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Development and validation of a high-throughput double solid phase extraction–liquid chromatography–tandem mass spectrometry method for the determination of tetrodotoxin in human urine and plasma

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ABSTRACT

A sensitive analytical method for the determination of tetrodotoxin (TTX) in urine and plasma matrices was developed using double solid phase extraction (C18 and hydrophilic interaction liquid chromatography) and subsequent analysis by HPLC coupled with tandem mass spectrometry. The double SPE sample cleanup efficiently reduced matrix and ion suppression effects. Together with the use of ion pair reagent in the mobile phase, isocratic elution became possible which enabled a shorter analysis time of 5.5 min per sample. The assay results were linear up to 500 ng mL^{-1} for urine and 20 ng mL^{-1} for plasma. The limit of detection and limit of quantification were 0.13 ng mL^{-1} and 2.5 ng mL^{-1}, respectively, for both biological matrices. Recoveries were in the range of 75–81%. To eliminate the effect of dehydration and variations in urinary output, urinary creatinine-adjustment was made. TTX was quantified in eight urine samples and seven plasma samples from eight patients suspected of having TTX poisoning. TTX was detected in all urine samples, with concentrations ranging from 17.6 to 460.5 ng mL^{-1}, but was not detected in any of the plasma samples. The creatinine-adjusted TTX concentration in urine (ranging from 7.4 to 41.1 ng μmol^{-1} creatinine) correlated well with the degree of poisoning as observed from clinical symptoms.

1. Introduction

Tetrodotoxin (TTX), a well known natural toxin, is a powerful sodium channel blocker [1,2] found in puffer fish and some gastropods. There are several other marine animals, such as Gobius cringer, horseshoe crabs, Costa Rican frog of the genus Atelopus, Australian blue-ring octopus and Californian newt, which, if eaten, can cause poisoning. TTX poisoning incidents are particularly frequent in Japan, Taiwan and Southeast Asia, where fugu or puffer fish is served as a human food. Onset of clinical symptoms usually occurs 10–45 min after ingestion, but may be delayed by 3 h or even more. Typical symptoms include perioral numbness, dizziness, nausea, vomiting, acroparesthesia, breathlessness and even death [3].

Previous studies indicated that TTX only remains in the plasma component of the blood for a short period (less than 24 h), but can be found in urine even on day 4 after ingestion [4]. This suggests that TTX in plasma is rapidly eliminated through the urine. In light of this, urine TTX determination could be a better choice, for the purposes of clinical laboratory diagnosis, to confirm TTX poisoning. In the literature, several analytical methods for tracing TTX in urine and blood samples of poisoned patients have been reported, including gas chromatography–mass spectrometry (GC–MS) [5], immunoaffinity chromatography [6], high performance liquid chromatography (HPLC) with post-column derivatization and fluorescence detection [4], HPLC with UV detection [7] and liquid chromatography–mass spectrometry (LC–MS) [8,9]. Among these methods, the GC–MS method entails a complicated extraction process and is considered time-consuming. HPLC methods are generally limited in their sensitivity. The immunoaffinity chromatography method shows relatively high sensitivity in detecting TTX in urine, but requires a costly monoclonal antibody.

Few reports were found in the literature describing the determination of TTX using LC–MS/MS. One relevant article [10] was found working in serum but not in the urine matrix. The new method described in this paper uses C18 and hydrophilic interaction liquid chromatography solid phase extraction (C18-HILIC SPE) to reduce matrix interference and overcome severe ion suppression of TTX, followed by LC–MS/MS detection. This validated method was used with clinical samples of both plasma and urine, to detect and quantify the presence of TTX.

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2. Experimental

2.1. Materials and reagents

Tetrodotoxin standard (99% HPLC) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Ammonium formate and formic acid were purchased from Sigma–Aldrich (Fluka, Switzerland). Heptafluorobutyric acid was obtained from Tokyo Chemical Industrial Co. Ltd. (Japan). Oasis MCX, HLB, MAX solid phase extraction (SPE) cartridges were purchased from Waters Co. Ltd. (USA). SeQuant ZIC-HILIC SPE cartridge was obtained from SeQuant (Sweden). Superclean LC18 SPE cartridge was supplied by Supelco (USA) and Sep-Pak SPE cartridge was purchased from Sep-Pak cartridge Vac, Waters (Australia). Methanol and acetonitrile with HPLC grade were purchased from J.T. Baker (Philipsburg, NJ, USA). Water used in this study was generated from a Millipore system (Bedford, MA, USA). All other chemicals were analytical grade.

Stock TTX solution at the concentration of 1 mg mL^{-1} in 2% acetic acid was prepared and stored at −20 °C. Working aqueous standards (10, 50, 100, 250, 400, 500 ng mL^{-1}) were prepared daily by diluting stock solutions with mobile phase. Both TTX-spiked urine and plasma calibrators were prepared by adding TTX to TTX-free urine and plasma at various concentrations. Data from the standard curves were subjected to linear regression analysis, with peak area (y) versus amount of TTX (x) plotted.

2.2. Patient samples

Both spot urine and heparinized whole blood samples were collected in a local hospital from patients suspected of TTX poisoning within 24 h (range 5.5–12.5 h) after ingesting puffer fish or soup containing puffer fish. Urine and plasma were stored at −20 °C until assayed.

2.3. Sample preparation for LC–MS/MS

The sample cleanup procedure was based on that developed by Akaki et al. [11] with modifications. In brief, 1 mL of sample was mixed with 4 mL 2% acetic acid, vortexed and centrifuged at 2000 × g for 5 min. The supernatant was passed through a preconditioned C18 cartridge. The C18 cartridge was pre-conditioned with 2 mL methanol followed by 2 mL water. The first 2-mL portion of eluant was discarded and the rest was collected. A 1 mL eluate from C18 cartridge was mixed with 10 mL methanol, and then loaded onto the HILIC SPE cartridge, which was preconditioned with 2 mL water, 5 mL acetonitrile and 5 mL 80% methanol. After complete loading, the HILIC cartridge was washed with 5 mL 80% methanol followed by 5 mL water. Tetrodotoxin was eluted by 3 mL 2% acetic acid. The eluate was evaporated to dryness at elevated temperature (90 °C) and then reconstituted in 100 μL mobile phase; 20 μL was injected into the LC–MS/MS system.

2.4. Determination of creatinine in urine samples

Creatinine determination was carried out by a kinetic colorimetric assay based on the modified Jaffe method [12] using the Roche Modular System (Roche Diagnostics, IN, USA), with an analytical range between 360 and 57,500 μmol L^{-1}.

2.5. Liquid chromatography

An Agilent 1200 series liquid chromatographic system equipped with an Atlantics dC18 column (2.1 mm × 150 mm, 5 μm) was used, with isocratic elution of a single mobile phase containing 10 mmol L^{-1} ammonium formate with formic acid [95:5, v/v], with 5 mM heptafluorobutyric acid and 2% acetonitrile. The flow rate was 200 μL min^{-1} with a total run time of 5.5 min per sample.

2.6. Mass spectrometry

This study was carried out by using a quadrupole mass spectrometer (Applied Biosystems 3200QTRAP) equipped with a Turbolon-Spray ionization source. To optimize mass spectrometer settings, TTX standard was infused with a syringe pump at 10 μL min^{-1} in the mobile phase. Positive ionization was performed with the following settings: ion spray voltage, 5500 V; curtain gas, 10; collision gas, medium; ion source gas 1 and 2 at 50 and 30 U, respectively; interface heater, on; and source temperature, 700 °C. To protect the detector from unnecessary exposure, LC eluent was diverted to the waste just before and immediately after the peak appeared.

2.7. Quantitative and qualitative identification

For every batch of patient samples, blank and calibrators at 50, 100, 250 and 500 ng mL^{-1} for urine were run, and blank and calibrators at 5, 7.5, 10 and 15 ng mL^{-1} for plasma were run. Mass transition for TTX, m/z 320.1 → 162.3 was used for quantitation; and mass transitions m/z 320.1 → 302.3 and 320.1 → 256.2 were used as qualitative ions for positive identification.

3. Results and discussion

3.1. Effect of ion pair reagent content on column retention

The effect of ion pair reagent content on retention was investigated by varying the amount of ion pair reagent in the mobile phase while keeping other components constant. At an ion pair reagent concentration of 20 mM, the retention time (RT) for TTX was 3.52 min. At 2 mM concentration, RT for TTX was 7.7 min. To compromise between the total run time and avoidance of co-elution of interference substances, an ion pair reagent concentration at 5 mM was used.

3.2. Ion suppression or enhancement

Severe ion suppression was encountered in the study. Various sample preparation procedures were therefore envisaged including single SPE of different composition of adsorption materials, varied washing solvents in preparation steps, etc. Although modifications of mobile phase and elution program were attempted, the ion suppression phenomenon persisted (Fig. 1). In light of this, double SPE was attempted to increase the efficiency in removing ion suppression. Significant ion suppression was still observed in MCX-HILIC (Fig. 2), MAX-HILIC, and HLB-HILIC. Better observations were made for both C18-HILIC (Fig. 3) and Sep-Pak-HILIC. It is probably due to the fact that both C18 and Sep-Pak cartridges could retain hydrophobic substances in the sample but could not retain TTX. These adsorption materials effectively separated/removed interferences that eluted close to TTX peak and therefore fixed the problem of ion suppression. Ion enhancement was not found for either C18-HILIC or Sep-Pak-HILIC. In our work, a better recovery was obtained using C18-HILIC and therefore it was used in subsequent method development.

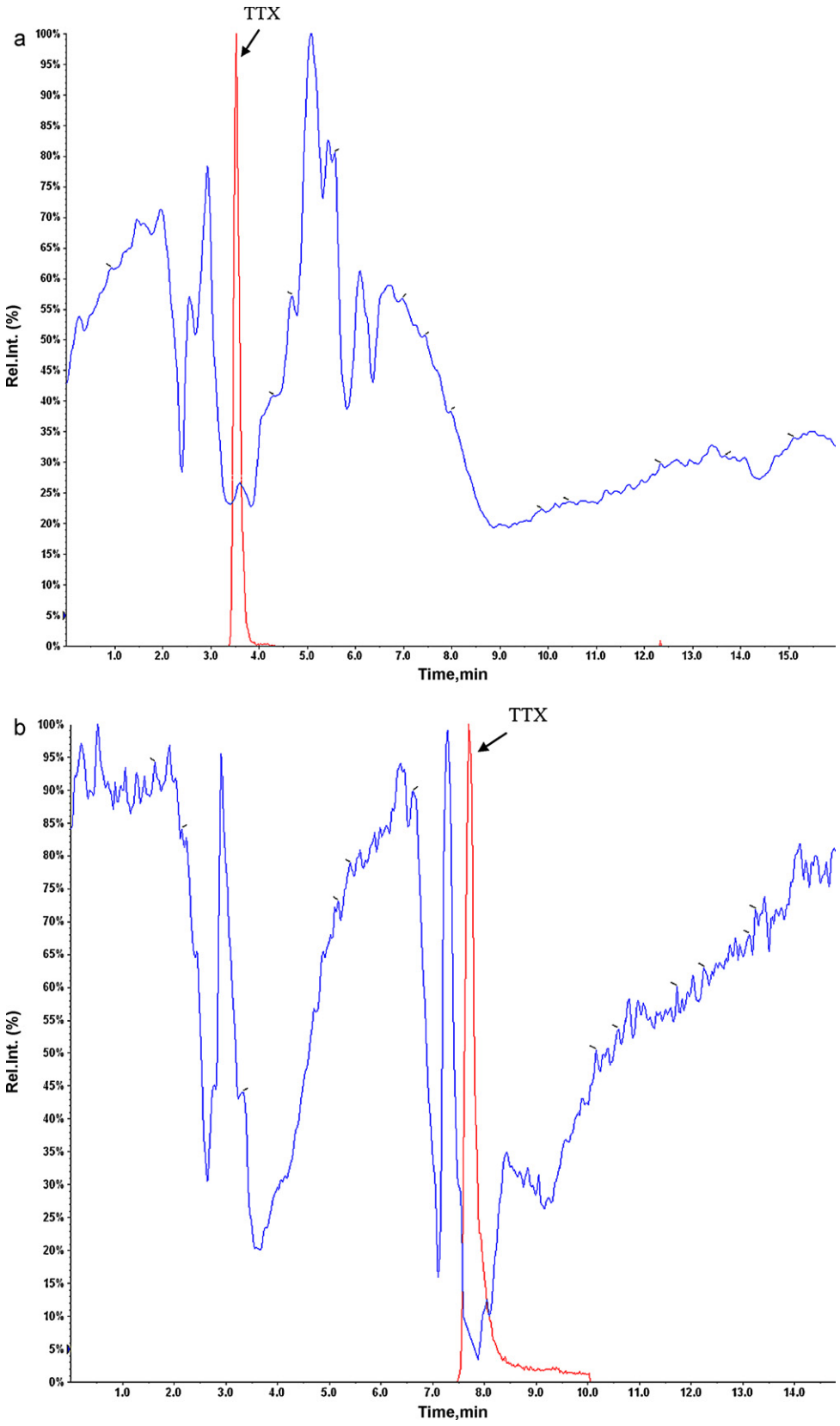

Fig. 1. Post-column infusion study: observed ion suppression even with mobile phase modification. Sample injected: TTX-free urine with single SPE sample preparation. (a) Running condition: HPLC mobile phase: 10 mmol L^{-1} ammonium formate with formic acid [95:5, v/v], with 20 mM heptafluorobutyric acid, 2% acetonitrile. Flow rate = 200 μL min^{-1}. Post-column TTX infusion rate = 10 μL min^{-1}. Retention time of TTX = 3.52 min (overlap chromatogram for easy reference). (b) Running condition: same as (a) except with 2 mM heptafluorobutyric acid. Retention time of TTX: 7.7 min (overlap chromatogram for easy reference).

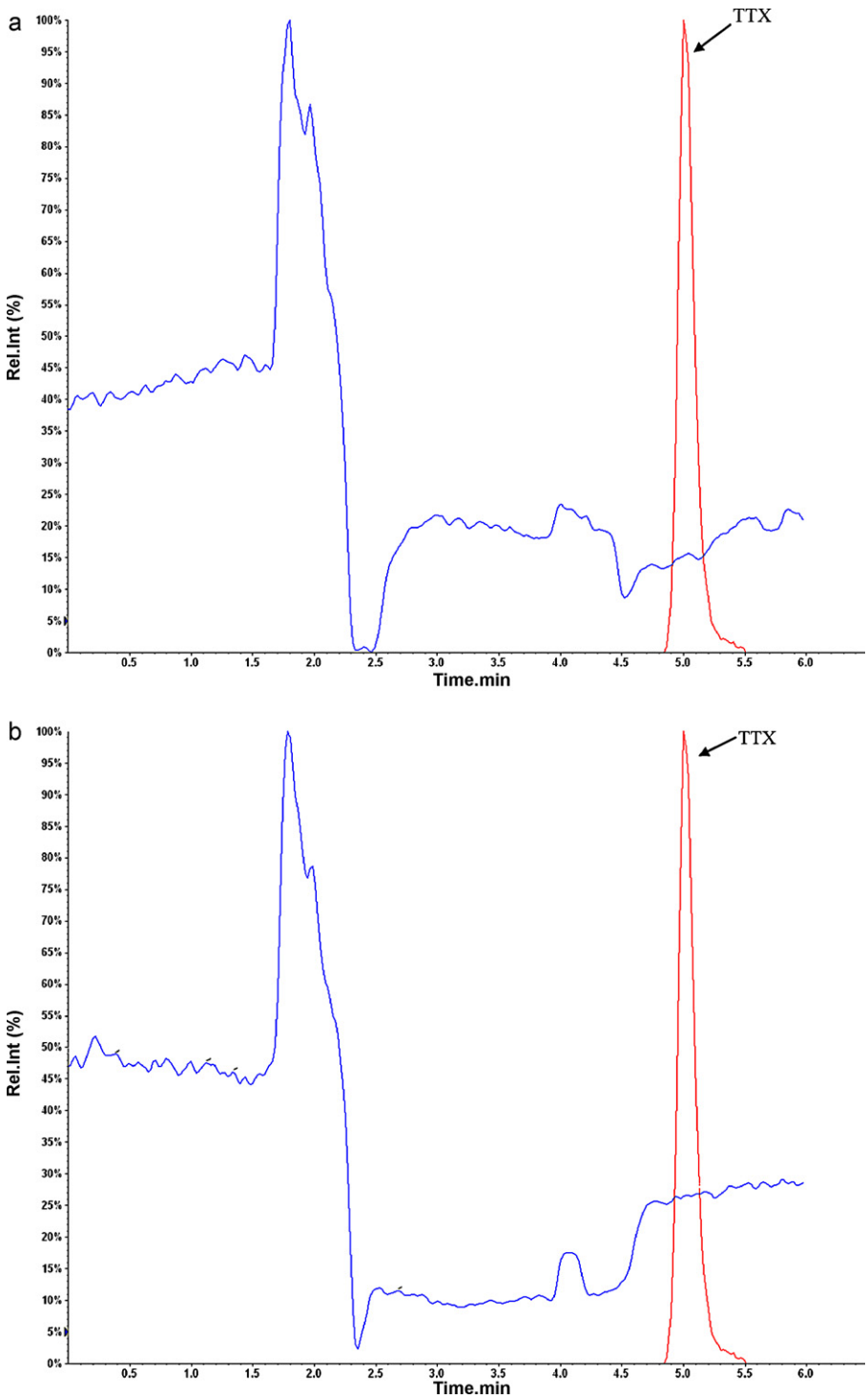

Fig. 2. Post-column infusion study: observed ion suppression in blank urine and plasma with MCX-HILIC sample preparation. Running condition: HPLC mobile phase: 10 mmol L^{-1} ammonium formate with formic acid [95:5, v/v], with 5 mM heptafluorobutyric acid, 2% acetonitrile. Flow rate = 200 μL min^{-1}. Post-column TTX infusion rate = 10 μL min^{-1}. Retention time of TTX = 5.05 min (overlap chromatogram for easy reference). (a) TTX-free urine with MCX-HILIC sample preparation. (b) TTX-free plasma with MCX-HILIC sample preparation.

3.3. Quantitative study of matrix effect

Table 1 summarizes the results of quantitative evaluation of the matrix effect by the post-extraction spike method. The matrix effect calculated for urine ranged from 97 to 103% (plasma, 95 to 104%). Negligible ionization enhancement for urine (103%) and plasma (104%) as well as ionization suppression for urine (97%) and plasma (95%) were encountered. Based on these results, using optimized experimental conditions, the present method was considered practically free from matrix effects [13].

3.4. Recovery

As for the choice of organic solvent to use in mixing with the eluate from C18 cartridge before loading onto the HILIC cartridge, we found that both methanol and acetonitrile gave similar recov-

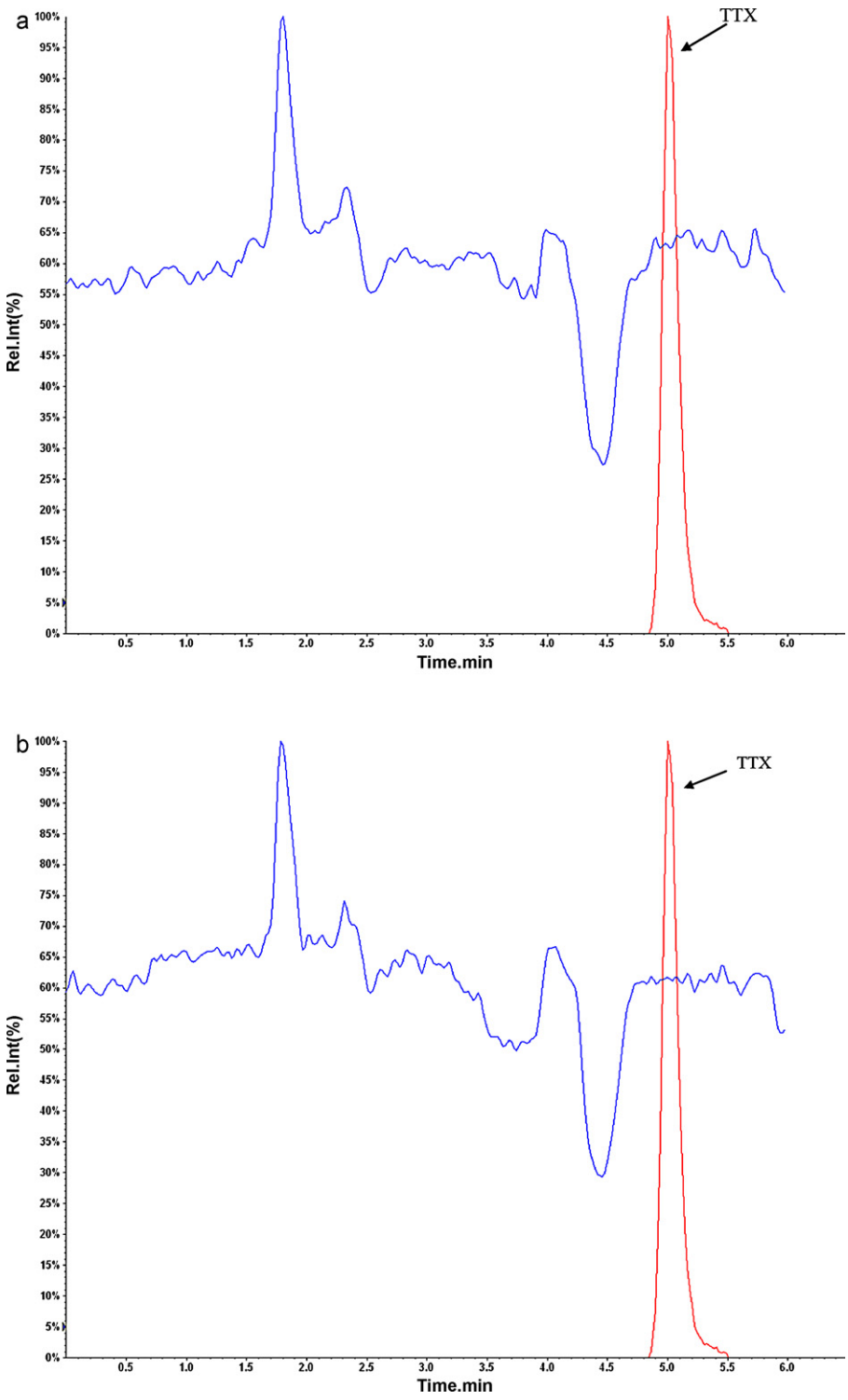

Fig. 3. Post-column infusion study: no ion suppression observed in blank urine and plasma with C18-HILIC sample preparation. Running condition same as in Fig. 2. Retention time of TTX = 5.05 min (overlap chromatogram for easy reference). (a) TTX-free urine with C18-HILIC sample preparation. (b) TTX-free plasma with C18-HILIC sample preparation.

Table 1
Quantitative matrix effect by post-extraction spike method and mean recovery of C18-HILIC SPE for urine and plasma.

	Urine						Plasma					
Spiked TTX (ng mL^{-1})	10	50	100	250	400	500	5	7.5	10	12.5	15	20
Matrix effect (%), $n = 6$	97.0	99.1	97.5	101.3	98.4	103.1	95.6	97.4	102.3	104.0	100.8	99.4
Mean recovery (%), $n = 5$	75.3	78.9	77.5	80.7	79.9	81.3	74.8	76.4	78.6	79.1	79.4	77.3
CV (%) for recovery	7.1	5.4	4.9	3.6	4.1	4.3	9.7	8.3	6.8	7.3	7.1	4.7

Table 2
Precision and accuracy characteristics of LC–MS/MS method for urine and plasma TTX (ng mL^{-1}).

Precision characteristics				
	Urine, $n = 10$		Plasma, $n = 10$	
	Mean	CV (%)	Mean	CV (%)
Inter-assay	48.2	9.1	6.5	14.7
	210.3	6.8	11.3	11.2
	392.7	5.7	15.7	9.5
Intra-assay	48.7	8.2	6.6	13.4
	208.1	5.7	10.8	10.6
	399.8	5.1	15.3	7.7

Accuracy characteristics			
Mean concentration, $n = 5$ (sample: target value)	Accuracy (%)	Mean concentration, $n = 5$ (sample: target value)	Accuracy (%)
48.9 (U1: 50)	97.8	8.1 (QC 1: 7.5)	108.0
413 (U2: 400)	103.3	13.8 (QC 2: 15)	92.0

ery values. In view of the global shortage of acetonitrile, methanol was used in our study. Results also showed that the best HILIC cartridge recovery of TTX was 1:10 (sample:methanol). Subsequent study also indicated that TTX was poorly retained on the HILIC cartridge when non-acidified solutions were used. A 2% acetic acid was found to be the best diluted acid to enhance TTX retention. We also found that recovery of TTX decreased when vacuum suction was used during the HILIC SPE steps, even at values as low as 5 mm Hg. Therefore, it was found necessary to carry out the HILIC SPE steps under gravity flow. With the above optimized conditions, the C18-HILIC SPE gave satisfactory recoveries of 75–81% for urine and plasma matrices (Table 1).

3.5. Linearity, LOD and LOQ

The standard curve of TTX was linear up to 500 ng mL^{-1} for aqueous standards ($y = 1187x - 6112$; $r^2 = 0.9987$). For TTX-spiked urine standards, the assay results curve was linear up to 500 ng mL^{-1} ($y = 326x - 2969$; $r^2 = 0.9967$). For TTX-spiked plasma standards, the curve was linear at least up to 20 ng mL^{-1} ($y = 216x + 2348$; $r^2 = 0.9948$). The LOD was found as 0.13 ng mL^{-1}, comparable with previous works of Jen et al. [10] and Akaki et al. [11]; but generally better than other methods [7,8,14]. The LOQ was 2.5 ng mL^{-1} for both urine and plasma.

3.6. Precision

The inter- and intra-assay precisions for TTX are summarized in Table 2. Inter-assay CVs ranged from 5.7 to 9.1% (intra-assay CVs: 5.1–8.2%) at concentrations ranging from 48 to 400 ng mL^{-1} for urine ($n = 10$); and 9.5 to 14.7% (intra-assay CVs: 7.7–13.4%) at concentrations ranging from 6.5 to 16 ng mL^{-1} for plasma ($n = 10$).

3.7. Accuracy

Accuracy determinations were done using TTX-free urine and plasma with appropriate amounts of TTX added (Table 2). The accuracy of the method was in the range of 92–108%.

3.8. Comparison with other methods

Although other methods have been reported in the literature for detection of TTX in human urine/plasma/blood/serum using LC/MS(/MS), the present method showed a better limit of detection [7,8,14]. Besides, the sample preparation procedure of the present work is comparatively simpler than others [8] in which freeze-drying and ultrafree microcentrifuge filtrations are always needed.

In a relevant work by Jen et al. [10], their method applied only on blood matrix but not for urine. It was likely that severe ion suppression was encountered. In fact, the present sample preparation procedure can be applied to both urine and plasma matrices without significant ion suppression or enhancement. Our modified method uses a minimal amount of organic solvent, and thus can be considered more environmentally friendly as well as more cost-effective. Unlike other similar studies [4,11,14] that adopted gradient elutions which demand chromatographic re-equilibration between runs, our method uses an isocratic elution which markedly reduces analysis time and thus increases sample throughput.

3.9. Patient samples for TTX determination

Table 3 gives a brief summary of TTX levels detected in urine and plasma of patients with TTX poisoning who were admitted to a regional hospital in Hong Kong in past few years.

Using the method described in this study, TTX was detected in all the urine samples but not in any blood plasma sample. This demonstrated that TTX only remains in the blood for a short time, and agrees with observations described in previous works [4,7,8]. In order to eliminate the effect of dehydration and variations in urinary output, creatinine adjustment was made for all urine samples. The creatinine-adjusted TTX concentrations in urine (UC-TTX) (ranging from 7.4 to 41.1 ng μmol^{-1} creatinine) correlate well with the degree of poisoning, as determined from clinical symptoms. The three cases (patients 3–5) with highest UC-TTX concentrations (ranging from 20.1 to 41.1 ng μmol^{-1} creatinine) were found to have liver derangement as evidenced by increases in alanine aminotransferase with levels at 174, 56 and 304 U L^{-1} (reference range: 6–53); and aspartate aminotransferase at 122, 37 and 168 U L^{-1} (reference range: 13–33). Other patients with UC-TTX of less than 20 ng μmol^{-1} creatinine did not present with liver derangement. Among the three cases with the highest UC-TTX concentrations, patients 4 and 5 presented with more severe clinical symptoms including markedly decreased muscle power and generalized muscle weakness, respiratory failure which required assisted ventilation and admission to intensive care unit for management. The patient with the highest UC-TTX (patient 4: 41.1 ng μmol^{-1} creatinine) also presented with a sinus rhythm of ST 1 mm elevation over V2–V4, as shown in an electrocardiogram, suggesting myocardial infarction of the anterior wall.

The severity of symptoms and prognosis generally depends on the amount of toxin ingested, and also can be influenced by

Table 3
Tetrodotoxin concentrations in urine and plasma for 8 patients (all male) with puffer fish poisoning.

Patient	Age	Plasma TTX ($ng\,mL^{-1}$)	Urine TTX ($ng\,mL^{-1}$)	Urine TTX ($ng\,\mu mol^{-1}$ creatinine)	Time of sample collection (post-ingestion hour)	Clinical features
1	37	<2.5	59.3	9.3	8	D, P, MW, SOB
2	54	<2.5	76.8	7.4	8.25	D, P, MW, SOB
3	55	<2.5	109.6	32.7	7.75	D, PO, P, MW, SOB, V, ABD
4-1	40	<2.5	460.5	41.1	6.5	PO, P, DS, MD, AV, ICU, ST
4-2	–	<2.5	351.9	36.9	12.5	–
5-1	39	<2.5	44.8	20.1	6.45	D, P, N, V, DS, MD, AV, ICU
5-2	–	<2.5	17.6	17.8	12.5	–
6	34	<2.5	30.7	18.9	8	D, PO, P, MW, SV
7	34	<2.5	48.2	12.7	9	D, PO, P, MW, SV
8	49	NS	88.2	8.9	5.5	D, P, MW, SOB

Patients 4 and 5: two samples received -1 denoted first sample and -2 denoted second sample.
NS, no sample; D, dizziness; PO, perioral numbness; P, paraesthesia over limbs; MW, mild weakness; SV, self-induced vomiting; V, vomiting; ABD, abdominal pain; DS, drooling of saliva; MD, marked decreased in muscle power; AV, assisted ventilation; ICU, intensive care; ST, ST elevation in electrocardiogram; N, nausea; SOB, short of breath; HD, headache.

the patient's overall health and/or other medical conditions, such as diabetic neuropathy, neural myoinositol and Na–K-adenosine-triphosphatase deficiency [15,16] as well as natural variation in individual sensitivity to the toxin. As urine is the primary route of TTX excretion for humans, renal function would affect the severity of symptoms and should be checked for all patients suspected of TTX poisoning. In this study, all eight patients showed no renal impairment and recovered without complications. For others with renal impairment, lesser amounts of TTX may result in more severe symptoms and may require ICU care even at UC-TTX levels less than 20 $ng\,\mu mol^{-1}$ creatinine.

4. Conclusion

Determination of small polar molecules in biological samples are often challenging due to the lack of retention on conventional reverse-phase columns. The development of the HILIC stationary phase, both as an analytical column or in sample preparation cartridge, has drawn much attention in recent years. Most published applications of HILIC have focused on peptides, carbohydrates and pharmaceuticals [17–23]. Here, we have developed a simple technique for the determination of a toxin, tetrodotoxin, in urine and plasma by using the C18-HILIC SPE LC–MS/MS, and we have used it to confirm TTX poisoning in eight patients who presented to a hospital following ingestion of puffer fish in Hong Kong. To the best of our knowledge, this is one of the first applications to use C18-HILIC as sample pretreatment for the determination of a toxin in biological samples. Another potential application of this method lies on the determination of ciguatoxin in human samples. Ciguatoxin shares some physical properties as TTX that it is very polar and heat resistant, and the extraction from human samples always poses challenge to scientists. Unlike TTX which remains in the blood for a short time, ciguatoxin is measurable in blood up to 3 days [24]. Although the application of HILIC to toxin analysis is still in its infancy, it will likely expand greatly in the future.

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