Identification of a Rapid Detoxification Mechanism for Brevetoxin in Rats

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Received January 19, 2005; accepted February 24, 2005

We examined detoxification of brevetoxin in rats through metabolic activities and key elimination routes by analyzing samples from individual rats exposed to two brevetoxin congeners (PbTx-2 and PbTx-3). Brevetoxins were detected by radioimmunoassay in methanolic extracts of blood within 1 h post intraperitoneal (ip) administration. The toxin assay response was about three times higher in PbTx-2-treated rats versus the same dose (180 μg/kg) of PbTx-3. This difference persisted for up to 8 h postexposure. When the blood samples were reextracted with 20% methanol to enhance recovery of potential polar brevetoxin metabolites, 25-fold higher assay activity was present in the PbTx-2-treated rats. Analysis of urine from the same animals identified 7-fold more activity in the PbTx-2-treated rats that accumulated over the course of 24 h. Radioimmunoassay-guided high performance liquid chromatographic analysis of urine from PbTx-2-treated rats yielded three major peaks of activity. The first peak was attributed to the two cysteine adducts, cysteine-PbTx sulfoxide and cysteine-PbTx (MH+: m/z 1034 and 1018). The second peak was attributed to the oxidized form of PbTx-2 (MH+: m/z 911) and its reduction product PbTx-3. The third peak remains unidentified. Brevetoxin cysteine conjugate and its sulfoxide product contributed nearly three-quarters of the brevetoxin immunoactivity. Our findings indicate the most commonly occurring PbTx-2 is rapidly transformed to a polar metabolite of reduced biological activity that appears in blood and remains for about three times higher in PbTx-2-treated rats versus the same dose (180 μg/kg) of PbTx-3. This difference persisted for up to 8 h postexposure. When the blood samples were reextracted with 20% methanol to enhance recovery of potential polar brevetoxin metabolites, 25-fold higher assay activity was present in the PbTx-2-treated rats. Analysis of urine from the same animals identified 7-fold more activity in the PbTx-2-treated rats that accumulated over the course of 24 h. Radioimmunoassay-guided high performance liquid chromatographic analysis of urine from PbTx-2-treated rats yielded three major peaks of activity. The first peak was attributed to the two cysteine adducts, cysteine-PbTx sulfoxide and cysteine-PbTx (MH+: m/z 1034 and 1018). The second peak was attributed to the oxidized form of PbTx-2 (MH+: m/z 911) and its reduction product PbTx-3. The third peak remains unidentified. Brevetoxin cysteine conjugate and its sulfoxide product contributed nearly three-quarters of the brevetoxin immunoactivity. Our findings indicate the most commonly occurring PbTx-2 is rapidly transformed to a polar metabolite of reduced biological activity that appears in blood and remains for up to 8 h, yet is cleared mostly to the urine within 24 h.

Key Words: brevetoxins; brevetoxin metabolism; Karenia brevis; harmful algal bloom.

Brevetoxins are neurotoxic polyether toxins produced by Karenia brevis (formerly known as Gymnodinium breve and Ptychodiscus brevis), a dinoflagellate originating in the Gulf of Mexico (Martin and Chatterjee, 1969; Steidinger et al., 1998). Other dinoflagellate species producing the same or similar toxins occur throughout the world, particularly in New Zealand (Ishida et al., 1996). When K. brevis blooms, brevetoxins can directly affect humans by inhalation of ocean aerosols or transfer directly from filter-feeding mollusks. Consumption of brevetoxin-contaminated shellfish causes neurotoxic shellfish poisoning (NSP), an illness characterized by gastrointestinal disturbances, progressive paresthesias, muscle weakness, and neurological and cardiovascular effects (McFarren et al., 1965). Brevetoxin activates voltage-gated sodium channels at normal resting potential and inhibits normal inactivation, leading to increased Na⁺ permeability of excitable cell membranes (Huang et al., 1984; Sheridan and Adler, 1989; Wang and Wang, 2003; Westerfield et al., 1977).

Recently, red tides appear to be increasing in incidence, duration, and geographic distribution (Tester and Steidinger, 1997; Van Dolah, 2000) and have been associated with significant environmental, human health, and economic impacts. Significant die-offs of fish, dolphin, endangered marine mammals, and birds, as well as adverse human health effects, have been reported annually due to brevetoxin exposure along the Gulf of Mexico coast (Kirkpatrick et al., 2004; Landsberg, 2002). Interestingly, the nature and the fate of brevetoxins responsible for human illnesses are largely unknown (Poli et al., 2000).

Karenia brevis produces at least nine brevetoxins, grouped according to their backbone structures (types A and B). Principal A-type brevetoxins are PbTx-1 and PbTx-7, and B-types are PbTx-2, PbTx-3, and PbTx-9. The major brevetoxin produced by K. brevis is PbTx-2, while PbTx-1 is the most potent (Landsberg, 2002). In shellfish, brevetoxins are found primarily in the form of metabolites (Dickey et al., 1999; Wang et al., 2004). The metabolism of brevetoxins was determined initially by fractionation and in vitro cytotoxicity assay of tissue extracts from oysters collected in the field during and after a K. brevis bloom. Studies exposing oysters to pure algal brevetoxins revealed that PbTx-2 was extensively metabolized in the oysters to the reduction product PbTx-3, and two cysteine conjugates named cysteine-PbTx and cysteine-PbTx sulfoxide (Plakas et al., 2002) (Fig. 1). A series of brevetoxin metabolites, including C-42 N-taurine conjugate of oxidized

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PbTx-2 (named BTx-B1) (Ishida et al., 1995), cysteine-PbTx sulfoxide (named BTx-B2) (Murata et al., 1998), N-myristoyl and N-palmitoyl-cysteine-PbTx sulfoxide conjugates (Morohashi et al., 1999), glutathione and related peptide conjugates, and a series of fatty acid–amino acid conjugates (Morohashi et al., 1995; Wang et al., 2004) were structurally determined.

Brevetoxin metabolites in contaminated oysters have shown a slow elimination. For example, field-exposed oysters were found toxic by mouse bioassay for up to 75 days after dissipation of a K. brevis bloom (Dickey et al., 1999). Moreover, in the laboratory, metabolites of PbTx-2 were very persistent and still detectable by LC/MS and cytotoxicity assay for up to 6 months after dosing (Plakas et al., 2004).

Recent studies have shown that brevetoxin metabolites are refractory molecules in intoxicated organisms, and they are transferred from one biological compartment to another—apparently as they acquire new structure/function changes. For example, brevetoxins undergo biotransformation in rodents and fish (Kennedy et al., 1992; Poli et al., 1990). In fish, the brevetoxins induce both cytochrome P450 and glutathione S-transferase with a variety of pathways for metabolism (Washburn et al., 1994, 1996). In evaluating PbTx-3 activity on the sodium channels of rat sensory neurons, it has been suggested that PbTx metabolites may be more potent than the PbTx parent compound in affecting sodium channels (Jeglitsch et al., 1998). Another investigation evaluating metabolites of the urine of three humans suffering from NSP after exposure via contaminated shellfish suggested that PbTx metabolites generated in shellfish and humans may be an additional cause of NSP and should be taken into account during regulatory testing (Poli et al., 2000).

Several studies have been carried out to understand the distribution and the elimination of brevetoxins in mammals (using rats) treated with PbTx-3 (Cattet and Geraci, 1993; Poli et al., 1990); however, no similar experiments have employed PbTx-2 as the major brevetoxin congener produced by K. brevis. In the present study, we examined the toxicokinetics and metabolism patterns of K. brevis major brevetoxin component (PbTx-2) compared to PbTx-3 after ip exposure of laboratory rats. Blood and urine were analyzed for brevetoxin elimination profile for 2 weeks after exposure. Detoxification was assessed by rapid metabolism and elimination, which varied according to the brevetoxin congener administered.

**MATERIALS AND METHODS**

**Chemical reagents.** Analytical grade solvents and chemicals used in this study were purchased from Sigma Chemical Company, Fisher Scientific, Burdick & Jackson, or otherwise as stated. PbTx-2 standards were isolated from toxic K. brevis (supplied by Dr. Steve Morton, CCEHBR Marine Biotoxins Program); PbTx-3 standards were purchased from Calbiochem, San Diego, CA; 3H-PbTx-3 (18 Ci mmol) was supplied by Amersham Biotech, Piscataway, NJ.

**Protocols of animal exposure to brevetoxin and samples collection.** Brevetoxin (PbTx-2 or PbTx-3) treatments were carried out at Argus (Charles River Laboratories, Inc., PA) using approximately 2-month-old Charles River male rats (Crl:CD®(SD)IGS BR VAF/Plus®, 400–425 g body weight). Rats were individually housed in stainless steel, wire-bottomed cages throughout the
study period. Food (Certified Rodent Diet #5002, PMI Nutrition International) and water were allowed ad libitum during collection intervals. Animals were separated into two groups (5 rats each): Group I and II (identified using Monel self-piercing ear tags).

Brevetoxins dissolved in 100% methanol (1mg/ml) were further diluted in phosphate-buffered saline (PBS; 137 mM NaCl, 8 mM NaHPO4, 1.5 mM KH2PO4, and 2.7 mM KCl) for intraperitoneal (ip) injections in a volume of 100 µl per 10 g body weight. The final dosage was 180 µg/kg of PbTx-2 (Group I) or PbTx-3 (Group II).

Blood samples (approx. 0.9 ml each) were collected from each rat assigned to the study. Control samples were collected prior to toxin exposure and post-treatment samples were collected at approximately 1, 4, 8, 24 h, and 2, 3, 7, 10, and 14 days following administration. To each circle on grade 903 filter paper blood collection cards (Schleicher & Schuell, Keene, NH), 100 µl of blood was applied as previously described (Fairey et al., 2001). The cards were then allowed to dry overnight in a cool, dark environment. Once the cards were dry, they were separated by 6” × 6” weighing paper and transferred to airtight plastic bags (VWR Scientific Products, Suwanee, GA) containing desiccant packages (Multisorb Technologies, Inc., Buffalo, NY) and humidity cards (Multisorb Technologies, Inc.). The cards were stored at −20°C until use. Both control blood and spiked blood (50 ng PbTx-3/ml) cards were used as inter-assay recovery tests.

Urine samples were collected at intervals between 0 and 4, 4 and 8, 8 and 24 h, and at 2, 3, 7, 10, and 14 days post dosing. Urine from untreated animals was used as the control and was also spiked with PbTx-3 (50 ng/ml) to be used as an inter-assay control. The experimental protocol was reviewed by the Institutional Animal Care and Use Committee (IACUC) for compliance with the Federal Animal Welfare Act (1988); 7 U.S.C.2131 as well as the National Research Council’s (NRC) Guide for the Care and Use of Laboratory Animals (National Academy Press, 1996).

**Brevetoxin extraction and cleanup from blood and urine.** Brevetoxin was extracted from blood card spots with 2 ml methanol (100%) per spot for 18 h. Blood spots were then subjected to a second extract with 2 ml methanol (20%) for 6 h to recover the relatively aqueous brevetoxin metabolites. Extracts were evaporated under nitrogen. The methanolic fractions were resolubilized in in assay buffer.

Urine samples were collected at intervals between 0 and 4, 4 and 8, 8 and 24 h, and at 2, 3, 7, 10, and 14 days post dosing. Urine from untreated animals was used as the control and was also spiked with PbTx-3 (50 ng/ml) to be used as an inter-assay control. The experimental protocol was reviewed by the Institutional Animal Care and Use Committee (IACUC) for compliance with the Federal Animal Welfare Act (1988); 7 U.S.C.2131 as well as the National Research Council’s (NRC) Guide for the Care and Use of Laboratory Animals (National Academy Press, 1996).

**Radioimmunoassay.** A radioimmunoassay (RIA) selective for toxins with the PbTx-2 type backbone was performed as described previously (Woofter et al., 2001). RIA’s were carried out in 12 × 75 borosilicate glass tubes using antibodies raised in sheep immunized with PbTx-2-fetuin conjugate (Garthwaite et al., 2001). Assays were conducted in RIA buffer: PBS containing 0.01% Emulphor-EL 620 (GAF, NY) with a final volume of 500 µl per test. Briefly, brevetoxin extracts (50 µl) or PbTx-3 standard set (from 0.01 to 100 nM) were incubated with 20 µl anti-PbTx antigen (1:4000) in RIA buffer for 1 h at 25°C. Thereafter, 0.4 nM of the [3H-PbTx-3 tracer was added, and the incubation continued for 1 h. Finally, 200 µl Sac-Cel (Alpeo Diagnostics, Windham, NH) was added to each of the assay tubes allowing the separation of the bound and unbound brevetoxin. The Sac-Cel was filtered onto 25 mm-diameter GF/B glass fiber filters (Whatman, Newton, MA). The filters were placed in 5 ml Scinti-verse (Fisher, Suwanee, GA) for 18 h, and the radioactivity was counted on a Tri-Crab 3100TR liquid scintillation counter (Packard Bioscience Company, Meriden, CT). Standard curves were constructed for each individual experiment, and results were expressed as PbTx-3 equivalents/ml original volume used from blood or urine.

**Receptor binding assay (RBA).** RBA provides a measurement of brevetoxins binding to the native receptor site on the voltage-sensitive Na+ channel and, compared with RIA, yields a biological versus immunological potency (Van Dolah and Ramsdell, 2001). Our experiments were carried out using the same per-tube assay volumes as described above in the RIA (Woofter et al., 2003). Extracts or PbTx-3 standards were incubated with rat brain membrane preparation (100 µg protein/ml) and [3H-PbTx-3 (1 nM) in ice cold binding buffer for 1 h at 4°C. The assay buffer consisted of 50 mM HEPES (pH 7.4), 130 mM choline chloride, 5.5 mM glucose, 0.8 mM magnesium sulfate, 5.4 mM potassium chloride, 1 mg/ml bovine serum albumin, and 0.01% Emulphor EL 620. Samples were then filtered through Whatman GF/C glass fiber filters. The filters were placed in 5 ml Scinti-verse for 18 h, and the radioactivity was counted as for the RIA.

**LC-mass spectrometry (LC-MS) analysis.** Liquid chromatography was performed on an Agilent 1100 LC system (Agilent Technologies, Palo Alto, CA) coupled to an Applied Biosystems/MDS Sciapi AXIO 4000 triple quadrupole mass spectrometer equipped with a Turbo Ion Spray ionization interface (Applied Biosystems, Foster City, CA). LC separation was performed on a YMC J sphere ODS-L80, 4 µ, 2.0 × 150 mm column (Waters, Milford, MA). The mobile phase consisted of water (A) and acetonitrile (B) in a binary system, with 0.1% acetic acid as an additive. The elution gradient was 2 min of 35% B, linear gradient to 80% B at 30 min, 95% B at 35 min, hold for 5 min, then return to initial conditions at 43 min and hold for 7 min before next injection. The flow rate was 0.2 ml/min.

The detection of brevetoxin congeners by mass spectrometer was achieved by selected ion monitoring (SIM) or multiple reaction monitoring (MRM). Nitrogen was used as the nebulizing gas, turbo gas, curtain gas, and collision gas. The ion spray voltage was 5 kV; the declustering potential was 55 V. The collision energy was adjusted (25–50 eV) as appropriate for each toxin congener in the MRM method. Injection of 0.12 ng of PbTx-3 on a YMC column produced a response for the protonated molecule (MH “: m/z 897) with signal/noise ratio of >8 by SIM method and >140 by MRM method. The LC fractions were also collected (one fraction per min) in separate injections for further RIA analysis.

**Data analysis.** PbTx-3 equivalents and half-maximal effective concentration (EC50) values were calculated using Prism Graph Pad 3.0 (GraphPad Software, Inc., San Diego, CA). Statistical analysis and graphs were processed using Microsoft Office Excel.

### RESULTS & DISCUSSION

In recent years, there has been enormous interest in brevetoxin metabolites amongst researchers in biochemistry, biomedicine, and seafood safety management. There are two major reasons for this increased attention. The first is to improve sample preparation techniques for regulatory screening, while the second is to better define the contribution of these metabolites to the composite toxin responses obtained by *in vivo* and *in vitro* assays and to understand their possible relation to human health effects.

To investigate the metabolism of brevetoxins in mammals, groups of 5 rats were ip treated with PbTx-2 or PbTx-3 (180 µg/kg body weight), and sequential blood and urine samples were collected from each animal. Brevetoxin immunoactivity was present in methanolic blood extracts of both treatment groups of rats within 1 h of administration; however, the level of toxin in blood (expressed as PbTx-3 equivalents) differed in magnitude by approximately a 3:1 ratio for PbTx-2-treated (113.2 ± 11.3 ng/ml)
versus PbTx-3-treated (41.7 ± 3.3 ng/ml) rats, respectively (Fig. 2A). This does not represent a differential affinity of the RIA antibody toward PbTx-2 versus PbTx-3, as shown by the PbTx-3 spiked control and as demonstrated in a previous investigation (Woofter et al., 2003). This difference persisted for 4 and 8 h post exposure; however, by 24 h the brevetoxin values were comparable between the two treatments and remained comparable through 14 days. These observations for the first 4 h are consistent with PbTx-2 forming a unique metabolite(s) that has either a longer half-life in blood or a higher affinity to the antiserum used for the radioimmunoassay.

In order to enhance the recovery spectrum of polar metabolites, dried blood initially extracted with 100% methanol was reextracted in 20% methanol, and this second extract was analyzed by radioimmunoassay. An additional 3-fold-higher amount of brevetoxin activity (369.2 ± 14.2 ng PbTx-3 equiv./ml) was recovered with the 20% methanol extract of the PbTx-2-treated rats at 1 h post administration (Fig. 2B). By comparison of the 20% methanol extract of the two treatment groups, an approximate 25:1 ratio for PbTx-2-treated:PbTx-3-treated rats was found for the first 8 h, and a 3:1 ratio was maintained for up two weeks (Fig. 2B). This finding showed that almost 75% of brevetoxin immunoactivity in PbTx-2-treated rats is more polar than the parent compound and had not been extracted by the 100% methanol procedure.

We next examined whether the polar brevetoxin activity identified by RIA had biological activity, using a radioligand binding assay (RBA) specific for toxins that bind to site 5 of the voltage-gated sodium channel. Brevetoxin activity was compared in both 100% and 20% methanolic extracts of blood. The 100% extracted, PbTx-2-treated rats had substantially lower biological- than immunological-based brevetoxin activity. The RBA to RIA activity ratios for 1 h post-exposure were approximately 1:7 for PbTx-2-treated rats and 1:2 for PbTx-3-treated rats (Fig. 3A). The difference between biological and immunological brevetoxin activity for the 20% extract was even greater (Fig. 3B), with RBA to RIA ratios of approximately 1:14 for PbTx-2-treated rats and 1:1 for PbTx-3-treated rats. These results indicated that the polar metabolite(s) of PbTx-2 has substantially lower biological to immunological activity, and it is better recognized by the antibody than the voltage-gated sodium channel-based assay. These results parallel the findings of Poli and coworkers, who fractionated two brevetoxin metabolites from shellfish that had a third less activity by receptor assay than by radioimmunoassay (Poli et al., 2000).

We next examined whether the brevetoxin immunoactivity behaved as a polar metabolite that is excreted in urine. Urine from PbTx-2-treated rats was found to contain large amounts of brevetoxin immunoactivity, reaching a peak of over 3 μg/ml in the first 24 h. As found for blood, PbTx-2-treated rats had higher levels (approximately 7-fold) of brevetoxin immunoactivity in urine than PbTx-3-treated rats (Fig. 4). The immunoactivity appeared in urine at maximal levels as early as 4 h, and the kinetics of elimination paralleled that of blood, with a short time shift. After two days, PbTx-2-treated rats had measurable, but substantially lower levels of brevetoxin immunoactivity in urine. These results indicated that the high levels of brevetoxin immunoactivity seen in PbTx-2-treated rats were rapidly eliminated in the urine in a manner consistent with a polar metabolite.

We also evaluated whether the urine contained polar species of brevetoxin metabolites by high performance liquid chromatographic (HPLC) fractionation of urine. Urine samples collected between 4 and 8 h following the exposure of rats to PbTx-2 or PbTx-3 showed multiple fractions, which were recognized by the brevetoxin RIA. Extracts of PbTx-2-treated rats yielded three peaks of activity (Fig. 5A) from which the
major peak (at 12–13 min) recovered about 73% of the total brevetoxin activity measured in the original sample. This peak was determined by HPLC-mass spectrometry to include the cysteine adduct of cysteine-PbTx sulfoxide (MH$^+$: m/z 1034) eluted at 11.4 min at a concentration of 276 ng PbTx-3 equiv./ml and cysteine-PbTx (MH$^+$: m/z 1018) eluted at 12.5 min at a concentration of 2,389 ng PbTx-3 equiv./ml (Figs. 5B and 5C). However, the peak retained at 24 min was determined to contain PbTx-3 eluted at 24.1 min with concentration of 1 ng/ml and an oxidized form of PbTx-2 (MH$^+$: m/z 911) that eluted at 24.4 min. The calculation of the concentration of two cysteine adducts was based on their peak areas against a standard peak area of PbTx-3 monitored by SIM. The two cysteine adducts were confirmed by LC/MS(/MS). Three characteristic fragments of m/z 929, 879, and 753 for the cysteine-PbTx precursor ion m/z 1018 and m/z 947, 879, and 753 for the cysteine-PbTx sulfoxide precursor ion m/z 1034 (Wang et al., 2004) were monitored by MRM (Fig. 6).

The low concentration of the cysteine adducts in the urine made detection by SIM difficult, but clear with MRM. The detection of PbTx-3 was based on the retention time of standard PbTx-3 and MRM transition of m/z 897 to 725; however, the detection of the oxidized form of PbTx-2, (a monoxygenated product previously identified by Ishida et al., 2004, as BTX-5) was based on its two MRM transitions of m/z 911 to 875 and to 455, and on the LC retention time of this compound in brevetoxin contaminated oyster samples (Wang et al., 2004). A hydrolytic compound of cysteine-PbTx conjugate (MH$^+$: m/z 1036) with A ring open was also detected by MRM method, in accordance with its two MRM transitions of m/z 1036 to 1000 and 947 (Wang et al., 2004); but did not show up in the RIA-guided LC fractionation. No PbTx-2, however, was detected. A peak of activity eluting substantially later than PbTx-3 was observed at 42 min. This compound has not been identified; however, lipophilic brevetoxin congeners have been identified in shellfish (Ishida et al., 2004).

Previous toxicokinetic studies using $^3$H- PbTx-3 have been conducted by intravenous, intratracheal and oral administration of PbTx-3 to laboratory rats (Benson et al., 1999; Cattet and Geraci, 1993; Poli et al., 1990). Poli and coworkers have found that approximately 90% of intravenously administered PbTx-3 in rats was cleared from circulation within 1 min (Poli et al., 1990). Elimination over a 24-h period was primarily through the feces and secondarily through urine. The liver was suggested to be a major organ of metabolism and pumping of metabolic products through biliary excretion; hence, several potential metabolites excreted in the bile were found in the feces. Cattet and Geraci (1993) orally administered PbTx-3 to...
rats and found wide distribution to all organs, with the highest concentrations in the liver for up to 8 days after exposure. Ingested PbTx-3 was eliminated approximately equally in urine and feces. More recently, Woofter et al. (2003) followed the kinetics of PbTx-3 in the blood of mice after intraperitoneal (ip) exposure, using radioimmunoassay for detection of toxin. Preliminary findings from the mouse study, comparing PbTx-2 and PbTx-3 methods of detection, revealed greater brevetoxin by RIA for mice exposed to PbTx-2 and served as the foundation for the current study (Woofter et al., 2002). The current study in rats provides clear demonstration that these two molecules are processed differently, with PbTx-2 being rapidly metabolized to a cysteine conjugate and eliminated in the urine. Obviously, the $\alpha,\beta$-unsaturated aldehyde group makes PbTx-2 more reactive toward glutathione (Michael addition) than PbTx-3. The glutathione conjugate is further metabolized to the cysteine conjugate, usually in kidney. An analogous investigation conducted in shellfish by Plakas et al. (2004) revealed that PbTx-2 was converted to both PbTx-3 and the cysteine conjugate; however, in this case the cysteine conjugate was not readily eliminated from shellfish. A recent study of aqueous exposure of brevetoxin (via algal cultures) to finfish showed that brevetoxin activity was detectable in blood for several weeks (Woofter et al., 2005). Whether this is in the form of PbTx-3 or a more polar conjugate remains to be determined.

In summary, the results presented here demonstrate that PbTx-2 is rapidly metabolized in mammals to a polar cysteine conjugate that is largely eliminated in urine over the course of a day (Fig. 6). The RIA appears to have very high sensitivity to PbTx metabolic products, presumably because conjugated toxin was the source of immunogen for the production of antibody (Garthwaite et al., 2001). Additional characterization of the biological activity of brevetoxin metabolites will require further purification; however, our findings indicate that the use of a radioimmunoassay may be highly advantageous for the analysis of brevetoxin metabolites in blood and urine specimens from humans and other mammals.

![FIG. 5. HPLC-RIA and LC/MS profiles of urine extracts collected at 4–8 h post-PbTx-2 or PbTx-3 treatments. For HPLC-RIA, 10 μl of urine was injected onto the YMC150 column at flow rate 0.2 ml/min. Mobile phase was acetonitrile/water gradient (0.1% acetic acid). Fractions (1 min) were screened for brevetoxin activity by RIA (A). In this assay, a decrease in radioactivity (i.e., decreased CPM) indicated a higher brevetoxin immunoreactivity. Control urine fractions exhibited no immunoactivity (data not shown). (B) and (C) LC/MS chromatograms show cysteine adducts and PbTx-3 MRM transitions of urine extracts of rats treated with PbTx-2 and PbTx-3, respectively.](https://academic.oup.com/toxsci/article-abstract/85/2/839/1668953)
ACKNOWLEDGMENTS

This work was performed while the author (F.R.) held a National Research Council Associateship Award at Marine Biotoxins Program, NOAA/NOS/CCHEBR. The authors would like to thank Ricky Woofter for assistance with the radioimmunoassay, Stephen Eaker and Robert Roberts for production of purified PbTx-2, and Drs. Christopher Miles and Lynn Briggs for providing the brevetoxin antiserum. This work was funded by the National Oceanic and Atmospheric Administration (NOAA-NOS). The National Ocean Service (NOS) does not approve, recommend, or endorse any proprietary product or material mentioned in this publication. No reference shall be made to NOS, or to this publication furnished by NOS, in any advertising or sales promotion which would indicate or imply that NOS approves, recommends, or endorses any proprietary product or proprietary material mentioned herein or which has as its purpose any intent to cause directly or indirectly the advertised product to be used or purchased because of NOS publication.

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FIG. 6. Extracted ion MRM chromatograms of cysteine adducts of PbTx-2-treated rat urine extract. Control urine extracts did not exhibit cysteine adduct peaks (data not shown).


