Traceability

Project 6.3 - Valid

Domoic acid analysis in bivalve molluscs

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Abstract

Domoic acid (DA) was identified as the toxin responsible for an outbreak of illness in Canada in 1987, caused by eating blue mussels that had accumulated DA as a result of the presence of *Pseudo-nitzschia pungens*. It is named amnesic shellfish poisoning (ASP). Since a regulatory level of 20 mg DA/kg of shellfish meat was established.
The methods of determination of domoic acid are reviewed: biological assays *in vivo* and *in vitro*, biochemical and chemical assays. The AOAC mouse bioassay, which is used for PSP, cannot detect domoic acid at the regulatory level of 20 mg/kg tissue and no interlaboratory validation of this method for domoic acid has been carried out. The Quillian method, a liquid chromatography-UV detection method is approved as European Norm by CEN and recommended by *Codex Alimentarius* as reference method. The European Commission Regulation stipulate that the reference method shall be the HPLC method and the Community Reference Laboratory on Marine Biotoxins (CRLMB) recommends Quillian method as routine method. Others protocols such as receptor binding assay, immunoassays and thin layer chromatography can be used as alternative for routine monitoring. The liquid chromatography-mass spectrometry method allows to confirm the presence of domoic acid isomers.

1. Introduction

Domoic acid (DA) was identified as the toxin responsible for an outbreak of illness in Canada in 1987, caused by eating blue mussels that had accumulated DA as a result of the presence of *Pseudo-nitzschia pungens*. Effects on both the gastrointestinal tract and the nervous system were observed. Since some of those affected experienced memory loss, the syndrome was named amnesic shellfish poisoning (ASP). As a result of the episode of human illness in Canada, a regulatory level of 20 mg DA/kg of shellfish meat was established. This level is adopted by the regulatory authorities in most other countries. The Council Directive 91/492/EEC implemented a maximum permitted level (MPL) of 20 mg DA/kg shellfish intended for human consumption, with a derogation (Commission Decision 2002/226/EC) for the species *Pecten maximus* and *Pecten jacobaeus*, the level may exceed 20 mg/kg but remain lower than 250 mg/kg with restricted conditions: a)after total removal of hepatopancreas, soft tissues and any other contaminated part the adductor muscle and/or gonads intended for human consumption must not contain more than 20 mg/kg of DA, b)DA concentration in the parts intended for human consumption, analysed separately, has to be lower than 4.6 mg/kg. The presence of DA in shellfish has been reported in various regions of the world. The toxin is not destroyed by cooking or freezing.

2. Analytical methods

2.1.1. *In vivo* Assays: PSP Mouse Bioassay

The AOAC mouse bioassay for PSP toxins (AOAC, 1990) can detect domoic acid (DA) at concentrations of approximately 40 mg/kg tissue. It involves acidic aqueous extraction of the tissue followed by intraperitoneal injection of 1 ml of the extract into mice. Although the AOAC extraction procedure can yield substantial recovery of DA, the limit of detection of the AOAC bioassay procedure is not low enough to be used with confidence for regulatory purposes to quantify this toxin. No interlaboratory validation of this method for DA has been carried out.
Ethical issues, relating to the use of live animals, affect the acceptance and use of mouse bioassay in some countries.

2.1.2. *In vitro* Assays: Receptor Binding Assays

A competitive microplate receptor binding assay for DA using frog (*Rana pipiens*) brain synaptosomes has been developed. The analysis of DA was based upon binding competition with radiolabelled-[^{3}H]-kainic acid for the kainate/quisqualate glutamate receptor. In 1997, Van Dolah *et al*.(1997) reported the further development of the receptor assay by replacement of frog brain by a cloned rat GLUR6 glutamate receptor to eliminate animals from the testing procedure. The limit of detection and selectivity of the assay were optimized through inclusion of the glutamate decarboxylase pretreatment step to eliminate potential interference due to high concentrations of endogenous glutamate in shellfish. The receptor binding assay of Van Dolah (1997) is suitable for analysis of DA in sea water extracts from algae and for analysis of DA in shellfish. No interlaboratory study of this method has been carried out. However, it is being considered for study by AOAC International.

3. Biochemical Assays

3.1. Immunoassays

Garthwaite *et al.* (1998) used ovine antibodies raised against DA to develop an indirect competitive ELISA for shellfish and seawater. This ELISA method has been commercialised by Biosense®, into a kit format, intended to be used in routine monitoring of DA in bivalve molluscs to check compliance with the regulatory limits (http://www.biosense.com/). The method has been validated by interlaboratory study. Results indicated that 14 of 16 laboratories were able to establish the method with good calibration curves and they reported valid concentration data for all study samples. The precision estimates for the ELISA data did not show a strong dependence on the DA concentration in the study samples. The analysis of shellfish samples spiked with certified reference materials demonstrated good method accuracy (recovery of 104%). This was supported by an excellent correlation slope of 1.015 (R2 0.992) for the determined vs. the expected values of DA in the spiked samples. There was also good correlation of the ELISA results with those for the instrumental liquid chromatography analyses of the same samples extracts. This method appears suitable for the routine quantitative determination and monitoring of DA in shellfish.

Another antibody-based technique employing lateral-flow strip test technology has been commercialised (Jellett Rapid Testing Ltd.) and evaluated (MacKintosh and Smith, 2002) for domoic acid. This approach has much potential for qualitative rapid screening of shellfish. The test is sensitive enough for screening for domoic acid well below the regulatory guideline of 20 mg/kg shellfish tissue.

Traynor *et al.* (2002) have described the detection of DA in bivalve molluscs with an immunobiosensor. In this application, DA is bound to the sensor surface and use is made of polyclonal antibodies. The assay was found suitable for rapid analysis of cockles, mussels, oysters and scallops. A limit of detection was found at 0.8 mg/kg, and an intra assay C.V. of 8 % was found at a level of 20 mg/kg, the current legal limit for whole body.

4. Chemical Assays

4.1. Thin layer chromatography (TLC)

DA can be determined by TLC as a weak UV-quenching spot that stains yellow after spraying with a 1% solution of ninhydrin (Quilliam *et al*., 1998). The detection is about 0.5 μg by this method, which permits detection in shellfish tissues at about 10 mg/kg. It is also possible to detect DA on the TLC plate using some other spray reagents. Quilliam further studied TLC as a separation technique to detect DA, after extraction with aqueous methanol followed by SAX-SPE cleanup. The method was successfully applied to scallop and razor clam samples contaminated with DA. It was concluded the method should prove successful for the routine screening of shellfish tissues in those laboratories not equipped with an LC system. It should also be useful as a chemical confirmation method for DA in samples tested positive by assay methods such as immunoassay. No in-depth quantitative studies have been reported for this method. However, it has potential as a relatively inexpensive screening technique.

4.2. Liquid Chromatography-UV detection (LC-UV)

LC-UV is currently the preferred analytical technique for the determination of DA in shellfish and a method is available, formally validated for mussels in an AOAC collaborative study (Lawrence *et al*., 1991). The LC-UV detection limit for DA is about 10-80 ng/ml, depending

on the sensitivity of the UV detector that is used. If crude extracts (either acidic or aqueous methanol) are analysed without cleanup, the practical limit for quantitation is about 1 μg/g. However, stability of DA in the extract is poor and the improved LC-UV method developed by Quilliam *et al.* (1995) using 50% methanol extraction is now preferred . In this procedure an aqueous methanol extraction is applied in combination with strong anion exchange-solid phase extraction cleanup, leading to chromatograms free from interferences. Other advantages of the method are: more stable extracts, higher recoveries and a lower limit of detection (0.02-0.03 mg/kg).
The Quilliam LC-UV method has been standardized by the working group on biotoxins of the European Committee for Standardization (CEN) and approved as European Norm EN 14176 (CEN, 2003). It is recommended by *Codex Alimentarius* as reference method. The method was found suitable for analysis of cockles, mussels, oysters, clams and razor clams.
The European Commission Regulation (2005/2074/EC) stipulate that the reference method shall be the HPLC method if the results are challenged method and the Community Reference Laboratory on Marine Biotoxins (CRLMB) recommends this protocol as routine method.

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

LC-MS techniques continue to make important strides in the detection and quantification of marine toxins. It is the only method at present that can detect all isomers of the domoic acid group and new isomers continue to be discovered by the approach (Holland *et al.* 2003a; Rhodes *et al.* 2003). An inter-laboratory study of an LC-MS method for determination of DA (and DSP toxins) in shellfish was carried out (Holland and McNab, 2003). The eight participating laboratories generally obtained consistent sets of data for the broad group of toxins down to low levels (< 5ng/ml, equivalent to 0.05 mg/kg). A method specifically for DA was developed and successfully submitted to single laboratory validation (Holland *et al*. 2003b). The LC-MS approach continues to evolve and improve for quantification of marine biotoxins and is increasingly being employed in developed countries for marine biotoxin analyses. These methods are suitable for screening and for confirmation, particularly where isomers of DA are present.

4.4. Capillary electrophoresis

Capillary electrophoresis (CE) is a relatively new technique that has gained acceptance in a number of areas. DA has been determined by CE (Pineiro *et al*.,1999; Zhao *et al.,* 1997) but the approach has not been widely used.

5. Management of analytical results 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).

Expression of Analytical Results
Analytical data for all methods should be expressed as mg DA equivalents per kg of whole flesh. Toxicity equivalence factors for the epi-DA and other isomers of DA are not currently available and are assumed as 1.0 in the interim.

Reference Materials
A calibration solution certified reference material (CRM) and a mussel tissue CRM are available at National Research Council of Halifax, Canada.

6. Conclusion

The AOAC mouse bioassay which is used for PSP cannot detect domoic acid at the regulatory level of 20 mg/kg tissue and no interlaboratory validation of this method for domoic acid has been carried out. The Quillian method, a liquid chromatography-UV detection method is approved as European Norm by CEN and recommended by *Codex Alimentarius* as reference method. The European Commission Regulation stipulate that the reference method shall be the HPLC method and the Community Reference Laboratory on marine biotoxins (CRLMB) recommends Quillian method as routine method. Others protocols such as receptor binding assay, immunoassays and thin layer chromatography can be used as alternative for routine monitoring. The liquid chromatography-mass spectrometry method allows to confirm the presence of domoic acid isomers.

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