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First Toxicity Report of Tetrodotoxin and 5,6,11-TrideoxyTTX in the Trumpet Shell *Charonia lampas lampas* in Europe

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Tetrodotoxin (TTX) is one of the most potent toxins already isolated, which occurs in a wide variety of animals. In this work, the occurrence of TTX and analogues was examined using mass spectrometry, confocal microscopy, liquid chromatography−mass spectrometry (LC−MS), and mouse bioassay in a trumpet shell (*Charonia lampas lampas*) and in the fluids of a patient poisoned by consuming this shell. Retention time data in the LC−MS system within the enhanced mass spectrum (EMS) mode indicated the presence of TTX and the analogue 5,6,11-trideoxyTTX; the enhanced product ion (EPI) mode confirmed the existence of both toxins with the formation of characteristic daughter ions from the fragment pattern of each molecule. TTX and 5,6,11-trideoxyTTX were only detected in the digestive gland of the trumpet shell and also in the urine and serum of the patient. The concentration of 5,6,11-trideoxyTTX checked in the samples by LC−MS was 3 times higher than TTX. However, the results obtained by mouse bioassay showed that the analogue is much less toxic than TTX. In vitro toxicity was checked using cerebellar cells; in these experiments the trumpet shell sample showed high toxicity, but the level was lower than in vivo results probably due to some competition between analogues. This paper shows for first time the presence and toxicity of TTX and 5,6,11-trideoxyTTX in a trumpet shell collected in the European coasts. The LC−MS method is a useful tool to confirm the presence of TTX and the further identification of TTX analogues.

Tetrodotoxin (TTX) is one of the most potent neurotoxins and is known to block sodium ion channels responsible for nerve and muscle excitability.[1] Its molecule has six hydroxyl residues at the C-4, C-6, C-8, C-9, C-10, and C-11 positions in addition to a guanidinium group (Figure 1A), which is positively charged in the biological pH range. Although the hydroxyls at C-9 and C-10 are the most important, those at C-4, C-6, and C-11 also make significant contributions to the binding to the channel as hydrogen bond donors.[2]

TTX usually exists as a mixture of its analogues (TTXs) in puffer and other tetraodontiforme fish[3] and causes paralytic poisoning and occasionally death in humans through ingestion.[1,4] The symptoms included numbness of the tongue, lips, paresthesia of the face and limbs, followed by a sense of light-headedness and floating, head- and stomachache, nausea, diarrhea, and vomiting. In severe cases, there is unconsciousness, respiratory paralysis, and convulsions.[5]

Many cases of TTX-food poisoning are reported in Southeastern Asia and, more specifically, Japan.[6] In addition to Japan, Taiwan, and Thailand, intoxication has also been reported from the South Pacific, Malaysia, Hong Kong, Singapore, Australia, Madagascar, China, and Bangladesh.[6] Since the description of TTX, several studies revealed its wide distribution in terrestrial as well as marine animals including the Anuran family *Brachycephalidae*,[7] newts *Notophathalmus viridescens*,[8] *Triturus* spp.,[9] goby *Gobius criniger*, xanthid crab *Atergatis floridus*, blue-ringed octopus *Octopus maculosus*, chaetognatha, starfish *Astropecten polycanthus* and *A. scoparius*, four species of gastropods *Zeuxis siquijorensis*, *Babylonia japonica*, *Tutufa lissostoma*, and *Niotha clathrata*, three species of nemerteans *Tubulanus punctatus*, *Lineus fuscoviridis*, and *Cephalothrix lineariz*, and the trumpet shellfish *Charonia sauliae*.[10]

Unlike the rest of biotoxins that accumulate in fishery products, the TTX is not produced by microalgae. Symbiotic bacteria have been suggested to be involved in TTX genesis for marine animals[9]

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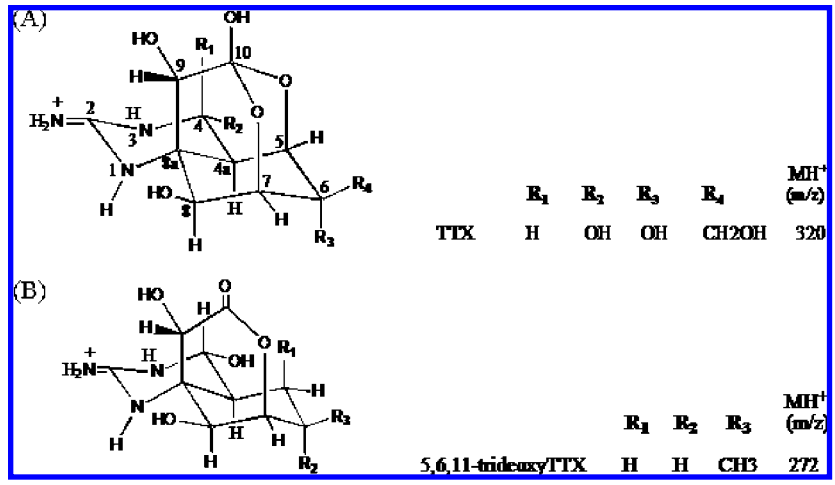

Figure 1. Chemical structures of TTX (A) and 5,6,11-trideoxyTTX (B).

and specifically have involved *Shewanella algae*, *S. putrefaciens*, *Vibrio* sp., *Pseudomonas* sp., and *Alteromonas tetraodonis* that accumulated in the subcutaneous mucus, or in the intestine, releasing the TTX,[11,12] which were later confirmed by the isolation of TTX-producing bacteria from different TTX-bearing animals.[10]

A food poisoning incident resulting from the ingestion of a trumpet shell of the species *Charonia lampas lampas*, involved a single person (49 year old man) in October 2007 (paper submitted). The symptoms began minutes after ingestion of the mollusk; symptoms included abdominal pain with nausea and vomiting, weakness, difficulty articulating words and keeping the eyelids open, and difficulty breathing. After 72 h, the symptoms were fully reversed. These symptoms were similar to those of TTX poisoning.

The species *Charonia lampas* (family: *Ranellidae*) is typical of the Western Mediterranean and the northeast Atlantic being very frequent in the Sea of Alborán (westernmost portion of the Mediterranean Sea, between Spain and Morocco). As a result of climate change and rising water temperatures, exotic species from the Red Sea or tropical habitats are colonizing the Mediterranean, and it is possible that TTX-bearing animals infected by TTX-producing bacteria have contaminated the species *Charonia lampas lampas* through the food chain.[13]

The identification and quantification of TTX and TTXs are important not only for food hygiene but also for the study of the biosynthetic pathway of TTX which has not yet been clarified.[14] In the present study, we report analytical and biological data on the presence of the TTX and 5,6,11-trideoxyTTX in the trumpet shell *Charonia lampas lampas* implicated in the human poisoning and the presence of these toxins in the urine and blood following the intoxication.

MATERIALS AND METHODS

Materials. TTX used as a calibrant was from Calbiochem Corporation. The HPLC grade methanol, acetonitrile, and acetic acid were from Panreac (Spain). Trimethylamine (25%) solution in water and ammonium formate were from Sigma (Spain). Formic acid was obtained from Merck (Spain).

The trumpet shell (24 cm shell length) was from the species *Charonia lampas lampas*, purchased in a Malaga market. Its harvest place was later traced back to the south coast of Portugal. The samples corresponding to the trumpet shell were sent from the Public Health Laboratory of Malaga.

Urine, blood, and serum samples were collected from the patient and frozen at −20 °C until the analysis was carried out. These samples were obtained to identify and describe the clinical case by Mr. Juan Francisco Fernández Ortega (paper submitted).

Samples of *Charonia lampas* and *Murex trunculus* were later collected at the same time from the Algarve offshore coast and Riá Formosa, respectively, at the south coast of Portugal. The samples were provided by the National Reference Laboratory of Marine Biotoxins (INRB/IPIMAR).

Sample Preparation of Trumpet Shell. The trumpet shell *Charonia lampas lampas* was boiled and then was divided into two parts: digestive gland (sample 1) and remaining tissues (sample 2). Both samples were extracted according to the official procedure for PSP (paralytic shellfish poisoning). Sample 1 was formed by a residue with pasty consistency and dark color that was the visceral mass at the end of the trumpet shell. In this sample (3 g) an initial 1/10 dilution to pH 2, adjusting the final volume to 30 mL with hydrochloric acid 0.1 M. Sample 2 was constituted of the meat of the trumpet shell after being crushed and homogenized. After homogenization, the sample (50 g) was extracted with 200 mL of hydrochloric acid to pH 3. An aliquot of 200 μL of each sample was vaccuum-dried and dissolved in 1000 μL of methanol. Aliquots were filtered through an Ultrafree-MC centrifugal filter (Millipore Corporation) and then analyzed.

The other samples of *Charonia lampas* and *Murex trunculus* were boiled for 15 min and then were dissected in visceral mass and remaining tissues. The samples (3−5 g) were extracted with 1% acetic acid and analyzed by LC−MS.

Biological Samples Preparation. The sample cleanup procedure used was developed before for TTX and saxitoxin analysis of biological samples.[4] Each sample of urine, blood, and serum (1.0 mL) was mixed with 500 μL of 0.5 M acetic acid. The samples were immediately centrifuged at 10 000*g* for 10 min. The super-

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natant was passed through a cartridge column (Supelclean LC-18 cartridges; Supelco) preconditioned with 10 mL of methanol followed by 10 mL of water. After the sample was applied into the cartridge, it was eluted with 10 mL of acetic acid (0.3%). The eluant was freeze-dried, dissolved in 2 mL of acetic acid (0.3%), and filtered through a 10 000 NMWL cutoff Ultrafree-CL centrifugal filter (Millipore Corporation). The filtrate was freeze-dried and dissolved in 250 μL of methanol and analyzed by LC−MS.

Confocal Microscopy Analysis. *Cell Cultures.* Primary cultures of CGC were obtained from cerebella of 7-day-old mice following previously described methods.[15,16] In brief, cells were dissociated by mild trypsinization with trypsin from bovine pancreas (0.002% w/v) at 37 °C, followed by trituration in a DNase solution (0.004% w/v) containing a soybean trypsin inhibitor (0.05% w/v). The cells were suspended in DMEM containing 25 mM KCl, 31 mM glucose, and 0.2 mM glutamine supplemented with *p*-amino benzoate (0.1% w/v), insulin (0.04% w/v), penicillin (0.03% w/v), and 10% fetal calf serum. The cell suspension was seeded in glass coverslips precoated with poly-L-lysine and incubated in 6-multiwell plates for 7−11 days in a humidified 5% CO_2/95% air atmosphere at 37 °C. Cytosine arabinoside, 20 μM, was added before 48 h in culture to prevent glial proliferation.

Determination of Plasma Membrane Potential (E_m). The coverslips plated with CGC were transferred to custom-made recording chambers, in which the cells were loaded with 20 nM [$DIBAC_4(3)$], to monitor the E_m.[17,18] In order to calculate the ability of different TTX concentrations and the TTX-containing samples to block voltage-activated sodium channels, the intensity of the fluorescence was evaluated after exposure of the neurons to veratridine alone (control values) or to veratridine after preincubation with different concentrations of TTX or the samples of trumpet shell (samples 1 and 2) evaluated in this work.

The $DIBAC_4(3)$ fluorescence was monitored using a Nikon C1 confocal microscope and Nikon Plan Apo Tirf 60×, NA 1.45 objective (Nikon, Melville, NY). The images of fluorescence emitted at 515 nm (after excitation at 488 nm) were collected every 5 s. The fluorescence intensities, measured in selected regions of interest, were analyzed off-line using the Nikon EZ-C1 viewer software.

High-Performance Liquid Chromatography−Mass Spectrometry (LC−MS) Analysis. The high-performance liquid chromatography (LC) equipment was formed by a binary system of LC-10ADVP pumps, an autoinjector (SIL-10ADvp) with refrigerated rack, degasser, column oven, and the system controller from Shimadzu (Japan). This system was coupled to a mass spectrometer (MS) QTRAP-2000 instrument from Applied Biosystems, which consists of a hybrid quadrupole-linear ion trap mass spectrometer equipped with an electrospray (ESI) source. The nitrogen generator is a Nitrocraft $NC_{LC/MS}$ from Air Liquide (Spain). The separation and identification of toxin was achieved in a Zorbax 300SB-C3 column (i.d. 4.6 mm × 150 mm) inside the column oven at 25 °C. The injection volume was 5 μL. The mobile phase for analysis was 1% acetonitrile, 10 mM trimethylamine (TMA), and 10 mM ammonia formate (pH 4.0 with formic acid) at an isocratic flow rate 0.4 mL/min. Analyst software was used for instrument control as well as data processing and analysis.

Extracts were analyzed with the ESI interface operating in the positive ion mode using the following parameters: curtain gas, 25; CAD gas, 6; IonSpray voltage, 4000 V; temperature, 500 °C; gas 1, 50; gas 2, 50; these parameters had been previously optimized using the toxin standard. The mass spectrometer was operated in the enhanced mass spectrum (EMS) mode to confirm the presence of TTX and in the enhanced product ion (EPI) mode to quantify the toxin. For EPI positive, the transitions selected were TTX, 320 → 302/320 → 162 (m/z range 2 amu).

Mouse Toxicity Assay. The lethal potency of samples of the trumpet shell was estimated by intraperitoneal (i.p.) injection of the extract or TTX standard into mice (19−23 g of body weight). The toxicity was determined by the time of death of six mice, according to the standard dose−lethal time plot prepared by using the commercial TTX. The amount of toxin was expressed in mouse units (MU, mean ± SD); one MU is defined as the amount of toxin required to kill an 18−23 g ICR mouse in 7−15 min after i.p. administration.[5,10]

RESULTS

The trumpet shell *Charonia lampas lampas* involved in the poisoning episode was purchased in the Malaga market, and its collection later traced back to the south coast of Portugal. It was sent to the Public Health Laboratory of Malaga, and there the trumpet shell was dissected into the digestive gland (sample 1) and the remaining tissues (sample 2). These samples were initially analyzed by mouse bioassay according to PSP protocol, as TTX was never reported in Europe before. The PSP concentration value obtained in sample 2 was 151 μg of saxitoxin equiv/100 g of flesh. Sample 1 checked by bioassay showed a very high PSP concentration (25 500 μg of saxitoxin equiv /100 g). To be able to quantify the samples, the extract was diluted 1/100 with buffered saline solution to pH 3.0. The time of death of the mice was approximately 7 min 10 s and 7 min 15 s, which indicated a very high toxicity.

Subsequently both samples were analyzed by the Lawrence[19] and Oshima[20] methods in the Community Reference Laboratory of Marine Biotoxins-CRLMB of Vigo and in the Department of Pharmacology of Lugo, respectively. The analyses indicated a total absence of PSP toxins by both methods. Because of the toxicity observed in the mouse bioassay and the patient symptoms, it was suspected that the samples could have TTX.

Samples of *Charonia lampas* and *Murex trunculus* collected from the south coast of Portugal were also analyzed by the Lawrence method[19] at IPIMAR laboratory, and PSP toxins were not detected although some traces appeared at the limit of detection.

Then, all samples were analyzed by mass spectrometry to check for the presence of TTX. The TTX standard solutions and samples of trumpet shell were dissolved in methanol and then

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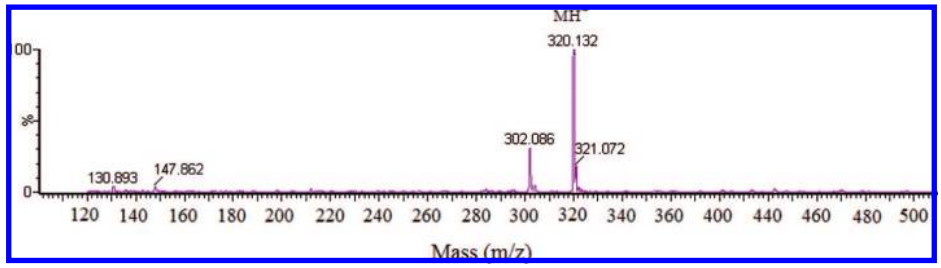

Figure 2. Mass spectrometry analysis by infusion of TTX standard.

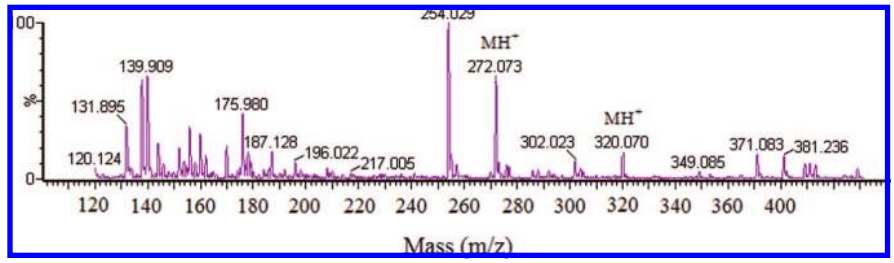

Figure 3. Mass spectrometry analysis by infusion of sample 1; the protonated molecular ion MH^+ of TTX was *m/z* 320 and of 5,6,11-trideoxyTTX was *m/z* 272.

directly injected into the MS system by infusion. A TTX standard was first injected, and a peak at *m/z* 320 was observed (Figure 2). The injection of a sample 1 extract provided two peaks, one at *m/z* 320 that corresponds to TTX and another peak at *m/z* 272 that corresponds to the analogue 5,6,11-trideoxyTTX[14] (Figure 3). However when sample 2 was injected, TTX and 5,6,11-trideoxyTTX were not detected.

Once we have confirmed the presence of TTX in the *Charonia* sample, the amount of toxin was quantified by LC−MS. The molecular ions corresponding to the $(M + H)^+$ ions of TTX (*m/z* 320) and 5,6,11-trideoxyTTX (*m/z* 272) were detected in the EMS and EPI mode by LC−MS. With the TTX standard, a calibration curve with six concentrations of TTX (10−1000 ng/mL) versus the EPI signal was done to transform the signal obtained in each sample to TTX concentration. Figure 4A shows the LC−MS chromatogram of a TTX standard, with only one peak at the retention time of 4.5 min. The mass spectrum of this peak is shown in Figure 4B where a high peak with *m/z* 320.1 is observed corresponding to the mass of TTX. Then this injection was repeated in the EPI mode; as shown in parts C and D of Figure 4 (LC−MS/MS chromatogram) with the formation of characteristic daughter ions 302.1, 284.3, 256.1, 178.0, and 162.1. In the same conditions, the samples of trumpet shell and the samples of urine and blood were analyzed. The LC−MS/MS chromatogram of sample 1, Figure 5A, shows a peak at retention time 4.5 min that corresponds to TTX; the mass spectrum of this peak, Figure 5B, shows the parent peak at *m/z* 320 and the daughter peaks *m/z* 302 and 162 indicating the presence of TTX. Figure 5C shows a peak at retention time 5.6 min that corresponds to 5,6,11-trideoxyTTX; the mass spectrum of this peak, Figure 5D, shows the parent peak at *m/z* 272 and the daughter peaks *m/z* 254 and 162 confirmed the presence of 5,6,11-trideoxyTTX. The same spectra were obtained after urine injection, Figures 6A−D. In addition, when the blood serum was injected, the same peaks were detected even though the signal was close to the detection limit. On the contrary, in sample 2, both toxins were again not detected. Samples of *Charonia lampas* and *Murex trunculus* collected by IPIMAR were also analyzed at the same conditions, but the analysis indicated total absence of TTX and TTXs.

The results obtained by LC−MS were transformed into the amount of TTX by using a calibration curve done with the transitions of the daughters ions (*m/z* 302 and 162) obtained in the EPI mode. Therefore, the amount of TTX in the samples calculated with these transitions was 31.5 mg/100 g in sample 1, 211.1 ng/mL in the urine sample, and 26.4 ng/mL in the serum sample. The quantification of 5,6,11-trideoxyTTX was calculated using the TTX pattern as a standard due to the unavailability of standards for this compound and assuming a relative response factor of 1:1. Since the analogue has different transitions than that of TTX, its quantification was done in the EMS mode with reference to the TTX peak. At these conditions, the amount of 5,6,11-trideoxyTTX was 100.4 mg/100 g in sample 1, 692.1 ng/mL in the urine sample, and 86.5 ng/mL in the serum sample. These results are shown in the Table 1.

In order to quantify the toxicity of the samples by a nonanimal assay, samples 1 and 2 from *Charonia lampas lampas* were analyzed by an in vitro functional assay. The purpose was to quantify the presence of TTX by a rapid and sensitive in vitro model previously employed to detect the presence of paralytic shellfish toxins[21] by measuring the ability of these types of toxins to block the veratridine-induced changes in membrane potential in primary cultured neurons. Veratridine is known to depolarize excitable cells by opening the voltage-dependent sodium channels and blocking its inactivation whereas TTX toxins block neuronal transmission through inhibition of voltage-gated Na^+ channels. Hence, the addition of veratridine (50 μM) to excitable cells increases $DIBAC_4(3)$ fluorescence and preincubation of the cells with TTX before addition of veratridine reduces the veratridine-induced depolarization. Figure 7 shows that TTX standard blocked the veratridine-induced depolarization with an IC50 of 4.7×10^{-9} M (95% confidence intervals from 3.2 to 6.8×10^{-9}M). In this in vitro model, sample 1 inhibited the VTD-induced depolarization

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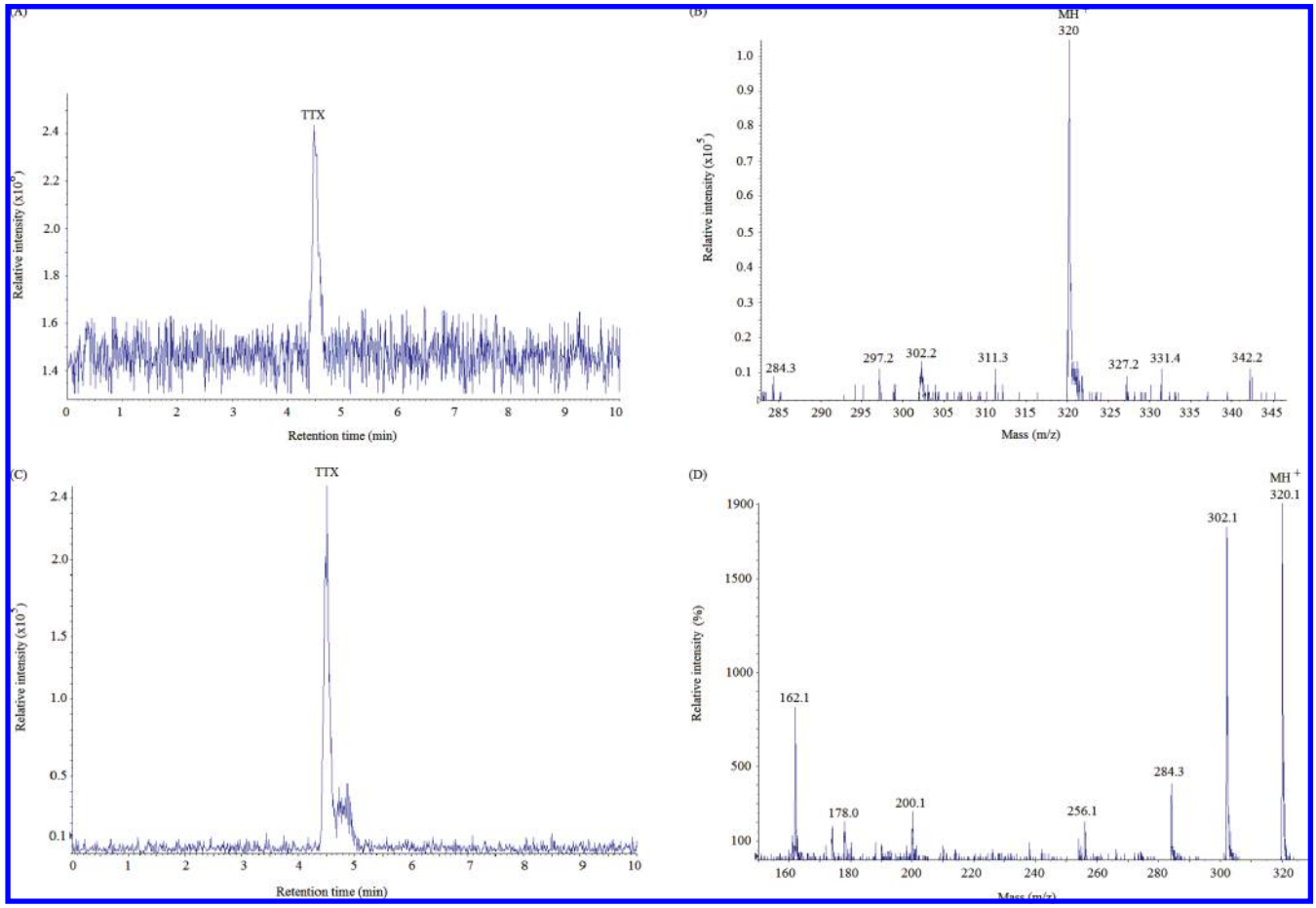

Figure 4. LC−MS analysis of a TTX standard. (A) LC−MS chromatogram of TTX obtained in the EMS mode. (B) LC−MS mass spectrum of TTX from part A. (C) LC−MS/MS chromatogram of TTX obtained in the EPI mode. (D) LC−MS/MS mass spectrum of TTX from part C.

by 33.3 ± 7.2%, which will be equivalent to the presence of approximately 10 nM TTX in the diluted sample, therefore equivalent to 3.2 mg in 100 g of tissue; whereas sample 2 did not show any effect on the veratridine-induced depolarization.

Finally, the toxicity of the samples was also determined by mouse bioassay. The equivalence was determined in μg/mL of TTX, in MU. Six mice injected i.p. with the extract of trumpet shell (1 mL/mouse) showed typical symptoms similar to those described for TTX and their analogue toxins in mice (paralysis of the hind legs and brief dyspnea), and in approximately 7 min they died. The TTX standard was injected, and the concentration of TTX in MU was equal to 0.355 ± 0.03 μg/mL of TTX. Then sample 1 was diluted 1:100, and a volume of 1 mL was injected into mice; the toxicity determined by the mouse bioassay was 1.53 MU. The concentration of toxin by LC−MS in this volume was 0.380 μg/mL of TTX and 1.213 μg/mL of 5,6,11-trideoxyTTX; 0.380 μg/mL of TTX produces 1.07 MU, therefore, the toxic value attributed to the analogue is 0.460 MU. These results are shown in Figure 8. The mouse bioassay evidences that the analogue is less toxic than TTX, and therefore it would require a concentration 3 times higher than that of 5,6,11-trideoxyTTX to show half of the toxicity of TTX.

DISCUSSION

LC−MS is a known and trusted technology to detect and quantify toxins in any biological sample.[6] With the use of this technique, high amounts of TTX and 5,6,11-trideoxyTTX were detected in both the digestive gland of the trumpet shell and in the patient body fluids. The percentage of toxin in urine versus blood after 33 h (paper submitted) was 90%. Similar results and technology were reported in other TTX human intoxication after fish consumption.[4] The rapid recuperation of the patient is more reliable with the elimination of TTX in urine, indicating that TTX is redistributed from the serum to its target sites at sodium channels.[22] Urine is the major excretion route of TTX intoxication and of PSP intoxication for human beings.[23]

From the results in the present study, it is suggested that the combination of a protocol that includes precleanup of the samples with LC−MS analysis is very useful in detecting TTX from urine samples of poisoned patients for diagnosis of TTX-food poisoning, as it had been previously reported for saxitoxin.[4]

The analysis done by confocal microscopy on plasma membrane potential changes indicated an amount of 3.2 mg of TTX/100 g of tissue. The fact that sample 1 shows a lower toxicity by this analysis than those obtained by LC−MS and mouse bioassay could be because the cell assay is underestimating the amount of toxin found in the sample, due to limitations of the fluorescent probe. However, another possibility is that 5,6,11-trideoxyTTX may

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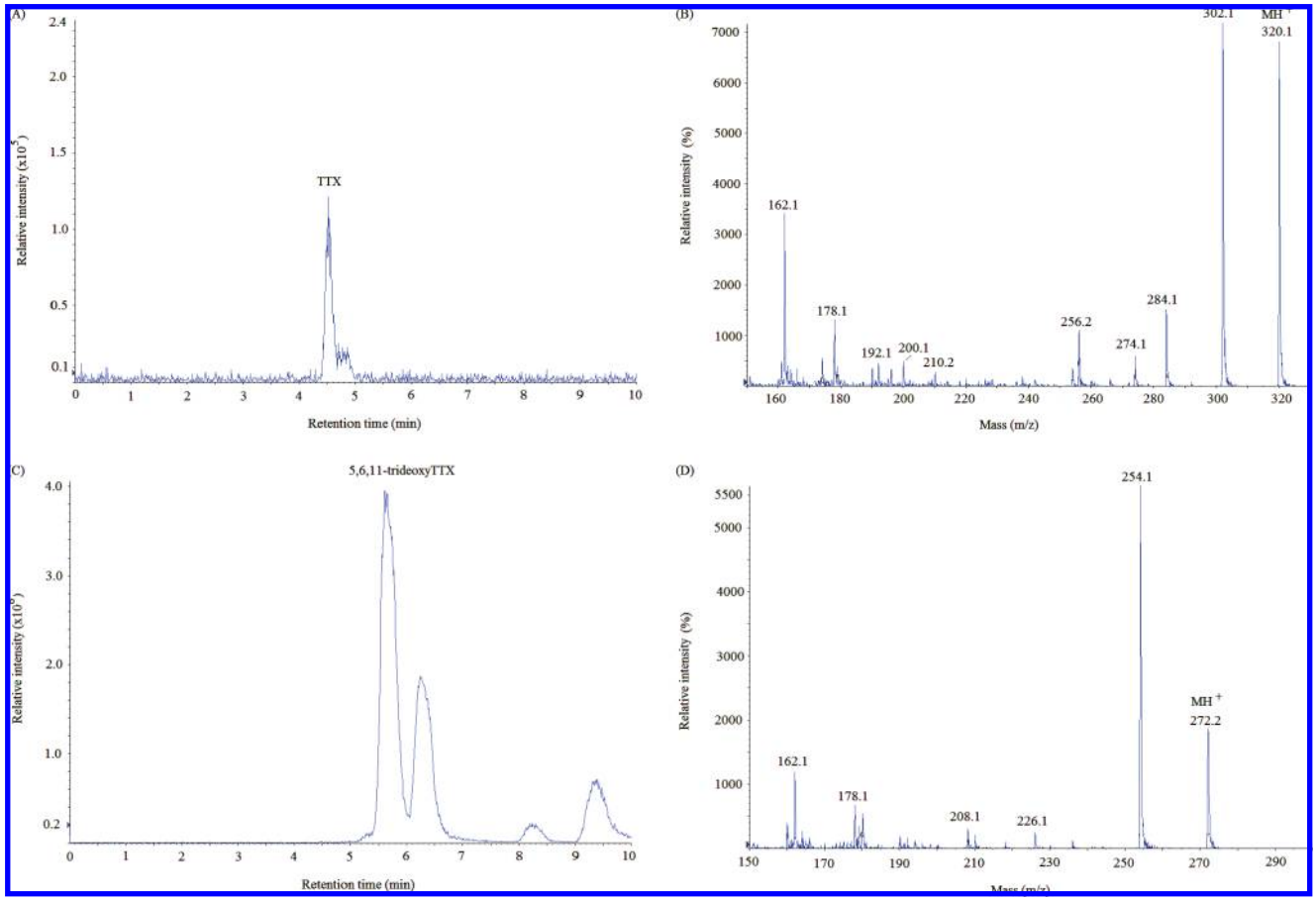

Figure 5. LC−MS/MS analysis in the EPI mode of sample 1. (A) LC−MS/MS chromatogram of sample for *m/z* 320. (B) LC−MS/MS mass spectrum from part A. (C) LC−MS/MS chromatogram of sample for *m/z* 272. (D) LC−MS/MS mass spectrum from part C.

be a partial agonist to TTX on the binding to the sodium channel, which induces a loss of TTX effect on the intracellular target. Also in the in vitro analysis we are isolating cerebellar cells, while in the mouse bioassay the whole body can be a target for TTX, so it is difficult to get similar results of toxicity by both methods. It is important to highlight the fact this paper reports for the first time this membrane potential assay as a nonanimal alternative method to detect TTX.

In the analysis carried out by LC−MS; the fragment ions at *m/z* 302 and 284 of TTX obtained by LC−MS/MS were due to elimination of one or two water molecules, respectively, but the ion at *m/z* 256 probably generated with the loss of 28 mass units from the ions of $[MH - H_2O]^+$ or $[MH - 2H_2O]^+$, could be accounted for by elimination of CO at C-10 as the result of the cleavage of the bonds between C-9 and C-10, C-10 and C-5−O, and C-10 and C-7−O, due to the α-hydroxy hemilactal structure.[14] The fragment ion at *m/z* 254 of 5,6,11-trideoxyTTX is due to the loss of one water molecule. In contrast, the specific fragment ions at *m/z* 162 and 178 that appeared on the spectra of TTX and 5,6,11-trideoxyTTX can be interpreted as 2-aminohydroxiquinazolines and 2-aminodihydroxyquinazolines, respectively (Figure 9). These structures probably are due to bond cleavage between C-8a and C-9, and between C-6 and C-11.[7,14]

TTX and 5,6,11-trideoxyTTX were detected in the digestive gland of the trumpet shell *Charonia lampas lamas*; however, both toxins were not detected in the others tissues of the trumpet shell. This agrees with previous results in other study where TTX was only detected in the digestive gland of the species *Charonia sauliae*.[11]

In the *Charonia lampas* sample collected by IPIMAR at the south coast of Portugal and unrelated to the poisoning episode, TTX and TTXs were not detected. As in the sales documents, the whelk species was referred to as *Murex* spp., samples of *Murex trunculus* (now *Hexaplex trunculus*) were also included in the analysis, but no TTXs were detected. The question remains about the exact origin of TTX in the food chain, because the ecological environments of TTX-bearing animals seem to have no common factor other than being closely implicated in an aquatic system; bacteria, the omnipresent organisms that commonly inhabit aquatic systems, were implicated as the primary source of TTX.[11] Also, if contamination exists in Mediterranean *C. lampas*, why do human outbreaks remain so rare? In this case, the patient reported having eaten the visceral mass. This portion of the animal usually is not included in common gastronomical preparations.

In this paper, 5,6,11-trideoxyTTX was found in an amount 3 times higher than TTX in all samples. With comparison of the concentrations of TTX and 5,6,11-trideoxyTTX obtained by LC−MS to concentrations of both toxins by mouse bioassay, it was demonstrated that the analogue is almost not toxic. This coincides with previous studies where several analogues including 5,6,11-trideoxyTTX are shown as having low toxicity.[3] 5,6,11-TrideoxyTTX can be less toxic because it has less hydroxyls groups compared with the TTX, and for this reason could have less affinity for the binding to the sodium channel. However, this analogue

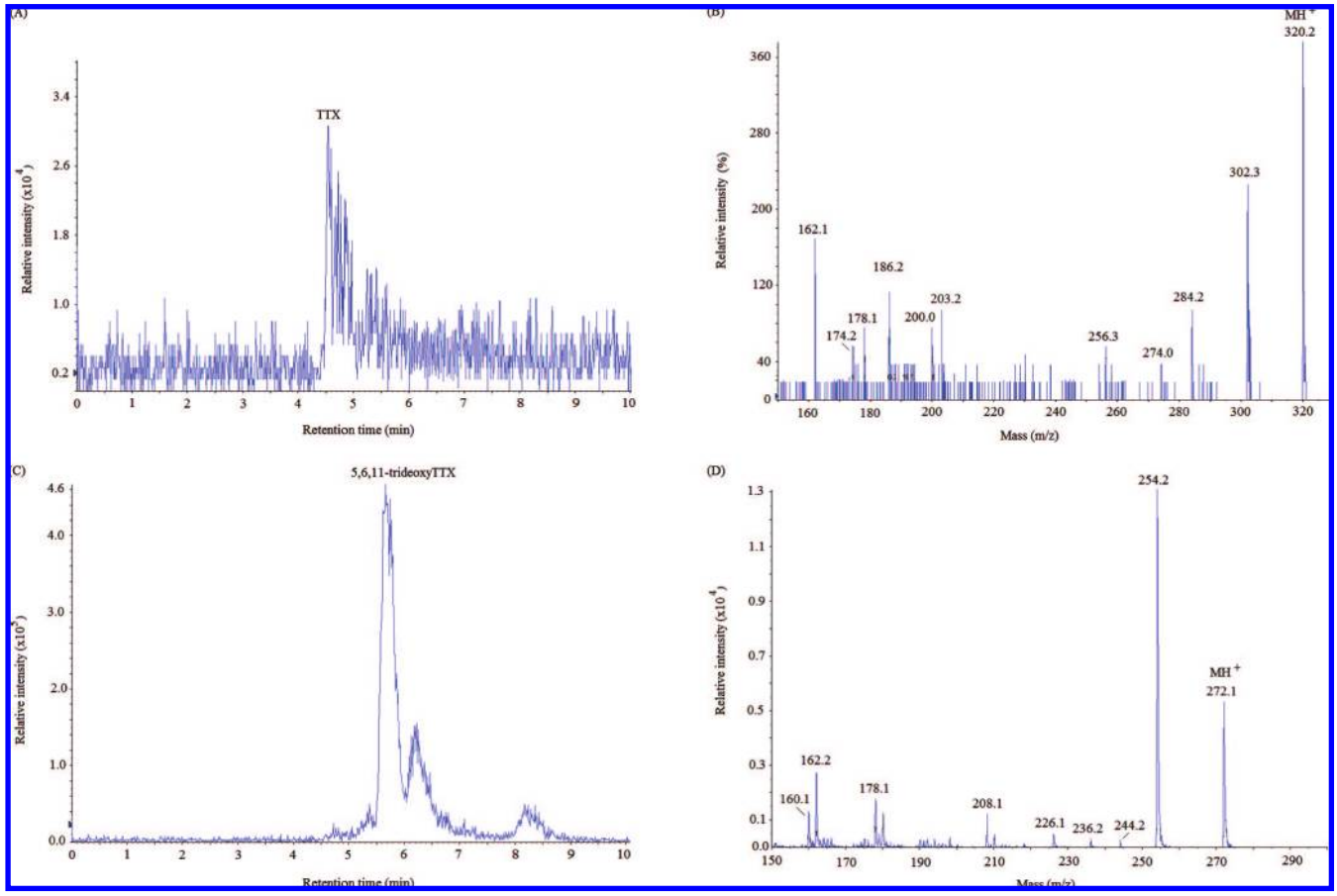

Figure 6. LC−MS/MS analysis in the EPI mode of a urine sample. (A) LC−MS/MS chromatogram for *m*/*z* 320. (B) LC−MS/MS mass spectrum from part A. (C) LC−MS/MS chromatogram for *m*/*z* 272. (D) LC−MS/MS mass spectrum from part C.

Table 1. Analysis by LC−MS

	TTX level	5,6,11-trideoxyTTX
sample 1 (digestive gland)	31.5 mg/100 g	100.4 mg/100 g
urine	211.1 ng/mL	692.1 ng/mL
serum	26.4 ng/mL[a]	86.5 ng/mL[a]

[a] Concentrations just at the limit of detection.

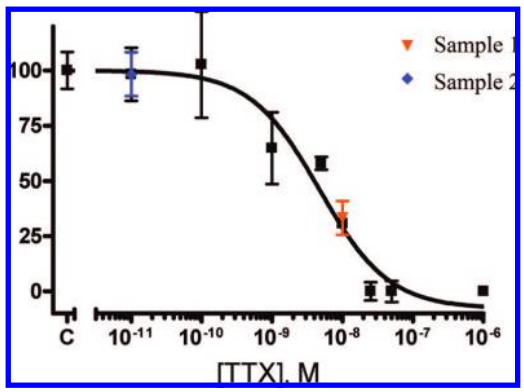

Figure 7. Confocal microscopy analysis of sample 1 (digestive gland) and sample 2 (meat) corresponding to the trumpet shell *Charonia lampas lampas*.

involves other complex changes including dehydrogenation of the C-10-hydroxyl, which is believed to be essential for the affinity,[2] competing with TTX on intracellular targets.

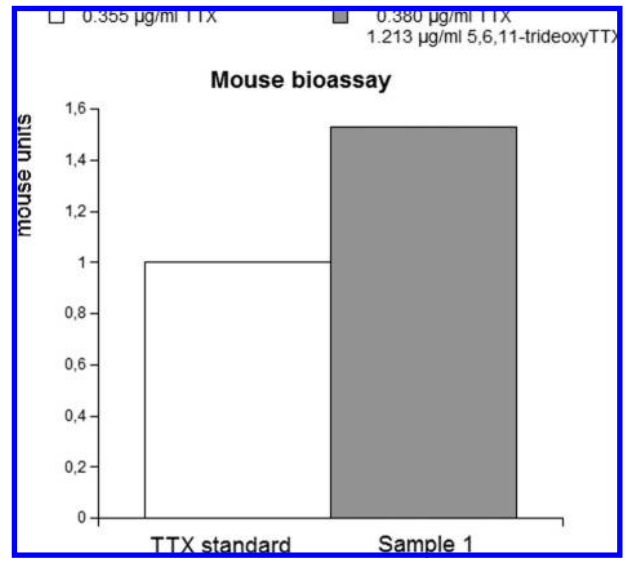

Figure 8. Mouse toxicity assay. TTX standard (Calbiochem Corporation) and extract of digestive gland (sample 1) injected i.p. into mice (19−23 g body weight).

This paper describes the analytical results of a TTX intoxication, from an instrumental, toxicological, and kinetic viewpoint. The most striking observation is the fact that a new, nonregulated toxin has appeared in gastropods in Europe. This is clearly a matter for concern, since this new phenomenon is probably to be attributed to a potential ecological change due to increased

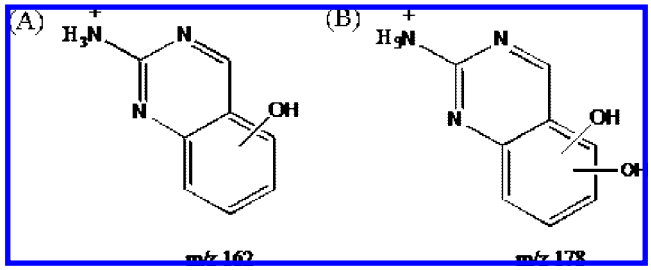

Figure 9. Fragment ions at *m*/*z* 162 (A) and *m*/*z* 178 (B) of MH^+ of TTX and 5,6,11-trideoxyTTX.

warm temperatures. This could pose a hint of potential consequences of global warming on food safety.

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