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SEPARATION OF TETRODOTOXIN AND PARALYTIC SHELLFISH POISONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH A FLUOROMETRIC DETECTION USING *o*-PHTHALALDEHYDE

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SUMMARY

Tetrodotoxin (TTX) and a variety of paralytic shellfish poisons (PSPs) were extracted from toxic specimens of puffer and scallop, and quantitated by high-performance liquid chromatography with a fluorometric detection using *o*-phthalaldehyde. Fluorescence spectra for the TTX- and PSP-fluorophors in 0.05 *M* borate buffer (pH 10) showed maxima at 453 nm with 332-nm excitation. The fluorescence intensity per n*M* of TTX was found to be 3 and those of PSP to be 4–12. This fluorometric technique may be useful for the simultaneous quantitation of TTX and PSP in small volumes of toxin extracts.

INTRODUCTION

A mouse bioassay test has been the preferred means for determination of toxicity of fish and shellfish containing tetrodotoxin (TTX) and paralytic shellfish poisons (PSPs). Although this test seems reliable, it may be difficult to obtain mice suitable for assay.

Some chemical assay techniques have previously been developed[1–6]. These involve conversion of TTX or PSP to a fluorogenic substance in the presence of alkali or hydrogen peroxide. However, in the case of PSP, hydrogen peroxide interferes with detection by producing bubbles when heated in the solution employed to carry out the reaction. The use of alkali is also limited because of its low sensitivity to these toxins.

We have applied *o*-phthalaldehyde (OPA), which yields fluorophors at room temperature on reaction with primary amines[7,8], for the determination of TTX and PSP occurring in toxic puffers and scallops. The application of OPA fluorometry coupled with high-performance liquid chromatography (HPLC) generally overcame the shortcomings encountered in the detection with alkali or hydrogen peroxide.

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EXPERIMENTAL

Chemicals and reagents

TTX was isolated from the puffer *Fugu vermicularis porphyreus*[9]. PSP standards were prepared from toxic specimens of the following: two toxins (PX_1 and PX_2) newly isolated from the oyster *Crassostrea gigas*[10,11] (Fig. 1); gonyautoxins 1–4 (GTX_{1-4}) from the scallop *Patinopecten yessoensis*[12,13] and saxitoxin (STX) and neosaxitoxin (neoSTX) from the crab *Zosimus aeneus*[14,15].

OPA was purchased from Wako (Tokyo, Japan), Brij 35 from Sigma (St. Louis, MO, U.S.A.).

Deionized glass-distilled water was used for the preparation of reagents. All glassware was washed by immersion in a sulphuric acid–dichromate cleaning solution.

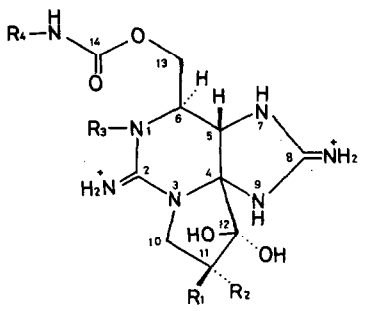
Instrumentation

HPLC was carried out on a Hitachi 638-50 analyzer with a 650-10 spectrofluorometer equipped with a 18-μl microflowcell. Peaks were recorded on a 056 recorder operating at 10 mV full scale. A stainless-steel column (150 × 4 mm I.D.) with a water jacket (45°C) was packed with Hitachi 3013 C ion-exchange resin at a pressure of 150 kg/cm^2. PTFE tubes (10 m × 0.3 mm I.D.) were used as the reaction coil.

Automated analysis

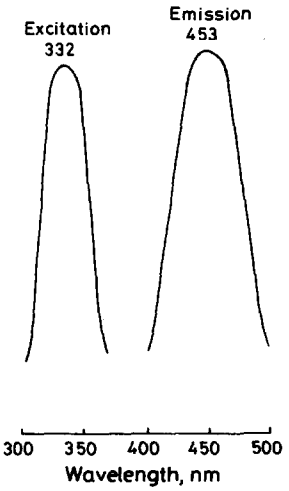
Ten microlitres of standard toxins including 1–5 n*M* PSP and 10–20 n*M* TTX per μl were placed on a Hitachi 3013 C column equilibrated with 0.005 *M* acetic acid. A three-step gradient elution with acetic acid was then applied: 0.005–0.015 *M*, 0–15 min; 0.015–0.15 *M*, 15–30 min; 0.15–0.50 *M*, 30–80 min. The flow-rate of the eluent and the column pressure were 1 ml/min and 110 kg/cm^2, respectively. The column was washed with 1 *M* acetic acid for 10 min, then with water and 0.005 *M* acetic acid for 20 min each. The column is then ready for the next run.

The OPA reagent for HPLC was prepared according to the method of Benson and Hare[16]. The fluorescence reaction was performed in a 55°C water-bath. The ratio of column eluate to OPA reagent at a mixing tee was 1:2. Toxins were monitored at 453 nm with 332-nm excitation. Peak areas were calculated by a data processing system of the analyzer.



	R_1	R_2	R_3	R_4
GTX_1	H	OSO_3^-	OH	H
GTX_2	H	OSO_3^-	H	H
GTX_3	OSO_3^-	H	H	H
GTX_4	OSO_3^-	H	OH	H
STX	H	H	H	H
neoSTX	H	H	OH	H
PX_1	H	OSO_3^-	H	SO_3^-
PX_2	OSO_3^-	H	H	SO_3^-

Fig. 1. Various forms of PSP.



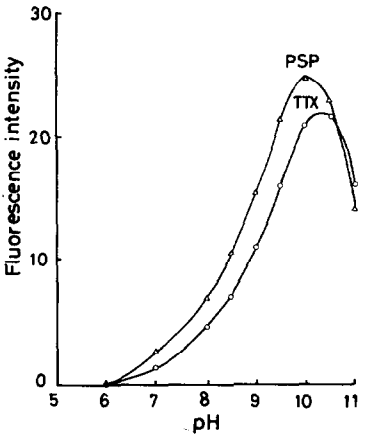


Fig. 2. Fluorescence spectra of OPA derivatives of TTX and PSP incubated in 0.05 *M* borate buffer (pH 10) at 45°C for 10 min.

Fig. 3. Fluorescence intensity as a function of pH. The toxin derivatives were incubated in 0.05 *M* each of acetate (pH 5–6), phosphate (pH 7) and borate (pH 8–11) buffers (45°C) for 10 min.

Bioassay

HPLC was carried out as above except that the flow of OPA reagent was cut of. Fractions of 0.5 ml were collected. The toxicity of each fraction was determined by the official method for PSP or TTX using 18–20 g male mice[17].

Thin-layer chromatography (TLC) and electrophoresis

The identification of toxins was made by TLC on Merck silica gel GF 254 plates, and electrophoresis on Chemetron cellulose acetate strips[18].

Fluorometry

One hundred microlitres of each of the toxins (0.2–0.5 μM) were mixed with 2 ml of Roth's OPA reagent[7], warmed either at 45°C for 10 min or at 55°C for 2 min and the fluorescence intensity measured on a Hitachi 650-40 spectrofluorometer with a 1-cm quartz cell. A blank was run at the same time.

RESULTS AND DISCUSSION

The successful use of OPA as a fluorogenic reagent for α-amino acids was first described by Roth[7]. This reagent reacts with primary amines in the presence of 2-mercaptoethanol to give a fluorescent product[8]. We have found that this reaction is also applicable to the detection of TTX and PSP.

Fluorescence spectra for the OPA products of TTX and PSP were identical to each other (Fig. 2), exhibiting maximum fluorescence at 453 nm with 332-nm excitation. Fluorescence intensities (relative units) of these toxins are shown in Table I. There was little difference between the molar intensities of all the toxins other than TTX, GTX_1 and GTX_4 which exhibited 30–40 % of the intensity of GTX_2 or GTX_3.

TABLE I

FLUORESCENCE INTENSITIES OF OPA DERIVATIVES OF TTX AND PSP

Toxin	*Fluorescence intensity*	
	per nM	*per MU*
TTX	3	2
GTX_1	4	2
GTX_2	10	16
GTX_3	10	6
GTX_4	4	6
STX	7	3
neoSTX	7	9
PX_1	12	867
PX_2	12	86

However, the intensities per mouse unit (MU) greatly differed among the toxins. For example, PX_1 fluoresced about 10 times as strongly as PX_2. The reason for this could be the varied specific toxicity; 30 or 300 MU/mg for PX_1 or PX_2.

Fig. 3 shows the effect of pH on the fluorescence intensity of TTX or PSP in acetate, phosphate and borate buffers. The optimal pH for the TTX- or PSP-fluorophores fell between 9.5 and 10.5. Neither of them fluoresced at pH <6.

These relationship between fluorescence and incubation temperature or incubation time is illustrated in Figs. 4 and 5. The fluorescence was maximal at 40–50°C, then decreased up to 60°C with a breakdown of some fluorophores; thereafter it again increased possibly due to a cleavage of the ring structures of the toxins. The fluorescence was maximized during 10 min of incubation at 45°C. Prolonged incubation beyond 60 min caused a gradual loss of fluorescence.

No marked fluorescence enhancement was observed on addition of 0.003% Brij in buffered OPA reagent.

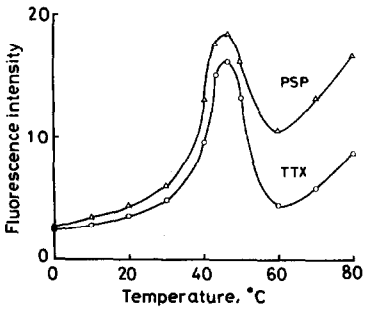


Fig. 4. Fluorescence intensity as a function of temperature. The toxin derivatives were incubated in 0.05 *M* borate buffer (pH 10) for 10 min at the indicated temperatures.

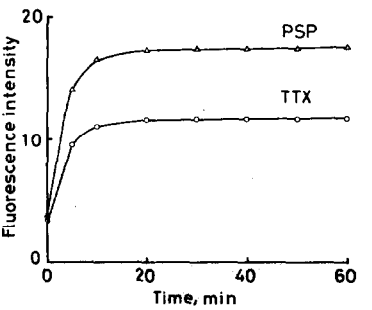


Fig. 5. Fluorescence intensity as a function of time. The toxin derivatives were incubated in 0.05 *M* borate buffer (pH 10) at 45°C for the indicated times.

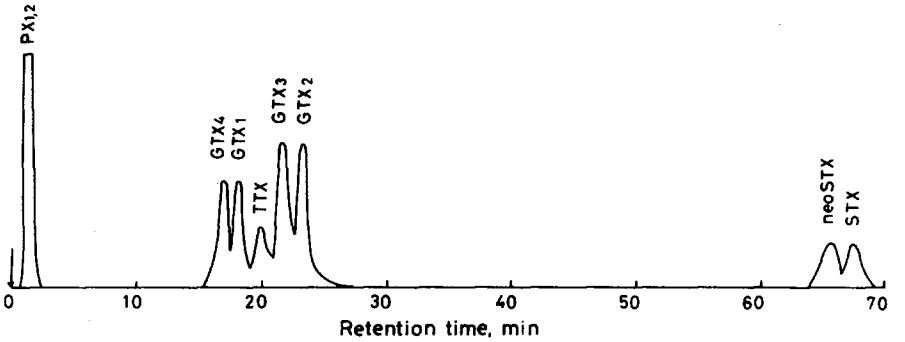


Fig. 6. Chromatogram of a standard mixture of toxins. The mixture (1 μl) contained 20 nM TTX, 1 nM of PX_1 and PX_2 and 5 nM each of GTX_1, GTX_2, GTX_3, GTX_4, STX and neoSTX.

Analysis of standard toxin mixture

A standard mixture of toxins including TTX and PSP was analyzed by HPLC. The chromatogram is shown in Fig. 6. Application of a three-step acetate gradient allowed a complete separation of TTX and PSP except for PX_1 and PX_2 which usually coeluted. The elution order of toxins was $PX_{1,2}$, GTX_4, GTX_1, TTX, GTX_3, GTX_2, neoSTX and STX.

The peak areas were proportional to the amounts of toxins applied. The linearity of this relationship was maintained up to 10 nM for PX_1 and PX_2, 50 nM for GTX_2, GTX_3, STX and neoSTX and 100 nM or more for GTX_1, GTX_4 and TTX. All PSP compounds showed higher fluorescence responses than did TTX.

The lower limit of OPA detection was 1–2 nM for TTX and 0.1–1 nM for PSP. The sensitivity of TTX to alkali (3 M NaOH, 80°C) in this system was about one-tenth of that to OPA.

Analysis of TTX or PSP in a crude toxin preparation from puffer or scallop

The samples used for HPLC were partially purified to remove the bulk of interfering substances.

Ovaries (100 g, 10,000 MU) of the puffer *F. vermicularis porphyreus* or hepatopancreas (5 g, 11,500 MU) of the scallop *P. yessoensis* were extracted with acidic

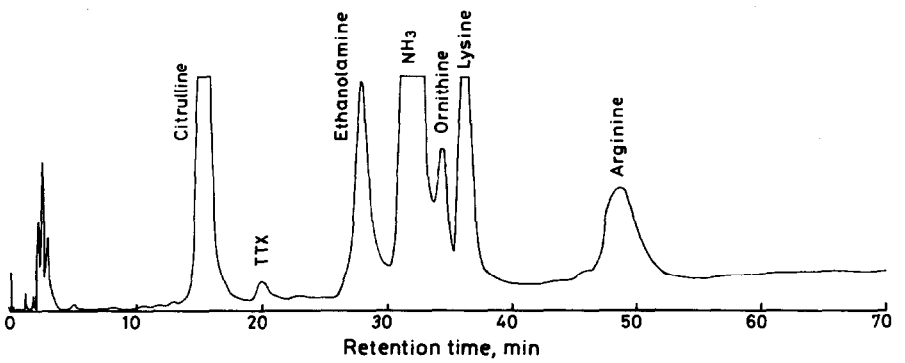


Fig. 7. Chromatogram of a crude toxin preparation (8 MU/μl) of puffer.

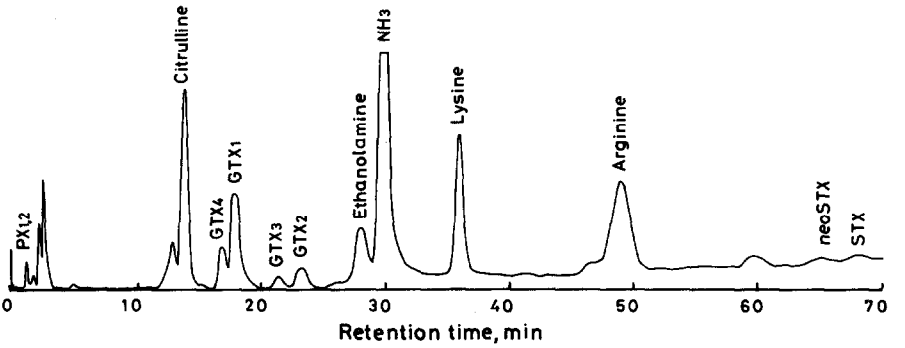


Fig. 8. Chromatogram of a crude toxin preparation (4 MU/μl) of scallop.

methanol or ethanol. Each extract, after being defatted with chloroform, was treated with activated charcoal. Puffer toxin (TTX) was then purified on a column of Amberlite IRC (NH_4^+) or CM-Sephadex C-25 (NH_4^+)[9]. On the other hand, scallop toxins (PSP) were purified on a Bio-Gel P-2 column[10]. Approximately 20–30% of the toxicity was lost during the purification steps. Both toxins were freeze-dried, each was dissolved in 1 ml of water and analyzed by HPLC as mentioned in the Experimental section. The chromatograms of both toxins from a Hitachi 3013 C column are shown in Figs. 7 and 8.

Although numerous peaks appeared for the crude puffer toxin, only one of them gave a retention time (20.0 min) compatible with that of TTX standard. Most of the toxicity was recovered from this peak. Similarly, scallop toxins gave four major peaks corresponding in their retention times to GTX_1, GTX_2, GTX_3 and GTX_4. The identity of the toxins and other main contaminants was confirmed by TLC, electrophoresis and amino acid analysis. The contents of TTX and PSP in both organisms are shown in Table II.

The puffer contained 62 n*M* TTX per g ovary on a wet weight basis. In the scallop hepatopancreas, GTX_2 was predominant, followed by GTX_1, GTX_3, GTX_4 and $PX_{1,2}$. STX and neoSTX were present in very small amounts.

TABLE II

TTX AND PSP CONTENTS IN PUFFER OVARY AND SCALLOP HEPATOPANCREAS

The figures were corrected for the toxicity loss (20–30%) during the preparation of crude toxins.

Toxin	*Per g of puffer*		*Per g of scallop*	
	nM	*MU*	*nM*	*MU*
TTX	62	100	–	–
GTX_1	–	–	590	1210
GTX_2	–	–	648	504
GTX_3	–	–	215	470
GTX_4	–	–	119	67
STX	–	–	5	8
neoSTX	–	–	3	24
$PX_1 + PX_2$	–	–	116	17

Interferences

Since OPA reacts with primary amines the presence of α-amino compounds in quantity interferes with the determination of toxins. Major interfering substances found in the crude TTX or PSP preparation were citrulline, ethanolamine, lysine, ornithine and arginine. The presence of more than 10 n*M* of these amines made difficult the detection of toxins because of their intense fluorescence. A similar effect was also noted with 100 μ*M* ammonium ions.

Precision

Five or more assays indicated that the relative standard deviation for TTX or GTX_1 was ±2.5% or ±3.5%. The errors for assay of other PSP components were within 5% in the concentration range of 1–50 n*M*.

Increased demand for chemical assay of TTX and PSP

Rapid and accurate determination of TTX and PSP occurring in a variety of marine organisms is becoming increasingly important from the public health standpoint, since food poisoning from ingestion of these toxins is often fatal to man.

The technique proposed here may be efficient to identify or quantitate such toxic compounds in marine organisms, even though some complexity still exists in the purification of toxins, which is necessary for removal of diverse interfering substances.

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