ELSEVIER

Journal of Chromatography A, 862 (1999) 191–197

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Analysis of anatoxin-a in freshwaters by automated on-line derivatization–liquid chromatography–electrospray mass spectrometry

Masahiko Takino*, Shigeki Daishima, Kenji Yamaguchi

Yokogawa Analytical Systems Inc., 3-3-11 Kinryo Bld. Niitaka, Yodogawa-ku, Osaka 532-0033, Japan

Received 11 June 1999; received in revised form 1 September 1999; accepted 1 September 1999

Abstract

Anatoxin-a is a toxin produced from cyanobacterial blooms in freshwaters. In order to determine trace anatoxin-a in freshwaters, an automated on-line derivatization procedure with fluorenyl methylchloroformate using liquid chromatography–electrospray ionization mass spectrometry was developed. Anatoxin-a was extracted using solid-phase extraction with adequate recovery (75.7±7.2%, $n=6$) at 20 ng/l. The limits of quantification and detection were calculated to be 15.2 ng/l and 2.1 ng/l, respectively, using selected ion monitoring.

Keywords: Water analysis; Anatoxins; Toxins

1. Introduction

The frequency of cyanobacterial (blue–green algal) blooms in freshwaters has dramatically increased throughout the world. It is estimated that 50% of cyanobacterial blooms are toxic, producing both hepatotoxins and neurotoxins. These toxic effects have caused many animal deaths and have also been implicated in cases of human illness [1]. The toxins involved are microcystins, nodularin, saxitoxin and anatoxins, and they are produced by *Anabaena*, *Microcystis*, *Oscillatoria*, *Aphanizomenoa*, *Nodularia* and *Cylindrospermopsis*, respectively [2]. Mycrocystins and nodularin are potent inhibitors of protein phosphatases PP1 and PP2A and they also have tumor-promoting properties [3].

Anatoxin-a was the first cyanobacterial toxin to be structurally elucidated and it is an alkaloid, 2-acetyl-9-azabicyclo[4,2,1]non-2-ene [4]. Anatoxin-a has a high toxicity (LD_{50} i.p. mouse 200 μg/kg), and it is a potent nicotinic agonist which acts as a post-synaptic, depolarizing, neuromuscular blocking agent. Typical symptoms in animals include muscle fasciculation, gasping and convulsion, with death due to respiratory arrest within minutes of drinking contaminated water. A number of chromatographic methods are available for the analysis of anatoxin-a in cyanobacterial bloom material and these include high-performance liquid chromatography (HPLC) [5,6] and gas chromatography–mass spectrometry (GC–MS) [7,8]. But HPLC methods have a lack of spectrometric information and GC–MS methods are time consuming because they need off-line derivatization prior to analysis. On the other hand, liquid chromatography–mass spectrometry (LC–MS) would offer significant advantages. In fact LC–MS

*Corresponding author. Fax: +81-6-6399-3716.

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00943-7

methods with thermospray ionization [9], and electrospray ionization (ESI) [10] have been used. ESI is suitable for the amines but it is difficult to analyze anatoxin-a by reversed-phase HPLC because of high water solubility. Ion-pairing chromatography is one of most commonly used techniques for the separation of both polar and ionic compounds. But in LC–ESI-MS, the utilization of ion-pairing reagents suppresses ion intensity. Another approach to analyze these compounds is on-line pre-column derivatization. In this study, LC–ESI-MS using a volatile ion-pair reagent and LC–ESI-MS with on-line pre-column derivatization were investigated to develop a high-sensitivity method for anatoxin-a in freshwater. Pentafluoropropionic acid (PFPA) was selected as the volatile ion-pairing reagent. 9-Fluorenyl methylchloroformate (Fmoc) was selected for pre-column derivatization because of its high and rapid reactivity with anatoxin-a which permits automated on-line derivatization [11].

2. Experimental

2.1. Instrumentation

The LC–MS system consisted of an LC pump, an autosampler, a thermostated column compartment, a Model 1100 UV detector, and an MSD bench top mass spectrometer (Hewlett-Packard, Palo Alto, CA, USA) with ESI interface.

LC was performed on a 150×2.1 mm I.D. column packed with 5 μm Inertsil ODS3 (GL Science, Tokyo, Japan).

2.2. Chemicals

Anatoxin-a hydrochloride was purchased from Sigma–Aldrich Japan (Tokyo,Japan). HPLC-grade methanol, acetonitrile, ammonium acetate and ammonium hydroxide were purchased from Wako (Osaka, Japan). Trifluoroacetic acid (TFA), PFPA, nonafluoropentanoic acid (NFPA) and tridecafluoroheptanoic acid (TDFHA) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Fmoc and borate buffer were purchased from Hewlett-Packard. Anatoxin-a standard solutions were prepared in HPLC-grade methanol and stored in the dark at 2°C.

2.3. Extraction of anatoxin-a from freshwater

A filtered water sample (200 ml) was adjusted to pH 10 with ammonium hydroxide before disk type solid-phase extraction (SPE) using a reversed-phase polymer, SDB-XD, 47 mm Empore disk (3M, USA). The SPE disk was conditioned with methanol (10 ml) and water (20 ml). The sample was transferred to the disk and washed with methanol–water (1:4, 10 ml) and dried under vacuum. The anatoxin-a was eluted using methanol containing 0.2% formic acid (10 ml) and the solvent was evaporated at 50°C under nitrogen. The samples were reconstituted in 1 ml of methanol–water (1:9, v/v).

2.4. LC–ESI-MS determination of anatoxin-a

MS was used with ESI. For optimization of ion source parameters, a calibration standard (Hewlett-Packard) was introduced with an automated delivery system. The optimization of drying gas and nebulizer gas was done to introduce anatoxin-a standard solution at 0.2 ml/min. The instrument was used in the positive ion mode using the following operating conditions: drying gas, 10 l/min at 350°C; nebulizer gas, 50 p.s.i.; capillary voltage, 4000 V; fragmentor voltage, 100 V; multiplier gain, 3 (1 p.s.i.=6894.76 Pa).

Full scan acquisitions were made over a mass range of 100–600 u. Selective ion monitoring (SIM) was performed at m/z 388 ($[M+H]^+$ of anatoxin-a-Fmoc) and 166 ($[M+H]^+$ of anatoxin-a), the dwell time was 0.5 s.

2.4.1. LC–ESI-MS with ion-pairing reagent

In the case of the analysis using the ion-pairing reagent, isocratic elution was performed with solvent A (acetonitrile–water, 15:85, v/v) containing 0.1% PFPA for 7 min and then the eluent was switched to 100% acetonitrile in 5 min. The column was conditioned with solvent A for 13 min before injection of the next sample.

2.4.1.1. Mass spectrometric data

The mass spectrum of anatoxin-a was obtained by direct flow injection without analytical column. The base peak in the mass spectrum of anatoxin-a was the protonated molecular ion at m/z 166 $[M+H]^+$

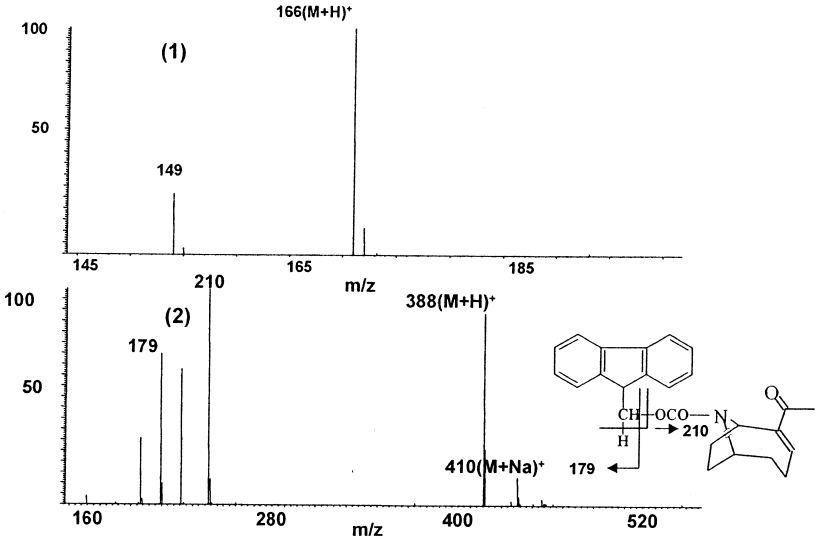

Fig. 1. Full scan mass spectra of anatoxin-a (1) and *N*-9-anatoxin-a-Fmoc (2).

and a characteristic fragment ion (*m*/*z* 149; possibly $[M+H-NH_3]^+$) was also present (Fig. 1).The base ion was selected for SIM mode.

2.4.2. LC–ESI-MS online derivatization with Fmoc-Cl

On the other hand, the automated on-line derivatization with Fmoc was done by running the following program on the programmable autosampler:

1. Draw 20 μl from the borate buffer vial
2. Draw 0 μl from the water vial to rinse the outside of the needle
3. Draw 1 μl from the Fmoc vial
4. Draw 0 μl from the water vial
5. Draw 10 μl from the sample vial
6. Mix with 30 μl of the air, five cycle times
7. Inject 10 μl

After derivatization, isocratic elution was performed with solvent B (acetonitrile–water, 50:50, v/v) containing 50 m*M* ammonium acetate for 8 min and then the eluent was switched to 100% acetonitrile in 5 min.The flow-rate was 0.2 ml/min in both cases.

2.4.2.1. Mass spectrometric data

The mass spectrum of *N*-9-anatoxin-a-Fmoc obtained by automated on-line derivatization is shown in Fig. 1. As can be seen, the strong protonated molecular ion at *m*/*z* 388 $[M+H]^+$, the weak sodium adduct ion at *m*/*z* 410 $[M+Na]^+$ and two major fragment ions at *m*/*z* 179, 210 were observed. The fragmentation giving *m*/*z* 179 and 210 involves homolytic cleavage of the bond between the β-carbon and the α-oxygen of ester. The protonated molecular ion was selected for the SIM mode.

3. Results and discussion

3.1. LC–ESI-MS with ion-pairing reagent of anatoxin-a

3.1.1. General aspects

While reversed-phase LC offers advantages with ESI, a problem occurs with the separation because anatoxin-a is a strongly basic compound. LC–ESI-MS requires that ions be generated in the spray chamber for MS detection. Analyzing strong basic compounds, like anatoxin-a presents a challenge to both processes; to separate mixtures and to prepare the analyte as an ion for MS detection. Anatoxin-a severely lacks chromatographic reliability or no separation occurs with reversed-phase columns. To eliminate the peak ‘‘smearing’’ and increase the retention time, ion-pair chromatography has been

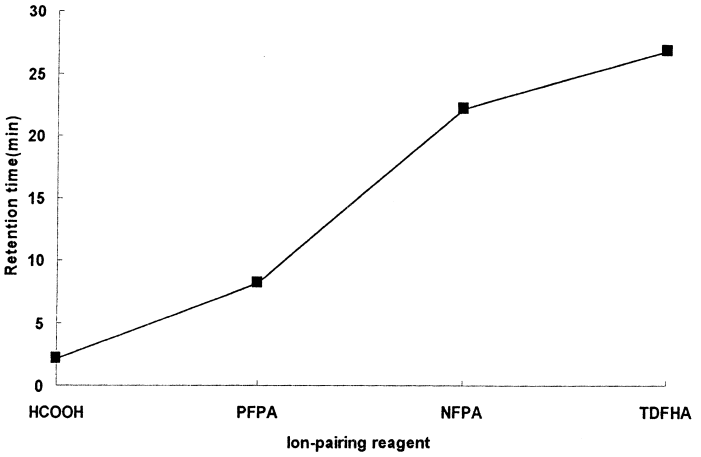

Fig. 2. Influence of ion-pairing reagent on retention time of anatoxin-a. Conditions: concentration of ion-pairing reagents, 10 mM; mobile phase, acetonitrile–water (15:85, v/v).

used. Typical ion-pairing reagents are alkylsulfonates. However, these reagents cannot be used for LC–ESI-MS because they are non-volatile and the non-volatile alkylsulfonates deposit in the spray chamber of the MS system. Hence, replacement of non-volatile substances by volatile equivalents is necessary [12,13]. In this study, the short-chain perfluorinated fatty acids TFA, PFPA, HFBA and TDFHA were used as volatile ion-pairing reagent for the analysis of anatoxin-a by LC–ESI-MS.

3.1.2. Influence of the ion-pairing reagent on retention of anatoxin-a

For the retention of anatoxin-a, the influence of four types of perfluorinated fatty acids was investigated. The concentration of the ion-pairing reagent was 10 m*M*. The eluents were mixtures of buffer containing different ion-pairing reagents and 15% (v/v) acetonitrile. The dependence of the retention time of anatoxin-a on the type of ion-pairing reagents is shown in Fig. 2. With increasing chain length of the ion-pairing reagents, an increase in retention was noted. NFPA and TDFHA were not suitable because retention times of the anatoxin-a were too long and the peak widths were very broad. Consequently, PFPA was selected as ion-pairing reagent. But the 10 m*M* PFPA required for optimal chromatography suppressed intensity of the anatoxin-a because PFPA produced the neutral anatoxin-a–PFPA complex. The limit of detection (LOD) using SIM, based on a signal-to-noise ratio of 3 was 3.3 pg/μl with a 10 μl injection. Fig. 3 shows the chromatogram of anatoxin-a standard solution using SIM at 10 pg/μl.

3.2. Automated on-line derivatization LC–ESI-MS of anatoxin-a

3.2.1. General aspects

For the analysis of the highly water soluble anatoxin-a, another approach is to develop an auto-

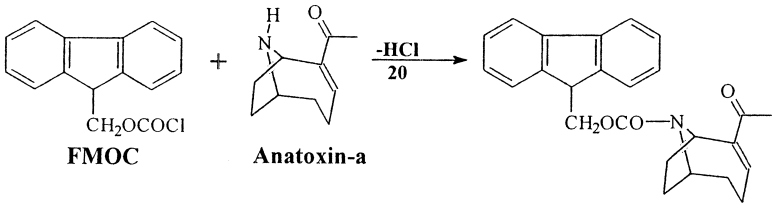

Fig. 3. Chemical reaction of anatoxin-a with Fmoc.

mated on-line derivatization–LC–ESI-MS method. This method is based on the derivatization of the analyte followed by the on-line processing of the sample solution with an autosampler. Fmoc is a very reactive reagent and in aqueous solution, the reagent rapidly converts onto Fmoc-OH and reacts with compounds containing a secondary amino group in borate buffer (pH 10) [11]. Fig. 3 shows chemical reaction of anatoxin-a with Fmoc at room temperature. Further, *N*-9-anatoxin-Fmoc shows adequate retention on the C_{18} column with ammonium acetate buffer because of its low polarity. This procedure without using an ion-pairing reagent has an additional advantage because ion-pairing reagents suppress the intensity of the anatoxin-a.

3.2.2. Development of automated on-line derivatization with Fmoc

In this study, the procedure of automated on-line derivatization with an autosampler was investigated. For the derivatization with Fmoc, conversion from Fmoc to Fmoc-OH is the first step. This step occurs in borate buffer at pH>8. Thus, the volume of borate buffer is a very important parameter. For the intensity of anatoxin-a-Fmoc, the influence of the volume of the borate buffer with a 10 μl sample and 1 μl Fmoc was investigated. The intensity of *N*-9-anatoxin-a-Fmoc reaches a maximum at 20 μl. Therefore 20 μl was chosen for the automated on-line derivatization. All steps with the autosampler for the derivatization are described in Experimental.

3.3. Comparison of both methods.

In this study, two analytical methods of the anatoxin-a were compared. The advantage of the method using the ion-paring reagent was that this method is simple and rapid; a disadvantage is its low sensitivity. On the other hand, the advantage of the method using automated on-line derivatization was a very high sensitivity and the disadvantage was the special instrumentation required. Fig. 4 shows two SIM chromatograms of the anatoxin-a using an ion-pairing reagent at 10 pg/μl and the *N*-9-anatoxin-a-Fmoc using automated on-line derivatization at 1 pg/μl. As can be seen, the LOD, based on a signal-to-noise ratio of 3 was 3.3 pg/μl and 0.5 pg/μl. This difference of sensitivity resulted from the molecular mass of the target compounds and the suppression effect of the ion-pairing reagent. Thus, the automated on-line derivatization method was chosen for the trace analysis of anatoxin-a in freshwater.

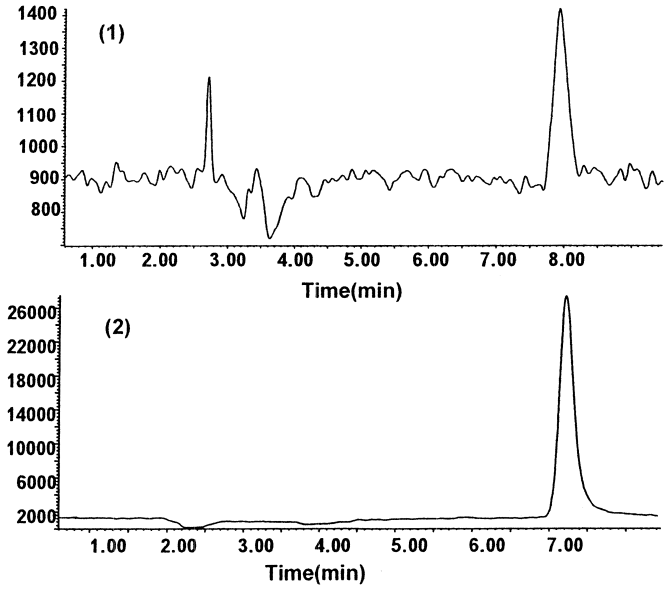

Fig. 4. LC–MS-SIM of anatoxin-a at 10 pg/μl (1) and *N*-9-anatoxin-a-Fmoc at 1 pg/μl (2).

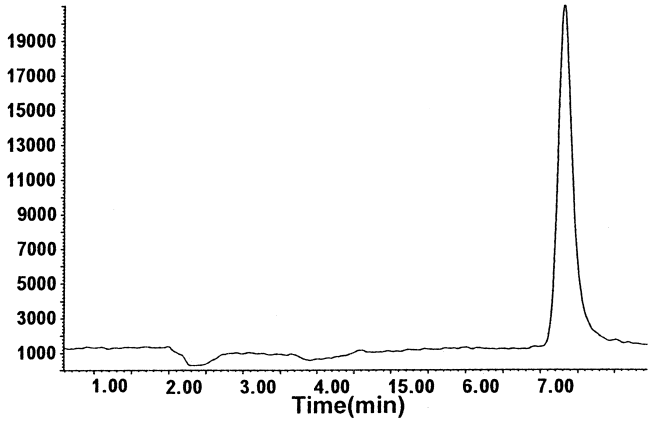

Fig. 5. LC–MS-SIM of anatoxin-a-Fmoc in freshwater (10 pg/μl).

3.4. Chromatography, validation and analysis of freshwater sample

In order to validate the method, a freshwater sample spiked with anatoxin-a was prepared and extracted with SPE. A chromatogram of anatoxin-a-Fmoc in freshwater at 10 ng/l, obtained using on-line derivatization–LC–ESI-MS with SIM is shown in Fig. 5. Anatoxin-Fmoc is resolved to baseline with a good peak shape and no interfering peaks. The calibration graph of anatoxin-a in freshwater was linear in the range 5–5000 ng/l with linear regression producing an equation of $y=35\,144x-15\,584$ with a correlation coefficient of 0.999 [where $y=$ peak area and $x=$concentration of anatoxin-a (ng/l)]. The mean recovery at 20 ppt was 75.7% whilst precision was acceptable (RSD 7.2%, $n=6$). By using replicate analysis and an RSD of 10%, a limit of quantification (LOQ) of 15.2 ng/l was obtained. The LOD, based on a signal-to-noise ratio of 3, was 2.1 ng/l (Fig. 6). This was considered sufficiently sensitive to enable the use of on-line derivatization–LC–ESI-MS for the determination of trace anatoxin-a in environmental waters.

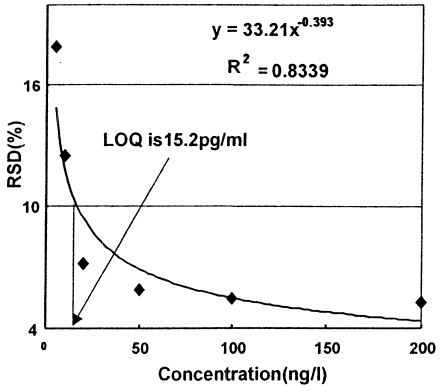

Fig. 6. Precision curve of anatoxin-a in freshwater.

4. Conclusion

In this study, two methods for the determination of anatoxin-a were investigated automated on-line derivatization was adopted for the analysis of trace amounts of anatoxin-a in freshwater. The method has been shown to have both good precision and accuracy, whilst also yielding a LOD and a LOQ that were adequate for the detection of freshwater concentrations. The method should be a valuable addition to existing analytical tools available for the determination of trace anatoxin-a in environmental waters.

References

[1] I.R. Falkoner, G.A. Codd, T.M. Jefferics, C.W. Keevil, E. Potter, in: Detection Methods for Cyanobacterial Toxins, Royal Society of Chemistry, 1994, p. 3.
[2] W.W. Carmichael, I.R. Falconer, Algal Toxins in Seafood and Drinking Water, Academic Press, London, 1991.
[3] C. MacKintosh, K.A. Bcaiije, S. Klumpp, P. Cohen, G.A. Codd, Fed. Evrion. Biochem. Soc. Lett. 264 (1990) 187.

[4] J.P. Devlin, O.E. Edwards, P.R. Gorham, N.R. Hunter, R.K. Pike, B. Stavric, J. Chem. 55 (1977) 1367.
[5] H.S. Wong, E. Hindin, J. Am. Water Works Assoc. 74 (1982) 528.
[6] K.J. James, A. Furey, I.R. Shelock, M.A. Stack, M. Twohig, F.B. Caudwell, O.M. Skulberg, J. Chromatogr. A 798 (1998) 147.
[7] K. Himberg, J. Chromatogr 481 (1988) 358.
[8] R.A. Smith, D. Lewis, Vet. Hum. Toxicol. 29 (1987) 153.
[9] K. Harada, H. nagai, M. Suzuki, Tetrahedron 49 (1993) 9251.
[10] G.K. Poon, L.J. Griggs, C. Edwards, M. Waris, Analyst 116 (1991) 265.
[11] E.A. Hogendoorn, F.M. Ossendrijver, E. Dijkman, R.A. Baumann, J. Chromatogr. A 833 (1999) 67.
[12] A.L.L. Duchateau, B.H.M. Munsters, G.T.C. Kwakkenbos, R.G.J. van Leuken, J. Chromatogr. 552 (1991) 605.
[13] R.G.J. van Leuken, G.T.C. Kwakkenbos, A.L.L. Duchateau, J. Chromatogr. 647 (1993) 131.