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Development of a manganese dioxide solid-phase reactor for oxidation of toxins associated with paralytic shellfish poisoning

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Abstract

Using manganese dioxide as a solid-phase oxidant, a post-column HPLC reactor for paralytic shellfish poison toxins was constructed and evaluated. Operating parameters such as reaction temperature and pH, flow-rate, reactor column size, and MnO_2 particle size were studied. Based on a 3:1 signal-to-noise ratio, the limits of detection for the non-hydroxylated toxins were in the sub-nanogram range, while the hydroxylated toxins were ten times less detectable. The limit of detection for saxitoxin was about 0.1 ng per injection. The repeatability of replicate injections was $\leq 10\%$ (relative standard deviation). The system was used to analyze extracts of shellfish and plankton, yielding results that were in agreement with those obtained by established analytical methods. Extracts of shellfish contaminated at the regulatory limit of 0.8 μg/g were successfully analyzed.

Keywords: Shellfish poisoning; Solid-phase reactors; Derivatization, LC; Toxins; Manganese dioxide

1. Introduction

Paralytic shellfish poison (PSP) intoxication is an illness with symptoms ranging in severity from nausea to respiratory failure and even death. It is caused by ingestion of shellfish contaminated with neurotoxins which are produced naturally by certain marine algae on which the mollusks feed [1]. The occurrence of these toxic algal blooms is difficult to predict, so an effective shellfish monitoring program is required to protect public health and to maintain a viable fishing industry. Since PSP contamination has become a world-wide problem, with cases being reported on nearly every continent, there is much interest in developing reliable analytical methods which are capable of detecting at least 80 μg of toxin in 100 g (0.8 μg/g) of shellfish, the current allowable limit in many countries.

The family of chemicals associated with PSP is shown in Fig. 1. These compounds are non-volatile and have no useful UV-absorbing chromophores, so analysis by conventional methods such as GC or HPLC with UV–Vis detection is not possible. The mouse bioassay [2] is the most widely used test for PSP toxins, but it is plagued by problems such as lack of sensitivity, limited range of linear response, poor reproducibility, and public opposition to mammalian bioassays. Liquid chromatography with fluorescence detection is the most common instrument-based method for PSP analysis. Most HPLC methods

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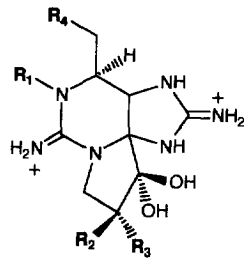

R1	R2	R3	Carbamate Toxins	Sulfocarbamate Toxins	Decarbamoyl Toxins
H	H	H	STX	B1	dc-STX
OH	H	H	NEO	B2	dc-NEO
OH	H	OSO_3^-	GTX1	C3	dc-GTX1
H	H	OSO_3^-	GTX2	C1	dc-GTX2
H	OSO_3^-	H	GTX3	C2	dc-GTX3
OH	OSO_3^-	H	GTX4	C4	dc-GTX4
		R4:	H_2N O– O	^-O_3S N O– O	HO–

Fig. 1. Structure of paralytic shellfish poison toxins. Saxitoxin (STX), neosaxitoxin (NEO), gonyautoxin (GTX).

involve the chemical oxidation of the toxins to fluorescent derivatives, a reaction first used by Bates and Rapoport [3]. Post-column reactors based on solution chemistry have been used successfully [4,5], but these systems require a great deal of daily maintenance and are adversely affected by minor fluctuations in the flow of the periodate oxidant. Because of its labour-intensive nature, the post-column system may not be practical for surveillance programs, especially on an occasional basis where equipment may have to be assembled and dismantled frequently. A highly sensitive pre-chromatographic oxidation method has been developed [6], but a major disadvantage to this method is that some toxins (e.g. NEO and B2) are not separated because their oxidation products are identical. This problem, however, can be overcome with the use of cleanup steps before the oxidation. Separation of the oxidation products of the optical isomer pairs (GTX1/4 and GTX2/3) can only be achieved with the use of chiral chromatography [7]. In a move towards a simpler, 'reagentless' post-column system, the use of electrochemical reactors for the oxidation of PSP toxins was recently evaluated [8,9]. Our current study, a variation on the 'reagentless' theme, employs a post-column solid-phase reactor using MnO_2 to oxidize the toxins. Solid-phase reactors have been used in a variety of reactions in HPLC [10], and manganese dioxide is a commonly used oxidant in organic synthesis [11–13]. The work presented here combines these two well-studied areas into a novel approach to the oxidation of PSP toxins.

2. Experimental

2.1. HPLC apparatus and conditions

The HPLC system consisted of two pumps (Beckman, Model 110B), a gradient controller (Model 421A), and an injection port with a 50-μl sample loop. A dual monochromator fluorescence detector (Jasco 820-FP), set at λ_{ex} 330 nm, λ_{em} 400 nm, and an integrator (BioRad 3392A) were used to monitor the effluent.

2.1.1. Batch experiments

The oxidation products were analyzed as outlined in the pre-chromatographic oxidation method [6]. Both mobile phases were comprised of 0.1 *M* ammonium formate, adjusted to pH 6.0 with acetic acid. Mobile phase B contained 10% acetonitrile (v/v) while A contained none. A two-part linear gradient program was used to increase %B from 0 to 5.3 in the first 8 min and then to 40 in the next 7 min. The column was a Supelcosil LC-18 from Supelco (5 μm, 15 cm×4.6 mm I.D.).

2.1.2. Post-column system

A schematic representation of the post-column system is provided in Fig. 2. Chromatographic conditions were similar to those used in the post-

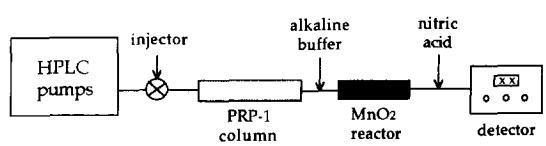

Fig. 2. Schematic representation of apparatus for post-column solid-phase reaction system.

Table 1
HPLC conditions for post-column reaction system. Both gradient programs are linear

	Gradient I	Gradient II
Mobile phase A	3.0 m*M* heptanesulfonate 1.5 m*M* ammonium phosphate, pH 7 1% (v/v) acetonitrile	3.0 m*M* heptanesulfonate 1.5 m*M* ammonium phosphate, pH 7
Mobile phase B	3.0 m*M* heptanesulfonate 6.25 m*M* ammonium phosphate, pH 7 25% (v/v) acetonitrile	3.0 m*M* heptanesulfonate 6.25 m*M* ammonium phosphate, pH 7 25% (v/v) acetonitrile
Gradient program	flow: 0.8 ml/min %B 3–23 in first 7.5 min 23–100 in next 8 min	flow: 0.8 ml/min %B 0–19 in first 8 min 19–100 in next 8 min

column periodate reaction system [4] and are outlined in Table 1. The gradient program had to be modified towards the end of this study in order to separate the earlier-eluting compounds (see gradient II, Table 1). Fresh mobile phases were prepared at least every second day. A Hamilton PRP-1 (10 μm, 15 cm×4.1 mm I.D.) was used as the analytical column and was maintained at 45°C with a column heater (Bio-Rad).

2.2. MnO_2 reactor

The solid-phase reactor was assembled using an empty HPLC column housing (7.5 cm×4.6 mm I.D.). The column was dry-packed by the tap-and-fill method with MnO_2 (Anachemia) that had been ground and sieved to a particle size range 45–75 μm. The packing was then compressed by flushing with mobile phase A, and the void volume at the head of the column was filled with additional MnO_2. This step was repeated until the formation of void volumes ceased. The reactor was heated to 85°C by a second column heater. Two post-column pumps (Altex 110B), each equipped with a pulse-dampener and an old PRP-1 column to reduce baseline noise, were used to introduce pH-adjusting solutions before and after the reactor. The first one delivered an alkaline buffer solution (0.1 *M* Na_2HPO_4, adjusted to pH 11.5 with KOH) at a rate of 0.3 ml/min, while the second pumped 0.08 *M* nitric acid, also at 0.3 ml/min. To maintain the integrity of the reactor, the mobile phases and alkaline buffer solution were purged continuously with helium. When not in use, the entire HPLC system was left in mobile phase A at 0.1 ml/min.

2.3. Batch reactions

The batch reactions were carried out under various conditions, but typical experimental parameters are given in parentheses. A 1.5-ml plastic microcentrifuge vial containing 200 μl 0.1 *M* phosphate buffer (pH 8) and 200 μl standard solution of a PSP toxin (0.1 ng/μl) was pre-heated in a water bath (60°C) for 1 min. A known mass of MnO_2 (0.01 g) was added, and the vial was shaken and returned to the water bath. The mixture was shaken briefly every 2 min for the duration of the reaction. At the end of the desired reaction time (10 min), 3 μl of acetic acid and 600 μl of water were added. The mixture was transferred using a pasteur pipette to a syringe fitted with a 0.45-μm syringe filter (Acrodisc, Gelman Sciences). After filtration, the solution was allowed to stand for 10 min and then analyzed by the HPLC method for oxidation products.

2.4. Materials

All solvents and reagents were analytical or HPLC-grade materials, and deionized water was used (Milli-Q water system, Millipore). Certified standard solutions of the following PSP toxins were obtained from the National Research Council of Canada (Halifax, Canada): saxitoxin (STX), neosaxitoxin (NEO), and gonyautoxins 2 and 3 (GTX 2/3). A mixture of GTX1-4 was received as a generous gift from Dr. Y. Oshima (Tohuku University, Japan).

2.5. Sample extraction

The extraction and cleanup of samples were performed using the procedure for the mouse bioassay [2]. Briefly, 10 g of homogenized shellfish tissue were boiled with 10 ml 0.1 *M* HCl for 5 min. The mixture was cooled and centrifuged. A 1-ml aliquot of the supernatant was passed through a C_{18} solid-phase extraction cartridge and the effluent collected. Following this, 2 ml water were added and the effluent combined with the first. An aliquot of this solution was diluted 7–10 times for analysis of samples near the regulatory guideline level of 0.8 μg/g PSP toxins.

3. Results and discussion

3.1. Batch experiments

Batch experiments were performed on STX, NEO, and GTX2/3 so that reaction conditions could be controlled and studied independently. Effects of pH, temperature, reaction time and quantity of oxidant were investigated. Retention times of the products of MnO_2 oxidation were compared to those of the periodate reaction [6]. The two reactions were found to produce similar purine derivatives. The products of the periodate reaction have previously been identified by LC–MS [14].

As the reaction temperature increased from 20 to 90°C, there was a higher yield of fluorescent products for STX and NEO. The yield also increased for GTX2/3, but more than one product was formed at elevated temperatures. Increasing pH from 8 to 10 led to higher yield for all toxins studied. At pH≥11, multiple products were formed in all cases and the yield decreased for STX.

More detailed studies were done with STX and NEO only; GTX2/3 were not included because formation of multiple products complicated the quantitation process. The effect of reaction time was studied (pH 8, 70°C). The rate of product formation was constant for STX from 2 to 25 min, but the NEO–MnO_2 reaction appeared to reach equilibrium after only 3 min (i.e. formation of fluorescent products stopped). When the surface area of oxidant available for reaction was increased, either by decreasing particle size or by adding more MnO_2, the product yield increased for STX. The reaction seemed to be first-order in MnO_2. In the case of NEO, however, there was no change observed except at very low quantities (0.1 mg) of large MnO_2 particles. The slight structural difference between STX and NEO, the N-1 hydroxyl group, seems to cause a notable difference in how the oxidation proceeds. The reason for this is unknown, but it may be related to the different pK_a values of the two analogues [1]. Only the fluorescent oxidation products have been considered so far, but preliminary studies indicate that there are no UV-absorbing (330 nm) compounds produced other than the fluorescent ones.

Since pre-column MnO_2 oxidation led to multiple products for most toxins and the proportion of each product changed with pH, temperature and time elapsed between derivatization and injection of sample, it would not be a useful analytical method. These studies indicated, however, that MnO_2 had the potential to be a solid-phase oxidant for the post-column oxidation of PSP toxins. The advantage of this mode is that multiple oxidation products need not be separated, identified and quantitated. Instead, the parent compounds are separated so that each individual PSP toxin elutes as a single peak, and then undergoes oxidation to one or more fluorescent products which are quantitated together.

3.2. Post-column system

With the knowledge gained from the batch experiments, a post-column reaction system was assembled as described in the Experimental section. Parameters such as reactor residence time, MnO_2 particle size and reaction pH and temperature were studied in detail.

Reaction time was studied by changing the flow-rate through the reactor and keeping the flow of the basic buffer at 0.3 ml/min. (The reaction pH did not change significantly despite the change in proportion of mobile phase to buffer.) When the flow of the mobile phase was increased from 0.75 to 1.2 ml/min, a 10% decrease in STX peak area was observed. A second investigation of reaction time was conducted by changing the size of the reactor while maintaining constant flow. Increased reaction bed

volume and the consequent increase in residence time led to greater peak areas for STX and GTX2/3. The response for NEO remained unchanged. These results support the findings of the pre-chromatographic batch experiments in which the NEO–MnO_2 reaction went to completion very quickly while the reaction of STX progressed at a constant rate for reaction times up to 25 min. The response factors for STX and GTX2/3 in the post-column reaction system changed proportionally with the changes in volume of the reactor. Unfortunately, the trade-off involved in using a larger reactor was band-broadening due to increased void volume.

The reaction pH was monitored by turning off the second post-column pump and was changed by altering the pH of the buffer solution. The concentration of the nitric acid had to be adjusted to ensure that the solution entering the detector was at pH 7, the optimum pH for fluorescence of the derivatives. The results of this study are shown in Fig. 3. Increasing the pH from 8.5 to 10.0 led to improved sensitivity for all the toxins investigated. The optimum reaction pH for STX and GTX2 was approximately 11.0, after which the sensitivity was reduced. The response for GTX3 did not reach a maximum in the pH range investigated. The response for NEO peaked at pH 10.0, decreased for one pH unit, and then began to rise again. Ideally, the optimum pH for NEO should be chosen, but attempts to operate the system at a reaction pH of 11.0 on a routine basis were hampered by excessive baseline noise. (This limitation was due to the increased HNO_3 concentration required and might be corrected by using more effective pulse-dampening equipment.) In the end, a reaction pH of 10.5 was chosen for all further studies. In order to achieve this, the phosphate buffer was adjusted to pH 11.5.

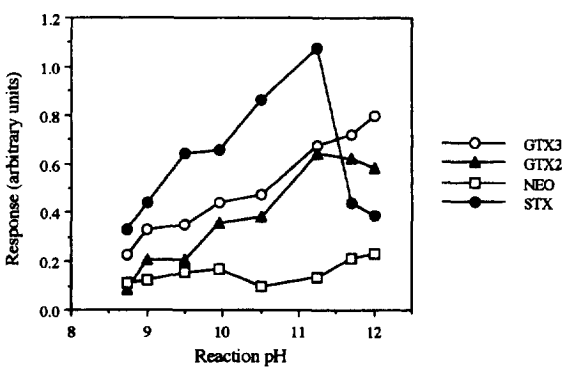

Fig. 3. Fluorescence detector response (per ng toxin injected) as a function of reaction pH for individual PSP toxins.

The yield of fluorescent products for each toxin increased with higher temperatures, which is the same trend observed in the batch experiments. It was decided to set the column heater at 85°C because, at higher temperatures, there was a greater chance of bubble formation in the detector. Based on the above results, the operating conditions described in the Experimental section were selected for use in all further studies.

The effects of band-broadening in solid-phase reactor beds are well-documented [10]. Despite our best efforts to minimize these effects by using a more uniform particle size and by checking for (and filling when necessary) void volumes that formed at the head of the MnO_2 column, there was still a noticeable amount of broadening due to the reactor. To quantitate this contribution, the HPLC system was set up to analyze PSP toxins in the pre-chromatographic mode [6] with all the post-column hardware still in place. A STX standard was derivatized and analyzed twice, once with the reactor on-line (MnO_2 and previously oxidized STX did not react) and the second time with a zero-dead volume union in place of the reactor. The peak width broadened by two times with the reactor in place.

When the reactor was stored in acetonitrile–water (1:1) overnight, a gradual reduction in sensitivity for STX and GTX2/3 was observed as the MnO_2 reactor aged. Compared to results obtained on the first day of use, the response for STX had decreased by 50% by day 4 and 90% by day 7. This problem was eliminated by flushing the entire HPLC system with helium-purged mobile phase A at 0.1 ml/min continuously when it was not in use. Under these overnight storage conditions, the reactor remained stable for at least eight days of use. Beyond this time, the reactor may lose its reactivity due to reaction with trace components of the mobile phase and native components of sample extracts. Long-term use of a single reactor for extensive sample analysis was not carried out. In practice, the reactors were changed weekly since they are inexpensive and easily prepared.

The estimated limits of detection (LODs), based

on a 3:1 signal-to-noise ratio, for the PSP toxins studied were from 0.1 to 0.3 ng/injection for the non-N-1-OH toxins and 0.4 to 2.0 ng/injection for the N-1-OH compounds. These LODs are comparable to those obtained using other post-column reaction systems. The response for the hydroxylated toxins was lower than that for the non-hydroxylated analogues by about ten times, a trend that has been observed with the pre- and post-chromatographic periodate oxidation and with electrochemical oxidation [4,6,9]. The response was linear for each toxin at levels at least 100 times the LOD, and the within-day repeatability was ≤10% (relative standard deviation) for all toxins.

Extracts of shellfish and plankton were analyzed using the post-column MnO_2 reactor. Chromatograms of standards and extracts, obtained using two different gradients, are shown in Figs. 4 and 5. Modification of the gradient program caused a noticeable change in sensitivity for some toxins. This might be explained by the change in composition of the mobile phase (percent acetonitrile) in which these toxins were eluted. In the batch experiments, it was shown that increased amounts of acetonitrile in the reaction mixture led to a lower yield of fluorescent products for saxitoxin at elevated temperatures.

The mussel extract shown in Fig. 4 was found to contain 0.1 μg/g STX, 0.6 μg/g NEO and 0.2 μg/g GTX2. This compares well with a total PSP toxin value of 1 μg/g as determined by the standard mouse bioassay [2]. The results of the clam sample in Fig. 5 (11 μg/g total PSP toxin) agreed well with data obtained on the same sample using other methods [4,6,9], including electrochemical oxidation (12 μg/g), pre-chromatographic periodate oxidation

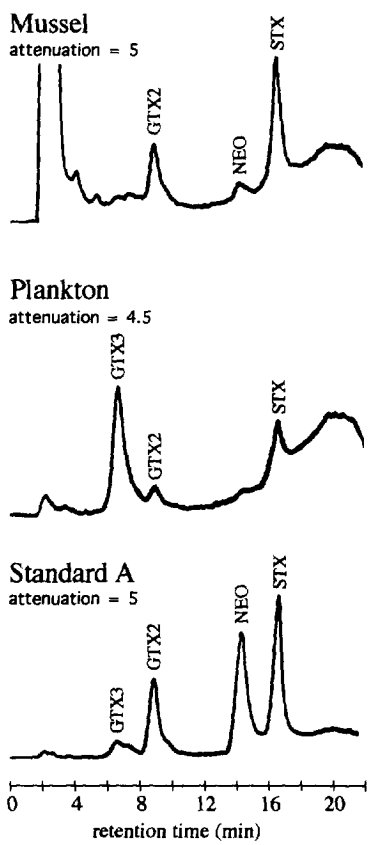

Fig. 4. Chromatograms obtained using gradient I (see Table 1 for conditions) The mussel sample had a total toxin concentration of 1 μg/g (extract diluted 7 times). The plankton extract (qualitative only) was diluted 300 times; ca. 1 ng of GTX3 injected. The amounts injected (ng) in the standard mixture were: GTX3, 0.45; GTX2, 1.8; NEO, 28; STX, 0.70.

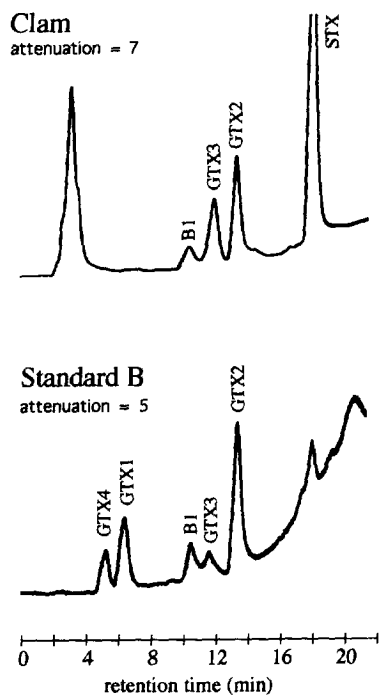

Fig. 5. Chromatograms obtained using gradient II (see Table 1 for conditions). The clam sample was contaminated at a level of 11 μg/g (extract diluted 10 times). The amounts injected (ng) in the standard mixture were: GTX4, 1.1; GTX1, 3.1; B1, 1.3; GTX3, 0.36; GTX2, 1.2.

(10 μg/g) and post-column periodate oxidation (13 μg/g). The minimum detectable concentrations in actual samples were estimated to be about 3-10 ng/g for the non-N-1-OH toxins and 15–50 ng/g for the N-1-OH toxins. These results indicate that the MnO_2 solid-phase reactor shows promise as an alternative post-column oxidation system for PSP toxins.

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