Traceability

Project 6.3 - Valid

Saxitoxin analysis in bivalve molluscs

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Abstract

Paralytic shellfish poisoning (PSP) is caused by consumption of a wide variety of shellfish which accumulated saxitoxins (STXs) from marine dinoflagellates (*Alexandrium minutum, A. tamarense*, *Gymnodinium catenatum*, *Pyrodinium bahamense*) and affect a wide variety of shellfish. There are about 20 saxitoxin analogs with closely related structures. A regulatory level of 0.8 mg/kg shellfish meat as STX equivalents has existed in North America and Europe for many years.

The methods of determination of saxitoxins are reviewed: biological assays *in vivo* and *in vitro*, biochemical and chemical assays. The mouse bioassay protocol has been widely used and has protected public health for over 50 years using an action level of 0.8 mg/kg STX.2HCL equiv. It is a routine method, and in EU it is the reference method if the results are challenged (Commission Regulation 2005/274/CE). However ethical issues, relating to the use of live animals, affect the acceptance and use of mouse bioassay in some countries.

A precolumn-derivatization liquid chromatography method (Lawrence method) is approved as European Norm by CEN and by AOAC as official method of analysis. Others techniques such as receptor binding assay (in vitro bioassay), immunoassay (commercial test kits) and postcolumn-derivatization liquid chromatography can be used as alternative method for routine monitoring. Capillary electrophoresis and liquid chromatography with mass spectroscopic detection are studied in research laboratories.

1. Introduction

Paralytic shellfish poisoning (PSP), associated with intake of toxins from the saxitoxin group (STX), has been known for a long time, and has caused many fatalities. It is neurotoxic and causes respiratory paralysis and other effects in mammals. Saxitoxins have been found worldwide. A regulatory level of 0.8 mg/kg shellfish meat as STX equivalents has existed in North America and Europe for many years, and the probability of suffering PSP from commercially harvested shellfish is extremely low. The same regulatory limit is presently used in many other countries.

2. Origins and chemical data

The saxitoxins are a group of low molecular weight (STX=299 dalton) non-protein toxins, with about 20 naturally occurring analogues. Saxitoxins have been found to occur worldwide. They are produced by dinoflagellates which include *Alexandrium minutum, A. tamarense*, *Gymnodinium catenatum*, and *Pyrodinium bahamense*. STXs affect a wide variety of shellfish.

Saxitoxin is heat and acid stable and does not alter the odour or the taste of food. This toxin cannot be destroyed by cooking or freezing.

3. Analytical methods

Because of the potential hazard to humans and animals, a quick, sensitive and specific method is needed to determine the presence of the toxins in shellfish. Traditionally, the presence of toxins has been determined using the mouse bioassay. However, the controversial issue of using mammals for testing in addition to the inherent problems and limitations of mammalian bioassays encourages the development of alternative assays such as pharmacological assays, immunoassays, chemical assays and alternative bioassays to detect marine toxins in seafood.
The development of analytical methods for the saxitoxin group is made challenging for several reasons, including: the presence of a large number of saxitoxin analogs with closely related structures; a great variation in toxicity (lethality to mice) among analogs; a wide variation in toxin composition in different shellfish species and in different geographic locations.

4. Bioassays

4.1. In vivo Assays: mouse biassay

The mouse bioassay (MBA) was first applied toxin-contaminated shellfish by Sommer and Meyer (1937). The bioassay procedures were standardized and now the AOAC protocol is widely used (AOAC Method 959.08).

However an interfering factor in the assay is the extractable materials co-existing with the toxins (Oshima 1995a, LeDoux and Hall 2000) and some tightening of the protocol (e.g. pH adjustment of the extract) has been recommended to improve the reproducibility (European Community Reference Laboratory and Asia Pacific Economic Cooperation). In France, a proficiency study was conducted, in which 8 laboratories applied the mouse assay for the

analysis of oyster samples, contaminated with PSP toxins at levels from non-detectable to levels of 1.53 and 3.35 mg STXequ./kg meat. Within-laboratory variations and between-laboratory variations ranged from 5-10 and from 8-40 % respectively (LeDoux and Hall, 2000). The detection limit of the MBA is 0.4 mg/kg STX.2HCl equivalents and considerable uncertainties exist at levels close to this limit.

The mouse bioassays is widely used as routine monitoring method and in EU it is the reference method if the results are challenged (Commission Regulation 2005/274/CE) but ethical issues, relating to the use of live animals, affect the acceptance and use of MBA in some countries.

4.2. In vitro assays: Receptor binding assays

Saxitoxins specifically bind on receptor site 1 of Na channels in a reversible manner and are used as a tool to investigate the mechanism of neurotransmission in neurophysiology. Davio and Fontelo (1984) were the first to use this binding activity for the detection of toxin. The binding of toxins on rat brain preparation was detected by displacement of radiolabeled STX (11-[^{3}H]STX). The method was later improved using microplate scintillation to give a high throughput operation and tested for toxin measurement in shellfish as well as dinoflagellates by many authors (Vieytes et al. 1993, Doucette et al. 1997, Velez et al. 2001).

Ruberu et al (2003) reported the optimisation of procedures for the receptor assay and the results of an interlaboratory comparison with high throughput using microplate scintillation counting. Shellfish samples tested (n=75) ranged from non-detectable by mouse bioassay, (<0.4 mg STXeq/kg) to 1.37 mg STXeq/kg. The detection limit of the optimized assay was 0.002 mg STXeq/kg with a between-assay relative standard deviation of 10%.

The receptor binding assay is sensitive and rather specific, but requires the use of radio-isotopes.

5. Immunoassays

Initially polyclonal antibodies have been obtained from rabbits injected by a STX-protein immunogenic conjugate (Carlson *et al.*, 1984 ; Yang *et al.*, 1987 ; Renz et Terplan, 1988, Usleber *et al.*, 1991 ; Cembella et Lamoureux, 1993 ; Usleber *et al.*, 1994 ; Huang *et al.*, 1996 ; Usleber *et al.*, 1997). Using the same type of immunogen, monoclonal antibodies were also produced (Dietrich *et al.*, 1996). Other polyclonal antibodies have been obtained from conjugates with others compounds than STX such as neoSTX (Huang *et al.*, 1996 ; Bürk *et al.*, 1995), or GTX2,3 (Frémy *et al.*, 1997). Regarding specificity, because the STX group can be divided according to the molecular formula in two sub groups, cross reactivity figures are different accordingly: antibodies obtained from a conjugate with STX cross react with the sub group involving STXOL, dcSTX, GTX2,3 and N-sulfocarbamoyl-STX (B1); antibodies obtained from a conjugate with neoSTX cross react with the sub group involving the N1-hydroxy STX compounds (neoSTX, GTX1,4) (Usleber *et al.*, 2001). For this reason it is difficult to expect a general quantitative immunoassay specific to all STX group toxins by using only one type of antibody.
Moreover, it has been noticed that monoclonal antibodies have a lower affinity than polyclonal. The lowest sensitivity obtained by an ELISA test was 0.0002 mg STXeq/kg of shellfish tissue (Usleber *et al.* 200I). Based on this principle a test kit is commercially available (R-Biopharm) and in-house validated.
Some others screening test kits have been developed using membrane principle such as the MIST Alert ^{TM} by Jellett *et al*, (1992, 1998) with a detection limit of 0.4mg STXeq/kg in less

than 20 minutes. RIDASCREEN Saxitoxin kit and MIST Alert were also evaluated with satisfactory results (Inami *et al.* 2004).
The results indicate that immunochemical method could be used as a screnning test within biotoxin monitoring.

6. Chemical assays

Saxitoxins show no specific UV absorption nor fluorescence which can be utilized for the detection. For the detection on liquid chromatography, oxidative conversion of saxitoxins to fluorescent derivatives is required. The used oxidative reagents are H_2O_2, tert-butylhydroperoxide and at present, periodate seems the most suitable for the chemical detection of the wide range of toxin analogs. However, it should be noted that fluorescence yield varied greatly with slight change of oxidizing conditions (species and concentration of oxidants, pH, temperature and reaction time). Another difficulty of chemical analysis is the large number of target molecules with different chemical natures, especially the wide range of charged states from -1 to +2 at pH 7, and the wide variation of specific toxicity. The toxin profiles (relative abundance of analogs) of contaminated shellfish greatly differ according to the toxin profiles of the causative organisms, and also by the time after accumulation.

For the application of both pre-column and post-column derivatization LC, a whole set of STX analogs is essential as external standards and when changing from one standard to another, as a discontinuity of data may occur. Concentration differences up to 20 % have been noticed between STX concentrations of three different suppliers (Quilliam et al., 1993).

6.1. Precolumn - derivatization liquid chromatography (LC)

Lawrence *et al*.(1991a, 1991b) developed a method to separate and detect the fluorescent compounds formed by the oxidation of toxins. The so called “pre-column oxidation method”, is instrumentally much simpler than the post-column derivatization method described below, by using a single reverse phased column chromatography system. The method was improved and submitted to interlaboratory studies. In Europe the method has been inter-lab studied on mussel and two toxins were analysed: saxitoxin and decarbomoyl saxitoxin. It was standardized by the working group on biotoxins of the European Committee for Standardization (CEN) and approved as European Norm EN 14526 (CEN, 2004). Another collaborative study (Lawrence *et al.* 2004) had wider scope: four matrices (mussels, clams, oysters and scallops) and 12 toxins (saxitoxin, neosaxitoxin, GTX2,3 (together), GTX1,4(together), decarbamoyl saxitoxin, B-1 (GTX5), C-1 and C-2 (together) and C-3 and C-4 (together). STX, NEO, dcSTX, GTX2,3, GTX1,4 and B1 were quantified at individual concentrations down to between one tenth and one twentieth of the common regulatory guideline level of 0.8 mg/kg saxitoxin equivalents. The C toxins were successfully quantified at levels down to about one fiftieth to one hundredth of the regulatory level in terms of saxitoxin equivalents although in terms of µg/kg concentration units they were the least sensitive. The method was approved Official First Action in June 2005 by AOAC. Accuracy and precision were good and correlation to MBA data was high. The limit of quantification is *circa* 0.1 mg/kg STX equivalents, dependent on the composition of toxins.

6.2. Postcolumn-derivatization liquid chromatography (LC)

The idea of continuous analysis of the toxins by post-column oxidation and subsequent detection of fluorescence was first proposed by Buckley et al (1978). Separation of the toxins was achieved to some extent with ion-exchange (Oshima et al. 1984, 1989, 1995b). Detection limits of post-column derivatization LC varied according to the chemical nature of

toxins, but were generally an order of magnitude lower than the mouse assay and correlations with mouse bioassay have been given favourable results. This method continues to be developed and improved. The so called "post-column derivatization method" is an automated method.

A post-column method was standardized by the working group on biotoxins of the European Committee for Standardization (CEN) and approved as European Norm EN 14194 (CEN, 2002).

6.3. Capillary electrophoresis

Analysis of STX and some analogs were resolved by capillary electrophoresis (Thibault et al. 1991, Locke and Thibault 1994, Pineiro *et al*. 1999). Applicability of the method for the shellfish extract was not tested much and was rather questionable due the limit sample loading and low sensitivity.

6.4. Liquid Chromatography-Mass Spectrometry (LC-MS)

Mass spectroscopy has been used as the fundamental tool for the structure elucidation of saxitoxin analogs as well as for the identification of purified toxins. There are some reports on the utilization of MS coupled with liquid chromatography for the quantitative analysis of STXs (Pleasance *et al*. 1992a, Quilliam *et al.* 1993, Lagos *et al*. 1999, Jaime *et al* 2001, Dell'Aversano *et al*. 2002). However, most of them are preliminary studies using mainly pure toxins and have not been proven useful for practical analytical methods of STXs in shellfish.

6.4.1. Management of Analytical Results, standards and reference material

Domain of application of the method
The analysis are performed on the edible parts of molluscs, the entire body or any part edible separately (2005/2074/EC).

Management of Analytical Results
Analytical data for all methods should be expressed as mg STX.2HCl equivalents per kg of whole flesh. The Oshima TEFs should be used with instrumental methods but accuracy of results may be limited by the availability of some of the standards necessary to evaluate total toxicity in shellfish.

Standards and Reference Materials
At the moment, certified standards of STX, neoSTX, GTX1/4, GTX 2/3, B1(GTX5) , C1/C2, dcGTX2/3, dcSTX and dcneoSTX are commercially available from National Research Council Halifax, Canada. An STX reference material for calibration is distributed by the Centre for Food Safety and Applied Nutrition, FDA, USA. Two mussel tissue certified reference materials are available at JRC/IRMM, Geel, Belgium.

7. Conclusion

The mouse bioassay protocol has been widely used and has protected public health for over 50 years using an action level of 0.8 mg/kg STX.2HCL equiv. It is a routine method, and in EU it is the reference method if the results are challenged (Commission Regulation 2005/274/CE); but ethical issues, relating to the use of live animals, affect the acceptance and use of MBA in some countries.

The Lawrence method, a liquid chromatography–Fluorescence detection method with pre-column derivatization is approved as European Norm by CEN and by AOAC as official method of analysis.

Others methods such receptor binding assay, immunoassay and postcolumn-derivatization liquid chromatography can be used for routine monitoring.
Capillary electrophoresis and liquid chromatography with mass spectroscopic detection are studied in research laboratories.

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