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Use of the receptor binding assay for determination of paralytic shellfish poisoning toxins in bivalve molluscs from Great Britain and the assessment of method performance in oysters

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ABSTRACT

A receptor binding assay (RBA) for the determination of paralytic shellfish poisoning toxicity is formally validated through collaborative study and approved for regulatory monitoring use in the US for mussels and clams. However, to date, the method has not been tested on bivalve molluscs originating from European waters and no validation studies have been conducted for oysters, a shellfish species of great importance globally. This study firstly reports the work conducted to assess the performance of the assay in comparison with a regulatory chemical detection method for a range of shellfish species originating from Great Britain. Data obtained showed a complete absence of false negative RBA results, with a tendency to over-estimate PSP toxicity for some shellfish species in comparison with liquid chromatography with fluorescence detection. Secondly, the performance of the RBA was assessed for oysters, with the analysis of a dilution series of oyster matrix certified reference materials. Method trueness, sensitivity and precision were found to compare well with results reported previously for other species. In addition, the RBA analysis of untreated and demetallated oyster extracts, provided good evidence that the RBA is not suppressed in the presence of high concentrations of zinc as reported previously for the mouse bioassay. Consequently, there is strong evidence from this study, that the RBA would be suitable for determination of PSP toxicity in bivalve molluscs from GB, with acceptable method performance in oysters. Further validation studies would be required for other shellfish species of interest before the method can be considered suitable for implementation in Europe.

1. Introduction

Paralytic shellfish poisoning (PSP) is an intoxication that occurs in humans following consumption of seafood containing saxitoxin (STX) and its congeners (Schantz, 1969; Halstead, 1978; Hallegreff, 2003; FAO, 2004; Wiese et al., 2010). Paralytic shellfish toxins (PST) are naturally produced by certain species of phytoplankton (Shumway, 1990, 1995) and can periodically accumulate to high concentrations most notably in filter-feeding bivalve molluscs (Llewellyn, 2006). Monitoring of PST in bivalve molluscs as well as monitoring of causative organisms in the water column is consequently a global regulatory requirement including in the European Union (EU) (Anon, 2004) and the US (ISSC, 2013) to help ensure consumer protection. The current regulatory action limit for PST in the EU as well as the US in shellfish is 800 μg STX eq/kg (CODEX, 2008).

Methods have been developed to quantify PSP toxicity in bivalve molluscs, with the EU and US reference method still currently a PSP mouse bioassay (MBA) based on AOAC Official Method (OM) 959.08 (Anon, 2005a, 2006). This method involves the direct determination of PSP toxicity following the injection of live mice with acidic extracts of shellfish tissue. The method has been replaced in some countries due to ethical, and method performance issues (Humpage et al., 2010; Turner et al., 2012). In the EU, including Great Britain (GB), as well as some other areas of the world such as New Zealand (Harwood et al., 2013), the alternative is a pre-column oxidation (pre-cox) liquid chromatography with fluorescence

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detection (LC-FLD) method as described by AOAC OM 2005.06 (Anon, 2005b). This and other LC methods quantify PST concentrations using known or assumed toxicity equivalence factors (TEF) to calculate total PSP toxicity from individual toxin congeners. The LC method has been subjected to a single and inter-laboratory validation (Lawrence and Menard, 1991; Lawrence and Niedzwiadek, 2001; Lawrence et al., 2005) and refined by Cefas with the validation extended to additional toxins and matrices (Turner et al., 2009, 2010; 2011; Turner and Hatfield, 2012; Harwood et al., 2013). Since 2008 the semi-quantitative (Turner et al., 2014a) and quantitative LC-FLD methods have been applied to the routine official control monitoring programmes within GB (Turner et al., 2014b).

An alternative regulatory approach in the US, involves the use of a receptor binding assay (RBA) (Anon, 2011). As a functional assay, the RBA also quantifies total PSP toxicity, specifically assessing the binding of saxitoxins to receptor site 1 of sodium channels (Davio and Fontelo, 1984; Negri and Llewellyn, 1998) with each PST analogue binding to the receptor with an affinity proportional to its toxicity (Usup et al., 2004). Radiolabelled STX (^{3}H-STX) is competitively displaced by the action of STXs from its receptor in rat brain membranes, resulting in a highly specific assay (Vieytes et al., 1993). Doucette et al. (1997) developed the assay to incorporate the RBA into a microtiter plate based format with liquid scintillation counting for endpoint determination. Since this time, the method has been further optimised, formatted for use as a high throughput and highly sensitive screening tool and validated for a range of algal and shellfish matrices (Srisuksawad et al., 2010), with others developing procedures for quality control enabling the method precision to remain acceptable (Ruberu et al., 2012). A full single-laboratory validation of the microplate RBA was conducted successfully by Van Dolah et al. (2009) following AOAC guidelines. The validation of the method was subsequently extended through a formal collaborative study incorporating nine participants who each analysed a total of 21 shellfish samples. Shellfish tissues were sourced from the US, Chile and New Zealand and species were primarily mussels (n = 13), with clams (n = 5) and scallops (n = 3) also included (Van Dolah et al., 2012). Validation results showed acceptable intra- and inter-laboratory repeatability and toxin recoveries, with a good correlation against MBA and LC-FLD, again showing a degree of over-estimation in PSP toxicity compared to both methods. More recently, the RBA has been tested on a larger number of mussel samples (n = 295) over a wider range of concentrations, with results again indicating a slight over-estimation in PSP toxicity by the RBA (18%), in comparison to the MBA (Ruberu et al., 2017).

Following publication as AOAC official method 2011.27 (Anon, 2011), the method was approved in the US as a regulatory monitoring tool for mussels, with limited use for clams and scallops (ISSC, 2013). The assay cannot be applied to other shellfish species in the US for regulatory purposes until validation has been extended to other matrices (ISSC, 2013). Currently, there is no specific approval for use of the RBA for regulatory testing in the EU (Anon, 2004, 2006). For potential future use of the RBA in the EU, validation of the method would be required for relevant shellfish species as a minimum. Notably, the method has so far not been extended to the analysis of oyster species. Investigations into the 2–3 fold under-estimation in total PSP toxicity by the MBA reference method in Pacific oysters (*Crassostrea gigas*) and native oysters (*Ostrea edulis*), showed the cause of this under-estimation to relate to the suppressive effects of high zinc concentrations occurring naturally in oyster tissues (Turner et al., 2012). Given that the RBA measures PSP toxicity through receptor activity, as with the MBA there is also the potential for divalent metal ions such as zinc and manganese to affect the performance of the RBA. Consequently, there is an important need to assess the performance of the RBA in oysters in comparison to methods such as LC-FLD which are not affected by high metal concentrations, as part of any matrix extension validations.

The objectives of this study were firstly to assess the applicability of the RBA for the analysis of shellfish samples obtained from GB, comprising seven different shellfish species. Secondly, the performance of the method was assessed in comparison with the LC-FLD for oysters, including the determination of PST in extracts both with and without high concentrations of zinc. Results were to be used to determine if zinc ions would potentially suppress the performance of the RBA and the degree of correlation between PSP toxicities determined by the RBA in comparison with the pre-cox LC-FLD GB official control monitoring method.

2. Materials and methods

2.1. Reagents and chemicals

HPLC-grade solvents and analytical grade chemicals were used throughout the study. Certified reference materials gonyautoxins 1 to 5 (GTX1-5), neosaxitoxin (NEO), dicarbamoyl saxitoxin (dcSTX), C toxins 1&2 (C1&2), saxitoxin (STX) di-hydrochloride (di-HCl), dicarbamoyl neosaxitoxin (dcNEO) and dicarbamoyl gonyautoxin 2&3 (dcGTX2&3) were obtained from the Institute of Biotoxin Metrology, National Research Council Canada (NRCC, Halifax, Nova Scotia, Canada). Primary toxin standards were diluted in water to form stock standard solutions prior to dilution in 0.1 mM acetic acid for calibration standard preparation. For MBA, toxicity was determined against NRCC STX di-HCl CRM calibration standards. For the RBA, [^{3}H] STX was obtained from American Radiolabeled Chemicals (St. Louis, MO 63146 USA). Saxitoxin standard curves were generated in 3 mM HCl from STX diHCl standard (NIST RM8642; nist.gov) to yield 10^{-6}, 10^{-7}, 3×10^{-8},10^{-8}, 3×10^{-9}, 10^{-9}, and 10^{-10} M in-well concentrations. This bulk standard curve was aliquotted and stored at 4 °C for use in each assay. From the same standard, an interassay quality control check sample (3 nM STX in 3 mM HCl) was made in bulk and stored at −80 °C. RBA assay buffer was 100 mM MOPS/100 mM choline chloride, pH 7.4. Rat brain membranes were prepared in bulk according to the method described in Van Dolah et al. (2012) and stored at −80 °C until use. Optiphase liquid scintillation cocktail (Perkin-Elmer Life Sciences, Downers Grove, IL USA) was used for scintillation counting.

2.2. Shellfish materials

Naturally contaminated shellfish samples were taken from the official control monitoring programmes of Great Britain, sampled between 2010 and 2012. Bivalve mollusc species included mussels (*Mytilus edulis*), Pacific oyster (*Crassostrea gigas*), native oyster (*Ostrea edulis*), cockle (*Cerastoderma edule*), king scallops (*Pecten maximus*), razor clams (*Ensis* sp.) and surf clams (*spisula solida*). Given the low number of contaminated oysters and cockles available, additional shellfish tissues were produced following the method described by Turner et al. (2011), feeding live shellfish with mass cultured *Alexandrium* strains. Pacific oyster, native oyster and cockle shellfish used for the feeding experiments were sourced from different geographical localities at different times of the year as described by Turner et al. (2011). A certified Pacific oyster matrix reference material (POCRM1, Cefas, United Kingdom) was also used for method performance assessment, with matrix dilutions employed using PST-free Pacific oyster tissue to generate a series of additional oyster tissues.