Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography B, 832 (2006) 75–80
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of tetrodotoxin in human urine and blood using C18 cartridge column, ultrafiltration and LC–MS

Y.H. Tsai [a], D.F. Hwang [b,*], C.A. Cheng [c], C.C. Hwang [b], J.F. Deng [d]

[a] *Department of Food Science and Technology, Tajen Institute of Technology, Pingtung, Taiwan, ROC*
[b] *Department of Food Science, National Taiwan Ocean University, Keelung, Taiwan, ROC*
[c] *Department of Food Engineering, National Kinmen Institute of Technology, Kinmen, Taiwan, ROC*
[d] *Division of Clinical Toxicology, Department of Medicine, Veterans General Hospital, Taipei, Taiwan, ROC*

Received 21 October 2005; accepted 21 December 2005
Available online 24 January 2006

Abstract

Six fishermen were victims (including one death) of food poisoning from unknown fish on their boat in central Taiwan Strait, in April 2001. The symptoms were like those of tetrodotoxin (TTX) poisoning. As there was no remaining fish, a new protocol was developed to determine TTX in the urine and blood of the victims. The urine and blood samples were cleansed using a C18 Sep-Pak cartridge column, and the toxin was extracted by methanol. The eluate was filtered through a microcentrifuge filter. The filtrate was freeze-dried, dissolved in distilled water, and determined by LC–MS. The recovery was more than 88.9%. The detection limit was 15.6 nM. A linear relationship between response and concentration was obtained between 93.75 and 9375 nM of TTX. It was shown that the urine and blood of the victims contained TTX. The range of TTX was 4.5–40.6 nM in blood and 47–344 nM in urine. Judging from the symptoms of the victims and the experimental data, the causative agent of the food poisoning was identified as TTX.

Keywords: Tetrodotoxin; Food poisoning; LC–MS

1. Introduction

A food poisoning incident resulting from the ingestion of unknown fish involving six Mainland Chinese fishermen occurred on a fishing boat in central Taiwan Strait in April 2001 [1]. All six patients were middle-aged men ranging from 32 to 49 years (mean 41 ± 4 years). Onset of symptoms began approximately 2–3 h after ingestion; symptoms included paresthesias, ataxia, dizziness, dysarthria, respiratory distress, nausea and vomiting. Among these victims, four men suffered from more serious symptoms and were treated with intravenous fluids, mechanical ventilation, and intensive treatment in hospital. One victim passed away 4 h after ingestion because of intractable bradycaria and multiple organ failure; the other three victims were then discharged and were healthy. These symptoms of food poisoning were similar to those of tetrodotoxin (TTX) poisoning.

Poisoning due to the ingestion of TTX-containing puffers has occurred frequently in Japan, and to a lesser extent in Taiwan, Hong Kong, Thailand, Singapore, Malaysia, Kiribati, Fiji, Australia, Papua New Guinea, Bangladesh, and the United States [2]. Especially, puffer-associated poisoning has been reported sporadically in Taiwan [3,4]. Recently, we also reported that gastropods, cooked fish liver and dried dressed fish fillet implicated in food poisoning contained TTX [5–9].

The cook on the fishing boat was very scared and discarded all suspected meal into sea after the incident, and no leftover of meal was retained. It was, thus, very important to measure TTX in the urine and blood of poisoned patients for the diagnosis of TTX-food poisoning. Until now, TTX-associated food poisonings were usually identified by judging from the toxin in causative food. The amount of toxin in the causative food is high enough to be easily detected by using high performance liquid chromatography (HPLC) [10] and to be identified by gas chromatography–mass spectroscopy (GC–MS) [11]. In this food poisoning incident, the amount of toxin in the urine and blood of the victims was expected to be low.

* Corresponding author. Tel.: +886 2 24622192x5103; fax: +886 2 24626602.
E-mail address: dfhwang@mail.ntou.edu.tw (D.F. Hwang).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2005.12.036

Hence, a new approach for examining TTX in the urine and blood was established by using C18 cartridge column, ultrafiltration and high performance liquid chromatography–mass spectrometer (LC–MS). The evidence indicated that the new approach for detecting TTX in the urine and blood of victims was validated.

2. Materials and methods

2.1. Materials

Urine and blood (50 ml/specimen) were collected from four patients who were admitted to a hospital; no biological specimens were obtained from two other persons who were not admitted but had significant symptoms (Table 1). The samples were frozen at −20 °C until the assay was carried out. Normal blood (100 ml) was purchased from Hospital of National Taiwan University. Fresh normal urine (100 ml) was collected from laboratory students. Authentic TTX (94.3% purity), obtained from the liver of the puffer *Takifugu oblongus*, was used as reference standard [12].

2.2. Sample preparation

The sample cleanup procedure used was based on the procedure developed by Andrinolo et al. [13] for saxitoxin (STX) poisoning in cats in vivo. Each (1.0 ml) of the urine and blood samples was thawed and mixed with 500 μl of 0.5 M acetic acid to keep it acidic. The samples were immediately centrifuged at 10,000 × *g* for 10 min. The supernatant was passed through a prepared cartridge column (C18 Sep-Pak cartridges, Millipore, Waters, MA). The prepared cartridges were previously washed with 10 ml methanol and then 10 ml water before use. After applying sample into the cartridge, 10 ml 0.3% acetic acid was eluted. The eluant was freeze-dried, dissolved in 2 ml 0.3% acetic acid and filtered through a 3000 MW cut-off Ultrafree microcentrifuge filter (Micron YM-3, Millipore, Waters). The filtrate was freeze-dried and dissolved in a small amount (1 ml) of water. Five microliters of the filtrate was subjected to high performance liquid chromatography–mass spectrometry analysis.

2.3. LC–MS analysis

In this method, combined LC–MS was performed using Agilent (Palo Alto, CA, USA) model 1100 series LC/MSD Trap system coupled to a mass spectrometer with a positive ion electrospray ionization (ESI) interface. The electrospray interface was typically operated using the following settings: fragmentor, 150 V; nebulizer, N_2 (60 psi); drying gas, N_2 (10 l/min, 350 °C); V-cap, 3500 V. HPLC system was equipped with a Zorax 300SB-C3 column (I.D. 4.6 mm × 150 mm) at room temperature. The injection volume was 5 μl. The mobile phase for TTX analysis was 1% acetonitrile, 10 mM trimethylamine (TMA), 10 mM ammonia formate (pH 4.0, flow rate 0.4 ml/min).

Table 1
Recovery of TTX when spiked to urine and blood sample and then treated with C18 cartridge column, ultrafiltration and LC–MS

Spiked concentration (μg/ml)	Recovery (%)	
	Urine	Blood
100	92.6 ± 1.6	90.4 ± 2.1
50	91.3 ± 1.8	90.5 ± 1.5
10	88.9 ± 1.3	90.8 ± 1.7
Mean ± S.D.	90.9 ± 1.4	90.6 ± 0.2

2.4. Standard curve, detection limit and recovery

The standard curve of TTX was prepared from 93.75, 468.8, 937.5, 4687.5 and 9375 nM of TTX. The determination was performed three times ($n = 3$). Data from the standard curve were subjected to linear regression analysis, and peak area (y) versus amount of TTX (x) was plotted. The detection limit that was evaluated according to the ratio of sample peak area to noise area was more than three times. Spiked samples were prepared by dissolving approximate 10, 50 and 100 μg of TTX in normal urine and blood (1.0 ml). The samples were mixed with 500 μl of 0.5 M acetic acid, centrifuged, passed through the cartridge column, and extracted with methanol as described above.

2.5. Reproducibility test

Intraday (running three times on the same day), and interday tests (running three times on seven successive days with at least 24-h intervals) were conducted. The reproducibility precision values were characterized by the coefficients of variation (CV, %).

2.6. GC–MS analysis

The preparation procedure of samples for GC–MS was derived from that of Narita et al. [11]. To confirm the presence of TTX in the urine and blood of victims, the filtrate (100 μl) of each sample was dissolved in 2 ml of 3N NaOH and heated in a boiling water bath for 45 min to obtain the C_9 base derivative (2-amino-6-hydroxymethyl-8-hydroxyquinazoline) of TTX and/or related substances. The above hydrolysate was adjusted to pH 4 with 1N HCl and extracted three times with 5 ml of 1-butanol. The extracts were combined and evaporated to dryness. Then trimethylsilylation was applied, and the products were subjected to GC–MSD on Agilent (Palo Alto, CA, USA) model 6890 series. Injector temperature was set as 180 °C. A column (HP-1, 0.25 mm × 30 m, Agilent Technologies Inc.) was used, and the temperature was raised from 165 to 230 °C at a rate of 3 °C/min. The carrier gas used was He at a flow rate of 1.0 ml/min. The ionizing voltage was kept at 70 eV at an ion source temperature of 200 °C. Scanning was carried out in a mass range of m/z 46–560 at 3 s intervals [11]. The injection volume was 1 μl in this study.

3. Results and discussion

The standard curve of TTX was prepared in the range of 93.75–937.5 nM. The correlation coefficient (r) and linear

Table 2
Reproducibility of intraday and interday analysis ($n = 3$)

Concentration (μg/ml)	Intraday[a]		Interday[b]	
	Mean ± S.D.	CV (%)	Mean ± S.D.	CV (%)
100	98.20 ± 0.21	0.21	96.93 ± 0.49	0.51
50	49.41 ± 0.52	1.05	48.31 ± 0.64	1.32
10	9.62 ± 0.22	2.28	8.92 ± 0.26	2.91
1	0.93 ± 0.02	2.13	0.91 ± 0.03	3.30

[a] Intraday: running three times within 24 h.
[b] Interday: running three times within successive 7 days with at least 24 h intervals.

regression equation for TTX were as follows: $y = 2305x + 1060$ ($r = 0.9965$). The detection limit was 15.6 nM of TTX. The recovery of TTX in urine and blood was more than 88.9% and 90.4%, respectively (Table 1).

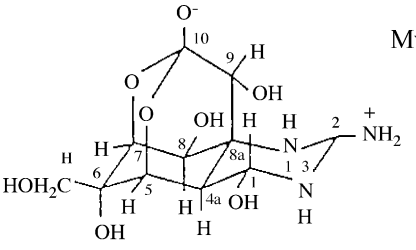
Reproducibility of the measurements is evaluated by intraday and interday analysis calculated from the results of three replicates and illustrated by the average, standard derivation trials (S.D.) and coefficient of variation (CV, %), as shown in Table 2. All repeated trails obtained CV values less than 3.5%, pointing out high degree of reproducibility.

TTX levels of the urine and blood samples of patient nos. 1–4 collected about 10 h after ingesting fish meat are shown in Table 3. TTX was detected in all the urine and blood samples. The levels of TTX in urine and blood ranged from 47 to 325 nM and from <15.6 to 40.6 nM, respectively. The level of TTX in urine was significantly higher than that in blood. This indicates that TTX is easily metabolized and excreted in urine because the sampling time was about 10 h after eating. According to the memory of victims, the amount of ingested fish meat for each victim was estimated as shown in Table 3. The ingested amount

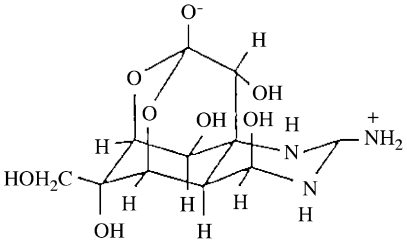
Table 3
Clinical features of victims and TTX level in the urine and blood of victims

Patient no.	Sex/age	Symptoms meat intake	Estimated fish (g)	TTX level (nM) by LC–MS		GC–MS	
				Urine	Blood	Urine	Blood
1	M/45	PNRVGD[a]	90[b]	325 ± 12	40.6 ± 2.5	+[c]	+
2	M/44	PNRG	70	190 ± 16	21.1 ± 1.2	+	+
3	M/49	PNRVG	100	344 ± 17	28.6 ± 1.5	+	+
4	M/38	PNRG	15	47 ± 5	4.5 ± 0.3	+	+
5[d]	M/40	PNRVG	30				
6[d]	M/32	PNG	10				

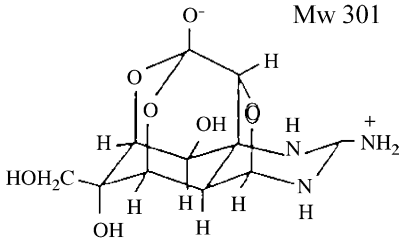
[a] P, paresthesias; N, neurological symptoms other than paresthesias (including ataxia, dissociative feeling, dizziness, dysarthria, dysphasia, or weakness); R, respiratory distress; V, mechanical ventilation; G, gastrointestinal symptoms (nausea or vomiting); D, death.
[b] The ingested amount of fish roughly estimated according to memory of victims.
[c] +: positive detected.
[d] Patient nos. 5 and 6 did not have urine and blood collected.



tetrodotoxin



4-*epi*-tetrodotoxin



anhydrotetrodotoxin

Fig. 1. Structures of TTX, 4-*epi* TTX and anh-TTX.

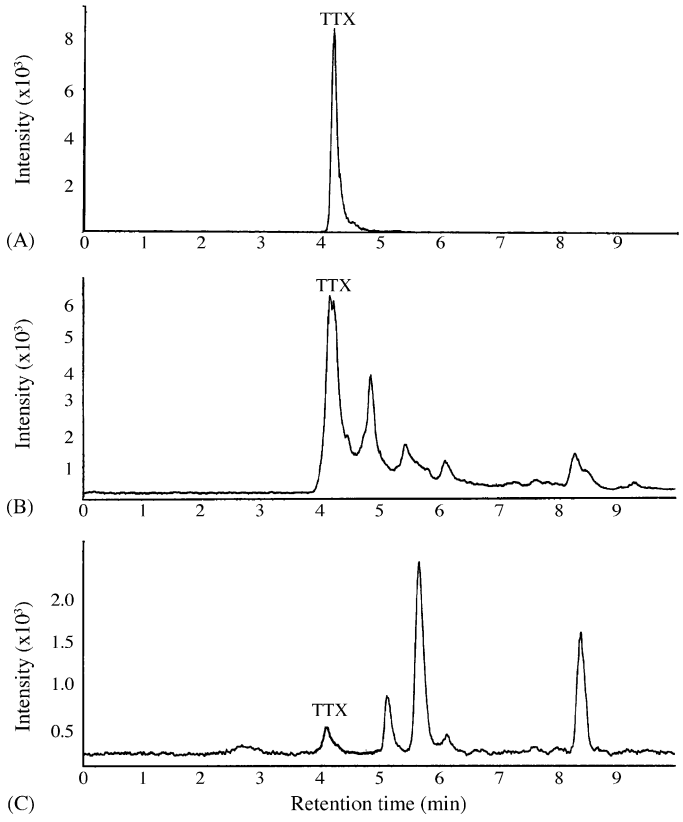


Fig. 2. LC–MS chromatograms of (A) authentic TTX (9375 nM) and (B) the urine (338 nM) and (C) blood (36 nM) samples of patient no. 1. The retention time for TTX was 4.5 min.

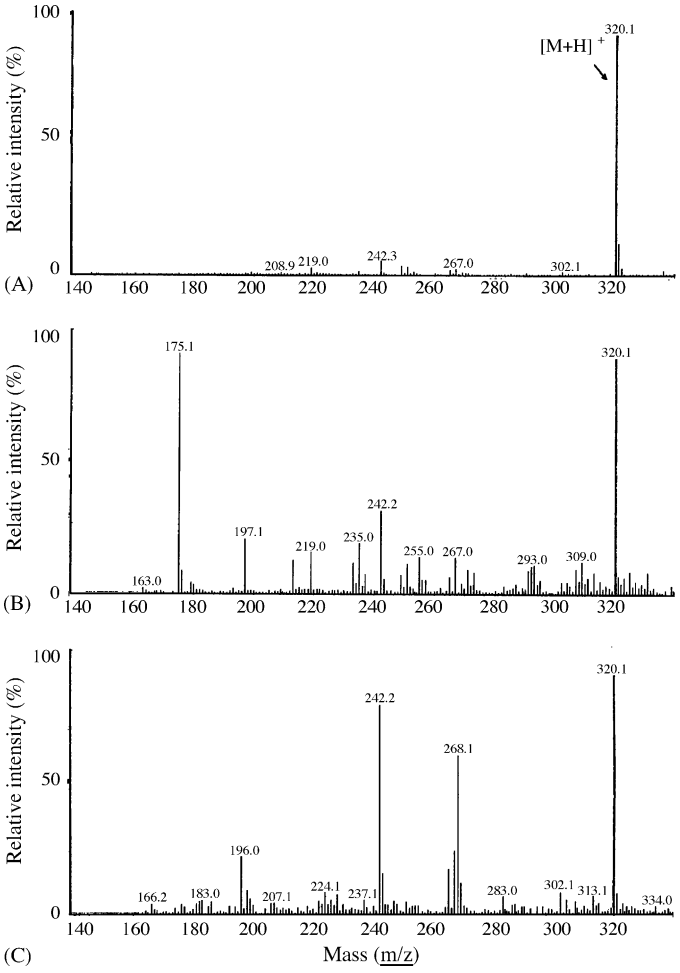


Fig. 3. LC–MS mass spectra of (A) authentic TTX and (B) the urine and (C) blood samples of patient no. 1. The retention time for TTX was 4.15 min and the protonated molecular ion $[M+H]^+$ of TTX was m/z 320.1.

was positively corelated with TTX levels in the urine and blood of each individual patient. Although patient no. 1 died soon after eating, the amount of fish meat ingested by him was less than that ingested by patient no. 3. The severity of the symptoms and prognosis generally depends on the amount of toxin taken, but the variation of individual sensitivity to toxin is an important factor that influences TTX intoxication. Fig. 1 shows the structure and molecule weight of TTX and other two compounds (4-*epi* TTX and anhydro-TTX). Fig. 2 shows LC–MS chromatograms of the urine and blood samples of patient no. 1; authentic TTX with an ion peak at $m/z = 320.1$ at the same retention time (4.15 min). Fig. 3 shows LC–MS mass spectra of the urine and blood samples of patient no. 1; authentic TTX displayed an ion peak at $m/z = 320.1$ at the same retention time (4.15 min). Furthermore, all samples exhibited the C_9-base derivative of TTX by using GC–MS method. Fig. 4 shows GC–MS chromatograms of the trimethylsilyl (TMS) derivative from the urine and blood of patient no. 1 and authentic TTX gave rise to three ion peaks at m/z 407, 392, and 376 at the same retention time (20.8 min). Fig. 5 shows GC–MS mass spectra of TMS derivatives from the urine and blood samples of patient no. 1 and authentic TTX displayed a parent peak at m/z 407, a base peak at m/z 392, and a fragment peak at m/z 376. Judging from these data, the causative agent of this food poisoning incident was identified as TTX. This study also documents the low blood toxin levels sufficient to cause serious human illness and establishes that urine is the primary route of TTX excretion for human beings.

The C-18 Sep-Pack cartridges could retain hydrophobic substances in the sample but would not retain TTX. Therefore, we spiked 10, 50, 100 μg of TTX to normal urine and blood (1.0 ml). We measured the concentration of TTX before and after loading the cartridge (C18 Sep-Pak cartridges). As a result, it was found that the concentration of TTX did not change, and TTX was not retained by the cartridge. The recovery of spiked TTX in urine and blood was better than 88.9%, indicating that ion suppression from matrix component could be ignored; this Sep-pak treatment was effective in removing interferences eluted close to TTX peak and hindered the detection and identification of TTX in urine and blood.

Although Kawatsu et al. [14] reported that immunoaffinity chromatography combining with fluorometric high performance liquid chromatography has been developed for detecting TTX from urine samples, monoclonal antibody against TTX is difficult to purchase and is very expensive. From the results in the present study, it is suggested that the combination of C18 Sep-Pak cartridge column, ultrafiltration with an Ultrafree microcentrifuge filter, and LC–MS is very useful in detecting TTX from the urine and blood samples of poisoned patients for the diagnosis of TTX-associated food poisoning. The cleaning methods

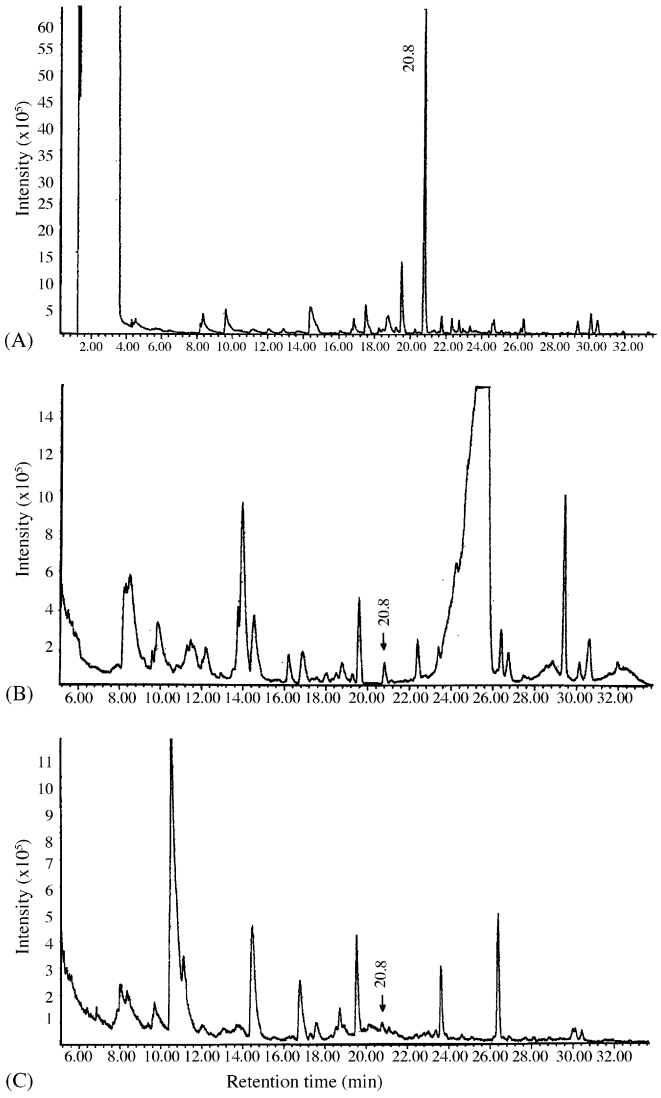


Fig. 4. GC–MS chromatograms of the TMS derivatives from (A) authentic TTX (468 nM) and (B) the urine (338 nM) and (C) blood (36 nM) samples of patient no. 1. The retention time for TTX was 20.8 min.

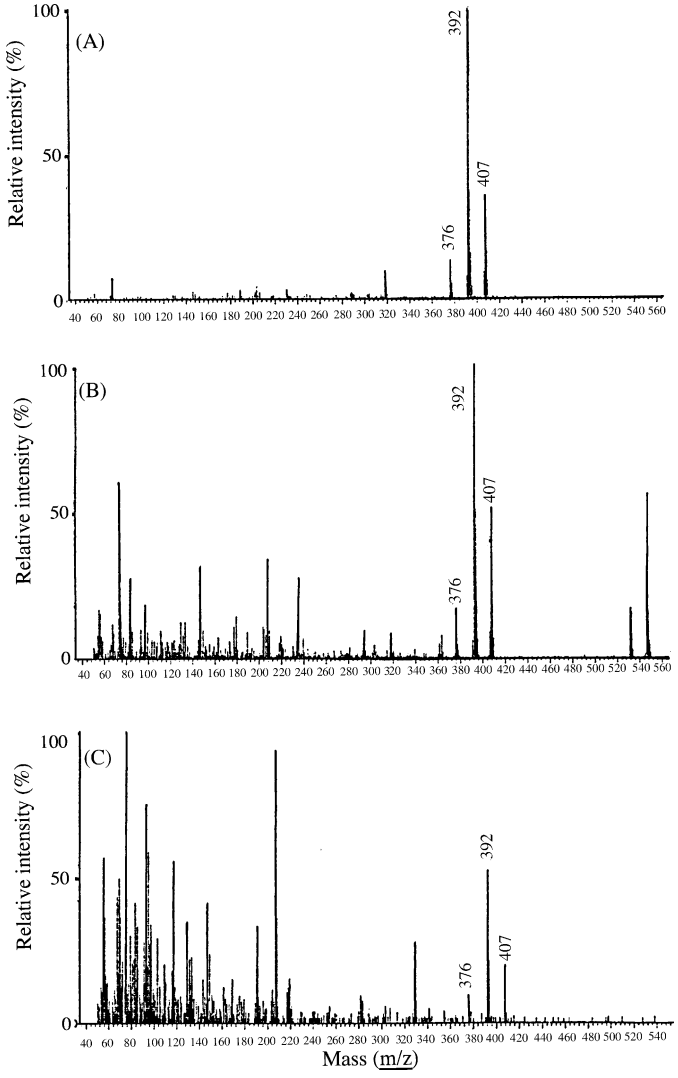


Fig. 5. GC–MS mass spectra of the TMS derivatives from (A) authentic TTX and (B) the urine and (C) blood samples of patient no. 1. The retention time for TTX was 20.8 min.

using a cartridge column and microcentrifuge filters together with HPLC method have been also reported to be useful for detecting paralytic shellfish poison (PSP) in cats [13].

The toxicity of 4-*epi*-TTX is considerably lower than that of TTX itself [15]. Moreover, the amount of 4-*epi*-TTX in the puffer-fish is commonly known to be lower than that of the main TTX component [15,16]. Although TTX standard preparations are commercially available, 4-*epi*-TTX standard preparations are not available so far. Therefore, this time, we decided to determine TTX quantitatively only.

Identification of TTX in seafood implicated in food poisoning has been studied extensively [5–9], but there are no reports in qualifying and quantifying TTX in urine and blood so far. In this study, we demonstrated that a combined protocol including C18 cartridge column, ultrafiltration and LC–MS could be validated in qualifying and quantifying TTX in the urine and blood of humans. Urine is the major excretion route of TTX intoxication and of PSP intoxication for human beings [17,18]. Although GC–MS is also useful for identifying the presence of TTX and/or related compounds, the method described here cannot be directly used in qualifying and quantifying TTX. TTX intoxication may occasionally occur due to cultural (as a delicacy in Japan), intentional (suicide or murder attempt), and inadvertent (accidental consumption) factors. The above procedure may be usefully applied in detecting TTX in the urine of victims for forensic toxicology.

The concentration of TTX in the blood of victims was between 4.5 and 40.6 nM, and the major concentration of TTX was in the urine (47–344 nM). It might be supposed that TTX had been distributed in the bodies of all the victims, and almost all of the TTX intake had been excreted from urine. The volume of human body fluid is usually 38 l [19]. Hence, the intake amount of TTX in the victims was estimated to be more than 54.5–492.2 μg. The toxicity of causative fish meat in this food poisoning incident was estimated to be about 4 μg TTX (20 MU/g). The minimum lethal dose and minimum acute dose of TTX to human are supposed to be 2 mg and 0.2 mg, respec-

tively [2]; so the above estimated intake amount of TTX in victims and the toxicity of causative fish meat accordingly from the blood data seemed to be low. How to estimate real TTX intake when TTX-associated food poisoning occurred should be further studied.

Acknowledgement

The study was supported by the National Science Council, Taiwan, ROC (Contract No. NSC 93-2120-M-019-001).

References

[1] C.K. How, C.H. Chern, Y.C. Huang, L.M. Wang, C.H. Lee, Am. J. Emerg. Med. 21 (2003) 21.
[2] T. Noguchi, J.S.M. Ebesu, J. Toxicol. Toxin Rev. 20 (2001) 1.
[3] D.F. Hwang, C.A. Cheng, Y.H. Tsai, S.S. Jeng, J. Nat. Toxins 4 (1995) 165.
[4] Department of Health (DOH), ROC, Occurrence of Food Poisoning in Taiwan, Taipei, 1997, p. 37.
[5] D.F. Hwang, C.A. Cheng, H.T. Tsai, D.Y. Shih, H.C. Ko, R.Z. Yang, S.S. Jeng, Fish. Sci. 61 (1995) 675.
[6] D.F. Hwang, Y.W. Hsieh, Y.C. Shiu, S.K. Chen, C.A. Cheng, J. Food Portect. 65 (2002) 389.
[7] Y.W. Hsieh, Y.C. Shiu, C.A. Cheng, S.K. Chen, D.F. Hwang, J. Food Sci. 67 (2002) 948.
[8] P.A. Hwang, Y.H. Tsai, Y.H. Lu, D.F. Hwang, Toxicon 41 (2003) 529.
[9] L.M. Shui, K. Chen, J.Y. Wang, H.Z. Mei, A.Z. Wang, Y.H. Lu, D.F. Hwang, J. Food Prot. 66 (2003) 110.
[10] C.A. Cheng, D.F. Hwang, Y.H. Tsai, H.C. Chen, S.S. Jeng, T. Noguchi, K. Ohwada, K. Hashimoto, Food Chem. Toxicol. 33 (1995) 929.
[11] H. Narita, T. Noguchi, J. Maruyama, Y. Ueda, K. Hashimoto, Y. Watanabe, K. Hida, Bull. Jpn. Soc. Sci. Fish. 47 (1981) 935.
[12] D.F. Hwang, T. Noguchi, O. Arakawa, T. Abe, K. Hashimoto, Nippon Suisan Gakkaishi 54 (1988) 2001.
[13] D. Andrinolo, L.F. Michea, N. Lagos, Toxicon 37 (1999) 447.
[14] K. Kawatsu, T. Shibata, Y. Hamano, Toxicon 37 (1999) 325.
[15] M. Nakamura, T. Yasumoto, Toxicon 23 (1985) 271.
[16] M. Horie, S. Kobayashi, N. Shimizu, H. Nakazawa, The Analyst 127 (2002) 755.
[17] B.D. Gessner, P. Bell, G.J. Doucette, E. Moczydlowski, M.A. Poli, F. Van Dolah, S. Hall, Toxicon 35 (1997) 711.
[18] L.E. Llewellyn, M.J. Dodd, A. Robertson, G. Ericson, C. de Koning, A.P. Negri, Toxicon 40 (2002) 1463.
[19] K.K. Rozman, C.D. Klaasen, in: C.D. Klassen (Ed.), Casarett and Doull's Toxicology, Mcgraw-Hill Co. Inc., New York, 1995, p. 101.