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Detection of Tetrodotoxin by High Performance Liquid Chromatography in Lined-Moon Shell and Puffer Fish

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Abstract

High performance liquid chromatography (HPLC) using fluorescent detection following post-column alkaline degradation and a sample preparation procedure for the analysis were established to quantitatively detect tetrodotoxins (TTXs) in gastropods and puffer fishes. The analysis showed 91% recovery of TTXs using the sample preparation described in this article and a wide (10-2000 ng) linear relationship between the tetrodotoxin amount and its fluorescent response. A good correlation between the results of mouse bioassay and HPLC was also obtained in this research. Each specimen of lined-moon shell, *Natica lineata* Roeding, collected from Tungkang from October 1994 to September 1995 was analyzed by HPLC to detect the presence and quantity of TTXs. Overall results showed that 45% of the gastropods contained TTXs with an average content of 54.5μg in toxic specimens. The rate of toxic specimens in the ten samples collected monthly varied, but there were no seasonal trends. Random sampling of cultured tiger puffer, *Takifugu rubripes rubripes* Temminck & Schlegel, and field-collected balloonfish, *Diodon holocanthus* Linnaeus, trace amounts of TTXs were found only in ovaries of balloonfishes. Muscle tissues of balloonfishes, and the livers, ovaries, and muscles of cultured tiger puffers showed no tetrodotoxin (< 0.2 μg/g tissue) in this screening procedure.

Key words: Tetrodotoxin; *Natica lineata*; *Takifugu rubripes rubripes*; *Diodon holocanthus*.

INTRODUCTION

Tetrodotoxin (TTX) is a potent marine neurotoxin which blocks the sodium channels of the neuron cell membrane of animals. It is found in various marine organisms, such as some species of gobies, octopuses, sea stars, crabs, gastropods in addition to the well-known puffers (Mosher and Fuhrman, 1984; Hashimoto *et al.*, 1990). Nevertheless, the reason for the wide distribution of these toxins in animals of such different taxa is still unclear. TTX and its derivatives have caused sporadic seafood poisonings in Taiwan (Hwang *et al.*, 1989a, b); therefore, there is always a risk of being poisoned by TTXs when consuming exotic or even ordinary seafood. This risk is evidenced by some reports showing that several species of the gastropods in Taiwan contain TTX (Hwang *et al.*, 1990a, b). To ensure the safety of seafood consumed, TTX monitoring needs to be implemented routinely on susceptible animals.

Mouse toxicity assay, similar to the method developed for PSP (paralytic shellfish poisoning) toxins monitoring (AOAC, 1995), has

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been applied for toxicity determination among puffers. The amount (0.22 μg) of TTX needed to kill, in 30 min, a male mouse of ddY strain weighing 20 g was determined as one mouse unit (MU) (Kawabata, 1978). An amount of 0.178 μg TTX was also determined equivalent to 1 MU for ICR strain mice (Hwang and Jeng, 1991). To achieve the high sensitivity and specificity in TTX monitoring and to avoid the excessive killing of mice, Yasumoto *et al.* (1982) and Yotsu *et al.* (1989) constructed a similar fluorometric TTX analyzer, by combining high performance liquid chromatography (HPLC) and a post column reaction with a hot NaOH solution, to detect tetrodotoxins and its derivatives. Such chemical methodology has resulted in the discovery of TTX-producing bacteria (Yasumoto *et al.*, 1986) and the identification of novel TTX analogs from puffers (Nakamura and Yasumoto, 1985). In this paper we report a procedure modified from the HPLC method described by Nagashima *et al.* (1987). The modified procedure was used to quantitatively detect TTX in the lined moon shell *Natica lineata*, balloonfish *Diodon holocanthus,* and cultured tiger fugu *Takifugu rubripes rubripes.*

MATERIALS AND METHODS

Toxin standard

TTX standard (L8503, citrate free crystals) with a purity of 96% (by HPLC, IR, and NMR) was purchased from Latoxan (France). Serially diluted aqueous solutions that ranged from 10 to 2000 μg/ml of TTX acetate were prepared. One microliter of each solution was injected into the HPLC column to determine the system sensitivity and linear range. Measurements were taken in triplicate and values of the peak areas were recorded as HPLC responses to the different amounts of authentic TTX injected. A linear regression line was plotted to show the correlation between peak areas and injected samples.

Sample organisms

Lined moon shells, *Natica lineata* Roeding, were collected from Tungkang, Pingtung Prefecture throughout the year from October 1994 to September 1995. Ten specimens were randomly sampled and analyzed each month for body toxicity. A depuration setup for the lined moon shell was constructed in an aquarium in the laboratory. The aquarium was continuously aerated and its temperature and salinity were kept at 25°C and 25 ppt, respectively. The water in the aquarium was renewed every 3-4 days and circulated through an aquarium filter. Twenty specimens of the shells were kept in the 15 cm deep beach sand bottom of the aquarium and fed with nontoxic hard clams (*Meretrix lusoria*) for one month. Efficiencies of depuration were measured from the reduction of toxicity in the shells. Randomly collected balloonfishes, *Diodon holocanthus* Linnaeus, from the coast of northern Taiwan and cultured tiger puffers, *Takifugu rubripes rubripes* Temminck & Schlegel, from Ilan Prefecture were examined for their TTX contents. Combined muscle (250 ± 10 g), liver (72 ± 5 g) and ovary (2.0 ± 0.3 g) samples from three tiger puffers, and muscle (600 ±10 g) and ovary (13.3 ± 0.3 g) samples from six ballonfishes (totally weighing 980 g) were analyzed separately. All animal samples were kept alive during transportation from the collection sites to the laboratory.

Sample extraction

Whole flesh bodies of lined moon shells and the different sorted tissues of balloonfishes and puffers were extracted with three volumes of methanol (containing 1% acetic acid) for 5 min in a tissue homogenizer. The extracts were centrifuged (900 g, 20 min), and the residues were extracted again with the same volume of an acidic methanol solution. The supernatants were combined, concentrated to dryness by rotary evaporator and then redissolved in 1% aqueous acetic acid. The aqueous extracts were then defatted with equal volume of chloroform. After removing the chloroform by centrifugation, the upper aqueous layer was filtered through an ultrafiltration membrane

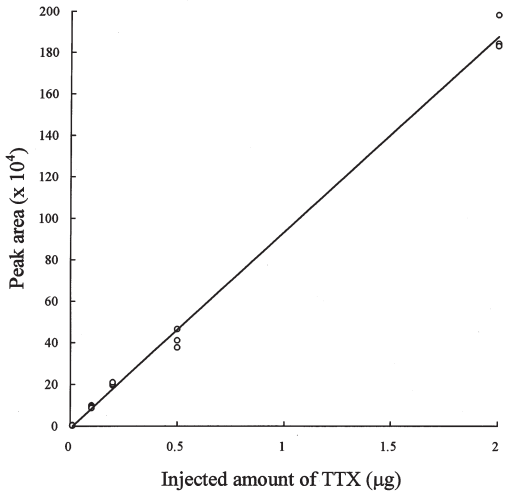

Figure 1. Calibration curve of the amount vs. peak areas for TTX.

(MWCO 10000) by the force of centrifugation. Subsequently, 10ml aliquots of each sample extract were subjected to HPLC analysis for TTX contents. Recovery of TTX in this sample preparation procedure was previously determined by blending the flesh of nontoxic shellfish with the authentic toxin in a quantity of 20 mg TTX/g shellfish. Part of the extract was further diluted into 1/10 and 1/100 solution for HPLC determination. Solutions of different concentrations were analyzed in triplicate. The recovery rate, 91.0 ± 5.2%, was then obtained for the most diluted toxin solution, and was further applied in all calculations of toxin content in shellfishes and puffers analyzed by HPLC.

HPLC

High performance liquid chromatography was run by a combination of Hitachi L-6200 Intelligent pump and F-1000 Spectrophotometer equipped with an L-6000 Reagent pump and a post-column reaction coil (10 m × 0.3 mm i.d.) in a 100°C water bath. The analysis used a Cosmosil 5C18-AR column, 250 × 4.6 mm (Nacalai Tesque, Japan) with a mobile phase of 2 mM heptanesulfonic acid (HSA) in 0.05 M potassium phosphate buffer (pH 7.0), flow rate 0.5 ml/min. Tetrodotoxin and its co-existing derivative, anhydro-tetrodotoxin, were detected by mixing 4 N NaOH with the eluate at a 1:1 ratio through the reaction coil. Fluorescent detection was observed at 505 nm with 381 nm excitation.

Mouse Bioassay

Mouse bioassay was performed according to the method described by Hwang and Jeng (1991). Mice of ICR strains (19-21 g) used in the toxicity assay were purchased from the Animal Laboratory of National Taiwan University Hospital. Shellfish extracts of ten samples with various known toxic levels by HPLC analysis were diluted with water and injected intraperitoneally into a group of three mice. The lethal potency was calculated as the time required to kill the mice and was expressed in mouse units (MU) according to the formulation defined by Hwang and Jeng (1991). Sample toxicities obtained from both mouse bioassays and HPLC analysis were compared with each other.

RESULTS

Quantitative analysis of TTX over a range of 10-2000 ng showed a good linear relationship between the fluorescence response (peak area on chromatograms) and the amount of injected authentic TTX (Fig.1). A regression line was plotted as Y = 94.254 X- 0.9925 (r^2 = 0.996), where X represents the amount of TTX in micrograms, and Y represents the peak area (x 10^4). This formula was applied to estimate the tetrodotoxin contents in extracts through the entire research using HPLC for quantitative analysis. The detection limit of TTX in the established analyzer was 10 ng which gave a signal to noise ratio (S/N) higher than 2. The co-existing anhydro-TTX which showed a contaminant peak 2.6 min after the major TTX peak was ignored in the quantification of TTX because a correction factor of 96% from the product purity label of the authentic standard (Fig. 2, upper chromatogram) was applied into the calculation.

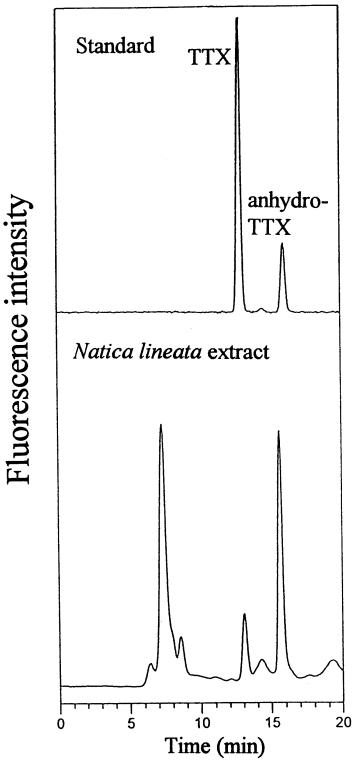

Figure 2. High performance liquid chromatograms of TTXs for authentic standard (upper plot) and extract from *N. lineata* (lower plot).

During the method development, the optimal ratio for alkaline reagent to eluate, and the reaction time were tested by adjusting the reagent flow rate and coil length. By comparing with the 100% peak area of TTX shown at a reagent flow rate of 0.5 ml/min, the peak area of TTX showed a decrease to 24.2%, 62.0%, 31.7%, and 21.7%, when the flow rate was set to 0.3 ml/min, 0.4 ml/min, 0.6 ml/min, and 0.8 ml/min, respectively. The highest detection value for the fluorescent TTX derivative was obtained with a reagent flow rate equal to the column elution. A reduced peak area (62.3 %) for TTX was also obtained when the reaction coil was cut in half.

Figure 2 (lower chromatogram) also shows a typical chromatogram of the toxic lined moon shell extract. A peak corresponding to anhydro-TTX was always found co-existing with TTX, and its peak area was higher than

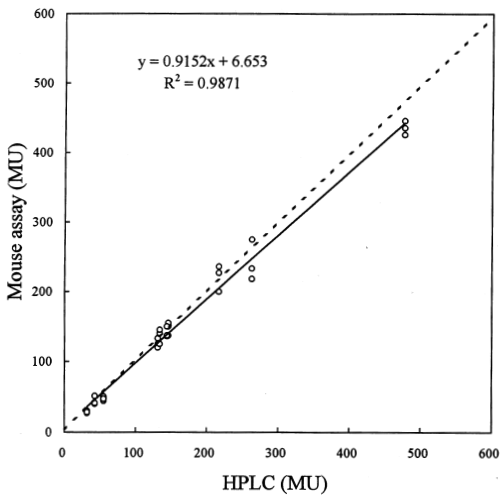

Figure 3. Correlation between MU data converted from HPLC analysis and mouse assay. The diagonal line represents good correlation between the two assay methods.

TTX in the extracts. In addition, it seemed another TTX derivative, similar to 4'-epi-TTX eluted out between TTX and anhydro-TTX (Nakamura and Yasumoto, 1985), was also co-existing in the toxic samples. Due to the lack of a stable anhydro-TTX or 4'-epi-TTX standard, their quantitative analysis was not possible in this research. Hence all conversions of MU toxicity from the HPLC peak area could not take the data of anhydro-TTX into account. Only the content of TTX was considered and a mouse toxicity assay proceeded in parallel to observe the contribution of anhydro-TTX to gastropod toxicity.

Ten gastropod extracts of different TTX contents known from HPLC analysis were also studied for toxicity by mouse assay. MU data converted from HPLC peak areas were compared with data obtained from the mouse assay for each toxic sample and showed a slight overestimate of toxicity (Fig. 3). A regression line, $Y = 0.9152\ X + 6.653$ ($r^2 = 0.987$) was plotted on all MU data in Fig. 3 to show a good correlation between these two methods. Anhydro-TTX did not seem to contribute any toxicity to the lined moon shell, although it showed a

Table 1. Monthly TTX monitoring of *N. lineata* collected from Tungkang, Pingtung Prefecture, from October 1994 to September 1995. Values are means ± SE of ten specimens.

Month of sampling	Average body weight (g)	Highest TTX content (μg/specimen)	Average TTX content in toxic specimens (μg/specimen)	No. of toxic specimens within the ten samples
Oct. 1994	14.0 ± 2.8	47.0	27.9 ± 9.8	8
Nov. 1994	14.0 ± 1.8	165.5	95.7 ± 44.1	6
Dec. 1994	14.2 ± 1.6	66.7	32.4 ± 13.5	4
Jan. 1995	17.9 ± 1.4	113.6	105.5 ± 11.5	2
Feb. 1995	16.8 ± 1.7	112.8	87.7 ± 24.8	5
Mar. 1995	15.5 ± 3.6	45.3	21.9 ± 18.5	5
Apr. 1995	14.2 ± 3.0	93.2	42.4 ± 29.5	5
May 1995	16.8 ± 1.7	442.0	160.1 ±139.0	6
Jun. 1995	15.7 ± 3.0	57.5	57.5 ± 3.9	10
Jul. 1995	13.6 ± 3.1	45.9	32.9 ± 11.1	6
Aug. 1995	14.5 ± 2.1	32.9	29.2 ± 3.9	4
Sep. 1995	12.5 ± 3.3	91.2	74.7 ± 23.4	2

larger quantity than did TTX in the extracts.

The ratios of toxic samples in the monthly collected gastropods are shown in Table 1. The ratio of TTX-containing lined moon shells in the year-round samplings from October 1994 to September 1995 was calculated as 45%. Toxic samples were found in every month during the year, although the ratio of toxic samples varied through the year. The existence of toxic gastropods does not seem to have any seasonal trend. The highest TTX content observed in this survey was 442.0 μg in one specimen of 19.0 g body weight, which equals 324 MU per gram of its flesh. The average body weight of the lined moon shells was 15.0 ± 3.0g in the market, and the average toxicity through the year was calculated to be 160.3 ± 73.6 MU per shell or 25.7 ± 22.4MU per gram of flesh. Twenty specimens of lined moon shells that were kept in laboratory and fed with toxin-free clam meat were analyzed for toxin contents and the ratio of toxic samples. It was found that only seven specimens remained alive after one month, and among them, five contained TTX. The TTX content of these five specimens showed an average of 46.6 μg/specimen, not much less than the yearly average of the toxic samples at 62.6 μg/specimen. The stocking of gastropods away from any possible intake of food-born TTX for a month seemed not to exclude or reduce the body toxin significantly.

HPLC analysis on randomly collected ballonfishes and cultured tiger puffers showed that TTX or its derivative, anhydro-TTX, was not present either in the liver, ovary, and muscle tissues of cultured *T. rubripes rubripes*, or in the muscle sample of wild *D. holocanthus*. However, the ovary of *D. holocanthus* showed the presence of both TTX and anhydro-TTX. It was also determined that the TTX content in the ovaries of balloonfishes was 0.5 μg /g.

DISCUSSION

The present work shows once more that the application of the fluorometric HPLC system

in the determination of TTX and its derivatives is very useful. This method provided higher sensitivity and accuracy than the mouse assay and did not require an animal colony. Previous mouse assays for the seafood toxicity test may give false positives due to high salt contents in marine organisms, because salts present in the meat are concentrated during sample preparation. The salt concentration has been reported to cause mouse death (Fernandez *et al.*, 1996). It was also reported that a 20% error exists between the mice using mouse toxicity assay (Shimizu and Ragelis, 1979). Chemical analysis, such as an HPLC system reported here, can solve these problems. Moreover, it can easily distinguish TTX from other toxins, *e.g.* paralytic shellfish poisons (PSP), which cause the same acute lethal effect to mice due to their common toxicological function. The postulated sample preparation combined with the known HPLC system has proven itself a useful tool in routine monitoring of susceptible seafoods. Although sample pretreatments, such as Bio-gel P2 (Hwang *et al.*, 1991) and Amberlite CG-50 (Yasumoto *et al.*, 1982; Yosumoto and Michishita, 1985) prewashing may clarify the chromatograms of the subsequent HPLC analysis, a non-disposable operation may cause some contamination among different samples. A disposable ultrafiltration tube operated by centrifugation following the defatting operation was found to be time-saving and to have a high recovery that is important when considering the sensitivity.

Anhydro-TTX and 4'-epi-TTX were always found co-existing with TTX in the gastropod or puffer sample extracts. However, the relative abundance of these toxins in lined moon shells was significantly different from that in the ovary of ballonfishes. It was reported that the relative abundance of TTX derivatives in the puffers, TTX (77%), 4'-epi-TTX (11%), and anhydro-TTX (12%), was similar to the result of anhydro-TTX treated with 5% HCl at ambient temperature overnight (Nakamura and Yasumoto, 1985). Our gastropod analysis did not give the same result as that of puffers; however, this may indicate that different organisms have different relative abundances. From an attempt to isolate individual toxins, combined gastropod extracts gave a composition similar to that of puffer after repeated gel filtration separation under acidic conditions. Since the metabolism and biogenesis of these toxins are still unknown, an immediate HPLC analysis after the extraction is needed for composition studies.

Ignoring the presence of anhydro-TTX did not show too much bias in the conversion of TTX contents to MU toxicity in spite of the large amount of anhydro-TTX in the lined moon shell. There is a good parallel correlation between the TTX data converted from HPLC analysis and the mouse bioassay (Fig. 3). This may be due to the 1/500 lower potency in toxicity of anhydro-TTX to TTX (Hwang and Jeng, 1991), or the fluorescent response of C9 base from anhydro-TTX being stronger than that from TTX. It was also reported that the toxicity of TTX (4500 MU/mg) is 49 times higher than the toxicity of anhydro-TTX (92 MU/mg) (Nakamura and Yasumoto, 1985).

The 10 ng detection limit of the postulated HPLC was found to be similar to the result of 9.6 ng reported by Yotsu *et al.* (1989). Higher sensitivity (< 0.2 μg TTX/g sample) than the mouse assay, 0.36 μg/g (Hwang *et al.*, 1994) and 1.1 μg/g (Saito *et al.*, 1991), gave a good opportunity for trace toxin analysis.

The ratio of TTX-containing lined moon shells in the year-round samplings from October 1994 to September 1995 was calculated as 45% which is lower than 95.1% in a previous report using mouse assay (Hwang *et al.*, 1991). The lower ratio may be due to false positive results caused by salts in the sample extracts (Fernandez *et al.*, 1996) or simply by the different occurrence of toxic samples from different samplings. Although winter samples of *N. lineata* were reported to have higher toxicity (Hwang *et al.*, 1991), a seasonal preference was not observed in this monitoring. The sporadic occurrence of toxic gastropods gave no evidence of endogenous origin of TTXs.

Laboratory stocking of lined moon shells for depuration was unsuccessful, although it was reported that gastropods are able to release tetrodotoxin after removal from seawater (Hwang *et al.*, 1990b). Several studies have pointed out that many bacteria strains isolated from toxic shells or fishes are TTX producers (Noguchi *et al.*, 1987; Yasumoto *et al.*, 1986), but the toxin content of the isolated bacteria seemed to be insignificant compared with the amount of toxin in their host animals. From our results, we are still unable to make any judgement on the origin of TTX.

Tiger fugu *T. rubripes rubripes* culture is a newly developed business in Taiwan. The wild tiger fugu was thought to be toxic and the toxicity of the puffers varied with the location where the puffers were caught (Matsui *et al.*, 1982b). Cultured puffers, due to the artificial feed on which they are fed, were observed to be nontoxic in the research of Matsui *et al.* (1982a) and Saito *et al.* (1984). We observed the same result for cultured specimens, but the sample size is rather small, and this result needs further confirmation. Randomly collected ballonfishes from the wild showed trace amounts of TTX in the ovary; however, the TTX contents have not been reported yet. Our study indicates that consumption of the puffer fish, *D. holocanthus* and lined moon shell, *N. lineata* is highly risky and we would like to suggest a ban on the consumption of these animals. The consumption of puffers is regulated in Japan; the safety criteria for consumption is 2.2 μg TTX/g flesh (Kawabata, 1978).

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