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Short communication

Screening of tetrodotoxin in puffers using gas chromatography–mass spectrometry

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ABSTRACT

Tetrodotoxin (TTX), a toxic compound found in some puffers can cause death to humans through consumption. We have developed a simplified method for the screening of TTX in puffers using GC–MS. A puffer tissue of 0.5 g was treated with 5 mL of 0.1% acetic acid, followed by alkaline hydrolysis, LLE or liquid–liquid extraction and N-methyl-N-TMS-trifluoroacetamide derivatization. The developed method used only a small sample and solvent, simplified LLE and derivatization procedures and short chromatographic analysis (8.2 min). All of these contribute to cost-saving, enhanced sample throughput and high sensitivity of the screening assay. The developed method was validated and proved to be within the acceptable range.

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1. Introduction

Tetrodotoxin (TTX) is a marine neurotoxin commonly found in puffer fish [1]. Consumption of toxic puffers by humans has resulted in deaths [2–6]. Studies reported that TTX level varies within and between species [1,7–11]. Accumulation of bacteria in the puffers, geographical habitats, seasonal change and ecological food chain contribute and influence the level of TTX in puffers [7–9,11–14]. As such, consumers are at serious risk since some species thought to be safe for human consumption may not be so. It is therefore vital that TTX levels in puffers are continuously monitored.

TTX levels in puffers are normally estimated through mouse bioassay [7–10,15]. However, this assay and other techniques such as thin layer chromatography (TLC), electrophoresis [7,16,17], liquid chromatography (LC) [10,16–20], spectrophotometry [7,12], enzyme immunoassays (EIA) [21–23] poses ethical concern, is not specific and lacked sensitivity at low concentration or of poor precision. Mass spectrometry (MS) coupled with LC or GC is a sensitive technique for identification of TTX. However, due to the complexity of sample matrices and insolubility of TTX in organic solvent, the LC–MS [8,9,11,15,18,24–27] or LC–MS/MS [4,6,13,15,18,27] is a preferred method than GC–MS. The drawback for LC–MS and LC–MS/MS analysis is that it involves the use of expensive instruments that require higher maintenance compared to GC–MS.

Analytical assay of TTX generally involves pre-extraction of the compound from fish tissues using acetic acid (HOAc) accompanied by a 5–10 min heating [4,10,11,17,19,25–27], or non-heating [6,7,18,20]. After centrifugation and/or evaporation, the recovered supernatant is subjected to sample cleanup using either solid-phase extraction (SPE) [6,7,25,26], preparative column [7,18], membrane or nylon filtration [2,4,11] or a combination of any of the two cleanup methods. In GC–MS analysis, structure modification of TTX is necessary as it is heat stable and not-volatile. This is accomplished by alkaline hydrolysis followed by trimethylsilylation to form trimethylsilyl derivative of 2-amino-6-hydroxymethyl-8-hydroxyquinazoline or better known as C_9-base [2,5,7,12,28]. With no reported validation and quantification of TTX in puffer tissues by GC–MS thus far, it is the authors' objective to develop and validate a GC–MS assay for this purpose, particularly involving species consumed by humans.

2. Experimental

2.1. Chemicals

All purchased certified reference material and chemical were of analytical grade: TTX (>96%, Ascent Scientific), N-methyl-N-TMS-

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trifluoroacetamide (MSTFA) and *n*-dodecane (Sigma), *n*-butanol (J.T. Baker), sodium hydroxide (Surachem), salicylic acid, acetic acid and hydrochloric acid (Merck).

2.2. Blank, standards, calibrators and validation samples

Sample was prepared by sonicating 0.5 g of minced fish muscle in 5 mL HOAc for 10 min. The extract was vortexed for 1 min and centrifuged for 10 min at 2500 rpm. The recovered supernatant was used for the extraction. Known blank matrix from marine fish muscle was treated as sample and the supernatant was used in the preparation of calibrators and validation samples. A working solution of 1.0 μg/mL was freshly prepared from the stock solution of TTX (0.04 mg/mL) in HOAc. Five duplicate calibrators were prepared daily by spiking 10, 20, 30, 60 and 100 μL of TTX working solutions into 100 μL blank matrixes. HOAc was added to make a total volume of 200 μL. The calibrators were equivalent to on-column detection at 0.1, 0.2, 0.3, 0.6 and 1.0 ng.

Three validation samples, equivalent to the detection of 0.1 ng, 0.5 ng and 1.0 ng of TTX were prepared by pipetting 25 μL, 125 μL and 250 μL of TTX stock, into respective volumetric flasks and diluted to 10 mL mark with blank matrix. Each of the 100 μL of validation samples, blank matrix and samples were added with 100 μL HOAc to make a total volume of 200 μL. An internal standard (IS) of 2.5 μg/mL salicylic acid in HOAc was also prepared. The concentration of HOAc used in the above preparations was 0.1%.

2.3. Sample extraction

A mixture of 200 μL sample, 50 μL sodium hydroxide (3 N) and 50 μL IS was boiled for 20 min, cooled to room temperature and defatted or cleanup using 0.5 mL *n*-butanol (BuOH). The aqueous layer was then added with 70 μL hydrochloric acid (2 N), 2 mL distilled and subjected to 1 min extraction with 0.5 mL BuOH and 5 min centrifugation at 2500 rpm. The recovered organic layer was dried in anhydrous sodium sulphate, evaporated to dryness and subsequently derivatized with 50 μL MSTFA at 60 °C for 30 min 50 μL of *n*-dodecane was added to the extract at room temperature, vortexed briefly and transferred into crimped-capped autosampler vials. The blank matrix, calibrators and validation samples were all treated similarly

2.4. Gas chromatography–mass spectrometry (GC–MS) conditions

A GC–MS system consists of HP6890 GC and HP5973 MS. One μL injection volume using splitless mode was injected into a fused-silica capillary column, HP-5MS (30 m × 0.25 mm i.d. 0.25 μm film thickness). The injector and interface temperatures were set at 250 °C and 280 °C, respectively. The oven temperature was programmed from 100 to 280 °C (1 min hold) at a rate of 25 °C/min. Quantifying ions used in selective ion monitoring mode were *m/z* 392 (TTX) and *m/z* 268 (IS). Column head was trimmed from time to time on evidence of active site formation. The total run time was 8.2 min.

2.5. Validation of GC–MS method

2.5.1. Linearity, sensitivity and recovery

Calibration curve was constructed on average peak area ratio of TTX/IS versus concentrations of 5 calibrators. Linearity was calculated based on the regression line and expressed as the correlation coefficient ($r^2 \geq 0.995$). Sensitivity of the assay was determined by limit of quantification (LOQ) and limit of detection (LOD). LOQ was the lowest concentration in a calibration curve with a signal to noise ratio of 5:1 and the relative accuracy and precision was <20%. A total of 15 replicates of LOQ were analyzed in 3 runs. The LOD was the concentration which had a signal to noise ratio of 3:1. The extraction recovery was determined by comparing the mean peak areas of TTX in fish tissues with the extracted standards in HOAc treated as the tissues [5,28]

2.5.2. Precision and accuracy

Within-day and between-day precisions and accuracy were calculated using validation samples by comparing data from within one and three runs, respectively. Precision was expressed as percent coefficient of variation and relative accuracy as the percent difference from the nominal values.

3. Results and discussion

TTX is extremely soluble in weak acid and as such, extraction of TTX from puffer tissue was carried out with only 5 mL of HOAc under sound sonication. The lengthy evaporation steps as practiced by others [6,25,29] was omitted. In several studies, the recovered supernatant was subjected to defatting procedure using dichloromethane [6,11,25], chloroform [29,30] or diethyl ether [28] followed by further cleanup procedures before the sample could be analyzed by analytical instruments. For over 30 years, SPE and preparative column have been used to extract TTX. Some commercialized SPE cartridges available for such purposes are Sep-Pak C18 [1,30], Bond Elut SCX [1], Sep-Pak PS-2, OASIS HLB [2], Shodex MSpak CX-4A [3], Bond-Elut C18 [17] and NHS-activated [9]. Self-packed preparative column using activated charcoal [6,7,9,25,30], Amberlite IRC-50 [18], Bio-Gel P-2 [4,11,25], Bio-Rex 70 [25], etc. have also been used to extract TTX. The SPE cartridges were either used singularly or with another cartridges, or in combination with self-packed column. Filtration using commercialized nylon or membrane filters were among the common methods used in the cleanup procedure [11,13,26,29]. In our method, the cleanup process was replaced with a two-step LLE, each step using a small amount of cheap solvent, i.e. 0.5 mL BuOH. The first step was for the removal of fats and BuOH soluble impurities. The second to extract TTX and IS from the remaining acidic supernatant. This approach was quantitative and could produce good separation and detection of TTX (Fig. 1).

Prior to LLE, hydrolysis of TTX was performed for 20 min, within the range of optimum time of 10–30 min [2]. Longer hydrolysis period could result in reduced TTX recovery [7]. The recovery (98.4%) (Table 1) of the developed assay was better than previously reported assays by HPLC (91%) [20], LC–MS (77.7–80.7%)[25] or LC–MS/MS (79–83%) [6]. The common derivatization procedure for GC–MS analysis of TTX involves the use of bistrimethylsilylacetamide (BSA), TMCS and pyridine mixture to form trimethylsilylated C_9-base (3TMS) [2,7,12,28]. Correct and careful use of TMCS and pyridine is vital as these chemicals are corrosive and can deteriorate and reduce the GC capillary column's lifespan. But with the absence of TMCS/pyridine, derivatization with BSA resulted in the formation of multiple C_9-base derivatives, i.e. 2-TMS and 3-TMS [5]. In our method, the derivatization procedure was simplified by using a single derivative agent, i.e. MSTFA. A heating at 60 °C for 30 min ensures the formation of 3TMS C_9-base derivative. Trimethylsilylation often cause blockages in injection needle but this can be overcome by using ethyl acetate as the washing solvent. The use of salicylic acid as IS (Fig. 1), which is extractable in acidic condition such as in TTX, also lend good precision and accuracy as demonstrated in this method. Dodecane has a higher boiling point (216.2 °C) than other TMS compatible solvents (such as hexane, dichloromethane, heptane, etc.) was added to TMS derivatives to facilitate the vaporization of C_9-base in the high temperature injector port. It was able to produce good precision and accuracy for the assay (Table 1)

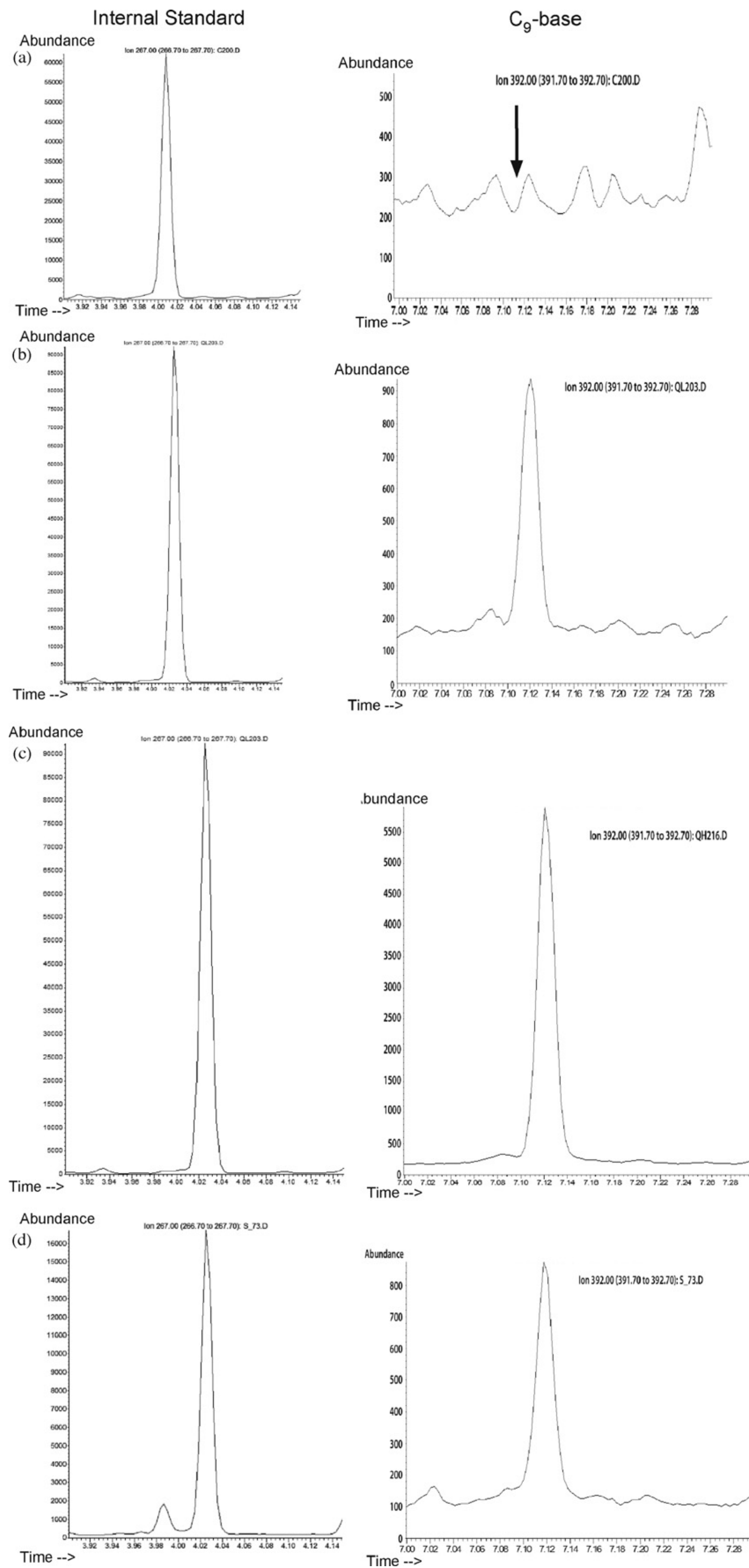

Fig. 1. Extracted ion chromatogram of internal standard and tetrodotoxin derivative (C9-base) in (a) Blank fish muscle (b) 10 ng spiked TTX (0.1 ng on-column) (c) 100 ng spiked TTX (1.0 ng on-column) and (d) TTX in muscle of *Tetrodon nigroviridis*.

Table 1
Recovery, within and between assays precisions and accuracies of TTX.

Concentration (ng)	Within-assay[a]			Between-assay[b]			Recovery[a] (%)
	Observed concentration (mean ± SD) (ng)	Precision (%)	Accuracy (%)	Observed concentration (mean ± SD) (ng)	Precision (%)	Accuracy (%)	
0.1	0.094 ± 0.007	8.21	5.64	0.102 ± 0.008	7.54	8.13	105.59
0.5	0.488 ± 0.056	11.58	2.32	0.453 ± 0.039	8.20	9.43	104.40
1.0	0.865 ± 0.105	12.23	13.46	0.935 ± 0.131	13.88	6.32	85.14
	Average	10.67	6.95	Average	9.87	7.58	98.38

[a] $n = 5$ for each concentration.
[b] $n = 15$ for each concentration.

The short GC run-time (8.2 min) with relative retention time of 0.56 was sufficient to produce good separations for TTX (Fig. 1), and also contributed to the high throughput of the assay. This run-time is shorter than other reported GC–MS and LC, LC–MS or LC–MS/MS assays which ranged from 15 to 50 min [7,11,12,16–20,25]. Three characteristic fragment ions were monitored in this assay, i.e. m/z 392 (100%), m/z 407 (35%) and m/z 376 (14%). The m/z 392 was derived from the loss of methyl group from the molecular ion of C_9-base; (m/z 407). It was selected as the quantitative ion due to its highest abundance and lowest background interference. High sensitivity assay is critical in screening the traces of TTX. The LOD achieved by this current assay was 0.05 ng (or 0.5 μg/g sample). The LOQ of 0.1 ng (or 1.0 μg/g sample) was established with precision and relative accuracy of <15.4%. Lethal potency of TTX is normally expressed using mouse unit (MU); one MU is approximately 0.22 μg TTX [25]. The sensitivity of this assay was estimated at 0.00023 MU, better than those of indirect competitive inhibition EIA (0.1 ng/mL or ~0.00041 MU) [23], electrophoresis and TLC (2 μg) [16], LC with fluorescent detectors (3.0, 4.4, 10.0 ng) [16,19,20], LC–MS assay (0.2 ng) [18], LC–MS/MS (0.09 ng) [4] and TLC/fast atom bombardment MS (0.1 μg) [30]. Nevertheless, by using larger quantities of starting material and HOAc together with commercialized cartridges, Horie et al. [25] reported similar on-column assay sensitivity as used in our method. The linearity of the assays performed in three consecutive days had an average correlation coefficient $r^2 \geq 0.998$ (Fig. 2). The working range (0.1–1.0 ng) is beneficial for quantifying the actual level of TTX in puffer species, especially those consume by human. The average within and between precision and accuracy assays was <11% (Table 1).

4. Application

Seven different puffer species from the Malaysian waters were collected between July 2009 to February 2010 (Table 2). Three replicates of the samples were analyzed according to the developed

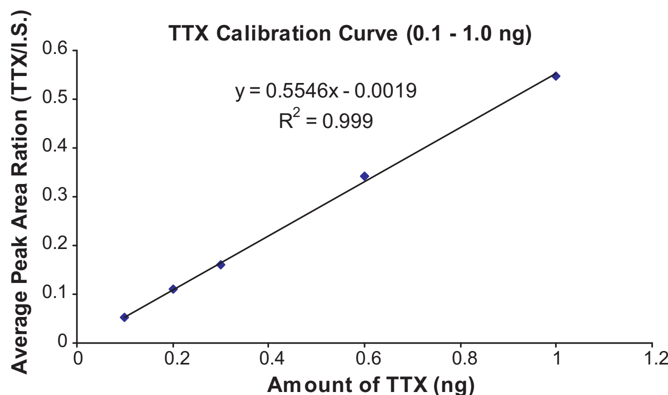

Fig. 2. Calibration curve of tetrodotoxin.

Table 2
Concentration of tetrodotoxin in various puffer species in Malaysia.

Species	TTX per gram muscle[a] (mean ± SD) (μg/g)	Calculated[b] (MU/g)
Tetrodon nigroviridis	1.34 ± 0.05	6.1
Arothron hispidus	4.11 ± 0.32	18.7
Takifugu oblongus	1.38 ± .0.11	6.3
Lagocephalus sceleratus	1.71 ± 0.28	7.8
Lagocephalus lunaris	3.47 ± 0.10	15.8
Lagocephalus spadiceus	ND	–
Xenopterus naritus	ND	–

[a] n=3.
[b] 1 MU = 0.22 μg TTX [25].

assay. *Lagocephalus spadiceus* was found to be non-toxic. However, *Arothron hispidus* and *Lagocephalus lunaris* are toxic for human consumption as the level was >2 μg/g or >10 MU/g [17]. These results are consistent with other studies [11,31,32]. The TTX levels were also consistent with reported mouse bioassay in *Lagocephalus lunaris* tissues (2–67 MU/g) [31] and LC–MS/MS assays in *Tetrodon nigroviridis* (0.19–98.79 μg/g) and *Takifugu oblongus* (1.64 μg/g or 2.8–6.3 MU/g) [4,27]. In our method, mild level (1.34–1.71 μg/g) of TTX was detected in *Lagocephalus sceleratus*, *Tetrodon nigroviridis* and *Takifugu oblongus*. These species must be closely monitored as excessive consumption from this group (>1 g) may pose serious health effect on humans.

5. Conclusion

We have developed a high throughput screening assay for quantification of TTX in puffer tissues. The assay is sensitive and simple. The LLE extractions and derivatization of TTX were done with the use of very little solvent and single derivatizing agent. The GC–MS analysis was carried out in 8.2 min. The results affirm that it is applicable for routine monitoring of TTX in puffers.

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