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Short communication

Effect of pH on the oxidation of paralytic shellfish poisoning toxins for analysis by liquid chromatography

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Abstract

The effect of pH on the oxidation of individual PSP toxins using both periodate and peroxide oxidations was studied. It was found that the optimum pH for individual toxins varied considerably. For periodate oxidations, pH 8.2 produced the maximum yield of fluorescent products for neosaxitoxin and GTX1/GTX4 while the non-hydroxylated toxins (saxitoxin, GTX2/GTX3, decarbamoyl saxitoxin, GTX5) showed optimum pHs from about pH 10–11.5. Neosaxitoxin and GTX1/GTX4 did not produce significant fluorescent oxidation products with peroxide oxidation at any of the pHs studied (pH 8.2–12.8). The non-hydroxylated toxins all showed optimum pHs above pH 12 with peroxide oxidation. Yields of fluorescent products of these toxins decreased substantially at pHs below pH 12. Neosaxitoxin and GTX1/GTX4 each produced three product peaks at pH 8.2 with periodate oxidation. There was no pH where these toxins produced predominantly a single oxidation product. Decarbamoyl saxitoxin always produced two oxidation products with both oxidation reactions at the pHs studied. However, the relative yields of the products changed with pH. At low pH the second eluting product predominated, while at higher pH values the first eluting product predominated. This pattern was observed for both oxidation reactions. The other non-hydroxylated toxins produced mainly single unique products with both oxidation reactions over the pH range studied. No single pH was found optimum for the oxidation of both hydroxylated and non-hydroxylated toxins without a significant compromise in yield of oxidation products. This has implications for the post column oxidation liquid chromatographic methods, since small changes in pH of the post column oxidant can both positively and negatively affect the yields of oxidation products of toxin mixtures leading to increased error in the subsequent quantitation of these compounds. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Toxins associated with Paralytic Shellfish Poisoning (PSP) are among the most acutely toxic substance known. The structures of these toxins are shown in Fig. 1. The contamination of shellfish with PSP is recognized as a worldwide problem. A number of methods for the determination of PSP toxins have been reported in literature. However, liquid chromatography (LC) with fluorescence

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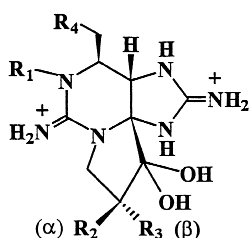

			R_4		
R_1	R_2	R_3	H_2N-C(=O)-O-	SO_3NH-C(=O)-O-	HO-
H	H	H	STX	GTX5	dcSTX
H	H	OSO_3^-	GTX2	C1	dcGTX2
H	OSO_3^-	H	GTX3	C2	dcGTX3
OH	H	H	neoSTX	GTX6	dcneoSTX
OH	H	OSO_3^-	GTX1	C3	dcGTX1
OH	OSO_3^-	H	GTX4	C4	dcGTX4

Fig. 1. Structures of PSP toxins.

detection has been the chemical method most commonly employed for the sensitive and selective analysis of these compounds [1–5].The ability of PSP toxins to be easily converted into the fluorescent derivatives, has been the basis for these methods. The original fluorescent method developed by Bates and Rappoport [6] used hydrogen peroxide as derivatization reagent to convert the PSP analogues to fluorescent products and the total fluorescence was measured as an indication of total PSP concentration. However, it was found that the *N*-1-hydroxy toxins did not yield fluorescent products with peroxide under the conditions employed. Sullivan [1] applied this approach to the development of an LC method employing post-column oxidation using periodate as the oxidant. This system could detect all of the PSP toxins then available. The post-column oxidation method was refined by Oshima [2] who employed three different isocratic LC systems to separate and quantify the toxins in groups such as the “C” toxins, the GTX group and the saxitoxin group. Another approach developed by Lawrence [3–5] involves prechromatographic oxidation followed by reversed-phase LC analysis of the oxidation products. Both approaches have their advantages and limitations.

The prechromatographic oxidation method [3–5], uses both peroxide and periodate oxidants. Most of this work focused on obtaining a single set of optimum oxidation conditions for all of the PSP toxins. However, the optimum oxidation conditions for individual toxins were not fully investigated. In the present work, we report on studies aimed at determining optimum oxidation conditions for a number of individual toxins. These data could be very useful in selecting the best oxidation conditions for both pre- and post-column oxidation LC methods based on the known historical profile of the toxins in a given geographical location. For example, if *N*-1-hydroxylated toxins are never present in a geographical region, then if using the post column LC method, there is no need to optimize the periodate oxidation for these analogues since that pH would diminish the sensitivity of the method to the non-hydroxy compounds. Instead, the oxidation conditions can be selectively changed to provide the best results for the toxin profile to be determined.

2. Materials and methods

2.1. Standards

Standard solutions of saxitoxin (STX), neosaxitoxin (neoSTX), GTX2/3 (mixture) and GTX1/4 (mixture) were kindly provided by Dr. M. Quilliam, NRC Halifax (Canada). Solutions of C1-C4, GTX1-5, and the STX group (STX, neoSTX and decarbamoyl saxitoxin (dcSTX)) were kindly provided by Dr. Y. Oshima, Tohoku University (Japan). Standard solutions of STX and dcSTX were also provided by RIVM-Bilthoven (Netherlands) as part of a BCR standard measurement and testing program certification study.

2.2. Reagents

All solvents and chemical reagents were LC or analytical grade. Hydrogen peroxide, periodic acid, acetic acid and sodium hydroxide were purchased from Panreac, potassium phosphate from Probus and ammonium formate from Sigma. Other reagents such as acetonitrile and methanol (from Panreac), were analytical grade and the water used was Milli-Q grade (Millipore Ltd.).

2.3. Apparatus

The LC system (Jasco PU-980) consisted of 2 single-piston pumps, a gradient controller and an injection port with a 20-μl loop. The column was a reversed-phase, Prodigy LC-18 (Phenomenex 250× 4.6 mm i.d, 5um). A filter fluorescence detector, Perkin-Elmer LC-10 (excitation maximum, 370 nm; emission range, 418–700 nm) was used to monitor the LC effluent.

2.4. Extraction of PSP toxins from shellfish

The extraction conditions used were those described for the AOAC mouse bioassay [7]. A 3 ml aliquot of the extract was cleaned up using a 3-ml C_{18} cartridge (Sep-Pak Plus C_{18} Waters) as described elsewhere [3].

2.5. Oxidation reactions

2.5.1. Effect of pH on the oxidation reaction

The studies on the effect of pH on the oxidation reaction for individual PSP toxins were carried out to determine the reaction conditions that would produce the maximum yield of fluorescent oxidation products for each toxin.

2.5.2. Periodate oxidation

Periodate oxidation of PSP toxins in standards and samples was carried out as described elsewhere [5]. All reagent solutions used in this reaction were prepared fresh weekly. The oxidation solution was prepared daily by mixing equal volumes of 0.03 *M* periodic acid, 0.3 *M* ammonium formate and 0.3 *M* potassium phosphate and adjusting the pH from 7.2 to 12.0 with 0.2 *M* NaOH. To 250 μl of oxidant solution, 50 μl of PSP standard or sample extract were added. The mixture was permitted to react for 1 min at room temperature. A 3-μl volume of concentrated acetic acid was added to stop the reaction and the solution was mixed well. The mixture was maintained for 10 min at room temperature before analysis.

2.5.3. Peroxide oxidation

Peroxide oxidation was carried out as described above for the periodate oxidation in Section 2.5.2 except that 3% aqueous hydrogen peroxide was used in place of periodic acid. The pH of the oxidant solution was adjusted from pH 8.2–12.8 with 0.2 *M* NaOH.

2.6. Liquid chromatography

Chromatographic separation of the oxidation products was performed by using reversed-phase chromatography based on the method described by Lawrence [5] with some slight modifications. The mobile phase program was a gradient of 0–4% (v/v) acetonitrile in 0.1 *M* ammonium formate, adjusted to pH 6 with acetic acid, as follows: 0–1% in the first 6 min, then 1–4% in the next 9 min and finally 4–0% in the last 3 min. The flow-rate was 1.5 ml/min.

3. Results and discussion

3.1. Periodate oxidation

The toxins, GTX1/4 (mixture), GTX2/3 (mixture), GTX5, dcSTX, STX and neoSTX were studied over the pH range of 7.2–12.2. Fig. 2 graphically shows the results obtained. It can be seen that a significant increase in the fluorescence response was observed with increased reaction pH for most PSP analogues with the exception of neoSTX and GTX1/4 (optimum pH, 8.2). (The results for GTX1/4 are not shown but the curve was virtually identical to neoSTX but with only one third the area per ng as neoSTX.) For the non-*N*-1-hydroxylated analogues studied, the optimum pH for each individual toxin varied from about pH 10 for GTX2/3 to about 11.5 for GTX5.

Decarbamoyl saxitoxin always produced two derivative peaks over the full pH range studied. However, the peak height ratio of the two peaks changed substantially in going from low to high pH. At pH 9.4 the second eluting peak predominated while at pH 10.5 and higher, the first eluting peak was the major product. It had been shown in earlier work [5] that with the periodate oxidation, the second peak predominated while with the peroxide oxidation, the first peak was the major product. The present work indicates that these results were not caused by the different oxidants employed but to a difference in the

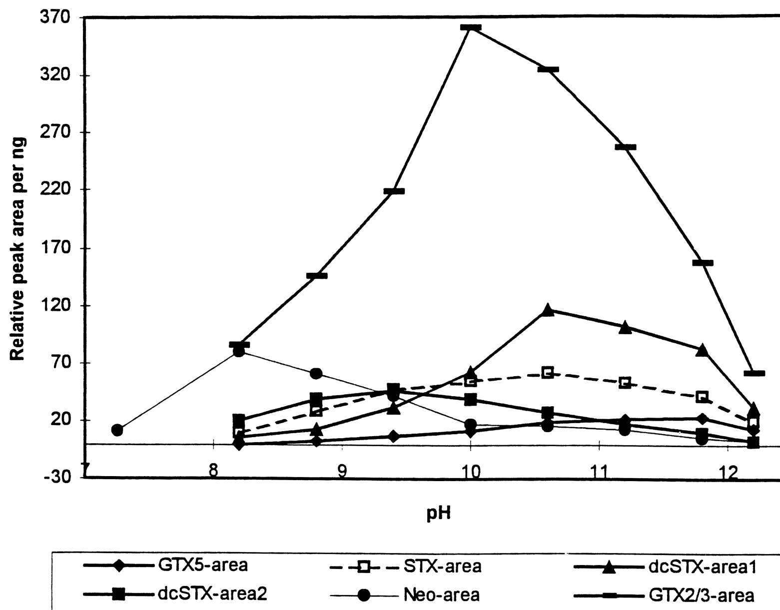

Fig. 2. Influence of pH on the yield of fluorescent products of PSP toxins using periodate oxidation, measured as relative peak areas per ng injected.

pH used in the oxidation reactions. Fig. 3 compares chromatographic results of periodate oxidation for dcSTX at pH 9.4 and 10.6. The change in relative peak heights at the different pHs can be readily observed over this relatively small pH range. For application to actual samples, a pH of 10.4 (from Fig. 2) for the reaction would be optimum using the first eluting peak for quantitation.

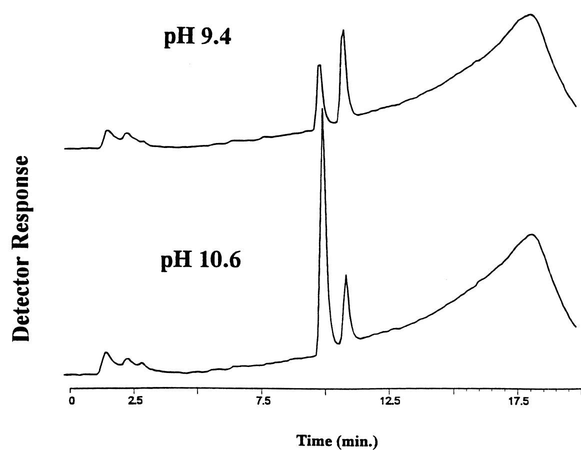

Fig. 3. Chromatograms of the oxidation products of dcSTX using periodate oxidation at pH 9.4 and 10.6.

Optimum oxidation pHs for the other toxins were about pH 10.0 for GTX2/3, pH 10.6 for STX, pH 11.5 for GTX5 and pH 8.2 for neoSTX and GTX1/4. With the exception of GTX5, the yield of fluorescent oxidation products of all toxins decreased as the reaction pH increased from about 10.6 to 12.2, the highest pH studied. (The yield for GTX5 decreased significantly from about pH 11.8 to 12.2.). It can be observed in Fig. 2 that the relative response of the GTX2/3 products are significantly higher than those obtained from the other toxins studied over most of the pH range. Similar results were reported earlier for the fluorescence response for these toxins under somewhat different oxidation conditions [8].

One of the reasons for studying different pHs for the *N*-1-hydroxylated toxins, neoSTX and GTX1/4, was to determine if there was a pH at which they would produce predominantly single fluorescent products. However, neoSTX and GTX1/4 both produced three product peaks at pH values from 8.2 to 10.5 The ratios of these peaks did not change substantially unlike the two peaks for dcSTX. The

second eluting peak for neoSTX was always the most predominant peak while the third peak was the highest for GTX1/4. However, above pH 8.2, the quantity of fluorescent products decreased substantially. Thus, for optimum results for neoSTX and GTX1/4, a pH of about 8.2 must be used. Since the yield of fluorescent products also decreased substantially at pHs below pH 8.2, it is important for the oxidation of neoSTX, that the pH be carefully controlled to ensure good repeatability. Although GTX6 (B2) was not evaluated in this work, it would be expected that it would behave similarly to neoSTX and GTX1/4.

Fig. 4 illustrates a comparison of the oxidation at pH 8.2 and 12.0 of a mixture of neoSTX (0.45 μg/g), GTX1/4 (0.51 μg/g), GTX2/3 (0.19 μg/g), GTX5 (0.61 μg/g) and STX (0.18 μg/g) spiked in an extract of mussels. At pH 8.2, the *N*-hydroxylated toxins, GTX1/4 and neoSTX, are readily detected but GTX5 is not. However, at pH 12, the hydroxylated toxin peaks decreased substantially while that for GTX5 increased and could be readily quantitated. The peaks for GTX2/3 and STX also changed but to a lesser extent.

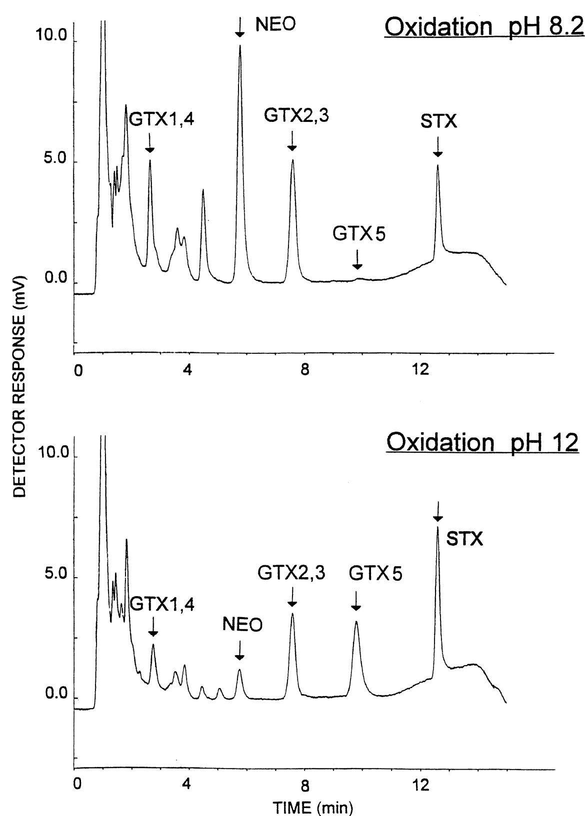

Fig. 4. Chromatograms after oxidation at pH 8.2 and 12 of a mixture of GTX1/4, NEO, GTX2/3, GTX5 and STX spiked in an extract of mussels. Conditions described in the text.

The results in Figs. 2 and 4 also illustrate one of the problems associated with trying to employ periodate oxidation for post-column oxidation of PSP toxins. It can be seen that there is no optimum pH suitable for the efficient oxidation of both *N*-1-hydroxylated- and non-*N*-1-hydroxylated toxins. The selection of any single pH for the oxidation would significantly reduce the sensitivity of one or the other groups of toxins. In addition, any compromise pH would likely be in an area of the curves where the oxidation yield for individual toxins is very sensitive to changes in pH which could lead to difficulties with repeatability of quantitation for replicate analyses for certain toxins.

3.2. Peroxide oxidation

Different pH conditions in the range 8.2–12.8 were evaluated for peroxide oxidation of the toxins using the same buffer and reagent conditions as used for the periodate oxidation described in Section 2.5.2. No analytically-useful fluorescent products were observed for neoSTX and GTX1/4 at any pH studied (including pH 8.2 which was found optimum using the periodate oxidation). This is consistent with results carried out earlier with the peroxide reaction where neoSTX has never been shown to yield sufficient quantity of fluorescent products suitable for analytical purposes [3–5,8]. Decarbamoyl saxitoxin produced the same two fluorescent products that were formed with periodate oxidation. The behavior of the two peaks was also similar to that observed for periodate oxidation, in that the first eluting peak increased with increasing pH of the oxidation reaction while the second peak decreased slightly at pH values above pH 10.

Fig. 5 compares the results obtained for the toxins studied. A substantial increase in the yield of fluorescent products was observed for all non-*N*-1-hydroxylated toxins over the pH range of 8.2 to about 12.2. This latter pH was optimal for STX and GTX2/3.

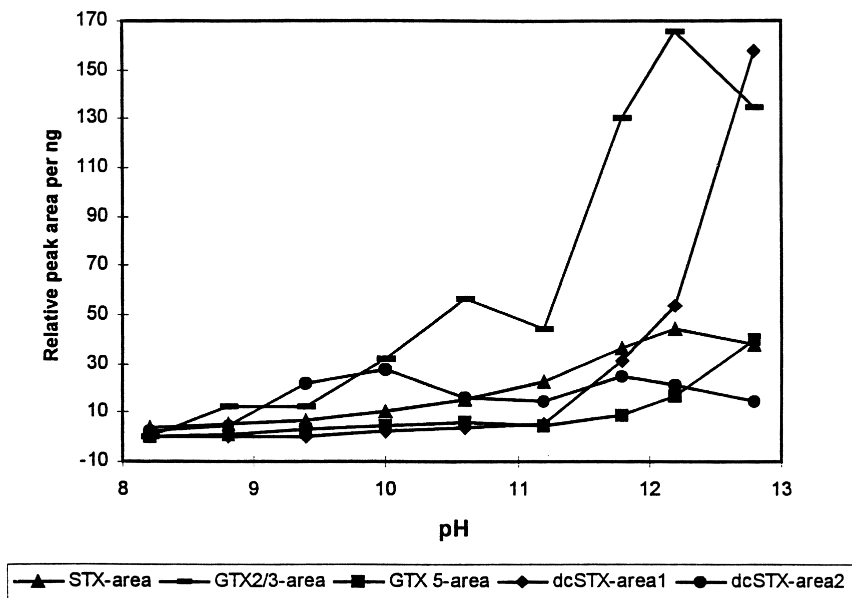

Fig. 5. Influence of pH on the yield of fluorescent products of PSP toxins using peroxide oxidation, measured as relative peak areas per ng injected.

The peak for GTX5 and the first eluting product peak of dcSTX continued to increase up to pH 12.8, the highest pH examined. From these results, it can be observed that no single pH is optimum for all of the non-*N*-1-hydroxylated toxins. For example, at pH 12.2, optimum for STX and GTX2/3, the yields for GTX5 and dcSTX (first eluting peak) are only about half of the yields obtained for these toxins at pH 12.8. But at pH 12.8, the yields for STX and GTX2/3 are slightly lower compared to pH 12.2. However, the decrease is much less than that for GTX5 and dcSTX mentioned above.

In comparing the results of the two oxidation reactions, it was observed that the optimum pH for each non-*N*-1-hydroxylated toxin was about 2 pH units higher for peroxide than for periodate. (The exception being GTX5 where the difference was only 1.3 pH units.) Under optimum conditions for each reaction, the peroxide oxidation produced higher quantities of fluorescent products for GTX2/3 (+50%), GTX5 (+100%) and dcSTX (+25%). For saxitoxin, the optimum yields of fluorescent product for both oxidation reactions were similar.

3.3. Influence of sample extract pH on PSP oxidation

Since the pH of shellfish extracts obtained using the AOAC mouse bioassay extraction procedure [7] is acidic and the optimum pHs for both peroxide and periodate oxidations are basic, it is important to ensure that the sample extract does not exert a negative influence on the oxidation reactions. With this in mind, adjustment of the pH of the sample extracts was studied in order to obtain the highest yield of fluorescent products using the periodate oxidation. These studies were carried out with extracts of contaminated samples containing mainly dcSTX. by adjusting the pH of the extracts to different values in the range of 5–8 with 1 *M* NaOH. The optimal response was achieved at pH 7 which was slightly better than that at pH 5 or 6. However, adjustment of the extracts to pH 8 led to a slight decrease in yield of fluorescent oxidation products. This may be the result of some instability of the toxins under basic conditions before oxidation, although this was not pursued further. Thus for routine

application, pH 7.0 was selected for best yield and repeatability.

Acknowledgements

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