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Human intoxication with paralytic shellfish toxins: Clinical parameters and toxin analysis in plasma and urine

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ABSTRACT

This study reports the data recorded from four patients intoxicated with shellfish during the summer 2002, after consuming ribbed mussels (*Aulacomya ater*) with paralytic shellfish toxin contents of 8,066 ± 61.37 μg/ 100 gr of tissue. Data associated with clinical variables and paralytic shellfish toxins analysis in plasma and urine of the intoxicated patients are shown. For this purpose, the evolution of respiratory frequency, arterial blood pressure and heart rate of the poisoned patients were followed and recorded. The clinical treatment to reach a clinically stable condition and return to normal physiological parameters was a combination of hydration with saline solution supplemented with Dobutamine (vasoactive drug), Furosemide (diuretic) and Ranitidine (inhibitor of acid secretion). The physiological condition of patients began to improve after four hours of clinical treatment, and a stable condition was reached between 12 to 24 hours. The HPLC-FLD analysis showed only the GTX3/GTX2 epimers in the blood and urine samples. Also, these epimers were the only paralytic shellfish toxins found in the shellfish extract sample.

Key terms: paralytic shellfish poisoning, PSP human intoxication, Chilean fjords

INTRODUCTION

Paralytic shellfish poisoning (PSP) corresponds to a syndrome produced by intoxication with paralytic shellfish toxins (PST). These toxins are all hydrophilic, of low molecular weight (under 500 Da) and have a skeleton of 3,4,6-trialkyl tetrahidropurine. Until now, around 26 different analogs of these toxins have been described, depending on modification of their functional groups, all with different specific toxicity in the mouse bioassay (Oshima, 1995; Onodera et al., 1997; Lagos, 1998).

Paralytic shellfish toxins shows high affinity for voltage-gated sodium channels, blocking the nerve impulse transmission (Henderson et al., 1973; Strichartz 1984; Guo et al., 1987; Hu & Kao 1991). The intoxication syndrome shows characteristic symptoms such as oral paresthesia, asthenia, disthonia, ataxia, dyspnea, hypotension, tachycardia, vomiting and muscular weakness. If the amount of PST is high enough, the intoxication can result in death. (Long et al., 1990; Montebruno, 1993; Lagos & Andrinolo 2002; García et al., 2004a). Paralytic shellfish poisoning has the highest mortality rate (13%) of all marine toxins (Lagos, 1998).

In Chile, PST can be found in contaminated shellfish in the fjords system in the southern part of the country. Until now, only the dinoflagellate *Alexandrium*

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catenella has been described as a PST producer in this geographical area (Lembeye et al., 1975; Lagos, 1998; Lagos 2003). In this southern part of the country, along a stretch of 1500 km of coast between 42° 00' 00" and 56° 00' 00" lat. S (Lagos, 2003), PST are responsible for health, economic and social losses in the isolated communities living in this area. This was the case of Castro City (42º 00´ lat. S / 71º 20´ long. W) and the community of Quellón (43º 15´ lat. S / 73º 45´ long. W) in February 2002 (Lagos, 2003; Molinet et al 2003; García et al ., 2004b).

During the spring-summer of 2002, an expansion of an *Alexandrium catenella* bloom took place around Chiloe Island, to 46º 00´ lat. S, the historical northern geographic limit of paralytic shellfish toxin blooms in this southern area. This bloom damaged the shellfish industry in the geographical areas of Quellón and Castro City on Chiloé Island (Garcia et al., 2004b) and caused 25 intoxication cases, with four reported fatalities. The affected geographic area was quickly declared under emergency by the national authorities due to the serious threat to public health (García et al., 2004b).

This study describes and explains data recorded from four patients intoxicated with PST-contaminated shellfish (*Aulacomya ater*) in Castro City, Chiloé Island, during the summer of 2002.

The patients arrived in critical clinical condition to the hospital, and after being treated according to the protocol proposed by the authors, they reached a clinically stable condition within an eighteen-hour period, during treatment at Castro City Hospital. Data associated with the clinical variables and PST content in the intoxicated patients' plasma and urine are shown in this paper.

Both the contents of toxin in the shellfish and in the body fluid samples of the patients are compared and discussed according to the symptoms shown by the intoxicated individuals. Clinical manifestations and medical parameters were recorded during the intoxication episode in an attempt to propose a treatment protocol for paralytic shellfish poisoning.

MATERIALS AND METHODS

Chemicals

1-heptenesulfonic acid sodium salt, periodic acid, potassium phosphate dibasic and tetrabutylammonium phosphate were purchased from SIGMA (Sigma Chemical Co., St. Louis, MO, USA). HPLC grade solvents (acetonitrile, HCl, acetic acid) were purchased from Fisher Scientific (New Jersey, USA). Phosphoric acid and ammonium hydroxide were purchased from Merck (MERCK, Darmstadt, Germany).

The Sep-Pak® cartridges for solid phase extraction of silica and C-18 were purchased from Waters Corporation (Division of MILLIPORE, Milford, MA, USA). Water of high purity grade was obtained by elution through an ion exchange cartridge and then by boiling for 2 hours with nitrogen bubbling.

Sample preparation

100 g of each mussel sample were homogenized in equal volumes by weight of 0.1 N HCl in a variable speed Tissue Tearor (Biospec Products, USA). The pH was adjusted to pH 4 and then extracted at 85 ºC for 10 minutes. The extract was centrifuged at 10,000 x g for 15 min. Twenty microliters of the filtrate were applied to the HPLC.

Human blood and urine samples were collected at 2, 6 and 8 hours after treatment began at Castro City Hospital. To keep the body fluid samples in acidic medium, 200 µl of 500 mM acetic acid were added to each collection vial. Two ml of the urine sample was immediately centrifuged at 10,000 x g for 5 minutes. The supernatant was passed through a cartridge column (C-18 Sep Pak®, Millipore, Waters, MA), previously regenerated with 10 ml methanol and equilibrated with 10 ml water. PST was collected immediately and the pigments retained in the cartridge. Ten microliters of the fraction eluted with acetic acid was used for HPLC analysis.

The intravascular blood samples were also collected at 2, 6 and 8 hours; samples were centrifuged at 10,000 x g for 5 min.

The serum fraction was mixed with 200 µl of acetic acid 500 mM, immediately frozen and stored at -20ºC for later analysis. After thawing, the samples were centrifuged to 10,000 x g for 2 min. The supernatant was filtered through 5,000 M.W. cut-off microcentrifuge filters (Ultrafree-MC C3GC, Millipore Corp., MA, USA). Ten microliters of the filtrate were injected into the HPLC (Andrinolo et al., 2002).

Chromatographic conditions for HPLC analysis of PSP toxins

Poison components were determined under the conditions described previously using ion-pairing chromatography with post-column derivatization (Lagos, 1998). Briefly, 20 µl treated samples were injected (Rheodyne model 7725i with a 20-µl loop) into a silica-base reversed phase column (Symmetry C-8, 4.6 x 150 mm, 5 µm Waters, USA). The following mobile phase of 2 mM 1-heptenesulfonic acid in 30 mM ammonium phosphate buffer pH 7.1: acetonitrile (100: 5), at a flow rate of 0.7 ml/min was used for detection and quantitation of Saxitoxin (STX) group of toxins.

For the Gonyautoxins (GTX) group of toxins, 2 mM 1-heptenesulfonic acid in 10 mM ammonium phosphate buffer pH 7.1 elution buffer was used. In both cases, the elution from the column was mixed continuously with 7 mM periodic acid in 10 mM potassium phosphate buffer pH 9.0, at 0.4 ml/min, heated at 65ºC by passing through a coil of Teflon tubing (0.5 mm i.d., 10 m. long), and then mixed with 500 mM acetic acid at 0.4 ml/min before entering the fluorescent detector.

The fluorescent detector was set at an excitation wavelength of 330 nm and an emission wavelength of 390 nm. For HPLC chromatographic equipment, a Shimadzu LC-10AD liquid chromatograph apparatus with an online Shimadzu RF-551 spectrofluorometric detector was used. Acid and the oxidizing reagent were pumped by a dual-head pump (model SP-D-2502, Nihon Seimitsu Kagaku). Data acquisition and data processing were performed with Shimadzu CLASS-CR10 software. Toxin concentrations were measured by comparing the peak areas for each toxin with those of the standard. Pure PST solutions calibrated by combustion analysis of nitrogen measurements and HPLC-MS were used as external standards (Lagos, 1998).

In order to avoid mistaking false peaks as toxins, the samples were reanalyzed with the same HPLC with fluorescence online detection (FLD) procedure, but replacing the oxidizing reagent by distilled water. Under these conditions, oxidation does not occur, but false peaks are detected (Lagos et al., 1999).

Clinical features

The clinical history was recorded by anamnesis. Monitoring of vital signs and evaluation of the patients' physiological condition were performed upon admission to the emergency room of Castro City Hospital, Chiloé Island. The time at which treatment with the proposed clinical protocol began was considered the starting time. Written informed consent was obtained from each patient in the same emergency room of Castro City Hospital.

RESULTS

Case report

Four male adults (from the 21 surviving intoxicated individuals), patients of 60, 62, 64 and 66 years old with an average age of 63 ± 1.29 years old (mean ± SEM, N = 4), from Quellón, Chiloé Island (43º15´ latitude / 73º45´ length), consumed PST-contaminated, fresh ribbed mussel. Approximately five minutes after ingestion, they presented characteristic symptoms of paralytic shellfish poisoning: oral paresthesia and vomiting, which resulted in their arrival at the emergency room in Quellón.

Symptoms of progressive respiratory failure and hypotension developed within the next 40 minutes. Increasing deterioration of clinical conditions after 50 minutes of shellfish ingestion led the

Quellón Emergency Room medical staff to transfer the patients to the nearest hospital, Castro City Hospital, 79 km north of Quellón (42º 00' lat. S / 71º 20' long. W). The PST analysis of the contaminated mussel sample showed a PST amount of 8,100 μg/100 gr. of shellfish tissue.

Patient's characteristics

When patients were admitted at the Quellón Emergency Room, they declared that after consumed only two raw ribbed mussels, they started to feel oral paresthesia. Although they were not particularly literate, they knew about the "Red Tide" phenomenon in that geographical area, and they also were aware of the dangers of eating PST-contaminated shellfish. Therefore, they decided to stop eating and sought medical assistance at the nearest emergency facility.

The symptoms referred to and detected by the patients were: oral paresthesia (100%), facial paresthesia (33.35%), weakness in the lower limbs (66.6%), weakness in the upper limbs (66.6%), vomiting (38.2%), headache (41%), abdominal pain (25%) and impaired consciousness (20%).

Clinical management

Upon their arrival to the emergency room all patients received gastric lavage. Great care was taken to prevent pulmonary aspiration of the return flow. Gastrointestinal decontamination with activated charcoal was performed afterwards. Both these procedures were performed during the first two hours after the onset of initial symptoms. All four patients suffered respiratory arrest, requiring orotracheal intubation and ventilatory assistance (500 m^3 Tidal volume and FiO2 30%) (Fig.1A).

The four patients were hydrated with 1.0 liter of ringer lactate solution, 1.0 liter of saline solution supplemented with 4.0 grams of NaCl and 2.0 grams of KCl. The vasoactive drug, Dobutamine (250 mg in 25 ml i.v. in isotonic saline serum) was required to revert the low arterial pressure (53.3 ± 2.73/36.3 ± 2.40 mmHg; mean ± SEM, N = 4) (Fig 1B). Furosemide (40 mg i.v.), a known diuretic, was used to stimulate renal clearance of the PST already impaired by hypotension (Fig. 1B), and Ranitidine (50 mg/ml, one ampoule i.v.) was used to inhibit the production of acid by parietal stomach cells. Since PST can be hydrolyzed in neutral-alkaline medium, the strength of the intoxication decreased (Lagos, 1998).

Hypoxia developed as a consequence of respiratory failure with arterial O_2 pressures of 53.0 ± 3.3 mmHg (mean ± SEM, N = 4) (Table 1), concomitant with bradycardia (38.33 ± 2.08 bpm; mean ± SEM, N = 4) (Fig. 1C). The arterial blood analysis showed low serum bicarbonate concentration, which effectively compensates the respiratory acidosis expected with the respiratory failure (Table 1).

After four hours from the beginning of clinical treatment (indicated with arrow in Fig. 1), the physiological condition of the patients began to improve, returning to normal 12 hours after the onset of the respiratory arrest and remaining stable thereafter. The respiratory rate 24 hours after the respiratory arrest was 18.67 ± 0.67 (breaths/min; mean ± SEM, N = 4) (Fig. 1A), arterial blood pressure was 118 ± 3.05/ 62 ± 4.04 (mmHg; mean ± SEM, N = 4) (Fig. 1B) and heart rate 57.67 ± 2.18 (bpm; mean ± SEM, N = 4) (Fig. 1C).

Shellfish toxin levels and toxicological variables

The shellfish toxin content, measured by HPLC-FLD, averaged 8,066 ± 61.37 μg/100 grams of shellfish meat. The PST profile of the shellfish extract showed the presence of GTX3/GTX2 epimers pair (Fig. 2B). Besides these epimers, no other PST was found in the shellfish extract.

The first urine and blood samples were obtained two hours after starting the clinical treatment in the Castro City Hospital (samples Nº 1), the second one was obtained at 6 hours (samples Nº 2) and then at 8 hours (samples Nº 3). The analysis of blood sample Nº 1 showed 4.9 ± 1.12 ng/ml (mean ± SEM, N = 4) of PST, while blood sample N°2 and Nº3 showed 4.31 ± 0.57 ng/ml and

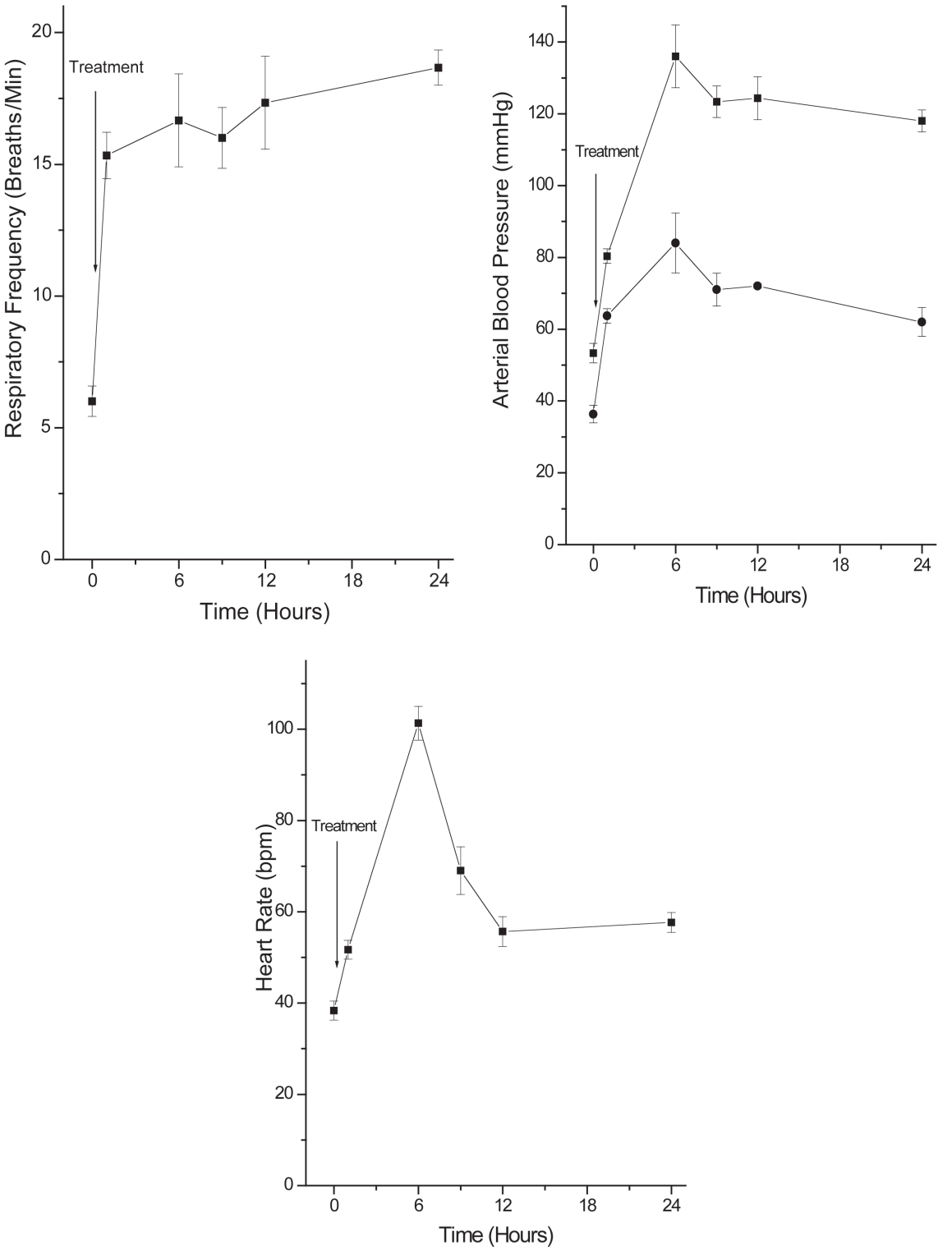

Figure 1. A. Record of respiratory frequency (breaths/min) during 24 hours of the 4 patients intoxicated with PSP-contaminated shellfish. Arrow shows the starting time of the treatment in the hospital facility. **B.** Record of arterial blood pressure (mmHg) during 24 hours of the 4 patients intoxicated with PSP shellfish. (■ systolic blood pressure; ● diastolic blood pressure). **C.** Record of heart rate (bpm). The values are Mean ± SEM, N = 4.

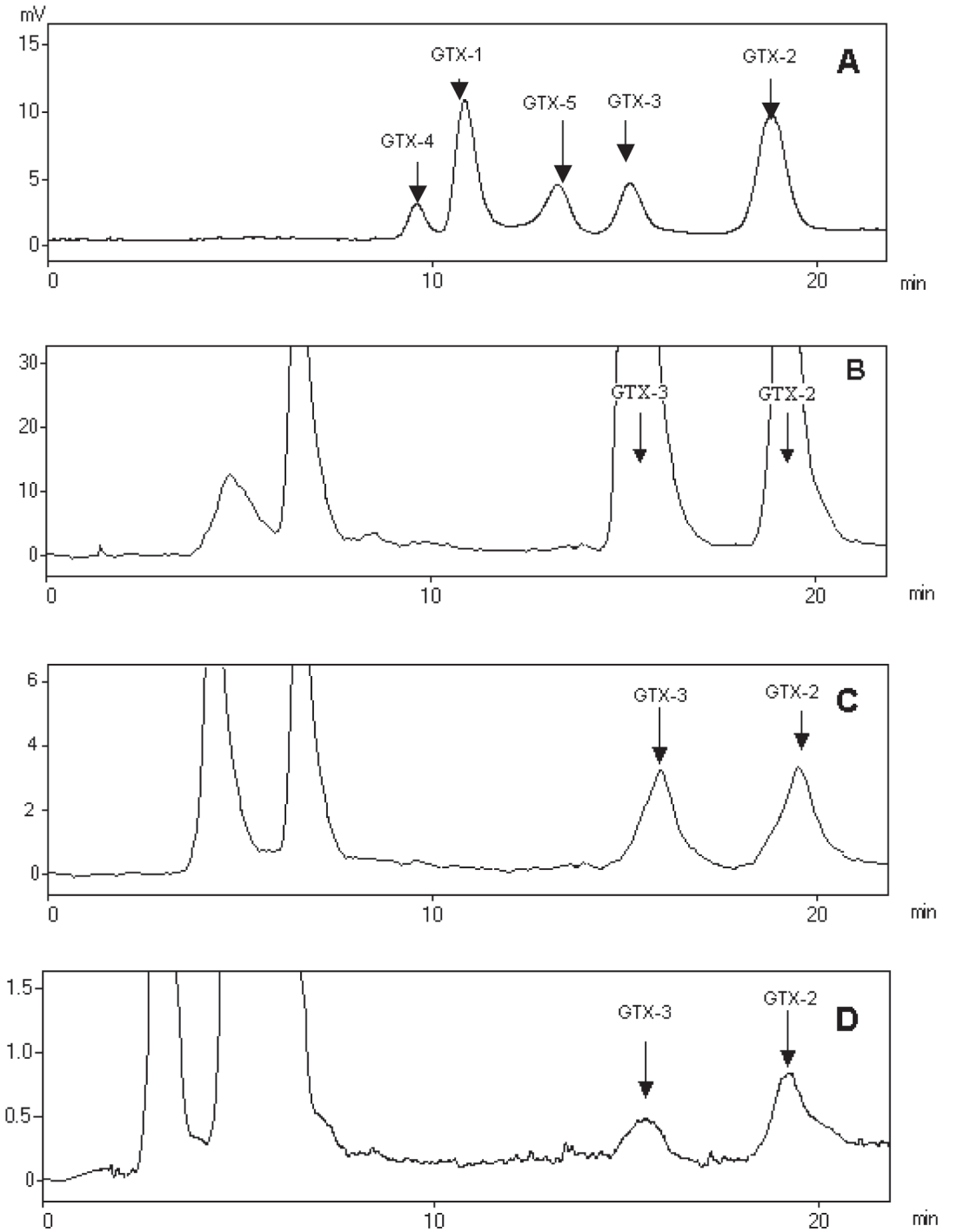

Figure 2.- HPLC chromatograms of PST detection (GTXs). **A**. Analytical standard mixture for Gonyautoxins group: GTX4 (Rt = 9: 02 minutes), GTX1 (Rt = 12: 12 minutes), GTX5 (Rt = 14: 01 minutes), GTX3 (Rt = 16: 59 minutes) and GTX2 (Rt = 18: 52 minutes). **B**. Ribbed mussels (*Aulacomya ater*) extract, showing only GTX3/GTX2 epimers with retention times of Rt = 16: 59 and Rt = 18: 55 minutes respectively. **C**. Chromatogram of urine human sample collected at six hours, GTX3/GTX2 epimers, Rt = 17: 02 and Rt = 19: 00 minutes respectively. **D.** Chromatogram of blood human sample collected at six hours, GTX3/GTX2 epimers, Rt = 17: 03 and Rt = 19: 02 minutes respectively.

3.61 ± 0.26 ng/ml (mean ± SEM, N = 4) PST respectively. PST contents in blood samples decreased steadily during the time of treatment (Table II), showing that the paralytic shellfish toxins were removed from the intravascular fluid.

TABLE I

Hemodynamic parameters in the intoxicated patients (Mean ± SEM, N = 4).

Arterial O_2 pressure	53.0 ± 3.3 mmHg
Arterial CO_2 pressure	33.5 ± 1.9 mmHg
Arterial O_2 saturation	94.2 ± 4.0 %
Arterial blood pH	7.3 ± 0.8
Serum bicarbonate concentration	19.2 ± 1.2 mEq/L
Base excess	-1.8 mEq/L

TABLE II

Toxin content in plasma and urine (Mean ± SEM, N = 4).

	GTX3/GTX2 content in plasma	GTX3/GTX2 content in urine
Time	ng/ml	ng/ml
2 hours	4.9 ± 1.12	12.22 ± 0.59
6 hours	4.31 ± 0.57	16.86 ± 0.48
8 hours	3.61 ± 0.26	10.59 ± 0.60

Mean ± SEM, N = 4
GTX3 = gonyaulatoxin 3
GTX2 = gonyaulatoxin 2

The high amount of toxin found in sample Nº 1 would explain the dramatic decrease in diuresis and the hypotension observed in all patients (Fig.1B). The homodynamic stabilization after the clinical treatment improved the diuresis, restoring the renal clearance of toxins from the bloodstream (Table II).

The urinary excretion of toxin within the two hours of clinical treatment was 12.22 ± 0.59 ng/ml (mean ± SEM, N = 4) and increased to 16.86 ± 0.48 ng/ml (mean ± SEM, N = 4) after six hours of treatment (Table II). This data correlates very well with the increase in blood pressure to 80.2 ± 1.89/50 ± 2.24 mmHg (mean ± SEM, N = 4) (Fig 1A), which facilitates the diuresis and therefore toxin renal excretion. The urinary PST level found at 8 hour was 10.59 ± 0.60 ng/ml PSP-toxins (mean ± SEM, N = 4) (Table II).

Besides GTX3/GTX2 epimers, no other paralytic shellfish toxins were found in urine or blood samples in all fours patients. Moreover, no transformation pattern of PST was detected during the 8 hours of experimental sampling in both blood and urine samples. This was demonstrated by comparison with the analytical standard solution that includes GTX4, GTX1, GTX5, GTX3 and GTX 2, which showed retention times (Rt) of 09: 02 min, 12: 12 min 14: 01 min, 16: 59 min and 18: 52 min respectively (Fig. 2A).

The presence of GTX3/GTX2 epimers was detected in all HPLC-FLD analysis performed, with Rt = 17: 02 min and Rt = 19: 00 min respectively in urine samples and Rt = 17: 03 min and Rt = 19: 02 min respectively in blood samples (Fig 2C and Fig 2D).

DISCUSSION

The patients intoxicated with paralytic shellfish toxins showed the classical symptoms described in the literature for paralytic shellfish poisoning: oral paresthesia, facial paresthesia, weakness in the lower limbs, weakness in the upper limbs, vomiting, headache, abdominal pain, impaired consciousness and finally, respiratory arrest. All four demonstrated classical PSP intoxication syndrome. Furthermore, patients evidenced hypoxia, with low arterial O_2 pressure concomitant with bradycardia as a consequence of respiratory failure. Also, the serum bicarbonate concentration was under the normal level, showing clearly that lowering the bicarbonate was used to compensate the respiratory acidosis produced by the respiratory failure.

The shellfish PST content measured by HPLC-FLD was 8,066 ± 61.37 μg/100 grams of shellfish meat, which is a very high toxic amount that contrast with the international safe limit of 80 μg/100 grams of shellfish meat and correlates well with

the severity of the clinical manifestations observed in the intoxicated patients.

Considering that patients declared that they ate only two mussels and stopped eating because they felt immediate oral paresthesia and knowing that the meat of each Chilean mussel, on average weighs 23 grams each, these patients were intoxicated with an oral toxic dose of approximately 3.7 milligrams.

Since the average weight of the patients was 70.2 ± 3.3 kilograms, the amount of PST per kilogram weight can be estimated around 53 μg/kg in the patients. This rather high amount explains the respiratory arrest they all suffered. These data are also in agreement with those published in 2002 by Andrinolo and colleagues who used an oral dose of 70 μg/kg of animal to induce classical PSP intoxication syndrome in cats. This *in vivo* experimental animal model allowed them to study the toxicokinetics and toxicodynamics of gonyautoxins 3 (GTX3) and 2 (GTX2) after an oral toxin dose in cat (Andrinolo et al., 2002).

The quantitative analysis of blood PST content showed that the amount of toxins declined during the time that the patients were medically treated and recovering from the poisoning episode. These data show clearly that PST are moving from the intravascular compartment into the intercellular compartment and consequently binding to all possible voltage-dependent Na^+ channels present there. Nevertheless, in the urine compartment the amount of PST increases after 6 hours, showing the recovery of blood pressure from the pronounced toxin-induced fall and consequent anuria, which has been described previously in human patients poisoned with PST (Montebruno, 1993) and also in cats (Andrinolo et al., 1999; 2002; Lagos & Andrinolo, 2000).

After 8 hours, patients were clinically stable, while normal level of blood pressure and normal urinary flow were recovered. Toxin content found in urine was even lower than that found at the time of the first sampling (two hours after treatment). These data also correlate well with those found in the *in vivo* experimental cat model, the only similar data published (Andrinolo et al., 2002). The amount of toxins found in the urine samples at the eighth hour clearly shows that toxin excretion through urine is a consequence of the hemodynamic stabilization of the patients.

Arterial pressure decrease observed in these patients two hours after eating the mussels also was described in cats intoxicated with an oral dose (Andrinolo et al., 2002). This hypotensive effect also has been described by saxitoxin administration in guinea pigs (Chang et al., 1992), dogs (Kao et al., 1967; Henderson et al., 1973; Nagasawa et al., 1971; Catteral et al., 1979; Strichartz, 1984; Moczydlowski et al., 1986; Guo et al., 1987; Hall et al., 1990), cats (Borinson et al., 1977; Borinson et al., 1980; Andrinolo et al., 1999) and in human intoxications with PST (Long et al., 1990; Montebruno, 1993).

Patient recuperation showed a compensatory increase of arterial pressure, which occurs after the depression stage produced during the respiratory arrest. A possible explanation for this result could be associated with the fact that a significant fall of arterial pressure activates central reflex mediated by carotid receptors, which produce, as a secondary response, an increase in arterial pressure until patients reach a physiologically stable condition.

The toxicological profile of the samples analyzed showed only the presence of the GTX3/GTX2 epimers, meaning that no toxin metabolization took place in the patients during the 8 hours of sampling. The enzymatic oxidation that produces the metabolic transformation of GTX3/GTX2 epimers in GTX4/GTX1 epimers, previously described by García and colleagues (2004a) in postmortem analysis of body fluid samples from human victims, was carefully investigated. However, the only PST found in blood and urine samples in the four patients for a period of 8 hours were the GTX3/GTX2 epimers. Perhaps a longer period of time is required to observe the metabolic transformation described in human postmortem analysis, however, as in that study samples were collected 48 hours after shellfish consumption (García et al., 2004a).

Finally, the reduction of the severe paralytic shellfish toxin intoxication

symptoms, with the clinical stabilization of patients in less than 24 hours, was achieved thanks to the previous experimental work done with the cat *in vivo* model, where for the first time the toxicokinetics and toxicodynamics of paralytic shellfish poisoning were described in mammals (Andrinolo et al., 1999; Lagos & Andrinolo 2002; Andrinolo et al., 2002).

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