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New fluorimetric method of liquid chromatography for the determination of the neurotoxin domoic acid in seafood and marine phytoplankton

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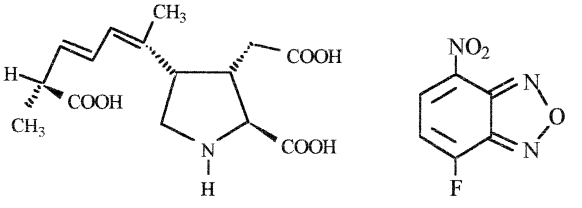
Abstract

Domoic acid (DA) is a neurotoxic amino acid that is responsible for the human toxic syndrome, amnesic shellfish poisoning (ASP). A new rapid, sensitive liquid chromatographic (LC) method has been developed for the determination of DA in various marine samples. DA in marine biological materials was derivatised with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) and analysed using isocratic reversed-phase LC with fluorimetric detection. The calibration, based on standard DA solutions, was linear in the range 0.04–2 μg/ml (r^2=0.998) and the detection limit (3:1, signal/noise) was better than 1 ng/ml. Using the certified reference material (MUS-1B), recoveries of DA from shellfish tissue were >95% (n=5). When a strong anion exchange SPE cartridge was used for sample clean-up the detection limit was 6 ng DA/g mussel tissue. Good reproducibility was achieved with RSD values ranging from 3% for 8 μg DA/g (n=5), to 5% for 0.04 μg DA/g (n=5). This new method was successfully applied to the determination of DA in naturally contaminated shellfish and in marine phytoplankton cultures of *Pseudonitzschia* sp.

Keywords: Derivatization, LC; Domoic acid; 4-Fluoro-7-nitro-2,1,3-benzoxadiazole

1. Introduction

A major toxic incident in Canada occurred in 1987, which was caused by the consumption of cultured blue mussels, affected more than 150 people and resulted in three deaths [1]. In addition to neurotoxic symptoms in victims, there was persistent short-term memory impairment in many individuals and this led to this syndrome being named amnesic shellfish poisoning (ASP). Domoic acid (DA) was identified (Fig. 1A) as the principal toxin responsible for ASP although other DA analogues have since been found [2,3]. This led to the rapid implementation of monitoring programmes to detect these compounds in shellfish and the development of several chromatographic methods for determining domoic acid which was critical in limiting more



A B

Fig. 1. (A) Structure of domoic acid. (B) Structure of derivatising reagent, NBD-F.

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widespread human intoxications. Domoic acid, and related amino acids, possess a conjugated diene moiety and regulatory agencies have employed a liquid chromatographic method with photodiode-array ultraviolet detection (LC–UV) which has a detection limit of 0.5 μg/g tissue, more than 100 times better than mouse bioassays [4]. Various procedures have been used for the extraction of domoic acid from shellfish [5], but a method that uses methanol–water and a strong anion exchange (SAX) for clean-up and analyte concentration, has recently been validated which a detection limit of 0.02–0.03 μg/g [6]. Capillary electrophoresis has also been applied to the determination of DA in shellfish but two solid-phase extraction (SPE) steps using SAX phases were required [7]. Liquid chromatography with mass spectrometric detection (LC–MS) has also been used for the determination of DA and its analogues in shellfish [8,9]. Subsequent to the initial outbreak in Canada, DA has been found in shellfish in the USA [10] and in Europe [11], as well as in other marine animals and seabirds [10].

DA accumulates in shellfish that have been grazing on marine phytoplankton belonging to the *Pseudonitzschia* sp. The derivatisation reagent, 9-fluorenylmethylchloroformate (FMOC), has been used for toxin analysis in phytoplankton and in sea water samples using fluorimetric detection, LC–FL. Although improved detection limits were reported, this method was not successful for the analysis of shellfish tissue [12]. The main problems with shellfish tissue analysis are associated with chromatographic interferences due to reagent products and other compounds in the matrix.

We now report the development of a rapid, sensitive, isocratic fluorimetric LC method for DA using 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) for derivatisation and the application of this method to the analysis of DA in marine phytoplankton and shellfish tissue samples.

2. Experimental

2.1. Chemicals

Purchased chemicals included 7-fluoro-4-nitro-2,1,3-benzoxadiazole (NBD-F) and trifluoroacetic acid (TFA), Aldrich (Dorset, UK), formic acid (Merck, Darmstadt, Germany). All solvents were LC grade and were purchased from Labscan (Dublin, Ireland). MUS-1B, certified reference shellfish material (38.3±0.8 μg domoic acid/g) and DACS-1C, certified calibration solution (100 μg domoic acid/ml) were obtained from the National Research Council, Halifax, Canada.

2.2. Shellfish sample preparation

Samples were extracted according to the procedure described by Quilliam et al. [6]. Briefly, 4 g of tissue homogenate were accurately weighed into a graduated centrifuge tube. 16 ml of methanol–water (1:1) were added and homogenised (3 min at 10 000 rpm). The resulting slurry was then centrifuged at 3000 rpm for 10 min. The supernatant was filtered through a 0.45-mm filter into a screw capped vial.

For the direct analysis, extracts (1 ml) were diluted to 10 ml with water and a 50-μl aliquot was used for derivatisation and LC–FL analysis. For clean-up and concentration of extracts, 5.0 ml of extract were subjected to solid-phase extraction (SPE) using a procedure similar to that which has been described previously [6,7]. Briefly, a strong anion exchange (SAX) cartridge (3 ml, J.T. Baker, Deventer, The Netherlands) was conditioned with methanol (6 ml), water (3 ml) and finally with methanol–water (1:1, 3 ml). Filtered supernatant (5 ml) was loaded onto the cartridge and washed with a 10% acetonitrile–water. 0.1 *M* formic acid (pH 2.92) was added to the cartridge, the first 0.5 ml was discarded and the following 3.0 ml collected for analysis.

2.3. Marine phytoplankton sample preparation

A culture sample of *Pseudonitzchia australis* (50 ml) was centrifuged (3000 rpm, 20 min). A 50-μl aliquot of the medium was analysed using NBD-F and LC–FL and was found to be negative for DA. The cell pellet in water (1 ml) was sonicated (1 min) to disrupt the cells after centrifuging again (3000 rpm, 10 min), the supernatant was removed and filtered (0.45 μm) into an amber vial (2 ml). A 50-μl aliquot was used for analysis by LC–FL.

2.4. Derivatisation of domoic acid with NBD-F

NBD-F in acetonitrile (1 mg/ml, 50 μl) and 0.1 *M* sodium borate (50 μl) were mixed with sample extract or standard solution (50 μl) in an amber vial (2 ml), at 20°C and the reaction was terminated after 3 min by adding 1 *M* HCl (50 μl) to the mixture. An aliquot (20 μl) of this derivatised mixture was analysed directly by LC–FL.

2.5. Liquid chromatography–fluorimetric detection (LC–FL)

The LC system (Shimadzu, Duisberg, Germany) consisted of a pump (LC-10AD), column oven (CTO-10A) and fluorescence detector (RF-551). Isocratic chromatography was performed using acetonitrile–water (40:60) containing 0.1% TFA, solvent flow (1.0 ml/min) with a Luna C_{18} column (250×4.6 mm, 5 μm, Phenomenex, Macclesfield, UK) and a precolumn (30×4.6 mm, 5 μm) at 35°C. Chromatographic data handling was performed using Unipac Class-VP software (Shimadzu). Fluorimetric detection was used (λ_{ex}=470 nm, λ_{em}=530 nm).

3. Results and discussion

Previous attempts to develop a sensitive fluorimetric LC method for the determination of domoic acid (DA, Fig. 1A) in shellfish have not been successful mainly due to interferences from fluorescent artefacts [12]. The primary aim of this study was to develop a robust chromatographic procedure that was sufficiently sensitive for the determination of DA in both marine phytoplankton and shellfish. We selected the reagent, 7-fluoro-4-nitro-2,1,3-benzoxadiazole (NBD-F, Fig. 1B), as a potential candidate because it does not have a strong fluorescent signal but is very reactive with amines under mild conditions and produces highly fluorescent products. NBD-F was previously applied as a derivatising reagent for the LC analysis of amino acids [13] and secondary amines. An example of the latter was the determination of the neurotoxins, anatoxin-a and analogues, in lake water and cyanobacteria [14]. Derivatisation with NBD-F is very straightforward and complete reaction occurred with DA in 3 min at room temperature.

3.1. LC–FL analysis of domoic acid in marine phytoplankton

In the first part of this study, a certified standard domoic acid (DACS-1C, 100 μg DA/ml), was used for method development. The fluorescent derivative with NBD-F (DA-NBD) was well separated from reagent peaks (Fig. 2A) using isocratic reversed-phase LC, eluting with acetonitrile–water (40:60) containing trifluoroacetic acid (0.1% TFA). However, the FMOC derivatisation method for DA required gradient LC to separate the derivatised analyte from matrix and reagent products [12]. The calibration, using standard DA solutions derivatised with NBD-F, was linear for the range 0.04–2.0 μg/ml (r^2=0.998) and the detection limit (3:1 signal to noise) was better than 1 ng/ml.

DA was previously identified in a clone culture of *Pseudonitzschia australis* obtained from a toxic bloom in Monterey Bay, CA [15]. A similar culture, sampled in Ireland, was examined in this study and the DA content was conveniently determined using a simple extraction procedure that involved sonication to disrupt the cells and did not require a clean-up or a concentration step prior to derivatisation. The chromatographic conditions were the same as those used for standard DA solutions (Fig. 2B) and this culture was found to contain 0.30 pg DA/cell.

3.2. LC–FL analysis of domoic acid in shellfish

For shellfish studies using this method, a certified reference mussel material containing DA (MUS-1B) was used for method development. The LC–UV analysis of domoic acid in marine material may sometimes be affected by interferences from other constituents, especially tryptophan, with similar chromatographic properties as domoic acid but such problems were not observed with this fluorimetric procedure. It was even possible to determine DA in a methanol–water extract [6] of this shellfish material without using any cleanup. Fig. 3A shows a typical chromatogram that was obtained for MUS-1B. However, to avoid column problems from repeated injection of crude samples, a solid-phase extraction

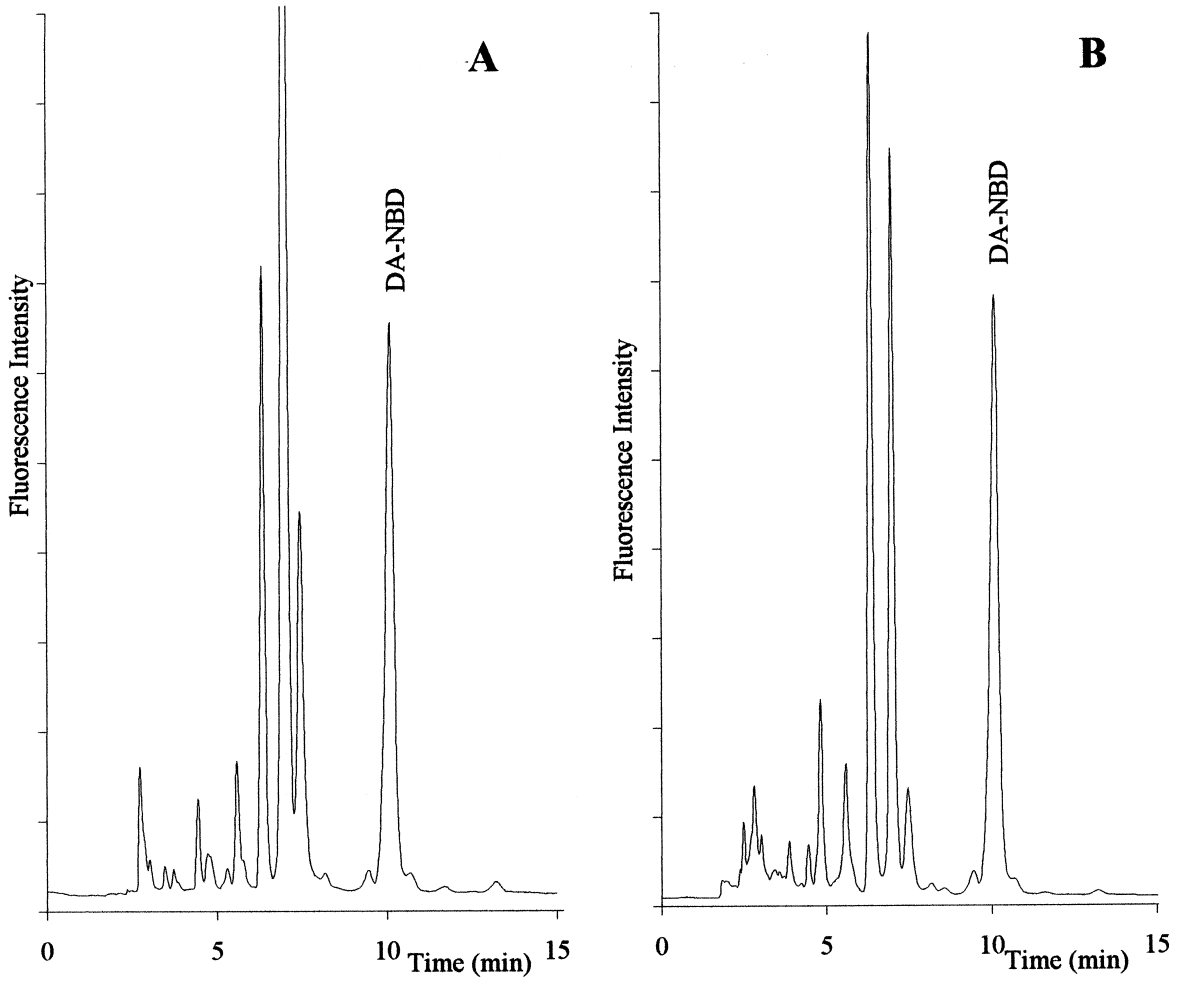

Fig. 2. (A) Chromatogram from the LC–FL analysis of the domoic acid (DA) certified standard, DACS-1C (100 μg/ml) following derivatisation using NBD-F. The standard was diluted to 1 μg/ml and the peak produced is equivalent to 5 ng on-column. The retention time for DA is 10 min Conditions: Luna C_{18} column (250×4.6 mm, 5 μm); mobile phase, acetonitrile–water (40:60) containing 0.1% TFA; temperature, 35°C; flow-rate, 1.0 ml/min; injection volume, 20 μl; fluorimetric detection (λ_{ex}=470 nm, λ_{em}=530 nm). (B) Chromatogram from the HPLC–FL analysis of a *Pseudonitzschia australis* culture, 6.9×10^6 cells/ml, without SPE, following derivatisation with NBD-F. The concentration of domoic acid was 2.05 μg/ml, which is equivalent to 10.2 ng DA on-column. Other conditions are the same as in (A).

(SPE) with a strong anion exchanger (SAX) was used [6,7]. Fig. 3B shows a chromatogram obtained using a mussel sample that did not contain DA, and this showed very little interference from artefact peaks in the region where DA-NBD is expected to elute. Fig. 3C shows a chromatogram from an extract of the MUS-1B standard material which had also been subjected to SPE clean-up. The average recovery of domoic acid from MUS-1B using SPE clean-up prior to derivatisation was 92% (n=5). Calibration curves using mussel tissue, *Mytilus edulis*, containing 1–30 μg DA/g, were linear (r^2= 0.992) with good reproducibility. RSD values ranged from 3% for 8 μg DA/g (n=5) to 5% for 0.04 μg DA/g (n=5).

This analytical method was also applied to a shellfish sample harvested in Spain. Razor clams (*Siliqua patula*), which were extracted and subjected

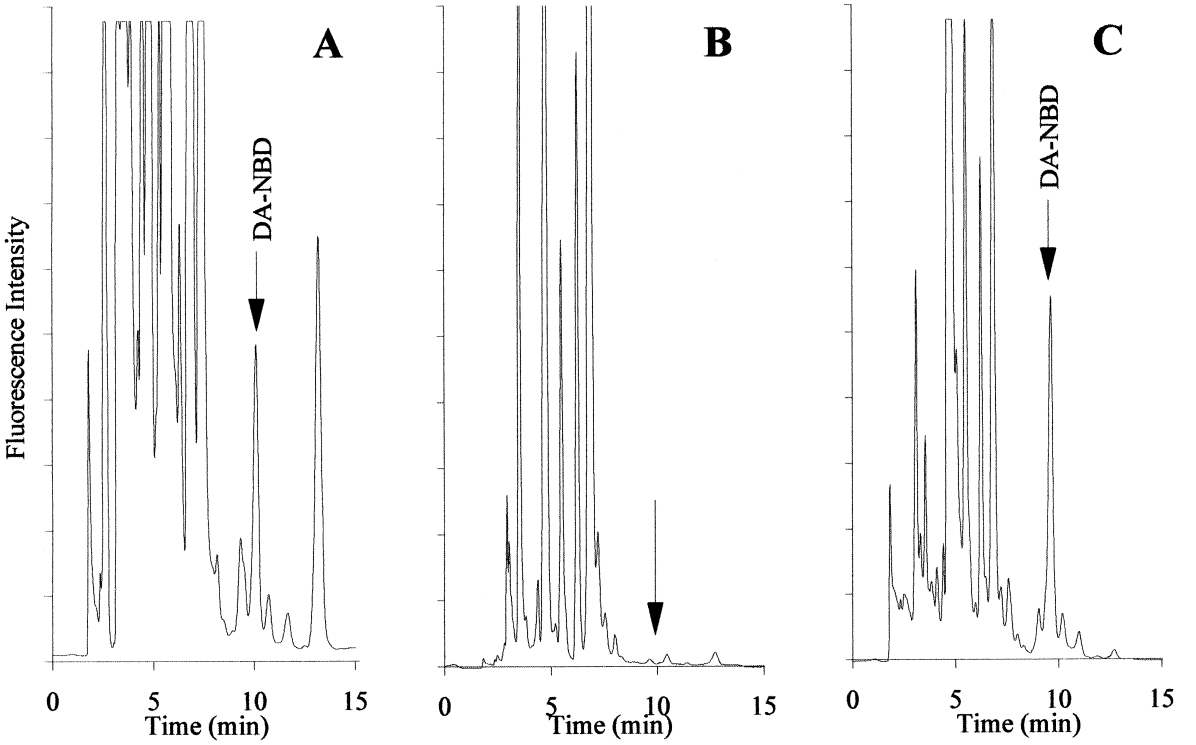

Fig. 3. (A) Chromatogram from the LC–FL analysis of the certified standard mussel material, MUS-1B (37.5±0.8 μg DA/g), without SPE, following derivatisation with NBD-F. The amount of domoic acid is equivalent to 8.1 ng on-column. For other conditions see Fig. 2A. (B) Chromatogram from the LC–FL analysis of a blank mussel sample, with SPE clean-up, following derivatisation with NBD-F. No signal detected at the expected time for DA (10 min). (C) Chromatogram from the LC–FL analysis of MUS-1B, with SPE clean-up, following derivatisation with NBD-F. The amount of DA is equivalent to 7.75 ng on-column. Chromatographic conditions are given in Fig. 2A.

to SPE as described above, produced a chromatogram in which there were more artefact peaks than with mussel samples but the DA-NBD derivative was well resolved (Fig. 4).

4. Conclusion

A fluorimetric LC method has been developed for the determination of DA in a variety of matrices and this should prove useful in environmental toxicological studies since it can be used to analyse both shellfish and marine algae samples. The method is sufficiently sensitive for potential applications in clinical studies of domoic acid intoxication. This is the first successful implementation of a fluorimetric LC method for DA in shellfish. The protocol is simple with a rapid derivatisation step and is readily automated. This highly sensitive method permits the isocratic LC determination of DA in phytoplankton without any clean-up or concentration steps.

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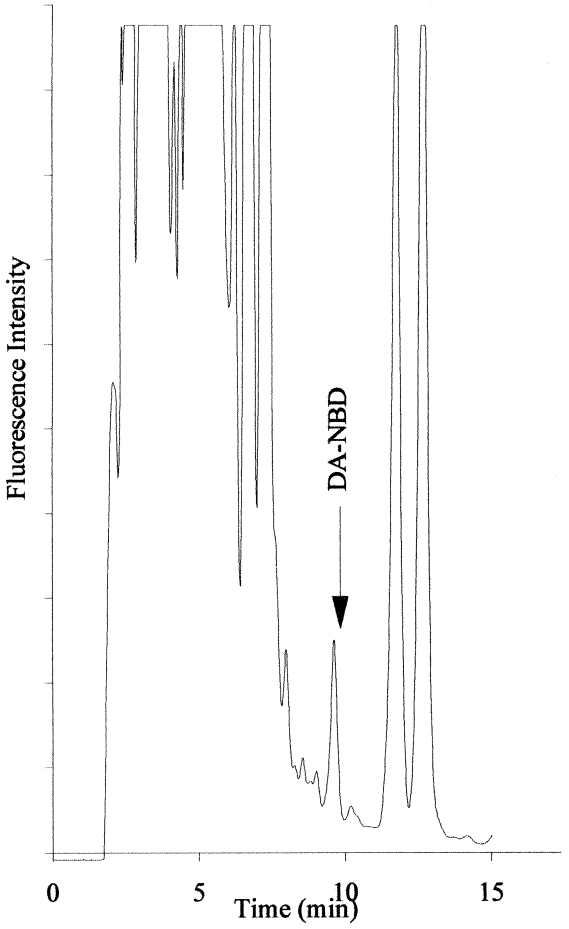

Fig. 4. Chromatogram from the LC–FL analysis of Spanish razor clams (*Siliqua patula*), with SPE treatment, following derivatisation with NBD-F The amount of DA was 3.2 mg/g which is equivalent to 2.6 ng on-column. Chromatographic conditions as in Fig. 2A.

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