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Neurotoxic shellfish poisoning and brevetoxin metabolites: a case study from Florida[1]

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Abstract

In June of 1996, three family members were diagnosed as suffering from neurotoxic shellfish poisoning (NSP) as a result of eating shellfish harvested from Sarasota Bay, Florida. Urine from two of these patients and extracts of shellfish collected from the same location were analyzed by radioimmunoassay (RIA) and by receptor binding assay. Activity consistent with brevetoxins was present in both urine and shellfish extracts. High performance liquid chromatographic (HPLC) analysis of shellfish extracts demonstrated multiple fractions recognized by specific anti-brevetoxin antibodies, suggesting metabolic conversion of parent brevetoxins. Affinity-purification of these extracts yielded four major peaks of activity. One peak was identified by HPLC-mass spectroscopy (HPLC-MS) to be PbTx-3, which was likely produced metabolically from the dominant parent toxin PbTx-2. No PbTx-2, however, was detected. Other peaks of activity were determined to consist of compounds of apparent masses of $[M+H]^+$ of 1018, 1034, and 1005. These higher masses are suggestive of conjugated metabolites, but their structures have yet to be determined. The material associated with these latter three peaks were recognized by both RIA and receptor binding assay, but they quantitated differently. This finding suggests that these metabolites react differently in the two assays, and this result may have important

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implications for seafood safety and regulation. We suggest these metabolites to be the true cause of NSP, and they should be taken into account during regulatory testing. Published by Elsevier Science Ltd.

1. Introduction

In June of 1996, three family members were diagnosed as suffering from neurotoxic shellfish poisoning as a result of eating shellfish harvested from Sarasota Bay, Florida. NSP is caused by the ingestion of brevetoxins (PbTx-1, PbTx-2, etc), cyclic polyether neurotoxins produced by the red tide dinoflagellate *Gymnodinium breve* (formerly *Ptychodiscus breve*) (Steidinger et al., 1973; Baden, 1983). Urine from two of these patients and extracts of shellfish collected from the same location were analyzed by radioimmunoassay and by receptor binding assay. Brevetoxin-like activity was present in both urine and shellfish extracts. In this paper, we unequivocally confirm brevetoxin exposure by these analytical methods. Further, we demonstrate the presence of brevetoxin metabolites in toxic shellfish and suggest the ingestion of these metabolites to be the true cause of NSP in humans. Finally, we demonstrate that these metabolites are recognized differently by radioimmunoassay and receptor binding assay, and we suggest that these differences be carefully considered by the regulatory community.

2. Materials and methods

On 15 June 1996, at approx. 8 p.m., three family members ate boiled whelks (*Busycon contrarium*) harvested from Sarasota Bay. The family members consisted of the father (age 49), and two young children (male, age 2 yr 9 mo and female, age 3 yr 9 mo). The father reportedly ate ''several'' whelks, ingesting the ''soft'' part of the meat. Amounts ingested by the children are unknown. Approximately 1 h after eating, the father developed perioral numbness, numbness of the extremeties and vomiting. The children were awakened from sleep and immediately complained of abdominal pain and began to vomit. All three victims were seen in the local emergency room approx. 3 h post-ingestion. The father was stable upon admittance and complained only of nausea and fatigue; he was discharged 7 h post-ingestion. The male child lost consciousness and experienced convulsions on the way to the hospital. The female child was actively vomiting, tachycardic (pulse 130) and short of breath upon admittance, and she experienced seizures approx. 30 min later. Both children were intubated and stabilized, and discharged from the hospital 2 (male) and 3 (female) days later. Urine and serum samples were collected from both children upon hospital admittance and again during a followup visit with their pediatrician 4 days later.

The shellfish was supplied to the family by a friend who routinely harvested from the bay for his own consumption. Subsequent interviews with the family and

supplier resulted in the location of harvest and an empty shell from the night of the offending meal. This empty shell was the basis of the taxonomic identification of the toxic organism. Approximately 1 month after the incident, additional whelks and two co-occurring species of clams were harvested from the same location, frozen, and shipped to the US Food and Drug Administration Office of Seafood for analysis.

2.1. Shellfish extraction

Two species of clams were collected: adult specimens of *Chione cancellata* and juvenile specimens of *Mercenaria* spp (probably either *campechiensis* or *mercenaria*, or a cross). Both clam species measured 2–2.5 cm in length. Only one species of whelk (*Busycon contrarium*) was found and collected. Shellfish were thawed and removed from their shells. Whelk digestive tissue was separated from that of the muscular feet and extracted separately. Each species of clam was extracted without division of tissues. Samples were homogenized in 2 volumes acetone and filtered through Whatman ♯1 filter paper. This extraction was repeated three times, and the pooled acetone fractions were evaporated to dryness and redissolved in aqueous methanol at 2 ml/g original tissue weight. This methanolic fraction was extracted 3 times with equal volumes of hexane to remove neutral lipids. The extracted methanolic fraction was then evaporated to dryness under vacuum and re-dissolved in 100% MeOH at 1 ml/g original tissue weight.

2.2. Urine extraction

Urine was extracted with C_{18} solid phase extraction (SPE) columns (Bond Elut, Analytichem International, Harbor City, CA, USA, 6 cm^3 size). The column was first pre-conditioned with 5 ml MeOH followed by 5 ml water. Urine (5 ml) was then slowly pushed through the column. After washing the column with 3 ml water followed by 3 ml 70% MeOH, the bound material was eluted with 3 ml 100% MeOH. This methanol fraction was then evaporated to dryness and re-dissolved in 2.5 ml phosphate-buffered saline (PBS) containing 0.01% emulsifier (Alkamuls EL-620, Rhone-Poulenc, Cranbury, NJ, USA).

2.3. Radioimmunoassay

This radioimmunoassay (RIA) is specific for brevetoxins sharing the PbTx-2-type backbone and is fully described elsewhere (Poli and Hewetson, 1992; Poli et al., 1995). Standard curves were constructed by incubating antiserum (1:7500 dilution in PBS containing 0.01% emulsifier) with increasing concentrations of PbTx-3 in the presence of a constant concentration (0.1 nM) [^{3}H]PbTx-9 (Chiral Corp, Miami, FL) in a total volume of 1 ml. After incubation for at least 1 h at 4°C, 0.5 ml of a 1:160 dilution of 10% dextran-coated charcoal in PBS was added, mixed, and incubated for an additional 15 min. Centrifugation for 15 min at 1500 ***g*** sedimented the charcoal and separated bound from free label. The clear

supernatant (1 ml) was tranferred to scintillation vials, acidified with 50 μl glacial acetic acid, and the bound radioactivity counted on a scintillation counter. Shellfish extracts were analyzed by drying aliquots of the methanolic extract in the tubes prior to addition of assay reagents. Urine extracts were tested directly. Results were quantitated by comparison of unknowns to a standard curve and expressed as PbTx-3 equivalents/ml.

2.4. Receptor binding assay

This assay measures binding of the toxins to the native receptor site on the voltage-sensitive Na^+ channel; it is described fully in Poli et al. (1997). The assay was performed in a binding buffer containing (mM): HEPES 50 (pH 7.4), choline chloride 130, glucose 5.5, magnesium sulfate 0.8 and potassium chloride 5.4. The binding medium also contained 1 mg/ml bovine serum albumin (BSA) and 0.01% Alkamuls EL-620. Standards or unknown samples were dried in 12 × 75 mm test tubes and then taken up in binding medium containing 0.25 nM (final concentration) [^{3}H]PbTx-9. Rat brain membranes (75–100 μg) suspended in 0.1 ml binding medium were added, mixed, and incubated for 1–2 h in an ice bath. Samples were then filtered through Whatman GF/F glass fiber filters and washed 3 times with 3 ml ice-cold wash medium consisting of (mM): HEPES 5 (pH 7.4), choline chloride 163, calcium chloride 1.8, magnesium sulfate 0.8, and 1 mg/ml BSA. The filters were transferred to scintillation vials and incubated overnight in 10 ml scintillation fluid (Ecoscint-A, National Diagnostics, Atlanta, GA). Bound radioactivity was then measured on a scintillation counter and quantitated by comparison to a standard curve. Results were expressed as PbTx-3 equivalents/gram original tissue weight.

2.5. Affinity column preparation

Brevetoxin metabolites were purified using a column constructed by coupling 10 mg of anti-brevetoxin antibody to 3 g of cyanogen bromide-activated Sepharose 4B. The antibody was a goat polyclonal preparation which is fully described elsewhere (Poli and Hewetson, 1992; Poli et al., 1995). Coupling to the gel was accomplished using standard coupling techniques. Briefly, 3 g of gel was hydrated with 10 ml of 1 mM HCl. After centrifugation at 3000 ***g*** for 15 min, the pellet was washed twice with, and finally resuspended in, 15 ml coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). Antibody (2 ml at 5 mg/ml in coupling buffer) was added and the solution rotated gently for 5 h at room temperature. After centrifugation for 15 min at 3000 ***g***, the pellet was resuspended in 15 ml blocking buffer (0.1 M $NaHCO_3$, 0.2 M glycine, pH 8.0) and gently rotated overnight at 4°C. The gel was poured into a column, washed with 3 column volumes of coupling buffer followed by 3 column volumes of PBS. The column was stored at 4°C in PBS containing 0.9% benzoyl alcohol.

2.6. Affinity purification of metabolites

Metabolites were affinity-purified from an extract of toxic oysters collected during a 1996 red tide in Texas and extracted as described above. The extract of 25 g of toxic oysters was subjected to HPLC separation as described below. Fractions were collected at 1 min intervals and assayed for brevetoxin activity by RIA. Fractions corresponding to peaks of brevetoxin-like activity were pooled and subjected to affinity chromatography.

Prior to purification, the column was flushed and equilibrated with PBS containing 0.01% Alkamuls EL-620. Active fractions were pooled, evaporated nearly to dryness, and taken up in 5 ml PBS/0.01% EL-620. This solution was allowed to flow onto the column by gravity, and the column was capped and incubated for 1 h at room temperature. The column was then rinsed with 12 ml PBS (1 ml/min). The buffer was changed to 70% ethanol in PBS. The first 5 ml of elution volume was discarded, and the next 15 ml was collected as the eluted fraction. This fraction was evaporated to dryness under vacuum, taken up in methanol, and subjected to HPLC analysis as described below.

2.7. High performance liquid chromatography

HPLC was performed on a Beckman System Gold (Beckman Instruments, San Ramon, CA, USA) consisting of a Model 126 programmable solvent module and a Model 168 photodiode array detector. Absorbance was monitored at 208 nm. A 4.6×250 mm J-sphere ODS-L80 column (YMC Inc, Wilmington NC, USA) was used for all analyses. The mobile phase consisted of a nonlinear gradient of acetonitrile (ACN) in water, with both phases containing 0.1% formic acid. Specific gradients used for each experiment are described in the figure legends. Fractions were collected every min, and the relative amount of brevetoxin activity present in each fraction was measured by RIA.

2.8. HPLC-mass spectrometry analysis (HPLC-MS)

A Hewlett–Packard (Palo Alto, CA) Model 1050 HPLC pump was used to provide linear gradients and a constant flow rate of 200 μl/min. All chromatography was performed on a YMC, Inc. (Wilmington, NC) ODS-L80 column (2×250 mm). Chromatography conditions started with 20% ACN followed by a linear gradient to 65% ACN in 45 min. The mobile phase pH was maintained with 0.1% formic acid. Mass spectrometry was performed on a Finnigan (San Jose, CA) Model TSQ-7000 triple-quadrupole mass spectrometer with the standard Finnigan electrospray ion source or APCI ion source. Nitrogen was used as the nebulizing gas and the inlet capillary temperature was 250°C for electrospray or 150°C for APCI. The instrument scanned over the range of 700–1300 amu at 1.5 s/scan. Under these conditions, injection of a 25 ng PbTx-3 standard produced a response for the ($[M+H]^+$ 897) ion with a signal/noise ratio of 20:1.

3. Results and discussion

3.1. Analysis of urine specimens

RIA analysis of urine specimens taken at the emergency room demonstrated the presence of 42 (±2) ng/ml brevetoxin-like activity in one patient (TD) and 117 (±30) ng/ml in the other (TCD). These concentrations were more than sufficient for analysis. Indeed, the urine extract from both victims required substantial dilution for accurate quantitation. No activity was present at a followup examination 4 days post-ingestion, suggesting that the majority of the toxin had been eliminated by that time. This time course is consistent with that seen in rats, where elimination was largely complete 3 days after either intravenous (Poli et al., 1990a,b) or oral (Cattet and Geraci, 1993) exposure. To our knowledge, these data represent the first confirmation of NSP in humans by analysis of clinical samples, and reconfirms the importance of sample collection by the medical caregiver. Although serum samples were collected from both children at the time of hospital admission, insufficient sample volume was available for analysis. Because brevetoxins occur in the serum only at very low levels and matrix effects require a 10-fold dilution of serum prior to analysis, urine remains the clinical sample of choice for confirmation of brevetoxin exposure by RIA.

HPLC-MS analysis demonstrated the presence of both PbTx-3 and PbTx-2 in the urine samples (data not shown).

3.2. Analysis of Sarasota shellfish extracts by RIA and receptor binding assay

Whelks and clams collected from the same location as the offending shellfish 1 month after the intoxication were still toxic. Results of the analysis of the various shellfish extracts by RIA and receptor binding assay are shown in Table 1. Whelks were the most toxic of the three species of shellfish collected, containing over 22 μg PbTx-3 equivalents/g digestive tissue by RIA. The feet contained only about 10% of the activity of the digestive tissue on a per-gram basis. Of the two species of clams inhabiting the same location, the juvenile specimens of *Mercenaria* spp contained significantly more activity (6.6 μg/g) than did adult *Chione cancellata* (0.4 μg/g). Results of the receptor binding assay were qualitatively similar to the

Table 1
Analysis of shellfish extracts by radioimmunoassay and receptor binding assay. Results are the mean (±SD) of 3 separate assays and are expressed as ug PbTx-3 equivalents/g original tissue weight

Sample extracted	RIA	Receptor
Busycon contrarium digestive (19.7 g)	22.0 (±5)	9.2 (±3.6)
Busycon contrarium feet (22.5 g)	2.5 (±0.5)	0.6 (±0.1)
Chione cancellata (10.1 g)	0.4 (±0.1)	0.7 (±0.2)
Mercenaria spp (8.3 g)	6.6 (±1.4)	4.3 (±2.0)

RIA, but differed in magnitude. By this assay, the whelk digestive tissue contained 9.2 μg/g brevetoxin-like activity and the feet 0.6 μg/g. Toxin levels were again higher in *Mercenaria* (4.3 μg/g) than *Chione* (0.7 μg/g).

The distinctly different levels of toxicity demonstrated by two different species

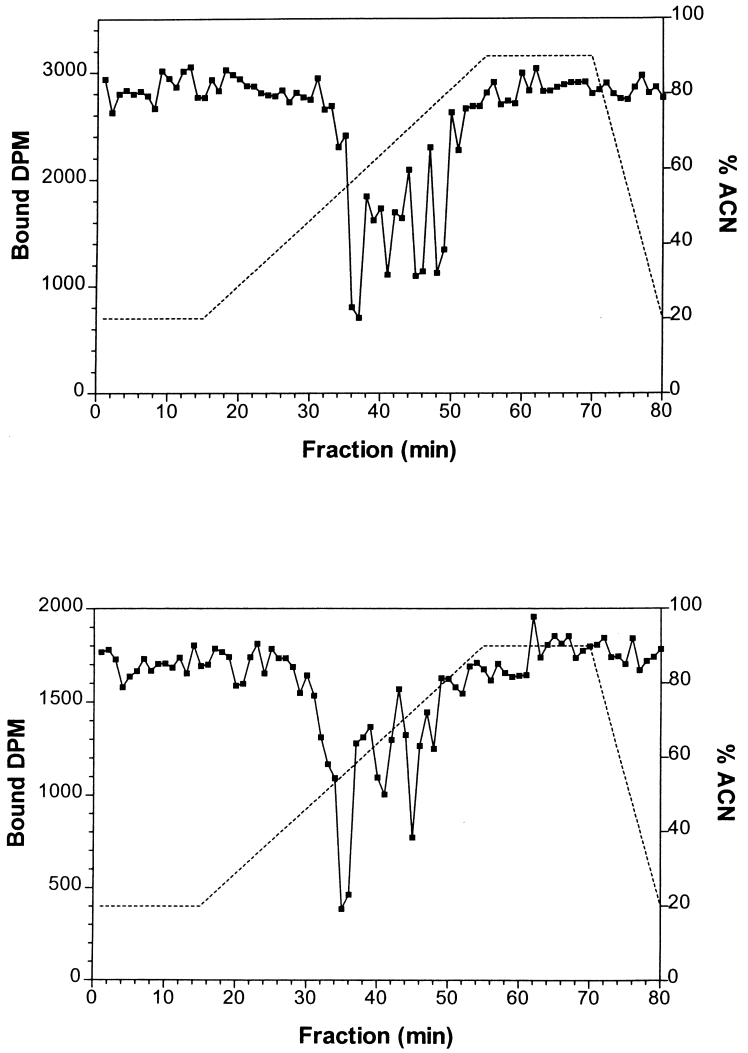

Fig. 1. Comparison of Florida whelk extract with Texas oyster extract. Extracts of toxic whelk from Sarasota Bay, Florida and red tide oysters from Texas were analyzed by HPLC. Column: J-Sphere ODS — L80, 4.6 × 250 mm. Flow rate: 1 ml/min. Mobile phase: ACN/water (0.1% formic acid). Percentage of ACN is indicated by dashed line. Top: 100 μL whelk extract. Aliquots (25 μL) of each fraction were tested for brevetoxin activity by RIA. In this assay, a decrease in bound radioactivity represents brevetoxin activity. Bottom: 100 μl Texas oyster extract analyzed as above.

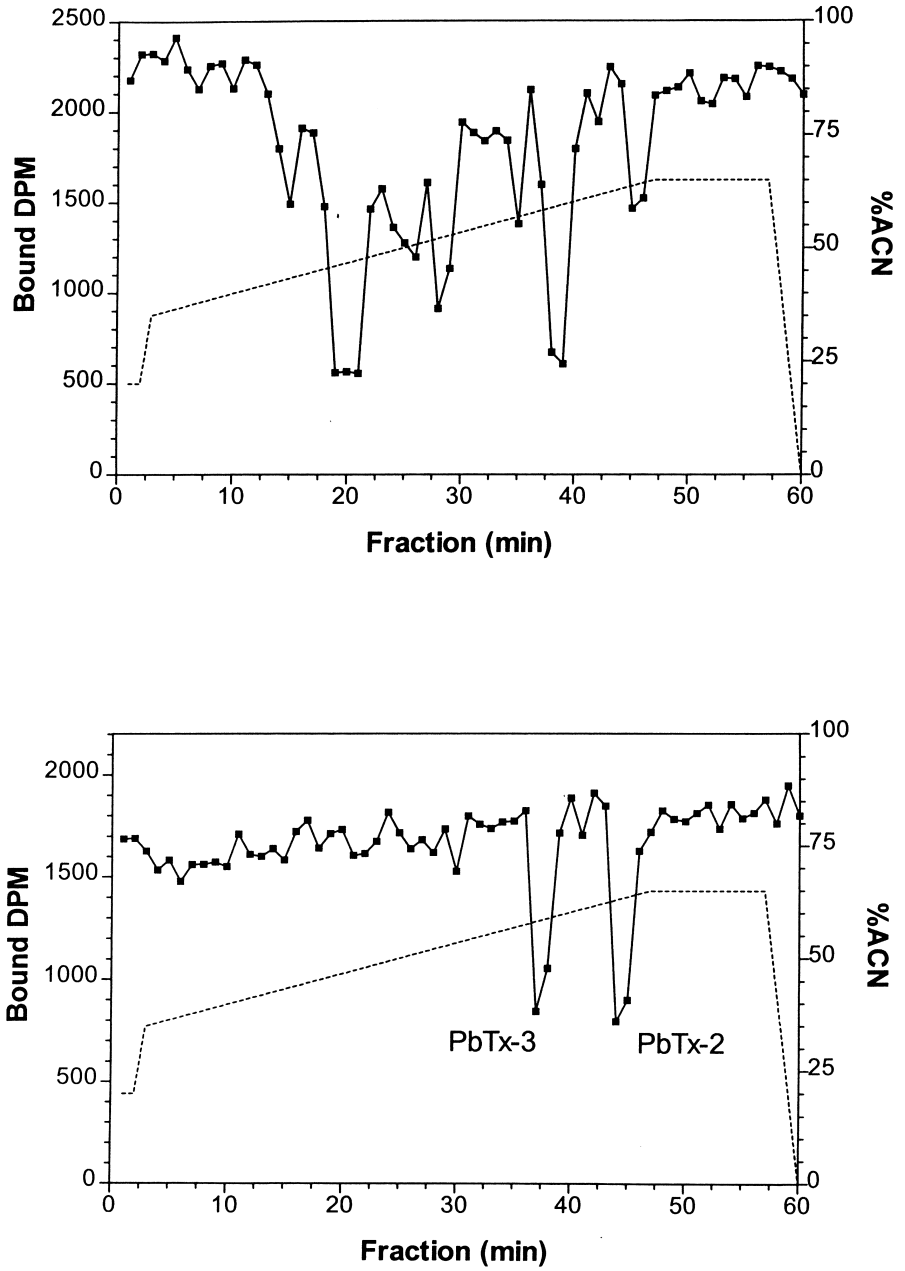

Fig. 2. HPLC profiles of toxic whelk extract and brevetoxin controls. Column: J-Sphere ODS-L80, 4.6 × 250 mm. Flow rate: 1 ml/min. Mobile phase: ACN/water (0.1% formic acid). Percentage of ACN is indicated by dashed line. Top: 100 µl whelk extract. Aliquots (50 µl) of each fraction were tested for brevetoxin activity by competitive RIA. In this assay, a decrease in bound radioactivity represents brevetoxin activity. Bottom: approx. 2 µg each PbTx-2 and PbTx-3 analyzed as above (10 µl aliquots were tested by RIA).

of filter-feeding bivalves coexisting at this location may reflect differences in feeding strategies, assimilation rates, or depuration rates. It is also interesting that whelks, which are predatory and known to feed on bivalve molluscs, contained a higher level of toxic activity than did the bivalves. This may suggest a food-chain magnification phenomenon in shellfish previously unreported for the brevetoxins. While this cannot be determined from the limited data contained here, it is an interesting area for further study.

The regulatory limit for brevetoxins in shellfish in Florida is 0.8 μg/g. Thus, by either assay, *Mercenaria* and *Busycon* would have failed regulatory testing while Chione would have passed. This situation highlights the risks involved in testing only a single species from a given location. Clearly, in the absence of information on retention and clearance rates, each species intended for consumption should be tested independently for maximum consumer safety.

3.3. Analysis of Sarasota and Texas shellfish extracts by HPLC

Analysis of whelk extract by HPLC revealed 4 major peaks of activity (Fig. 1, upper panel). Interestingly, a comparison of the whelk extract to an extract prepared from oysters collected during a Texas red tide revealed the same 4 bands, although two bands (2 and 4) were present in lower amounts in the oysters (Fig. 1, lower panel). This may suggest that the active fractions are products of metabolic reactions common to many molluscs. Modification of the linear portion of the elution gradient from 20%–90% ACN to 35%–65% ACN separated the peaks of activity even further, and revealed several additional minor peaks of activity (Fig. 2, top panel). Two major peaks (peaks 3 and 4), migrated in this system with retention times similar to PbTx-3 and PbTx-2 standards (Fig. 2, lower panel). HPLC-MS analysis of both clam and whelk extracts from Sarasota demonstrated the presence of PbTx-3 and PbTx-2. No PbTx-1 or other brevetoxins were found. In contrast, no PbTx-2 was detected in the Texas oyster extract.

3.4. Affinity purification of metabolites from Texas oyster extract

There was a significant difference between the toxicity values determined for each of the Sarasota shellfish extracts by RIA and by the receptor assay. With the exception of *Chione cancellata*, the receptor assay consistently measured toxin levels lower than the RIA. This is suggestive of the presence of brevetoxin derivatives which react differentially in the two assays. If so, this could weigh heavily in the decision of which assay is best suited for regulatory testing.

To test this hypothesis, we undertook affinity purification of active fractions from an extract of toxic oysters collected during a 1996 Texas red tide. This effort yielded 4 major peaks of activity. HPLC-MS analysis of these peaks revealed the major components to consist of compounds of $[M+H]^+$ m/z 1018, 1034, 1005, and the parent compound PbTx-3. Interestingly, unlike the Sarasota Bay whelk extract, no PbTx-2 was found either in the crude extract or the purified active

fractions. The purified fractions were then quantified by both RIA and membrane assay (Table 2). Consistent with earlier published data (Poli and Hewetson, 1992; Poli et al., 1995), PbTx-3 was recognized equally by both assays. However, each of the three major metabolites was recognized more strongly by the RIA than by the receptor binding assay. The ratio of binding activity favored the RIA by approx. 3-fold for the 1018 and 1034 metabolites and 10-fold for the 1005 metabolite.

Gymnodinium breve cultures produce PbTx-2 as the primary toxin and much smaller amounts of the others. In the log phase of growth, the organism produces PbTx-2, PbTx-1, and PbTx-3 in the approximate ratio of 20:4:1, respectively, with all other toxins present in only trace amounts (Roszell et al., 1989). In the lag phase of growth, the ratio of PbTx-2:PbTx-3 decreases, but PbTx-2 always remains the dominant toxin. The absence of PbTx-2 in the Texas oyster extract and the presence of significant amounts of PbTx-3 may suggest that PbTx-2 is metabolically converted to PbTx-3. Alternately, PbTx-3 could be metabolically stable and preferentially retained, although this is unlikely.

The comparative assay data suggest that the metabolic conversions undergone by these compounds occur at a region of the molecule which affects binding to the endogenous receptor to a greater extent than to the antibody. Most likely these metabolic reactions occur at the K-ring region of the molecule (Fig. 3). Modification of this region has been shown to have no effect on antibody binding (Poli et al., 1995). In addition, the primary alcohol on PbTx-3 makes it a likely substrate for metabolic enzymes, and metabolic conversion of PbTx-3 in rat hepatocytes has been reported (Poli et al., 1990). The double bond on the eight-membered H-ring of PbTx-3 is another potential site of metabolism which would not affect antibody binding. In mammals, this is a potential target for cytochrome P-450-mediated epoxidation reactions. Although the lactone function on the A-ring is fairly stable to enzymatic reduction by ketone reductases, this reaction, mediated by intestinal microflora, has been shown to occur in rats (Testa and Jenner, 1976). If this were to occur, however, binding by the antibody would likely be adversely affected to a substantial degree and the corresponding metabolite may not be effectively isolated by the affinity-purification technique used here.

It is interesting that PbTx-2 was noted in the whelk collected from Sarasota

Table 2
Analysis of affinity-purified brevetoxin metabolites by radioimmunoassay and receptor binding assay. Results are the mean (±SD) of 3 separate assays and are expressed as μg/ml PbTx-3 equivalents

Metabolite (m/z)	Radioimmunoassay	Receptor binding assay
1,034	1.22 (±0.07)	0.37 (±0.05)
1,018	0.92 (±0.07)	0.34 (±0.01)
1,005	1.77 (±0.25)	0.17 (±0.06)
PbTx-3	0.97 (±0.08)	1.00 (±0.08)

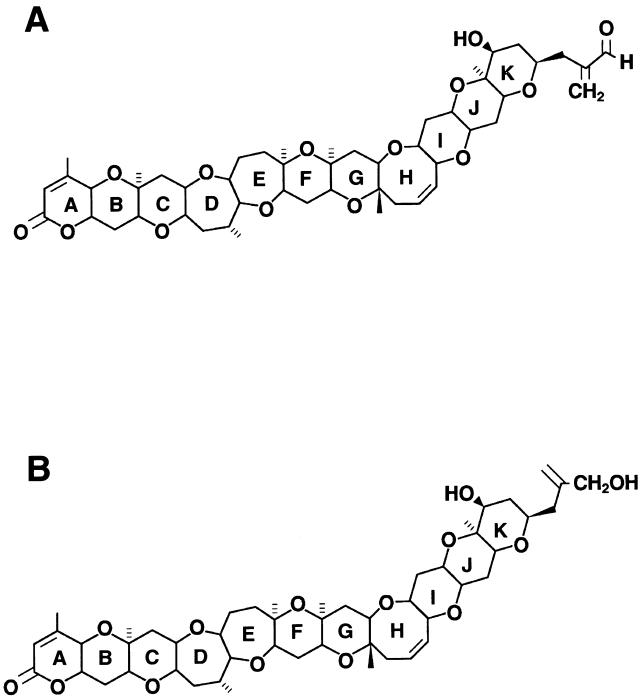

Fig. 3. The neurotoxins PbTx-2 (A) and PbTx-3 (B) from *Gymnodinium breve*.

Bay, yet absent from the Texas oyster extract. It is possible, although unlikely, that this reflects different toxin profiles elucidated during the different blooms. More likely, however, is that the toxin profile of metabolites in the shellfish changes over time. Metabolic conversion of PbTx-2 to PbTx-3 may be the first step in the production of conjugated phase-2 metabolites. The concentration of PbTx-2 in shellfish may slowly decrease during the depuration process and reach zero while the other metabolites are still present. Monitoring the toxin profiles in different species over the course of a red tide will be required to resolve this issue.

4. Conclusion

We describe here an outbreak of neurotoxic shellfish poisoning which followed the ingestion of whelks (*Busycon contrarium*) from Sarasota Bay, Florida. Brevetoxin-like activity was confirmed in the urine of two victims by RIA, confirming this technique as a viable method of analyzing brevetoxin exposure in humans. Analysis of shellfish collected subsequently from the same location demonstrated toxic activity in two filter-feeding species of bivalves, *Chione cancellata* and *Mercenaria* spp which are likely prey species for the whelk. Thus, a

putative food chain from *Gymnodinium breve* to human victims of NSP has been established.

HPLC analysis of the whelk extract revealed multiple toxic fractions which likely reflect metabolic conversion of the parent brevetoxin(s). Activity in the receptor binding assay demonstrated these derivatives to be biologically active. The HPLC profiles of toxic activity appeared to be similar in both the whelk extract from Sarasota and a toxic oyster extract collected from a red tide event in Texas. Affinity purification of active fractions from the Texas oyster extract yielded four major toxic compounds. HPLC-MS analysis yielded masses corresponding to $[M+H]^+$ m/z 1,018, 1,034, 1005, and the brevetoxin PbTx-3. No PbTx-2 was detected in the oyster extract, suggesting that PbTx-3 is also metabolicaly derived from PbTx-2.

Each of these major metabolites was quantitated by both RIA and receptor binding assay. With the exception of PbTx-3, which measured identically in each assay, all metabolites measured higher in the RIA than the receptor binding assay. No metabolites were detected which were active in either the receptor assay or RIA alone. This explains why the results of analysis of shellfish extracts has always differed between the two assays. The magnitude of the difference will depend upon the relative ratios of each metabolite, which likely varies according to species and when the samples were collected. These differences may have important implications for seafood safety and regulation. Because these active metabolites are likely to be the true cause of neurotoxic shellfish poisoning, and they appear to be under-reported by the receptor binding assay, use of this assay in the regulatory process may be questionable. Before this decision can be made, additional information on structure and in vivo toxicity is urgently needed. Towards that end, further purification and characterization of these metabolites is underway.

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