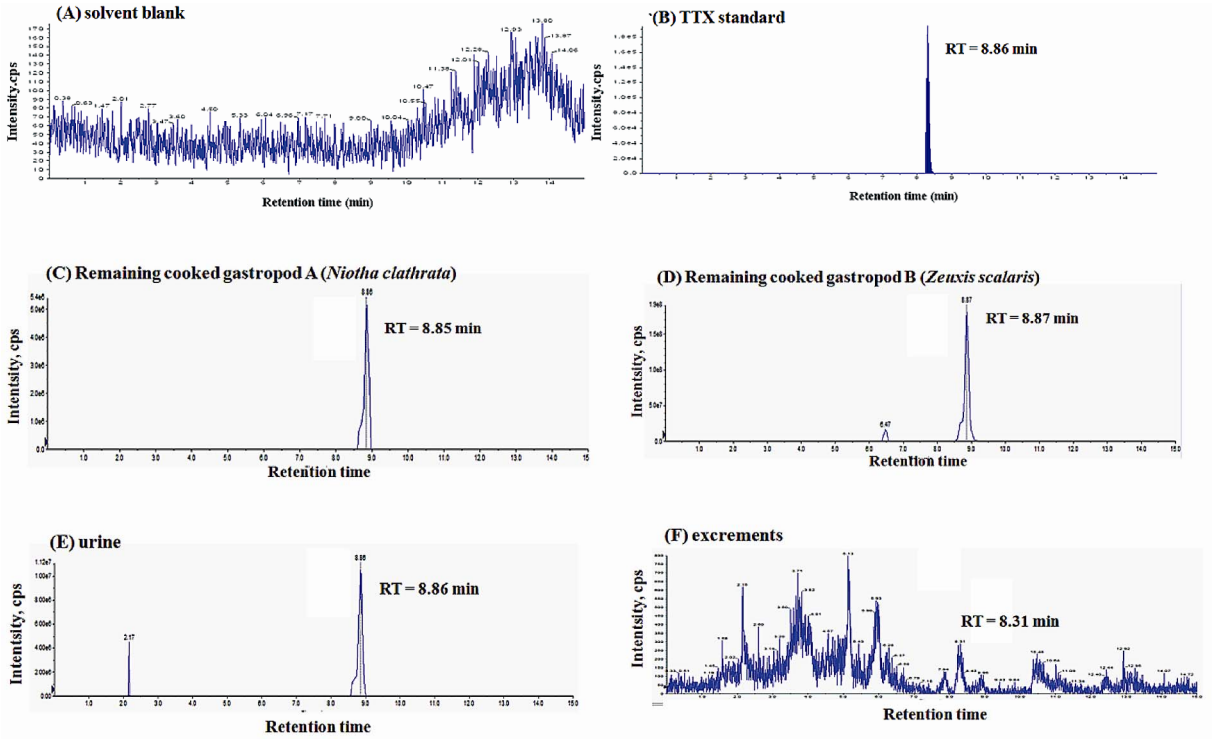

Fig. 1. Full scan TIC chromatography of TTX analyzed by LC-MS/MS in different samples.

CE 40 eV), *m/z* 320 → *m/z* 162 (DP 89 Ev; CE 40 Ev) for TTX. Victim's clinical samples (urine, excrements) and remained cook gastropod extracts were analyzed through application to the LC-MS/MS in the ESI mode detecting at *m/z* 320-162 for TTX. As shown in Fig. 2, all data were cnofirmed by LC-MS/MS analysis to contain TTX. By searching the specific selective ion *m/z* 162, 256, 302 and 320 Da corresponding to the ions of TTX fragmentation, the product ion *m/z* 162 was monitored because it had the most abundant and stable ion for the TTX analysis.

IV. DISCUSSION

Poisoning due to ingestion of TTX containing puffer has frequently occurred in Asia areas, Australia and U.S.A. [7, 11, 19, 20]. The incident consuming TTX-containing gastropods was first reported in Japan [16]. The incidents of gastropod poisoning have occasionally occurred in Taiwan, including *Oliva miniacea*, *O. mustelina*, *O. nirasei*, *O. hirasei*, *Z. scalaris* and *N. clathrata*, associated poisoning has been reported [4, 10]. The responsible toxin in the gastropods has been established to be TTX and PSP. Similarly, the toxin involved in the gastropod poisoning, more than 40 incidents, in Mainland China occurred from 1977 to 2001 was caused by TTX [21].

The present study reported a case, caused a by two causative food gastropods *N. clathrata* and *Z. scalaris* which contained only TTX. This result closely resembled the profile of toxicity and toxin components of *N. clathrata* and *Z. scalaris* toxin collected in South Taiwan [6, 4, 8, 13], the average toxicity of live specimen was 345 ± 192 in *N. clathrata* and 98 ± 46 MU in *Z. scalaris*, respectively [8]. Hwang and Noguchi [7] indicated the victim died due to ingestion of a total toxicity score of more than 10,000 MU (about 2 mg TTX). Because the specimens many appear when the indicated dose of TTX is over 1,000 MU, We supposed that the victim has eaten about 4-40 specimen of toxic gastropods. In addition, *N. clathrata* and *Z. scalaris* have been reported to contain minor amount of paralytic shellfish poisons (PSP) in the spring season [4]. Hwang *et al*. [6] also reported that specimen of gastropod *N. clathrata*. Specimens showed a wide individual variation in toxicity. In 2006, specimens of gastropod *Niotha clathrata* implicated to a food paralytic poisoning. 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 [12]. In Australia, some gastropods including T*ectus fenestratus*, *T. niloticus*, *T. pyramis*, *T. hanleyanus* and *Turbo argyrostomus*, contained PSP only [16]. But several gastropods including *N. papillosus* [12], *N. clathrata* [3], and *N. lineate* [1] contained TTX and PSP.

In this study LC-MS/MS method is capable of detecting small quantities of TTX and derivatives in remained cook gastropods, blood and excrements. Comparison of toxicity of remain of cook gastropods and victim's clinical samples (blood and excrements) by mouse bioassay and LC-MS/MS, the contents of TTX in poisoning sample by mouse bioassay and LC-MS/MS analysis were almost the same. The low detecting limits are 0.178 μg/g for bioassay and 0.1 ng/g for

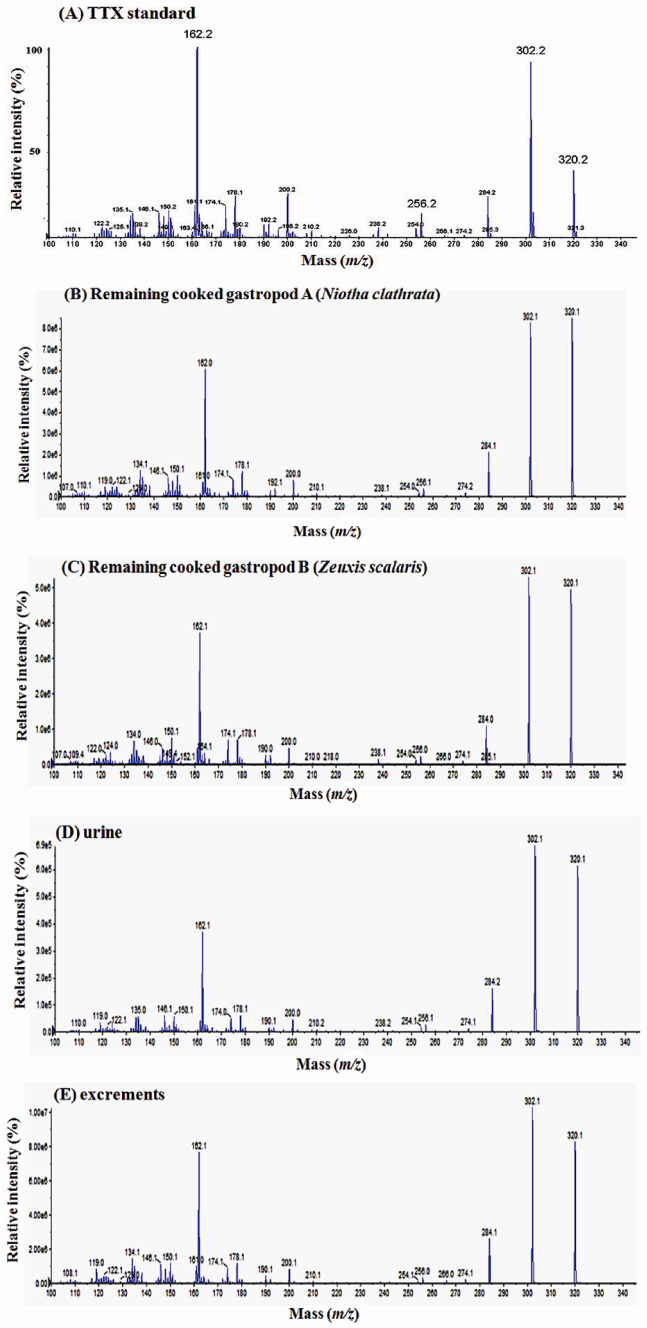

Fig. 2. Fragmentation ion profile of TTX analyzed by LC-MS/MS in different samples.

LC-MS/MS, respectively. Thus, LC-MS/MS method is a useful tool to detect TTX and its derives.

V. CONCLUSION

Food paralytic poisoning incident of Nassariidae species *Niotha clathrata* and *Zeuxis scalaris* occurred in Kaohsiung was found to be caused by TTX using mouse and LC-MS/MS method. The result from the mouse bioassay and LC-MS/MS showed a strong linear correlation, indicating that LC-MS/MS may represent an effective method in identifying toxin from causative gastropod.

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