Journal of Chromatography B, 876 (2008) 245–251

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Determination of domoic acid in mussels by HPLC with post-column derivatization using 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) and fluorescence detection

Marios Maroulis[b], Ioannis Monemvasios[a], Elisabeth Vardaka[a], Pantelis Rigas[a,*]

[a] *Biotoxins and Analytical Chemistry Laboratory, Department of Fisheries and Aquaculture Technology, Technological Educational Institute of Thessaloniki, N. Moudania, Greece*
[b] *Analytical Chemistry Laboratory, Chemistry Department, Aristotle University of Thessaloniki, 541 24 Thessaloniki, Greece*

ARTICLE INFO

Article history:
Received 25 May 2008
Accepted 30 October 2008
Available online 5 November 2008

Keywords:
Domoic acid
Kainic acid
Shellfish poisoning
Biotoxins
Post-column detection
NBD-Cl
HPLC post-column fluorescence detection

ABSTRACT

A new, sensitive method was developed for the determination of the neurotoxin domoic acid (DA) using a reversed phase separation followed by post-column derivatization (PCD) with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) and subsequent fluorescence detection. The PCD conditions which involves a two-step reaction was fully optimized for the lowest detection limit. The first reaction occurs between DA and NBD-Cl while the second makes possible the detection of the derivative causing the destruction of the interfering fluorescent 4-hydroxy-7-nitrobenzo-2-oxa-1,3-diazole (NBD-OH) which is the hydrolysis product of NBD-Cl. Kainic acid a similar base structure compound with DA was used as an internal standard. The developed post-column method provides the ability for a fully automated analysis, low detection limits (LOD 25 ppb in real samples of mussel extracts), it requires less sample preparation, and it gives clean simple chromatograms without chromatographic interferences from coeluting compounds such as tryptophan. The method was successfully applied to for the quantitative determination of DA in mussel tissues at quantities as low as 75 μg/kg tissue.

1. Introduction

In 1987, 153 people suffered from intoxication after consuming blue mussels (*Mytilus edulis*) from Prince Edward inland and it was shown that the poisoning was caused by the neurotoxin, domic acid (DA) [1,2]. Since then DA has been detected in razor clams and crabs [3] and is being monitored in shellfish, mainly mussels, clams and oysters [4–6]. DA, which is produced by algae Pseudo-nitzschia [7,8] is ingested by Phytoplankton Shellfish and accumulates in their edible tissue. Consuming contaminated shellfish causes Amnesic shellfish poisoning to mammals, which in turn can cause permanent or short term memory loss as well as other neurological symptoms, such as severe headaches, loss of balance like nausea, vision disturbances, disorientation, etc. [9,10]. DA accumulates in small fish that feed on the algae.

In the European Union as well as Canada and the USA the legal limit for DA is 20 μg DA/g in edible tissues. High performance liquid chromatography (HPLC) with photodiode array detection (LC-UV) is the most popular method used for the determination of DA [11]. Bioassays can also be used for higher concentrations of DA but are lacking in sensitivity and selectivity with up to 20% variation in results.

One of the most common LC methods for the determination of DA in shellfish tissue is the one developed by Quilliam et al. in 1989 [1] using a reversed phase column, under isocratic elution, and an optical absorbance detection at 242 nm. For the trace determination of DA in seawater and phytoplankton (where DA levels are lower than the shellfish) the method developed by Pocklington et al. in 1991 with pre-column derivatization with fluorenylmethoxycarbonyl chloride (FMOC-Cl) and fluorescence detection following a gradient reversed phase separation, is often used [12]. This method can achieve a very low detection limit (15 ppb) but cannot be used for the analysis of DA in shellfish tissues due to interferences from compounds present in the shellfish matrix. One such strong interferent is the, tryptophan which elutes close to DA. A procedure using precolumn derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) was developed and applied to the determination of DA in phytoplankton at 1 ppb [13]. A precolumn derivatization method based on a 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F) was shown to be suitable for the determination of DA

* Corresponding author. Tel.: +30 2373026457; fax: +30 2373026450.
E-mail address: rigas@aqua.teithe.gr (P. Rigas).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.10.053

[14]. Unfortunately, matrix co-eluted compounds are present in the chromatograms that might interfere with DA peak and the NBD-F is commercially available at very high cost and consequently limits its use in post-column applications. LC coupled to mass spectrometry (MS) detection has also been used for the determination of DA [15]. Although the LC-MS method is sensitive and selective, the overall methodology is complex and costly.

4-Fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F) and 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) were reported previously to be useful post-column reagents for the fluorometric detection of amino and imino acids after their separation by ion-exchange chromatography [16–18]. This work is focused on the development of a procedure for the LC reversed phase separation of DA followed by post-column derivatization (PCD) using NBD-Cl with subsequent fluorescent detection. PCD methods typically require less sample preparation and clean–up than precolumn derivatization methods, have less interferences from the reagents employed, and the results are overall more reproducible. Furthermore, NBD-Cl is commercially available at modest cost compared to that of the NBD-F. All parameters affecting the reversed phase separation of DA and those that affect the post-column derivatization of DA with NBD-Cl were identified and optimized in order to obtain the most favorable detection limits for the DA. When applied to mussel matrix samples and using kainic acid as an internal standard it was possible to determine DA at a detection limit of 25 ppb.

2. Experimental

2.1. Reagents

Domoic acid (DA) a certified calibration solution (CRM-DA-e) at a concentration of 99.4 μg DA/L in 1:9 acetonitrile:water solution and CRM-ASP-Mus-b certified reference shellfish material (36 μg DA/g) were obtained from National Research Council, Nova Scotia, Halifax, Canada. Water was produced by a SG Water Ultra Clear Water purification system. HPLC grade acetonitrile and methanol and kainic acid (>99%) were obtained from Sigma–Aldrich, St. Louis, MO, USA Ethyl acetate p.a. was purchased from Fluka, St. Louis, MO, USA. HCl (37% solution) and trifluoroacetic acid PB were obtained from Panreac, Barcelona, Spain, while 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) was purchased from Fluka, St. Louis, MO, USA. All reagents were used as received.

2.2. Equipment

The LC system (Shimadzu Corporation, Kyoto, Japan) is composed of a Model LC-10ADvp solvent delivery module with FCV-10AL quaternary valve and DGU-14A degasser, a model SIL-10ADvp autosampler, a CTO-10ACvp column oven, a SCL-10Avp controller, a SPD-M10A VP Diode array detector, and a RF-10Axl fluorescence detector. Class-VP chromatography software was used for data collection/handling. The LC column was a Nucleosil C18, 250 mm × 4.6 mm, 5 μm, 100 Å column obtained by Macherey Nagel, Dueren, Germany. The post-column derivatization system (PCD) used was the model Prometheus PCD 300 Plus manufactured by Rigas Labs, Thessaloniki, Greece and included two heated reaction coils, as well as two reagent delivery Marathon I pumps. For sample preparation an IKA (Staufen, Germany) Ultra Turrax T25 blender, an Eppendorf (Hamburg, Germany) 5810R Centrifuge, and an Alltech Vacuum Manifold equipped with Alltech (Deerfield, IL, USA) solid phase extraction cartridges (500 mg, 3 mL SAX) and 0.45 μm nylon membranes were used.

2.3. Sample preparation

The procedure for handling mussel tissue was based on that published by Quilliam et al. in 1995 [19,20] and was followed with minor modifications. DA was extracted from the mussel tissue by homogenization with methanol-water (1:1, v/v). Approximately 50 g of mussel tissue was homogenized with the blender. A portion of this homogenized tissue was weighed directly on a precision balance. A 4.0-g homogenated tissue was extracted with 15.0 mL extraction solvent (1:1 methanol–water) and homogenized again for 10 min at 6500 rpm. The centrifuged sample was then filtered through a 0.45-μm membrane and was purified further using the following SPE procedure.

2.3.1. Cartridge conditioning

Initially 6 mL of methanol passed through the cartridge, followed by 3 mL water, and finally 3 mL extraction solvent (1:1 methanol–water) through the SAX cartridges.

2.3.2. Cartridge cleanup/elution

A 5.0-mL fraction of the filtered crude extract was loaded onto the cartridge in a flowrate of about one drop per second. The flow was stopped when the fluid's meniscus reached the top of the cartridge packing. Then the effluent was discarded. The cartridge was washed at about one drop per second with 5 mL wash solution (1:9 acetonitrile–water). Again the flow was stopped when the fluid's meniscus reached the top of the cartridge packing. Then the effluent was discarded and 0.5 mL of formic buffer eluent (0.5 M, pH 3.2 ± 0.2, adjusted with sodium hydroxide) were added and allowed to flow until the fluid's meniscus reached the top of the cartridge packing. Then the effluent was discarded again. A 2-mL volumetric tube was placed under the cartridge and 2 mL formic buffer were loaded onto the cartridge and allowed to flow at a rate of one drop per second. Exactly 2 mL of the final eluted DA extract were collected and then stored for up to 1 day.

An alternative method without SPE treatment is based using a 4.0-g homogenated tissue, extracted with 15.0 mL solvent (5:1 methanol–water) and homogenized again for 10 min at 6500 rpm. The centrifuged sample was preconcentrated under nitrogen to a final volume of 2 mL. This solution was then filtered through a 0.45-μm membrane and then injected to HPLC without any further clean-up steps.

2.4. Liquid chromatography – post-column derivatization

2.4.1. LC separation of DA

The mobile phase conditions and reversed phase column for the separation of DA were 0.1% trifluoroacetic acid (TFA) in 87:13 water:acetonitrile with a flow rate of 0.7 mL/min and Nucleosil C18 column, respectively. The composition of TFA and the percent acetonitrile in the eluent are the parameters affecting mostly the separation of the analytes (DA and Kainic acid (IS) a compound with similar base structure with DA) that for this reason they were fully optimized in order to obtain the best separation from the sample. Detection of the analytes was achieved with a fluorescence detector using a post-column derivatization reaction with two reagents. The detector was set at 469 nm λ_{ex} and 529 nm λ_{em} (the λ_{ex} and λ_{em} are the optimum excitation and maximum emission wavelengths for the derivative between the NBD-Cl and DA) with gain at 4×. Data were collected and processed with the Shimadzu Class-VP chromatography software.

2.4.2. Post-column derivatization conditions

The PCD system reagent 1 was optimized to be 9.0 mM NBD-Cl in methanol with the borate buffer adjusted to pH 10.0. The output