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Rapid screening and multi-toxin profile confirmation of tetrodotoxins and analogues in human body fluids derived from a puffer fish poisoning incident in New Caledonia

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ABSTRACT

In August 2014, a puffer fish poisoning incidence resulting in one fatality was reported in New Caledonia. Although tetrodotoxin (TTX) intoxication was established from the patients' signs and symptoms, the determination of TTX in the patient's urine, serum or plasma is essential to confirm the clinical diagnosis. To provide a simple cost-effective rapid screening tool for clinical analysis, a maleimide-based enzyme-linked immunosorbent assay (mELISA) adapted for the determination of TTX contents in human body fluids was assessed. The mELISA was applied to the analysis of urine samples from two patients and a response for the presence of TTX and/or structurally similar analogues was detected in all samples. The analysis by LC-MS/MS confirmed the presence of TTX but also TTX analogues (4-*epi*TTX, 4,9-anhydroTTX and 5,6,11-trideoxyTTX) in the urine. A change in the multi-toxin profile in the urine based on time following consumption was observed. LC-MS/MS analysis of serum and plasma samples also revealed the presence of TTX (32.9 ng/mL) and 5,6,11-trideoxyTTX (374.6 ng/mL) in the post-mortem plasma. The results provide for the first time the TTX multi-toxin profile of human samples from a puffer fish intoxication and clearly demonstrate the implication of TTX as the causative agent of the reported intoxication case.

1. Introduction

Tetrodotoxin (TTX) is one of the most potent low-molecular-weight marine neurotoxin, well-known for its distribution in puffer fish, but also present in many other organisms, including amphibians, echinoderms, cephalopods and bivalve molluscs (Noguchi and Arakawa, 2008; Turner et al., 2017). It is believed to be primarily produced by certain marine endosymbiotic bacteria and to accumulate through the food webs and enter into other organisms, eventually reaching humans (Pratheepa and Vasconcelos, 2013). TTX and TTX analogues selectively bind to site one of voltage-gated sodium channels, blocking the influx of sodium ions into the nerve cells and affecting neuromuscular transmission, causing progressive paralysis and even death due to a failure of the respiratory system (Lee and Ruben, 2008).

Puffer fish food poisoning cases have been reported worldwide. A poisoning incidence reported by the Territorial Hospital of New Caledonia in August 2014 resulting from the ingestion of an *Arothron nigropunctatus* puffer fish (Maillaud et al., 2015) is addressed in this work to demonstrate the implication of TTX in this intoxication. At around 6 p.m. on the 27th of August, four men aged 33, 34, 37 and 38 years old, with no known medical history, consumed a boiled fish that the oldest man had captured whilst fishing. The older fisherman ate most of the liver and the gonads in their totality. One of the other men consumed, the flesh in great quantity and, the other man consumed, a small portion of the liver. The fourth and youngest man only ingested a small part of the flesh. Rapidly, the first three consumers presented with gastrointestinal signs such as nausea, vomiting and diffuse abdominal pain, together with neurological signs such as peribuccal, facial and

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extremal paraesthesia and ataxia-like sensations. The three men lay down and fell asleep. On the morning of the 28th of August, the fisherman was found deceased in his bed and the other two men showed regressed digestive signs and appearance of tetraparesis. The fourth consumer returned home with no signs of intoxication. The two men that presented with neurological signs were hospitalised, one patient in the intensive care unit (Pt#1) and the other one (Pt#2) in the short stay hospitalisation unit of the Territorial Hospital of New Caledonia. The neurological symptoms with associating tetraparesis, tetraparesthesis, ataxia and deep sensitivity disturbance dissipated after 48 h and disappeared in a few days.

Although the causative agent of the food poisoning was established from the patients' symptoms and the identification of the species responsible through the photos of the fish provided by the medical team to one of the patients, the puffer fish could not be analysed for the identification of the toxin involved in this intoxication case. Thus, the detection of TTXs in clinical samples of the poisoned patients can be essential to confirm TTX intoxication. As TTXs are very polar compounds, the ingested toxins are mainly eliminated in urine. Previous studies have indicated that TTXs only remain in blood for a matter of hours (less than 24 h), but they can be found in urine even on day 4 after ingestion (O'Leary et al., 2004).

Chromatography-based methods have been to date the most widely used techniques for the determination of TTXs in the urine and blood of patients from poisoning incidents. However, determining TTX intoxication from body fluids faces certain challenges. First, unlike in food, the amount of toxin in the urine and blood of a patient who has been intoxicated is typically low; therefore, a sensitive method is required. Secondly, because TTXs are very polar compounds, there will be low retention on conventional reverse-phase columns; thus, the use of another type of analytical column or modification of the mobile phase composition is needed. Third, endogenous metabolites of the biological samples may lead to matrix effects. Ion suppression is a big challenge in mass spectrometry (MS) analysis, requiring efficient clean-up procedures before sample analysis (Leung et al., 2011).

Enzyme-linked immunosorbent assays (ELISAs) are rapid, sensitive, cost-efficient and easy-to-use methods of analysis that do not require sophisticated instrumentation and highly trained personnel. The use of ELISAs as alternative or complementary to conventional methods for the determination of TTXs in biological samples can provide expedient solutions for rapid and reliable diagnosis in food poisoning incidences, and favours their implementation at hospitals where patients are treated. A maleimide-based ELISA (mELISA) for the detection of TTXs in mussels and oysters was recently developed at IRTA (Reverté et al., 2018). The TTX was immobilised on self-assembled cysteamine in a stable, ordered and optimally-oriented way that provided a long-term stability for storage of the modified microtiter plates, allowing the assay to be performed in less than 2 h with the use of these pre-coated plates. To the best of our knowledge, only one work has used an immunoassay for the analysis of human body fluids for TTX monitoring, but no details are provided on the method or its performance (Islam et al., 2011). Here, the application of the mELISA to the screening of TTXs in clinical samples was assessed and fully characterised by the analysis of blank and TTX-spiked samples. Moreover, urine samples from the two intoxicated patients were analysed and the multi-toxin profile (TTX and TTX analogues) in urine, serum and plasma samples was evaluated by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The results obtained by both techniques were compared, demonstrating the applicability of the immunoassay and the complementarity of the techniques based on different recognition principles.

2. Materials and methods

2.1. Reagents and materials

Pure TTX standard was purchased from Tocris Bioscience (Bristol, UK) and standard solutions were prepared at 1 mg/mL in 10 mM acetic acid (AA). The anti-TTX monoclonal antibody (mAb) TX-7F was produced as described in Kawatsu et al. (1997). Pierce maleimide-activated plates were obtained from Thermo Fisher Scientific (Madrid, Spain). Cysteamine hydrochloride, formaldehyde solution, anti-mouse IgG (whole molecule)-horseradish peroxidase antibody produced in rabbit (IgG-HRP), bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), 4-morpholineethanesulfonic acid (MES) hydrate, potassium phosphate dibasic, potassium phosphate monobasic and 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate, ammonium hydroxide solution (NH_4OH, 25%), ammonium acetate and amorphous graphitized polymer carbon Supelco ENVI-Carb 250 mg/3 mL cartridges were supplied by Sigma-Aldrich (Tres Cantos, Spain). HPLC-grade acetonitrile (ACN), glacial acetic acid (AA) and methanol (MeOH) were obtained from Chem-lab (Zedelgem, Belgium). Ultrapure Milli-Q water (18.2 MΩ/cm^2) was used for the preparation of solutions (Millipore Iberica Ltd., Madrid, Spain).

2.2. Human samples

Urine, serum and plasma samples were collected and stored at −20 °C until their analysis (Table 1). Urine and serum samples from patient 1 (Pt#1) were collected the 28th and 29th of August 2014, coinciding with ~17 h and ~45 h after the ingestion of the boiled puffer fish; urine and serum samples from patient 2 (Pt#2) were taken only the second day of hospitalisation, approximately 42 h after the ingestion of the fish. Three plasma samples were collected from Pt#1: the first one was collected at the moment of hospitalisation, ~17 h after the puffer fish consumption; two additional samples were collected ~38 and ~43 h after the ingestion; only one plasma sample was collected from Pt#2, ~23 h after the fish consumption. A post-mortem plasma sample was collected from the fisherman, ~17 h after the puffer fish ingestion.

The creatinine concentration in urine samples was determined by Echevarne Laboratory (Barcelona, Spain) using Jaffe's reaction.

Table 1
Human body fluid samples collected from the two hospitalised patients and the deceased fisherman.

	Date	Time (hours after ingestion)	Samples
Pt#1	28/08/14	17 h	Urine, serum and heparinized plasma
	29/08/14	38 h	Heparinized plasma
	29/08/14	43 h	Heparinized plasma
	29/08/14	45 h	Urine and serum
Pt#2	28/08/14	23 h	Heparinized plasma
	29/08/14	42 h	Urine and serum
Fisherman	28/08/14	17 h	Post-mortem plasma (with fluoride)