Detection of Paralytic Shellfish Poisoning (PSP) Toxins in Philippine Mussel Samples by Electrospray Mass Spectrometry

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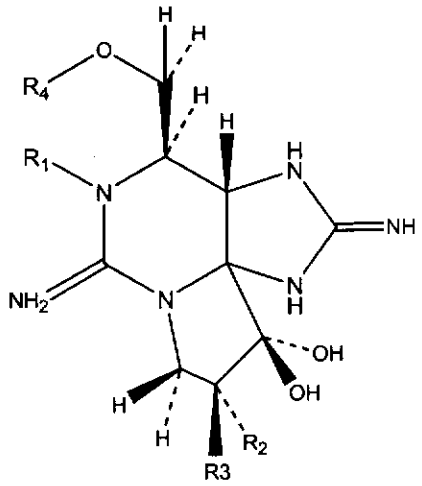
The occurrence of toxic red tide outbreaks is an environmental and public health hazard in the Philippines. Thus, it is necessary to develop monitoring programs to protect the shellfish industry and the general public. Previous methods for the detection of paralytic shellfish poisoning (PSP) toxins make use of mouse bioassays and/or fluorescence detection through High Performance Liquid Chromatography (HPLC). The mouse bioassay, while cheap and rapid, requires a large amount of sample, and is capable of detection of toxin concentrations that are already near the regulatory limit. Fluorescence HPLC analysis of derivatized PSP samples is destructive and is not sufficiently reproducible. We report a new procedure for the rapid detection of components of samples contaminated by Pyrodinium bahamense var compressum using a combination of reverse-phase HPLC and electrospray mass spectrometry. The procedure is fast and requires minimal amounts of sample, so that purified toxins need not be derivatizated as a prerequisite for its detection. In addition, results from this study complement earlier findings that the main toxic components of Philippine toxic red tide are neosaxitoxin, decarbamoylsaxitoxin, and gonyautoxin.

INTRODUCTION

The existence of toxic red tide compounds was demonstrated when saxitoxin (STX) was first isolated by Sommer and Meyer by ion exchange chromatography[1]. Due to its highly polar and nonvolatile nature, and the absence of a chromophore in its structure, it was not until almost forty years later before Henry Rapoport, Jon Bordner, and coworkers were able to purify and crystallize saxitoxin in order to determine its structure[2].

The mouse bioassay was first developed by Hermann Sommer as a means to monitor the presence of paralytic shellfish poisoning toxins (Figure 1) and is still widely adopted for red tide monitoring purposes[1]. The mouse bioassay is significantly limited in its minimum detection level of approximately 37 µg/100g meat, which is close to the maximum allowed level of 80 µg/100 g meat[3]. Nevertheless, the assay is simple and cheap to operate. Moreover, it is a direct measure of toxicity that is an important consideration for seafood safety.

The need for rapid and sensitive methods for the detection of red tide toxins led to the development of monitoring techniques utilizing High Performance Liquid



Toxin	R_1	R_2	R_3	R4	Molecular Weight
Carbamate Toxins				$OCONH_2$	
STX	H	H	H		301.31
NEO	OH	H	H		317.31
GTX I	OH	H	OSO_3^-		412.36
GTX II	H	H	OSO_3^-		396.36
GTX III	H	OSO_3^-	**H**		396.36
GTX IV	OH	OSO_3^-	**H**		412.36
Sulfamate Toxins				$OCONHOSO_3^-$	
B1	H	H	H		380.36
B2	OH	H	H		396.36
C3	OH	H	OSO_3^-		491.41
C1	H	H	OSO_3^-		475.41
C2	H	OSO_3^-	**H**		475.41
C4	OH	OSO_3^-	**H**		491.41
Decarbamyl Toxins				OH^-	
dc-STX	H	H	H		258.28
dc-NEO	OH	H	H		274.28
dc-GTX I	OH	H	OSO_3^-		369.33
dc-GTX II	H	H	OSO_3^-		353.33
dc-GTX III	H	OSO_3^-	**H**		353.33
dc-GTX IV	OH	OSO_3^-	**H**		369.33

Figure 1. Structures of the paralytic shellfish toxins

Chromatography (HPLC) with fluorescence detection[4,5,6]. Depending on the toxins present, the limit of detection for HPLC can be as low as 10-30μg STX/100g and accuracy can be +/ -10%[4,5,6]. When toxin levels are approximately 200μg or below by the mouse bioassay, the fluorescence HPLC method for toxin detection is used. However, this method is destructive to the toxins, thus the need to have large amounts of samples in order to isolate and purify them[4,5,6].

The first reported outbreak of toxic red tide in the Philippines occurred in June 1983 in Maqueda Bay, in Samar[7]. This was followed by several incidents of toxic red tide in various locations later in the year. Analysis of the causative organism by Professor Rudolf Hermes revealed the presence of *Pyrodinium bahamense var compressum*[7]. Since the red tide outbreak of 1987, toxic red tide blooms have occurred annually in the Philippines. During this time, several methods for the detection of toxic red tide have been adopted. Among these methods are the estimation of cell density from plankton sampling, the mouse bioassay, the fluorescence HPLC assay, and the blowfly bioassay[8]. In the Philippines at present, the methods employed for red tide monitoring are the mouse bioassay and the estimation of cell density from plankton sampling[9].

Rubinson suggested an HPLC method for separation of toxins generated from synthetic reactions by using an analytical C-18 HPLC column and a refractive index detector[10]. The mobile phase used was water with 25 mM formic acid and 3 to 4 mM pentanesulfonic acid. With a flow rate of 1.5 mL/min the separation can be completed in about 10 minutes. The solutes in the effluent were detected using a Waters model R401 to measure differential refractive index. The results of Rubinson's study showed that there were about 2 unresolved products, one of which is saxitoxin as shown by bioassay[10]. We substituted the ion pairing reagent, 0.1% heptanesulfonic acid in water for our mobile phase and monitored our samples using a wavelength of 235 nm.

The use of mass spectrometry coupled to HPLC as a tool for the rapid detection of red tide toxins is fast gaining acceptance[11,12]. Accordingly, we now apply the use of this technique for the detection of paralytic shellfish poisoning toxins in Philippine mussel samples. In this study, we report the detection of decarbamoylsaxitoxin (dcSTX) and neosaxitoxin (NEO) in mussel samples (*Perna viridis*) contaminated with the toxic red tide causative organism *Pyrodinium bahamense var compressum*. Samples were analyzed by a combination of reverse-phase HPLC and electrospray mass spectrometry for the rapid detection of components of paralytic shellfish poisoning contaminated sample. Unlike previous methods for the isolation of PSP toxins, this technique is rapid, requires a small amount of sample, and allows for the simultaneous determination of structurally related compounds. Thus this procedure can be used for monitoring contaminated mussel samples as well as for the preparation of purified PSP toxins.

MATERIALS

Mussel samples were a generous donation from Dr. Lourdes J. Cruz and Ms. Cecile Conaco of Marine Science Institute, U.P. Diliman. Samples were taken from a toxic red tide outbreak which occurred in July 1993 in Limay, Bataan. Three-week old white mice were purchased from BioResearch. Hydrochloric acid (HCl), dichloromethane (DCM), and Sodium Chloride (NaCl) were of analytical grade and were purchased from Sigma Chemical Corp. (St. Louis, MO).

METHODS

Sample Preparation and Toxin Isolation

Mussel meat (wet weight: 391.42 grams) was homogenized in 0.1 M HCl using a Waring Blender. The slurry was filtered through Whatman Filter Paper. An aliquot of the supernatant fluid was set aside for mouse toxicity assay. Nonpolar organic constituents were removed by extracting with dichloromethane

before the aqueous layer was concentrated by using a rotary evaporator and purified by ion exchange chromatography. Aliquots from each layer were tested for toxicity by mouse bioassays.

Ion-Exchange Chromatography

The crude contaminated extract was purified initially by ion-exchange chromatography on an Amberlite strong cation exchanger (column dimensions: 450 x 3.5 cm) equilibrated with 1% acetic acid. The crude extract was eluted by using a concentration gradient of 1% sodium acetate in 1% acetic acid. Fractions (25 ml) were monitored by UV-Vis spectrophotometry (Hitachi U2000) at 235 nm and 330 nm. Equal number of fractions were combined to form four pools (125 ml per pool). All pools were dried by evaporation in an oven (80 °C) before analysis by mass spectroscopy and further purification by HPLC.

High Performance Liquid Chromatography

Dried pooled fractions were redissolved in solvent buffer and filtered through a Sep-Pak column prior to injection into a Merck Lichrosphere analytical reverse phase C18 column (LKB Bromma 2248 HPLC system). Samples were eluted with 0.1% heptanesulfonic acid at a flow rate of 1 mL/min and monitored at 235 nm. Individual peaks from each run were collected from HPLC and submitted for MS analysis.

Mass Spectroscopy Analysis

All samples from HPLC were injected directly into a Finnigan-Mat LCQ LC-MS electrospray mass spectrometer.

RESULTS

Figure 2 shows the results from ion exchange chromatography. Using the plot at 253 nm wavelength, three peaks were observed. The first peak (the highest peak) was observed to be in fractions 1 to 5. Each of the peaks were tested for their toxicities through the mouse bioassay method (Table 2). Only peak 1 demonstrated toxicity in mouse bioassay, although mice injected with aliquots from peak 2 suffered from uncontrollable scratching and shivering which eventually wore off.

Peak 1 was further purified using HPLC. The HPLC chromatogram of peak 1 is shown in Figure 3. Good resolution of the components was achieved although some of these components were not fully resolved. Figure 4 shows the HPLC trace of 3 nanomoles (3 x 10^{-9} mol) saxitoxin standard using HPLC conditions identical to that used in the contaminated mussel samples. Data shows the presence of two small, unresolved peaks eluting at 8.847 and 10.140 minutes which is believed to be saxitoxin and neosaxitoxin, respectively[10]. The

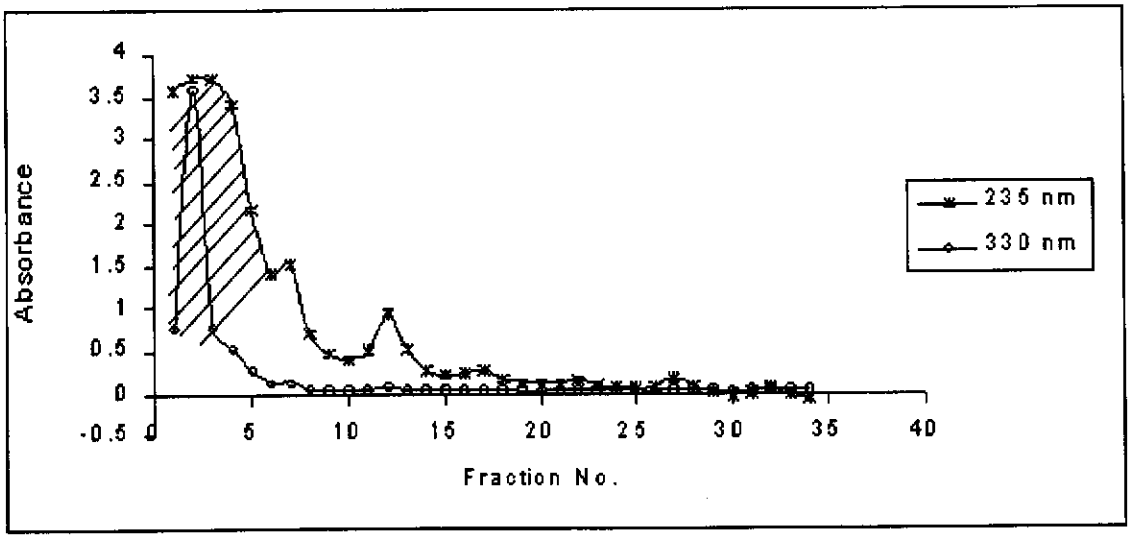


Figure 2. Plot of the absorbances of the fractions from ion-exchange chromatography at 235 nm and 330 nm. Hatched peak in this chromatogram denotes the toxic fractions from mouse bioassay.

Table 1. Purification Table of Mussel Samples

Sample	Volume of Sample Injected (mL)	Weight of Mouse (g)	Death Time	Mouse Units (MU)	Total MU
Homogenate	1	14.30 13.24 12.10	0:14 6:39 5:55	* 0.965 1.017 0.991	1486.5
Aqueous Layer	1	10.06	1:37	6.950	5212.5
Ion-Exchange					
Pool 1	1	10.99	12:01	0.588	17640.0
Pool 2	1	8.21	—	inactive	
Pool 3	1	8.99	—	inactive	
Pool 4	1	8.93	—	inactive	

retention time of the two peaks (denoted by arrows) from Figure 3 are consistent with published results [10].

Each individual peak from HPLC were submitted for electrospray mass analysis (ESI-MS). The mass spectral profile of the hatched peak from Figure 2 is shown in Figure 5. A m/z at 265.8 and 318.9 was observed which corresponds to $[dcSTX+Na]^+$ and $[NEO+H]^+$.

DISCUSSION

Toxin amounts of 9.05×10^{-9} g can thus be detected using HPLC and electrospray mass spectrometry. In comparison, the detection limit for the mouse bioassay is 37×10^{-6} g, although results can be obtained within 6 minutes. Quantitative and qualitative data for the presence or absence of PSP toxins can be obtained within a time span of ten minutes, using a sample volume of 20×10^{-6} liter. In contrast, quantitative analysis can be obtained in the mouse bioassay only after repeated injection into mice.

The results from this study is in agreement with earlier findings on the presence of decarbamoylsaxitoxin (dcSTX) in Philippine mussel samples[13]. In addition to decarbamoylsaxitoxin, neosaxitoxin (NEO) was also detected in the same sample. Oshima theorized that the absence of neosaxitoxin in Philippine mussel samples was due to the instability of the toxin and the inherent inability of the fluorescence HPLC method to detect for this particular compound. Thus, the presence of neosaxitoxin could only be conjectured based on the toxin profiles of Borneo and Palau[13]. A study on the toxin profile of *Pyrodinium bahamense var compressum* demonstrates the presence of neosaxitoxin, decarbamylsaxitoxin, and gonyautoxin[14]. Our results are in agreement with these findings. Furthermore, the mass spectral profile of peak 1 (prior to injection intoHPLC) shows m/z peaks at 282.9, 318.7, and 412.0 which corresponds to [dcSTX+Na]+, [Neo+H]+, and [GTX+H]+ (data not shown).

In summary, the use of reverse phase HPLC and electrospray mass spectrometry is an acceptable alternative for the rapid detection of samples contaminated with the toxic red tide causative organism *Pyrodinium bahamense var compressum*. Data obtained is in agreement with previous findings using the mouse bioassay technique and the fluorescence HPLC technique. A more compelling evidence for the presence of these toxins could be provided by the use of tandem mass spectrometry.

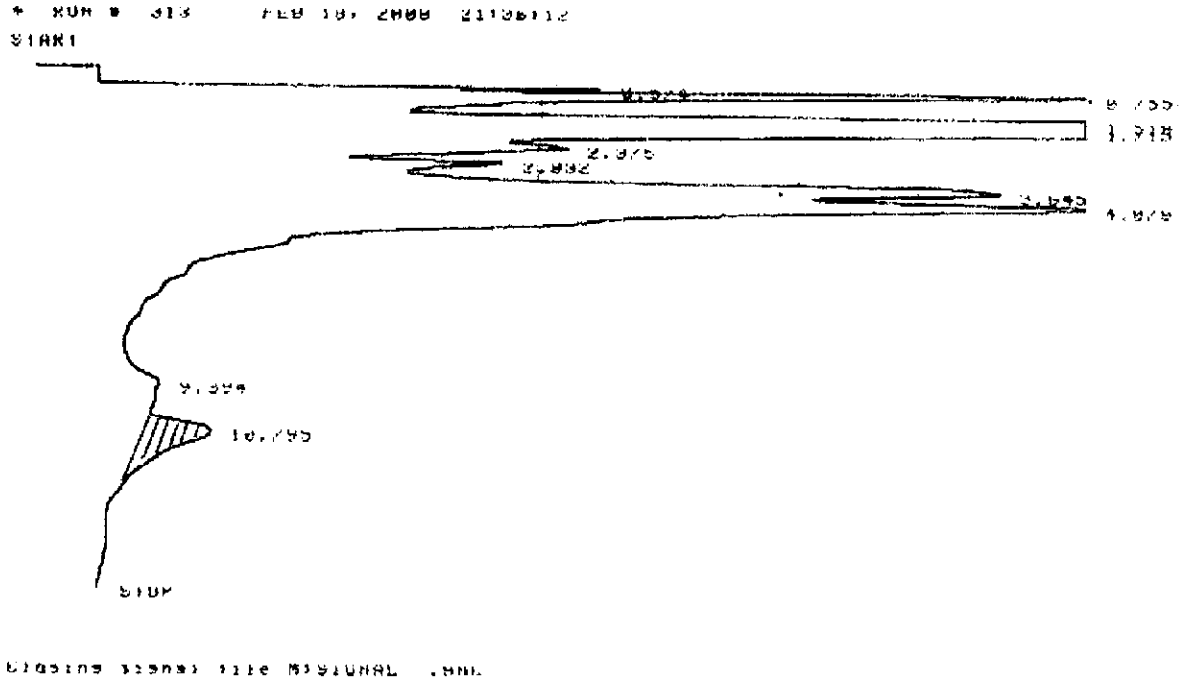


Figure 3. HPLC chromatogram of peak 1 from ion exchange chromatography. Hatched peak eluting at retention time of 10.799 minutes denotes location of neosaxitoxin from mass spectrometry.

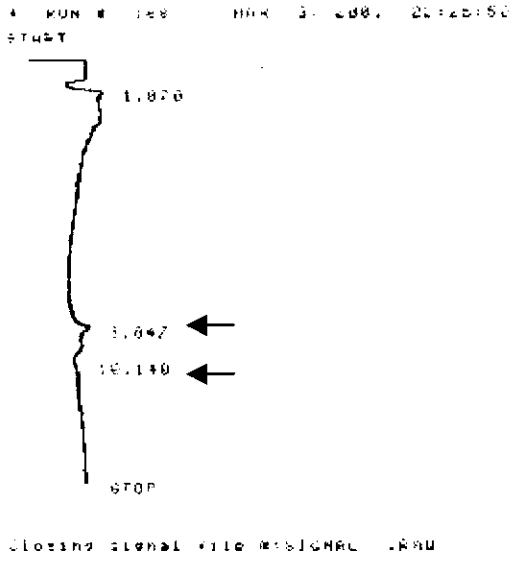


Figure 4. HPLC profile of 3 nanomoles saxitoxin standard.

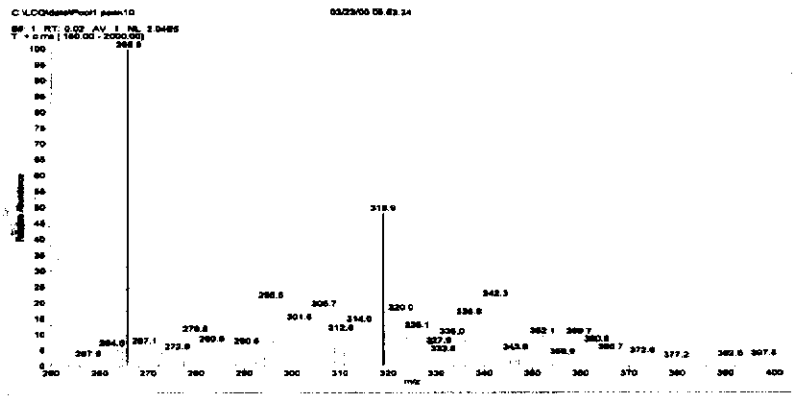


Figure 5. Electrospray Mass Spectra of hatched peak from Figure 3. M_1 denotes decarbamysaxitoxin (dcSTX) while M_2 denotes neosaxitoxin (neo).

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