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Short communication

Liquid chromatographic determination of okadaic acid and dinophysistoxin-1 in shellfish after derivatization with 9-chloromethylanthracene

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Abstract

The reagent 9-chloromethylanthracene was evaluated for derivatization of the diarrhetic shellfish poisons, okadaic acid and dinophysistoxin-1 (DTX-1), to form fluorescent products separable by liquid chromatography. The toxins were reacted with the reagent in acetonitrile in the presence of tetramethylammonium hydroxide for 1 h at 90°C. The products were purified by using two silica solid-phase extraction cartridges before being determined by reversed-phase liquid chromatography with fluorescence detection. The results are comparable to those obtained using 9-anthryldiazomethane (ADAM) for okadaic acid and DTX-1 in mussel tissue. Detection limits were estimated to be about 70–100 ng/g hepatopancreas (equivalent to 12–20 ng/g whole tissue) for each toxin.

1. Introduction

Okadaic acid (OA) and dinophysistoxin-1 (DTX-1) are lipophilic polyether compounds that are part of a group of toxins responsible for diarrhetic shellfish poisoning (DSP) [1]. DSP is considered to be a problem throughout the world and has recently been found on the east coast of Canada [2]. The most commonly used technique for determining OA and DTX-1 in shellfish has been liquid chromatography after conversion of the toxins to fluorescent products with the reagent 9-anthryldiazomethane (ADAM) [3]. A number of variations of this technique have been reported in the literature. Quilliam [4] recently carried out a comprehensive study on the extraction and cleanup of shellfish tissue for the determination of OA and DTX-1 using the ADAM method as well as liquid chromatography–mass spectrometry. Replacements for the ADAM reagent are desirable because ADAM is very unstable, costly and requires special low-temperature storage conditions (−70°C). Marr et al. [5] recently carried out a comprehensive investigation of several derivatization reagents which had potential for use in the determination of OA and DTX-1, and compared them to the ADAM reaction. They found that the ADAM reagent provided the best reaction selectivity. Other aryldiazomethane reagents proved to be less suitable. Several coumarin reagents were also evaluated with 4-bromomethyl-6,7-dimethoxycoumarin (BrDMC) being found to perform

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the best but with less selectivity than the ADAM reagent.

Another reagent which has been used for fatty acids [6] as well as OA [7] is 9-chloromethylanthracene. However, its application to okadaic acid [7] was found to have a detection limit of about 1 μg/g in mussel hepatopancreas (or 200 ng/g on a whole tissue basis) which is the level of concern in Canada. In order to implement a monitoring program for DSP, the detection limits of the method should be at least ten times less than the concern level. We felt that, because of its advantages of being very stable and relatively inexpensive, 9-chloromethylanthracene was worth investigating further as a reagent suitable for the quantitative determination OA and DTX-1. The following is the result of our study.

2. Experimental

2.1. Reagents

Calibration solutions of okadaic acid were purchased from the Institute for Marine Biosciences, National Research Council of Canada, Halifax, Nova Scotia, Canada. A dilute solution of DTX-1 was received as a generous gift from Dr. M.A. Quilliam (IMB, NRC, Halifax). The reagents, 9-chloromethylanthracene (CA) (Aldrich, USA) and tetramethylammonium hydroxide (TMAH) (25% w/v in methanol) (Aldrich) were used as received. All other solvents were HPLC or distilled-in-glass grade. Doubly deionized water was used throughout. All standard and reagent solutions were refrigerated when not in use. Mussels (*Mytilus edulis*) were purchased locally in the Ottawa area. They were cultured in Prince Edward Island on the east coast of Canada. The mussel hepatopancreas reference material (MUS-2) was purchased from the National Research Council of Canada, Halifax, Canada.

2.2. Sample extraction

The hepatopancreas was carefully removed from whole mussels and homogenized (Polytron). A 1-g subsample of the homogenate was extracted according to a procedure described elsewhere [5] with minor modification. Briefly, the sample was homogenized with 6 ml aqueous methanol (80%, v/v) and then centrifuged. The supernatant was collected and the residue suspended in 2 ml 80% methanol. The mixture was centrifuged again and the supernatant collected. The combined supernatants were transferred to a small separatory funnel and extracted with 3×15 ml 15% (v/v) dichloromethane in hexane which were discarded. After this, 5 ml of H_2O were added to the aqueous phase in the separatory funnel and the mixture extracted with 3×15 ml of 50% (v/v) dichloromethane in hexane. The dichloromethane phases were combined and dried with anhydrous Na_2SO_4. The liquid was decanted from the Na_2SO_4 into a 100-ml round-bottom flask. The Na_2SO_4 was rinsed with a small volume of dichloromethane which was added to the extract in the round-bottom flask. The contents of the flask were then evaporated to dryness using rotary vacuum evaporation at 40°C. The residue was dissolved in 1 ml of methanol to yield a concentration of 1 g hepatopancreas per ml.

2.3. Derivatization

A 25–50 μl aliquot of sample extract in a Reactivial was evaporated to dryness at room temperature using a stream of nitrogen. To the residue was added 400 μl of 0.8 m*M* TMAH solution (diluted in acetonitrile). The mixture was warmed for 2 min at 40°C to ensure that the residue was dissolved and then the mixture was evaporated again to dryness at 40°C. A 400-μl volume of 0.8 m*M* CA in acetonitrile was added and the vial cap replaced. The contents were permitted to react at 90°C for 1 h. After this, the mixture was cooled and evaporated to dryness under a stream of nitrogen at room temperature. The residue was dissolved in 300 μl of 50% (v/v) dichloromethane in hexane for silica gel cleanup. Standards (0.1–10 μg) of OA and DTX-1 were derivatized in exactly the same way except that 200-μl volumes of 0.4 m*M* solutions of CA and TMAH were used.

2.4. Silica gel cleanup

The derivatized extract (300 μl) was transferred to a 3-ml volume silica gel solid-phase extraction (SPE) cartridge (Supelco, USA) containing 500 mg of sorbent [previously conditioned with 6 ml dichloromethane followed by 6 ml of 50% (v/v) dichloromethane in hexane]. The sample vial was rinsed twice with 300 μl of 50% (v/v) dichloromethane in hexane and the rinsings transferred to the SPE cartridge. The effluent was discarded. The cartridge was then washed with 6 ml 50% (v/v) dichloromethane in hexane and 7 ml of 1% methanol in dichloromethane, all of which were discarded. A second silica SPE cartridge (conditioned with 6 ml of dichloromethane only) was placed directly under the first cartridge. The first cartridge was then eluted with 7 ml of 5% methanol in dichloromethane with the effluent passing directly into the second cartridge. The effluent from the second cartridge was collected and evaporated to dryness. The residue was dissolved in 2 ml of mobile phase and 50 μl injected into the LC system for analysis.

2.5. Liquid chromatography

The LC system consisted of a single pump (Beckman Model 114M) set at a flow-rate of 1.0 ml/min; a variable-wavelength fluorescence detector (Jasco, Model 821-FP) set to 365 nm excitation and 412 nm emission; an injection port (Beckman Model 210) with a 50-μl loop. A reversed-phase C_{18} column (15 cm × 4.6 mm I.D., 5 μm, Supelco) was employed for the separations. The mobile phase was acetonitrile–H_2O (75:25, v/v). An electronic integrator was used to measure peak areas.

3. Results and discussion

During our studies on optimization of the reaction conditions, we found that the quantity of reagents used greatly affected the yield of fluorescent product for OA and the ease of cleanup before LC analysis. Excessive amounts of TMAH caused a decrease in yield of product with standard solutions. In spiked extracts of hepatopancreas, a larger amount of reagents was required to ensure a high and reproducible yield of OA product in the presence of competing free fatty acids which would also be present in the extract because of the extraction procedure employed. It has been shown that concentrations of free fatty acids in mussel hepatopancreas can vary greatly depending upon geographical location and time of year [8,9]. Thus, in unknown samples where the amount of free fatty acids is unknown it is difficult to determine exactly the optimum quantity of reagents to employ to obtain a high and consistent yield of fluorescent products for OA and DTX-1. We found that the reaction conditions employed elsewhere for okadaic acid and fatty acids [6,7] were not optimum nor reproducible enough to be acceptable at regulatory guideline levels of OA or DTX-1 in the mussel hepatopancreas samples available to us. We thus reevaluated the reaction conditions to enable detection of the toxins at levels well below the concern levels with acceptable repeatabilities and recoveries.

3.1. Reaction conditions

We evaluated TMAH and the crown ether 18-crown-6 as base catalysts for the reaction. Many experiments were carried out to optimize the reagent concentrations, temperatures and times for the derivatization reactions. While both catalysts functioned reasonably well, the reproducibility of the reactions was less than satisfactory and different results were obtained when using different quantities of hepatopancreas extract in the reaction. For example, 50 μl of extract (50 mg tissue) gave lower yields of OA product than if 25 μl (25 mg tissue) were employed under the same reaction conditions. After many experiments, we found that the base concentration was particularly critical. To alleviate this problem, the approach used by Korte [6] was evaluated and finally employed. Rather than add the base and the reagent together for reaction, we added base, TMAH, first to the extracts and then evaporated the solution to dryness leaving the carboxylic acids as their tetramethylammonium salts while the remaining ex-

cess TMAH was removed by the evaporation. The CA reagent was then added and permitted to react with the carboxylic acid salts. We found that under these conditions a temperature of 90°C was required for a high yield of product within a 1-h reaction time. Lower temperatures required longer reaction times and did not produce better yields of fluorescent product. The 90°C temperature did not affect the yield of product indicating that OA and its fluorescent product are stable under the reaction conditions used. The optimum reaction conditions (as described in the Experimental section) were found to give consistent results with quantities of hepatopancreas extract up to 100 mg of tissue. Larger quantities were not tested. Usually 25–50 mg tissue were used for the derivatizations.

3.2. Sample cleanup

Since CA yields the same fluorescent products of OA and DTX-1 as the ADAM reaction, we initially employed the same silica gel SPE cleanup as used for the ADAM products [3,10]. However, we found that these were not entirely satisfactory. Since the cleanup of the derivatized extracts was a critical part of the method, we carried out many studies to optimize this step by employing various solvent combinations and different sorbents. The most successful approach was to use two silica SPE cartridges as described in the Experimental section. This modification significantly improved the chromatograms. Fig. 1 shows typical results obtained for an extract of spiked mussel hepatopancreas (1 μg/g, okadaic acid) carried through the entire analytical procedure. Okadaic acid is clearly detected at this level with the cleanup procedure employed.

3.3. Quantitation

We found that the reaction and cleanup conditions employed here resulted in very consistent results with standards of okadaic acid on a day-

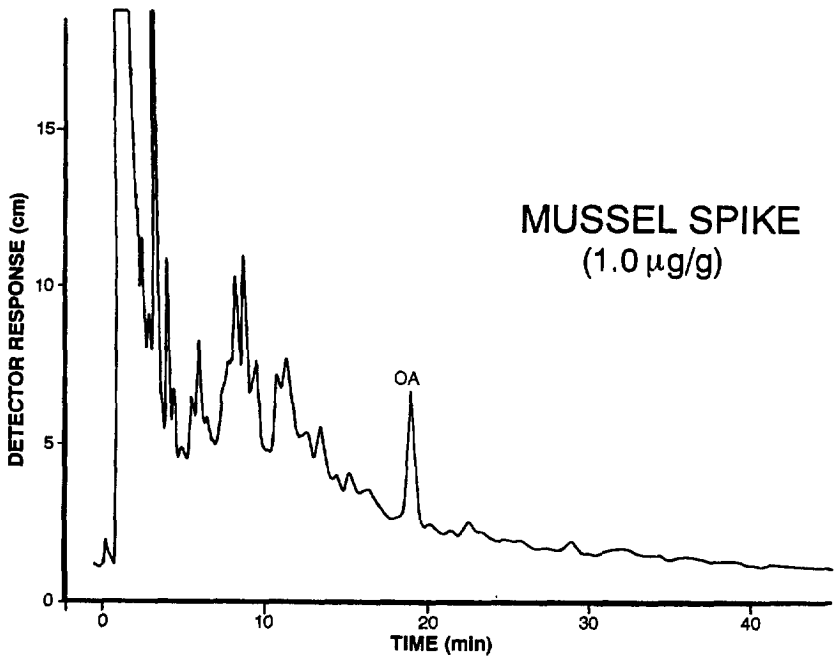

Fig. 1. Typical chromatogram of an extract of mussel hepatopancreas spiked with 1.0 μg/g okadaic acid (OA) and carried through the reaction and cleanup procedure. Conditions are described in the Experimental section. Equivalent of 1.3 ng OA injected.

to-day basis. In fact, the OA product was stable enough that a single reaction solution could be used for a week or more with minimal degradation. This was particularly useful for method development purposes since daily reactions with fresh OA were not needed saving time and OA consumption.

The minimum detectable quantity of OA as the fluorescent product was about 100 pg per injection. In mussel hepatopancreas the minimum detectable concentration (at a signal-to-noise ratio of 3) was about 70 ng/g or about 15 ng/g on a whole tissue basis. The repeatability (coefficient of variation) of triplicate reactions on aliquots of the same sample extract containing 1 μg/g OA was ±5%. Similar results were obtained for DTX-1. Major modifications to the initial sample extraction procedure were not made since that has yielded fairly good recoveries as found by others employing the ADAM derivatization procedure. We obtained a recovery of OA (0.5–20 μg/g) through the entire analytical procedure consistently higher than 80%. In order to assess the accuracy of the procedure, we analysed a mussel tissue reference material (MUS-2, National Research Council of Canada) which contained OA certified at 11 μg/g and DTX-1 at 0.9 μg/g (uncertified). Unfortunately, our reference material was stored at −15°C in our laboratory for about 1 year before analysis. Our results after correcting for recovery were only about 75% of the expected values for both OA and DTX-1. It is possible that some decomposition may have occurred during the prolonged storage. Otherwise the chromatograms were very similar to that obtained with the ADAM reaction as reported elsewhere for the same reference material [4,5]. Fig. 2 shows results obtained on MUS-2 with CA derivatization. OA is readily detected at 11 μg/g. For improved quantitation of DTX-1 at the 1 μg/g concentration level a larger aliquot of the de-

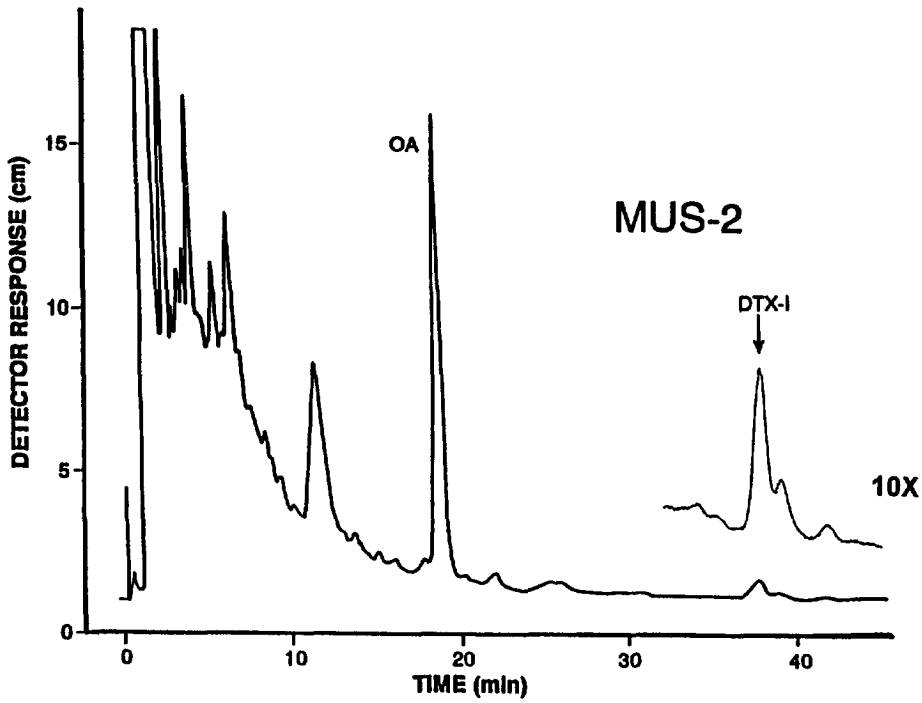

Fig. 2. Chromatogram of a mussel tissue reference material (MUS-2) containing 11 μg/g okadaic acid (OA) and 0.9 μg/g DTX-1. Conditions are described in the Experimental section. 10× = scale expanded ten times.

rivatized extract was injected. The chromatogram in the area of DTX-1 was clean enough to enable injection of an increased quantity of extract to detect DTX-1 as low as 0.1 μg/g or less in the hepatopancreas.

In conclusion, CA has been shown to be as effective as ADAM as a fluorescent labelling reagent for OA and DTX-1 in mussel tissue, provided suitable reaction and cleanup conditions are employed. The big advantage of CA is that it is relatively inexpensive and it is shelf-stable. Even the reagent solutions once prepared are stable for at least a week if refrigerated when not in use. In any analytical method employing chemical derivatization, it is essential that the reagents perform as expected. Unstable reagents such as ADAM require special storage conditions and constant tests to ensure the efficacy of the reagent. The use of CA for derivatization of OA and DTX-1 should significantly reduce these requirements while still maintaining good reproducibility and similar detection limits as the ADAM reaction.

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