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Determinations of MC-LR and [Dha^7] MC-LR Concentrations and Physicochemical Properties by Liquid Chromatography-Tandem Mass Spectrometry

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Abstract A liquid chromatography electrospray mass spectrometry (LC/ESI/MS) method working in multiple reactions monitoring mode for the determination of trace amounts of microcystin variants (MC-LR and [Dha^7] MC-LR) in waters was developed. The limit of quantification was 0.05 μg/L and the limit of detection was 0.015 μg/L for MC-LR and [Dha^7] MC-LR, respectively. Recoveries for MCs were in the range of 68%–81%. MC-LR and [Dha^7] MC-LR were chemically stable with similar physiochemical behavior.

Keywords MC-LR · [Dha^7] MC-LR · LC-MS · Physiochemistry

Microcystins (MCs) are increasingly a problem for both wildlife and human health as a consequence of toxic cyanobacteria occurrences in aquatic ecosystems (Codd et al. 2005; Chen et al. 2009). Almost 80 structurally different MCs have been discovered (Pérez and Aga 2005). MC-LR has been found to be the most commonly occurring heptapeptide hepatotoxin (Fastner et al. 2002), and [Dha^7] MC-LR was found in the replacement of the methylene group of the Mdha of MC-LR by an ethylidene group of Dha.

There have been many analytical methods for MCs including enzyme-linked immunosorbent assay (ELISA) (Rapala et al. 2002) and HPLC-linked protein phosphatase bioassay (Li et al. 2004), HPLC with mass spectrometry (LC/MS), including fast atom bombardment (FAB) (Kondo et al. 1995), electrospray ionization (Spoof et al. 2003). Liquid chromatography coupled with ion trap mass spectrometry (LC-IT-MS) is another alternative technique to identify and quantify small peptides (Dai et al. 2008). It is capable of determining trace amounts of each MCs separately at the same time and provides more detailed structural information. Usually, environmental behavior of compounds can be predicted from various physicochemical data which are generally measured in lab under well defined condition, i.e., stability in water, water-octanol partition coefficient, etc. Although the chemical structures of MC-LR and [Dha^7] MC-LR have been well documented, little is known about their physicochemical properties (Pérez and Aga 2005; Codd et al. 2005).

The aims of this study were to develop an appropriate analytical procedure, based on solid phase extraction (SPE) and liquid chromatography electrospray mass spectrometry (LC/ESI/MS) for the determination of trace amounts of MC-LR and [Dha^7] MC-LR in water samples and to evaluate the physicochemical properties of MC-LR and [Dha^7] MC-LR.

Materials and Methods

The samples of MC-LR and [Dha^7] MC-LR in this study were isolated from a water bloom dominated by *Microcystis aeruginosa* in Dianchi Lake, Yunnan of China. Standard

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MC-LR was purchased from Wako Pure Chemical Industries (Japan). All reagents used were of analytical grade or higher. Microcystin LR and [Dha^7] microcystin-LR are abbreviated as MC-LR and [Dha^7] MC-LR, respectively.

The collected water samples were stored in pyrex borosilicate glass bottles and kept at 4°C until analysis. Separation, identification and quantification of the microcystin variants were performed using a Finnigan LC-MS system comprising a Surveyor HPLC system (Thermo Electron Corporation, San Jose, CA, USA) equipped with an electrospray ionization source (ESI). The instrument control, data processing, and analysis were conducted by using Xcalibur software. At analysis stage the analytics were eluted with the following linear gradient program: 0 min 5% B, 0.5 min 30% B, 3 min 40% B, 6 min 70% B, 14.5 min 70% B, 14.6 min 5% B, 20 min 5% B. Sample injection volume was typically 10 μL and triplicate injections were used for each sample. The temperatures of vial tray and column oven in the autosampler were set to 10 and 25°C, respectively. The traces monitored, relevant collision energy and isolation width were shown in (Table 1).

The evaluation of the analytical curves' linearity was done based on injections of the standard solutions of MC-LR and [Dha^7] MC-LR with blank water sample extracts cleaned up by SPE to form a series of concentration with 0.01, 0.05, 0.1, 0.5, 1.0 and 2.5 μg/L MC-LR and [Dha^7] MC-LR, where this sequence was injected three times (n = 3). Calculations were performed using average peak areas, relative standard deviations (RSD) and calibration curve equations, and the determination coefficients (γ^2) and linear ranges were also determined for each analysis. The extraction recovery expressed as mean ± SD (standard deviation) was determined by calculating the ratio of the amount of extracted water spiked with known amounts of MC-LR and [Dha^7] MC-LR to the amount of compound added at the same concentrations in the mobile phase solution. To determine the limits of detection (LOD) and the limits of quantification (LOQ), sub-samples of a grab sample were spiked with the analytes and prepared for analysis. By determining the increase in response between spiked and unspiked samples, the LODs were calculated as a signal-to-noise (S/N) ratio of 3:1 and the LOQs could be estimated as a signal-to-noise (S/N) ratio of 10:1. To assess intra-day precision, five spiked samples at three concentration levels each, 0.05, 0.25, and 1.25 μg/L, were prepared and analyzed using LC/MS. Procedure was repeated on three different days to determine inter-day and intra-day precision.

Table 1 MS/MS parameters for MC-LR and [Dha^7] MC-LR

Compound	MC-LR	[Dha^7] MC-LR
Molecule weight	995	981
Isolation width (*m*/*z*)	1.0	1.0
Collision energy (eV)	37	37
Parent ion	995.5	981.5

NaCl was added to distilled water at final concentration of 0.1 M. The pH of prepared NaCl solution was then adjusted between 1.0 and 12 with an increment of 0.5 by addition of HCl or NaOH. Triplicate measurements were performed at pH = 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0. To measure the concentration of MCs in water and n-octanol phase, a method based on HPLC-ESI-MS/MS technology was developed.

The stability experiment was performed at 10, 20, 30, 40, 60, 80, 100, 150 and 200°C in triplicates, respectively. A mixture of MC-LR and [Dha^7] MC-LR was dissolved in 200 μL of methanol, and the solution was evaporated to dryness. A triplicate set of each tube was kept in the dark at each temperature in 2-mL test tubes for 6 h. Samples were taken at regular intervals and the residue was dissolved in 1 mL distilled water.

Triplicate measurements were performed at pH = 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0. A mixture of MC-LR and [Dha^7] MC-LR was dissolved in 40 μL of water and the solution was evaporated to dryness, and then dissolved in 100 μL each pH solution. A triplicate set of each tube was kept in the dark at each pH in 2-mL test tubes for 24 h at 25°C.

Results and Discussion

In this work, a ODS cartridge (500 mg/6 mL, Waters, Milford, MA, USA) was employed for the sample pretreatment, which had been preconditioned by 10 mL 100% methanol and 10 mL distilled water. The column containing sample was eluted with 10 mL 100% MeOH. The eluant collected from the C18 cartridges was evaporated to dryness. The residue was dissolved in 1 mL 100% MeOH and this elution fraction was also evaporated to dryness and the residue was dissolved in 100% MeOH. Finally, the extract was evaporated to dryness and redissolved in 100 μL of the LC mobile phase and transferred to HPLC autosampler vials. The aliquots (10 μL) were injected into the LC-MS system.

Separation was carried out under the reversed phase on Agilent ZORBAX SB-C18 column (2.1 mm i.d. × 100 mm, 3.5 μm, Agilent Corporation, USA). Solution A of the elution system was formic acid-water solution (0.05%, v/v) while solution B was formic acid methanol solution (0.05%, v/v). Better separation for both groups was generated by linear gradient elution. Thus the linear gradient elution as described above was applied.

Table 2 Calibration curve equations for [Dha^7] MC-LR and MC-LR

Compound name	Regression equations	Linear range (μg/mL)	γ^2
MC-LR	$Y = 93416.1 + 9.61145e + 006 \times X$	0.025–2.5	0.9981
[Dha^7] MC-LR	$Y = 334.756 + 6.16112e + 006 \times X$	0.025–2.5	0.9980

X: concentration μg/mL, *Y*: peak area

MS was set to ESI+ mode and MS tuning and optimization were achieved by infusing MC-LR with ion of $[M + H]^+$ at *m/z* of 995.5. Product ions (*m/z*) included 599.3, 553.2, 866.4, 967.4 and 977.4, which are identical with that of the standard sample (MC-LR, Wako Pure Chemical Industries, Japan). Quantification of MCs was achieved through total signal of MS/MS. Precursor ion was $[M + H]^+$ at *m/z* of 995.5 for MC-LR, while precursor ion was $[M + H]^+$ at *m/z* of 981.5 for [Dha^7] MC-LR. It can also be assigned as [Dha^7] MC-LR. [Dha^7] MC-LR is identical with the product ions at *m/z* 599.3, 539.3, 852.4, 937.6 and 953.5. Collision energy was 37% for both [Dha^7] MC-LR and MC-LR. Focused on these precursor ions and productions, the MS/MS parameters were optimized, as described above. All the values present in the text were measured by ESI-LC/MS^2.

Excellent linearity was obtained for compounds between peak areas and concentrations of [Dha^7] MC-LR and MC-LR were shown in Table 2. Their linear range and correlation coefficients (γ) were almost identical.

A fresh water sample spiked with standard MCs at the levels that produced distinguishable signals from the baseline noise was used for determination. The limits of detection (LOD) was considered as 3 times the signal/noise ratio (S/N) and the LOQ was 10 times the S/N ratio. The LOD (limits of detection) were calculated to be 0.015 and 0.015 μg/L and the LOQ (limits of quantification) were calculated to be 0.05 and 0.05 μg/L for [Dha^7] MC-LR and MC-LR, respectively.

The procedure was repeated on intra-day and inter-day on the same spiked standard series. The relative standard deviations (RSDs) for the intra- and inter-day precisions of the method are presented in (Table 3). The data indicate that the precision of the method were acceptable.

Table 3 Intra- and inter-day RSD for [Dha^7] MC-LR and MC-LR

Item	Intra-day RSD (%, n = 5)			Inter-day RSD (%, 3 days)		
	Low[a]	Medium[b]	High[c]	Low	Medium	High
MC-LR	12.6	9.4	2.1	2.1	6.8	3.6
[Dha^7] MC-LR	13.4	3.6	3.4	13	3.2	4.5

[a] Low fortification: 0.05 μg/L for [Dha^7] MC-LR and MC-LR

[b] Medium fortification: 0.25 μg/L for [Dha^7] MC-LR and MC-LR

[c] High fortification: 1.25 μg/L for [Dha^7] MC-LR and MC-LR

The accuracy of the developed method was validated with spiking-recovery tests. The recovery was determined for fresh water spiked with low, medium and high concentrations of MC-LR and [Dha^7] MC-LR with three replicates, respectively. The results were summarized in Table 4. The average recovery of MC-LR spiked with low, medium and high concentrations was >73% and of [Dha^7] MC-LR >74%.

The determined log D_{ow}s of MC-LR and [Dha^7] MC-LR at the different pHs were shown in Fig. 1. It can be seen that the log D_{ow} of MC-LR ranged from 1.63 at pH = 1 to −1.50 at pH = 12. The log D_{ow} of [Dha^7] MC-LR decreased from 1.60 at pH = 1 to −1.57 at pH = 12. The result is different from (De Maagd et al. 1999), but similar to other ionic compounds such as pentachlorophenol (Jafvert et al. 1990).

After an incubation of 6 hours, both MC-LR and [Dha^7] MC-LR were still stable when the temperature was less than 100°C. From 10 to 150°C, neither MC-LR nor [Dha7]

Table 4 Recoveries for the method validation of two MCs (mean ± SD, n = 3)

Compound name	Spiked (μg/L)	Mean ± SD (%)
MC-LR	0.05	68 ± 11.3
	0.25	76 ± 4.8
	1.25	77 ± 2.4
	0.05	65 ± 14.6
[Dha^7] MC-LR	0.25	75 ± 5.2
	1.25	81 ± 3.8

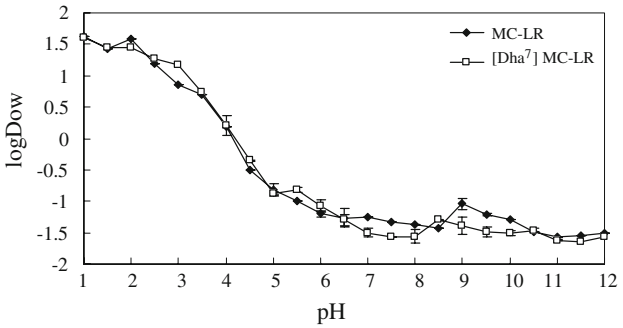

Fig. 1 pH-dependent log D_{OW} of [Dha^7] MC-LR and MC-LR. *Vertical bars* indicate the standard deviations (n = 3)

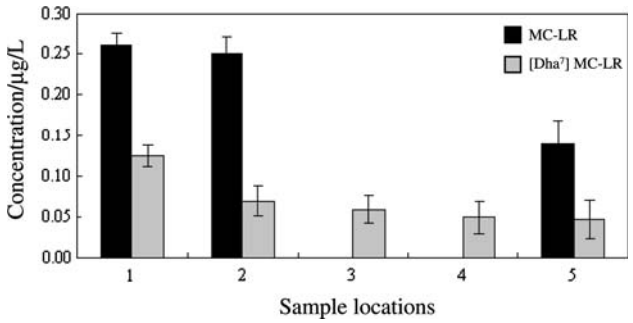

Fig. 2 Concentrations of MC-LR and [Dha7] MC-LR in the water at different locations of Lake Donghu, Wuhan, China

MC-LR was degraded, whereas the half-lives of MC-LR and [Dha^7] MC-LR at 200°C were less than half an hour (8%), which differs from a previous report (Wannemacher 1989) that MC-LR was stable at 300°C.

Changes from pH 1 to pH 12 had little impact on either MC-LR or [Dha^7] MC-LR. Similarly the results indicated that both MC-LR and [Dha^7] MC-LR were stable when kept in the auto-sampler.

The developed method was applied to analyze the microcystin-polluted surface water taken from Donghu Lake in Wuhan City of China at October, 2007. Water samples were collected from different locations. These water samples were pre-treated and analyzed with the method described above. The results were shown in Fig. 2. The concentration of the [Dha^7] MC-LR in the water differed greatly from each other.

Conclusion

The simple extraction procedure for sample cleaning-up, the acceptable recoveries of analytes and the validation results demonstrate that this method can be used to determine MCs in water samples. The developed liquid chromatography coupled with electrospray ionization-ion trap mass spectrometry method for MC-LR and [Dha^7] MC-LR have been detected. This method is not only sensitive and selective but also reproducible. It is also applicable for detecting MCs in field samples. Both MCs wer stable with similar physiochemical behavior. Because they are very stable, both MCs are expected to accumulate in aquatic creatures such as fish.

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