Toxicon 51 (2008) 1264–1268

Contents lists available at ScienceDirect

Toxicon

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

Effective determination method for a cyanobacterial neurotoxin, β-*N*-methylamino-L-alanine ☆

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ARTICLE INFO

Article history:
Received 12 October 2007
Received in revised form
10 January 2008
Accepted 20 February 2008
Available online 29 February 2008

Keywords:
Cyanobacteria
Neurotoxin
BMAA
LC–ESI-MS
HILIC
SIM

ABSTRACT

We developed a simple and effective analysis procedure that includes pretreatment and determination methods for β-*N*-methylamino-L-alanine (BMAA), a cyanobacterial neurotoxin. BMAA may be produced by all known groups of cyanobacteria living in freshwater as well as marine environments. In this paper, we report a novel determination method for BMAA. A cation-exchange resin was effective for the selective concentration of BMAA from cyanobacterial extracts and yielded a high recovery rate. Moreover, liquid chromatography (LC)–electrospray ionization mass spectrometry with a hydrophilic LC column was effective for determining BMAA levels. The quantitation limit for BMAA based on selected ion monitoring (SIM) was determined as 0.5 ng at a signal/noise ratio of 5.

1. Introduction

A number of toxic compounds have been detected in the cyanobacteria present in freshwater lakes and reservoirs as well as marine environments worldwide (Codd et al., 2001; Elder et al., 1993; Hunter, 1992; Watanabe et al., 1992). Microcystin is the best-known hepatotoxic cyclic peptide produced by *Microcystis*, *Anabaena*, *Planktothtix*, *Hapalosiphon* and *Nostoc*. Correspondingly, other hepatotoxins and neurotoxins, such as cylindrospermopsins, noduralins, anatoxin-a, anatoxin-a(s) and saxitoxin have also been identified from the cyanobacteria present in fresh or marine waters.

β-*N*-Methylamino-L-alanine (BMAA) (Cruz-Aguado et al., 2006; Lobner et al., 2007; Rao et al., 2006; Santiago et al., 2006) is one of the neurotoxins produced by cyanobacteria and is a possible cause of amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS/PDC). In particular, the Chamorro people of Guam exhibit a higher incidence rate of ALS than that observed anywhere else in the world (Spencer et al., 1987). In some previous reports, the authors suggested that BMAA was concentrated by the food chain (Banack et al., 2003; Cox and Sacks, 2002; Cox et al., 2003; Murch et al., 2004). The high incidence rate of ALS among the Chamorro people is attributable to the presence of a root symbiont of the genus *Nostoc* in cycad seeds. Additionally, Cox et al. have detected BMAA in the brain tissues of Canadian Alzheimer's patients. This is very interesting because cycads are not part of the Canadian flora. Therefore, the production of BMAA by several cyanobacterial sources was investigated and confirmed (Cox et al., 2005). These results are enormously important and demonstrate the possibility of the presence of BMAA in all types of cyanobacteria worldwide. Consequentially, we need to establish a simple and effective method for determining the presence of BMAA from the viewpoint of environmental risk management.

☆ *Ethical statement*: (1) This material has not been published in whole or in part elsewhere; (2) This paper is not currently being considered for publication elsewhere; (3) All authors have been personally and actively involved in substantive work leading to the paper, and will hold themselves jointly and individually responsible for its content.

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0041-0101/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2008.02.015

Some methods have been reported for the analysis of BMAA (Banack and Cox, 2003; Cox et al., 2005; Guo et al., 2007; Pan et al., 1997). The separation procedure involves liquid chromatography (LC) using C_{18} columns, which are usually utilized without pretreatment, including selective concentration. Moreover, the presence of BMAA is determined by fluorescence detection after derivatization or mass detection. As expected, we cannot obtain accurate results by using fluorescence detection after derivatization because several derivatizations include contaminants, such as other amino acids present in environmental samples or other cyanobacterial extracts (Cox et al., 2005). Additionally, we suggest that hydrophilic LC (HILIC) is more effective than reverse-phase LC, such as those using C_{18} columns, because of the higher ionization efficiency due to the high content of organic solvents.

In this study, we report a novel procedure for the determination of BMAA, including a simple pretreatment using a cation-exchange resin as well as effective detection using LC–electrospray ionization (ESI) mass spectrometry (MS) (LC–ESI-MS) with an HILIC column.

2. Experimental

2.1. Chemicals

Standard BMAA was purchased from Sigma Aldrich (USA). $(NH_4)_2CO_3$, HCl, methanol (MeOH) and trifluoroacetic acid (TFA) were purchased from Wako Chemicals (Osaka, Japan). Moreover, acetonitrile (MeCN) and pure water of LC–MS grade were purchased from Wako Chemicals.

2.2. LC– ESI-MS analysis

LC–MS analyses of BMAA were performed using a system that comprised a LC (Shimadzu LC10Avp; Shimadzu Corporation, Kyoto, Japan) coupled to a MS (Shimadzu LCMS 2010A; Shimadzu Corporation, Kyoto, Japan) with an ESI interface. The electrospray interface was operated using the following settings: polarity, positive and nebulizer, N_2 (1.5 L min^{-1}; drying gas, N_2 at 0.1 MPa). Full-scan MS was performed from m/z 50 to 150 at 0.5 s scan^{-1} in the continuum mode. Selected ion monitoring (SIM) was performed at m/z 119.1 $(M+H)^+$. LC conditions were as follows: column, TSK-gel Amide-80 (150 mm × 2.0 mm i.d.; Tosoh Co. Japan) (Dell'Aversano et al., 2004); mobile phase, 90–60% aqueous linear gradient of MeCN for 20 min, then 60% aqueous MeCN maintained for 10 min; flow rate, 0.2 mL min^{-1}; and column temperature, 40 °C.

To obtain an analytical curve and to determine the quantitation limit for BMAA, standard BMAA solutions (0.1 μg mL^{-1} to 0.1 mg mL^{-1}), which were prepared using MeCN/water (v/v, 1/1) as the solvent, were evaluated by LC–MS.

2.3. Concentration of BMAA

To confirm the selective concentration of BMAA, solid-phase extraction (SPE) of the standard BMAA solutions was performed using a C_{18} cartridge (Sep-pak C18), hydrophobic polymer cartridge (Oasis HLB) and cation-exchange resin (Oasis MCX) purchased from Waters. After conditioning each cartridge, the sample solution (50 mL of 0.1 μg mL^{-1} aqueous BMAA) was passed-through the cartridges, and each cartridge was washed with water. Then, the adsorbate was eluted with organic solvent (MeOH) or aqueous ionic solution. The passed-through, washed, and collected fractions were evaluated by LC–ESI-MS.

2.4. Analysis of cyanobacterial extracts

A strain of *Microcystis aeruginosa* (N-88) and one of *Cylindrospermopsis raciborskii* (CRJ-1 = AWT205) were obtained from Microbial Culture Collection (MCC-NIES, Japan). The lyophilized cells (50 mg) were extracted with 0.1 M HCl (50 mL) using ultrasonication for 10 min. After centrifugation (3500 rpm for 20 min), the supernatant was collected. Then, an authentic standard BMAA solution was added to the extracts at 50 ng mL^{-1}. The BMAA-spiked sample and non-spiked sample (20 mL) were passed-through the cation-exchange cartridge, and the cartridge was washed with MeOH (1.0 mL). Finally, the adsorbate was eluted with an aqueous solution of 1.0 M $(NH_4)_2CO_3$ (2.0 mL). After evaporation of the collected fraction, the residue was re-dispersed into MeCN/water (v/v, 1/1) (1.0 mL). The concentration of BMAA was determined by LC–MS for all the fractions, i.e., the non-treated sample and the passed-through, washed (MeOH) and collected ($(NH_4)_2CO_3$) fractions. Furthermore, the cell extracts that did not contain additional BMAA were also evaluated using the same procedures to confirm the original amount of BMAA in cells.

3. Results and discussion

C_{18} columns are generally used in BMAA analyses. However, the direct detection of BMAA without additional derivatization, such as fluorescence labeling, is much more difficult when C_{18} columns are used. Furthermore, characterizations such as the content of octadecyl groups and remaining silanol groups cannot be performed using the commercially available C_{18} columns. In fact, we tested the efficiency of some C_{18} columns for BMAA separation and found that BMAA could either not be retained at all or was retained to a slight extent on the columns as broad peaks. These results depended on the octadecyl content and the remaining silanol groups. Therefore, we suggest LC-ESI MS with an HILIC column as a novel method for the analysis of BMAA. We selected TSK-gel Amide-80 (Tosoh, Japan) as the HILIC column because other HILIC columns had a lower separation efficiency for BMAA. The SIM chromatogram obtained at m/z +119,1 and the peak at 13.4 min on the MS spectra are shown in Fig. 1. Thus, effective separation was achieved using authentic