Detection of the Neurotoxic Amino Acid β-N-methylamino-L-alanine in Axenic Cultures of Cyanobacterial Isolates by the Application of Underivatized Method of Analysis

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Abstract

There is a growing demand to establish reliable method for the detection of cyanobacterial toxins in order to be well informed on the prevalence of cyanotoxins in our environments. This study employed the underivatized method of analysis to detect the production of BMAA by the axenic cultures of cyanobacterial strains *Synechocystis* NPLB 2 and *Nostoc* MAC PCC 8009 using liquid chromatography-electrospray ionization - ion trap mass spectrometry (LC/ESI-ITMS). Cyanobacterial cultures were grown in the laboratory for 12 weeks after which BMAA was extracted using methanol and analysed by LC/ESI-ITMS. Based on retention time, mass-to-charge ratio, and ratio of the product ions, the underivatized method of analysis employed in this study showed that both *Synechocystis* NPLB 2 and *Nostoc* MAC PCC 8009 strains produced BMAA. The results of this study suggests that the LC/ESI-ITMS is a promising method for the analysis of BMAA in cyanobacterial matrices.

Keywords: BMAA; Cyanobacteria; ESI-ITMS; Nostoc; Synechocystis; Underivatized

1. Introduction

The prevalence of harmful algal blooms in our environment could potentially increase the risk of human exposure to cyanobacterial toxins [1]. β-N-methylamino-L-alanine (BMAA) is a non-protein amino acid that is linked to the ontology of neurodegenerative diseases [2,3]. *Nostoc* sp. was the first cyanobacteria reported to produce BMAA [4], and this has led to more studies on cyanobacterial production of BMAA. It has now become clear that all known cyanobacterial groups could produce BMAA [5]. There is a growing demand to establish reliable methods for the detection of cyanobacterial toxins in order to be well informed on the prevalence of cyanotoxins in our environments.

BMAA can exist in variety of different forms and this caused analytical challenges such as false negatives during analysis [6]. Therefore, there is need for a proper and appropriate sample preparation and instrumentation to reliably detect BMAA in complex sample matrices. Most methods of BMAA detection employed techniques that involves derivatization using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) as derivatizing agent prior to detection analysis. However, some studies have reported that AQC methods can cause false positive results due to inefficiency in the specificity of method and technical expertise for the derivatization [7-9]. There are very limited literature reports on the application of LC/ESI-ITMS method of analysis to analyse the production of BMAA in cyanobacteria by the application of underivatized method of analysis. The aim of the present study is to employ the underivatized method of analysis to assess the production of BMAA in the axenic cultures of cyanobacterial strains *Synechocystis*, NPLB 2 and *Nostoc* MAC PCC 8009 by liquid chromatography-electrospray ionization ion trap mass spectrometry (LC/ESI-ITMS) method.

2. Materials and Methods

2.1. Materials

BMAA standard was obtained from Sigma-Aldrich, formic acid from Sigma-Aldrich, methanol from BDH Analar, hydrochloric acid (HCl) from Fisher Scientific, ammonium hydroxide from Acros Oganic.

2.2. Methods

2.2.1. Sample Preparation

The cyanobacterial strains, *Synechocystis* NPLB 2 and *Nostoc* MAC PCC 8009 were generously provided by Nigel J. Robinson (Institute of Cell & Molecular Biosciences, Medical School, Newcastle University, UK). Cultures were grown individually in sterilized BG11 media (Sigma BG11, fresh water) under 12-hour light/12-hour darkness in a shaking incubator at 28 °C over twelve (12) weeks period after which the cells were harvested by centrifugation.

2.2.2. Amino Acids Extraction

Amino acid extraction was carried out based on the previous protocol [10] with some few alterations. Cells were harvested by centrifugation and the cell pellets were dissolved in 500 µl of 80% methanol (v/v) and sonicated (Soniprep 150, MSE) under four cycles of 30 seconds at 70% intensity.

2.2.3. Solid Phase Extraction (SPE)

The solid phase extraction column (Thermo Scientific HyperSep Verify™-CX, 130 mg) was pre-equilibrated with 1 ml methanol followed by 1 ml water. Thereafter, the column was conditioned with 0.1 % formic acid in water (1mL). An aliquot of 200 µl sample was mixed with 800 µl formic acid (0.1 %) and loaded on to SPE column. The column was washed with 1 ml formic acid in water (0.1 %). The elution was conducted, first with

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1 ml of 0.1 % formic acid in 25 % methanol, followed by the second step elution with 2 % ammonium hydroxide in 100 % methanol. The eluted samples were evaporated completely for at least 4 hours in a vacuum centrifuge at 50°C and subjected to HPLC-ESI-ITMS analysis.

2.2.4. Analytical HPLC-ESI-ITMS
Analytical HPLC was conducted with a Surveyor HPLC system (ThermoFinnigan Hemel Hempstead, UK) fitted with a Phenomenex (Macclesfield, UK) Gemini C18 3µm HPLC column (150 mm 2.0 mm I.D) and a security guard column of the same material. Partial separation was achieved at 300 µl/min flow rate and 30 ºC column oven temperature. Two different solvents gradients (solvent A: 0.1% formic acid in 93:7 water/acetonitrile) and (solvent B: 0.1% formic acid in acetonitrile) were used. 10 µg/ml in 50/50 acetonitrile/water and 15 µl for BMAA standard and samples respectively, were injected individually. LC-ESI-ITMS was performed with ThermoFinnigan LCQ ion trap mass spectrometer equipped with an ESI source operated in positive ion mode. The capillary temperature was set at 280 ºC, spray voltage at 4.0, sheath gas flow at 30 and auxiliary gas at 2 (arbitrary units). The instrument was tuned using solutions containing both BMAA with m/z 119 as the target ion. Detection was achieved at an isolation width of m/z 3.0 and fragmentation with normalised collisional dissociation energy of 25%. The activation Q value (parameter determining the m/z range of the observed fragment ions) was set at 0.25 producing fragment ions in the range m/z 50-150. LC-ITMS was carried out in MS/MS scan mode with 1 scan event only: MS² of m/z 119.

3. Results and Discussions
3.1. Results
BMAA was determined using the three documented protocols of retention time, mass-to-charge ratio, and ratio of the product ions [11,12]. BMAA was first identified by comparing the retention time of the samples with that of the standard acquired under the same conditions. The chromatograms obtained from the LC separation showed that BMAA standard exhibits a retention time of 1.13 min, while Synechocystis NPLB 2 and Nostoc MAC PCC 8009 samples exhibit retention times of 1.42 min and 1.34 min, respectively (Figure 3.1). The mass-to-charge ratio of the acquired spectra for the underivatized cyanobacterial samples were compared with that of the BMAA standard in order to identify the presence of the analyte. The m/z of the product ions for the BMAA standard are 73.0, 76.0, 88.0, 101.0, 102.0, and 119.0 (Figure 3.2). Synechocystis NPLB 2 exhibits the characteristic product ions of 73.0, 74.1, 88.0, 100.8, 102.0 and 118.7 m/z (Figure 3.2). Likewise, Nostoc MAC PCC 8009 exhibits the product ion fragments of 73.0, 74.2, 76.0, 88.3, 101.0, 102.0 and 118.5 m/z (Figure 3.2).

The product ion ratio, which involves the comparison of the ratio of certain product ions to others in each of the analytes is one criterion that is commonly used for detection of BMMA. The BMAA standard exhibit 100% relative abundance of 101.0 m/z and high intensity of 102.0 m/z. Synechocystis NPLB 2 exhibit high abundance of 102.0 than 100.8 m/z while Nostoc MAC PCC 8009 exhibit high abundance of 102.0 m/z than 101 m/z. As reported previously, the fragment ions specific for BMAA are 88 m/z and 76 m/z (Rosen and Hellenas, 2008; Faassen et al, 2009; Kruger et al, 2010). Both cyanobacterial samples exhibit these specific fragment ions, although fragment ion 76 m/z was not seen in the spectra of Synechocystis NPLB 2.
Figure 3.1. LC-ESI-ITMS chromatograms of BMAA standard (10 µg/ml), Synechocystis NPLB 2 (15 µl) and Nostoc MAC PCC 8009 samples (15 µl). ESI full MS² scan of the precursor ion 119 m/z (black). ESI full MS² scan of the product ion 100.5-101.5 m/z (red). ESI full MS² scan of the product ion 101.5-102.5 m/z (green). The retention time for BMAA standard is 1.13 min (indicated by arrow), while the retention time for BMAA in Synechocystis NPLB 2 and Nostoc MAC PCC 8009 samples are 1.42 min and 1.34 min respectively.

Figure 3.2. LC-ESI-ITMS spectra of BMAA standard, Synechocystis NPLB2 and Nostoc MAC PCC 8009 samples. Specific product ion fragments are indicated by arrows.
3.2 Discussions
This study has detected BMAA in cyanobacterial samples by the application of LC-ESI-ITMS detection of underivatized analyte. Several methods of detection of BMAA in cyanobacteria have been developed based on derivatization of the amino acid [5, 10, 12-22]. These methods of derivatization have reported the cyanobacterial production of BMAA. On the other hand, developed methods of detecting underivatized cyanobacterial BMAA failed to detect the presence of BMAA in cyanobacterial matrix [11, 23-26]. However, Faassen and colleagues reported that 9 out of the 21 cyanobacterial isolates under study, produced BMAA by the application of detection method of underivatized BMAA [27]. More interestingly, a recent study showed that BMAA is produced by Synechocystis sp. FACHB-898 and Nostoc sp. FACHB-632; this study also used the detection method of underivatized BMAA [28]. Despite the fact that the mechanism of cyanobacterial production of BMAA is not well established, and the conditions under which BMAA production is favoured in cyanobacteria are not properly documented, some environmental factors may play a role in cyanobacterial BMAA production. The presence of BMAA in wild and laboratory cultures of cyanobacteria have been reported. Cox and colleagues reported variation of BMAA content among the same species of cyanobacteria during growth phase [5]. Similarly, other researchers in their study, found differences in BMAA content even within the same species of cyanobacteria either sampled at different period or from different location [27]. In addition, Jonasson’s group reported a seasonal variation in BMAA content produced by cyanobacteria samples from the Baltic Sea [19]. With these in mind, it can be seen why BMAA is detected in some cyanobacteria and not detected in some.

This study has detected BMAA in cyanobacterial matrix by the application of underivatized method of detection using LC-ESI-ITMS methodology. Li and colleagues [25] studied the production of BMAA by cyanobacteria using ESI-ITMS methodology by the application of underivatized method of detection but the underivatized BMAA was not detected from the cyanobacterial samples. Results of the present study showed that underivatized BMAA of cyanobacterial samples can be detected by LC-ESI-ITMS analytical method but the limitation of this method is the poor LC separation which hindered quantification of the detected analyte of interest. This method requires further validation in order to make it more reliable method of detecting underivatized analytes.

4. Conclusions
BMAA a non-protein neurotoxic amino acid was detected in both Synechocystis NPLB 2 and Nostoc MAC PCC 8009 strains of cyanobacteria. Anthropogenic factors and climate change are anticipated to increase the rate of proliferation of harmful algal blooms which could increase the risk of human exposure to cyanobacterial toxins such as the β-N-methylamino-L-alanine. Regulating the prevalence of these emerging toxins is the way forward towards making an appropriate decision on the risks implicated with their presence.

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References


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