Immuonoassay Methods for Paralytic Shellfish Poisoning Toxins

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The current status of immunochemical techniques for analysis of paralytic shellfish poisoning (PSP) toxins is summarized. Important aspects regarding production of the biological reagents necessary for immunochemical methods, the characteristics of polyclonal and monoclonal antibodies against saxitoxin and neosaxitoxin, and the importance of test sensitivity and specificity are discussed. Applications of immunochemical techniques for PSP toxins include microtitre plate enzyme immunoassays and enzyme-linked immunofiltration assays for toxin detection, and immunoaffinity chromatography (IAC) for sample extract cleanup. A major advantage of enzyme immunoassay (EIA) is simplicity and rapidity of the test procedure, and higher sensitivity than other methods. However, quantitative agreement between EIA and mouse bioassay is dependent on antibody specificity and the toxin profile in the shellfish; thus, both over- and under-estimation of total toxicity may occur. For screening purposes, however, EIAs offer major advantages over the mouse bioassay, which is criticized in Europe because of animal welfare. A major application of antibodies against PSP toxins is their use for extract cleanup by IAC, which gives highly purified extracts, thereby enhancing determination of PSP toxins by conventional physicochemical methods such as liquid chromatography. IAC can also be used to isolate PSP toxins for preparation of analytical standard solutions.

The health risks associated with paralytic shellfish poisoning (PSP) toxins in food have triggered development of analytical techniques, including biological, physicochemical, and immunochemical methods. Updates of these methods appear in the J. AOAC Int. annual referee report on shellfish toxins. The present report summarizes and reviews the current status of immunochemical techniques for PSP toxins. Important aspects of how to produce specific antibodies against PSP toxins are described. Antibodies against PSP toxins have been developed primarily for analytical purposes. Production of polyclonal (Pab) and monoclonal (Mab) antibodies as toxin-binding antidotes (1–3) or production of anti-idiotype antibodies as candidate vaccines (4) are not yet available for practical use. Present applications of anti-PSP toxin antibodies to analyze and purify target analytes are emphasized along with respective advantages and limitations of these techniques.

Antibodies Against PSP Toxins

Because PSP toxins are low molecular weight compounds (haptons), they must be conjugated to convert them to an immunogenic form. Johnson et al. studied the antigenic properties of saxitoxin-protein conjugates in the mid-1960s (5). Their method was used 20 years later when competitive enzyme immunoassays for saxitoxin were first described.

A major aspect in preparing protein conjugates (immunogens, labeled antigens) of saxitoxin (STX) or other PSP toxins (Figure 1) is the limited availability and the high price of toxin standards. Conjugates have predominantly been described for STX and neosaxitoxin (NEO). A suitable conjugation method must be efficient and straightforward, avoiding purification (and loss) of toxin derivatives. The carbamate toxins possess an amino group at N-21 which enables use of amino-reactive conjugation procedures. However, because guanidine and imine groups may also react during such procedures and the toxin present in the carrier molecule cannot be characterized, the true reaction pathway is not known. Decisive proof of successful conjugation is provided by demonstrating binding by specific anti-PSP antibodies, which presented a kind of vicious circle until antibodies became available.

We successfully used 3 methods to prepare STX and NEO conjugates: formaldehyde condensation according to the Mannich reaction (5, 6), reductive alkylation (periodate method; 7, 8), and glutaraldehyde reaction (9, 10). However, glutaraldehyde reaction requires large amounts of toxin because the molecular ratio toxin:carrier must be at least 100:1, and generally gives conjugates with weak specific activity. Therefore, the use of this method is not recommended.

For rabbit antisera against STX, an efficient strategy is the combination of formaldehyde treatment for immunogen synthesis and reductive alkylation for preparation of the labeled antigen used in the enzyme immunoassay. For formaldehyde treatment, about 300 µg toxin is required to prepare 2–5 mg ac-
tive immunogen, e.g., STX coupled to keyhole limpet hemocyanin STX-KLH. By using reductive alkylation, small amounts of STX (100 μg) can be used to prepare about 10 mg STX-horseradish peroxidase (HRP) conjugate, with a working concentration in EIA of 100 ng/mL or less. This combination was used for a competitive direct EIA for STX and resulted in a significantly greater sensitivity than assays using formaldehyde synthesis of both the immunogen and labeled antigen (7, 11).

The periodate method is an excellent alternative to formaldehyde treatment for conjugation of STX or NEO to glucose oxidase (GOx), a glycoprotein with a molecular weight of 186 000 and a neutral sugar content similar to that of HRP. This approach was used to induce immune response against STX in mice (12), because immunogens prepared by formaldehyde gave very poor immune responses in mice in earlier experiments (3, 13). Reductive alkylation conjugation may also be used when only trace amounts of toxin standard are available to prepare an immunogen. For example, a total amount of >300 mL antiserum against NEO was produced after preparation of 5 mg immunogen (NEO–GOx) from only 28 μg NEO (14).

**Assay Formats**

**Microtiter Plate Enzyme Immunoassays**

Although radioimmunoassays were developed in the mid-1980s (15, 16), they are rarely used any more. The most widespread immunoassay technique in food and environmental analysis today is the microtiter plate enzyme immunoassay (EIA). This test format, usually performed in 96-well plates with instrumental absorbance measurement (ELISA-reader), is a highly standardized technique that includes method validation.
Table 1. Immunoassays for PSP toxins

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Immunogen</th>
<th>Assay system</th>
<th>Sensitivity $IC_{50}$, ng/mL</th>
<th>Specificity (toxins detected with &gt;1% relative cross-reactivity)</th>
<th>Detection limit in shellfish tissue, ng/g</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>STX</td>
<td>Pab (rb)</td>
<td>STX'ol-CDI-BSA</td>
<td>RIA</td>
<td>Not indicated</td>
<td>STX, STX'ol</td>
<td>Approx. 20</td>
<td>15, 16</td>
</tr>
<tr>
<td>STX</td>
<td>Pab (rb)</td>
<td>STX-PA-BSA</td>
<td>CD-EIA</td>
<td>0.28</td>
<td>STX, GTX 1/4</td>
<td>0.2</td>
<td>11</td>
</tr>
<tr>
<td>STX</td>
<td>Pab (rb)</td>
<td>STX-PA-KLH</td>
<td>CD-EIA</td>
<td>0.140</td>
<td>STX, dc-STX, GTS 2/3, NEO, B1, C 1/2, GTX 1/4</td>
<td>20</td>
<td>38</td>
</tr>
<tr>
<td>STX</td>
<td>Mab</td>
<td>STX-PJ-GOx</td>
<td>CD-EIA</td>
<td>0.015</td>
<td>B1, C ½, GTX 1/4</td>
<td>3</td>
<td>7, 8, 25</td>
</tr>
<tr>
<td>STX</td>
<td>Pab (rb)</td>
<td>STX-GA-poly-alanine lysine</td>
<td>CD-EIA</td>
<td>Not indicated</td>
<td>STX, NEO, GTX 2/3</td>
<td>Not indicated</td>
<td>9, 10</td>
</tr>
<tr>
<td>STX</td>
<td>Mab</td>
<td>STX-PJ-GOx</td>
<td>CD-EIA</td>
<td>1.5</td>
<td>STX, GTX 2/3, dc-STX, b1, NEO, GTX ¼, C 1/2</td>
<td>Not tested</td>
<td>12</td>
</tr>
<tr>
<td>NEO</td>
<td>Pab (rb)</td>
<td>NEO-FA-KLH</td>
<td>CD-EIA</td>
<td>0.9</td>
<td>NEO, GTX</td>
<td>Not tested</td>
<td>39</td>
</tr>
<tr>
<td>NEO</td>
<td>Pab (rb)</td>
<td>NEO-FA-KLH</td>
<td>CD-EIA</td>
<td>0.18</td>
<td></td>
<td>0.2</td>
<td>11</td>
</tr>
<tr>
<td>NEO</td>
<td>Pab (rb)</td>
<td>NEO-FA-BSA</td>
<td>CD-EIA</td>
<td>0.076</td>
<td>NEO, GTX, STX 1/4, STX 1/4</td>
<td>Not tested</td>
<td>14</td>
</tr>
</tbody>
</table>

$^a$ Abbreviations: CDI, carbonyl diimidazole; FA, formaldehyde condensation; GA, glutaraldehyde reaction; PJ, reductive alkylation with periodate; STX'ol, saxitoxinol; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; GOx, glucose oxidase; rb, rabbit.

Because the competitive format applies to small molecules such as PSP toxins, the measured signal is inversely proportional to the concentration of the analyte. The negative control results in the strongest signal ($B_0$), and the test signals ($B$) for standard concentrations or sample extracts are expressed as a percent of the negative control ($B/B_0 	imes 100$), to standardize the dose–response relationship. Because of antigen–antibody binding and enzyme reaction kinetics, the standard curves of EIAs are not linear but follow a sigmoid shape (Figure 2). Both competitive direct (CD–EIA) and indirect EIA (CI–EIA) test formats have been developed for STX and NEO. Immunochemical methods described for PSP toxins are listed in Table 1.

In CD–EIA, antitoxin antibodies are immobilized at the solid phase (the polystyrene surface of the microtiter plate well). Free toxin and labeled toxin (STX or NEO coupled to HRP) compete for antibody binding sites, and color is developed by the specific activity of bound HRP. Although NEO–HRP and other PSP toxin–HRP conjugates have been used (8, 11), the use of STX–HRP for tests based on antibodies against STX and NEO is the most convenient and cost-efficient strategy. For color development, a system of enzyme-substrate (hydrogen peroxide) and oxidation of a chromogen, e.g., 3,3',5,5'-tetramethyl benzidine (17), is used, and after a stop solution (sulfuric acid) is added, the absorbance is measured at 450 nm.

In CI–EIA, a toxin–protein conjugate, i.e., STX or NEO coupled to bovine serum albumin (BSA) or polylysine, forms the solid phase. STX standard solution may also be directly bound to microtiter plates (18), but this procedure would only be cost-efficient if abundant amounts of inexpensive toxin standard were available. Free toxin and solid-phase toxin compete for antibody binding sites, and after a wash step, bound antitoxin antibody is detected by using a second, enzyme-labeled antibody. Color development is identical to the direct test format.

Different approaches are used to determine the sensitivity (detection limit) of competitive EIAs; therefore, the toxin concentration resulting in 50% binding inhibition ($IC_{50}$) of the labeled (or solid phase) antigen to antibodies is the preferred method for comparing EIA sensitivities. Depending on the absorbance measurement range ($\Delta A$) and the coefficients of variation for standard replicates, the absolute detection limit of an EIA standard curve is usually 1/2–1/4 of the $IC_{50}$ concentration of (75–80% $B_0$). We evaluated the results of microtiter EIAs with a software program designed for competitive EIAs (19), which uses a cubic spline function for calculation of the standard curve (4-fold determinations of all standard concentrates). The program also calculates the detection limit (Student’s $t$, 95% confidence limit) and the $IC_{50}$ concentration for each standard curve. To compensate for between-day variations, data for at least 20 (up to 50) standard curves performed over 4–6 weeks are pooled, and the mean detection limit ($\pm$ standard deviation) is calculated for each EIA. In most cases this approach leads to a mean detection limit corresponding to 75–80% $B_0$. This conservative approach gives a more realistic estimate than other calculations and reduces the frequency of false-positive results.

All EIAs for PSP toxins described so far (Table 1) have a high sensitivity for their target toxin (STX or NEO), several orders of magnitude better than that of the mouse bioassay, which detects STX at about 200 ng/mL (20). When both CI–EIA and CD–EIA were established with the same antibodies, the CD–EIA format was 5–15 times more sensitive, because different conjugation procedures were used for immunogen and labeled antigen synthesis, avoiding toxin-carrier bridge recognition effects. All but one of the available EIAs use rabbit Pab for toxin detection. High-affinity Mabs against STX have been produced only recently (12). Typical standard curves of EIAs developed by our group are shown in Figure 2. The Pab-based CD–EIA for STX has an $IC_{50}$ of 15 pg/m and a mean detection limit of 7 pg/mL (2.3 ×
which is close to the maximum sensitivity that may currently be achieved with competitive EIAs using HRP as the enzyme label.

Besides test sensitivity, an important aspect determining the practical use of immunoassays for PSP toxins is test specificity. Specificity is expressed as the IC$_{50}$ dose of a cross-reacting compound compared with that of the reference compound (expressed as % cross-reactivity). The relative cross-reactions of the EIAs that we established using toxin standards prepared by a Canadian research group (21) are shown in Figure 3. Obviously, from an immunological point of view, PSP toxins must be divided into the STX and NEO groups. Antibodies against STX usually have strong cross-reactions with N1-hydrogen analogs, such as decarbamoyl-STX (dc-STX), gonyautoxins 2/3 (GTX 2/3), and N-sulfocarbamoyl-STX (B 1), and both Pab and Mab show very similar behavior. Inversely, the test system for NEO predominantly recognizes the N1-hydroxy toxins (NEO, GTX 1/4). Because GTX 2/3 and GTX 1/4 were available only as epimeric mixtures, the individual cross-reactivities could not be determined, although data published by others (22) suggest that cross-reactivity of STX EIA is higher for GTX 2 than for GTX 3. This differentiation between N1-hydrogen and N1-hydroxy toxins means that, compared with results of the toxicity-based mouse bioassay, the toxin content of a sample will be underestimated by STX EIAs if the toxin composition is dominated by NEO or GTX 1/4. Inversely, the NEO EIA will underestimate STX and GTX 2/3. Therefore, the use of a combination of both tests has been suggested (23). However, it must be remembered that EIAs are measured in the pg/mL range, and a cross-reaction of only 3%, such as that of NEO in the STX EIA, still corresponds to a detection limit for NEO of about 200 pg/mL. For the NEO EIA, the situation is similar. Therefore, the presence of all PSP toxins will be reliably detected qualitatively by both assays at threshold levels that are lower than can be attained by the mouse bioassay.

Evaluation of EIAs by comparison with other detection techniques for naturally contaminated material is mandatory to provide insight into their true performance. However, such studies are complicated because the only method that is recognized worldwide, the mouse bioassay, is far from being a reliable quantitative method and is rarely performed exactly as described in AOAC Official Method 959.08 (20), simply to reduce the number of mice killed. Furthermore, animal protection laws in Germany and other European countries restrict the use of the mouse bioassay. Liquid chromatographic (LC) methods have problems with separating compounds of the PSP toxin complex, and quantitation is not possible for many compounds because no standards are available. Some available data are summarized below.
Chu et al. (23) compared the EIA and mouse bioassay using various combinations of their CD–EIAs for STX and NEO. A total of 1540 shellfish samples, PSP positive and negative as determined by mouse bioassay, were re-analyzed by EIAs. No false negatives were found, and agreement between EIA and bioassay was relatively good in a lower concentration range (<400 ng/g). However, correlation between results from one EIA (STX or NEO) and the bioassay was only moderate, whereas the combined data of both EIAs improved the quantitative agreement. The authors concluded that using both the STX and the NEO EIA in a monitoring system could eliminate about 80–85% of tests requiring the bioassay.

Inside a project focused on production of PSP toxin reference material, candidate materials were also analyzed by the CD–EIA for STX developed in our laboratory. Compared with LC results for STX, the EIA globally overestimated the toxin content, probably due to the presence of other PSP toxins (dc-STX, B1) which were present in the sample (24).

Comparison of a commercial CD–EIA test kit for STX with the mouse bioassay gave good qualitative agreement between both methods for mussels and scallops from the British North Sea coast (25). No false-negative results were obtained by EIA (n = 45), and only one false positive was obtained out of 15 bioassay-negative samples. At lower PSP toxin levels (<800 μg/kg by bioassay), the EIA overestimated the toxin content, whereas at high levels (800–9000 μg/kg), toxin was underestimated for 4 out of 32 samples. When CD–EIA was used for NEO (14), no NEO or GTX 1/4 was detected in any samples.

In a similar study, O’Neill et al. (26) compared the results of this EIA for samples of mussel (n = 45) and king scallops (n = 46) from the UK North Sea coast with those from the mouse bioassay and LC. There was better agreement for king scallops than for mussel because of a higher proportion of NEO and GTX 1/4 in the latter. Although false-negative results did not occur for samples with PSP levels >800 μg/kg, at levels between 400 and 800 μg/kg only 80% (king scallops) and 50% (mussel) of samples were correctly placed by EIA, and both under- and overestimation occurred. However, the authors concluded that although further validation is necessary, the EIA has the potential as a prescreen of shellfish.

Kasuga et al. (22) analyzed 2 samples of scallops from Japan (396 and 1730 μg STX equivalents per kg, determined by mouse bioassay) and found 50% lower results with this EIA test kit. The authors assumed that mouse bioassay toxicity was primarily due to GTX toxins.

Besides analysis of shellfish, crab meat, and similar tissues, EIA has been used to detect STX in mackerel at pg/g levels (27). In that study mackerel fed in captivity accumulated STX via the food chain, and, indeed, trace levels (0.02–0.64 ng/g) were found in samples associated with scombrotoxicosis, a food poisoning usually related to histamine. However, confirmation of such low levels was not possible because of the lack of sufficiently sensitive methods. Immunoaffinity chromatography (discussed below) may be helpful in such cases in the future.

The EIA test kit was also used as a qualitative screening method for PSP toxin production in bacterial cultures associ-
ated with *Alexandrium* spp. (28). Because of its tremendous sensitivity, this test can also be used to check the toxigenicity related to STX and other PSP toxins in freshwater cyanobacteria. For example, in August 1997 we collected water samples from 13 different lakes in southern Bavaria (Germany), some highly eutrophic. Water samples were directly analyzed by CD–EIA for STX, without any cleanup needed. At a detection limit of 7 ng/L for STX, no evidence of PSP toxin contamination was detected. Because false-negative results are highly unlikely with competitive EIAs, this method is suitable for the screening of environmental water samples (unpublished data).

**Rapid Visual Detection Techniques**

Besides microtiter plate EIAs, we also developed membrane-based EIAs that are designed as rapid visual tests. Both dipstick and enzyme-linked immunofiltration assay (ELIFA) formats for STX have been established (7, 29, 30). The ELIFA is the more convenient format in terms of test handling and test time. Sample and reagent solutions are simply dropped onto the antibody-coated membrane of a self-designed test device, and the visible result (blue color) of the coated area can be read after a total test time of 10 min. Although the sensitivity of this visual test (4 ng/mL) was much lower than that of the microtiter plate EIA (7 pg/mL), more concentrated sample extracts could be applied to the ELIFA, resulting in a relatively sensitive detection limit for STX in shellfish of 80 ng/g. Detection limit for dc-STX, GTX 2/3, and NEO in shellfish was estimated to be 250, 800, and 2500 ng/g, respectively. Although the ELIFA did not meet legal requirements for PSP toxins in shellfish (total STX equivalents < 800 ng/g), it could still be used for on-site monitoring of environmental samples, e.g., during toxic algal blooms.

**Immunoaffinity Chromatography (IAC)**

The use of IAC columns for sample cleanup is of increasing importance for trace level determination of analytes in foods and environmental samples, and has opened new analytical horizons. For example, reliable routine quantification of some mycotoxins (aflatoxins, ochratoxin A) by LC methods at the pg/g level has become feasible only with the availability of IAC columns (31–34).

Unlike the situation in a competitive EIA, antigen binding, or the efficiency of analyte retention in the IAC column, is not
limited by cross-reaction of a specific compound relative to the reference compound, but is determined only by affinity of the antibodies. Therefore, all compounds for which the absolute affinity constant is sufficiently high (approximately $10^7 \text{L/mol}$) are bound by IAC columns, provided that the capacity is high enough. This enables multianalyte cleanup with group-specific antibodies. Although IAC columns may be prepared with polyclonal antisera, Mabs are preferable because of the homogeneity of the antibody binding sites, and because large amounts of antibodies are needed.

Dietrich et al. (12, 35) first described Mab against STX which fulfilled the quality requirements for IAC of PSP toxins. The Mab reacted with all other PSP toxins for which standards were available for testing (Figure 3) and with others (e.g., dc-GTX 2/3), as found by LC after IAC of naturally contaminated shellfish samples. In experimental IAC of PSP toxins at a concentration of 400 ng/mL, $>75\%$ of STX, dc-STX, GTX 1/4, GTX 2/3, B1, and C 1/2 were retained on the columns, whereas NEO was incompletely bound (27.1%). A major advantage of these columns is their reusability (up to 25 times) when toxins are eluted with 0.1M glycine–HCl buffer (pH 2.5). The total capacity per column was about 3 μg PSP toxin, which should be sufficiently high for most LC detection systems.

After IAC cleanup, purified shellfish extracts analyzed by LC (36) were of standard solution quality (Figure 4). Therefore, this IAC method could greatly improve physicochemical detection methods for PSP toxins. This technique could also be helpful in preparing PSP toxin standard mixtures from naturally contaminated material.

**Conclusions**

Immunoassay techniques offer the advantages of standardized test format, simplicity, and speed. The high sensitivity enables detection of PSP toxins in shellfish and other sample materials far below toxic levels. Underestimation of the toxin content, compared with the mouse bioassay, may represent a disadvantage for legal purposes. However, as a simple and rapid screening system, EIAs are very useful and reliable tools for routine control of PSP toxins. Immunoaffinity chromatography provides an excellent cleanup for physicochemical methods, in particular for determination by LC. Taking all advantages and disadvantages into account, immunochemical techniques will never fully replace existing analytical methods. However, within a monitoring system for PSP and other shellfish toxins (37), immunoassays could help to reduce the number of bioassays, establish enhanced screening strategies, and thus contribute to the improvement of food safety.

**References**


(20) *Official Methods of Analysis* (1995) 16th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method 959.08


