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Radioimmunoassay of Paralytic Shellfish Toxins in Clams and Mussels

G. C. Yang,[1] S. J. Imagire,[1] P. Yasaei,[1] E. P. Ragelis,[1] D. L. Park,[1] S. W. Page,[1] R. E. Carlson,[2] and P. E. Guire[2]

[1]Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, Washington, DC 20204 and [2]Bio-Metric Systems, Inc., Eden Prairie, Minnesota 55344

Shellfish contaminated with paralytic shellfish poisons (PSP) compromise human health. The threat of this contamination results in enormous economic losses in the recreational and commercial exploitation of shellfish resources in the affected areas. Most states deal with the PSP problem either by prohibiting the collection of shellfish during certain time periods or by instituting monitoring programs. The only recognized method of analysis for PSP that is currently and routinely used in monitoring programs is the time-of-death mouse bioassay (Williams 1984). This assay is significantly limited by its pronounced variability and by its limit of determination of approximately 37 µg PSP/100 g meat, which is close to the maximum allowed level of 80 µg PSP/100 g. Its limited throughput, because it is labor-intensive, and the resulting expense are also factors in restricting more extensive monitoring programs. A recently developed high-performance liquid chromatographic (HPLC) method of analysis for PSP toxins is promising for use as a routine screening method (Sullivan and Wekell 1984). However, this method requires rather expensive instrumentation and relies on a relatively difficult post-column derivatization procedure. Converting this method to a "field" procedure (either in the shellfishing or wholesale market areas) would present a number of problems.

Several attempts to develop simple and highly specific biochemical assays for the detection and quantitation of the PSP toxins have been reported (Johnson et al. 1984; Davio and Fontelo 1984). More recently, much improved immunoassays have been developed (Carlson et al. 1984; Chu and Fan 1985). To evaluate the validity and usefulness of the immunoassay for the determination of PSP toxins, we have used extracts of shellfish gathered from Maine and Connecticut to compare the results of the mouse bioassay and HPLC methods with the immunoassay developed by Carlson et al.

MATERIALS AND METHODS

PSP standards of saxitoxin (STX); its 11-hydroxy sulfate derivatives, gonyautoxins 2 (GTX2) and 3 (GTX3) and neosaxitoxin

(NEO); and the 11-hydroxy sulfate derivatives of NEO, gonyautoxins 1 (GTX1) and 4 (GTX4), and 21-sulfo derivatives of STX, NEO, GTX2, and GTX3 (B_2, B_1, C_1, and C_2, respectively) were supplied by Dr. S. Hall, Center for Food Safety and Applied Nutrition, Food and Drug Administration.

Saxitoxinol (STXOL, Figure 1), obtained from the reduction of the hydrated ketone group at C-12 in STX, was coupled to a large protein molecule, bovine serum albumin, to elicit immunogenicity. Conjugation chemistry, rabbit immunization, antibody characterization, and tritium labeling have been detailed in a previous publication (Carlson et al. 1984). The assay uses a 1.5-mL Eppendorf microcentrifuge tube containing anti-saxitoxin serum, protein A (IgG Sorb, The Enzyme Center, Boston, MA), co-lyophilized with the tritium-labeled STXOL (specific activity 138 dpm/pmol).

Saxitoxins were extracted from clam and mussel meats with 0.1N HCl solution (Williams 1984). A portion of this acidic extract was injected intraperitoneally into the test mice; the time of death of each mouse was observed. This time was converted into equivalent toxin concentration by comparison with a standard time-of-death curve for mice by using known amounts of STX standard (Williams 1984).

For the radioimmunoassay (RIA) measurements, a known quantity of shellfish extract was evaporated to dryness under nitrogen and reconstituted with 1 mL of phosphate buffer solution (pH = 7.2, 0.02M). This solution was transferred to a microcentrifuge tube containing antibody, radiolabeled STXOL, and protein A. The centrifuge tube contents were thoroughly mixed by gentle shaking for 10-20 min and centrifuged at 3,000 rpm for 30 min (Sorvall RC-5B, Dupont Instrument Co., Wilmington, DE). A portion of supernatant (0.8 mL) was removed from the centrifuge tube, and radioactivity (dpm) was determined (Tri-Carb 460 CD, Packard Co., Downers Grove, IL).

A standard curve was obtained by adding known amounts of saxitoxins ranging from 5 to 1000 pmol to the microcentrifuge tube as described above (instead of shellfish extracts), counting the radioactivity of each addition, and plotting the percent binding against concentration of added standards. Concentrations of the unknown can thus be obtained by comparing the measured percent bindings to the standard curve values. Cross-reactivities of the anti-STXOL antibody against GTX2, GTX3, GTX4, C_1, B_2, and NEO for the same antibody were obtained by a similar procedure.

The HPLC procedure used in this study was developed by Sullivan and Wekell (1984). It involves the separation of saxitoxins on a polystyrene divinylbenzene resin column (Hamilton, PRP-1, Reno, NV), by using heptane and hexane sulfonic acids as ion-pairing reagents. A Shimadzu HPLC system (LC4A, Columbia, MD) equipped with a post-column reaction system (Kratos URS-051, Ramsey, NJ) followed by a fluorescence detector (Shimadzu, RF-530, Columbia,

MD) was used to separate and identify PSP toxins. The PSP toxin fractions (0.5 mL of each fraction) for subsequent RIA determinations were collected as they eluted from the HPLC column (before the post-column oxidation).

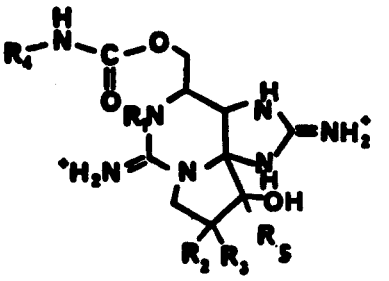
RESULTS AND DISCUSSION

Standard curves for STX-related compounds (GTX2, GTX3) showed considerable cross-reactivity with STX (Figure 2), while the neosaxitoxin compounds (NEO, GTX4) showed almost no cross-reactivity against STX with anti-STXOL antibody and ^{3}H-STXOL tracer. Table 1 illustrates the amounts of various PSP toxins needed to displace 50% of bound ^{3}H-STXOL. Specificity of the harvested antibody can be further illustrated by first separating a standard mixture of PSP toxins by HPLC and collecting various fractions to be examined by RIA. Chromatograms (Figure 3) obtained by both fluorescence and RIA techniques demonstrate that this antibody has stronger affinity toward STX, the N_1-hydrogen type of PSP, than toward the N_1-hydroxyl type. To further test the applicability and validity of this RIA technique, two collections of East Coast shellfish, 12 from Connecticut estuaries and 68 from Maine, were extracted and examined by both mouse bioassay and RIA techniques. Selected shellfish were also analyzed by the HPLC method.

Except at high PSP concentrations, the RIA gives highly reproducible results (Carlson et al. 1984). In our routine analyses of duplicate portions, the variability of the RIA results was 10-15%. The results of the mouse bioassay, the HPLC analyses, and the RIA analyses for shellfish from Connecticut waters are compared in Table 2. Table 3 shows the results from the 68 shellfish collected from the Maine seacoast. The qualitative agreement between these RIA results and both mouse bioassay and HPLC procedures supports the potential utility of immunoassay methods for PSP analysis. However, these correlations are partly due to the fortuitous general absence of NEO in the shellfish extracts in this study. Of the toxic components observed (STX, GTX2, GTX3, and NEO; cf Figure 3), only NEO was not detected by RIA.

Table 1. Cross-reactivity, as reflected by the 50% displacement of ^{3}H-STXOL by various STX derivatives, using an anti-STXOL-BSA antibody

PSP	pmol/tube
STX	85
GTX2	150
GTX3	400
GTX4	3,000
C_1	> 10,000
B_1	>100,000
NEO	>100,000



Compound	R1	R2	R3	R4	R5
Saxitoxin (STX)	H	H	H	H	OH
Gonyautoxin 2 (GTX2)	H	H	OSO_3^-	H	OH
Gonyautoxin 3 (GTX3)	H	OSO_3^-	H	H	OH
Neosaxitoxin (NEO)	OH	H	H	H	OH
Gonyautoxin 1 (GTX1)	OH	H	OSO_3^-	H	OH
Gonyautoxin 4 (GTX4)	OH	OSO_3^-	H	H	OH
Saxitoxinol (STXOL)	H	H	H	H	H
B_1	H	H	H	SO_3^-	OH
C_1	H	H	OSO_3^-	SO_3^-	OH
C_2	H	OSO_3^-	H	SO_3^-	OH
B_2	OH	H	H	SO_3^-	OH
C_3	OH	H	OSO_3^-	SO_3^-	OH
C_4	OH	OSO_3^-	H	SO_3^-	OH

Figure 1. Structure of PSP toxins.

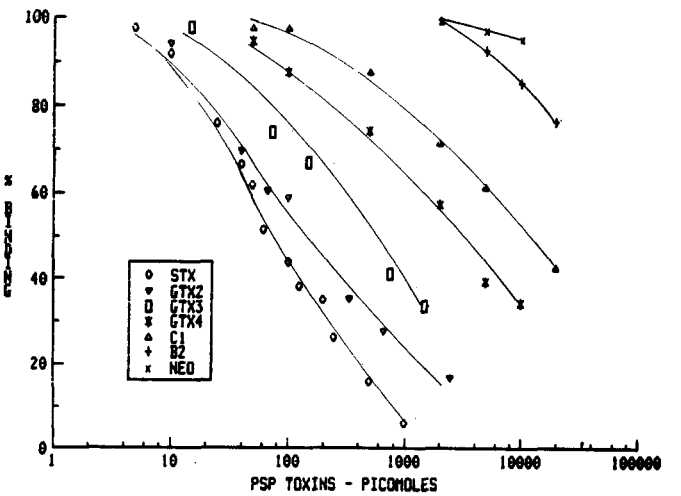


Figure 2. Cross-reactivity curves for various PSP toxins against anti-saxitoxinol antibody with ^{3}H-saxitoxinol tracer.

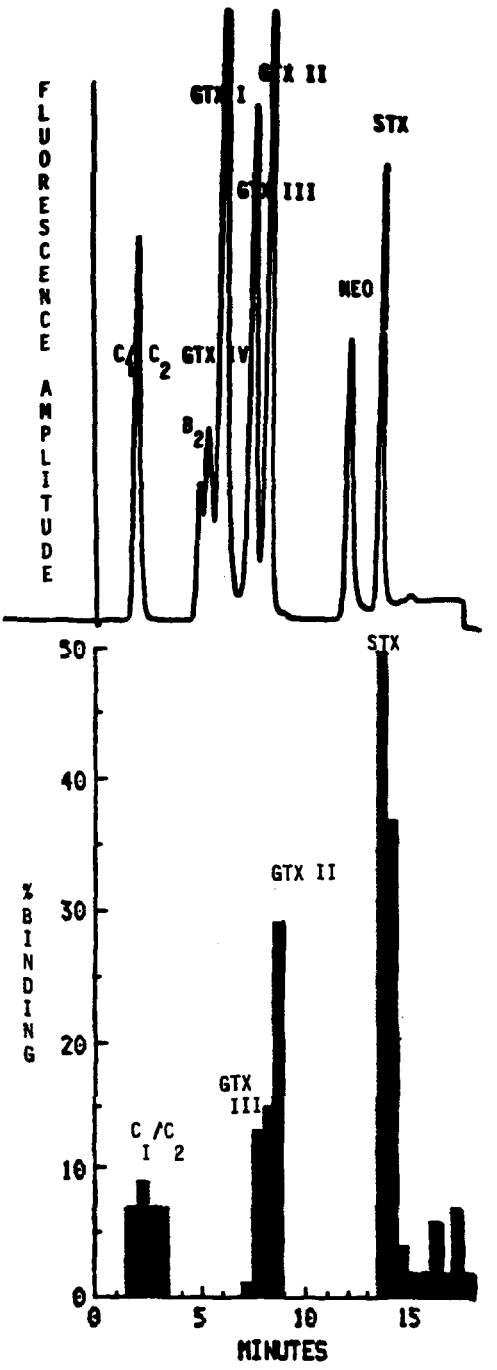


Figure 3. Chromatogram of East Coast shellfish extract with both fluorescence and RIA as detectors.

Table 2. Comparison of HPLC, mouse bioassay, and RIA results from Connecticut shellfish (µg/100 g meat)

Shellfish ID	HPLC	Mouse bioassay	RIA
8053187	257	207	167
8053380	77	< 37	< 17
8053184	491	346	325
8052988	81	64	56
8053043	113	136	77
8043044	70	103	79
8053042	143	151	88
8053410	131	126	97

Table 3. Comparison of HPLC, mouse bioassay, and RIA results from Maine shellfish (µg/100 g meat)

Shellfish ID[1]	HPLC[2]	Mouse bioassay	RIA
BA 514	27	< 37	29
BA 612	122	71	96
BA 712	-	115	154
BA 724	84	45	62
BE 612	-	195	177
BE 626	-	165	172
BE 717	210	259	198
BE 724	-	49	133
GE 514	-	< 37	< 17
GE 619	-	< 37	103
GE 709	-	125	175
GE 703	252	103	112
GE 716	207	58	181
GE 724(1)	82	45	49
MA 709	132	63	>496
MA 716	207	129	142
MA 723	-	67	>186
MA 730	197	52	150
ME 604	-	44	59
ME 716	-	606	584
ME 723	397	204	342
ME 730	268	220	268
MO 709	-	305	326
MO 716	-	357	296
MO 730	-	340	532
OE 516	-	< 37	< 8
OE 523	-	< 37	32
OE 608	-	73	44
OE 606	-	< 37	53
OE 610(1)	-	41	32

Table 3. (continued)

OE 709(L)	136	106	125
OE 705(H)	-	61	83
OE 725(L)	18	< 37	< 17
OE 816(B)	77	41	63
PA 515	-	< 37	0
PA 718	-	51	79
PA 712	-	73	113
RA 507	82	< 37	31
RA 514	31	38	107
RA 528	56	< 36	40
RA 521	48	40	47
RA 604	34	-	50
RA 611	47	< 37	27
RA 621	-	< 37	17
RA 625	94	-	62
RA 621	-	< 37	32
RA 705	-	91	325
RA 709	117	69	90
RA 723	64	55	57
RA 716(1)	-	104	201
RE 507	-	44	30
RE 514	-	-	28
RE 528	56	< 37	28
RE 604	-	42	0
RE 611	87	96	93
RE 618	-	129	235
RE 625	235	277	248
RE 621	209	250	179
RE 709	-	220	367
RE 705	-	830	175
RE 702	-	532	120
RE 730	-	63	275
VA 710	52	< 37	< 42
VA 723	15	< 37	17
VE 710	72	45	89
VE 716	31	< 37	30
VE 710	80	41	83
WA 725	7	< 37	23
WE 711	43	< 37	35
WE 801	21	< 37	25
YE 625	-	298	563
YE 702	444	333	377
YE 712	73	< 93	80
YE 725	-	< 37	< 42

[1]The second letter "A", "E", and "O" in shellfish code denotes Mya arenaria; Mytilus edulis, and Ostrea enfis species, respectively.

[2](-) = not determined.

This study indicates that the immunoassay developed by Carlson et al. (1984) has excellent potential for use as a screening method for PSP toxins in shellfish from Maine and Connecticut waters. It could not be used reliably for shellfishing areas where the shellfish contain significant levels of NEO or GTX1 and GTX4. If antibodies for a NEO conjugate can be obtained, a mixed (STX and NEO) assay would provide a method of analysis for all major PSP toxins. Further validation studies to determine the performance characteristics of this method will be conducted when antibodies with the appropriate cross-reactivities are available.

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