Short communication

A method for acetonitrile-free microcystin analysis and purification by high-performance liquid chromatography, using methanol as mobile phase

Esme L. Purdie*, Fiona M. Young, Diana Menzel, Geoffrey A. Codd

Division of Molecular Microbiology, College of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, Scotland, United Kingdom

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Abstract

Cyanobacteria produce a wide range of potent toxins, including hepatotoxic microcystins. HPLC methods for microcystin analysis and purification almost invariably include acetonitrile in the elution gradient mobile phase. The recent, acute, global acetonitrile shortage requires that adequate methods are available for microcystin analysis and purification without the need for acetonitrile. Here we present a convenient methanol-based method for effective HPLC analysis and purification of the toxins, with full separation of a range of microcystin variants.

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Cyanobacteria (blue-green algae) produce a wide range of toxins (hepato-, neuro-, cytotoxins, irritants, and gastrointestinal toxins), the most often implicated in cyanobacterial toxicoses being the microcystins (MC) (Codd et al., 2005; Carmichael, 2008). These cyclic heptapeptide, tumour-promoting hepatotoxins are produced by members of several cyanobacterial genera including Microcystis, Anabaena, Planktothrix (Oscillatoria) and Nostoc. The general MC structure is cyclo (d-Ala-l-X-D-erythro-methylAsp-l-Z-Adda-o-Glu-N-methyldehydro-Ala), where Adda is the unique β-amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienic acid (e.g. Spoof and Meriluoto, 2002).

MC detection and analysis occupy key positions in basic and applied research on the production, toxicology, toxicology and fates of cyanotoxins (Meriluoto and Codd, 2005). Although antibody-based methods are increasingly being used for MC detection and quantification (Metcalf and Codd, 2003), high-performance liquid chromatography (HPLC) with refinements, has been- and continues to be the principle laboratory method used (e.g. Lawton et al., 1994; Spoof and Meriluoto, 2002). The purification of MCs, for use as analytical and calibration standards, and to provide research reagents, also predominantly involves HPLC (e.g. Fastner et al., 2002; Meriluoto and Codd, 2005). Over 80 structural variants of MC have been recognised and their toxicities vary widely. It is necessary to be able to separate, identify and quantify MC variants to understand the toxin profile of a cyanobacterial bloom. The structural variations mainly relate to changes in l-amino acid residues 2 and 4 of the cyclic heptapeptides, as indicated by a two-letter suffix. For the most commonly found variant, MC-LR, these indicate leucine (L) in position 2 and arginine (R) in position 4.

The Adda residue and N-methyldehydroalanine are the main MC chromophores and strongly absorb at 238 nm. Single wavelength detection, or preferably detection and characterisation by UV photodiode-array absorption, thereby provide means of MC quantification by HPLC. Whether using isocratic or gradient HPLC methods, most
procedures include acetonitrile (see Spoof and Meriluoto, 2002; Spoof, 2005). A typical procedure may include an acetonitrile (ACN) gradient mobile phase with per-fluorinated alkyl carboxylic acids, including trifluoroacetic acid (TFA) (Lawton et al., 1994; Fastner et al., 2002; Meriluoto and Codd, 2005). These conditions provide a well-tried and satisfactory separation and quantification of MCs in environmental and laboratory matrices. However a severe, global shortage of ACN availability developed in 2008, imposing limitations on MC analysis by HPLC (as in numerous other procedures throughout chemistry and the life sciences). This shortage resulted in the need to develop effective methods for HPLC-based MC analysis and purification which do not require ACN, as a viable alternative.

ACN is produced as a co-product of acrylonitrile, used in the production of acrylic fibres and acrylonitrile-butadiene-styrene (ABS) resins. The use of these acrylonitrile products is predominantly in the manufacture of cars, electronics and small appliances (Tullo, 2008). Beginning in late 2008, ACN availability substantially fell worldwide for several reasons, primarily the current global economic slowdown which has resulted in a reduced demand for acrylonitrile products. In addition, a major US ACN production facility on the Gulf Coast was shut down due to damage from Hurricane Ike, resulting in increasing difficulties in sourcing ACN. ACN costs for analytical purposes increased at least 5-fold in Europe early in 2009, and locations to laboratories by suppliers were reduced by up to 80% (personal communication by UK suppliers).

Alternative non-ACN methods for MC analysis and purification published to date have included complex mobile solvent phases including phosphate-buffered methanol; methanol:sodium sulphate; and dichloromethane–methanol–acetic acid (TFA) (Lawton et al., 1994; Fastner et al., 2002; Meriluoto and Codd, 2005). These conditions provide a well-phobic analogues (Meriluoto and Codd, 2005).

All reagents used in the development of this method were analytical or HPLC grade: methanol (MeOH) and acetonitrile (ACN) (Rathburn, Walkerton, UK); trifluoroacetic acid (TFA) of protein sequencing grade (Fisons, Loughborough, UK); water purified to 18.2 MΩ cm on a Milli-Q system (Millipore Co., Bedford USA). HPLC analysis was performed using Waters Corporation (Milford, MA, USA) instrumentation consisting of a Model 600E solvent delivery system, a Degasus 4 Channel Degasser (Jaytee Biosciences Ltd, Kent, UK), and a Model 717 WISP autosampler with detection using a Model 991 photodiode-array (PDA) detector at 200–300 nm with 3 nm resolution. A Cosmosil C₁₈ column (300 × 3.9 mm i.d.; Nacalai USA Inc, San Diego, CA, USA) was maintained at 40 °C using a Waters temperature control module. Mobile phases of Milli-Q-purified water plus 0.01% TFA (A) and MeOH/ACN plus 0.01% TFA (B) were used with a flow rate of 3 ml min⁻¹. Absorbance data were analysed using Waters Millenium 4.0 software.

Crude extracts of Microcystis PCC 7813 were used for method development, and were spiked with purified MC standards to characterise separation efficiency. Microcystin variants were extracted and purified according to Lawton et al. (1994). Calibration graphs were determined from triplicate injections at concentrations between 0.01 and 10.0 μg ml⁻¹ with initial stock concentrations confirmed by spectrophotometry at 238 nm. Of a wide range of MeOH–water gradient conditions tested ( Waters 600E operating conditions), the scheme summarised in Table 1 gave optimal results. TFA is still required to achieve good separation of MC variants, but it is possible to use a low TFA concentration (0.01% v/v), reducing the risk to MC stability and the extent of clean-up needed on the final fractions. Under the conditions given in Table 1, full separation was obtained of MC-LR; -RR; -YR; -LW; -LF; -LY; and -D-Asp³-RR, in addition to nodularin-R. Examples of the characteristic absorption spectra of the separated MCs are given in Fig. 1. MC-LR and -RR are representative of the most common MC UV spectra, with an absorbance maximum at 238–240 nm. MC-YR is representative of tyrosine (Y)-containing MCs with a flatter absorbance maximum at 230–240 nm, and MC-LW is representative of the tryptophan (W)-containing MCs with an absorption max at 222–223 nm and a shoulder at 238–240 nm (Meriluoto and Codd, 2005).

A full method comparison was performed against the previously used ACN-based method described in Meriluoto and Codd (2005), using the same HPLC apparatus and column, and maintaining the 0.01% TFA content. There were no changes to the absorption maxima or characteristic absorption spectra of any of the MC variants compared in this study. Linear calibration graphs allowed accurate quantification (R²: 1) from 12.5 ng using the water:MeOH procedure (Table 1) validated by spectrophotometric quantification at 238 nm, whereas with the standard water:ACN protocol, accurate quantification (R²:1) was achieved from 2.5 ng MC-LR.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>% Solvent A</th>
<th>% Solvent B</th>
<th>Gradient curve</th>
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<tr>
<td>0</td>
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* Waters HPLC 600E programmed gradients.
When using MeOH as the mobile phase instead of ACN, microcystin variant retention times were delayed by around seven minutes for variants: for example MC-LR eluted at 19.4 min; MC-RR at 14.5 min; and MC-YR at 11.5 min compared to 12.2, 6.3 and 11.5 min respectively. Peak width was compared for microcystin variants at 125 ng concentrations, using both MeOH and ACN mobile phases. MC-LR peak width broadened from 20.0 s with ACN to 25.5 s with MeOH. There was no broadening of the MC-RR peak, maintaining a constant 23.5 s for both solvents. Conversely, the width of the MC-YR variant was reduced from 19.7 s with ACN to 16.3 s with MeOH.

The quality of separation was measured by resolution ($R_s$), which is equal to the distance between peak centres divided by the average bandwidth (Snyder et al., 1997). In method development, an $R_s$ value of 2.0 or greater is desired for optimum separation. For MC-RR and MC-LR, resolution was reduced from 21.02 with ACN, to 18.15 with MeOH; MC-RR and MC-LR resolution was reduced from 25.19 with ACN to 24.02 with MeOH; MC-LR and MC-YR resolution was improved by the use of MeOH by increasing resolution from 3.2 with ACN, to 5.3 with MeOH.

Previous studies have indicated that the use of ACN in MC analysis is preferable to methanol in terms of chromatographic peak separation in HPLC (Spoof and Meriluoto, 2002). The only apparent remaining drawback to the use of MeOH as compared to ACN for microcystin analysis and purification, is the increase in back-pressure, which contributes to broader peaks, signal:noise ratio, and may impact upon the long-term running efficiency of HPLC components. It seems likely that ACN-containing methods would continue to be favoured for the analysis and purification of MCs and nodularins if acetonitrile were readily available. However, the recent global shortage of ACN presented supply problems to laboratories and efforts to develop ACN-free HPLC methods for cyanotoxin analysis may be increasingly necessary. The present study has developed the use of MeOH, with 0.01% TFA, as an effective alternative solvent for HPLC analysis.
Acknowledgements

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Conflict of interest

The authors declare that there are no conflicts of interest.

References


