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Route of metabolization and detoxication of paralytic shellfish toxins in humans

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ABSTRACT

Paralytic shellfish toxins (PST) are a collection of over 26 structurally related imidazoline guanidinium derivatives produced by marine dinoflagellates and freshwater cyanobacteria. Glucuronidation of drugs by UDP-glucuronosyltransferase (UGT) is the major phase II conjugation reaction in mammalian liver.

In this study, using human liver microsomes, the *in vitro* paralytic shellfish toxins oxidation and sequential glucuronidation are achieved. Neosaxitoxin (neoSTX), Gonyautoxin 3/2 epimers (GTX3/GTX2) and Saxitoxin (STX) are used as starting enzymatic substrates. The enzymatic reaction final product metabolites are identified by using HPLC-FLD and HPLC/ESI-IT/MS. Four metabolites from GTX3/GTX2 epimers precursors, three of neoSTX and two of STX are clearly identified after incubating with UDPGA/NADPH and fresh liver microsomes. The glucuronic-Paralytic Shellfish Toxins were completely hydrolysed by treatment with β-glucuronidase. All toxin analogs were identified comparing their HPLC retention time with those of analytical standard reference samples and further confirmed by HPLC/ESI-IT/MS. Paralytic Shellfish Toxins (PST) were widely metabolized by human microsomes and less than 15% of the original PST, incubated as substrate, stayed behind at the end of the incubation.

The apparent V_{max} and Km formation values for the respective glucuronides of neoSTX, GTX3/GTX2 epimers and STX were determined. The V_{max} formation values for Glucuronic-GTX3 and Glucuronic-GTX2 were lower than Glucuronic-neoSTX and Glucuronic-STX ($6.8 \pm 1.9 \times 10^{-3}$; $8.3 \pm 2.8 \times 10^{-3}$ and $9.7 \pm 2.8 \times 10^{-3}$ pmol/min/mg protein respectively). Km of the glucuronidation reaction for GTX3/GTX2 epimers was less than that of glucuronidation of neoSTX and STX (20.2 ± 0.12; 27.06 ± 0.23 and 32.02 ± 0.64 μM respectively). In conclusion, these data show for the first time, direct evidence for the sequential oxidation and glucuronidation of PST *in vitro*, both being the initial detoxication reactions for the excretion of these toxins in humans. The PST oxidation and glucuronidation pathway showed here, is the hepatic conversion of its properly glucuronic-PST synthesized, and the sequential route of PST detoxication in human.

1. Introduction

Paralytic Shellfish Toxins (PST) are a collection of over 26 structurally related imidazoline guanidinium derivatives produced by some marine dinoflagellates and freshwater cyanobacteria (Sato et al., 2000; Onodera et al., 1997; Lagos, 1998, 2003). Eventually, when the PST-contaminated seafood is consumed by humans, the well known paralytic shellfish poison (PSP) syndrome may occur, resulting in severe public health problems (Hallegraeff, 1995; Lagos, 1998; García et al., 2004). The most characteristic

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symptoms of Paralytic Shellfish Poison (PSP) syndrome are: lips paresthesia, astenia, distonia, ataxia, dyspnea, hypotension, tachycardia, vomiting and muscular weakness. The PSP is the only Harmful Algae Bloom (HAB) poison which is lethal in South America (Long et al., 1990; Montebruno, 1993; Lagos and Andrinolo, 2000; García et al., 2004).

While being the only lethal HAB poison, still there is not direct evidence or clear knowledge about PST detoxication in humans. There are two pioneer studies, an *in vivo* animal model (Andrinolo et al., 1999, 2002; Lagos and Andrinolo, 2000) and a post-mortem analysis of tissue and body fluid samples obtained from intoxicated and deceased patients (García et al., 2004), that showed indirect evidence of PST metabolization in mammalian.

The post-mortem analyses of tissue and body fluid samples from people who died poisoned by consumption of PST-contaminated shellfish in the Patagonia fjords (Southern Chile), have shown biotransformation of some of these toxins. The detected biotransformation depended on the time that toxins remained in the organism. Detected oxidation of Saxitoxin (STX) into Neosaxitoxin (neoSTX) was reported (García et al., 2004).

Recently, *in vitro* experiment using human liver microsomes, showed N_1-oxidation of the GTX3 and GTX2 into the corresponding hydroxylamine analogs GTX4 and GTX1. This was the first direct evidence of biotransformation of GTX3/GTX2 epimers into GTX4/GTX1 epimers (García et al., 2009). They are the most common epimers found in PST shellfish toxin profile worldwide (Shunway, 1995; Lagos, 1998, 2003).

There is also indirect evidence of Gonyautoxins (GTXs) biotransformation by molluscs. In this case, Gonyautoxins into both Saxitoxin (STX) or Neosaxitoxin (neoSTX) had been described when GTXs were incubated with homogenate of the toxin-accumulating bivalve *Placopecten magellanicus*, suggesting that the conversion detected was catalyzed by enzymes within the shellfish homogenate (Shimizu and Yoshioka, 1981; Kotaki et al., 1985).

Liver microsome enzymatic preparation is advantageously used to determine *in vitro* metabolism of drug candidates, since it is easier than the whole animal assay (Wrighton et al., 1995). The *in vitro* fresh preparation should, therefore reflect the *in vivo* metabolism, detecting molecular species differences and predicting compound clearance.

Glucuronidation represents one of the major conjugation reactions involved in the metabolic conversion of xenobiotics and numerous endogenous compounds, both converted to polar water-soluble metabolites. The resulting glucuronides, which are frequently the end product of metabolism, are removed from the body either via urine or via bile. This two-stage process of glucuronidation followed by excretion is quantitatively one of the more important pathways of detoxication in man (Burchell et al., 1995). Numerous compounds once within 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, and hydrolytic modifications.

The present study using human liver microsomes, shows direct evidence of *in vitro* sequential oxidation and glucuronidation of neoSTX, GTX3/GTX2 epimers and STX, the major toxins of the PSP. For the first time, the mass spectrum of Glucuronic-neoSTX, Glucuronic-GTX3/GTX2 epimers and Glucuronic-STX is shown. Even more, the identities of these molecular ion peaks were further confirmed by the daughter ion spectra, the main decomposition molecular fractions in the mass spectrum are also identified. The oxidation and the sequential conjugation reactions of PST by human liver microsomes are demonstrated.

2. Materials and methods

2.1. Chemicals

1-Heptensulfonic acid sodium salt, periodic acids, potassium phosphate dibasic, tetrabutylammonium phosphate, and HPLC grade solvents were purchased from Fisher Scientific (New Jersey, USA), phosphoric acid, 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). UDP-Glucuronic acid, β-glucuronidase, D-saccharic 1,4-lactone monohydrate (saccharolactone) and alamethicin were purchased from SIGMA (Sigma Chemical Co., St. Louis, MO, USA).

2.2. Microsomes isolation from human liver

Human liver samples were obtained from liver donors, all patients of liver transplant procedures, with the protocol approved by the University of Chile Clinic Hospital Ethics Committee. None of the donors were treated or received drugs likely to interfere with drug metabolizing enzyme activities. Rifampicin, known to induce P-450 proteins and UDP-glucuronosyltransferase, was not ingested by any patient. The preparation of isolated microsomes was performed as described by Hiller et al. (1999). Briefly, livers were excised, washed in isotonic saline, and then blotted to dryness. All of the following steps were performed at 4 °C. Approximately, 3 g of frozen human liver were thawed and homogenized in 5 ml of homogenization buffer (154 mM KCl, 1 mM EDTA, 50 mM Tris/HCl pH 7.4) using a mechanical tissue tearor at 17,000 rpm for 45 s. A post-mitochondrial supernatant was prepared by centrifugation of the homogenate at 10,000*g* for 20 min. This supernatant was centrifuged at 202,000*g* for 70 min and microsomes sediment pellet was washed once in 15 ml of 0.1 M $Na_2P_2O_7$ (pH 7.8) using a tissue homogenizer. After centrifugation at 202,000*g* the pellet was resuspended in approximately 1 ml of 100 mM KH_2PO_4/K_2HPO_4 (pH 7.4), 1 mM EDTA (pH 8.0), 20% (v/v) glycerol. Aliquots of the microsomal suspension – with a protein concentration of 25 mg/ml – were fast-frozen in liquid nitrogen and stored at −80 °C.

Microsomes purity was assessed by a marker-enzyme analysis (Benedetti et al., 1988). Activities of glucose-6-phosphate, cytochrome c oxidase, and 5′-nucleotidase as markers for endoplasmic reticulum, mitochondria and

plasma membrane, respectively, were determined in total tissue homogenate and in the microsomal fraction. Also, the percentage of recovery was calculated. The integrity of the microsomal membranes was assessed using the mannose-6-phosphatase assay (Burchell et al., 1988), which showed latency greater than 95%.

As a control, we performed thermal inactivation studies. So, human liver microsomes were preincubated at 50 °C for 2 min in the absence of NADPH to inactivate FMO enzymes, and control reactions were preincubated at 37 °C for 2 min (Grothusen et al., 1996).

2.3. neoSTX, GTX3/GTX2 epimers and STX glucuronidation assay

Unless otherwise indicated, 0.5 mg of human liver microsomes, 0.1 M potassium phosphate buffer (pH 7.1), mixed with 25 μg of alamethicin were placed on ice for 15 min. Then $MgCl_2$ (1.0 mM in incubation), saccharolactone (5.0 mM in incubation), and acetaminophen (5.0 mM in incubation) were added and the mixture was preincubated at 37 °C for 3 min. To initiate the reaction, UDPGA (5.0 mM in incubation) was added to give a 200-μl final volume. Blank incubations were performed without UDPGA. Preliminary experiments indicated that the reaction was linear during 30-min incubation and up to 0.5 mg of protein. The reaction was stopped with 15 μl of ice-cold acetonitrile, the vials placed on ice for 30 min, and then they were centrifuged until the pellet of precipitated protein was formed. Next, 20 μl of the supernatant were injected for HPLC analysis. Standard curve correlation coefficients (r^2) were ≥0.99.

2.4. neoSTX, GTX3/GTX2 epimers and STX oxidation and glucuronidation

Typically, 0.5 mg of human liver microsomes, 0.1 M potassium phosphate buffer (pH 7.1) and 50 μg of alamethicin/mg microsomes were mixed and placed at 4 °C for 15 min. $MgCl_2$ (1.0 mM in incubation), saccharolactone (5.0 mM in incubation) and GTX3/GTX2 epimers, neoSTX or STX (15 μM final incubation concentration) were added and the mixture was preincubated at 37 °C for 3 min. To initiate the reaction, NADPH alone or with UDPGA was added to give a final volume of 200-μl and final concentrations of 1 and 5 mM, respectively. The reaction was stopped with 20 μl of methanol. Blank control incubations were performed without cofactor. Analysis was performed by HPLC. Experiments were performed from 7 human livers samples, with each of the values of a single set of experiments corresponding to the mean of a minimum of seven determinations ± SE.

Metabolite formation was quantitatively detected by comparing peak area ratios with standards containing known amounts of metabolite. Standard curve correlation coefficients (r^2) were ≥0.99.

2.5. Hydrolysis with ι-glucuronidase

An aliquot of 50 μl from sample incubates was taken and the solvent removed by heating at 70 °C and blowing off with a stream of nitrogen. To the residue, 1000 units of β-glucuronidase in 50 mM acetate buffer pH 5.0 were added to a final volume of 100 μl. Samples were incubated for 2 h at 37 °C. Blank control samples without β-glucuronidase were run in parallel. After incubation, samples were centrifuged at 5000*g* for 20 min in a Sorvall MC 12 V (DuPont) centrifuge and then 20 μl of the supernatant were directly injected into the HPLC.

2.6. HPLC analysis with fluorescence detection on line

Paralytic shellfish toxins were determined under previously described conditions, 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) to a silica-base reversed phase column (Symmetry C-8, 4.6 × 150 mm, 5 μm. Waters, USA). A 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 Gonyautoxins (GTXs) group of toxins, an elution buffer made of 2 mM 1-heptenesulfonic acid in 10 mM ammonium phosphate buffer pH 7.1 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 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 0.4 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. A Shimadzu LC–10AD liquid chromatograph apparatus with an on line Jasco FP 2020 Plus spectrofluorometric detector was used. Data acquisition and data processing were performed with ChromPass Chromatography Data System. Peak to concentration linear correlation, with an r^2 coefficient of 0.99, was found. The limit of detection set to a signal to noise ratio of 1:3. Toxin internal standards are normally run as routine control, starting in the morning before sample injections, at midday working day and after the last sample injected as a final control of the chromatographic run.

2.7. HPLC–MS

Samples obtained from each experiment were analyzed by HPLC–MS. Analysis was performed using a C-8 column (Waters 5 μm, 4.6 × 150 mm) at room temperature and isocratic conditions with mobile phase of aqueous 90% acetonitrile at a flow rate of 1 ml/min. The system was equipped with an electrospray mass spectrometer with ionic trap Esquire 4000 ESI-IT (Bruker Daltonics, Inc., MA, USA). As an interface, high purity 99% nitrogen was used as the nebulising gas (ca 0.2 L/min). A potential of 2.5 kV was applied at the corona and voltage cones of 5 and 50 V were used. The source temperature was set at 350 °C and the probe temperature was set at 150 °C. The negative ion mode was used with a full scan from 0 to 1000 *m/z*. Mass calibration of the instruments was carefully checked (Lagos et al., 1999; García et al., 2009).

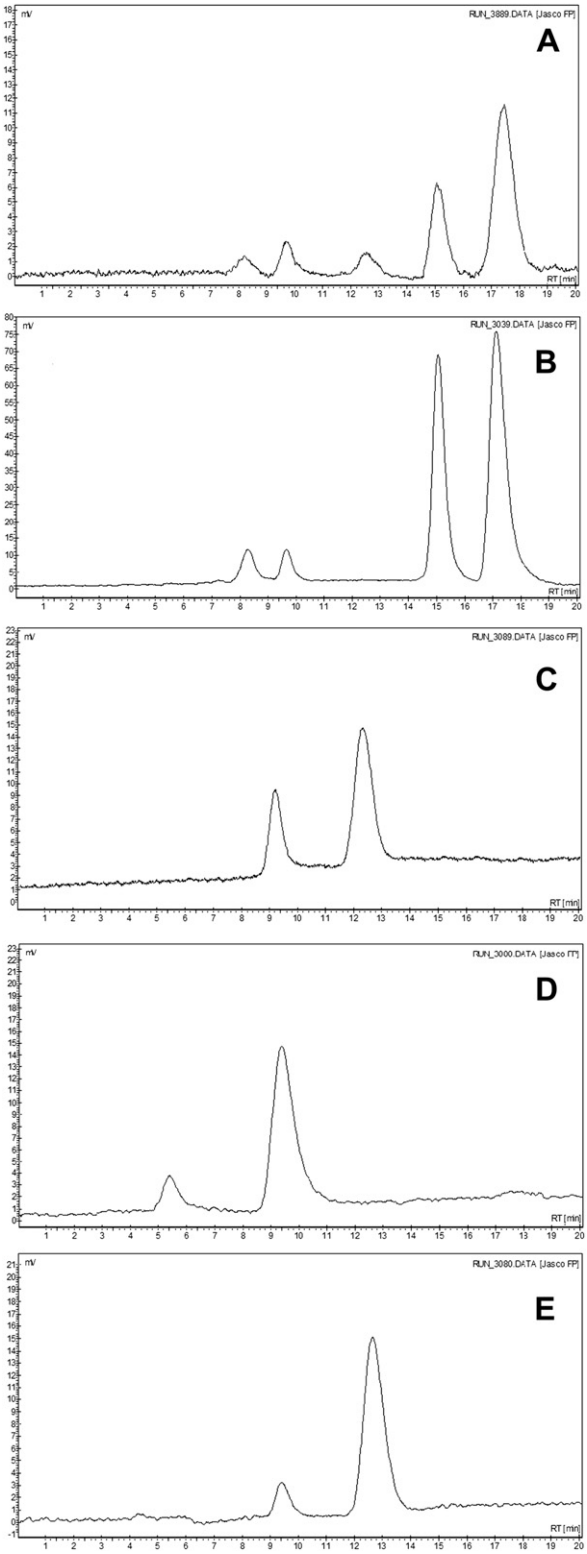

Fig. 1. Representative HPLC chromatograms with fluorescent detection of samples containing 15 μM PST analytical standards (neoSTX and STX; GTX4/GTX1 epimers and GTX3/GTX2 epimers) after incubation with UDPGA/NADPH. A. Analytical standards of Gonyautoxins 15 μM; B. HPLC-FLD chromatograms of GTX3 and GTX2 epimers after incubation with UDPGA/NADPH and microsomes for 5 h. C. Analytical standards of Neosaxitoxin and Saxitoxin 15 μM. D. HPLC-FLD chromatograms of Neosaxitoxin sample injected to the HPLC after the standard incubation of neoSTX with UDPGA/NADPH and microsomes for 5 h. E. HPLC-FLD chromatograms of Saxitoxin sample injected to the HPLC after the incubation with UDPGA/NADPH and microsomes for 5 h.

2.8. Analysis of the stability of glucuronides in the conditions of the experiments

Molecular integrity of the glucuronides was continuously controlled during the transport experiment. Samples were assessed using HPLC/ESI-IT/MS to detect the incidental appearance of glucuronate, which would have indicated degradation. At the end of each experiment, total samples were taken and analyzed the same way. The initial and final amounts of glucuronides were also compared to observe any loss. The degradation of all glucuronides used in this work remained under 1% (Csala et al., 2004).

2.9. Statistical analysis

Experiments were performed in triplicate, with each of the values of a single set of experiments corresponding to the mean of a minimum of three determinations ± SE.

Metabolite formation was quantitated by comparing peak areas in incubates to a standard curve containing known amounts of metabolite. Standard curve correlation coefficients (r^2) were 0.99.

3. Results

Fig. 1, shows HPLC-FLD chromatograms of samples containing 15 μM of pure PST as analytical standard and reference material (Fig. 1A. GTX4/GTX1 and GTX3/GTX2 epimers and Fig. 1C. neoSTX and STX show the analytical standard mixture). This figure, also shows samples after incubation with UDPGA/NADPH and the microsomal fraction. PST was incubated for 5 h and aliquots were taken every 1 h to a total of 5 h, starting 30 min after beginning of incubation. The Gonyautoxin standard mixture, exhibits five peaks GTX4, GTX1, GTX5, GTX3 and GTX2 with retention times of 8:11, 9:72, 12:75, 15:07 and 17:29 min respectively (Fig. 1A) and the neoSTX and STX standard mixture shows two peaks with retention times of 9:62, 12:31 min respectively (Fig. 1C).

Fig. 1B, shows the HPLC-FLD chromatograms of samples after incubation of GTX3/GTX2 epimers with UDPGA/NADPH and microsomes for 5 h. This figure shows four peaks, the larger two correspond to GTX3 and GTX2 with retention times of 15:03 and 17:25 min respectively. Other two smaller peaks shown would correspond to GTX4 and GTX1, because they exhibit similar retention times as the GTX4/GTX1 epimers standard (Rt = 8:18 and 9:68 min respectively, Fig. 1B). Fig. 1D, shows HPLC-FLD chromatograms of samples of neoSTX with UDPGA/NADPH and microsomes for a 5-h incubation time. The highest peak corresponds to neoSTX (Rt = 9:72 min) and the other, with a retention times of 5:13 min, corresponds to an oxidation product of neoSTX. Fig. 1E, shows HPLC-FLD chromatograms of samples after 5 h incubation of STX with UDPGA/NADPH

and microsomes. The peak with longer retention time corresponds to STX (Rt = 12:29 min) and the other, retention time of 9:67 min corresponds to neoSTX, according to the analytical standard mixture reference. This neoSTX is an oxidation product of STX.

Control experiments, without microsomes or UDPGA did not show oxidation or glucuronidation products and post-incubation chromatograms were the same, showing identical amount of GTX3/GTX2 epimers, neoSTX or STX with no biotransformation. Nevertheless, the reaction of any PST with glucuronic acid resulted in a significant conjugation of toxins. The glucuronidation reaction was further confirmed by HPLC/ESI-IT/MS analysis.

PST were extensively oxidized and glucuronodized by human liver microsomes. Less than 15% of the original PST remained after 5 h of incubation.

The new toxin analogs coming from neoSTX, GTX3/GTX2 epimers or STX due to the oxidation or glucuronidation reactions were further identified using HPLC/ESI-IT/MS. Results are summarized in Table 1. Molecular ions of each compound are shown: GTX3/GTX2 epimers, Glucuronic-GTX3/GTX2 epimers, GTX4/GTX1 epimers, Glucuronic-GTX4/GTX1epimers, neoSTX, Glucuronic-neoSTX, STX, Glucuronic-STX and UDPGA. All molecular ions were detected in the negative ion mode. The HPLC/ESI-IT/MS analysis also revealed that all the glucuronodized metabolites were monoglucuronides of the corresponding aglycones

The mass spectra of all these PST metabolites, coming as final products of glucuronidation and oxidation reactions, are shown in Figs. 2–5.

For each reaction was used UDPGA standard, which indicates mass fragmentation patterns of direct identification of each reaction glucuronidation. The spectra obtained in negative polarity signal is observed 579 *m/z* corresponds to the way no-ionic (species $[M+H]^-$), plus a series of signals that correspond to adducts such as 597 *m/z* (species $[M+NH_4]^-$), 618 *m/z* (species $[M+K]^-$), 1158 *m/z* (species $[2M+H]^-$) 1176 *m/z* (species $[2M+NH_4]^-$) and other signals fragments correspond to stable from the main compound: 323 *m/z* and 403 *m/z* (Table 1).

Fig. 2A, shows a control experiment, and the classical mass spectrum of the GTX3/GTX2 epimers with a negative Zwitterion of M^- 394 *m/z*, and Fig. 2B, shows a major peak – the Zwitterion of M^- 570 *m/z* – which corresponds to

Table 1
HPLC/ESI-IT/MS analyses of proposed PST metabolites.

Compound	Molecular weight	HPLC/ESI-IT/MS	
		Molecular ion	Fragments (*m/z*)
GTX3/GTX2	395	394	297.5, 311.1, 365.9
Gluc-GTX3/GTX2	571	570	194.1, 322.6, 395.2
GTX4/GTX1	411	410	210.8, 254.9, 323
Gluc-GTX4/GTX1	589	588.4	255.1, 323.3, 403.3
neoSTX	315	314	99, 175, 603, 619, 625
Gluc-neoSTX	493	492	226, 342, 379, 418, 438
STX	299	298	99, 137, 160
Gluc-STX	476	475	163, 288, 316, 388, 410, 418
UDPGA	580	579	124, 323, 403, 597, 618, 1158, 1176

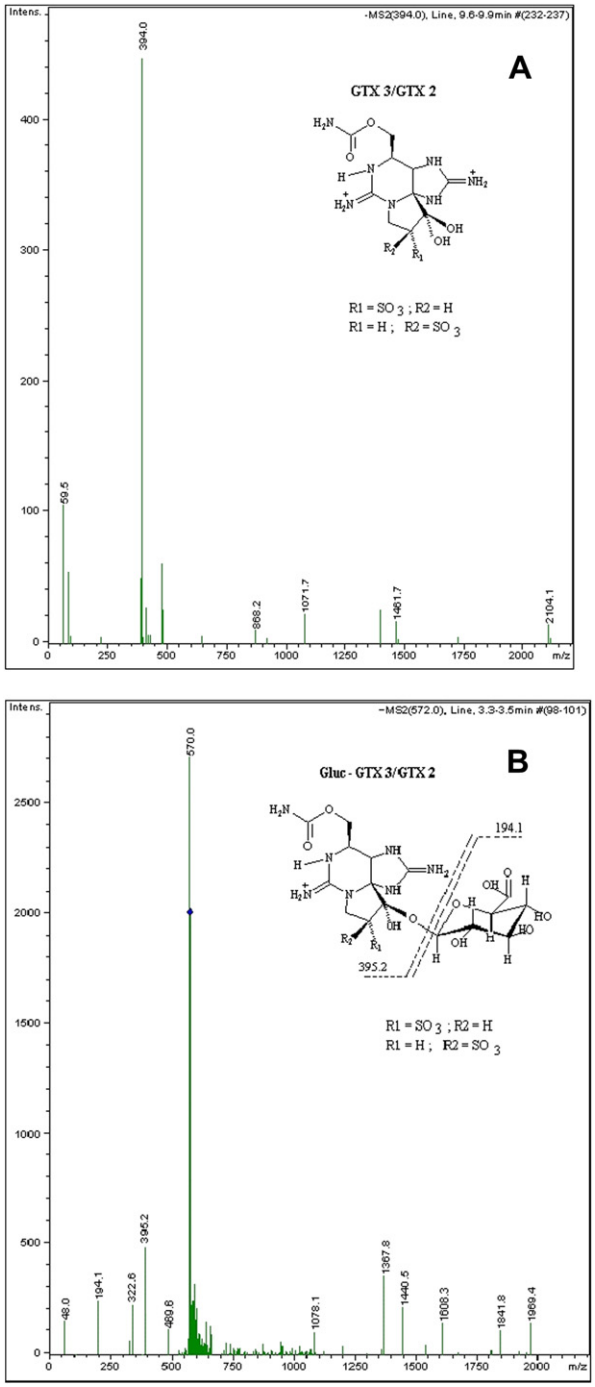

Fig. 2. Negative ion electrospray mass spectrum of GTX3/GTX2 isomers. A. Negative mass spectrum of GTX3/GTX2 isomers with M^- 394 *m/z* and B. HPLC/ESI-IT/MS ion electrospray mass spectrum of the Gluc-GTX3/GTX2 isomers with M^- 570 *m/z* showing the fragmentation pattern with the daughter ions at 194.1, 322.6, 395.2 *m/z*.

Glucuronic-GTX3/GTX2 epimers. This figure also shows the daughter ion spectrum with peaks at 194.1; 322.6; 395.2 *m/z*. Daughter ion spectrum of M^- 194.1 *m/z* is the glucuronic ion (Fig. 2B).

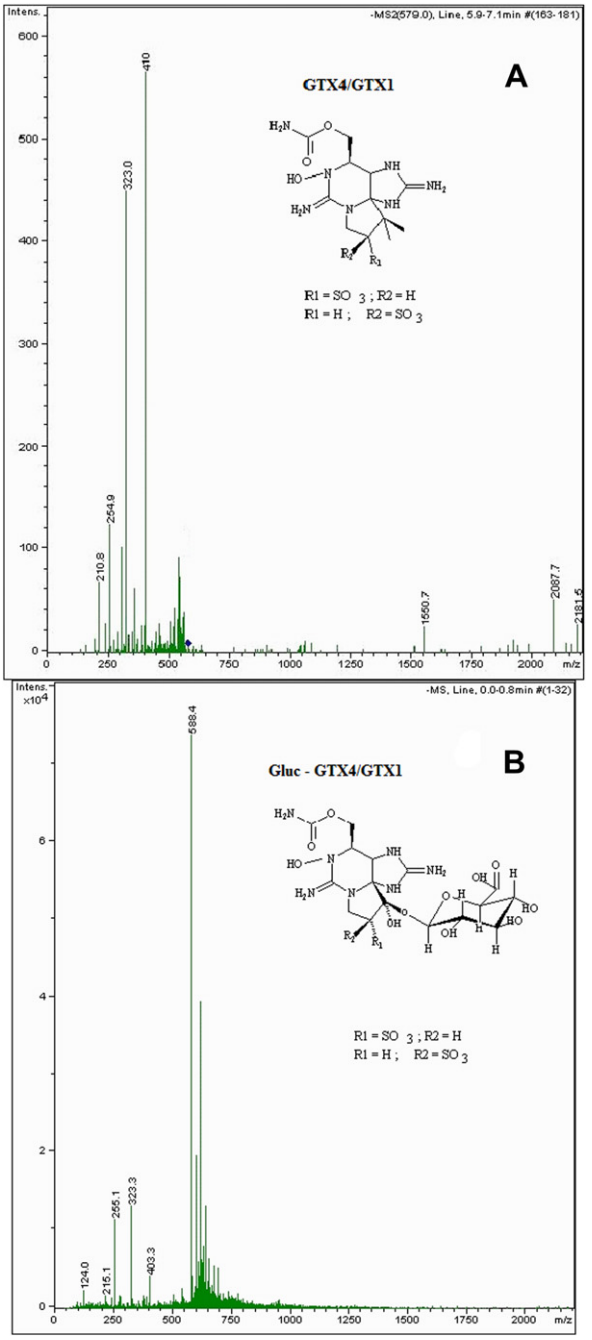

Fig. 3. Negative ion electrospray mass spectrum of GTX4/GTX1 isomers. Negative ion electrospray mass spectrum of the GTX4/GTX1 isomers was obtained after oxidation reaction done in presence of the incubation reaction with human liver microsomal mixture. A. Negative mass spectrum of GTX4/GTX1 isomers with M^- 410 *m*/*z* B. HPLC/ESI-IT/MS ion electrospray mass spectrum of the Glucuronic-GTX4/GTX1 isomers with M^- 588 *m*/*z* and showing the fragmentation pattern with the daughter ions at 255.1, 323.3, 403.3 *m*/*z*.

Fig. 3A shows a control experiment with GTX4/GTX1 epimers. It exhibits a negative Zwitterion ion of M^- 410 *m*/*z*. Fig. 3B shows the Glucuronic-GTX4/GTX1 epimers, which identify molecular ion peak M^- 588.4 *m*/*z*. The Glucuronic-

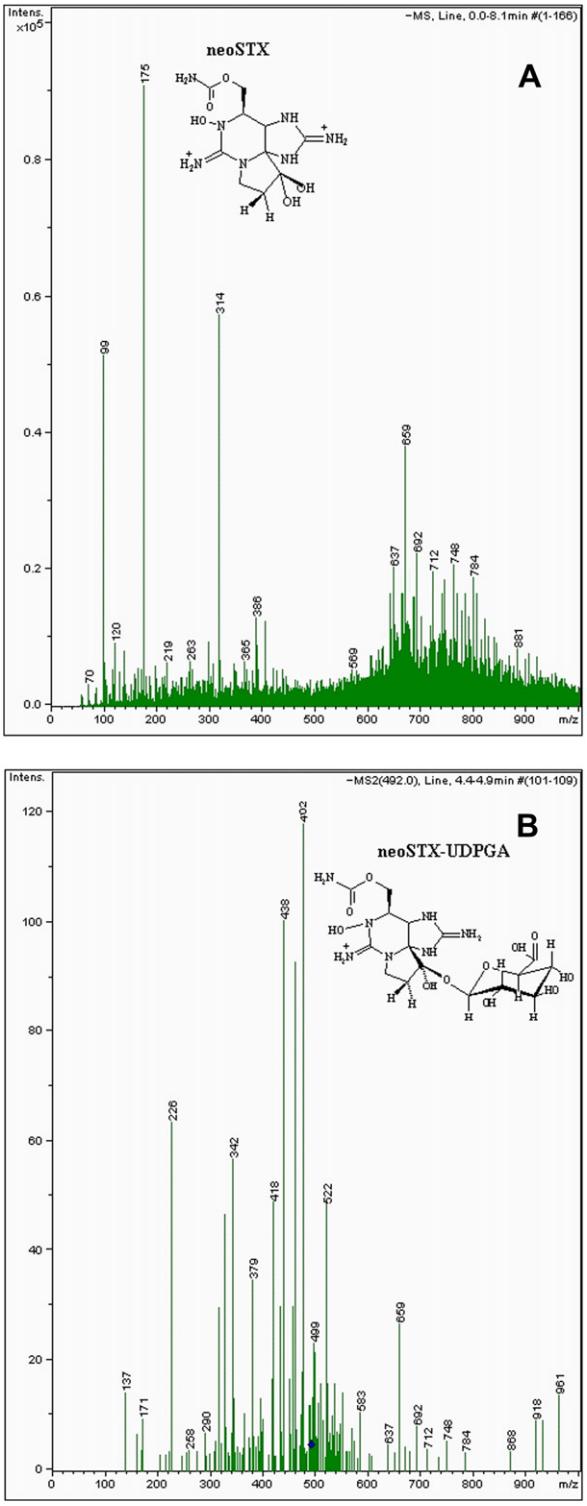

Fig. 4. Negative ion electrospray mass spectrum of Neosaxitoxin. A. Negative mass spectrum of neoSTX with M^- 314 *m*/*z* and B. HPLC/ESI-IT/MS ion electrospray mass spectrum of the Glucuronic-neoSTX with M^- 492 *m*/*z* showing the fragmentation pattern with the daughter ions at 226, 342, 379, 418, 438 *m*/*z*.

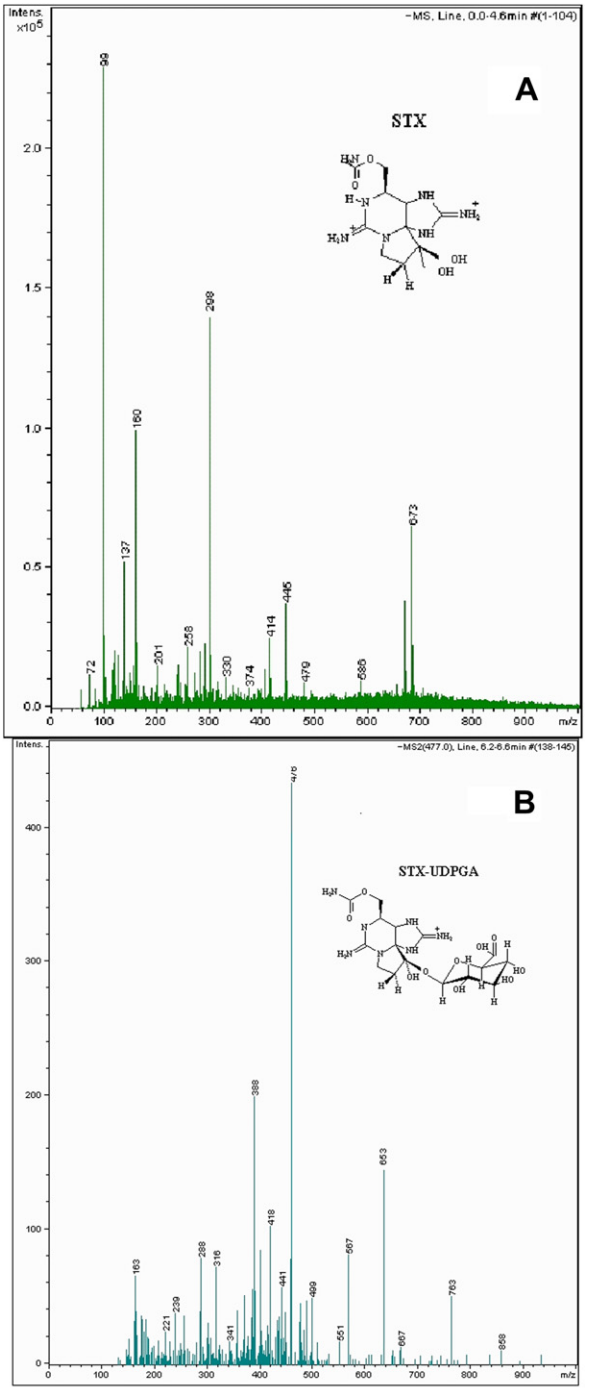

Fig. 5. Negative ion electrospray mass spectrum of STX. Negative ion electrospray mass spectrum of the STX was obtained after oxidation reaction done in presence of the incubation reaction with human liver microsomal mixture. A. Negative mass spectrum of STX with M^- 298 *m*/*z*. B. HPLC/ESI-IT/MS ion electrospray mass spectrum of the Glucuronic-STX with M^- 475 *m*/*z* and showing the fragmentation pattern with the daughter ions at 163, 288, 316, 388, 410, 418 *m*/*z*.

GTX4/GTX1 epimers were further confirmed by the daughter ion spectrum, which gave peaks at 255.1; 323.3; and 403.3 *m*/*z*.

Fig. 4A – with a negative Zwitterion ion of M^- 314 *m*/*z* – is a control experiment, showing the typical mass spectrum of neoSTX. Identity of this molecular ion peak was further confirmed by the daughter ion spectrum, which main decomposition consisted of 99 and 175 *m*/*z* (Fig. 4A). The mass spectrum of Glucuronic-neoSTX was confirmed with a negative Zwitterion of M^- 492 *m*/*z* (Fig. 4B). The identity of this molecular ion peak was further confirmed by the daughter ion spectrum, which gave peaks at 226, 342, 379, 418 and 438 *m*/*z*. Although the signal 580 *m*/*z* for UDPGA was not observed, looking at very low intensity as their adducts: 603 *m*/*z* (species $[M + Na]^-$), 619 *m*/*z* (species $[M + K]^-$), 625 *m*/*z* (species $[M + 2Na - H]^-$) 663 *m*/*z* (species $[M + 2H + ACN]^-$) indicating the indirectly the presence of UDPGA (Fig. 4B).

Finally, Fig. 5A shows the mass spectrum of STX with the classical negative Zwitterion of M^- 298. The product ion spectrum from fragmentation of $[M + H]^-$ was dominated by 99, 137 and 160 *m*/*z*, produced by the cleavage of the STX molecular structure (Fig. 5A). The mass spectrum of Glucuronic-STX showed a distinctive negative Zwitterion of M^- 475 *m*/*z*. The identity of this molecular ion peak was further confirmed by the daughter ion spectrum, with representative peaks at 163, 288, 316, 388, 410 and 418 *m*/*z*. The last one (M^- 418 *m*/*z*), should be STX with a double glucuronidation, one at $C_{12,}$ as it usually happens in other PSTs and a second one at N^+_9-glucuronide (Fig. 5B).

Kinetics Constants of the glucuronidation reaction were determined from the glucuronidation rate versus substrate concentration plots of three substrates: neoSTX, GTX3/GTX2 epimers and STX. The glucuronidation reactions of the three paralytic shellfish toxins substrate followed a typical Michaelis–Menten kinetics (Fig. 6). The V_{max} of Glucuronic-GTX3/GTX2 epimers synthesis was lower than that of glucuronidation of both neoSTX and STX ($6.8 \pm 1.9 \times 10^{-3}$; $8.3 \pm 2.8 \times 10^{-3}$ and $9.7 \pm 2.8 \times 10^{-3}$ pmol/min/mg proteins respectively). Also, the Km for the glucuronidation reaction of the GTX3/GTX2 epimers was less than Km for neoSTX and

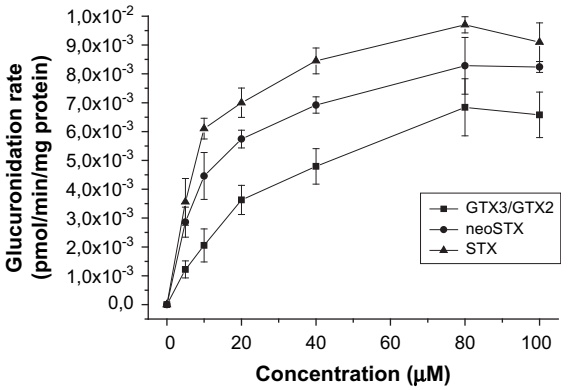

Fig. 6. Rate of *in vitro* formation of Glucuronic-GTX3/GTX2 (–■–), Glucuronic-neoSTX (–●–) and Glucuronic-STX (–▲–) when incubated with human liver microsomes. Each point represents the mean (±SEM) of the three replicates.

STX reactions (20.2 ± 0.12; 27.06 ± 0.23 and 32.02 ± 0.64 μM respectively). Both reactions present a coefficient of variation (CV) of 13% (Fig. 6).

4. Discussion

Removal and metabolization of paralytic shellfish toxins are the most important processes to understand the biotransformation of these compounds when humans are intoxicated by consumption of contaminated shellfish. Until now, there was no available data to understand how these toxins are metabolized by mammalian, humans included. The present study was undertaken to characterize the metabolic pathway of neoSTX, GTX3/GTX2 epimers and STX, the most common and abundant paralytic shellfish toxins worldwide, often above 70% of the total toxin composition of the Chilean contaminated shellfish samples (Lagos, 1998, 2003; Compagnon et al., 1998).

These toxins upon incubation with UDPGA/NADPH in presence of human liver microsomes undergo an immediate oxidation and successive glucuronidation reactions. HPLC/ESI-IT/MS analysis shows conclusive data revealing *in vitro* biotransformation of these compounds.

The first indirect evidence of PST metabolization in humans was reported in 2004 from the analysis of tissue and body fluid samples obtained from patients intoxicated by consumption of contaminated shellfish with PSP. Post-mortem analysis showed the oxidation of STX to neoSTX in liver, kidney and lung tissue samples. Furthermore, oxidation of GTX3/GTX2 epimers occurred in spleen, pancreas, bile, urine, brain and heart, showing as metabolic product

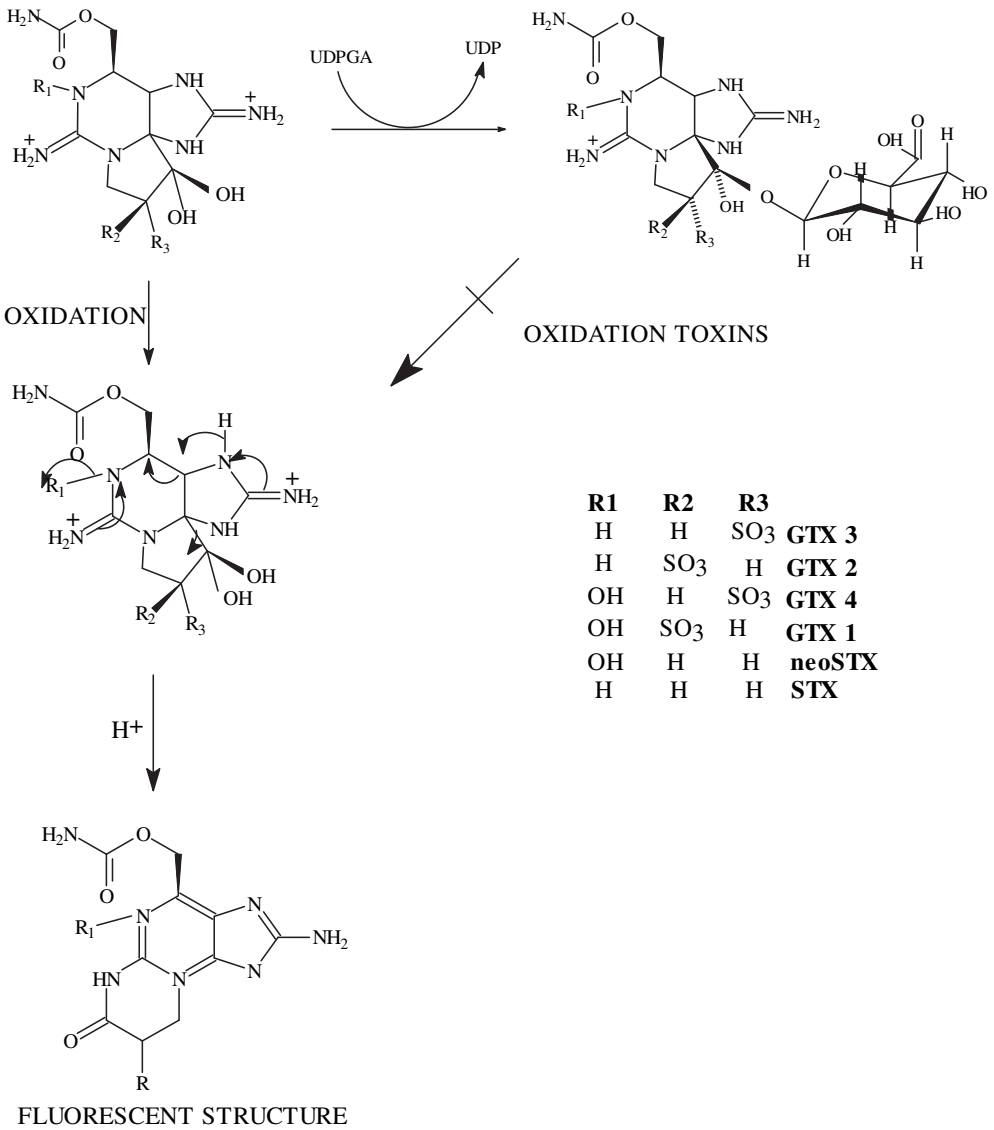

Fig. 7. Conjugated glucuronic acid from the toxin in the hydroxyl carbon 12 of the tetrahydropurine structure, resulting in the conjugation of the structure. The conjugation does not allowed the specific post-column oxidation reaction in the tetrahydropurine nucleus, absolutely required to acquire a fluorescent signal.

the GTX4/GTX1 epimers, which are the oxidative form of GTX3 and GTX2 (García et al., 2004).

Recently, García et al. (2009) reported indirect evidence of a possible glucuronidation reaction of PST. In that report, authors demonstrated glucuronidation by an indirect method, treating toxins previously glucuronodized, with the enzyme β-glucuronidase, quantifying the glucuronidation rate by measuring the increase of fluorescent signal after β-glucuronidase hydrolysis of the glucuronic-PST. Basically, the idea was to release the conjugated glucuronic acid from the toxin, since the glucuronic acid attacks PST in the hydroxyl carbon 12 of the tetrahydropurine structure, resulting in the conjugation of the structure. The conjugation blocks the specific post-column oxidation reaction in the tetrahydropurine nucleus, required to produce a fluorescent signal. The action of β-glucuronidase results in a deconjugation of the glucuronide, therefore releasing the toxin and allowing the post-column oxidation necessary for the fluorescent signal (Fig. 7).

The PST glucuronidation reported here is confirmed by HPLC/ESI-IT/MS analysis by mass spectra of glucuronic-neoSTX, glucuronic-GTX3/GTX2 epimers and glucuronic-STX, showing Zwitterion peaks of M^- 492 *m*/*z*, 570 *m*/*z* and 475 *m*/*z* respectively.

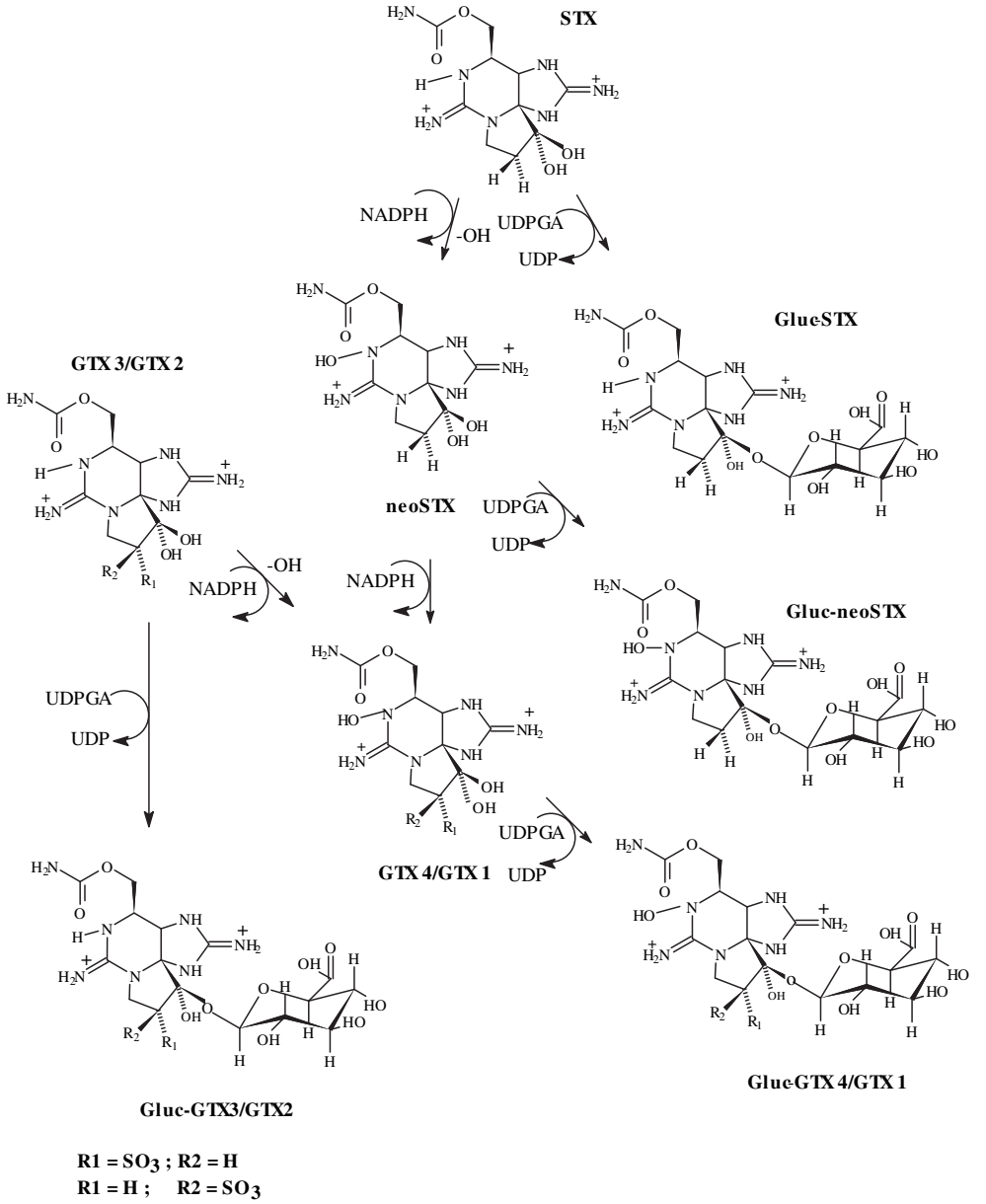

Fig. 8. Metabolic pathway proposed for the oxidation and glucuronidation reactions of paralytic shellfish toxins (PST): neoSTX, GTX3/GTX2 epimers and STX. Gluc-STX = Glucuronic-Saxitoxin. Gluc-neoSTX = Glucuronic-neoSaxitoxin. Gluc-GTX3/GTX2 = Glucuronic-Gonyautoxins 3 and 2. Gluc-GTX4/GTX1 = Glucuronic-Gonyautoxins 4 and 1.

The oxidation and glucuronidation of neoSTX, GTX3/GTX2 epimers and STX are of toxicology and clinical relevance. They provide direct evidence of metabolization of these toxins by humans, and that enzymes that prepare foreign substances for excretion (detoxication enzymes), are present in the microsomal fraction of fresh human liver tissue. Glucuronidation reaction is the major phase II conjugation reaction and, as is clearly shown in this study, also takes place in humans during metabolization of PSTs.

The glucuronidation rate depends merely on the activity of UDPGTs, which are located in the endoplasmic reticulum, and on the availability of UDPGA (Belanger et al., 1990). In general, UDPGTs have high Km values, but also a high capacity with broad and overlapping substrate specificities (Visser et al., 1993). This is also confirmed by our data and the Kinetics Constants reported here.

Conjugation by glucuronidation is the major route of metabolization of all xenobiotics with functional hydroxyl, thiol, amine or carbonyl groups (Burchell and Coughtrie, 1989). As a result, substances become more hydrophilic, which enhances their renal excretion (Kroemer and Klotz, 1992). In this study, the oxidation and sequential glucuronidation reactions using PST as substrates are demonstrated.

Previous data using an animal model (cats) *in vivo* established that the intoxication potency of STX was significantly higher than neoSTX and GTX3/GTX2 epimers (Andrinolo et al., 1999, 2002; Lagos and Andrinolo 2000). The highest toxicity showed by STX in this model *in vivo* is easily explained since cats do not have capability to develop glucuronidation reaction. With this exception, liver of mammals produces glucuronides as a mayor metabolic product (Kasper and Henton, 1960).

In conclusion, we suggest that the sequence showed in Fig. 8, should be the obligatory sequence for the detoxication and metabolization of PST in human, being both oxidation and glucuronidation the initial detoxification reactions for the excretion of these toxins. As a result, the PST oxidation and glucuronidation are the hepatic conversion of STX and GTX3/GTX2 epimers into neoSTX or GTX4/GTX1 epimers respectively. Synthesis of the glucuronic-PST complex is the starting point of PST detoxification in humans, just like any other xenobiotic.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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