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A Chemical Assay for Saxitoxin, the Paralytic Shellfish Poison

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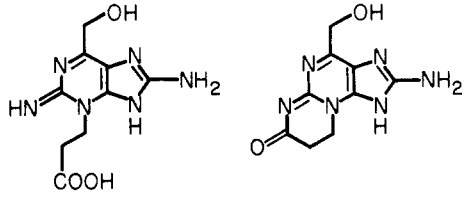
A chemical assay for saxitoxin, the paralytic shellfish poison, has been developed. The technique involves alkaline hydrogen peroxide oxidation of saxitoxin to 8-amino-6-hydroxymethyl-2-iminopurine-3(2*H*)-propionic acid (1), the fluorescence of which is measured at pH 5. This chemical assay is 100 times more sensitive than the existing bioassay and eliminates various problems associated with the bioassay, particularly at low levels of toxin. The method was developed initially with pure saxitoxin and has been applied to a number of samples of marine bivalves *Saxidomus giganteus* and *Mytilus californianus*.

Saxitoxin, which is one of the most toxic substances known, occurs in various bivalves endemic to the Pacific coast of America. The toxin originates in *Gonyaulax catenella* and is concentrated by shellfish which feed on these microscopic dinoflagellates (Schantz *et al.*, 1966). Human consumption of shellfish contaminated with saxitoxin causes poisoning and sometimes death; the human lethal dose is estimated to be ~1 mg, orally. This has necessitated the imposition of a permanent quarantine on clams in Alaska and on mussels from May through October in California.

Saxitoxin in shellfish is presently detected *via* mouse bioassay, which is sensitive to as little as 0.3 μg of saxitoxin per g of shellfish (Schantz *et al.*, 1958). In practice, however, this lower limit cannot be reached, since the presence of sodium ions in the shellfish counteracts the effect of saxitoxin. Several other problems are associated with the bioassay. (a) Before the concentration can be measured with any accuracy, several dilutions must be prepared and assayed. (b) A number of mice of the proper weight must be used for significant results, and there are differences in the susceptibility of different mouse strains. (c) The bioassay is not particularly sensitive and is not specific for saxitoxin.

An antigen–antibody assay has been attempted; however, the results were not promising (Johnson and Mulberry, 1966). A chemical assay utilizing a picrate complex has been reported (McFarren *et al.*, 1958) but it is nonspecific and less sensitive than the bioassay. Another procedure (Neve, 1972), in which saxitoxin is coupled with 1-fluoro-2,4-dinitrobenzene, appears to possess the same disadvantages.

The structure of saxitoxin was recently elucidated (Wong *et al.*, 1971b) and in the course of these studies, saxitoxin was degraded to 2-amino-8,9-dihydro-4-hydroxymethylpyrimido[2,1-*b*]purin-7(1*H*)-one (2) (Wong *et al.*, 1971a). Actually, the initial product of alkaline hydrogen peroxide oxidation of saxitoxin is 8-amino-6-hydroxymethyl-2-iminopurine-3(2*H*)-propionic acid (1) which forms 2 upon acid isolation. The structure of 1 suggested that it should be possible to design a chemical assay for saxitoxin based on the ultraviolet absorption or fluorescence of 1.



1 2

EXPERIMENTAL SECTION

Saxitoxin dihydrochloride was isolated from *Saxidomus giganteus* by the standard procedure (Schuett and Rapoport, 1962) and stored at −13° in a sealed ampoule as an ethanol solution. Final purification of a standard for both chemical and bioassay was carried out as follows.

Saxitoxin (22 mg, 67% pure by bioassay) was dissolved in pH 5 sodium acetate buffer (0.2 *M*, 1.5 ml). The water was evaporated *in vacuo* and the saxitoxin redissolved in the same volume of water. This sample was then applied to a column (0.6 × 21 cm) of Bio-Rex 70, 50–100 mesh, H^+ form (prepared by careful washing, then elution with 1 *M* HCl followed by H_2O until the effluent pH was 5.0), and rinsed with 10 ml of H_2O. Saxitoxin was eluted with 0.05 *M* acetic acid (45–90 ml, 0.25 ml/min). The solvent was evaporated and the saxitoxin redissolved in aqueous HCl (pH 2) repeating this process three times. Finally, the dry saxitoxin was dissolved in H_2O and the pH was adjusted to 5 with AGl-X8, OH^- form. After filtering and evaporating the solvent, the saxitoxin was redissolved in a small volume of 85% EtOH, and purified by chromatography on acid-washed alumina. The ethanol solvent was removed by codistillation with water, and the saxitoxin was dried to constant weight to give an analytically pure sample (12 mg, 80% recovery) of saxitoxin dihydrochloride.

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Table I. Concentration of Saxitoxin Detected in Shellfish Samples

Origin	Date collected	Saxitoxin concn, μg/g of meat: Chem assay	Saxitoxin concn, μg/g of meat: Bioassay
Saxidomus giganteus (from Alaska)			
Douglas Island	7/31/73	0.40	0.36[a]
Eagle Harbor	8/09/73	0.12	
Oliver Inlet	10/04/73	1.17	0.84[a]
Tanakee Beach	12/09/73	2.62	2.1[b]
Mytilus californianus (from California)			
Rockaway Beach	9/23/73	0.67	0.81[c]
Monterey	10/27/73	0.023	
Santa Cruz	11/24/73	0.009	
Seal Rocks	12/12/73	0.013	
Ft. Bragg	4/13/74	0.077	
Salt Point	4/27/74	0.031	
Outlet Cove	5/03/74	0.029	
Bodega Bay	5/03/74	0.013	
Salt Point	7/13/74	0.008	
Rockaway Beach	7/14/74	0.003	
Montara	7/14/74	0.008	

[a] Albino mice. [b] BDF_1 mice. [c] C57B mice.

Bioassay and optical rotation indicated 100% purity within the experimental limits of the methods.

The standard bioassay (Schantz *et al.*, 1958) for saxitoxin was performed using albino mice weighing about 20 g except where noted otherwise. Three mice were generally used to obtain the bioassay data as presented in Table I.

Fluorescence measurements were obtained with a Perkin-Elmer MPF-2A equipped with a UV-VIS photomultiplier tube (Hamamatsu TV R106). Initially a 1 × 1 cm cuvette was used; however screw cap vials were satisfactory for routine assays. There is a linear relationship between the concentration of aqueous solutions of **1** at pH 5 and the observed fluorescence from 10^{-9} to 10^{-5} *M*. When excitation is 330 nm and emission 380 nm, interference of the water Raman peak is minimized. The intensity remains the same between pH 4.7 and 6.0. In 0.5 *M* NaOH, a fluorescence emission maximum at 445 nm is observed. However, this fluorescence is more than an order of magnitude less intense than that observed at pH 5, and colloidal particles interfere with the measurement; thus observation at pH 5 is preferred. For fluorescence measurements, excitation and emission slits were set at 4 nm.

The molar extinction coefficients in the uv spectrum of **1** [335 nm (ε 14,800), 235 (15,400), in 0.5 *M* NaOH] were determined from the spectrum of **2** [347 (17,400), 281 (6400 sh), 254 (14,000), 218 (10,400) in 0.1 *M* NaOH], since the tendency of **1** to form **2** upon drying precluded direct measurement on **1**. A solution of **1** in 1 *M* HCl forms **2** in quantitative yield after 24 hr at 20°, and the reaction is reversed in 0.2 *M* NaOH after 11 hr. The identity of **1** obtained by oxidation of saxitoxin with that obtained by hydrolysis of **2** was confirmed by uv absorption, fluorescence emission, and liquid chromatography [*tert*-butyl alcohol–acetic acid–water (1:1:1), 20 μ of Spherisorb Silica].

The optimum conditions for formation of **1** from saxitoxin are oxidation in 0.5 *M* NaOH with 0.1% H_2O_2 at 20°. A yield of 63.3% is observed by uv (335 nm) after 0.5 hr. Thus, an absorbance of 1.00 will result from a concentration of 39.7 μg/ml (1.068×10^{-4} *M*) of saxitoxin ($C_{10}H_{15}N_7O_3 \cdot 2HCl \cdot H_2O$; mol wt 372). A slow destruction of **1** takes place when it remains in contact with alkaline peroxide for additional time. The yield is independent of the saxitoxin concentration up to 0.003 *M*.

Samples of *Saxidomus giganteus* from Alaska were dug at low tide, frozen in fresh water, and kept frozen until use. Samples of *Mytilus californianus* were collected near San Francisco and kept at 0° for several hours, then maintained at −13° until used. A sample of *Merceneria merceneria* was purchased live, locally, and then kept frozen at −13°.

CHEMICAL ASSAY PROCEDURE

(1) Remove meat from frozen shellfish, cut into chunks, and grind in blender *ca.* 1 min until a homogeneous consistency results. (2) Add 2.0 ml of 0.5 *M* trichloroacetic acid (freshly diluted from 2 *M* TCA) to 2.0 g of ground shellfish meat and mix with glass rod. (3) Heat to internal temperature of 85–90° for 10 min. Mix after 5 and 10 min. (4) Cool in ice bath to 20°. Add *ca.* 0.2 ml of 10% NaOH with stirring until a constant pH of 5.0–5.5 is reached. (5) Centrifuge at 12,000*g* for 10 min. (6) Apply supernatant to a 6 × 75 mm column of 2 ml of Bio-Rex 70, 50–100 mesh resin previously equilibrated with 0.2 *M* pH 5 sodium acetate buffer and discard the eluent. Prepare resin by rinsing several times with HCl and NaOH, rinse with H_2O, and suspend in several volumes 0.2 *M* acetic acid and adjust pH to 5.0 with HCl. Resin may be recycled after use. (7) Elute with 30 ml of 0.2 *M* pH 5.0 sodium acetate buffer, 25 ml of H_2O, and 1.0 ml of 0.5 *M* HCl. Discard eluents. (8) Elute with 4.0 ml of 0.5 *M* HCl and collect eluent in a centrifuge tube. Mix, then divide into two equal volumes in centrifuge tubes. (9) Add 2.0 ml of 1.2 *M* NaOH and 0.05 ml of 10% H_2O_2 to one portion and mix. Substitute H_2O for H_2O_2 in the other. (10) Centrifuge at 1000*g* for 1 min and transfer supernatants into cuvettes. (11) Forty minutes after H_2O_2 was added, neutralize to pH 5 with *ca.* 0.15 ml of glacial acetic acid. (12) Measure fluorescence of oxidized portion and subtract that of the unoxidized blank, using excitation at 330 nm and reading emission at 380. The Raman peak of pure water (excitation 330, emission 371) is useful for routine calibration and corresponds to approximately 0.017 μg of saxitoxin (mol wt 372) per gram of shellfish, or 7×10^{-9} *M* **1**. The relationship between saxitoxin concentration and fluorescence is completely linear. The instrument is conveniently calibrated with a solution of **1**, freshly prepared by oxidation of purified saxitoxin. The concentration of **1** is determined by uv, then this solution is appropriately diluted (pH 5, 0.1% H_2O_2) and its fluorescence is measured against the Raman peak. (13) An abbreviated assay useful for routine applications is possible if the Bio-Rex 70 chromatography and elution (steps 6, 7, and 8) are deleted. A significant amount of time is saved, and limited experience shows that the blank increases only 50%.

RESULTS

The chemical assay for saxitoxin has been successfully applied to a number of shellfish samples. As a procedural test, the nontoxic Atlantic Cherrystone clam, *Merceneria merceneria*, was subjected to the chemical assay. As expected, no saxitoxin was detected. When the ground clam meat was treated with amounts of saxitoxin down to 0.004 μg/g, the recovery was complete according to chemical assay.

The chemical assay was performed on several samples of *Saxidomus giganteus* (Alaska butter clam) and *Mytilus californianus* (California mussel). The results are reported in Table I, in conjunction with mouse bioassays of those samples containing sufficient saxitoxin.

The results show that while only one California mussel sample was particularly toxic, the Alaska butter clam

samples were all quite toxic. The clam sample from Tanakee Beach, Alaska, which was the most toxic sample encountered, was taken 2 months after a number of people became seriously ill by consuming clams collected at that location.

The fluorescence of the unoxidized blank generally corresponds to less than 0.02 μg of saxitoxin per g of shellfish for samples of *S. giganteus*. A somewhat higher blank is found of *M. californianus* and for clam samples subjected to prolonged cold storage. Reproducibility better than ±5% is easily obtained for the fluorescence of the oxidized portion and the unoxidized blank. The presence of compounds in the shellfish extracts capable of quenching the fluorescence of 1 was ruled out by adding a known amount of 1 to the fluorescent solution. No quenching was ever observed.

The bioassay compares reasonably well with the chemical assay, considering the modest number of test animals used and the detrimental effect of NaCl on the bioassay. The chemical assay is at least as expeditious as the bioassay, it eliminates the problems inherent in the bioassay, it is 100 times more sensitive, and it can detect any concentration of saxitoxin likely to be encountered without dilution.

The chemical method described here should allow for the specific rapid, routine, and reliable assay of shellfish for toxicity as a desirable substitute for quarantine by calendar.

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The Detection and Quantitative Determination of Ipomeamarone in Damaged Sweet Potatoes (*Ipomoea batatas*)

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A relatively simple procedure is described for the quantitative determination of ipomeamarone in damaged sweet potatoes. This compound is produced along with other toxic furanoterpenoid compounds as a general response of the sweet potato to certain exogenous stimuli. The toxic compounds were extracted from the tissue with ether and separated by column chromatography, using increasing percentages of ethyl acetate in hexane as the eluting solvent mixture. Thin-layer chromatography was used for qualitatively analyzing the separation fractions. Those fractions containing ipomeamarone were analyzed quantitatively by gas–liquid chromatography, using hexadecane as the internal standard. The lower limit of detection of ipomeamarone by this procedure was 2 ng.

Ipomeamarone is one of several toxic furanoterpenoid compounds produced in sweet potatoes in response to various exogenous stimuli such as fungal infection (Kubota, 1958), insect invasion (Akazawa *et al.*, 1960), and the presence of certain chemicals (Uritani *et al.*, 1960). Ipomeamarone is a hepatotoxic agent (Watanabe and Iwata, 1952) and is usually produced in larger amounts than the other furanoterpenes which are produced simultaneously. These compounds are not found in sound, healthy sweet potatoes; however, their presence in some sweet potatoes that contained minor blemishes and the observation that they are not destroyed by normal cooking procedures (Wilson *et al.*, 1970) suggested that they might pose a health hazard for humans. It was therefore of interest to develop a relatively simple, yet quantitative procedure for screening sweet potatoes for the presence of ipomeamarone and other furanoterpenes. Although procedures have been reported for several of these (Boyd and Wilson, 1972), our procedure involves several modifications of the one reported by Boyd and Wilson (1971) so that it could be used for our specific needs.

Thin-layer chromatography (tlc) was used to detect the presence of the toxin in crude extracts; column chromatography and gas–liquid chromatography (glc) employing an internal standard were used for isolating the ipomeamarone and determining its concentration.

EXPERIMENTAL SECTION

The sweet potatoes used in this study were carefully selected and purchased from local grocery stores. Sound, healthy sweet potatoes without any apparent blemishes or bruises were used for control purposes, while those that contained blemishes and showed evidence of bruises or other damages were used as possible sources of ipomeamarone. In some instances, peelings of healthy sweet potatoes (skin plus 2–3 mm of adjoining tissue) were sliced into small pieces, placed in a beaker, and covered loosely with aluminum foil overnight. The beaker plus contents were stored 5–7 days at room temperature in a sealed

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