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Paralytic shellfish poisoning: post-mortem analysis of tissue and body fluid samples from human victims in the Patagonia fjords

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Abstract

In July 5, 2002 fishermen working in harvesting sea urchin (*Loxechinus albus*) in the Patagonia Chilean fjords were intoxicated by consumption of filter-feeder bivalve *Aulacomya ater*. After the ingestion of 7–9 ribbed mussel, two fishermen died 3–4 h after shellfish consumption. The forensic examination in both victims did not show pathological abnormalities with the exception of the lungs conditions, crackling to the touch, pulmonary congestion and edema. The toxic mussel sample showed a toxicity measured by mouse bioassay of 8575 μg of STX (saxitoxin) equivalent by 100 g of shellfish meat. Using post-column derivatization HPLC method with fluorescent on line detection was possible to measure mass amount of each paralytic shellfish poisoning (PSP) toxin yielding individual toxin concentrations. These PSP toxins were identified in the gastric content, body fluids (urine, bile and cerebrospinal fluid) and tissue samples (liver, kidney, lung, stomach, spleen, heart, brain, adrenal glands, pancreas and thyroids glands). The toxin profiles of each body fluid and tissue samples and the amount of each PSP toxin detected are reported. The PSP toxins found in the gastric content, were STX and the gonyautoxins (GTX4, GTX1, GTX5, GTX3 and GTX2) which showed to be the major amount of PSP toxins found in the victims biological samples. The PSP toxin composition in urine and bile showed as major PSP toxins neoSaxitoxin (neoSTX) and GTX4/GTX1 epimers, both STX analogues with an hydroxyl group (–OH) in the N_1 of the tetrahydropurine nucleus. The neoSTX was not present in the gastric content sample, indicating that the oxidation of N_1 in the STX tetrahydropurine nucleus resulted neoSTX, in a similar way that GTX3/GTX2 epimers were transformed in GTX4/GTX1 epimers. Beside this metabolic transformation, also the hydrolysis of carbamoyl group from STX to form its decarbomoyl analogue decarbamoylsaxitoxin was detected in liver, kidney and lung. These two findings show that PSP toxins went under metabolic transformation during the 3–4 h of human intoxication period, in which PSP toxins showed enzymatic oxidation of N_1 in the tetrahydropurine nucleus, producing neoSTX and GTX4/GTX1 epimers starting from STX and GTX3/GTX2 epimers, respectively. This study conclude, that PSP toxins are metabolically transformed by humans and that they are removed from the body by excretion in the urine and feces like any other xenobiotic compound.

Keywords: Paralytic shellfish poisoning; Paralytic shellfish toxins; High performance liquid chromatography analysis; Casualties; Patagonia fjords; Chile

1. Introduction

The continuous augment in the number of toxic microalgal species coupled with increased incidence of

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outbreaks of these species presents a constant threat to public health worldwide. Blooms of toxic microalgae, popularly known as red tides, represent an expanding threat to human health and fisheries resources throughout the world (White, 1988; Smayda, 1992; Hallegraeff, 1993). The impacts of this phenomena goes from illness and death of human consumers of contaminated shellfish to ecosystem alteration and massive mortality of fish, seabirds and marine mammals (Geraci et al., 1989; Anderson, 1989; Smayda, 1992; Hallegraeff, 1993; Lagos, 1998, 2003).

Until now, six human illnesses associated with microalgae toxins have been described (Hallegraeff, 1993; Yasumoto and Murata 1993; Falconer, 1996) from them, paralytic shellfish poisoning (PSP) poses the most serious threat to public health worldwide, because of its high mortality rate (Lagos, 1998) and in the case of Chile, due to the high toxicity found in the Patagonia fjords molluscs (Lagos et al., 1996; Compagnon et al., 1998; Lagos, 1998, 2003). PSP is formed by at least 26 paralyzing shellfish toxin analogues which occur naturally (Oshima, 1995a; Onodera et al., 1997; Lagos, 1998, 2003; Molica et al., 2002).

PSP has been recognized for over a century as a clinical entity in the austral part of South America. In 1908 P.A. Segers documented periodic mass native people poisoning due to mussels consumption in Ushuaia (Beagle Channel). This is the earliest report of PSP in this area. Until now, only the dinoflagellate *A. catenella* has been described as the PSP toxins producer, this species is mainly found in the southernmost regions of Chile, 1500 km long distance between 42°00′00″ 56°00′00″ Lat. S. (Lagos, 2003).

Since 1972, toxic blooms of *A. catenella* have resulted in the intoxication of 527 people with 32 fatalities (official records of the Health Environmental Department, Chilean Health Ministry). All of them intoxicated by the consumption of shellfish harvested in the three most austral Regions in the Chilean Patagonia (Montebruno, 1993; Lagos, 1998, 2003). The highest PSP contaminated shellfish had been reported in this part of South America (Benavides et al., 1995; Compagnon et al., 1998). Since 1993, the appearance of PSP is considered endemic in the southern Chilean fjords (Uribe 1993; Compagnon et al., 1998; Lagos, 1998, 2003).

We report here two human casualties which occurred in the Patagonia fjords, close to Magellan strait, including the post-mortem analysis of PSP toxins in tissues and body fluids of the victims using a post-column derivatization method of High Performance Liquid Chromatography (HPLC) and fluorescence detection (Lagos, 1998). With this analytical tool, it was possible to obtain the PSP toxin content and profiles in tissue and body fluid samples from victims intoxicated with PSP toxins. This study overcomes all the technical problems to measure these toxins in human biological samples, showing the more complete data of PSP toxins analysis in human post-mortem body fluids and tissues until now reported.

2. Materials and methods

2.1. Chemicals

1-Heptenesulfonic acid sodium salt, periodic acid, potassium phosphate dibasic, 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, ammonium hydroxide were purchased from Merck (MERCK, Darmstadt, Germany). Water of high purity grade, was obtained by elution through an ion exchange cartridge and then by boiling for 2 h with nitrogen bubbling.

2.2. Post-mortem samples preparation

Twenty grams of each tissue or 5 ml of each body fluid samples, were homogenised in equal volumes by weight of 0.1N HCl in a variable speed Tissue Tearor (Biospec Products USA). The pH was adjusted between 3 and 4, then extracted at 85 °C for 10 min. The extract was centrifuged to $10,000g$ for 2 min, the supernatant was concentrated in a speed vac plus SC210A (SAVANT) then filtered through 5000 M.W. cut-off microcentrifuge filters (Ultrafree-MC C3GC, Millipore Corp., MA, USA). Twenty microliters of the filtrate were applied to the HPLC.

The human body fluid and tissue samples were collected by the official medical examiner of the Servicio Médico Legal, XII Región, Punta Arenas, Chile. This study was conducted with the approval of the University of Chile, Faculty of Medicine Ethic Committee, Santiago, Chile.

2.3. High performance liquid chromatography analysis of PSP toxins

PSP toxins were measured under the conditions described previously using post-column derivatization HPLC with a fluorescence on line detection method (HPLC–FLD) (Andrinolo et al., 1999). Briefly, 20 μl of body fluids (bile, cerebrospinal fluid, urine and vitreous humour) or tissues extracted samples (liver, papillary muscle, kidney, spleen, thyroids glands, stomach, gastric content, pancreas, adrenal glands, grey substance, white substance, pericardium, myocardium, endocardium, aorta and lung) were injected (Rheodyne model 7725i with a 20 μl loop) into a silica-base reversed phase column (Supelcosil 5 μm, C-8, 46 × 150 mm, SUPELCO, Bellefonte, PA, USA) and the following mobile phases were used: For the STXs group, 2 mM 1-heptenesulfonic acid in 30 mM ammonium phosphate buffer pH 7.1: acetonitrile

(100:3), a flow rate of 0.7 ml/min and for the gonyautoxins group, 2 mM 1-heptenesulfonic acid in 10 mM ammonium phosphate buffer pH 7.1. The column eluted fractions were mixed continuously with 7 mM periodic acid in 10 mM potassium phosphate buffer pH 9.0, at 0.4 ml/min, heated at 65 °C 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 0.3 ml/min before entering the monitor. The fluorometric monitor was set at an excitation wavelength of 330 nm and an emission wavelength of 390 nm. For HPLC procedure, a Shimadzu LC-10AD liquid chromatograph apparatus on line with a Shimadzu RF-551 spectrofluorometric detector was used. The oxidising reagent and the acid was pumped by a dual head pump (model SP-D-2502, Nihon Seimitsu Kagaku). Data acquisition and data processing were performed with a Shimadzu CLASS-CR 10 software. Toxin concentrations were measured by comparing the peak areas for each toxin with those of the standards. Pure toxins solutions calibrated by combustion analysis nitrogen measurements and HPLC-MS were used as external standards. (Lagos, 1998; Andrinolo et al., 2002).

In order to avoid false identification of the PSP toxins, the samples were reanalyzed by the HPLC procedure replacing the oxidizing reagent by distilled water, where only mild oxidation occurred (Onodera et al., 1996; Lagos et al., 1999).

3. Results

3.1. Case report

On July 5, 2002, in Augusta island (51°15′00″S; 75°05′00″ W) close to San Blas Channel and 140 miles from Puerto Natales, Chile (Fig. 1). A group of three fishermen harvesting sea urchin (*Loxechinus albus*), decide around 02:30 P.M. to harvest *Aulacomya ater*, a Chilean filter-feeder bivalve (ribbed mussel). Other fishermen vessels were also in the same location harvesting sea urchin. All of them were aware that the filter-feeder bivalves harvesting was officially prohibit in area since 1994. Only, the scuba diver decide not join to shellfish consumption, he

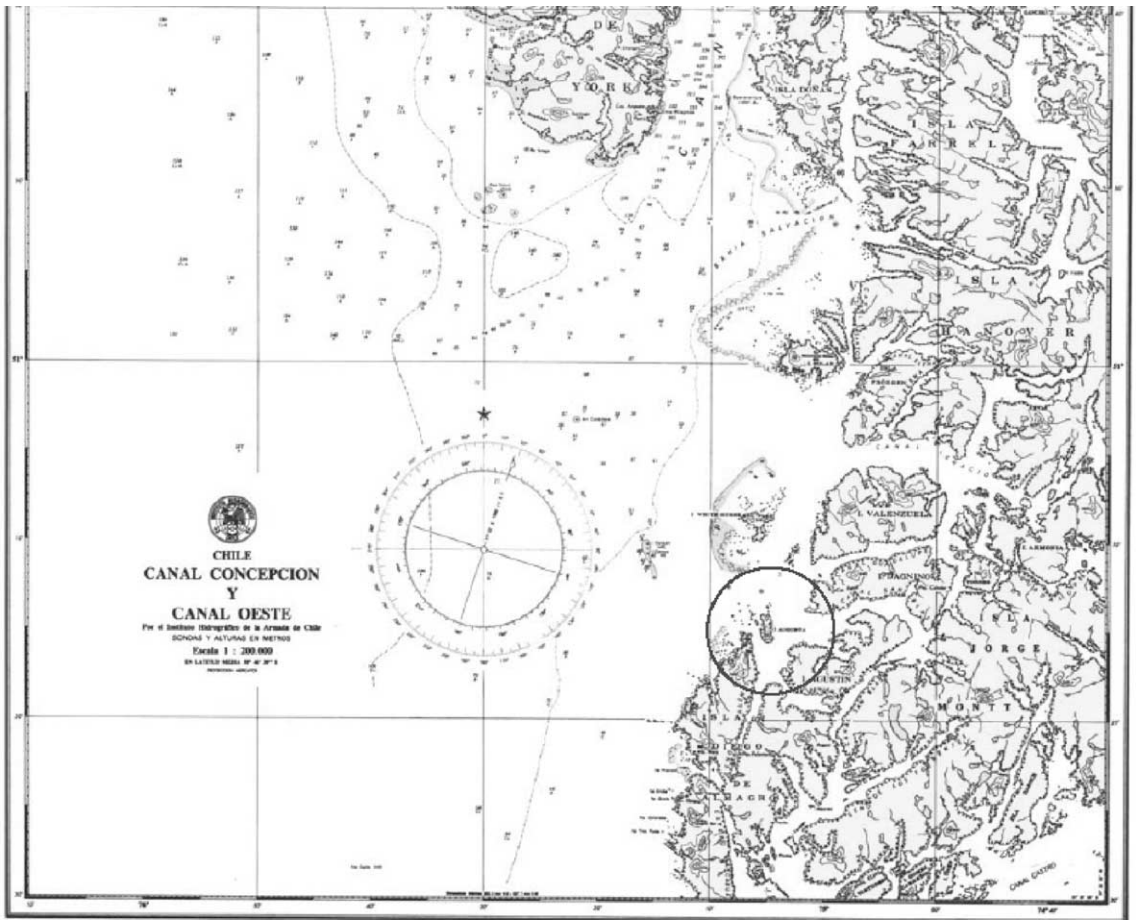

Fig. 1. Map and geographical location of Augusta island (51°15′00″ S; 75°05′00″W). San Blas Channel, Chilean Patagonia fjords.

was the only survivor. According to the police official declaration done by the scuba diver, after 30 min of shellfish consumption, one of the fishermen started to show intoxication symptoms: lips paresthesia, nausea and muscular weakness, immediately after the vessel patron get back to Puerto Natales looking for medical aid. Around 05:00 P.M., the intoxicated died and in parallel the vessel patron started to show upper extremity weakness and tongue immobilization, an hour later he was dead too. The official declaration informed that both casualties ate from 7 to 9 ribbed mussel. The diver was rescued around 10:00 P.M. by a Chilean navy vessel. A day after, when the vessels arrived to Puerto Natales, the contaminated shellfish was confiscated by the police and a sample was sent to the Red Tide Laboratory where a regulatory mouse bioassay of the shellfish sample consumed by the victims was performed. The laboratory reported 8575 μg of STX equivalent per 100 grams of shellfish meat. The international regulatory safe limit is 80 μg of STX equivalent per 100 g of shellfish meet.

3.2. Forensic examination

From the pathological examination of both victims (Table 1), the most remarkable findings were associated to the lungs conditions, crackling to the touch, pulmonary congestion and edema. These findings are indicative of serious effects on the respiratory muscles, due to the toxin paralyzing mechanism. The opaque aspect and low urine volume found in the bladder of both victims correlated very well with the finding reported by Andrinolo et al. (1999) in an in vivo cat experimental model, anaesthetized and permanently assisted by artificial ventilation, where STX intravenous injection drastically reduced blood pressure with the consequent diuresis fall (Andrinolo et al., 1999, 2002; Lagos and Andrinolo, 2000).

Table 1
Forensic and pathological examination

Victim N° 1
Exit of bloody liquid for nostrils
Heart 482 g, cardiac and coronary valves without injuries
Lungs of 1100 g each one, crackling to the tact, pulmonary congestion and oedema
Kidneys with normal aspect
Bladder with opaque urine
Normal liver without apparent injuries
Gastric Content mellow, mucous congestion with chunks of shellfish
Diffuse visceral congestion
Victim N° 2
Heart 371.5 g, cardiac and coronary valves without injuries
Lungs of 1300 g each one, crackling to the tact, pulmonary congestion and oedema
Kidneys without pathological findings
Bladder with scanty urine in its interior
Normal liver without apparent injuries
Visceral diffuse congestion
Mucous gastric hoemorragic
Gastric moderate content of color greenish clear brown with chunks of shellfish

3.3. HPLC analysis and PSP toxins content in tissues and body fluids samples

HPLC with on line fluorescent detection (FLD) is the most suited approach for PSP toxin quantitative analysis. Moreover, this method is the only one that allow to obtain sample toxin profile, thus given extra information, like metabolic transformations of toxins with modification of PSP toxin profiles (Oshima, 1995a; Lagos, 1998). The urine and blood samples normally show fluorescent compounds that get excited and emit fluorescent signal in the same wave range of 330 nm (excitation) and 390 nm (emission) used for PSP toxins detection. For that reason the matrix clean up procedures developed by Andrinolo et al. (1999) was used in order to avoid the fluorescent pigments in biological samples. Fig. 2 shows examples of HPLC–FLD chromatograms of some tissues and body fluids extracts, together with those of standards. The standard mixture, show three peaks: GTXs, neoSTX and STX with retention times of 6:99, 9:44 and 14:24 min, respectively (Fig. 2A). Fig. 2B, illustrate that bile extract also show the same three peaks with identical retention times. In this chromatographic run, the mobile phase used (2 mM 1-heptanesulfonic acid in 30 mM ammonium phosphate buffer, pH 7.1, containing 3% of acetonitrile v/v) only resolve the PSP toxins that belong to the group of STXs and these ones are neosaxitoxin (neoSTX), decarbamoylneosaxitoxin (dcneoSTX), STX and decarbamoylsaxitoxin (dcSTX), from them, neo-STX (Rt = 9:39 min) and STX (Rt = 14:21 min) are clearly seen. Afterwards, an extra peak just after the initial pigments with a Rt = 6:97 min was observed, this one correspond to the gonyautoxins, a group of PSP toxins that will be resolved with other mobile phase (see Section 2). For gonyautoxin, other standard mixture was used as is shown in Fig. 3.

The PSP toxin chromatograms of adrenal gland and aorta tissues are also shown in Fig. 2C and D, respectively, both are examples of typical chromatographic runs of victims tissue samples. In the adrenal gland sample, two peaks were seen, STX (Rt = 14:30 min) and the one corresponding to the mixture gonyautoxins (Rt = 6:95 min) (Fig. 2C). In the case of aortic tissue, only gonyautoxins (Rt = 6:95 min) were detected (Fig. 2D).

The amount of each toxin of the STXs group detected in body fluids and tissue samples are shown in Table 2. The quantity of each toxin was obtained by comparing the peak areas of each toxin with those of the standards (Lagos, 1998). For the tissue samples the amount are expressed as micrograms per gram of tissue and in the case of body fluid samples in micrograms per milliliter of body fluid.

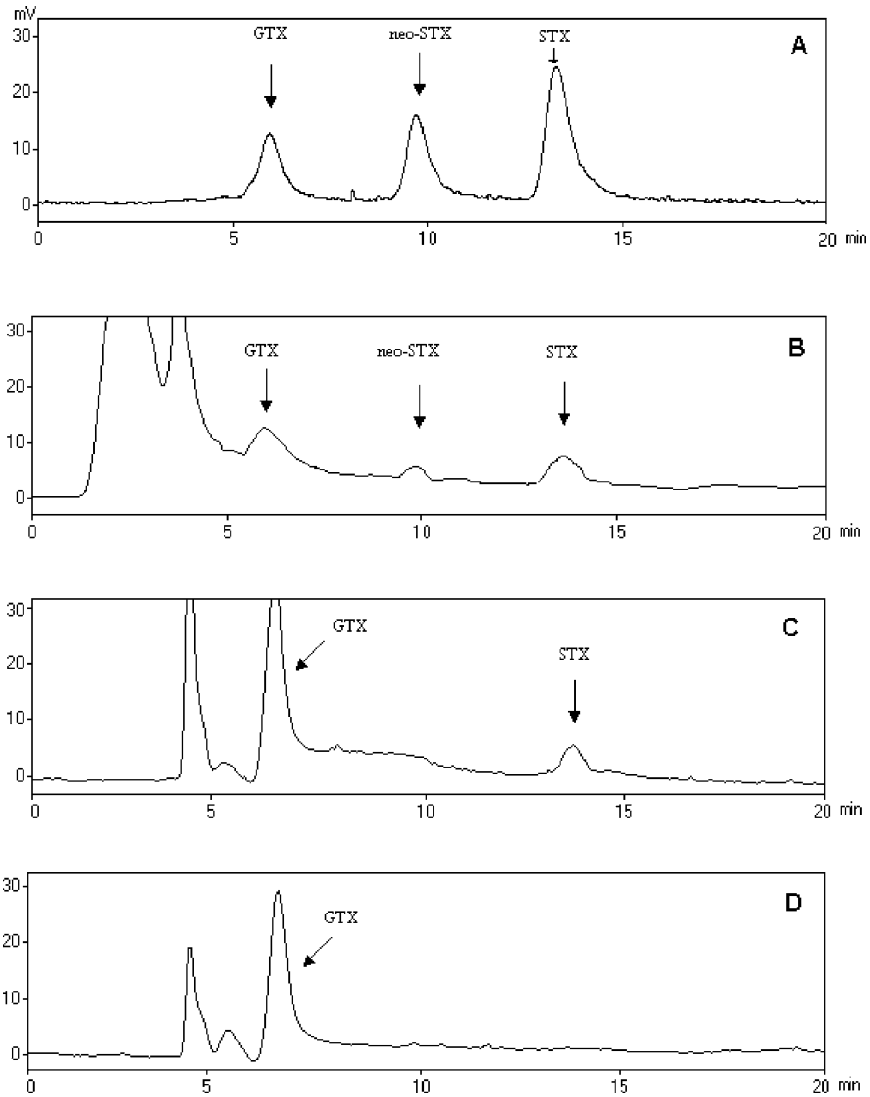

Fig. 2. Examples of HPLC–FLD chromatograms of PSP toxins using the mobile phase and conditions for the analysis of the STXs group. A. Standards chromatogram including STX (STX = 9.4 μM), neosaxitoxin (neoSTX = 14.7 μM) and gonyautoxin (GTX = 1.94 μM). B. Bile sample chromatogram. C. Adrenal glands tissue sample chromatogram and D. Aorta tissue sample chromatogram.

The major amount of PSP toxins were found in the gastric content (39.69 μg of STX/g of solid), this sample was obtained during the forensic pathological examination, collected directly from the mid stomach and antrum regions, most of it was solid undigested food and shellfish. Because, sample show up more solid than liquid, it was weighted and the toxin content was expressed as micrograms of toxin per gram of solid. In the analysis of this sample we looked carefully for neoSTX, but it was not found. The gut content toxin profile is important, because the gastric content correspond to the image of what was present in the original contaminated shellfish. Is important to remark that both victims died 3–4 h after shellfish consumption and the gastric secretion in the stomach kept food in acidic condition, which is the best condition to maintain these toxins chemically stable. PSP toxins are very stable from the chemical point of view, they are stable in boiling 0.1N HCl. This is the routine extraction procedure performed by the regulatory mouse bioassay (Cunniff, 1995).

The body fluids: urine, bile and cerebrospinal fluid and the tissues spleen and pancreas were the samples that showed the presence of neoSTX (Table 2). In order to avoid neoSTX false identification, further analysis were carried out according to Lagos et al. (1999). In all samples where neoSTX was detected, the purified samples were reanalyzed by the same HPLC procedure but now replacing the oxidizing reagent with distilled water. As was expected, the fluorescent intensity of the neoSTX peak increased

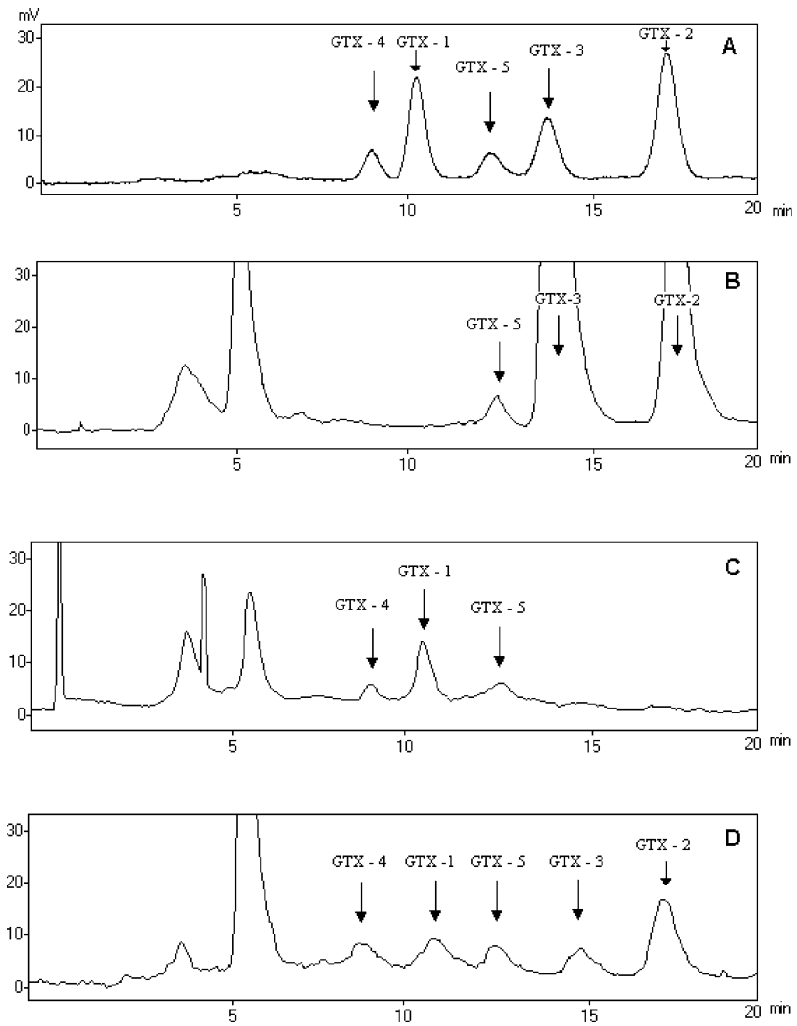

Fig. 3. Examples of HPLC–FLD chromatograms of PSP toxins under conditions appropriate and mobile phase for gonyautoxins analysis. A. Gonyautoxins standards chromatogram including gonyautoxin 4 (GTX4 = 0.52 μM), gonyautoxin 1 (GTX1 = 1.52 μM), gonyautoxin 5 (GTX5 = 0.70 μM), gonyautoxin 3 (GTX3 = 0.15 μM) and gonyautoxin 2 (GTX2 = 0.44 μM). B. Stomach tissue sample chromatogram. C. Spleen tissue sample chromatogram and D. Kidney tissue sample chromatogram.

several folds, so did the standard toxin. This behavior is typical of PSP toxin which has a hydroxyl group (–OH) in the Nitrogen 1 (N_1) of its chemical structure, as it is the case of neoSTX (Lagos et al., 1999). So, the peak with a retention time of 9:39 min correspond to neoSTX.

The liver, kidney and lung tissue samples showed a small amount of dcSTX (Table 2), this toxin was not present in the gastric content sample. Normally, the presence of this toxin is associated with STX and metabolic transformation between them has been reported (Sullivan et al., 1983). STX was the PSP toxin most frequently detected in all tissue samples and in most of the cases was the major one, also STX was the PSP toxin that showed the higher amount in the gastric content.

As examples, Fig. 3 shows the HPLC–FLD chromatograms of some tissue samples, including the chromatographic run of Gonyautoxin standards (Fig. 3A). In these runs, the mobile phase used was 2 mM 1-heptanesulfonic acid in 10 mM ammonium phosphate buffer, pH 7.1. Under this condition it is possible to resolve only the PSP toxins that belong to the group of gonyautoxins (GTX4/GTX1 epimers, GTX5 and GTX3/GTX2 epimers). These five PSP toxins are resolved in one HPLC run of 20 min as is shown in Fig. 3A. Here the chromatogram shows five peaks corresponding to GTX4, GTX1, GTX5, GTX3 and GTX2 with retention times of 9:43, 10:90, 13:80, 14:80 and 17:24 min, respectively (Fig. 3A). The stomach tissue sample chromatogram shows three peeks corresponding to GTX5 (Rt = 13:76 min) and the GTX3/GTX2 epimers (Rt = 14:87 and Rt = 17:27 min), the latter being the major toxin detected in this sample (Fig. 3B). Fig. 3C, show the chromatogram of spleen tissue sample, this

Table 2
Saxitoxins profiles from body fluids and tissues samples of PSP victim

Samples	neo-STX (μg/g tissue)	dc-STX (μg/g tissue)	STX (μg/g tissue)
Thyroids glands	n.d.	n.d.	2.86
Stomach	n.d.	n.d.	14.24
Gastric content	n.d.	n.d.	39.69
Spleen	0.22	n.d.	0.43
Liver	n.d.	0.04	0.55
Pancreas	1.30	n.d.	8.18
Kidney	n.d.	0.01	0.26
Adrenal glands	n.d.	n.d.	1.52
Fluids			
Bile[a]	0.69	n.d.	1.53
Cerebrospinal fluid[a]	0.77	n.d.	*n.d*
Urine[a]	22.33	n.d.	1.80
Vitreous humour[a]	n.d.	n.d.	n.d.
Brain			
Grey substance	n.d.	n.d.	0.65
White substance	n.d.	n.d.	0.08
Heart			
Pericardium	n.d.	n.d.	0.37
Miocardium	n.d.	n.d.	0.67
Endocardium	n.d.	n.d.	n.d.
Papillary muscle	n.d.	n.d.	0.63
Aorta	n.d.	n.d.	n.d.
Lung	n.d.	0.06	0.75

n.d., none detected; neoSTX, neoSaxitoxin; dcSTX, decarbamoylSaxitoxin; STX, Saxitoxin.
[a] μg/ml.

one also shows three peeks, but now the GTX4/GTX1 epimers (Rt = 9:42 and Rt = 10:98, respectively) as the major PSP toxins and with nonappearance of GTX3/GTX2 epimers. Both samples showed the presence of GTX5 (Rt = 13:77 min). Finally, Fig. 3D shows kidney sample chromatogram, here five gonyautoxins were detected showing similar chromatogram to the standards with practically the same retention times (Fig. 3A). This method clearly resolved the five gonyautoxins in tissue and body fluid samples. The gonyautoxins eluted far from the run front where extra peaks can be seen, these ones correspond to pigments present in the samples and they do not have relation with PSP toxins.

The gonyautoxin standard mixture resolved in the chromatogram showed in Fig. 3A was used for determination of the gonyautoxin peaks resolved for each body fluid and tissue samples. The quantities for each GTXs are shown in Table 3, the toxin amount was obtained by comparing the peak areas of each toxin with those of the standards (Lagos, 1998). For the tissue samples the amount are expressed as micrograms per gram of tissue and in the case of body fluid samples in micrograms per milliliter of body fluid (Table 3).

The fives gonyautoxins were detected only in gastric content, liver and kidney. The gastric content sample showed the major amount of gonyautoxins (Table 3). The three samples showed GTX5 as the minor gonyautoxin in the samples. Lung tissue samples showed both GTX4/GTX1 and GTX3/GTX2 epimers, but no GTX5 was detected in these samples. GTX5 was the minor gonyautoxin detected in the gastric content. Stomach tissue sample shows GTX5 and the GTX3/GTX2 epimers, these toxins correspond to the one found in the gastric content with GTX3 as the major one in both samples, resembling the gonyautoxin composition of the gastric content. The other body fluid and tissue samples only showed GTX4/GTX1 epimers (Table 3).

4. Discussion

The analysis of PSP toxins in body fluids and tissues samples have been complicated due to the low sensitivity and lack of specificity of the methods and procedures used to detect and quantify these toxins. In this paper, a post-column derivatization HPLC method with on FLD to quantify mass amount of PSP toxins was used. The HPLC–FLD is an analytical method that has the ability to measure each PSP toxin in samples of small size (20 μl), which yield a single toxin concentration. This method together with cleaning procedures using cartridge columns and microcentrifuge filters, allowed measurements of as low as 1 pmol of PSP toxin in body fluids and tissues samples. This procedures, developed by Andrinolo et al. (1999) proved to be quite powerful in the analysis of human biological specimen samples as is shown in this paper.

Considering the gastric content toxin profile, as the one in the contaminated shellfish, this one contain STX, GTX4, GTX1, GTX5, GTX3 and GTX2 as the PSP toxins. This profile correspond to the average PSP toxin composition (mol%) found in *Mythilus chilensis* (blue mussel); other similar filter-feeder bivalve also present in the area, which our laboratory has been monitored since 1994 (Lagos et al., 1996; Lagos, 1998, 2003).

In the enzymatic basis of liver detoxication, glucuronidation represents one of the major conjugation reaction involved in the metabolic conversion of xenobiotics and of numerous endogenous compounds to polar water-soluble metabolites. The resulting glucuronides, which are frequently the end product of metabolism, are removed from the body via either the urine or the bile. These two-stage process of glucuronidation followed by excretion are quantitatively the more important pathways of detoxication in humans and most mammals. Numerous compounds, when taken into the body, are not directly glucuronidated but instead must be first metabolically transformed to yield a suitable acceptor for conjugation. Initial reactions involved in the early metabolism of xenobiotics have classically been termed phase I reactions and encompass oxidative, reductive

Table 3
Gonyautoxins profiles from body fluids and tissues samples of PSP victim

Samples	GTX-4 (μg/g tissue)	GTX-1 (μg/g tissue)	GTX-5 (μg/g tissue)	GTX-3 (μg/g tissue)	GTX-2 (μg/g tissue)
Thyroids glands	0.31	2.33	n.d.	n.d.	n.d.
Stomach	n.d.	n.d.	0.22	1.04	0.57
Gastric content	1.29	1.26	0.10	2.64	1.15
Spleen	0.23	0.01	0.29	n.d.	n.d.
Liver	0.34	0.13	0.21	0.01	0.01
Pancreas	0.10	1.44	n.d.	n.d.	n.d.
Kidney	0.14	0.07	0.06	0.01	0.05
Adrenal glands	0.13	0.25	n.d.	n.d.	n.d.
Fluids					
Bile a	0.41	0.09	n.d.	n.d.	n.d.
Cerebrospinal fluid[a]	0.02	0.06	n.d.	n.d.	n.d.
Urine[a]	2.14	0.03	n.d.	n.d.	n.d.
Vitreous humour[a]	n.d	n.d.	n.d.	n.d.	n.d.
Brain					
Grey substance	0.05	0.65	n.d.	n.d.	n.d.
White substance	0.04	0.57	n.d.	n.d.	n.d.
Heart					
Ericardium	0.02	0.65	n.d.	n.d.	n.d.
Miocardium	0.05	1.52	n.d.	n.d.	n.d.
Endocardium	0.10	2.25	n.d.	n.d.	n.d.
Papillary muscle	0.06	1.03	n.d.	n.d.	n.d.
Aorta	0.28	0.57	n.d.	n.d.	n.d.
Lung	0.14	0.16	n.d.	0.05	0.03

n.d., none detected; GTX1, Gonyautoxin 1; GTX2, Gonyautoxin 2; GTX3, Gonyautoxin 3; GTX4, Gonyautoxin 4; GTX5, Gonyautoxin 5.
[a] μg/ml.

and hydrolytic modifications, while metabolic reactions producing the actual conjugate have been termed phase II reactions.

Phase I reactions are normally considered as functionalization reactions that prepared all xenobiotics for the next step of enzymatic detoxication. Considering PSP toxins as xenobiotics, these ones should go through these phase I reactions when arrived to the liver and for that reason they should also been chemically transformed. The enzymatic oxidation of N_1 in the tetrahydropurine nucleus of STX could have generate neoSTX and a similar functionalization reaction should transform the GTX3/GTX2 epimers into GTX4/GTX1 epimers.

Because xenobiotic are mainly excreted by urine and feces, these body fluid samples were analyzed with particular caution. Both samples showed GTX4/GTX1 epimers, neoSTX and STX. The epimers and neoSTX, belong to the group of STX analogues that have a hydroxyl group (–OH) in the N_1 of the tetrahydropurine nucleus common for all PSP toxins. Both samples did not show GTX3/GTX2 epimers, the most abundant gonyautoxins found in gastric content. Moreover, both excretion body fluids showed neoSTX, a PSP toxin that was also not detected in the gastric content. In the case of urine the amount of neoSTX (22.33 μg/ml) is amazingly high, only comparable with the amount of STX found in the gastric content, the highest amount of PSP toxin measured in all samples analyzed in this paper.

On the other hand, most of the tissue samples including the cerebrospinal fluid only showed the presence of neoSTX and the GTX4/GTX1 epimers and again, none of the GTX3/GTX2 epimers was detected in those samples. These two findings clearly show that PSP toxins went under metabolic transformation, in which most of the PSP toxins showed enzymatic oxidation of N_1 in the tetrahydropurine nucleus, producing neoSTX and GTX4/GTX1 epimers starting from STX and GTX3/GTX2 epimers, respectively. STX does not has hydroxyl group in the N_1 of the tetrahydropurine nucleus, not only necessary for next step of enzymatic transformation, but it also constitute an important mechanism of PSP toxins excretion, as it is showed by the high amount of neoSTX found in the urine (22.33 μg/ml, Table 2).

Hines et al. (1993) using [^{3}H] Saxitoxinol, a tritiated-reduced STX, made the first effort to study whole animal STX distribution, They showed that [^{3}H] Saxitoxinol was eliminated by urine and that it was not metabolized during the experimental period, also they did not find [^{3}H] radioactivity in feces.

Andrinolo et al., (1999 and 2002) using cat as in in vivo model under controlled conditions (mechanical ventilation) showed that cats with normal cardiovascular parameters and diuresis, excrete STX and GTX3/2 epimers by glomerular filtration. During the four hours of experimental time, no PSP toxins other than STX (Andrinolo et al., 1999) or GTX3/2 epimers (Andrinolo at al., 2002) were detected in the body fluids and post-mortem tissues samples analyzed. The authors concluded that cats could not metabolize PSP toxins and they were excreted only by urine. Moreover, they did not found PSP toxins in bile. Knowing that phase I reaction is normally considered as functionalization reaction that prepared all xenobiotics for the next step of enzymatic detoxication and that cat is the mammals exception in having the metabolic glucoronidation (Kasper and Henton, 1980), is possible to understand that STX and GTX3/GTX2 epimers did not showed any metabolic transformation in cat in vivo studies.

From medical records and samples analysis of PSP intoxicated people during 1994 on Kodiak Island, Gessner et al. (1997) reported differences between toxin composition in mussels, urine and serum. They suggested that PSP toxins human metabolism may occur. More recently, post- mortem analysis of urine and serum samples from one human victim of a fatal poisoning caused by xanthid crab, allowed the authors to conclude that PSP toxins metabolization occurred, suggesting that toxin conversion began at victim's intestinal level (Llewellyn et al., 2002). Until now, there is no report of mammalian metabolic studies associated to PSP toxins, so there is no direct evidence of PSP transformation in mammals, as has been already demonstrated in micro-algae, bacteria and shellfish (Shimizu and Yoshioka, 1981; Sullivan et al., 1983; Shimizu, 1993; Oshima, 1995b).

Beside the metabolic enzymatic change of N_1 group oxidation to form the hydroxyl analogue, which transforms STX to neoSTX and GTX3/GTX2 epimers to GTX4/GTX1 epimers showed in this paper, also the hydrolysis of the carbamoyl group to form the decarbamoyl analogue of STX (dcSTX) was detected. dcSTX was identify in liver, kidney and lung samples, all tissues very active in metabolic enzymatic transformations of one particular PSP toxin into another with the maintenance of the tetrahydropurine nucleus.

Secretion of bile is necessary for the proper digestion and absorption of lipids. It is also required for the elimination of endogenous products (e.g. cholesterol and bile pigments), as well as exogenously administered chemicals or xenobiotics (e.g. drugs and toxins). In this paper we show the presence of neoSTX, STX and GTX4/GTX1 epimers in the bile sample, showing for the first time that PSP toxins also are excreted by feces in significant amounts.

According to the post-mortem analysis of tissues and body samples from human victims, this study conclude, that PSP toxins are metabolically transformed by humans and that they are removed from the body by excretion in the urine and feces like any other xenobiotic. Despite of these advances it is still important to demonstrate that these toxins are also as glucuronides which are the most frequent end product of xenobiotics metabolism.

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