A Competitive ELISA to Detect Brevetoxins from *Karenia brevis* (Formerly *Gymnodinium breve*) in Seawater, Shellfish, and Mammalian Body Fluid

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We developed a competitive enzyme-linked immunosorbent assay (ELISA) to analyze brevetoxins, using goat anti-brevetoxin antibodies obtained after immunization with keyhole limpet hemocyanin-brevetoxin conjugates, in combination with a three-step signal amplification process. The procedure, which used secondary biotinylated antibodies, streptavidine-horseradish peroxidase conjugate, and chromogenic enzyme substrate, was useful in reducing nonspecific background signals commonly observed with complex matrices. This competitive ELISA detected brevetoxins in seawater, shellfish extract and homogenate, and mammalian body fluid such as urine and serum without pretreatment, dilution, or purification. We investigated the application of this technique for shellfish monitoring by spiking shellfish meat with brevetoxins and by analyzing oysters from two commercial shellfish beds in Florida that were exposed to a bloom of *Karenia brevis* (formerly *Gymnodinium breve*). We performed brevetoxin analysis of shellfish extracts and homogenates by ELISA and compared it with the mouse bioassay and receptor binding assay. The detection limit for brevetoxins in spiked oysters was 2.5 µg/100 g shellfish meat. This assay appears to be a useful tool for neurotoxic shellfish poisoning monitoring in shellfish and seawater, and for mammalian exposure diagnostics, and significantly reduces the time required for analyses. *Key words*: antibody, brevetoxin, detection, ELISA, immunoassay, neurotoxic shellfish poisoning, PbTx, seawater, serum, shellfish, urine. *Environ Health Perspect* 110:179–185 (2002). [Online 17 January 2002]
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The bloom-forming dinoflagellate *Karenia brevis* (formerly *Gymnodiniuim breve*) produces a family of neurotoxins known as the brevetoxins. These lipid-soluble polyether toxins, like the ciguatoxins, exert their toxicity by activating voltage-sensitive sodium channels (*1*). Human consumption of shellfish contaminated with brevetoxins leads to a nonlethal form of food poisoning (*2,3*) called neurotoxic shellfish poisoning (NSP). During *K. brevis* blooms, high concentrations of brevetoxins in seawater also affect fish (*4–6*), birds, and marine mammals (*7–9*), causing massive epizootic events. Fish kills during *K. brevis* blooms in the Gulf of Mexico have been reported in the scientific literature for over 40 years and known anecdotally for over 200 years (*10*).

Shellfish exposed to 5×10^3 *K. brevis* cells per liter for longer than 24 hr are toxic to humans (*11*). Effective water monitoring programs and shellfish bed closures following blooms have allowed few reported cases of brevetoxin intoxication in humans from consumption of contaminated shellfish. However, the actual monitoring of shellfish by mouse bioassay is slow, with a low throughput, causing delays in the reopening of shellfish beds. Development of rapid alternative methods for brevetoxin detection in seafood products is important for those involved in seafood regulation and the shellfishing industry as well as for those concerned with public health.

Brevetoxins induce toxicity at very low concentrations. Therefore, monitoring requires an analytic procedure having sufficient sensitivity and specificity to detect toxin at subsymptomatic levels. Because the matrices in which the toxins are found (algae, shellfish, body fluid, and seawater) are diverse and complex, current methods of quantitative analysis are laborious and imprecise, requiring many steps of extraction and partial purification of toxins before analysis. The difficulties in quantifying brevetoxins in biologic samples have led us to pursue a more simple and universal analytic approach that is applicable to all relevant matrices.

Radioimmunoassays and enzyme immunoassays are sensitive methods for quantifying many biologically active small molecules. During the last 15 years, intensive efforts have developed both polyclonal (*12–23*) and monoclonal antibodies (*24*) raised against brevetoxins. Additionally, competitive receptor binding assays using radiolabeled brevetoxins have shown promise (*25*). However, application of these assays for brevetoxin analysis in complex matrices such as seafood, seawater, or even mammalian body fluid has not been completely successful.

In this study we describe a quick, sensitive, and accurate ELISA method to quantify brevetoxins in seafood, seawater, and biologic mammalian fluids. Results indicate that brevetoxins can be detected accurately at very low levels in all matrices tested, with very little sample manipulation. This procedure appears to be a good candidate for an alternative method for brevetoxin monitoring in shellfish, significantly reduces assay time when used on homogenates, and could also be used to confirm exposure to brevetoxins in suspected cases of neurotoxic shellfish poisoning.

Materials and Methods

All brevetoxins were produced and purified in this laboratory from cultures of the Wilson clone of *K. brevis*. All chemical reagents, unless otherwise stated, were purchased from Sigma Chemical Company (St. Louis, MO, USA). HPLC grade solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). All immunochemical reagents, unless otherwise stated, were purchased from Pierce (Rockford, IL, USA).

Sample Preparation

Shellfish homogenization procedure. Toxin-free oysters from North Carolina used in this study were purchased live at a seafood market in Wilmington, North Carolina. We shucked 100-g portions of toxin-free oyster meat and combined them in a glass commercial Waring blender without additional aqueous medium and blended for 5 minutes at high speed. We used the resulting slurry for all subsequent preparation of standard

spiked homogenate and negative control homogenates. For analysis of potentially toxic samples, we substituted field collections made by Florida Marine Research Institute staff for nontoxic oyster meat.

Brevetoxin-spiked standard homogenates. Nontoxic homogenate prepared as described above was aliquoted into glass vials (5 g meat/vial). The samples were spiked with standard brevetoxins ranging from 0 to 4.0 µg/vial to generate final toxin concentrations of brevetoxins ranging from 0 to 80 µg/100 g shellfish meat in log 2 dilutions. Spiked samples were incubated for 1 hr at room temperature and were then homogenized with 200 mL of phosphate-buffered saline (PBS) for 3 min at high speed on a commercial blender to generate a final dilution of the homogenate at 0.025 g/mL. The homogenates were then frozen at –20°C until use (*26,27*).

Shellfish extraction procedure. We prepared homogenates of nontoxic controls (prepared previously), spiked homogenates, and authentic field samples for analysis using two methodologies, one involving use of the homogenate without solvent extraction and a second involving homogenization followed by solvent extraction. Further, we used two separate solvent extraction procedures: the Association of Official Analytical Chemists-approved ether extraction or acetone extraction.

For the ethyl ether extraction, we added 5 g NaCl and 1 mL 6 N HCl to 100 g shellfish homogenate as prepared above; the substances were mixed and then boiled for 5 min. The mixture was then transferred to a centrifuge bottle, combined with 100 mL ethyl ether, capped, and shaken vigorously for 5 min. The upper clear green ether phase was carefully decanted into a separatory funnel and transferred to a pre-weighed round-bottom flask. The lower solid phase was extracted three more times in the same manner. All ethyl ether extracts were combined and dried down under a stream of nitrogen gas.

For the acetone extraction, we extracted shellfish homogenate (100 g) 2 times using acetone (400 mL). The acetone extracts were then filtered and reduced *in vacuo*.

Brevetoxin-spiked extracts. We reconstituted both extracts (ethyl ether and acetone) in acetone:methanol (70:30). Aliquots (extracts equivalent to 5 g meat) were spiked with a mixture of PbTx-2, PbTx-3, and PbTx-9 (100:10:1) to generate final toxin concentrations of 80 µg/100 g shellfish meat, which is the actual regulatory limit based on 20 MU/100 g shellfish meat (1 MU = 4.0 µg PbTx) (*28*). Extracts were then diluted in 200 mL 0.1M PBS (pH 7.4) to generate a final dilution of the extract at 0.025 g meat equivalent/mL.

Brevetoxin-spiked standards in seawater, human urine, and rabbit serum. We centrifuged Atlantic Gulf Stream seawater at 800 × g for 10 min, decanted the supernatant solution, and used it for all further seawater samples. We used seawater, human urine, and rabbit serum undiluted or diluted as sample vehicles. Dilutions (1/2, 1/4, 1/8) were made in PBS containing 0.1% Tween 20 and 0.5% gelatin (PGT). Pure or diluted samples were then enriched with a mixture of three brevetoxins (PbTx-2, PbTx-3, and PbTx-9, 100:10:1, w:w:w). The final toxin concentration for liquid samples was between 0.1 and 20 ppb.

Antibodies

We produced and purified the anti-brevetoxin goat polyclonal antibodies using PbTx-3-KLH constructs as described previously by Trainer and Baden (*17*).

Enzyme Immunoassay Development

Flat-bottomed 96-well polystyrene immunoplates (NUNC, Maxisorp; Nalge NUNC, Rochester, NY) were coated with 100 µL of PbTx-3-bovine serum albumin (BSA) conjugate (*24,29,30*) (250 ng/mL) or BSA alone by incubating for 1 hr at 25°C or overnight at 4°C in PBS. After coating, the plates were washed 3 times with PBS to remove excess antigen or carrier protein. The remaining active sites on the plates were blocked by addition of 250 µL of blocking buffer (superblock in Tris-buffered saline; Pierce), followed by three washes with PBS containing 0.1% Tween 20 (PBS-T). We added 100 µL serially diluted anti-brevetoxin antibodies labeled with horseradish peroxidase (1:100 to 1:64,000 in PGT) to the each test well, incubated them for 1 hr at room temperature, and then washed them three times with PBS-T. Bound antibodies were visualized by the addition of 100-µL *o*-phenylenediamine (OPD; Sigma). To increase the sensitivity, we also tested two-step and three-step amplification procedures. We applied 100 µL diluted goat serum (1:100–1:64,000 in PGT) to each well of the plate. We achieved two-step amplification using the successive addition of rabbit anti-goat HRP conjugate (1:10,000 dilution in PGT) with visualization using OPD. Three-step amplification employed rabbit anti-goat biotinylated secondary antibody (1:10,000 dilution in PGT), followed by streptavidin-HRP conjugate (1:1,000 dilution in PGT) and OPD for visualization.

For all procedures, we measured the product absorbance at 492 nm using a microplate reader (FL600 Microplate Reader; Bio-Tek Instruments, Winooski, VT) after a 30-min incubation. Results are expressed as relative absorbance units.

We studied the specificity of the antisera further using a competitive ELISA. The goat anti-brevetoxin serum (1:4,000 final dilution) was first preincubated 1 hr at 25°C with an equal volume of PbTx-2, PbTx-3, PbTx-9, individually or in a mixture (0–20 ng/mL) as the inhibitor. We then transferred samples of these mixtures into wells of PbTx-3-BSA–precoated plates (100 µL/well). After incubation and washing, the antibodies associated with the plates were visualized using the three-step amplification procedure described above. The signal obtained in presence of the inhibitor and absence of the inhibitor (maximal signal) are referred to as B and Bo, respectively. Plotting the percent inhibition (100%–% B/Bo) generated inhibition curves against the log of the free brevetoxin concentration.

Brevetoxin Analysis of Seawater and Mammalian Body Fluid

We incubated 200 µL seawater, human urine, or rabbit serum enriched with individual brevetoxins or the mixture described earlier (0.1–20 ng/mL) for 1 hr at 25°C with the same volume of goat anti-toxin antibodies (1:2,000 dilution with PGT). The antibody–sample mixtures were transferred into wells of PbTx-3-BSA–pre-coated plates (100 µL/well) and incubated for 1 hr. Bound antibodies were visualized using the three-step amplification procedure described earlier. We calculated inhibitions as described above and defined the working range of the assay as the concentration of brevetoxins inhibiting between 20% and 80% of the signal.

Analysis of Shellfish Extracts and Homogenates

For shellfish sample analysis after solvent treatment, we diluted extracts 2-fold in PGT to obtain a final concentration of 0.0125 g meat equivalent/mL. Samples were then serially diluted (log 2 dilution, 0–1:128) in shellfish extract buffer (toxin-free shellfish extract at 0.0125 g meat equivalent/mL) to maintain matrix composition with the dilution. We preincubated an aliquot of 200 µL of each dilution for 1 hr at 25°C with the same volume of goat anti-brevetoxin antibodies (1:2,000 in PGT).

For homogenate analysis without solvent extraction, we diluted homogenates 2-fold in PGT to obtain a final concentration of 0.0125 g meat/mL. Samples were then serially diluted in shellfish homogenate buffer (toxin-free shellfish homogenate at 0.0125 g meat/mL) to maintain matrix composition along the dilution series. We then incubated an aliquot of 200 mL of each dilution for 1 hr at 25°C with the same volume of goat anti-brevetoxin antibodies (1:2,000 in PGT).

In both cases, the antibody-sample mixtures were transferred into wells of PbTx-3-BSA–precoated plates (100 µL/well) and incubated for 1 hr. Bound antibodies were visualized using the three-step amplification procedure described earlier. Results were expressed as percent inhibition.

Receptor binding assays were conducted as per Van Dolah et al. (*25*). We dissolved shellfish extracts in 2 mL acetone (100%), of which 1 mL was dried down and resuspended in a HEPES binding buffer (50 mM, pH 7.4). We performed competitive binding assays in 96-well polystyrene plates (*25*). PbTx-3 competition curves were generated using 35 µL [^{3}H]PbTx-3 (10 nM), 35 µL unlabeled PbTx-3 (10^{-6}–10^{-11} M), and 140 µL synaptosome preparation (*25*). For unknowns, we added 35 µL extract in binding buffer in place of unlabeled PbTx-3. The plates were then incubated for 1 hr at 4°C. After incubation, the mixtures were filtered onto Unifilter-96 GF/B filterplates (Packard Instruments, Perkin-Elmer, Boston, MA). Liquid scintillation cocktail was added to each well (30 µL Microscint 20; Packard), and the plates were counted using a TopCount Microplate Scintillation Counter (Packard). Competition curves were created and unknowns determined using GraphPad Prism (version 3.0; GraphPad Software, Inc., San Diego, CA).

Analysis of Field Samples

Two oyster collections (samples 2 and 3; each > 100 g of oyster meat) were made by the Florida Marine Research Institute, the organization charged with shellfish analysis in Florida, in December 2000 after a bloom of *K. brevis*. An additional sample of the same species (sample 1) was acquired at the same time from a nonexposed area in North Carolina. Mouse bioassays were conducted on ether extracts of each of these three samples at the Florida Marine Research Institute as per the FDA-approved protocol (*27*). Residual oyster meat was used for ELISA and receptor binding analysis, whereby the oyster meat was defrosted and aliquoted into two portions: One portion was extracted with acetone and the other used as homogenate.

Results

ELISA Development

One limiting factor when using goat serum polyclonal antibodies to detect brevetoxins by ELISA has been the nonspecific binding of antibodies to nontarget antigens in direct ELISAs. The indirect, competitive ELISA presented here circumvents this problem while retaining high sensitivity. To develop this competitive assay, we varied the antibody concentration and the number of amplification steps to find the best combination resulting in high sensitivity while maintaining a low background signal (Figure 1). A problem specific to shellfish sampling and quantification for brevetoxins concerns the current necessity to employ solvent extraction before analysis. The homogenate assay circumvents the need for solvent extraction and thereby shortens the time necessary to complete a test. Taken together, these two modifications produce a test that requires half the time and reduces background nonspecific noise.

ELISA plates first sensitized with a protein-brevetoxin conjugate (BSA-PbTx-3) or the protein alone (BSA) provide, respectively, a specific and a nonspecific binding matrix for goat anti-brevetoxins antibodies. Anti-brevetoxins antibodies at increasing dilution incubated in wells of the sensitized plates were successful in quantitative detection using all three signal amplification procedures described in "Materials and Methods."

As shown in Figure 1, a serum dilution factor of 4,000 reduces nonspecific background signal, regardless of the amplification method. However, at the same serum dilution factor, the three-step amplification procedure produced the highest specific signal (and hence greatest sensitivity) with BSA-PbTx-3 target antigen. We selected these conditions (serum dilution factor of 4,000 coupled to the three-step amplification procedure) to assess further the capacity of the antibodies to recognize brevetoxins in solution or suspension using a competitive ELISA format.
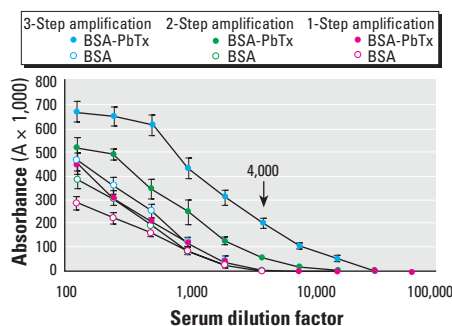
Competitive experiments performed using three different brevetoxins (PbTx-2, PbTx-3, and PbTx-9) alone or in mixture-produced the same inhibition curves (Figure 2). Although this result is not unexpected given the epitopic recognition of the H-K ring system (identical in each), it is nonetheless important to establish for regulatory purposes. We observed maximum (100%) inhibition when the total toxin concentration in the preincubation buffer was ≥ 5 ng/mL.

Matrix Effects

Various factors such as salinity, pH, and lipids modify antigen/antibody interactions in ELISA, making detection of specific substances in biologic samples difficult. In this study, we tested several different matrices (seawater, human urine, rabbit serum, and seafood) using the three-step amplification procedure described earlier, and none appeared to significantly hamper detection (Figures 3 and 4).

Liquid matrices. Seawater, urine, and rabbit serum used unmodified or diluted in buffer (PBS), and enriched with varying quantities of PbTxs before analyses, led to results more favorable than those obtained in pure buffer. As shown in Figure 3, the nature of the samples did not modify the recognition of brevetoxins by the antibody, evidenced by the identical inhibition curves for biologic matrices and buffer controls. The working range of the assay was 0.2–2 ng/mL for diluted and undiluted samples of seawater, urine, and rabbit serum.

Application to seafood matrices. Potential shellfish matrix effects were also a concern of

Figure 1. Influence of amplification procedure on signal specificity in direct ELISA. Toxin-protein conjugates (BSA-PbTx, 250 ng/mL in PBS) or control (BSA, 250 ng/mL PBS) were adsorbed to plastic wells of immunoplates before blocking. Diluted serum or anti-brevetoxins horseradish peroxidase antibodies were incubated on the plate and presence of immobilized antibodies was revealed using 3 different amplification procedures (*n* = 3, error bars indicating ± SD). An arrow points to the serum dilution factor (4,000) that produced a nondetectable background signal (BSA) and a sensitive specific signal (BSA-PbTx) with the three-step amplification procedure.
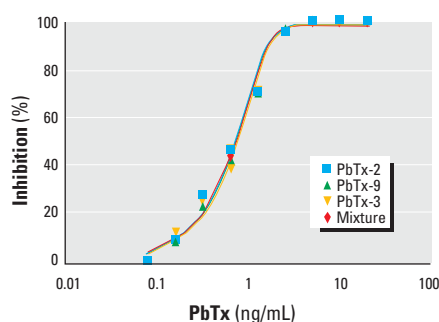
Figure 2. Competitive inhibition of goat antibody binding to BSA-PbTx-coated plates by free brevetoxins. Toxin-protein conjugates (BSA-PbTx, 250 ng/mL in PBS) were adsorbed to immunoplates before blocking. Antibodies (1/4,000 in PGT) in presence of PbTx-2, PbTx-3, PbTx-9 or a mixture of the three toxins (100:10:1) were incubated on the plate and after washing steps, immobilized antibodies were revealed using the three-step procedure. Results are expressed as inhibition percent from control (*n* = 2, error bars within symbols size; in all cases SD was < 5% of the value).
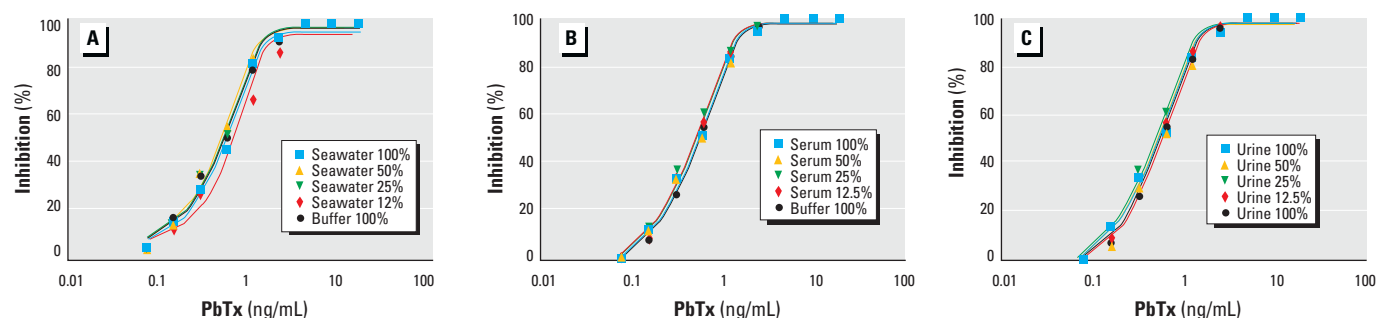
Figure 3. Brevetoxin determination in seawater (*A*), serum (*B*), and urine (*C*) at different dilutions by competitive ELISA. Toxin-protein conjugates (BSA-PbTx, 250 ng/mL in PBS) were adsorbed to immunoplates before blocking. Antibodies were mixed with pure or diluted samples spiked with various amounts of PbTx-3 and then incubated on plates. Results are expressed as percent inhibition from control ($n = 2$, ± SD).
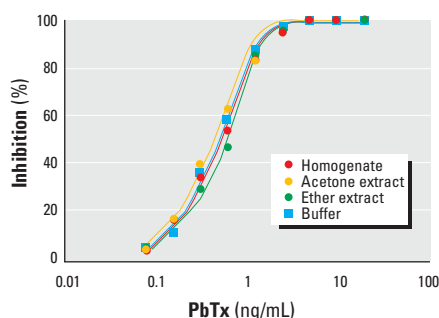

Figure 4. Comparison of the brevetoxin analysis performance in enriched oysters matrices. Immunoplates were sensitized with toxin-protein conjugates (BSA-PbTx, 250 ng/mL in PBS) before blocking the remaining active site. Antibodies were mixed with diluted samples (oyster homogenate, oyster acetone extract, oyster ether extract, and buffer) enriched with varying amount of brevetoxins, and then incubated on plates. Immobilized antibodies were visualized as previously described. Results are expressed as percent inhibition from control; each point represents the mean of duplicate determination with error bars indicating ± SD.

Figure 5. Brevetoxin recovery in shellfish ether extract, acetone extract, homogenate, and buffer assessed by ELISA. ELISA buffer (1 mL) and oyster meat (1 g) were spiked with 800 ng PbTx-9, -3, and -2 (1:10:100) and incubated 1 hr at room temperature. Shellfish meat was then extracted with ether or acetone or homogenized in buffer. Brevetoxin concentrations were determined by ELISA analysis, and results are expressed as percent recovery = 100 × (brevetoxins concentration measured by ELISA/brevetoxins added by spiking) ($n = 2$ with error bars indicating ± SD).

this study. Nontoxic shellfish extracts and homogenates spiked with brevetoxins showed identical ELISA responses to buffer spiked with the same amount of brevetoxins (Figure 4). In all cases, signal inhibition was correlated with brevetoxin concentration with no dampening of response. Brevetoxins in shellfish extracts, homogenate, or ELISA buffer exhibited 100% signal inhibition at toxin concentrations of 5 ng/mL and greater, each with the same working range. Absence of matrix effects provides a significant opportunity to reduce the time required to measure brevetoxins in shellfish without sacrificing precision or accuracy.

An earlier study demonstrated that brevetoxin recovery from shellfish is affected by the solvent used to perform the extraction (*31*). To define the best strategy for brevetoxin monitoring in shellfish, we spiked oyster meat with brevetoxins before extraction by ethyl ether or acetone or simply homogenization in buffer. Analysis of homogenates, extracts, and buffer measured by ELISA
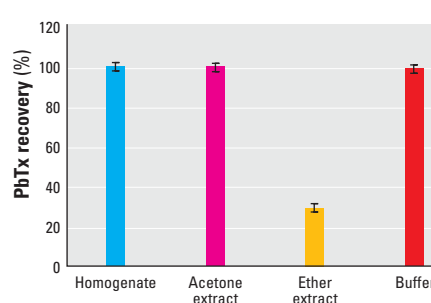
analysis and compared to the original amount of toxins incubated with the shellfish meat showed that acetone extraction produced a total recovery of the toxins whereas ethyl ether extracted only 25% of the amount present (Figure 5). Brevetoxin signals from acetone extracts were identical to those from spiked homogenate. Hence, brevetoxin analysis by ELISA can be conducted on both shellfish homogenates and shellfish acetone extracts with equivalent results, whereas the samples analyzed using ether extracts consistently underestimated the total amount of toxin present.

Two oyster samples (samples 2 and 3) collected by regulatory personnel from waters exposed to the *K. brevis* bloom were deemed unsafe for human consumption based on the mouse bioassay results performed by the Florida Marine Research Institute, whereas a control sample from an unexposed area (sample 1) was found to be safe by the mouse bioassay (Figure 6A). ELISA analysis of homogenates and acetone extracts of these same samples showed the

presence of brevetoxins in samples 2 and 3 while sample 1 was toxin-free (Figure 6B). Both samples that had greater than the regulatory limit of brevetoxins according to the mouse bioassay (samples 2 and 3) also showed high levels of brevetoxins by ELISA and receptor binding assay (RBA). There were no differences in ELISA-measured brevetoxin concentrations between shellfish homogenates and acetone extracts, indicating that a simple homogenization of shellfish meat is adequate for quantitative brevetoxin monitoring. RBA analysis showed a very good correlation with the brevetoxin concentrations determined by ELISA (Figures 6B, 6C).

Discussion

Enzyme immunoassays are used widely in both clinical and research fields because they are rapid, simple, accurate, and specific. They are also a cost-effective way to quantify many biologically important molecules such as steroids (*32*), peptides, and nucleic acids (*33,34*). However, the assays require specific antibodies for an epitope on the analyte that does not change within toxic forms of the analyte. Tests also require matrix adaptation especially for rare and low-molecular-weight lipid haptens such as hormones, drugs, and marine phycotoxins that may be masked by interfering substances in the matrix. The polyclonal antibodies used in the present study have been characterized as to their epitopic recognition of the H-K ring region of the brevetoxin type 2 molecule (*20,21*). These latter antibodies were first incorporated into radioimmunoassays (*12*) and then into enzymatic immunoassays (*17*), both of which have shown high affinity for brevetoxins. The above assays were effective in detecting brevetoxin in *K. brevis* cells and/or in buffer (*16,18*).

Brevetoxins are found in a variety of sample types in nature, ranging from seawater to mammalian body fluids that often cause high background noise when using traditional ELISAs and RIAs. Assays that have

attempted to use direct analysis in matrices have previously failed. The format in the present study circumvents matrix problems and nonspecific color development by employing a competitive assay. The success of the assay is accomplished by preincubation of samples with antibody before addition to microtiter plates.

The goat antibodies used in this study have a high affinity for brevetoxin (10^{-9} M). With the three-step amplification procedure, the competitive ELISA can be conducted with limited amounts of specific anti-brevetoxins antibodies. The working range of the assay was between 0.2 and 2 ng/mL, a level of sensitivity one order of magnitude better than HPLC-UV detection limit (35). Unlike HPLC analysis, the competitive ELISA does not differentiate among type-2 brevetoxins (PbTx-2, PbTx-3, PbTx-9) but does recognize all of these toxin substructures, consistent with the earliest observation made with polyclonal and monoclonal antibodies (21,24).

With synthetic brevetoxin analogues, the antibodies showed epitopic specificity for the H-K ring part of the molecule, the essential binding region for all type-2 brevetoxins (20,21). Type-2 brevetoxins account for 90–95% of the toxins produced in a brevetoxic bloom and all toxic forms possess the intact H-K ring system. Therefore, the complex matrix samples were spiked with a mixture of PbTx-2, PbTx-3, and PbTx-9 in the ratio found in cultured *K. brevis* and represent a likely spectrum of potential human toxicants.

Using a direct ELISA method, we found that the sample composition (e.g., seawater, urine, serum, or shellfish extract) modified the antigen/antibody interaction. At low dilution, the presence of components in the biologic samples reduced the binding of the antibodies to the antigen, as has been shown previously for brevetoxins in shellfish extracts (18) and for domoic acid in urine (36). To measure brevetoxins and minimize matrix effects, we performed competitive experiments varying toxin concentration in the sample by dilution and comparing them with the results from matrix controls without toxin. In all cases, 5 ng/mL of brevetoxin induced a total inhibition of the signal, the working range of the assay was between 0.2 and 2 ng/mL, and analysis can be performed without dilution and/or purification of sample (seawater, serum, and urine) (Figure 3). Thus a substantial time element is reduced by enabling direct measurement of brevetoxins in biologic liquids.

The limit of toxicity in humans upon ingestion of shellfish is presumed to be 20 MU/100 g shellfish meat or about 80 µg PbTx-2. This assay provides the potential to evaluate more closely the actual toxin concentrations that may lead to human illness.

Previous studies have shown that in rodents, 15–28% of the brevetoxins are excreted in urine after administration (37,38). Assuming the same depuration rate in humans, 120–240 ng of toxin would be expected to be found in 10 mL of human urine if 80 µg toxin had been ingested. The competitive ELISA sensitivity clearly would
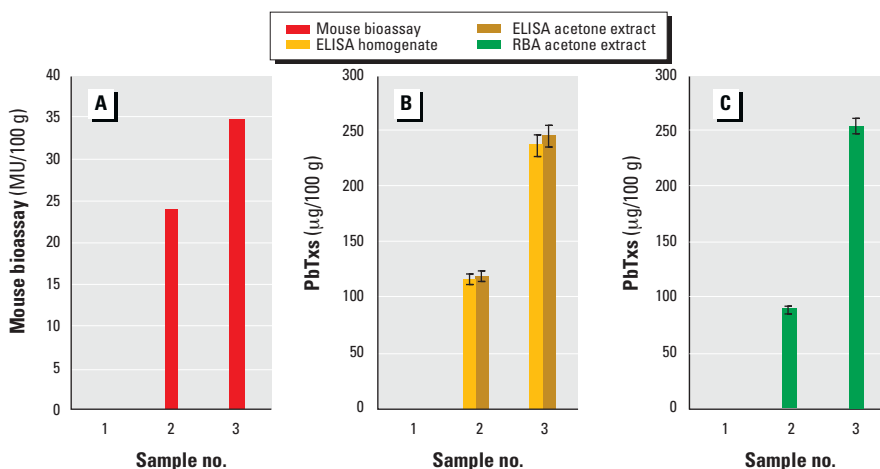

Figure 6. Comparison of brevetoxin determination in oyster samples from Florida by mouse bioassay (*A*), ELISA analysis of acetone extract and homogenates (*B*), and receptor binding assay of acetone extracts (*C*). Toxic field samples (sample 2 and 3) were collected in December 2000 after a bloom of *K. brevis*. Sample 1 (control) was collected at the same time from a nonexposed area. Mouse bioassay results were obtained according to the standard protocol for shellfish monitoring ($n = 3$; error bars indicate ± SD).
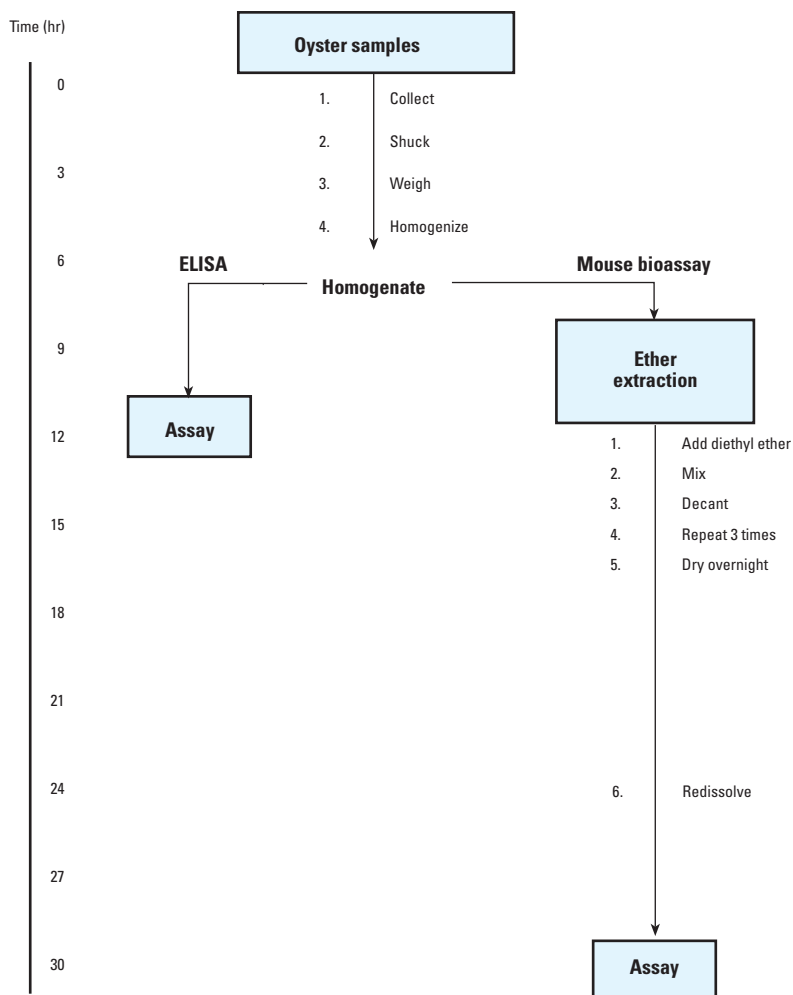

Figure 7. Time schedule for ELISA analysis versus mouse bioassay.

permit its use in diagnostic cases. The only reported case of brevetoxin detection in human urine of intoxicated people involved brevetoxin concentrations of 83–235 ng/mL in the urine of two children who had consumed contaminated shellfish (*39*). The competitive ELISA could have been used to measure brevetoxins in this study quickly and accurately, without sample pretreatment or dilution.

These results are encouraging for the development of a new method to replace the mouse bioassay. In the present study, we compared different formats of the assay for brevetoxin analysis in seafood matrices, analysis of homogenates, and analysis of shellfish extracts. Recently, a thorough comparison of different extraction processes indicated that the recovery of brevetoxins from shellfish depends on the extraction protocol used (*25*). Results from the present study clearly demonstrate that the presence of shellfish homogenate or shellfish extracts does not interfere with the performance of the assay (Figures 3 and 4). In both matrices, the signal inhibition correlated with the amount of toxin present in the sample, and no inhibition was observed when toxins were absent. The working range of the assay, as with all other matrices, was 0.2–2 ng/mL, corresponding to 0.8–8 µg brevetoxins/100 g shellfish meat (10–100 times below the national regulatory limit). The field oyster samples from three different shellfish beds analyzed by mouse bioassay, ELISA, and receptor binding assay showed strong agreement and correctly distinguished toxic from nontoxic samples.

Recent work by Poli et al. (*40*) has demonstrated that PbTxs are metabolized in shellfish and are implicated in shellfish toxicity. At least three derivatives have recently been identified (*40*). These compounds possess the PbTx type-2 backbone but a modified side chain. The antibodies used in this study are specific for the last H-K rings of the PbTx type-2 backbone excluding the side chain (*15*), so these antibodies will also recognize PbTx metabolites. As shown on Figure 6 and also reported by others (*31*), ether extraction is an inefficient method for extracting brevetoxins from shellfish meat, further increasing the value of measuring total toxin backbone in shellfish homogenate by ELISA. The presence of these metabolites may cause underestimation of the true toxicity of shellfish if only ether extracts are analyzed. Being sensitive, rapid, and accurate with a simple shellfish sample homogenate, this *in vitro* assay appears to be a good candidate for rapid shellfish monitoring.

The time required for analysis of shellfish samples (Figure 7) has been a perplexing problem for regulatory officials. Many variations for rapid assays have been proposed,

but most require extensive preparation of the food source of interest. As has been aptly pointed out time and again, "if the sample preparation time cannot be reduced, rapid assays present no true advantage over the presently available assays" (*41*). Aside from collection of the samples, preparation including solvent extraction represents the principal impediment to rapid analysis.

The industry concurs that if a particular method takes 6–8 hr to produce a sample suitable for testing, there is little value in the subsequent test format, even if it takes an additional 30 sec to complete. The fact remains that preparation of the sample represents the principal time constraint, as has been the case for Florida red tide brevetoxin testing since the 1950s. Samples are shucked, homogenized, and extracted with ether. The ether is dried, the extract redissolved in vegetable oil, and injected into animals. Animals are observed for 6 hr (a distinct improvement over the previous 24-hr observation time). However, even in its present regulatory form, only a limited-size set of analyses can be examined by mouse bioassay in a 24-hr period.

The shucking and homogenization aspects remain in the present assay. But all of the subsequent steps have been replaced by a 6-hr three-step procedure that overall decreases the amount of time required for analysis by 12 hr. The sample size also has been diminished substantially, allowing many more samples to be handled at one time. This in effect provides for multiple parallel analyses of different (or replicate) samples.

Thus, the present assay, being useful and applicable to crude homogenate samples, addresses the principal time constraint to Florida Red Tide toxin testing—sample preparation time. Coupled with the exquisite sensitivity of the ELISA amplification technique, this represents the most significant advance in the analysis of suspect brevetoxic seafood since the radioimmunoassay in 1984.

In conclusion, this competitive ELISA is a very sensitive method for detecting brevetoxins in complex matrices. It is very powerful tool not only for reducing the incidence and improving the diagnosis of brevetoxin intoxication of human and other animals, but also for studying transfer and impact of brevetoxins in the environment.

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