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Sensitive determination of anatoxin-a, homoanatoxin-a and their degradation products by liquid chromatography with fluorimetric detection

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Abstract

Cyanobacterial neurotoxins have been implicated in animal deaths resulting from drinking contaminated water. Anatoxin-a (AN) and homoanatoxin-a (HMAN) have previously been analysed using high-performance liquid chromatography (HPLC) with UV detection, but this procedure is insufficiently sensitive and is subject to interferences. A sensitive fluorimetric (FL) method for determining AN was recently developed using derivatisation with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) and this has been applied to the simultaneous determination of AN, HMAN and their epoxy and dihydro degradation products. Microscale syntheses were used to prepare the dihydro and epoxy derivatives from AN and HMAN. These compounds were produced in high yields, as confirmed by electrospray MS and HPLC–FL of their benzoxadiazole derivatives. All six NBD derivatives were readily separated using isocratic reversed-phase HPLC. The recoveries of these compounds from spiked water samples, using weak cation-exchange (WCX) solid-phase extraction (SPE), were 83.2–84.9% at concentrations of 10 μg/l. The R.S.D. values were 1.7–3.9% ($n=8$) and the limits of detection were better than 10 ng/l for all six compounds, illustrating the high sensitivity of the method. This methodology was successfully applied to the analysis toxin degradation products in natural samples. Dihydroanatoxin-a (0.8 mg/g) was isolated from a benthic *Oscillatoria* bloom from Caragh Lake, Ireland, and was found to contain two isomers but their ratio was different from that found in the synthetic material.

Keywords: Derivatization, LC; Water analysis; Anatoxins; Toxins; Neurotoxins; Homoanatoxins

1. Introduction

Toxic cyanobacteria (blue-green algae) have been identified in lakes and drinking water sources in Europe, North America, China, South Africa and Australia [1]. Their toxic effects have caused many animal deaths and have also been implicated in cases of human illness [2,3]. The main genera responsible for toxin production include *Anabaena*, *Microcystis*, *Oscillatoria*, *Aphanizomenon*, *Nodularia* and *Cylindrospermopsis*, which produce hepatotoxins and neurotoxins [4].

The hepatotoxin class of toxins include microcystins, nodularins and cylindrospermopsin. Over

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60 microcystins have been isolated to date and they are the most abundant cyanobacterial toxins. Microcystins are monocyclic heptapeptides with a potent toxicity that has been attributed to their specific inhibition of protein phosphatases 1 and 2A [5], and studies with laboratory animals have shown that microcystins are also potent tumour promoters [6,7]. Recently, cylindrospermopsin has been isolated from *Cylindrospermopsin raciborskii* and was shown to be a new hepatotoxin possessing a tricyclic guanidine moiety combined with hydroxymethyluracil [8].

The cyanobacterial neurotoxins include the saxitoxins, anatoxin-a(s) and the anatoxins. Saxitoxin and neosaxitoxin were detected in *Aphanizomenon flos-aquae* which was until recently believed to be the only cyanobacterial species that produced saxitoxins [9]. However, it has been shown that saxitoxin and related neurotoxins occurred in blooms of *Anabaena circinalis* in rivers and storage reservoirs in Australia [10]. In 1990, a bloom of *Anabaena* in the Darling river was responsible for the deaths of approximately 1600 cattle and sheep and the saxitoxin group were implicated [11].

Anatoxin-a(s) is a neurotoxin, the only naturally occurring organophosphate anticholinesterase inhibitor found in nature, and the terminology (s) was used to denote the viscous salivation caused in laboratory mice. Anatoxin-a(s) is a unique *N*-hydroxyguanidine methyl phosphate ester and is both chemically and physiologically different from anatoxin-a (Fig. 1A, R=CH_3) [12].

Anatoxin-a (AN) was the first cyanobacterial toxin to be structurally elucidated and it is an alkaloid, 2-acetyl-9-azabicyclo[4.2.1]non-2-ene [13]. AN has a high toxicity (LD_{50} i.p. mouse 200 μg/kg) [14], and it is a potent nicotinic agonist which acts as a post-synaptic, depolarising, 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 [14]. AN is produced by the genera *Anabaena flos-aquae*, *Oscillatoria*, *Anabaena circinalis*, *Aphanizomenon flos-aquae* and *Cylindrospermum* spp. [15]. A methylene analogue (Fig. 1A, R=C_2H_5), homoanatoxin-a (HMAN), was synthesised and predicted to be a natural neurotoxin [16] which was confirmed by its subsequent isolation from *Oscillatoria formosa* [17]. The toxicity of HMAN is similar to that of AN [18].

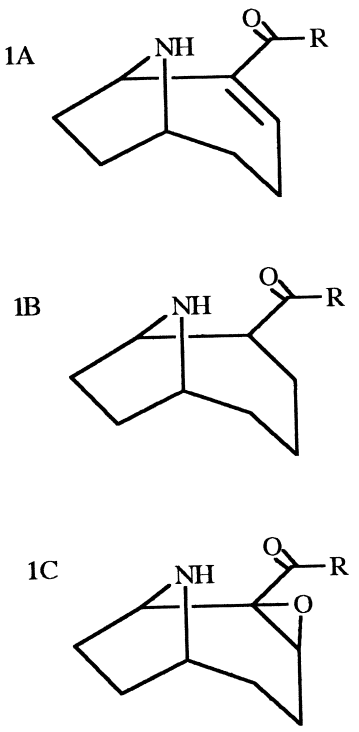

Fig. 1. Structures of the neurotoxins, anatoxin-a (A, R=CH_3), homoanatoxin-a (A, R=C_2H_5), and their degradation products, dihydroanatoxin-a (B, R=CH_3), dihydrohomoanatoxin-a (B, R=C_2H_5), epoxyanatoxin-a (C, R=CH_3) and epoxyhomoanatoxin-a (C, R=C_2H_5).

AN degrades readily, especially in sunlight and at high pH, to non-toxic degradation products [19]. The stable non-toxic alkaloid, dihydroanatoxin-a (Fig. 1B, R=CH_3), was identified from an ageing bloom of *Anabaena flos-aquae* [20]. Recently, anatoxin-a epoxide (Fig. 1C, R=CH_3), another non-toxic metabolite of AN has been identified from various blooms [21].

A number of chromatographic methods are available for the analysis of AN in cyanobacterial bloom material and these include high-performance liquid chromatography (HPLC) with ultra-violet detection [22], thin-layer chromatography [23] and liquid chromatography–mass spectrometry (LC–MS) using electrospray ionisation [24]. Although the determination of AN in natural waters poses greater analytical problems due to the typically low concen-

tration of this toxin, derivatisations followed by gas chromatography (GC) with electron-capture [25] or mass spectrometric (MS) detection [20,26–28] have been successfully applied.

We have recently devised a highly sensitive HPLC method for the determination of AN, using fluorimetric derivatisation with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) [29,30], and this method has now been further developed for the simultaneous determination of AN, HMAN and their dihydro (Fig. 1B) and epoxy (Fig. 1C) analogues.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a pump, (LC-10AD), column oven (CTO-10A) and an RF-551 fluorescence detector (Shimadzu, Duisberg, Germany) with an autosampler (ISS-100, Perkin-Elmer, Überlingen, Germany). The analytical HPLC columns used were Prodigy C_{18}, 5 μm, 250×3.2 mm; Ultremex C_{18}, 5 μm, 250×3.2 mm; Atlantis C_{18}, 5 μm, 250×3.2 mm (Phenomenex, Macclesfield, UK); and a Vydac, protein and peptide C_{18} column, 5 μm, 250×4.6 mm (Supelco, Hesperia, CA, USA); and all columns were used with the manufacturer's recommended precolumns. Weak cation-exchange cartridges (3 ml, Supelco, Poole, UK), C_{18} Mega Bond Elut (10 g, Varian, Habor City, CA, USA), Whatman 110-mm GF/C filter papers were used.

Solvent evaporation under nitrogen was carried out using a Turbo Vap LV Evaporator (Zymark, Warrington, UK). Sample preparation required the following equipment: centrifuges (Beckman model J2-21, High Wycombe, UK; and Easyspin, Sorvall Instruments, Stevenage, UK), vortex mixer (Maximix 11, Thermolyne type 37600), and a sonic bath (Sonicor SC-42, Sonicor Instrument, Copiague, NY, USA).

Chromatography data handling was performed using an Axxi-Chrom 717 chromatography data station (Axxiom chromatography, Gloucester, UK) or Unipac Class-VP software (Shimadzu) and data were transferred to Microsoft Excel for graphical presentation.

2.2. Standard anatoxin-a

Anatoxin-a (99%, Calbiochem-Novabiochem, Nottingham, UK) was purchased and used as a reference standard in this study. Homoanatoxin-a was isolated from a freeze-dried culture of *Oscillatoria formosa* NIVA-CYA 92 [17]. The quantitative data for the dihydro and epoxy analogues of anatoxin-a and homoanatoxin-a were determined using fluorimetric HPLC and related to this standard anatoxin-a.

2.3. Chemicals

4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was purchased (Aldrich, Gillingham, UK). Water, acetonitrile and methanol were of HPLC grade (Labscan, Dublin, Ireland), trifluoroacetic acid (TFA) was HPLC/spectrograde (Pierce, Chester, UK).

2.4. Extraction of anatoxins from water

Using a procedure based on that developed by Harada et al. [31], a filtered water sample (10 ml) was adjusted to pH 7 before solid-phase extraction (SPE) using a weak cation-exchange material, WCX, 3 ml cartridge (Supelco, Poole, UK). The SPE cartridge was conditioned with methanol (6 ml) and water (6 ml). The samples were transferred to the cartridge and washed with methanol–water (1:1, 3 ml) and air dried. The anatoxins were eluted using methanol containing 0.2% trifluoroacetic acid (10 ml) and the solvent was removed at 50°C, under nitrogen, using a TurboVap LV evaporator. The samples were reconstituted in methanol and transferred to 2-ml amber vials and evaporated prior to derivatisation.

2.5. Extraction procedure for the analysis of anatoxins in cyanobacteria

Freeze-dried cyanobacterial material (100 mg) was extracted by stirring with methanol (10 ml) containing hydrochloric acid (1 *M*, 100 μl) for 1 h. The solution was centrifuged at 3000 *g* for 10 min and the supernatant was collected. The pellet was re-extracted twice and the combined extracts were

evaporated at 50°C under nitrogen. The samples were reconstituted with water (5 ml) and the SPE procedure was similar to that for water samples except that 100% methanol was used in the SPE wash instead of methanol–water (1:1).

2.6. Derivatisation of anatoxins with NBD-F and HPLC–FL analysis

Sample material or standards, containing up to 100 ng of each anatoxin, were reconstituted with sodium borate (0.1 *M*, 100 μl) in a 2-ml amber vial. NBD-F in acetonitrile (1 mg/ml, 50 μl) was added and the mixture was allowed to stand (10 min) in the dark at room temperature. Hydrochloric acid (1 *M*, 50 μl) was added to terminate the reaction and HPLC–FL was performed directly on the products (20 μl injection) using Ultremex or Prodigy C_{18} columns (5 μm, 250×3.2 mm, Phenomenex) at 35°C. The mobile phase was acetonitrile–water (45:55), with a flow-rate of 0.5 ml/min, and fluorimetric detection (λ_{ex}=470 nm, λ_{em}=530 nm).

2.7. Analysis of anatoxin-a and homoanatoxin-a using HPLC–UV

HPLC analysis was performed using an Atlantis, C_{18} column (5 μm, 250×3.2 mm, Phenomenex, at 35°C with acetonitrile–water (5:95) containing 0.05% TFA as the mobile phase (flow-rate of 0.5 ml/min). A Waters 994 photodiode array detector (Millipore, Milford, MA, USA) was used with UV absorption monitored in the region, 200–300 nm, since the λ_{max} for anatoxin-a is 227 nm. This procedure was also used to purify small quantities (50–100 μg) of homoanatoxin-a.

2.8. GC–MS analysis of acetylated anatoxins

AN, HMAN and their dihydro products were derivatised overnight at 60°C using pyridine–acetic anhydride (1:1, 200 μl) according to the procedure of Edwards et al. [26]. Analysis of the acetyl anatoxins was performed using a Hewlett-Packard 5890 series II gas chromatograph, coupled to a mass detector 5971A, at 70 eV ionisation energy. The analytical column was a Hewlett-Packard HP1, methyl silicone, cross-linked (50 m×0.20 mm, 0.25 μm film.)

2.9. The isolation of anatoxins from cyanobacterial bloom material

The following is a typical procedure which was used to isolate anatoxins from an *Oscillatoria* sp. from Caragh Lake, Kerry, Ireland.

Freeze-dried algae (5 g) was extracted (3 h) with water (2×25 ml), adjusted to pH 4. The extract was filtered, pH adjusted to 7 and refiltered, and aliquots (5 ml) were then subjected to SPE using a weak cation-exchange (WCX) medium (3 ml, Supelco). The combined extracts were evaporated to dryness, reconstituted in water, adjusted to pH 9, and filtered prior to C_{18} SPE treatment (10 g, Mega Bond Elut, Varian). The cartridge was preconditioned with methanol (100 ml) followed by water (100 ml), the extract was then applied to the cartridge which was washed successively with methanol–water (20, 40, 60, 80% v/v, 10 ml each), methanol 10 ml fractions were collected. An aliquot (5 μl) of each fraction was derivatised and analysed by fluorimetric HPLC.

The final step in the purification required C_{18} HPLC (protein and peptide, C_{18}, 5 μm, 250×4.6 mm, Vydac). A gradient of acetonitrile–water both containing 0.1% TFA (0–30% acetonitrile over 15 min, followed by 30–40% acetonitrile over 35 min) was used for the separation of the anatoxins (1 ml/min, 0.5-ml fractions). Aliquots (5 μl) were analysed using fluorimetric HPLC following derivatisation with NBD-F.

2.10. Synthesis of dihydroanatoxins

AN or HMAN (100 μg) was dissolved in acetic acid (100 μl) and platinum V oxide (100 μg) was added. Hydrogen gas was passed through the mixture for 2–3 h. The mixture was evaporated under nitrogen, reconstituted in water (1 ml), adjusted to pH 7, and subjected to SPE cleanup. HPLC–FL, following derivatisation of an aliquot with NBD-F, showed that the yields of the dihydro products were >90%. GC–MS of the acetyl derivatives showed that two products with similar mass spectra were produced and these data were similar to the published spectrum of *N*-acetyldihydroanatoxin-a [26].

N-Acetyldihydrohomoanatoxin-a gave the following: *m*/*z* (% relative abundance) 223 (M^+, 84%), 208 (52%), 194 (12%), 167 (34%), 166 (19%), 124 (100%).

2.11. Synthesis of epoxyanatoxins

AN or HMAN (100 μg) was dissolved in acetone (200 μl). Sodium hydroxide (2 *M*, 20 μl) and hydrogen peroxide (30%, 20 μl) were added. After stirring for 35 min at 45°C, the mixture was evaporated under nitrogen, reconstituted in water (1 ml) and subjected to SPE. The yields and recoveries were >95%, based on HPLC–FL analysis.

2.12. Electrospray mass spectrometric analysis of anatoxin-a, homoanatoxin-a and their derivatives

Acetonitrile–water (1:1) with 0.1% formic acid was used as the mobile phase for the flow injection analysis experiments. A Masslynx software package (Fisons, Wythenshawe, UK) was used for instrument control, data acquisition and data processing.

3. Results and discussion

To adequately assess the environmental impact of the neurotoxins belonging to the anatoxin class, it is necessary to determine both the intracellular and extracellular toxin levels and to study the relationship between toxin production by cyanobacteria and their biodegradation products [15,32]. We recently developed a protocol using NBD-F (Fig. 2) for the determination of AN and used this to obtain the first evidence of the presence of this toxin in several lakes in Ireland [29,30]. NBD-F was first proposed as a reagent for the HPLC analysis of amino acids [33] and the use of benzoxadiazole reagents has been reviewed for the analysis of amines [34] and for general applications in the biosciences [35].

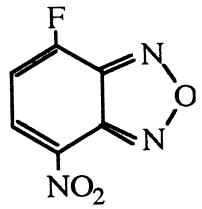

Fig. 2. 4-Flouro-7-nitro-2,1,3-benzoxadiazole (NBD-F).

AN has recently been implicated in incidents of fatal canine neurotoxicosis in Ireland [30] and in these studies, we also observed the presence of other alkaloid compounds, based on their signal responses using fluorimetric HPLC. The main aim of this study was to develop a robust, sensitive analytical protocol for the simultaneous determination of AN, HMAN and their degradation products. The rapid photodegradation of AN to non-toxic products has been demonstrated in laboratory studies [19] and natural degradation products include dihydroanatoxin-a (Fig. 1B, R=CH_3) [20] and epoxyanatoxin-a (Fig. 1C, R=CH_3) [31]. Most studies of freshwaters, contaminated by anatoxins from cyanobacterial blooms, have been carried out using HPLC–UV, but the toxin degradation products cannot be detected by this method. The forensic analysis of these toxins and their decomposition products is valuable in the investigation of sudden animal deaths. Thus, the determination of the degradation products provides useful evidence of the former toxic status of a waterbody long after the toxicity has dissipated.

3.1. Preparation of analytical standards

AN is commercially available, but is expensive, and HMAN was isolated from a culture of *Oscillatoria formosa* [17]. The purity of HMAN was examined using HPLC–UV and the UV spectrum was comparable to that of AN (Fig. 3B). There was no evidence of the presence of AN in the isolated material (Fig. 3A) and degradation products were not observed using HPLC–FL (data not shown). Since AN and HMAN were only available in small quantities, a programme was implemented to devise microscale synthetic procedures to obtain reference standards of the expected degradation products from these toxins.

Using 20–100 μg of AN or HMAN, hydrogenation using ADAM's catalyst produced the dihydro derivatives, and the epoxy derivatives were prepared by oxidation using basic hydrogen peroxide. The high sensitivity of the HPLC–FL method was used with advantage to conveniently follow these reactions, after derivatisation of aliquots with NBD-F.

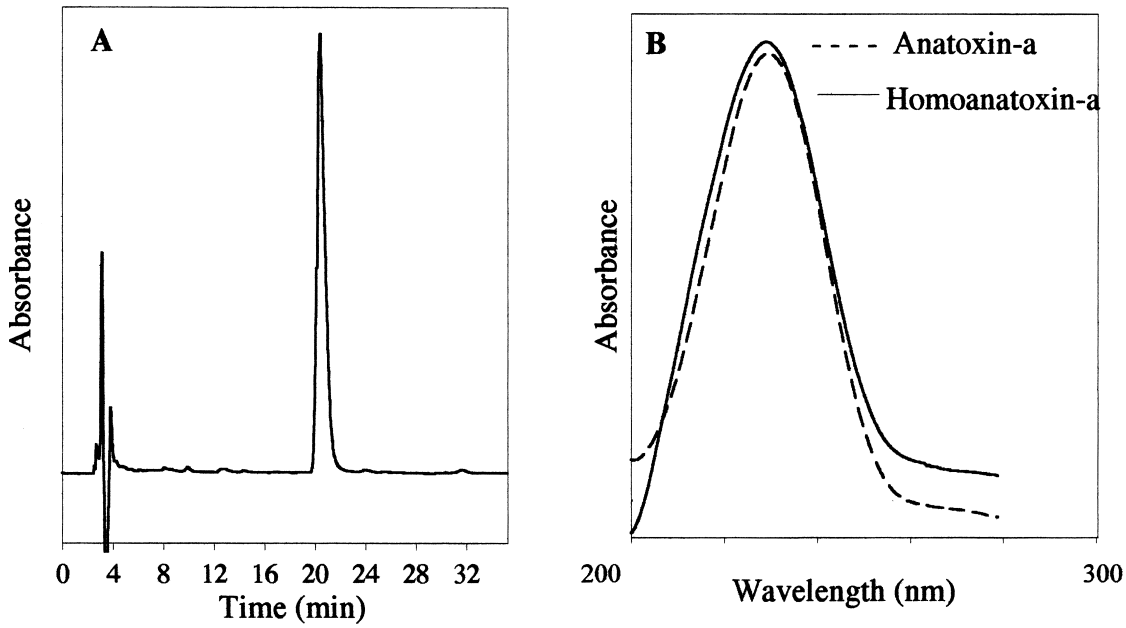

Fig. 3. Chromatogram of homoanatoxin-a isolated from *Oscillatoria formosa*. (A) Homoanatoxin-a (20.3 min, 1 μg). HPLC conditions: 5 μm Atlantis C_{18} column (250×3.2 mm); mobile phase, acetonitrile–water with 0.05% TFA (5:95, v/v); temperature, 35°C; flow-rate, 0.5 ml/min; detection, diode-array 200–280 nm. (B) Comparison of UV absorption spectra of anatoxin-a (– – –) and isolated homoanatoxin-a (———).

In most cases, clean-up of reaction products using weak cation-exchange SPE, similar to the procedure for anatoxin isolation, was sufficient to produce a purity >95%. Confirmation of the structures of the dihydro products was obtained from the mass spectral data of their *N*-acetyl derivatives. Dihydrohomoanatoxin-a is a novel compound but its MS fragmentation of the acetyl derivative showed the expected sequential cleavage of the side chain; *m/z* (% relative abundance) 223 (84%, M^+), 208 (52%, $[M-CH_3]^+$), 194 (12%, $[M\cdot C_2H_5]^+$), 166 (19%, $[M\cdot COC_2H_5]^+$). The epoxy products did not give satisfactory data following acetylation and GC–MS analysis. However, satisfactory data were obtained using electrospray MS for all the compounds used in this study (Table 1). In addition, the NBD

Table 1
Electrospray MS data for the anatoxins, degradation products and NBD derivatives

Compound	$[M+H]^+$	
	Calculated	Found
Anatoxin-a (Fig. 1A, R=CH_3)	165.1	165.8
Homoanatoxin-a (Fig. 1A, R=C_2H_5)	179.1	179.8
Dihydroanatoxin-a (Fig. 1B, R=CH_3)	167.1	167.9
Dihydrohomoanatoxin-a (Fig. 1B, R=C_2H_5)	181.2	181.9
Epoxyanatoxin-a (Fig. 1C, R=CH_3)	181.1	181.6
Epoxyhomoanatoxin-a (Fig. 1C, R=C_2H_5)	195.1	195.9
NBD–anatoxin-a	328.1	328.9
NBD–homoanatoxin-a	342.1	342.9

products of AN and HMAN were isolated by preparative HPLC and the electrospray MS data were in good agreement with calculated values (Table 1).

3.2. Calibration data for the analysis of anatoxins using HPLC–FL

Spiked water samples (10 μg/l) were prepared for each of the six anatoxins (Fig. 1). The recoveries of each anatoxin, using weak cation- exchange (WCX) SPE, was determined by comparing the peak areas using HPLC–FL with an equivalent sample that was directly derivatised without SPE. Acceptable mean recovery values, 83.2–84.9% (n=7), were obtained. The R.S.D. values were 1.7–3.9% (n=8) for concentrations of 10 μg/l and the limits of detection were better than 10 ng/l for all the anatoxins. Detailed calibration data on anatoxin-a analysis using NBD-F has previously been reported [30].

3.3. Isolation of dihydroanatoxin-a from cyanobacteria

The anatoxins were extracted from freeze-dried samples of cyanobacteria collected from Caragh Lake, with water at pH 4. After the standard clean-up using weak cation-exchange SPE, reversed-phase SPE with a MegaBond Elut (Varian) cartridge was used with a step gradient of 20–100% methanol–

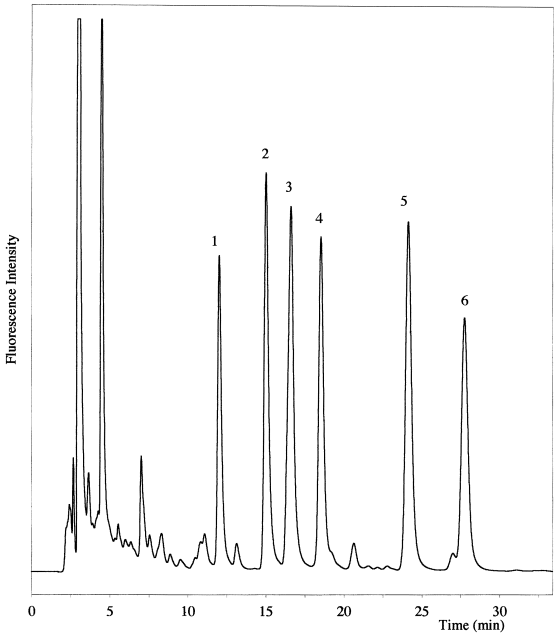

Fig. 4. Chromatogram from the HPLC–FL analysis of the six anatoxin standards following derivatisation with NBD-F. (1) NBD–epoxyanatoxin-a (12.0 min, 3.5 ng), (2) NBD–anatoxin-a (15.0 min, 5.0 ng), (3) NBD–dihydroanatoxin-a (16.6 min, 6 ng), (4) NBD–epoxyhomoanatoxin-a (18.5 min, 4.7 ng), (5) NBD–homoanatoxin-a (24.1 min, 6.1 ng) and (6) NBD–dihydrohomoanatoxin-a (27.7 min, 4.8 ng). HPLC conditions: 5 μm Ultremex C_{18} column (250×3.2 mm); temperature, 35°C; mobile phase, acetonitrile–water (45:55, v/v); flow-rate, 0.5 ml/min; fluorescence detection, λ_{ex}=470 nm, λ_{em}=530 nm.

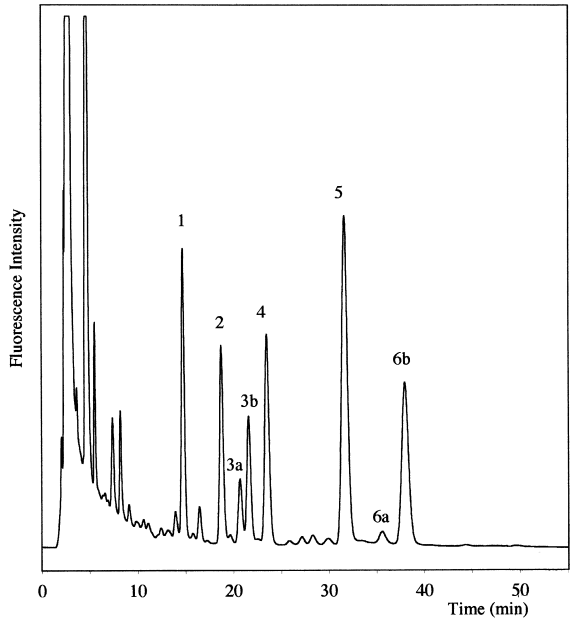

Fig. 5. Chromatogram from the HPLC–FL analysis of the six anatoxin standards following derivatisation with NBD-F. (1) NBD–anatoxin-a epoxide (14.7 min, 4.1 ng), (2) NBD–anatoxin-a (18.7 min, 3.5 ng), (3a) NBD–dihydroanatoxin-a (20.7 min, 1.2 ng), (3b) NBD–dihydroanatoxin-a (21.6 min, 2.5 ng), (4) NBD–homoanatoxin-a epoxide (23.5 min, 4.3 ng), (5) NBD–homoanatoxin-a (31.6 min, 8.8 ng), (6a) NBD-dihydrohomoanatoxin-a (35.5 min, 0.4 ng), (6b) NBD–dihydrohomoanatoxin-a (37.9 min, 5.2 ng). HPLC conditions: 5 μm Prodigy C_{18} column (250×3.2 mm); for other conditions, see Fig. 4.

water. The final step in the purification required C_{18} HPLC with a protein and peptide column (Vydac). A gradient of acetonitrile–water, containing 0.1% TFA, was used and the separation was monitored by analysing aliquots using HPLC-FL, following derivatisation with NBD-F.

3.4. HPLC–FL analysis of anatoxin-a, homoanatoxin-a and degradation products

After reacting a standard mixture of AN, HMAN, dihydro-AN, epoxy-AN, dihydro-HMAN and epoxy-HMAN (Fig. 1) with NBD-F, and clean-up using SPE, the benzoxadiazole derivatives were readily separated using isocratic reversed-phase HPLC (Fig. 4). The sensitivity of these measurements can be appreciated from the fact that almost full-scale fluorescence signals were produced for $\leq$5 ng of each compound on-column. However, using an Ultremex C_{18} column (Phenomenex), we observed that the retention time for synthetic dihydro-AN was slightly different from that for the isolated dihydro-AN. This anomaly was resolved when we found that there were two isomers of dihydro-AN, in different ratios, in the synthetic and natural materials. However, using the same mobile phase with a phase of different selectivity, Prodigy C_{18} (Phenomenex), was sufficient to separate the dihydro isomers of AN and HMAN (Fig. 5). A detailed investigation of the dihydroanatoxin isomers revealed that the synthetic material had an isomer ratio of ca. 1:3, whereas the natural material had a reverse ratio of ca. 3:1 (Fig. 6). GC–MS of the *N*-acetyl derivatives of these products confirmed that they contained isomers with virtually identical mass spectra corresponding to published data [26] for dihydroanatoxin-a (Fig. 7).

3.5. HPLC–FL for the analysis of anatoxins in cyanobacterial samples

To demonstrate the utility of this analytical protocol, using NBD-F with HPLC–FL, for the determination of anatoxin-a and degradation products in environmental samples, an ageing bloom was examined. A sample of a benthic *Oscillatoria* sp. that displayed no toxicity in animal bioassays, was extracted and derivatised. Evidence was obtained to show that there had been toxicity previously and, as shown in Fig. 8, dihydroanatoxin-a was present at 55 times the level of anatoxin-a.

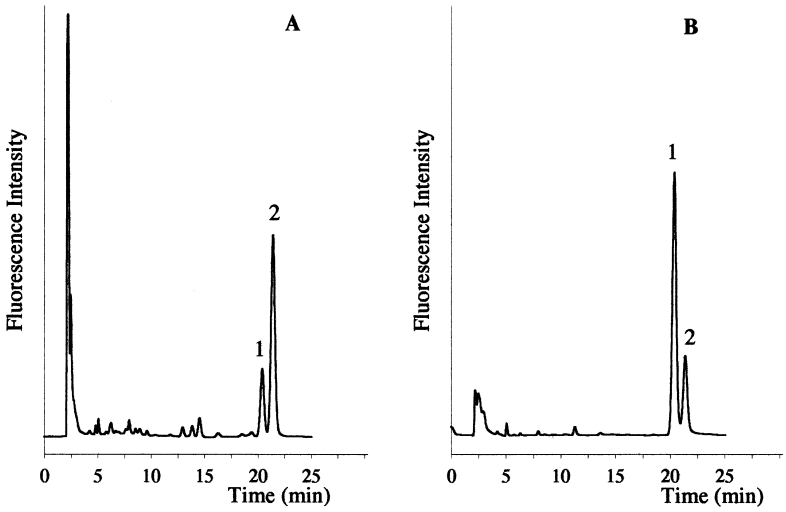

Fig. 6. Sample chromatograms of synthetic and natural dihydroanatoxin-a following derivatisation with NBD-F showing the presence of two isomers, 1 and 2. (A) Synthetic dihydroanatoxin-a: (1) NBD–dihydroanatoxin-a (20.3 min, 2.4 ng), (2) NBD–dihydroanatoxin-a (21.4 min, 7.6 ng). (B) Natural dihydroanatoxin-a: (1) NBD–dihydroanatoxin-a (20.3 min, 9.3 ng), (2) NBD–dihydroanatoxin-a (21.4 min, 3.2 ng). HPLC conditions: 5 μm Prodigy C_{18} column (250×3.2 mm); eluent was acetonitrile–water (44:56, v/v); for other conditions, see Fig. 4.

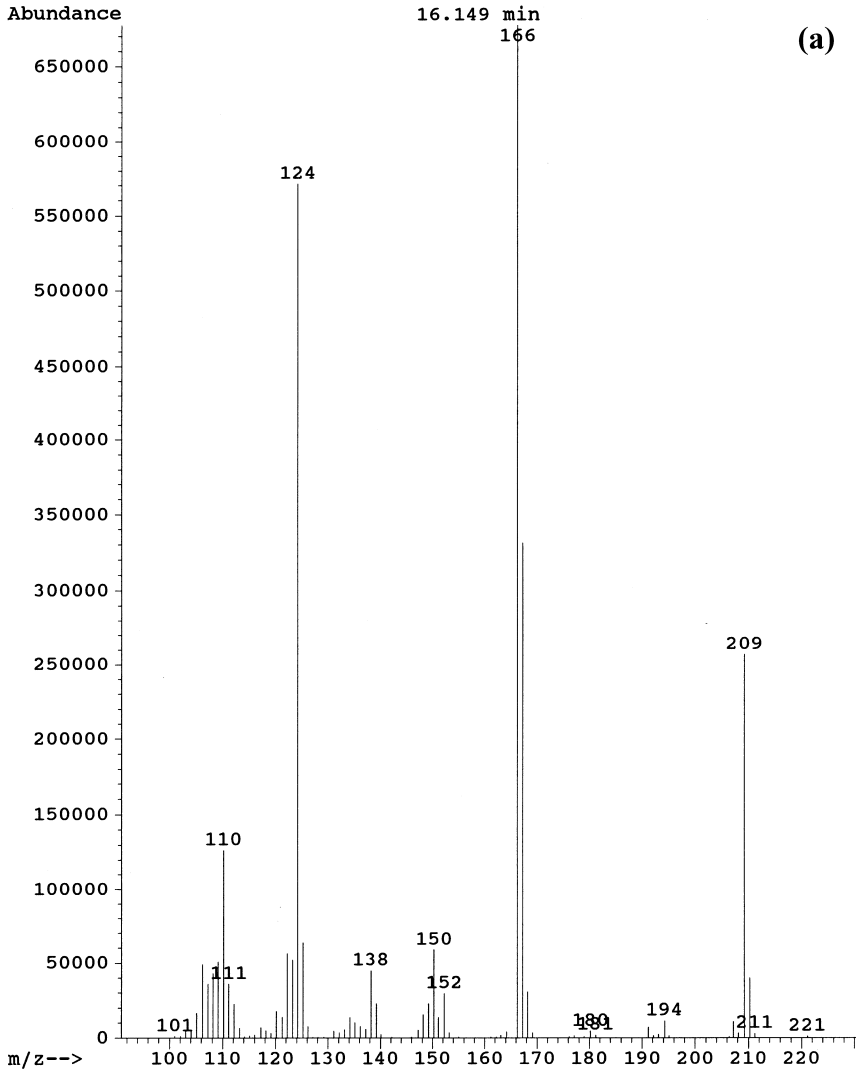

Fig. 7. GC–MS spectral data of an acetylated extract of dihydroanatoxin-a isolated from Caragh Lake. (a) TIC mass spectrum of peak 1, acetyldihydroanatoxin-a (16.15 min); (b) TIC mass spectrum of peak 2, acetyldihydroanatoxin-a (16.56 min). GC–MS conditions: 70 eV ionisation energy; transfer line and ion source temperatures 180 and 280°C, respectively; column HP1 (Hewlett- Packard), methyl silicone, cross-linked (50 m×0.20 mm, 0.25 μm film).

4. Conclusions

A fluorimetric HPLC protocol has been developed for the simultaneous determination of the two known anatoxins, AN and HMAN, the known degradation products of anatoxin-a, dihydro-AN and epoxy-AN, and the predicted degradation products of homoanatoxin-a, dihydro-HMAN and epoxy-HMAN. The limits of detection were better than 10 ng/l for all six compounds. As HMAN has only recently been identified in nature, it is understandable that there have been no reports of the occurrence of

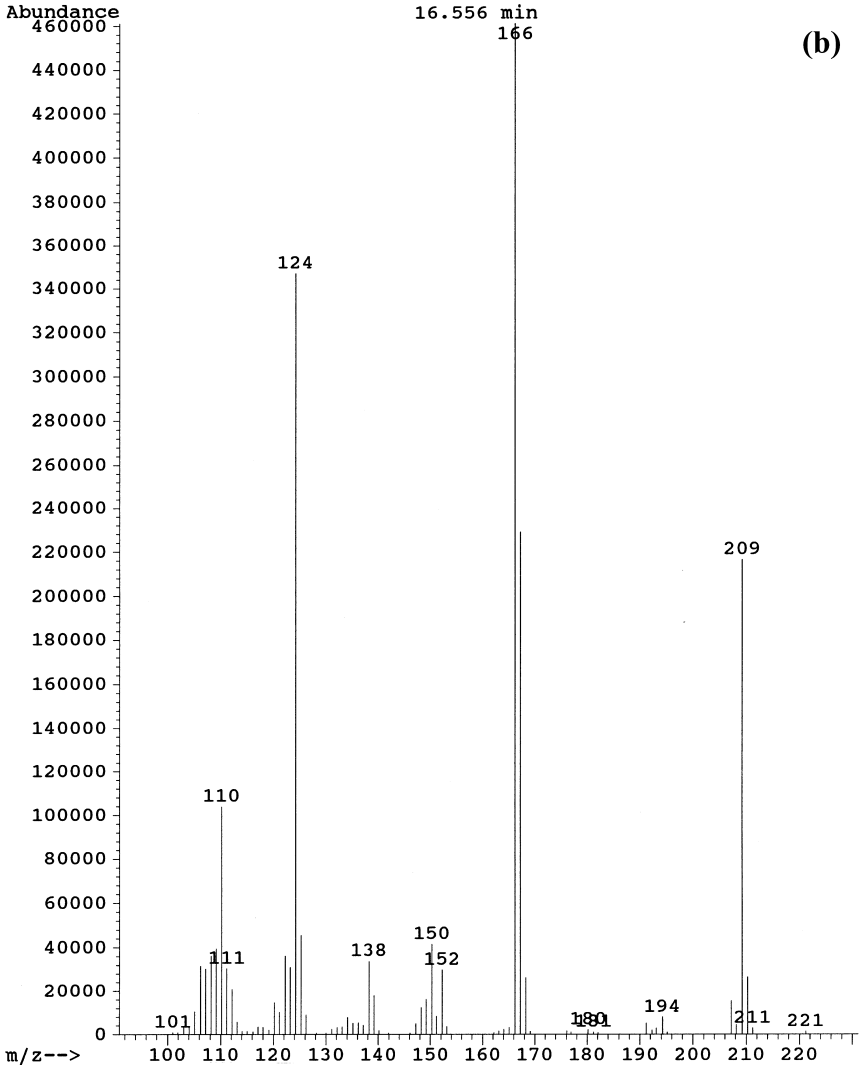

Fig. 7. (*continued*)

dihydro and epoxy products of HMAN. The use of synthetic standard materials should be useful for the investigation of natural samples for the presence of these compounds. In addition, detailed studies on the dynamics of toxin production and degradation can now be readily undertaken using the analytical protocols that have been developed in this study.

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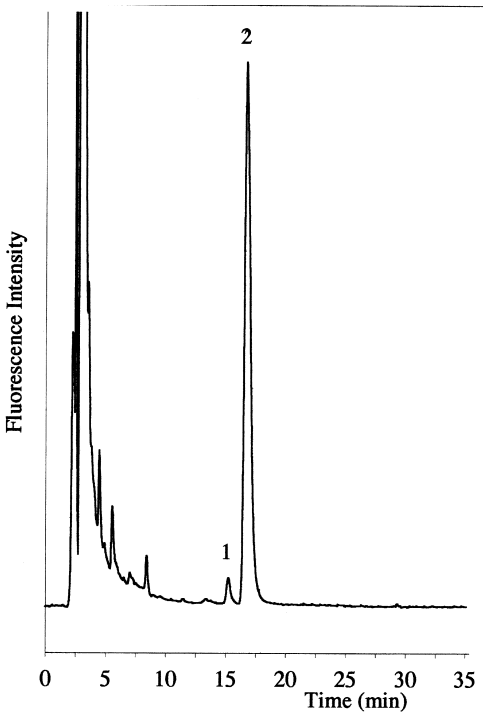

Fig. 8. Chromatogram from the HPLC–FL analysis of a freeze-dried algae sample from Caragh lake following derivatisation with NBD-F. (1) NBD–anatoxin-a (15.2 min, 0.06 ng), (2) NBD–dihydroanatoxin-a (16.6 min, 3.3 ng). For HPLC conditions, see Fig. 4.

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