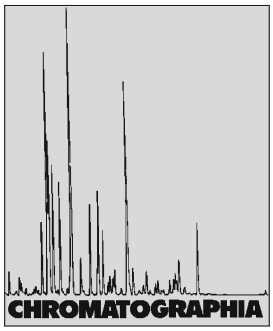
Quantitative Determination of Microcystins in Rat Plasma by LC–ESI Tandem MS



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Abstract

A rapid and sensitive method was developed and validated for the determination of MCYST (microcystin)-RR, -LR, and [Dha^7] MCYST-LR in rat plasma by liquid chromatography-tandem mass spectrometry. The analytes were extracted from rat plasma by protein precipitation, followed by solid-phase extraction. Liquid chromatography with electrospray ionization mass spectrometry, operating in selected reaction monitoring (SRM) mode, was used to quantify MCYST-RR, -LR, and [Dha^7] MCYST-LR in rat plasma. The recoveries for each analyte in rat plasma ranged from 70.8 to 88.7%. The calibration curve was linear within the range from 0.005 to 1.25 μg mL^{-1}. The limit of detection were 1.4, 1.0, 0.6 ng mL^{-1} for MCYST-RR, -LR, and [Dha^7] MCYST-LR. The overall precision was determined on three different days. The values for within- and between-day precision in rat plasma were within 15%. This method was applied to the identification and quantification of microcystins in rat plasma with acute exposure of microcystins via intravenous injection.

Keywords

Column liquid chromatography–mass spectrometry
Quantitative determination
Microcystins in rat plasma

Introduction

With the progress of eutrophication in freshwaters worldwide, the frequency and intensity of cyanobacterial bloom have increased greatly. Cyanobacteria toxins have quickly risen in infamy as important water contaminants that threaten human health [1]. Among these toxins, the hepatotoxic microcystins (MCYSTs) are considered to be one of the most dangerous groups [2]. Due to universality and durative, as well as biological activities [3, 4], MCYSTs are receiving much attention around the world as a public health concern [5, 6], especially after the disaster in Taihu Lake (the third largest freshwater lake in China) caused by the large bloom of blue-green algae. MCYSTs are monocyclic heptapeptides with the general structure of: cyclo- (D-alanine^1-*X*^{2}-D-MeAsp^3-*Z*^{4} -Adda^5-D-glutamate^6-Mdha^7) in which *X* and *Z* are variable L-amino acids, D-MeAsp^3 is D-erythro-*β*-methylaspartic acid, and Mdha is *N*-methyldehydroalanine. The amino acid Adda, (2*S*, 3*S*, 8*S*, 9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, is the most unusual structure in this group of cyanobacterial cyclic peptide toxins [7, 8]. Up to now, about 70 structural variants of MCYSTs have been successively isolated and identified [9], among which MCYST-RR, -LR, and [Dha^7] MCYST-LR (Fig. 1) are commonly found in freshwater cyanobacteria in China [10]. MCYST-LR was the most toxic followed by [Dha^7] MCYST-LR and MCYST-RR [11]. In recent studies, it has been found MCYSTs severely inhibit protein phosphatases 1 (PP-1) and 2A (PP-2A) and have a tumor-promoting activity in the rat liver [12, 13]. Following acute exposure to high doses, they cause death from liver hemorrhage or from liver failure, and may promote the

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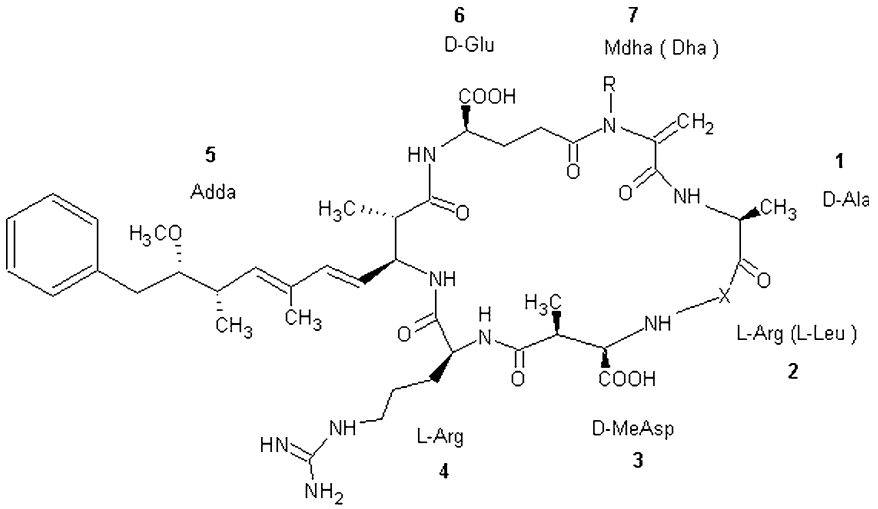

Fig. 1. Chemical structures of MCYST-RR, -LR, and [Dha^7] MCYST-LR

Table 1. Characteristic fragments ions of MCYSTs used for quantitation

Analyte	*m/z*	Fragment ions
MCYST-RR	520.0	$[M+2H]^{2+}$
	440.2	[Glu-Mdha-Ala-Arg + H]$^+$
	452.8	$[M+2H\text{-}134]^{2+}$
	887.3	$[M+H\text{-}134\text{-}NH_3]^+$
MCYST-LR	995.5	$[M+H]^+$
	599.2	[Arg-Adda-Glu + H]$^+$
	865.3	[Ala-Mdha-Glu-Adda-Arg-MeAsp-NH_3 + H]$^+$
	967.5	$[M+H\text{-}CO]^+$
[Dha^7] MCYST-LR	981.5	$[M+H]^+$
	599.2	[Arg-Adda-Glu + H]$^+$
	851.3	[Ala-Dha-Glu-Adda-Arg-MeAsp-NH_3 + H]$^+$
	953.4	$[M+H\text{-}CO]^+$

growth of liver and other tumors following chronic exposures to low doses [14, 15]. Many incidents of poisoning in humans and livestock caused by MCYSTs have been reported, among which the deaths of 50 Brazilian hemodialysis patients exposed to MCYSTs are the most famous [16, 17].

For better understanding of their pharmacokinetic action, clearance mechanisms and bioconversion process in vivo, the development of analytical methods with high sensitivity and selectivity for determination of MCYSTs in biological fluids and tissues was required. However, the assay and validation of MCYSTs by LC–MS in rat plasma has not been reported. Several analytical methods for MCYSTs, including protein phosphatase inhibition assay (PPIA) [18], enzyme linked immunosorbent assay (ELISA) [19], liquid chromatography (LC) [20–22], capillary electrophoresis (CE) [23, 24] and liquid chromatography-mass spectrometry (LC–MS) [25–27] have been reported. Because of its high specificity and sensitivity, liquid chromatography–tandem mass spectrometry (LC–MS–MS) has become the method of choice for quantitative determination of analytes in biological samples [28–30].

In this work, for the first time, a simple, sensitive, selective liquid chromatography–tandem mass spectrometry (LC–MS–MS) method was developed and validated for determination of MCYST-RR, -LR, and [Dha^7] MCYST-LR in rat plasma. The bioanalytical methodology was validated considering the specificity, linearity, precision, and limit of detection. The LC–MS–MS method was applied to investigate the plasma concentration–time profile of MCYST-RR, -LR, and [Dha^7] MCYST-LR in Wistar rats after the intravenous injection of MCYSTs extracted from surface blooms.

Experimental

Chemicals

MCYST-RR, -LR, and [Dha^7] MCYST-LR were isolated and purified from surface blooms collected from Lake Dianchi in China using an improved Ramanan method [31]. Acetonitrile was of LC grade (TEDIA, Fairfield, OH, USA). Formic acid (>99% purity) was purchased from Acros Organics (Geel, Belgium). Water was purified in a WaterPro PS system (Labconco, Kansas City, MO, USA). Other reagents were all analytical grade.

Instrumentation and Analytical Conditions

The liquid chromatography system (Thermo Electron, USA) consisted of a Surveyor LC pump, a Surveyor autosampler and a Surveyor photoelectric diode array (PDA) detector. The mass spectrometer was an LCQ Advantage MAX ion trap mass spectrograph (Thermo Electron, USA) equipped with electrospray ionization interface. Data acquisition was performed with Xcalibur 1.1 software. Peak integration and calibration were performed using Finnigan LCquan software.

An Agilent StableBond C18 column (2.1 × 150 mm, particle diameter 3.5 µm, Agilent, Santa Clara, CA, USA) was applied. The oven temperatures was set at 25 °C. Solution A of the elution

Table 2. Recoveries, precision for the different analytes in rat plasma

Spiked ($\mu g\ mL^{-1}$)	MCYST-RR			MCYST-LR			[Dha^7] MCYST-LR		
	Recovery (%)	RSD (%)		Recovery (%)	RSD (%)		Recovery (%)	RSD (%)	
		Inter-assay	Intra-assay		Inter-assay	Intra-assay		Inter-assay	Intra-assay
0.05	70.8 ± 4.7	10.1	7.2	75.1 ± 0.8	9.2	4.0	76.3 ± 5.5	9.1	7.1
0.25	87.0 ± 10.6	4.0	10.2	88.7 ± 4.2	11.4	2.7	84.1 ± 4.4	8.7	5.4
1.25	80.3 ± 9.6	5.8	10.5	85.8 ± 5.0	5.0	8.3	77.7 ± 7.1	3.0	2.9

system was formic acid–water solution (0.05%, *v*/*v*) while solution B was formic acid–acetonitrile solution (0.05%, *v*/*v*). Gradient conditions were initially 25% B to 55% B at 8 min, then 55% B to 60% B from 8 to 13 min at a flow rate of 200 $\mu L\ min^{-1}$. The injection volume was 10 μL.

Electrospray ionization (ESI) in positive ion mode was used. The optimized ionization conditions were: the sheath gas flow rate 20 units; the heated capillary temperature 250 °C; the spray needle voltage 4.5 kV for MCYSTs. Selected reaction monitoring (SRM) mode was chosen for quantitative analysis. The precursor ions were isolated and activated to produce fragment ions with the optimum relative collision energy from 37 to 42% for the analytes. The transitions were monitored at *m*/*z* 520.0 → 440.2 + 452.8 + 887.3 for MCYST-RR, *m*/*z* 995.5 → 599.2 + 865.3 + 967.5 for MCYST-LR, and *m*/*z* 981.5 → 599.2 + 851.3 + 953.4 for [Dha^7] MCYST-LR.

Clean-Up Procedure

Plasma samples (0.2 mL) were spiked with 0.2 mL of EDTA-Na_2 saturated aqueous solution. Then 0.8 mL of ice-cold acetonitrile were added. The combined samples were vortex-mixed for 1 min. After precipitation by acetonitrile, the samples were centrifuged at 13,000*g* for 15 min at 4 °C. The supernatant was transferred to another clean tube and evaporated to dryness. The residue was reconstituted in 200 μL of distilled water and applied to an Oasis HLB cartridge (60 mg/3 mL, Waters, Milford, MA, USA), which had been preconditioned by 100% methanol and distilled water. The column containing sample was washed with 5% MeOH 1 mL and then eluted with 100% MeOH 2 mL. Finally, the eluant collected from the HLB cartridges was evaporated to dryness and redissolved in 100 μL of the LC mobile phase. The aliquots (10 μL) were injected into the LC–MS system.

Preparation of Samples

Stock solutions of MCYST-RR, -LR, and [Dha^7] MCYST-LR were prepared by dissolving 2 mg of MCYST-RR, -LR, and [Dha^7] MCYST-LR in pure water. Quality control (QC) samples were prepared from the processed extract of plasma samples (0.2 mL) spiked with low, medium and high concentrations (0.05, 0.25, and 1.25 $\mu g\ mL^{-1}$). All solutions were stored at −20 °C before use.

Results and Discussion

Method Development

The positive and negative ion modes were compared and the MS parameters were optimized to obtain protonated ions of the analytes. By the positive mode, higher base peak intensities were obtained than those in the negative mode. In positive ion mode, MCYST-RR formed protonated ions $[M + 2H]^{2+}$ at *m*/*z* 520.0, MCYST-LR and [Dha^7] MCYST-LR formed protonated ions $[M + H]^{+}$ at *m*/*z* 995.5 and *m*/*z* 981.5 as major ion peaks, which were chosen as the precursor ions for the analytes. Three of the most abundant product ions for analytes were chosen in the SRM acquisition for MCYST. The main fragment ions used for quantitation are shown in Table 1. An Agilent StableBond C18 column was applied for the chromatographic separation. The mixture of acetonitrile and water with 0.05% formic acid was adopted as the mobile phase for the chromatographic separation. Acetonitrile was found to be more favorable for the chromatographic separation of the three analytes than methanol, which provided lower background noise, stable MS signal and higher sensitivity.

Sample preparation plays an important role for determination of MCYSTs in biological samples. The combination of sample precipitation and consecutive SPE was chosen to obtain clean extract of MCYSTs in rat plasma. Acetonitrile was chosen as precipitation agent. And the volume ratio of acetonitrile to the water phase is 2:1. Experiments showed that addition of EDTA-Na_2 to plasma could reach a higher recovery which indicated that MCYSTs might interact with the metal ions (e.g., Ca^{2+}) in plasma. After the sample precipitation, the Oasis HLB cartridge was applied. Compared to the clean-up by C18 cartridge for serum samples in the literature [32], the HLB sorbent gave higher and more reproducible recovery for MCYSTs. In the sample preparation, MCYSTs were added to whole blood and stirred uniformly. The samples were used directly or separated into plasma and cell pellet. The recovery of MCYSTs in whole blood and its different fractions (plasma and cell pellet) was analyzed. Results indicated that few MCYSTs were detected in the cell pellet and higher recovery was obtained for the plasma than that for whole blood. On the other hand, the matrix effect experiment also showed that the cellular compound (in the cell pellet) may suppress the ionization of the analytes (data not shown).

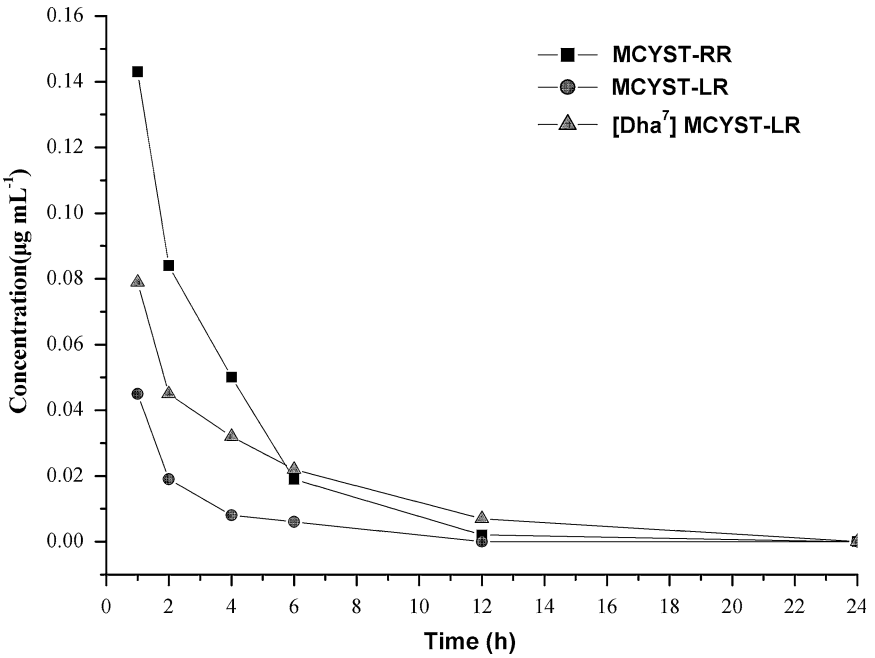

Fig. 2. The mean ($n = 3$) plasma concentration–time profiles for MCYST-RR, -LR, and [Dha^7] MCYST-LR after the intravenous injection of single dose of MCYSTs to Wistar rats

Selectivity and Matrix Effect

The specificity of the method was demonstrated by comparing chromatograms of blank samples and spiked rat plasma samples. Few interferences were detected from endogenous substances with MCYST-RR, -LR, and [Dha^7] MCYST-LR. The retention times were 8.47 min for MCYST-RR, 11.30 min for MCYST-LR and 11.33 min for [Dha^7] MCYST-LR. The matrix effect experiments indicated that no endogenous compounds significantly influenced the ionization of MCYST-RR, -LR, and [Dha^7] MCYST-LR.

Linearity

To evaluate the linearity of the LC–MS–MS method, the calibration curves were created for MCYST-RR, -LR, and [Dha^7] MCYST-LR in plasma matrices. The correlation coefficient (r^2) values for the calibration curves were both >0.99. The assay was proved to be linear and acceptable. Good linearity was observed over the concentration ranges of 0.005–1.25 μg mL^{-1} for MCYST-RR, -LR, and [Dha^7] MCYST-LR.

Precision and Detection Limits

The precision of the method was assessed in plasma samples by performing replicated analyses of spiked samples against calibration standards. The within-day and between-day precision of the method are presented in Table 2. The data indicate that the precision of the method were acceptable. The limit of detection (LOD) at signal-to-noise = 3 were 1.4, 1.0, 0.6 ng mL^{-1} for MCYST-RR, -LR, and [Dha^7] MCYST-LR. Although the levels found in our work were slightly higher than the results assayed by ELISA for serum samples (the LOD for MCYST-LR was 0.05 ng mL^{-1}) [33], LC-MS was more selective, robust and accurate.

Recovery

The recovery was determined for rat plasma spiked with low, medium and high concentrations of MCYST-RR, -LR, and [Dha^7] MCYST-LR with three replicates, respectively. The results were summarized in Table 2. The average recovery of three MCYSTs in rat plasma spiked with low, medium and high concentrations were all $>70\%$, which indicated that the recovery of MCYSTs from rat plasma was concentration-independent in the concentration range evaluated.

Stability

The freeze and thaw stability of the analytes were tested. The results showed that MCYST-RR, -LR, and [Dha^7] MCYST-LR were stable after three freeze–thaw cycles in 72 h. The short-term temperature stability experiments showed that of MCYST-RR, -LR, and [Dha^7] MCYST-LR at low and high concentrations in rat plasma samples were stable at room temperature for at least 24 h. The post-preparative stability of QC samples kept in the autosampler which was set 4 °C for 24 h was also assessed. The mean recoveries of the low and high QC level in rat plasma were all more than 90%, which suggested that MCYST-RR, -LR, and [Dha^7] MCYST-LR could remain at 4 °C for at least 24 h. Therefore, MCYST-RR, -LR, and [Dha^7] MCYST-LR could be considered to be stable in rat plasma matrices under frozen storage and assay processing.

Application

The method was applied to analyze the rat plasma samples obtained from Wistar rats after the intravenous injection of microcystins extracted from surface blooms collected from Lake Dianchi in China. The mean ($n = 3$) plasma concentration-time profiles for MCYST-RR, -LR, and [Dha^7] MCYST-LR are shown in Fig. 2. In plasma, the maximum concentrations of MCYST-RR, -LR, and [Dha^7] MCYST-LR were 0.143, 0.045, 0.79 μg mL^{-1} and all at 1 h. At 24 h, few MCYSTs could be detected.

Conclusion

An LC–MS–MS bioanalytical method was developed and validated for the simultaneous determination of MCYST-RR, -LR, and [Dha^7] MCYST-LR in rat

plasma. It was shown to be selective, sensitive and reproducible. Acceptable recoveries of analytes were obtained at three concentrations (low, medium and high), which were in the range 70.8–88.7% for the MCYSTs. Validation results demonstrated that this method can be used for determining low concentrations of MCYSTs in complex biological matrices such as rat plasma. It was successfully applied to determine the concentration–time profiles in pharmacokinetic studies.

Acknowledgments

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