Resolution of Ciguatera-Associated Toxins Using High-Performance Liquid Chromatography (HPLC)

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ABSTRACT—There is little doubt that the human illness, ciguatera, results from ciguatoxin in contaminated fish. That toxins other than ciguatoxin may be present in some fish and may also be isolated from the putative ciguatoxin progenitor, Gambierdiscus toxicus, *has complicated studies in this area. A method is proposed that fractionates the toxic moieties present in crude fish or dinoflagellate extracts based on their relative polarities and provides a tentative identification of these toxins. Four distinct toxic entities have been identified by this method. Each of four cultured* G. toxicus *strains yielded a single, chromatographically identical toxin (putative maitotoxin).* Ostreopsis lenticularis *cultured cells yielded a much more polar toxin that eluted in the void volume. Extracts of ciguatoxic fish harvested from the Caribbean yielded a single toxic component that co-chromatographed with purified ciguatoxin. An aliquot of an extract from a ciguatoxic fish caught from the waters off Tahiti yielded two distinct toxic fractions: One fraction that co-migrated with purified ciguatoxin and a second less polar fraction presumed to be the interconvertible form of ciguatoxin, termed scaritoxin. The chromatographic mobilities of these toxins relative to various markers illustrates the usefulness of this method in providing a tentative identification of the toxins present in crude extracts of suspect fish or dinoflagellates.*

Introduction

The predominant and perhaps sole toxin responsible for the clinical manifestations of ciguatera is ciguatoxin (Scheuer et al., 1967). The toxin is a colorless solid with a molecular weight of 1112 (Tachibana, 1980) and has been the only ciguatera-associated toxin purified and chemically characterized. Two additional toxins, however, have been isolated from suspect fish; these have been termed maitotoxin (Yasumoto et al., 1976) and scaritoxin (Bagnis et al., 1974). The marine organism responsible for the biosynthesis of ciguatoxin and maitotoxin appears to be the dinoflagellate, *Gambierdiscus toxicus* Adachi et Fukuyo (Yasumoto, et al., 1977). Laboratory cultures of the dinoflagellate, however, have yielded a significant level of toxicity attributable to maitotoxin but little, if any, toxicity associated with the "ciguatoxin fraction."

Since research efforts in the area of ciguatoxin are dependent on obtaining a reasonable supply of ciguatoxin, several laboratories have initiated programs for culturing *G. toxicus* or other suspect dinoflagellates with the expectation of acquiring sufficient quantities of ciguatoxin. However, convincing evidence has yet to be presented that ciguatoxin can be isolated from laboratory culture systems. Instead, a number of toxic moieties have been reported (Dickey et al., 1984; Miller et al., 1984; Withers, 1984), which may or may not be identical and associated with the illness ciguatera.

To help resolve the identity of these toxins and to provide unambiguous data necessary to define them, a relatively simple technique involving high performance liquid chromatography (HPLC) was developed. The method described does not require extensive preparation of the cell-free extracts before chromatography and lends itself to a preparative procedure for purification.

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Materials and Methods

Dinoflagellate Toxin Source

All dinoflagellate cultures used in this study were clonal cultures maintained and harvested as described elsewhere (Sawyer et al., 1984; Babinchak et al., 1986). *G. toxicus* T-39 was isolated from Tern Island by Withers (1984), and cultured cells of this strain were supplied by Richard York (Hawaii Institute of Marine Biology, University of Hawaii) or John Babinchak. *G. toxicus*, CD-series, were cultured from clones isolated from the Florida Keys. All dinoflagellates were extracted with methanol:water (80:20) for a minimum of 24 hours at room temperature, filtered, dried under nitrogen, and stored as a stock solution in absolute methanol at 4°C. One additional laboratory-cultured dinoflagellate isolated from Puerto Rican waters and possessing limited toxicity (Ballantine et al., 1986; Tosteson et al., 1986) was *Ostreopsis lenticularis*, submitted by T. Tosteson (University of Puerto Rico).

Fish Toxin Source

Partially purified extracts of ciguatoxic fish were kindly supplied by Joseph McMillan (College of the Virgin Islands). The fish were identified as

kingfish, *Menticirrhus* sp., and barracuda, *Sphyraena barracuda*, and were caught off the coast of St. Thomas, U.S. Virgin Islands, and the extracts (McMillan et al., 1980) were pooled. An aliquot of a crude extract of ciguatoxic fish from the waters off Tahiti was supplied by Raymond Bagnis (Institute of Medical Research, Papeete, Tahiti) and extracted according to Pompon and Bagnis (1984). All fish extracts were dissolved in acetone and stored at 4°C.

HPLC Method and Conditions

Chromatographic fractionation of the dinoflagellate or fish components in the crude extract was accomplished using a C_8 silica-based reverse phase column (4.6×250 mm with 5 μ particle size; Altech Assoc.[1], Deerfield, Ill.) equilibrated in methanol:water (50:50) and protected with an appropriate guard column. Dupont Instruments 8800-series Gradient Controller, Gradient Pump, and UV Spectrophotometer (Du Pont Co., Wilmington, Del.) were used. All dinoflagellate and fish toxin samples were filtered and applied in 50 percent aqueous methanol. The eluant was monitored at 215 nm and absorbance recorded on a Shimadzu C-R3A Integrating Recorder (Shimadzu Corp., Kyoto, Japan). The eluant was collected in 1 minute fractions using a Gilson Model FC-80K Fractionator (Gilson Medical Electronics, Middletown, Wis.). At zero time, a 50 μl sample was injected and a linear gradient of methanol:water (50:50 to 100:0) was applied with a segment length of 25 minutes, after which absolute methanol was introduced. The flow rate was maintained at a constant 1.0 ml/minute.

HPLC Standards

To establish uniform HPLC operating conditions, 10 μl of a mixture of six standards or markers were run before and after each dinoflagellate or fish toxin sample. These standards included phenol (0.15 mg/ml), p-bromophenol (0.50 mg/ml), 1-chloro-4-nitrobenzene (0.20 mg/ml), toluene (0.25 mg/ml), precocene II (0.10 mg/ml), and naphthalene (0.10 mg/ml). The detection of these markers was monitored by absorption at 215 nm.

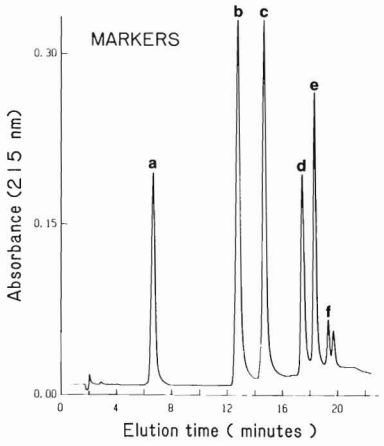

Figure 1.—U.V. profiles of selected markers used in standardizing the chromotographic conditions employed (see Materials and Methods section). Markers were phenol (a), p-bromophenol (b), 1-chloro-4-nitrobenzene (c), toluene (d), prococene II (e), and naphthalene (f).

The conditions described herein were established to optimize efficiency, selectivity, and resolution in the separation of toxicity associated with the particular test solutions. Six markers were selected based on their relative extinction coefficient at 215 nm and their relative residence time under the conditions employed. As evident from their typical chromatographic profile (Fig. 1), good separation and a distinctive elution pattern were obtained. Occasionally, an additional absorption peak at about 17.7 minutes appeared, but this peak corresponded to a contaminant in the "HPLC-Grade" water and was observed when the solvent gradient alone was run. The markers were routinely applied to the HPLC system within 3 hours before and after each toxic test sample run and over a 4-month period. The deviation in elution time over this time was minimal (Table 1) and attests to the reproducibility of the conditions employed. Because of the minimal variation in the mobility of phenol, it was used for determining the comparative elution time for the dinoflagellate and fish toxic components.

Table 1.—Retention time of markers.

Marker	Elution time (min.)[1]
Phenol	6.68 ± 0.24
p-Bromophenol	12.95 ± 0.38
1-Chloro-4-nitrobenzene	14.90 ± 0.41
Toluene	17.66 ± 0.30
Precocene II	18.52 ± 0.26
Naphthalene	19.85 ± 0.34

[1] $\bar{X}$ ± SD, n = 21

Toxicity Assay

Column fractions were placed in a stream of nitrogen until visibly dry and then transferred to a vacuum dessicator overnight. The samples were reconstituted with Tween 80 (5 percent; 0.5 ml) in phosphate-buffered saline (PBS) immediately before the assay.

Our routine bioassay for ciguatera-associated toxins was described at this Conference (Kelley et al., 1986). Each suspended fraction was administered intraperitoneally (i.p.) to two female, ICR mice (0.2 ml/mouse). For positive and negative controls, animals received either the crude extract or the Tween 80 in PBS solvent. The mice were observed for 48 hours, and their body temperature recorded at various intervals (Sawyer et al., 1984). The animal response that defined toxicity of a fraction was limited to those fractions wherein both animals died within the 48-hour test period.

Results

Separation of mixture components by C_8 columns is achieved by reverse phase partitioning between the stationary hydrophobic octasilane phase bonded to the silica gel matrix and the moving hydrophilic solvent. Residence time of a particular component on the column depends principally on its relative solubility in the stationary hydrophobic and moving hydrophilic phases. Separation of the components in the mixture in reverse phase HPLC is therefore related to their partition coefficients with the more polar substances being eluted first.

One of the first strains of *G. toxicus* placed into culture was the cloned

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

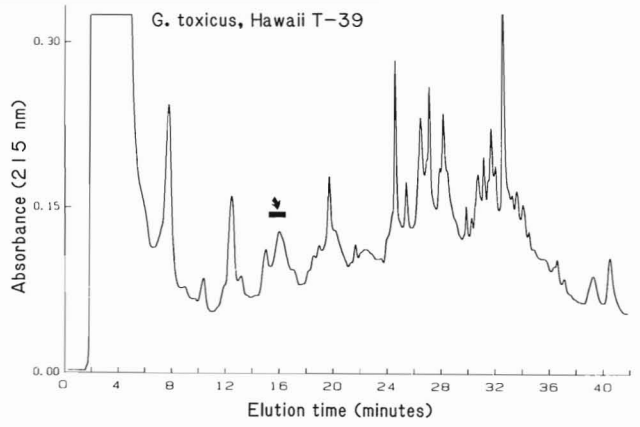

Figure 2.—Chromatographic profile of an extract of *G. toxicus*, clone T-39, isolated from Hawaiian Archipelago. Bar indicates the eluant fraction with toxicity when 1-minute fractions were bioassayed.

Hawaiian strain, T-39, isolated from Tern Island. Simple methanol extraction of these cells resulted in a cell-free extract with at least a 50 percent recovery of toxicity based on a standardized LD_{50} curve (McMillan et al., 1980), using whole cells and extracts thereof. During fractionation of the extracts from this strain, 1-minute fractions were collected. The UV elution profile was monitored and each fraction was assessed for toxicity (Fig. 2). With T-39, all the toxicity was eluted between 15 and 17 minutes.

Of the six Floridian strains (Babinchak et al., 1986), three strains were sufficiently toxic to permit testing. Figures 3, 4, and 5 illustrate the UV pro-

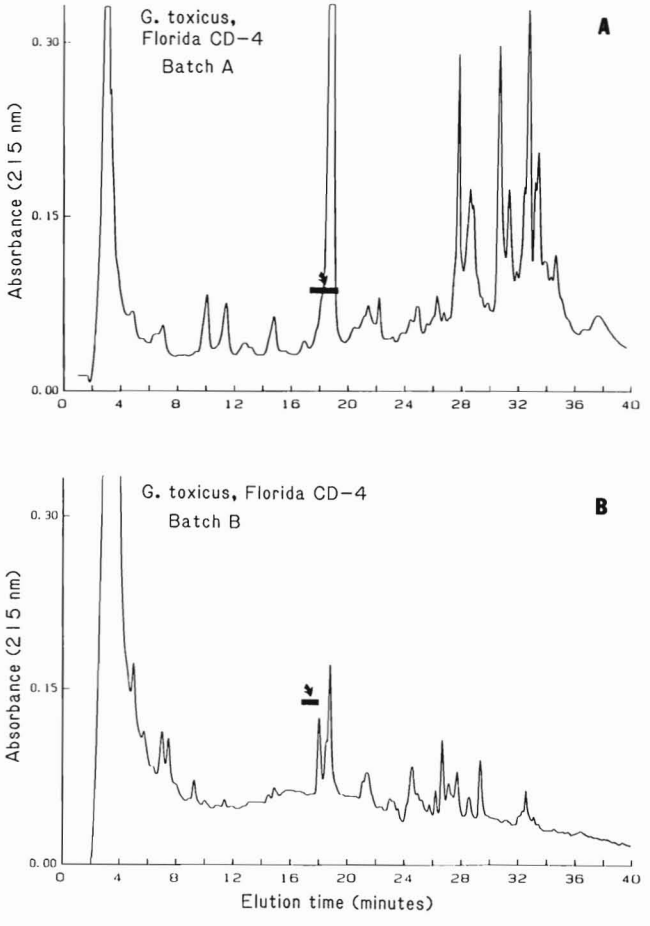

Figure 3.—Chromatographic profiles from two batches of cells of *G. toxicus*, clone CD-4, isolated from the Florida Keys. The two batches were grown under similar conditions, but at different times. Bar indicates the eluant fraction with toxicity when 1-minute fractions were bioassayed.

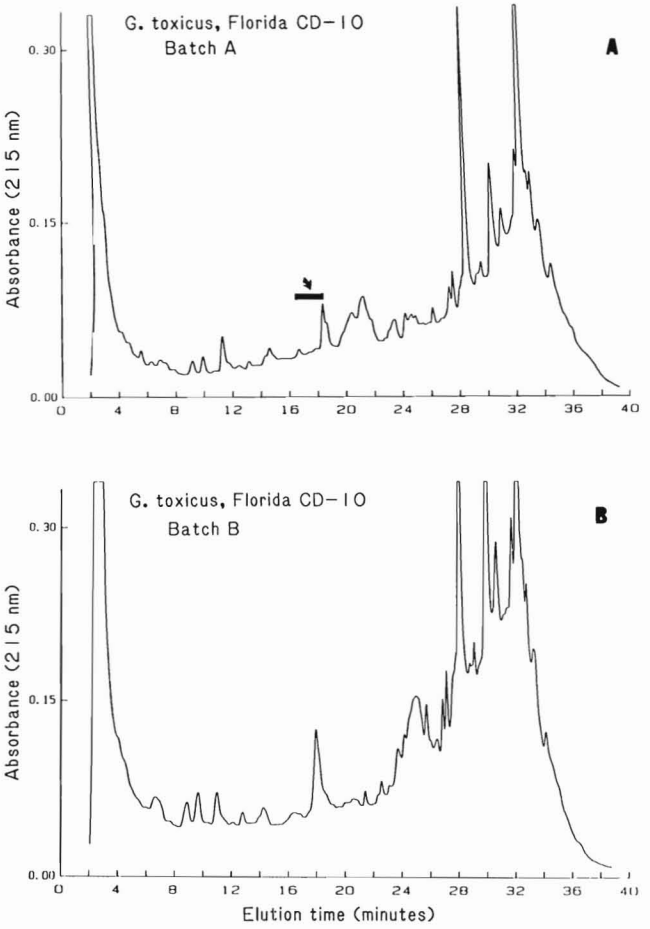

Figure 4.—Chromatographic profiles from two batches of cells of *G. toxicus*, clone CD-10, isolated from the Florida Keys. The two batches were grown under similar conditions, but at different times. Bar indicates the eluant fraction with toxicity when 1-minute fractions were bioassayed.

files obtained from strains CD-4, CD-10, and CD-20, respectively. Panels A and B of Figures 3 and 4 represent the same respective strain but were extracts from two different cell "batches" harvested from cultures having similar culture conditions. The two UV profiles for CD-4 (Fig. 3A, B) showed very little similarity, while the two profiles for strain CD-10 (Fig. 4A, B) were almost identical. In both cases, however, toxicity was limited to the same fraction regardless of the UV profile pattern exhibited by the cellular constituents harvested from different culture batches. In all three Floridian strains, toxicity was limited to a single area eluting as fraction 17 and/or 18.

Figure 6 illustrates the UV profile obtained with the methanol extract of *Ostreopsis lenticularis*. All the toxicity was eluted with the solvent front, i.e., the eluant fraction that had little or no interaction with the column's stationary phase.

The relationship between the toxic component(s) of *G. toxicus* and the ciguatoxin in fish flesh is not well understood. Extracts of ciguatoxic fish from Caribbean waters were supplied by Joseph McMillan. The extracts were dried and prepared for HPLC in the same manner as the dinoflagellate extracts. The UV profile of a typical fish extract is presented in Figure 7. When individual fractions were tested for toxicity, only fractions 26 and 27 were positive in the mouse bioassay.

To determine if the toxicity in the Caribbean fish extract was ciguatoxin, an aliquot of purified ciguatoxin (Scheuer et al., 1967; Nukina et al., 1984)

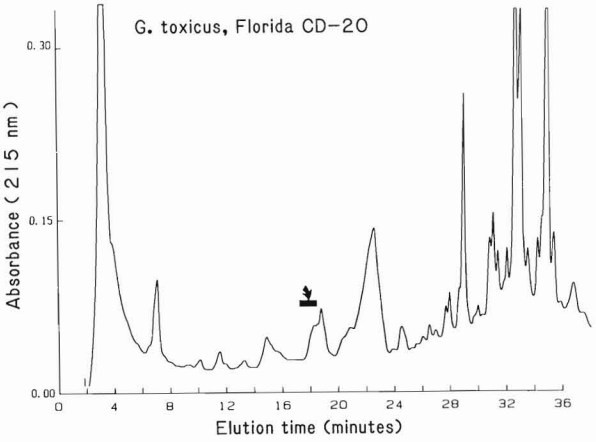

Figure 5.—Chromatographic profile of an extract of *G. toxicus*, clone CD-20, isolated from the Florida Keys. Bar indicates the eluant fractions with toxicity when 1-minute fractions were bioassayed.

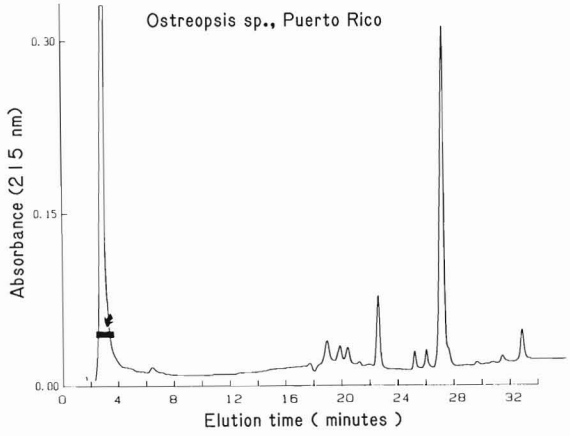

Figure 6.—Chromatographic profile of an extract of *O. lenticularis* isolated from Puerto Rico. Bar indicates the eluant fraction with toxicity when 1-minute fractions were bioassayed.

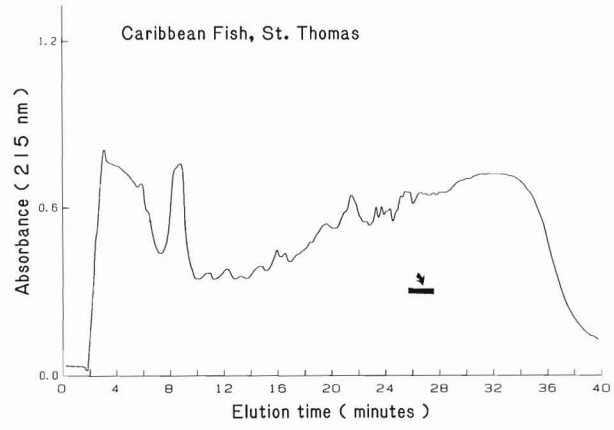

Figure 7.—Chromatographic profile of an extract of ciguatoxic fish caught around St. Thomas, U.S. Virgin Islands. Bar indicates the eluant fraction with toxicity when 1-minute fractions were bioassayed.

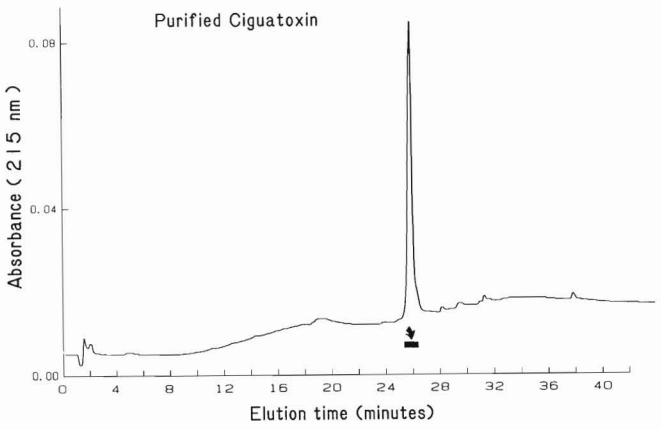

Figure 8.—Chromatographic profile of purified ciguatoxin. Bar indicates the eluant fraction with toxicity when 1-minute fractions were bioassayed.

was dried, dissolved in aqueous methanol, and applied to the chromatographic system (Fig. 8). A single UV-absorbing peak could be detected in the eluant. Moreover, the mouse bioassay revealed a single fraction of toxicity which eluted in tube 26. Chromatography of a fish harvested from the waters near Tahiti revealed a distinctive UV profile (Fig. 9) and two areas of toxicity. One of the toxic fractions corresponded to the toxic fraction obtained with purified ciguatoxin (Fraction 26). A second toxic fraction was observed, and it was eluted in Fraction 29.

Table 2 summarizes the results of this study and defines the migration of the toxic components in terms of their time of elution relative to that of the phenol marker run immediately before and after the test sample. At least four distinct toxins were evident. A very polar toxin was detected in extracts of *O. lenticularis*. Laboratory cultures of *G. toxicus* produced a second toxin that was more polar than the third toxin detected, ciguatoxin. An additional nonpolar toxin was present in the Pacific fish sample; the Caribbean fish sample lacked this component.

Table 2.—HPLC elution time of toxicity relative to phenol (R_t).

Source of material	R_t[1] (min.)
O. lenticularis, Puerto Rico	0.44
G. toxicus T-39, Hawaii (Batch A)	2.25
G. toxicus T-39, Hawaii (Batch B)	2.14
G. toxicus CD-4, Florida (Batch A)	2.81
G. toxicus CD-4, Florida (Batch B)	2.62
G. toxicus CD-10, Florida (Batch A)	2.63
G. toxicus CD-20, Florida	2.74
Fish, St. Thomas	4.01
Fish, Tahiti	3.95 and 4.40
Purified ciguatoxin	3.94

[1]Ratio of the mid-point of the fraction(s) exhibiting toxicity to the retention time of phenol.

Discussion

Unfortunately, biologists have not had a reasonable means to distinguish the toxins associated with ciguatera. As a result, many broad-based assumptions have appeared. For example, it has been assumed that the toxin isolated from Caribbean fish involved in clinical cases of ciguatera is the same toxin originally defined by Scheuer et al. (1967) as ciguatoxin, even though toxin from a Caribbean fish source has never been purified and chemically characterized. This report presents data providing the first strong evidence that the toxin isolated from Caribbean fish may be the same chemical entity previously described as ciguatoxin. Obviously, definitive arguments will require the structural elucidation of the purified toxins from each of the two geographical sources.

In addition to ciguatoxin, another toxin was present in the Pacific fish sample. Interestingly, this toxin produced similar symptomology as purified ciguatoxin and the dinoflagellate toxin when administered i.p. to mice, indicating biological similarities among all three ciguatera-associated toxins. This very nonpolar toxin may be related to the second chemical form of ciguatoxin recently reported by Nukina et al. (1984). This less polar toxin may be similar to scaritoxin isolated from some toxic fish of the Pacific Islands (Bagnis et al., 1974) and which has been shown to interconvert to ciguatoxin in vitro (Nukina et al., 1984).

To date, unequivocal evidence has not been presented that the dinoflagellate, *G. toxicus*, when grown in the laboratory, contains ciguatoxin. Currently, an effort is being made to collect a sufficient quantity of cells of this dinoflagellate from their natural habitat to determine if "wild" cells of this organism produce detectable levels of ciguatoxin, as reported by Yasumoto et al. (1979). It is interesting to note that the Hawaiian strain may be producing a slightly more polar toxin than the Floridian strain; however, additional samples need to be analyzed before a statistical evaluation of any differences can be reported.

The method described herein should be viewed as a reliable means to provide preliminary and tentative identification of the ciguatera-associated toxins. This method is relatively simple to perform and does not require extensive purification of the toxin sample. The detection of biological activity purposely rests with the mouse bioassay, a very reliable and noncontroversial assay of toxicity when performed correctly. The inclusion of markers into the chromatographic runs insures uniformity of conditions and permits different laboratories an element of standardization. This is exceedingly important for those laboratories lacking the chemical expertise and/or the quantity of toxin necessary for purification. Hopefully, use of this or a similar method will result in a universally acceptable standard for defining those toxins potentially involved in ciguatera seafood poisoning.

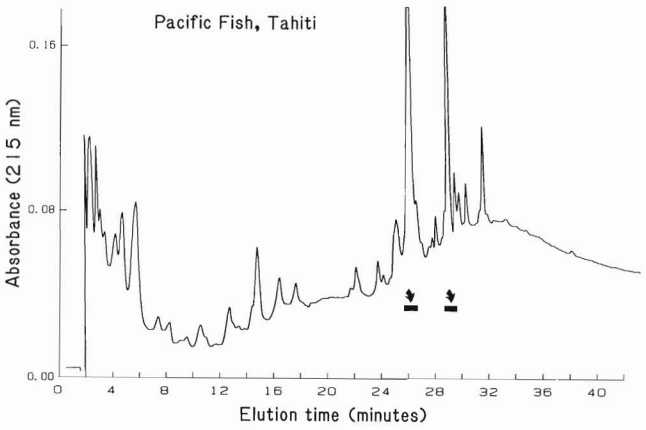

Figure 9.—Chromatographic profile of an extract of ciguatoxic fish caught around Tahiti. Bar indicates the eluant fraction with toxicity when 1-minute fractions were bioassayed.

Acknowledgments

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