An HPLC-Fluorescence Method for Identifying a Toxic Fraction Extracted from the Marine Dinoflagellate *Gambierdiscus toxicus*

L. V. SICK, D. C. HANSEN, J. A. BABINCHAK, and T. B. HIGERD

ABSTRACT—A high performance liquid chromatograph using fluorescence detection was employed for quantitatively monitoring the toxic fraction extracted from the marine dinoflagellate, Gambierdiscus toxicus. *The toxin in this extract is probably a precursor of or closely related chemically to ciguatoxin, a toxin found in several species of reef fish and the cause of one of the more potent and treacherous toxicities that can result from the consumption of seafood. Using continuous flow, post-column derivatization of the chromatographically separated algal toxin, the method yielded statistically significant increases in analytical precision and decreases in time for analysis and detection limit compared with the more traditional mouse bioassay. Determination of molecular weight of the toxin extracted from* G. toxicus *indicated that this toxic material had a molecular weight of about 38,000 daltons.*

Introduction

Although there have been previous attempts to develop quantitative chemical assays for ciguatoxin, no analytical method, having high analytical precision and a low detection limit, presently exists. A radioimmunoassay method (Hokama et al., 1977) was reported using immunoglobulin isolated from sheep that had been immunized with a conjugate of ciguatoxin human serum albumin. The assay (Hokama et al., 1977) proved useful as a relatively crude screening technique for fish suspected of being ciguatoxic. Using the same antisera, an enzyme immunoassay was also recently reported to yield relatively accurate but nonquantitative detection of ciguatoxin in fish (Hokama et al., 1984, 1985). Emerson et al. (1983) used a simplified counterimmunoelectrophoretic assay using nonimmunized sera and were able to distinguish toxic from nontoxic fish flesh. The method, however, could not be used to quantitate toxin concentration.

Routine detection of ciguatoxin and possibly related toxins (e.g., maitotoxin) has been by bioassay methods. As summarized by Withers (1982), a variety of animals and tissues have been used to bioassay for ciguatoxin and toxin(s) extracted from the dinoflagellate *Gambierdiscus toxicus*. Such methods have included frog sciatic nerve tissue and whole animal assays using cats (Hessel et al., 1960), the mongoose (Banner and Boroughs, 1958), ducks (Ross, 1947), chickens (Larson and Rivas, 1965), turtles and crayfish (Banner et al., 1960), brine shrimp (Grande et al., 1976), and, most extensively used, the mouse (e.g., Ohshika, 1971). Although these bioassays have been nonquantitative assays, Sawyer et al. (1984) attempted to quantitate the mouse bioassay by constructing LD_{50} dose response curves.

The objective of the present study was to develop an HPLC method using fluorescence detection for detecting and quantitating the toxic fraction extracted from the dinoflagellate *G. toxicus*.

Materials and Methods

Based on previous results indicating that ciguatoxin and its presumed progenitor *G. toxicus* toxin absorb light within UV or IR ranges relatively poorly (Tachibana, 1980), experimentation and instrumentation were designed to investigate fluorescence absorption as a means of analytically detecting these toxins. Previously, Tachibana (1980) reported relatively weak UV absorbance of semi-purified ciguatoxin at 215 nm. This absorbance was partially attributable to some chromophore associated with the toxic moiety. Because ciguatoxin, either purified or in the form of a crude solvent extract from fish tissue, was not available for the present study, methanol extracts from *G. toxicus* were used.

An extract was prepared by concentrating cultures of *G. toxicus* via filtration and freeze-drying the cells. A known weight of dried cells was extracted with 80 percent aqueous methanol for 48 hours using a wrist arm shaker. Extracts were weighed and brought to a volume of 1 ml with 80 percent aqueous methanol. Culture conditions for growing *G. toxicus* were as described by Babinchak et al. (1986). A stock solution of *G. toxicus* extract used to construct a calibration curve had a concentration, in mouse units (MU), of 1.21 MU/μl. A MU was defined as the dry weight of methanol extracted *G. toxicus* necessary to produce a statistically computed LD_{50} when intraperitoneally injected into $\approx$ 20 g mice. The calibration curve for toxic extract was linear in a range of 15-200 MU, had a slope of 0.428, a Y intercept of 13.08, and a regression coefficient of 0.987. Purified saxitoxin, used to standardize analytical response, was obtained from the U.S. Food and Drug Administration. Toxic extract was chromatographically

L. V. Sick, D. C. Hansen, and J. A. Babinchak are with the Charleston Laboratory, Southeast Fisheries Center, National Marine Fisheries Service, NOAA, P.O. Box 12607, Charleston, SC 29412-0607. T. B. Higerd is with the Department of Basic and Clinical Immunology and Microbiology, Medical University of South Carolina, Charleston, SC 29425.

separated from the crude methanol extract of *G. toxicus* using a DuPont[1] model 820 high performance liquid chromatograph (HPLC). An Alltech 25 cm column packed with 10μ, CN (cyano) substrate was used.

An initial effort was made to separate chromatographically precolumn, derivatized *G. toxicus* extract. Many biological toxins, such as paralytic shellfish toxins, form fluorescent derivatives when oxidized (Buckley et al., 1978). Oxidized derivatives of *G. toxicus* that had been semipurified by HPLC were formed by alkaline oxidation. Periodic acid and ammonium hydroxide were used to achieve batch mixtures of alkaline oxidized derivatives using 0.065 M and 2.0 M concentrations, respectively. The reagents and sample were mixed for about 1 minute and maintained at pH 9.8. Maintenance of pH was critical to insure oxidation of substrate without obtaining a large quantity of precipitation. Before injecting the sample for HPLC analyses, the pH of the oxidized sample was adjusted in the range of 5.0-5.5 with 6.0 M acetic acid to maximize fluorescence detection (Sullivan and Iwaoka, 1983). Elution of a toxic fraction was achieved using a 20-80 percent water-methanol linear gradient run at a ramp rate of 4 percent/minute. An Aminco-Bowman Spectrophotofluorometer was used as a fluorescence detector. All analyses were conducted using excitation and emission wavelengths of 340 and 410 nm, respectively.

Post-column derivatization was accomplished using an adaptation of a continuous-flow system designed by Sullivan and Iwaoka (1983) for analyses of paralytic shellfish toxins. Three reagent reservoirs containing periodic acid, ammonium hydroxide, and acetic acid (0.065 M, 2.0 M, and 4.0 M, respectively) were connected to the post-column effluent line (Fig. 1). Ammonium hydroxide and acetic acid were pumped using a Technicon Auto Analyzer peristaltic pump, while periodic acid was pumped using a Milton Roy LDC high pressure pump. The reaction

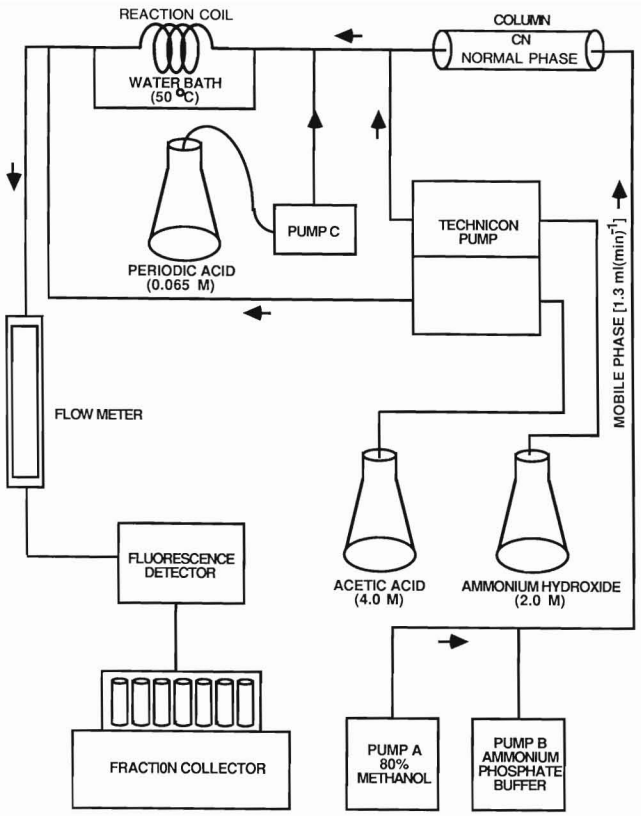

Figure 1.—Schematic diagram of the HPLC-fluorescence system including the post-column derivatization apparatus.

coil consisted of 0.56 mm i.d. stainless steel tubing with an internal volume of $\approx$ 1.5 ml. Residence time, using a column flow rate of 1.8 ml/minute, was $\approx$ 60 seconds. Flow rate for each reagent was regulated by a series of check valves and clamps to insure a pH of 9.0-9.8 in the reaction coil and 5.5-5.7 before the mobile phase entered the fluorometer. Optimum reagent flow rate, temperature, and pH of both the reaction coil and fluorometer cell for greatest analytical precision are given in Figure 2. The optical chamber of the spectrophotofluorometer was fitted with an American Instrument Company 9 μl continuous flow cell, Model J4-7484. Mobile phase A was HPLC grade 80 aqueous percent methanol and mobile phase B was a potassium phosphate buffer (pH 7.2). A linear elution gradient of 20-80 percent aqueous methanol was programmed at a ramp rate of 4 percent/minute, a 1-minute delay after initial injection and a 5-minute hold at the end of the gradient.

The molecular weight of the toxic moiety extracted from *G. toxicus* was estimated by first separating the toxic fraction chromatographically. The toxic fraction was eluted from a CN column, after the post-column apparatus had been disconnected, and 2-minute fractions collected with a Gilson fraction collector. The elution gradient was the same as described for post-column derivatization. The toxic fraction, identified from post-column derivatized fluorometry by corresponding retention time, was dried under nitrogen and brought to a volume of 20 μl with 80 percent aqueous methanol. Using a Varian gel permeation column (TSK Gel SW 2000 in tandem with a TSK Gel SW 3000, each 30×0.75 cm, Fair and Sick (1984)) and the same elution gradient as

[1]Reference to trade names or commercial firms does not imply endorsement by the National Marine Fisheries Service, NOAA.

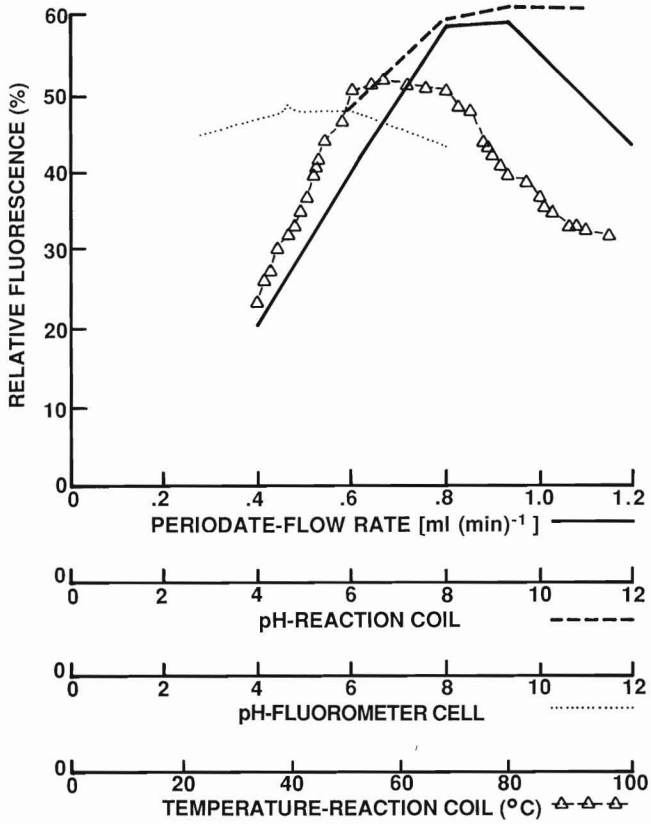

Figure 2.—Selected parameters that were examined to obtain optimum fluorescence detection of *Gambierdiscus toxicus* toxin extracted (from a stock solution having a concentration of 1-12 MU/μl). Subsample size injected for all tests was 5 μl.

Table 1.—Results obtained using HPLC-fluorescence versus mouse bioassay to quantitatively analyze a stock solution of *Gambierdiscus toxicus* extract.

Toxin conc. in dilutions[1] from stock (MU/20 μl)	Toxin conc. by HPLC[2] (MU/20 μl)	Toxin conc. by mouse[3] (bioassay (MU/20 μl)
1.00	1.15 ± 1.3	0
2.75	3.1 ± 0.8	4.55 ± 7.1
3.4	3.9 ± 0.7	5.5 ± 0.1
4.2	5.3 ± 0.6	10.73 ± 5.6
5.0	4.8 ± 0.3	7.50 ± 2.5
5.5	6.1 ± 0.8	17.24 ± 12.7
13.8	14.9 ± 2.7	45.4 ± 21.6
Mean	5.61	14.42
Estimate of analytical precision[4]	6%	0.5%
Estimate of detection limit[5]	0.81 MU	9.7 MU

[1]The concentration of toxin (as estimated from measurements of toxicity) in a stock solution of *G. toxicus* extract was determined using five replicate mouse bioassays (four mice per assay) of a given volume of the stock extract. Concentrations given were established using dilutions from the stock extract solution having a concentration of 1.12 MU/μl. Stock solution was made from *G. toxicus* extract having a concentration of 20.1 MU/mg dried extract. *G. toxicus* extracts were obtained from stock cultures averaging 12,600 cells/mg dry weight.

[2]Values are the average and standard deviation from five duplicate injections made up from the stock *G. toxicus* extract.

[3]Values are the average and standard deviation based on three replicate intraperitoneal injections of a given volume of *G. toxicus* stock solution. Each respective volume injected was adjusted to correspond to established concentrations cited in column one.

[4]Analytical precision was defined as the reciprocal of the variance among seven replicate determinations and expressed as a percentage.

[5]Detection limit was defined as the sample concentration yielding a peak area having twice the standard deviation of a series of blanks.

previously described, the toxic fraction was separated on the basis of molecular weight exclusion. The elution profile for respective fractions collected was calibrated for molecular weight using albumin (66,000), ovalbumin (43,000), ribonuclease A (13,700), phenylalanine (165), and histidine (151) as standards.

Data expressed as percent of total fluoroscence was converted to mouse units of toxicity by constructing a calibration curve comparing percent fluorescence versus toxicity (from mouse bioassays). Analytical standardization of the curve, subsequent conversion to toxicity units, and statistical treatment of the data are described in Table 1. LD_{50} values and subsequent MU values and constants for mouse bioassays were determined using a "time-to-death" dose response analysis based on four mice for each dose. Using the results of this bioassay, toxicity was calculated as follows:

$$MU = [K_1]\,[(TD)^{K_2}]^{-1},$$

where

K_1 = 80.07,
K_2 = 1.41, and
TD = time-to-death.

Analytical precision for the method for the dose response curve, using subsamples from a stock solution, was 2.35 percent.

Results

Attempts to tag extracted toxins via spiking the primary HPLC mobile phase (potassium phosphate buffer) with aniline, a commercially available fluorophore, resulted in fluorometrically detectable peaks and acceptable baseline noise. Using a methanol extract of *G. toxicus* known to be toxic, based on mouse bioassay, retention time of the fluorometrically detectable peak coincided with the area of the chromatogram previously demonstrated to have the toxic moiety. However, reproducible, quantifiable results using the standard additions technique were not obtained. In addition, the analytical precision (defined as the reciprocal of the variance among seven replicate determinations

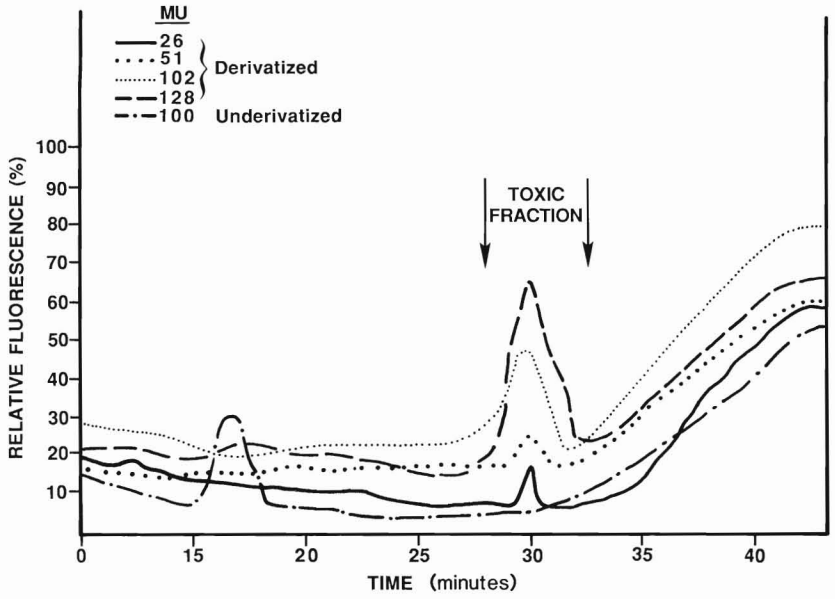

Figure 3.—Chromatographic comparison of HPLC-fluorescence separations of underivatized versus precolumn derivatized extracts from *Gambierdiscus toxicus*. Extracts used for these comparisons had an average dry weight of 0.068 ± 0.009 mg/MU.

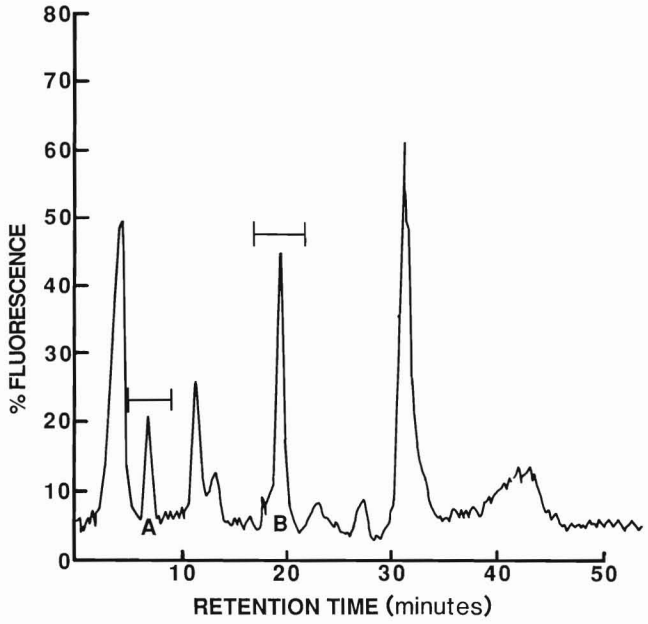

Figure 4.—Chromatographic results from HPLC-fluorescence analyses of *Gambierdiscus toxicus* extract using continuous-flow, post-column derivatization. Peaks identified by mouse bioassay as being toxic were A, saxitoxin standard, and B, peak assumed to contain the toxic moiety of *G. toxicus* extract. The chromatograph was obtained using a 5 μl subsample taken from a stock extract solution having a concentration, estimated from mouse bioassay, to be 1.12 MU/μl.

and expressed as a percentage, Steel and Torrie (1960)) of the technique was much poorer than obtained with the mouse bioassay. The calculated detection limit (defined as the sample concentration yielding a peak area having twice the standard deviation of a series of blanks) was >200 MU.

Derivatives of *G. toxicus* extract, formed in batch preparations by alkaline oxidation, were subjected to HPLC fractionation and the eluant detected fluorometrically (Fig. 3). Although fluorescence response was linear for extract doses >26 MU, attempts to detect concentrations of toxic extract <26 MU resulted in nonreproducible peaks and were calculated to be below the calculated analytical detection limits of 26.82 MU.

Using HPLC separation and post-column derivatization of a combined standard of saxitoxin and *G. toxicus* extract, relatively sensitive fluoremetric detection of corresponding toxic fractions from both saxitoxin standards and crude methanol extracts of *G. toxicus* was obtained (Fig. 4). Based on mouse bioassays of 2-minute fractions, the toxic moiety from *G. toxicus* extracts eluted in a fluorometrically detectable peak at ≈ 18 minutes. Similarly, a purified saxitoxin fraction, identified by mouse bioassay, eluted in a well resolved peak at 6-7 minutes. Based on a calibration curve established with the time-to-death mouse bioassay, the estimated detection limit by HPLC analysis for crude *G. toxicus* toxin was ≈ 0.81 MU (Table 1). Detection limit for the mouse bioassay, using the time-to-death analysis, was calculated to be 9.70 MU.

Molecular weight analyses, using HPLC size exclusion chromatography, indicated that the toxic fraction of *G. toxicus* extract had a molecular weight of 38,000 to 40,000 daltons. This analysis was conducted using *G. toxicus* extract that had been fractionated by HPLC-affinity chromatography and then refractionated by size exclusion chromatography.

Discussion

Toxin extracted from *G. toxicus* was considered appropriate for use in the

present study even though the ultimate research objective concerns development of a rapid, highly sensitive analytical method for detecting ciguatoxin. First, *G. toxicus* extract may in fact be the toxin described by Yasumoto et al. (1976) as maitotoxin and may be closely related to ciguatotoxin. Second, Sawyer et al. (1984) have demonstrated that a *G. toxicus* extract injected intraperitoneally into mice yields physiological symptoms that are empirically indistinguishable from those observed in mice injected with ciguatoxin. In addition, a physiological response thought to be specific for ciguatoxin, a lowering of body temperature by ≈ 10°C (Sawyer et al. 1984), was also observed in mice injected with *G. toxicus* extract.

Several attempts have been made to develop fluorescence detection methods for marine biotoxins. For example, Buckley et al. (1978) were able to separate oxidized derivatives of paralytic shellfish toxins by HPLC and detect toxic eluents using fluorescence detection. Sullivan and Iwaoka (1983), Sullivan et al. (1983), and Sullivan and Wekell (1984) have used alkaline oxidized derivatives of paralytic shellfish toxins to fluorometrically detect toxic fractions eluted from a HPLC column at concentrations of <1 MU.

Although parameters used for post-column derivatization of PSP toxins (Sullivan and Iwaoka, 1983) were generally adaptable for post-column analyses of *G. toxicus* extracted toxin, several differences in parameterization were found (Fig. 2). Using the dimensions recommended by Sullivan and Iwaoka (1983) for the reaction coil and other components of post-column tubing (Fig. 1), approximately twice the flowrate (0.81 vs. 0.42 ml/minute) of the oxidant, periodic acid, was required for derivatization of *G. toxicus* extract relative to derivatization of PSP toxins. Optimum derivatization of *G. toxicus* extract occurred using a reaction coil temperature of 55°-60°C. In contrast, Sullivan and Wekell (1984) recommended a reaction coil temperature of 75°C. Perhaps the use of more oxidant for *G. toxicus* extract derivative formation, relative to that used for derivatization of PSP toxins, resulted in overoxidation of algal toxin at temperatures appropriate for PSP derivatives. Similarly, using a column flow rate of 1.3 ml/minute, as reported by Sullivan and Wekell (1984) for PSP post-column derivatization, resulted in poorly resolved peaks. Maximum peak resolution and detection sensitivity for *G. toxicus* extracted toxin was obtained in the present study using a column flow rate of 1.5-2.0 ml/minute.

A comparison between HPLC and mouse bioassay methods for detecting and quantitating *G. toxicus* extract indicated that the HPLC method yielded greater precision, lower detection limits, and significantly lower average mean values than the mouse bioassay method (Table 1). The detection limit for *G. toxicus* extracted toxin, as determined for HPLC analyses, was 0.81 and 9.70 MU for the mouse time-to-death analysis. At a concentration of 1 MU (20 μl/injection), for example, detection of *G. toxicus* extract was obtained by HPLC, but no detection of toxin was obtained by mouse bioassay (Table 1). Analytical precision was 6 percent for HPLC analyses and 0.5 percent for the bioassay technique. A two-tailed T test between means of the results from the HPLC and mouse bioassays indicated that the mouse assay yielded significantly higher results at the 95 percent confidence level. Similarly, results of the two assay methods were found to be significantly different at the 95 percent confidence level using analysis of variance.

Although the chemical nature of purified *G. toxicus* toxin is not known, the toxic moiety analyzed in this study is probably the same toxin or closely related to the toxin reported as maitotoxin by Yasumoto et al. (1976). Maitotoxin was more polar than ciguatoxin when isolated from the surgeonfish *Ctenochaetus striatus* (Yasumoto et al., 1976). Based on chemical and physiological characteristics, Yasumoto et al. (1976) speculated that maitotoxin was either identical or closely related to an ichthyotoxin produced by the dinoflagellate, *Prymnesium parvum*. In addition, Yasumoto et al. (1984) suggested that the molecular weight of maitotoxin is significantly larger than that of ciguatoxin (1000 to 1500 daltons (Tachibana, 1980)). In the present study, *G. toxicus* toxin was determined to have a relatively large molecular weight (≈ 38,000 daltons), was relatively polar (eluted rapidly in a water-methanol gradient), and was the only toxic fraction found in the solvent extract of *G. toxicus*.

Use of an HPLC-fluorescence technique for monitoring toxicity offers a monitoring method having the sensitivity necessary for conducting further ciguatoxin research as well as monitoring the potential health risks of consuming selected seafood. Because ciguatoxin and maitotoxin are recognized as among the most potent, nonproteinaceous toxins (Tachibana, 1980), concentrations of toxin used in most experimental designs and concentrations lethal to test organisms and to humans are in the nanogram range. Higerd (1984), for example, estimated that if man is assumed to be twentyfold more sensitive to ciguatoxin than the mouse, a dose of as little as 1.8 μg would be lethal. Even using only semi-purified preparations of toxin, as in the present study, it was necessary to monitor as little as 50 μg (the determined LD_{50} of *G. toxicus* extract for mice) to conduct experiments using *G. toxicus* extract. If *G. toxicus* extract is used in purified form for developing assays, such as proposed immunological studies that could evolve into "marketplace" screening procedures for ciguatoxin fish (Higerd, 1984), the ability to monitor the toxic extract at nanogram concentrations by HPLC-fluorescence, as demonstrated in the present study, could be an essential tool.

Oxidation products formed in the alkaline oxidative derivatization of *G. toxicus* extract have not been determined. The fluorescent compound formed by peroxide oxidation of a PSP toxin, saxitoxin, was determined by Wong et al. (1976) to be an aminopurinyl propionic acid derivative. Furthermore, gonyautoxin, another PSP toxin, was found to yield more than one fluorescent product when oxidized (Shimizu et al., 1976). Both derivatized products formed from gonyautoxin were similar to aminopurinyl propionic acid. Although the chemical structure of ciguatoxin and maitotoxin are not known, their olefinic-polyether nature

(Tachibana, 1980) may render them structurally similar to the PSP toxins.

Literature Cited

Babinchak, J. A., D. J. Jollow, M. S. Voegtline, and T. B. Higerd. 1986. Toxin production by *Gambierdiscus toxicus* isolated from the Florida Keys. Mar. Fish. Rev. 48(4):53-57.

Banner, A. H., and H. Boroughs. 1958. Observations on toxins of poisonous fishes. Proc. Soc. Explor. Biol. Mediterr. 98:776-778.

_____, P. J. Scheuer, S. Sasaki, P. Helfrich, and C. B. Alender. 1960. Observations on ciguatera-type toxin in fish. Ann. N.Y. Acad. Sci. 90:770-787.

Buckley, L. J., Y. Oshima, and Y. Shimizu. 1978. Construction of a paralytic shellfish toxin analyzer and its application. Anal. Biochem. 85: 157-164.

Emerson, D. L., R. M. Galbraith, J. P. McMillan, and T. B. Higerd. 1983. Preliminary immunological studies of ciguatera poisoning. Arch. Intern. Med. 143:1931-1933.

Fair, P. H., and L. V. Sick. 1984. The role of digestion in the black sea bass, *Centropristis striata*, on the chemical speciation of organically bound cadmium. Comp. Biochem. Physiol. 79C(2):265-270.

Granade, H. R., P. C. Cheng, and N. J. Doorenbos. 1976. Ciguatera I. Brine shrimp (*Artemia salina*) larval assay for ciguatera toxins. J. Pharm. Sci. 65:1414-1415.

Hessel, D. W., B. W. Halstead, and N. H. Peckham. 1960. Marine biotoxins. I. Ciguatera poison: Some biological and chemical aspects. Ann. N.Y. Acad. Sci. 90:786-797.

Higerd, T. B. 1984. Ciguatera food poisoning: No ready answer yet. Proc. Gulf Caribb. Fish. Inst. Conf., 21 Nov. 1984, Cancun, Mex.

Hokama, Y., A. H. Banner, and D. B. Boylan. 1977. A radioimmunoassay for the detection of ciguatoxin. Toxicon 15:317-325.

_____, L. H. Kimura, M. A. Abad, L. Yakoch, P. J. Scheuer, M. Nukina, T. Yasumoto, D. G. Baden, and Y. Shimizu. 1984. An enzyme immunoassay for the detection of ciguatoxin. *In* E. P. Ragelis, editor, Seafood toxins, p. 307-320. Am. Chem. Soc. Symp. Ser. 262, Wash., D.C.

_____, A. M. Osugi, S. A. Honda, and M. K. Matsuo. 1985. Monoclonal antibodies in the detection of ciguatoxin and other toxic polyethers in fish tissues by a rapid poke stick test. Proc. 5th Int. Coral Reef Congr. 4:449-455.

Larson, E., and L. R. Rivas. 1965. Ciguatera poisoning from barracuda. Q. J. Fla. Acad. Sci. 28:172-184.

Ohshika, H. 1971. Marine toxins from the Pacific. IX. Some effects of ciguatoxin on isolated mammalian atria. Toxicon 9:337-343.

Ross, S. G. 1947. Preliminary report on fish poisoning at Fanning Island (Central Pacific). Med. J. Aust. 2:617-621.

Sawyer, P. R., D. Jollow, P. Scheuer, R. York, J. McMillan, N. Withers, H. Fudenberg, and T. Higerd. 1984. The effect of ciguatera associated toxins on body temperature in mice. *In* E. P. Ragelis, editor, Seafood toxins, p. 321-329. Am. Chem. Soc. Symp. Ser. 262, Wash., D.C.

Shimizu, Y., L. J. Buckley, M. Alam, Y. Oshima, W. E. Fallon, H. Kasai, I. M. Ung, V. P. Gullo, and K. Nakanishi. 1976. Structures of gonyautoxin II and III from the east coast toxic dinoflagellate *Gonyaulax tamarensis*. J. Am. Chem. Soc. 98:5414-5416.

Steel, R. G. D., and J. H. Torrie. 1960. Principles and procedures of statistics. McGraw-Hill Book Co., Inc., N.Y., p. 89.

Sullivan, J. J., and W. T. Iwaoka. 1983. High pressure liquid chromatographic determination of toxins associated with paralytic shellfish poisoning. J. Assoc. Off. Analyt. Chem. 66 (2):297-303.

_____, N. G. Simon, and W. T. Iwaoka. 1983. Comparison of mouse bioassay methods for determining PSP toxins in shellfish. J. Food Sci. 48:1312-1314.

_____, and M. M. Wekell. 1984. Determination of paralytic shellfish poisoning toxins by high pressure liquid chromatography. *In* E. P. Ragelis, editor. Seafood toxins, p. 197-205. Am. Chem. Soc. Symp. Ser. 262, Wash., D.C.

Tachibana, K. 1980. Structural studies on marine toxins. Ph.D. Thesis, Univ. Hawaii, Honolulu, 157 p.

Withers, N. W. 1982. Ciguatera fish poisoning. Ann. Rev. Med. 33:97-111.

Wong, J. L., N. S. Brown, K. Matsumoto, R. Oesterlin, and H. Rapoport. 1976. Degradation of saxitoxin to a pyrimido (2, 1-b) purine. J. Am. Chem. Soc. 93:4633-4634.

Yasumoto, T., R. Bagnis, and J. P. Vernoux. 1976. Toxicity of the surgeonfishes - II. Properties of the principal water-soluble toxin. Bull. Jpn. Soc. Sci. Fish. 42:359-365.

_____, U. Raj, and R. Bagnis. 1984. Seafood poisonings in tropical regions. Lab. Food Hyg., Fac. Agric., Tohoku Univ., p. 15.