Determination of Domoic Acid in Phytoplankton by High-Performance Liquid Chromatography of the 6-Aminoquinolyl-*N*-hydroxysuccinimidyl Carbamate Derivative

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Domoic acid is the toxin that can cause amnesic shellfish poisoning. In this work, a new precolumn derivatization reagent, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, was used to react with domoic acid to form a stable unsymmetrical urea derivative, which was readily analyzed by reversed-phase HPLC with fluorescence detection. The one-step derivatizing method is rapid and straightforward. The method was applied to detect domoic acid in phytoplankton using a 3.9 × 150 mm Nova-Pak, 4 μm, C_{18} column.

Keywords: *Domoic acid; AQC derivatization; reversed-phase HPLC; fluorescence detection; amnesic shellfish poisoning (ASP)*

INTRODUCTION

Domoic acid, a secondary amino acid (Figure 1), is a naturally occurring neurotoxin. Before 1987, the only previously reported sources of domoic acid were the macroalgae, *Chondria armata Okamura* (Takemoto and Daigo, 1958) and *Alsidium corallinum* (Impellizzeri et al., 1975), both in the family Rhodomelaceae. The first report of intoxication of domoic acid in a human being was made in the fall of 1987, in Canada, in which 153 people suffered from acute intoxication after eating cultured blue mussels (*Mytilus edulis*) and 3 people died (Wright et al., 1989). Symptoms of the disease, named amnesic shellfish poisoning (ASP), included nausea and accompanying diarrhea, followed by confusion, disorientation, loss of memory, and sometimes coma. As a neurotoxin, domoic acid probably acts as an agonist to glutamate, a neurotransmitter in the central nervous system (Nakajima et al., 1985).

Domoic acid has been found in the toxic blooms of several *Pseudo-nitzschia* species (Bates et al., 1989; Hallegraeff, 1994). Mussels can ingest the toxic diatoms and accumulate domoic acid at higher levels than in the sample of phytoplankton. Therefore, it is important to monitor the toxin level in mussels before they are harvested for human consumption.

Reversed-phase liquid chromatography with ultraviolet (UV) diode array detection (DAD) was developed to determine domoic acid (Quilliam et al., 1989; Dhoot et al., 1993a,b). This method has a detection limit of 0.3 ng, which is not satisfactory for the determination of domoic acid in the case of phytoplankton or seawater, which generally contains a much lower level of domoic acid. Therefore, precolumn derivatization with 9-fluorenylmethylchloroformate (FMOC-CL), followed by reversed-phase HPLC with fluorescence detection, was developed, which has a higher detection limit (Pocklington et al., 1990). However, there are some disadvantages in using the FMOC reagent. The derivatization method involves two steps: first, domoic acid reacts with FMOC; second, the excess reagent of FMOC was extracted to avoid its interference with derivatized domoic acid in the determination by HPLC. FMOC-CL has been reported to yield multiple derivatives. Significant interference due to the reagent artifacts (e.g., FMOC-OH) was observed with derivatives made from FMOC-CL even when the excess reagent was extracted prior to chromatographic analysis. The efficiency of derivatization is highly dependent on both the buffer concentration and the sodium chloride level. Derivatizing reagents for amino acid analysis have been developed. In recent years, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) has been demonstrated to be an excellent derivatizing reagent for amino acid analysis. Unlike the FMOC reagent, the excess AQC reacts with water to form products that have little interference with the fluorescent domoic acid derivative (Figure 2), so it is unnecessary to extract the excess AQC reagent, making the derivatization process easy and convenient. The unique fluorescent properties of AQC-related compounds permit the analysis of derivatized samples without prior reagent removal yet with minimal reagent interference. Reactions are quantitative, reproducible, stable, and linear over a wide dynamic range. In comparison to many precolumn amino acid derivatization procedures, the reaction is relatively

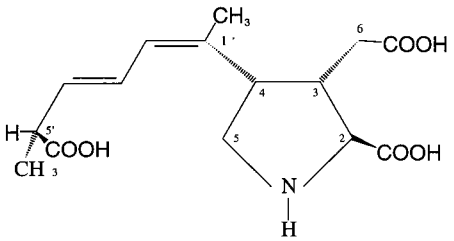

Figure 1. Structure of domoic acid.

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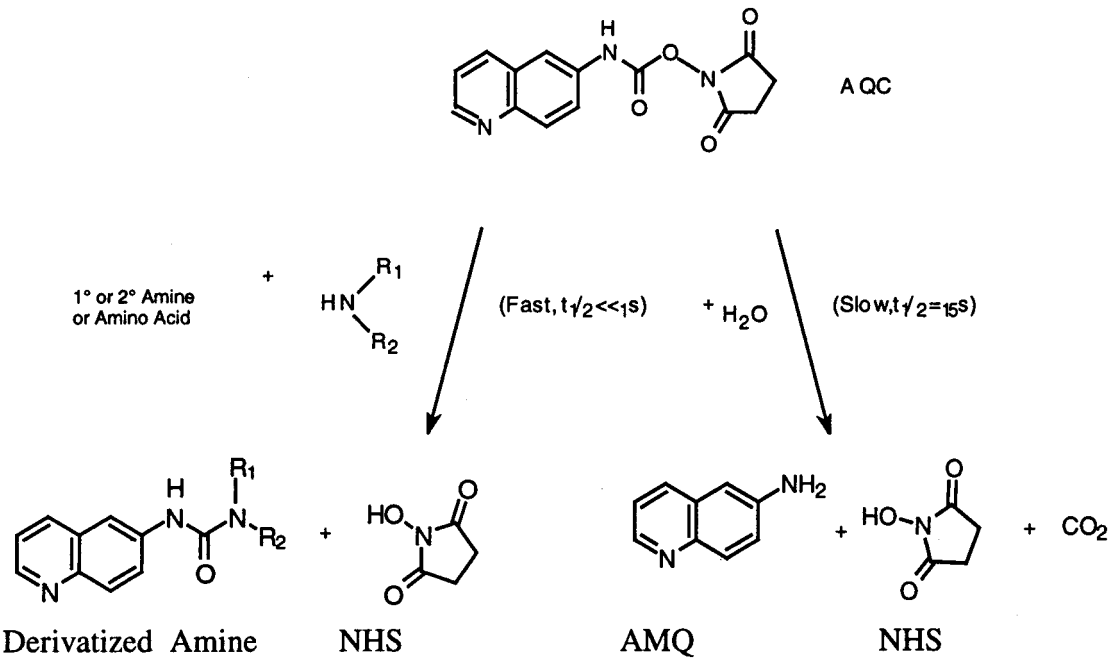

Figure 2. Reaction procedure of AQC with amino acids. AQC can react rapidly with amino acid to form the derivative, while the excess AQC can react with water to form AMQ. [Reprinted with permission from Cohen and Michaud (1993). Copyright 1993 Academic Press.]

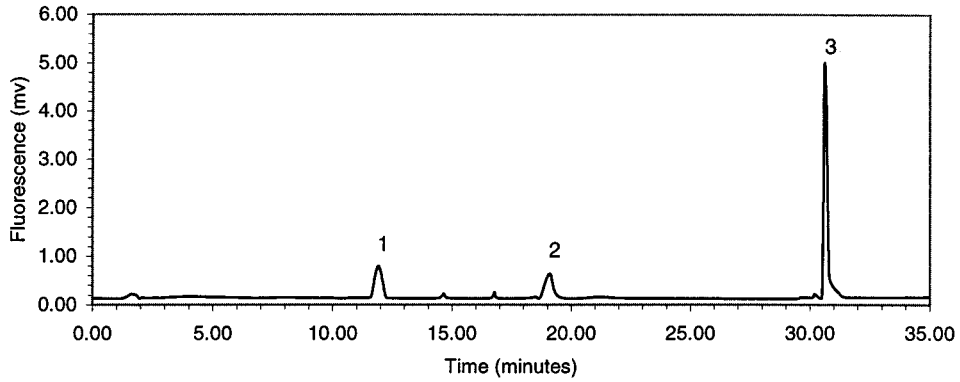

Figure 3. Gradient elution of AQC-derivatized domoic acid (DA) standard (10 ng injected). Peaks: 1, AMQ; 2, NH_3; 3, domoic acid. Conditions: Nova-Pak, 4 μm, C_{18} column, 3.9 × 150 mm, thermostated at 37 °C with a flow rate of 1.0 mL/min.

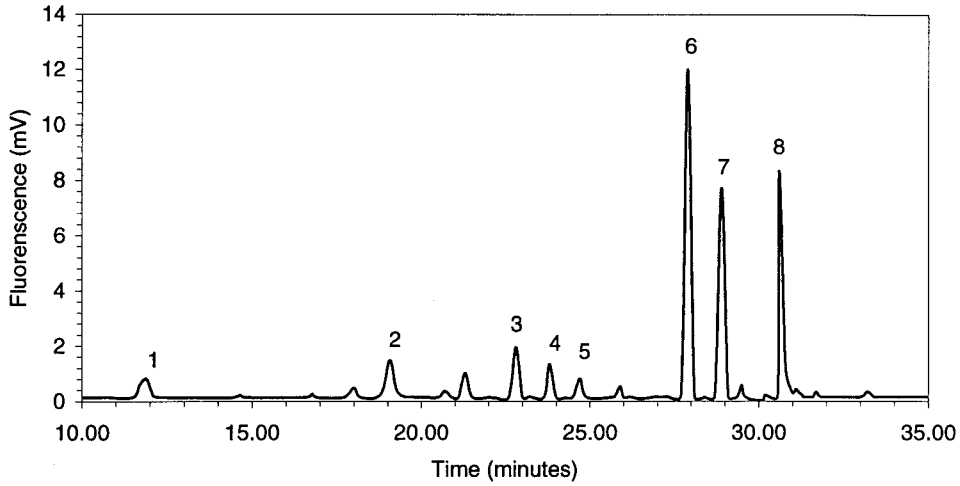

Figure 4. Gradient elution of AQC-derivatized sample of *Pseudo-nitzschia pungens* f. *multiseries*. Peaks: 1, AMQ; 2, NH_3; 3, Arg; 4, Thr; 5, Ala; 6, Val; 7, Met; 8, domoic acid. Conditions: Nova-Pak, 4 μm, C_{18} column, 3.9 × 150 mm, thermostated at 37 °C with a flow rate of 1.0 mL/min; injection volume, 10 μL.

insensitive to common buffers and salts and can be carried out in the presence of detergents (Cohen and Michard, 1993).

AQC is also a promising derivatizing reagent for domoic acid analysis. In this research, AQC was first used as a derivatizing reagent for domoic acid analysis

in phytoplankton, which provides improvement on the derivatizing method of domoic acid followed by HPLC determination.

MATERIALS AND METHODS

Chemicals. The derivative reagent, including AQC and borate buffer, was purchased from Waters Corp. (Milford, MA). AQC was powder in shipment. After reconstitution according to the product specification, the content was 10 mM AQC in acetonitrile. The reagent was stored in 2-mL glass vials with Teflon-lined caps in a desiccator at −20 °C.

An instrument calibration solution of domoic acid was purchased (DACS-1B, 99.5 μg/mL, Canadian National Research Council, Institute of Marine Biosciences, Halifax, NS, Canada). A calibration solution of domoic acid was prepared by diluting the primary calibration solution into distilled water.

Eluent A concentrate was purchased from Waters Corp. consisting of sodium acetate trihydrate, phosphoric acid, triethylamine, and sodium azide.

Phytoplankton Sample. Cultures of *Pseudo-nitzschia pungens* f. *multiseries* were initiated from samples grown at 20 °C in a growth cabinet under an irradiance level of 110 μEinstein/m^2 with a 10:14 h light/dark cycle. The flasks were rotated daily. A homogeneous subsample (10 mL) of whole culture was sonicated for 1 min at 100 W using a 1-cm-diameter probe (Braun-sonic 1510) and centrifuged; the supernant was filtered and ready for determination.

Derivatization. Domoic acid standard or sample (20 μL) was first mixed with 0.2 M borate buffer (60 μL) in a 0.5 mL tube on a Vortex mixer for 10 s. The AQC reagent (10 mM in 10% aqueous acetonitrile) (20 μL) was added, and the contents were mixed for 10 s and then transferred to a sample vial for HPLC analysis. Contaminants should be controlled during the whole process.

Chromatography Apparatus and Operation Conditions. The HPLC apparatus included two Waters Model 600 HPLC pumps, a Model 717 WISP autosampler, a Model 470 fluorescence detector, a TCM column heater, and a Nova-Pak 4 μm, C_{18} column, 3.9 × 150 mm. The column was kept at 37 °C. The flow rate was 1.0 mL/min.

Mobile phase A was prepared by mixing 100 mL of eluent A concentrate with 900 mL of deionized water. Mobile phase B contained 60% acetonitrile, 0.3% acetone, and deionized water. Acetonitrile and acetone were of HPLC grade (Baxter Healthcare Corp., Burdick and Jackson Division, Muskegon, MI). Gradient conditions were as follows: initial = 100% A, 17 min = 91% A, 24 min = 75% A, 32 min = 68% A, 34 min = 68% A, followed by a wash of 100% B for 2 min and maintained for 12 min before the program returned to initial conditions at 100% A. The total cycle time was 48 min. Injection volume was 10 μL. The excitation wavelength was 250 nm, and the emission wavelength was 395 nm.

Safety. Domoic acid is a neurotoxin; ACQ and acetonitrile are also toxic, so they should be handled with caution. All of these reagents are harmful if swallowed, inhaled, or absorbed through the skin.

RESULTS AND DISCUSSION

Derivatization. AQC reacts rapidly with domoic acid to yield highly stable ureas that fluoresce strongly at 395 nm. The derivatives are easily separated by reversed-phase HPLC. Excess reagent is consumed during the reaction to form aminoquinoline (AMQ), *N*-hydroxysuccinimide (NHS), and carbon dioxide. The destruction of excess reagent is complete within a minute. The major hydrolysis product, AMQ, has spectral properties that are significantly different from those of the derivatized domoic acid and other amino acids. This allows one to program a detector wavelength that maximizes the spectral emission response of the derivatives while minimizing the response of the AMQ. AMQ fluoresces weakly at 395 nm and produces a small peak that is easily resolved chromatographically; NHS and carbon dioxide do not interfer with the analysis.

Experiments at different pH values showed that there is little effect of derivatization buffer pH on recoveries in the range 8.0−9.6. Buffer concentration had little effect on the yield while the reaction pH was maintained in the optimal range.

The reaction product of domoic acid and AQC is stable for at least 1 week at room temperature, which allows for overnight automated chromatographic analysis.

In the chromatography of domoic acid standard, the derivative of domoic acid eluted at 30.6 min, well separated from AMQ and NH_3 at 11.8 and 19.2 min, respectively (Figure 3), so the excess reagent need not be extracted. The phytoplankton sample was analyzed using this method, and the peaks of domoic acid and several amino acids were well resolved. The amino acids presented in the sample included Arg, Thr, Ala, Val, and Met (Figure 4).

Recovery. Phytoplankton samples, spiked with domoic acid at concentrations of 0.01. 0.1, and 1.0 μg/mL, were extracted of domoic acid using the method described under Materials and Methods. Sample was derivatized with AQC to determine domoic acid by HPLC; the recoveries of domoic acid in the extraction method were 94 ± 2.2, 96 ± 1.2, and 97 ± 1.1%, respectively.

Reproducibility. The reproducibility of peak areas for repeat injections of one derivatized calibration solution (1 ng) was 1% relative standard deviation (RSD) for 1 day and 3.6% between days.

Detection Limit and Linearity. In this experiment, the detection limit for domoic acid was 0.001 ng. To get a standard curve, serial dilutions of domoic acid standard with injection amounts of 0.01, 0.1, 1, and 10 ng were used. Peak areas (mV × second) demonstrated good linear responses, which were 3.57 ± 0.037, 11.63 ± 0.313, 23.38± 0.981, and 304.18 ± 3.56 (mean ± standard error), respectively ($n = 3$, $R^2 = 0.998$, $P < 0.001$).

In this paper, the elution program was similar to that used for other amino acids. The elution time was 48 min and is longer than necessary. Because only domoic acid is under investigation, it is necessary to change the elution program by decreasing the eluting time of domoic acid and the total cycle time. This method can be optimized in future work.

Conclusions. The derivatization procedure of AQC with domoic acid is easy and straightforward. AQC can be used as a new derivatizing reagent for domoic acid analysis by HPLC because of its advantageous aspects of good detection limit, linearity of calibration curve, and relative standard deviation. This method is applicable to the monitoring of domoic acid concentration in phytoplankton.

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