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Simultaneous determination of microcystin-LR and its glutathione conjugate in fish tissues by liquid chromatography–tandem mass spectrometry

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Abstract

A sensitive and selective liquid chromatography–tandem mass spectrometry method was developed and validated for the simultaneous quantitative determination of microcystin-LR (MC-LR) and its glutathione conjugate (MC-LR-GSH) in fish tissues. The analytes were extracted from fish liver and kidney using 0.01 M EDTA–Na_2–5% acetic acid, followed by a solid-phase extraction (SPE) on Oasis HLB and silica cartridges. High-performance liquid chromatography (HPLC) with electrospray ionization mass spectrometry, operating in selected reaction monitoring (SRM) mode, was used to quantify MC-LR and its glutathione conjugate in fish liver and kidney. Recoveries of analytes were assessed at three concentrations (0.2, 1.0, and 5 $\mu g\,g^{-1}$ dry weight [DW]) and ranged from 91 to 103% for MC-LR, and from 65.0 to 75.7% for MC-LR-GSH. The assay was linear within the range from 0.02 to 5.0 $\mu g\,g^{-1}$ DW, with a limit of quantification (LOQ) of 0.02 $\mu g\,g^{-1}$ DW. The limit of detection (LOD) of the method was 0.007 $\mu g\,g^{-1}$ DW in both fish liver and kidney. The overall precision was determined on three different days. The values for within- and between-day precision in liver and kidney were within 15%. This method was applied to the identification and quantification of MC-LR and its glutathione conjugate in liver and kidney of fish with acute exposure of MC-LR.

Keywords: Microcystin-LR; Glutathione conjugate; LC–MS; Quantitative determination

1. Introduction

Microcystins (MCs) are potent monocyclic heptapeptides produced by many members of cyanobacteria, including *Microcystis*, *Anabaena*, and *Planktothrix* [1,2]. They have a common moiety composed of five amino acids: 3-amino-9-methoxy-l0- phenyl-2,6,8-trimethyldeca-4,6-dienoic acid (Adda), *N*-methyldehydroalanine (Mdha), D-alanine, β-linked D-erythro-β-methylaspartic acid and γ-linked D-glutamic acid, and two L-amino acids as variants [3]. Microcystins are named according to their variable L-amino acids, for example, MC-LR contains leucine (L) and arginine (R) [4]. Over 70 MCs have been successively isolated and identified [5], among which MC-LR is one of the most commonly occurring species [6]. To date, most of the works on microcystins have been conducted using the two variants L-amino acids as because of its presence in most countries reporting toxic episodes [7].

MC-LR has a relative molecular mass of about 995. Among the microcystins reported, MC-LR is one of the most toxic, with a median lethal dose (LD_{50}) of 50 $\mu g\,kg^{-1}$ body weight (BW) [1]. In recent studies, it has been found MC-LR is a potent inhibitor of protein phosphatases 1 and 2A and has a tumor-promoting activity in the rat liver [8,9]. Microcystins, especially MC-LR, cause adverse effects on mammals, birds, and fish and have been recognized as a potent stress factor and health hazard factor in aquatic ecosystems with heavy blooms occurring [10], and also represent a threat to human health through drinking water and the food chains [11]. In order to withstand increasing chemical stressors, many organisms have developed a complex detoxication metabolism, consisting of three phases: (1) activation (P450-monooxygenases); (2) conjugation (glutathione S-transferases, and other transferases); and (3) further processing, deposition, or excretion [12]. Microcystins are metabolized primarily by glutathione S-transferases (GSTs) [13]. The major

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GST-derived metabolites of MCs are the glutathione conjugates. This conjugation appears to be the first step of detoxication of MCs in aquatic organisms [14]. Thus, to better understand the mechanism of detoxification of MCs in aquatic organisms, the simultaneous determination of MCs and their glutathione conjugate is of great importance and urgency.

Due to the low concentrations of the MC-LR and its glutathione conjugate, and much disturbance species in fish tissues, it is needed to establish a selective and sensitive analytical technology for their identification and/or quantification in metabolic organs, such as liver and kidney. There have been many analytical methods for MC-LR, including protein phosphatase inhibition assay (PPIA) [15], enzyme-linked immunosorbent assay (ELISA) [16], liquid chromatography (LC) [17–19], capillary electrophoresis (CE) [20,21] and liquid chromatography–mass spectrometry (LC–MS) [22–24]. 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 [25–27]. For MC-LR-GSH, the previous studies primarily focused on the qualitative analyses and no quantitative analyses have been developed [14,28,29]. Kondo et al. [28] described the qualitative detection of the metabolites of microcystin-RR and -LR formed in vivo in mouse and rat liver by both HPLC and Frit-FAB LC/MS. Pflugmacher et al. [14] identified an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin MC-LR by HPLC and MALDI–TOF-MS. Ito et al. [29] applied an immunostaining method to the study of absorption and distribution of MC-LR and its glutathione conjugate in different tissues in mice. So far, there have been no literatures to report simultaneous quantitative determination of MC-LR and its glutathione conjugate in fish or other animal tissues.

This paper detailed, for the first time, an analytical method for the simultaneous quantitative determination of MC-LR and its glutathione conjugate in fish liver and kidney using SPE, and LCQ Advantage MAX ion trap LC/MS with ESI(+) and SRM. The extraction procedure was optimized in order to obtain sufficiently high recoveries for MC-LR and its glutathione con-

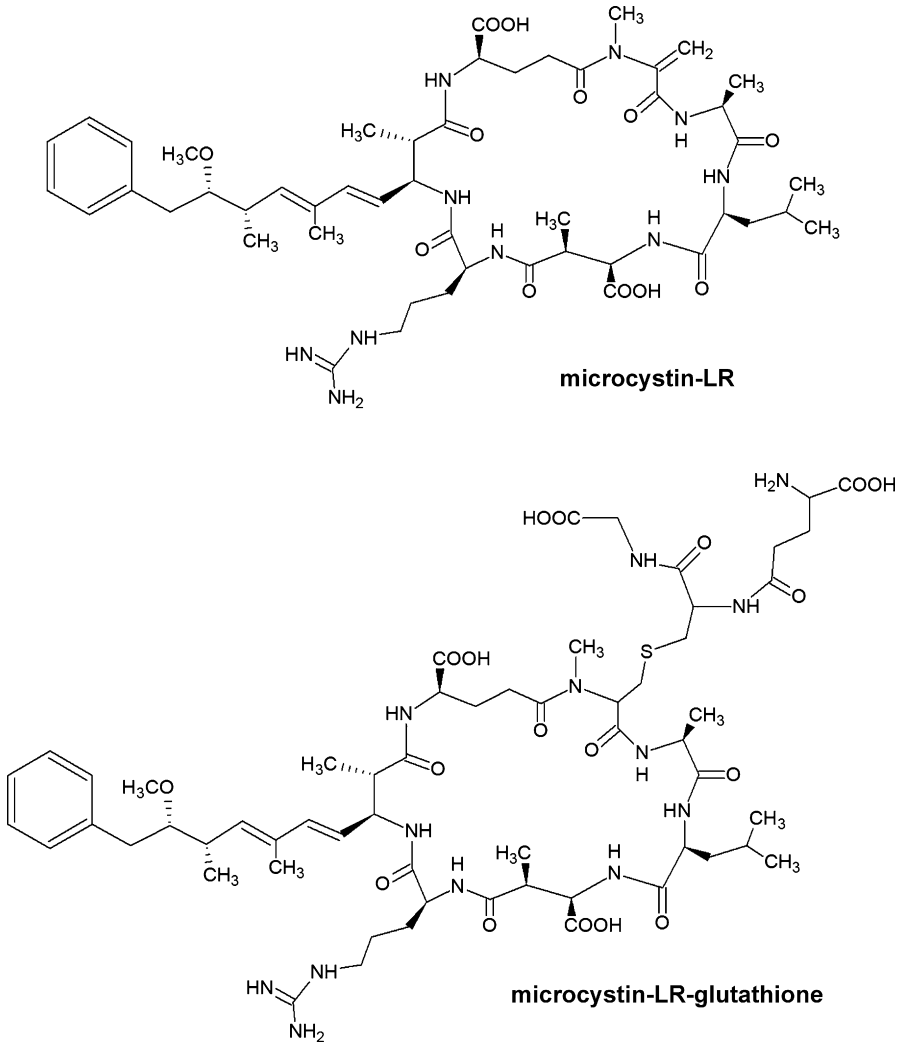

Fig. 1. Chemical structures of microcystin-LR and microcystin-LR-glutathione.

jugate, and the LC and MS/MS conditions were also investigated in order to detect these compounds at a very low concentration.

2. Experimental

2.1. Chemicals

MC-LR (Fig. 1) was isolated and purified from surface blooms collected from Lake Dianchi in China using an improved Ramanan method [30]. In this method, the extraction of microcystis cells was sequentially applied to an octadecylsilyl (ODS) cartridge and semi-prep-LC (Waters 600, USA). The content of purified MC-LR was over 95% and its identity was confirmed by LC–MS. MC-LR-GSH (Fig. 1) was prepared by modification of the method of Kondo et al. [31]. Briefly, MC-LR reacted with GSH in 5% potassium carbonate aqueous solution while stirring for 2 h at room temperature. The reaction mixture was neutralized with 0.2 M hydrochloric acid and applied to an ODS cartridge. The cartridge was rinsed with water and elute by methanol to give the reaction product. The reaction product was purified further by semi-prep-LC (Waters 600, USA). The content of purified glutathione conjugate of MC-LR was over 95% and confirmed by HPLC and LC–MS. The chemical characterization of MC-LR-GSH was performed by using multiple stages of MS^n. Glutathione (GSH) was purchased from Acros Organics (Geel, Belgium) and its purity was greater than 99%. Methanol was of HPLC grade (TEDIA company, INC., Fairfield, OH, USA). Water was purified in a WaterPro^{TM} PS system (Labconco Company, Kansas City, MO, USA). Other reagents were all analytical reagent grade.

2.2. Instrumentation

A Surveyor HPLC system (Thermo Electron Corporation, San Jose, CA, USA) including a Surveyor LC pump, a Surveyor autosampler and a Surveyor photoelectric diode array (PDA) detector combined with a LCQ Advantage MAX mass spectrograph (Thermo Electron Corporation, San Jose, CA, USA) equipped with electrospray ionization probe was used for all analyses.

An Agilent StableBond C18 column (2.1 mm i.d. × 150 mm length, 3.5 μm, Agilent Corporation, Santa Clara, CA, USA) was applied. 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). At analysis stage the analytes were eluted with the following gradient program: 0 min (95% A, 5% B), 0.5 min (65% A, 35% B), 14 min (25% A, 75% B), 21 min (25% A, 75% B). The total flow rate was held at 0.2 mL min^{-1} at analysis stage. After the analysis stage, the percentage of solution B was adjusted to 5% and the flow rate was increased to 0.3 mL min^{-1} for 4 min before the next injection to renew the initial condition rapidly. The injection volume was 10 μL. The temperatures of vial tray and column oven in the autosampler were set to 10 °C and 25 °C, respectively. The effluent was transferred on-line to ESI–MS system without splitting.

Table 1
MS/MS parameters for MC-LR and MC-LR-GSH

	MC-LR	MC-LR-GSH
Nominal molecular mass (Da)	994.5	1301.8
Isolation width (*m/z*)	2.0	2.0
Normalized collision energy (%)	36	24
Precursor ions (*m/z*)	995.5	652.2
Product ions (*m/z*)	599.2; 977.4	587.3; 1168.4

Infusion into the ion trap tandem mass spectrometer was performed as follows: the flow of standard compounds (5 μg mL^{-1}) coming from an integrated syringe pump at a flow rate of 5 μL min^{-1} for tuning the mass spectrometer and optimizing the ESI source. The ESI source and MS/MS parameters were automatically optimized and saved in a tune file. Spray needle voltage was set at 4.5 kV for MC-LR and MC-LR-GSH, automatic gain control (AGC) was on, maximum isolation time was 300 ms, and three microscans per scan were acquired. Voltages on capillary and tube lens were 45.5 and 55 V for MC-LR, and 30.2 and 50 V for MC-LR-GSH, respectively. These were set by automatic optimization using the LCQ autotune program on the mass spectrometer. Nitrogen was used as a sheath and auxiliary gas. Helium was used as a collision gas in the ion trap. The sheath gas flow rate was set at 20 units (a scale of arbitrary units in the 0–100 range defined in the LCQ system), the auxiliary gas was turned off, and capillary temperature was 250 °C for MC-LR and MC-LR-GSH. MS/MS parameters for MC-LR and MC-LR-GSH, including their precursor and product ions, collision energy and isolation width were summarized in Table 1.

2.3. Materials

Crucian carp (*Carassius auratus*), weighing 265 ± 22.6 g were obtained from a fish hatchery affiliated to College of Fisheries, Huazhong Agricultural University in Wuhan City, China. The liver and kidney were obtained from several untreated healthy fish, immediately frozen and freeze-dried for analytical method development. Before being used as matrices, the tissue samples were analyzed using the developed method in this study and no MC-LR and MC-LR-GSH were detected.

2.4. Sample preparation

The lyophilized liver and kidney samples (50 mg) were spiked with three concentration levels of MC-LR and MC-LR-GSH. The low, medium, and high levels consisted of 0.01, 0.05, 0.25 μg of MC-LR and MC-LR-GSH, respectively. Aliquots of the spiked lyophilized samples were homogenized in a mortar and added to centrifuge tubes. Fifty milligram samples were extracted three times with 5 mL water with EDTA–Na_2 (0.01 M)–5% acetic acid for sonicating 3 min (30% aptitude, 60 W, 20 KHz, Branson Digital Sonifier, Danbury, CT, USA) at 0 °C and then centrifuged at 12,000 × *g* (BR4, Jouan,Winchester, VA, France) at room temperature with three times repeated. Stock solutions were prepared by dissolving 2 mg of MC-LR and 2 mg MC-LR-GSH in pure water. Work-

ing standard solutions were prepared by serial dilutions of stock solutions using pure water. Quality control (QC) samples were prepared from the SPE extract of lyophilized liver and kidney samples (50 mg) spiked with low, medium and high concentrations (0.2, 1.0, and 5 $\mu g\,g^{-1}$ dry weight [DW]). All solutions were stored at −20 °C before use.

2.5. Cleanup procedure

The samples were purified according to the method of Xie et al. [32] with the replacement of the solid-phase extraction (SPE) sorbent from C18 to Oasis HLB cartridges. The newly optimized purification procedure was described as follows. The spiked liver or kidney sample (50 mg) was first applied to an Oasis HLB cartridge (500 mg/6 mL, Waters, Milford, MA, USA), which had been preconditioned by 100% methanol and distilled water. The column containing sample was washed with 20% MeOH 20 mL and then eluted with 100% MeOH 20 mL. The eluant collected from the HLB cartridges was evaporated to dryness. The residue was dissolved in 100% MeOH 2 mL and the solution was applied to a Sep-Pak silica gel (2 g/12 mL, Waters, Milford, MA, USA) cartridge which had been preconditioned by 100% MeOH. The silica gel column containing the analyte was washed with 20 mL of 100% MeOH and then eluted with 20 mL of 70% MeOH. 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.

2.6. Method validation

Calibration samples in liver and kidney were prepared by mixing a series of solutions at varying concentrations of MC-LR and MC-LR-GSH with fish blank liver and kidney sample extracts cleaned up by SPE to form a series of concentrations with 0.02, 0.10, 0.2, 1.0, 2.0, and 5.0 $\mu g\,g^{-1}$ MC-LR and MC-LR-GSH. Precision of the assay was determined by performing replicated analyses of QC samples against calibration standards. The precision of the method was calculated as the relative standard deviation (RSD) of observed concentration. The extraction recovery expressed as mean ± standard deviation (SD) was determined by calculating the ratio of the amount of extracted compound from drug-free liver and kidney spiked with known amounts of MC-LR and MC-LR-GSH to the amount of compound added at the same concentrations in the mobile phase solution. The stability of samples was also investigated by measuring lyophilized liver and kidney samples spiked with low and high concentrations and QC samples. Freeze–thaw stability, short-term temperature and post-preparative stability were determined according to analysis of biological samples guidelines by the FDA (available from URL:http://www.fda.gov/cder/guidance/4252fnl.htm). The limit of detection (LOD) was considered as three times the signal/noise ratio (S/N) and the LOQ was 10 times the S/N ratio. The selectivity of the method was characterized by assessing accurate quantification of MC-LR and MC-LR-GSH in the presence of endogenous compound, which was confirmed by the analysis of blank and QC samples.

2.7. Pharmacokinetic studies

The LC/MS/MS method was applied to pharmacokinetic studies of MC-LR and its glutathione conjugate in crucian carp (*C. auratus*). Crucian carp allowed to acclimate for 14 days, prior to experiment, and were fasted for 48 h before the initiation of the experiment. After healthy crucian carp were intraperitoneally injected with 100 μg MC-LR kg^{-1} BW, the liver and kidney were collected at 0, 1, 3, 12, 24, and 48 h postinjection, respectively. Tissue samples were sealed and stored at −20 °C until the analysis. Tissue samples were extracted and cleaned up following the methods described above. MC-LR and MC-LR-GSH concentrations in liver and kidney were determined by the LC–MS and three replicated analyses were performed.

3. Result and discussion

3.1. Characterization of MC-LR and its glutathione conjugate

MC-LR and its glutathione conjugate were first analyzed by LC–MS using the ion trap. For MC-LR, precursor ion was $[M+H]^+$ at *m/z* 995.5. Collision energy was 42%. 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). For MC-LR-GSH, the precursor ion was at *m/z* of 1302.8, which was consistent with the $[M+H]^+$ for MC-LR-GSH formed via addition of GSH to MC-LR and a two electron oxidation. The doubly protonated ion ($[M+2H]^{2+}$, *m/z* 652.0) was also observed (Fig. 2A). The $[M+H]^+$ at *m/z* 1302.8 was then used as precursor ion for a product-ion MS–MS scan. For subsequent MS^n experiments, MS^3 was carried out on *m/z* 1173.5 and MS^4 was carried out on *m/z* 995.5. And collision energy for MS^2, MS^3 and MS^4 were 30%, 34%, 42%, respectively. The resulting product ion mass spectra for MC-LR-GSH are shown in Fig. 2. Fig. 2B demonstrated the abundant product ion at *m/z* 1173.5, which was formed from the ion at 1302.8 by loss of a neutral fragmentation 129 Da. Fig. 2C gave two prominent fragmentation ions at *m/z* 1155 and 995. The ion at *m/z* 1155 was formed from 1173 by loss of H_2O. The ion at *m/z* 995 was formed from 1173 by loss of 178 Da (Gly–Cys). It can also be assigned as $[M+H-GSH]^+$. Fig. 2D showed product ions at *m/z* 599.3, 553.2, 866.4, 967.4 and 977.4, which are identical with the product ions of MC-LR. These data obtained from dissociating the ion sequentially using MS^n were also consistent with the structure of MC-LR-GSH described in Fig. 1. The characteristic fragment ions in the (+)-ESI–MS^n spectra of MC-LR-GSH are shown in Table 2.

3.2. Method development

In the sample preparation, we compared the effect of different extraction solvents on recovery of analytes in liver and kidney (Fig. 3). The extraction solvents evaluated were acidi-

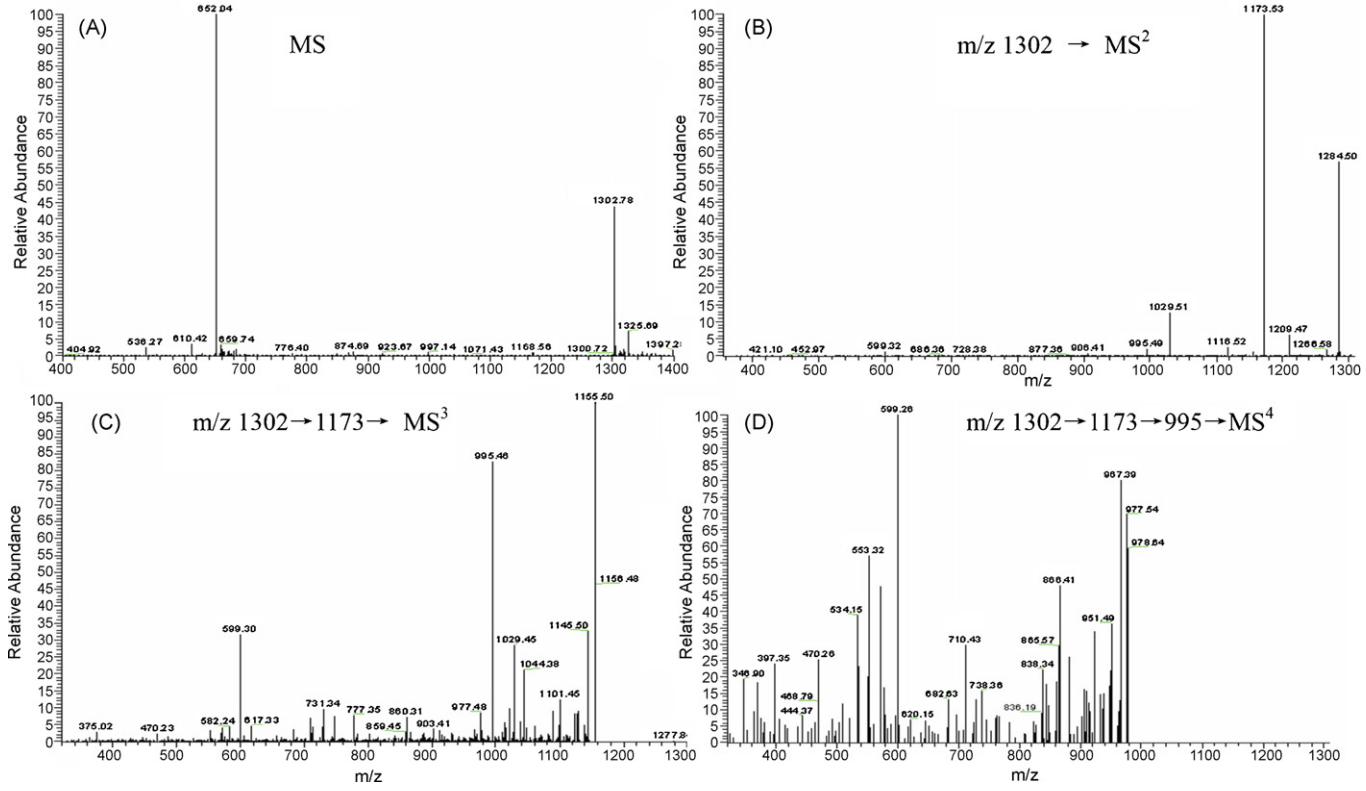

Fig. 2. (+)-ESI–MS^n spectra of microcystin-LR-glutathione. (A) MS scan, (B) MS^2 scan, (C) MS^3 scan, (D) MS^4 scan.

fied methanol (0.05% TFA, v/v), EDTA–Na_2 (0.01 M)–acidified methanol (0.05% TFA, v/v), 5% acetic acid and EDTA–Na_2 (0.01 M)–5% acetic acid. Experiments performed in triplicate showed that EDTA–Na_2 (0.01 M)–5% acetic acid yielded the best recovery (over 90% for MC-LR and nearly 70% for MC-LR-GSH) (Fig. 3.). The unsatisfied results for acidified methanol (0.05% TFA, v/v) and EDTA–Na_2 (0.01 M)–acidified methanol (0.05% TFA, v/v) were speculated to be the presence of much more endogenous compounds which resulted in the obvious ion suppression in the assay processing. Our results also indicated that MC-LR-GSH might interact more intensively with protein and/or metal ions than MC-LR because of the added –COOH and $-NH_2$ (Fig. 1), which resulted in lower recoveries. Therefore, the EDTA–Na_2 (0.01 M)–5% acetic acid system was chosen and tested as an efficient solvent.

Table 2
Characteristic fragment ions in the (+)-ESI–MS^n spectra of MC-LR-GSH

Fragment ions	*m/z*
$[M+H]^+$	1302.8
$[M+2H]^{2+}$	652.0
$[M-Glu+H]^+$	1173.5
$[M-H_2O+H]^+$	1284.5
$[M-H_2O-Glycine+H]^+$	1209.5
[Ala–Leu–MeAsp–Arg–Adda–Glu–Mdha–SH + H]$^+$	1029.5
$[M-Glu+Gly+H]^+$	1116.5
$[M-Glu+H_2O+H]^+$	1155.5
$[M-Glu+CO+H]^+$	1145.5
[Ala–Leu–MeAsp–Arg–Adda–Glu–Mdha + H]$^+$	995.5
[Arg–Adda–Glu + H]$^+$	599.3
[Ala–Leu–MeAsp–Arg–Adda–Glu–Mdha $-$ H_2O + H]$^+$	977.5
[Ala–Leu–MeAsp–Arg–Adda–Glu–Mdha $-$ CO + H]$^+$	967.4
[Mdha–Ala–Leu–MeAsp–Arg–Adda + H]$^+$	866.4
[Mdha–Ala–Leu–MeAsp–Arg + H]$^+$	553.3

After the sample preparation, a cleanup method utilizing a combination of HLB and silica gel cartridge was chosen to obtain clean extract of MC-LR and MC-LR-GSH. The HLB sorbent was a copolymer designed to have a hydrophilic–lipophilic balance that gave higher and more reproducible recoveries for MC-LR and MC-LR-GSH than C18 sorbent.

In this process, the suppression of ionization was evaluated by comparing the absolute peak areas of the extracts of liver and kidney spiked with a known amount of analytes to neat standard injected directly in the same reconstituted solvent. Results indicated that combination of HLB and silica gel cartridge improved the sample clean-up and thereby decreased the amount of matrix injected onto the column and the ion suppression effect was minimized to less than 12%. While the HLB cartridge was used separately, the ion suppression effect was more than 50%.

An Agilent StableBond C18 column was applied for the chromatographic separation and a C18 guard column was used to protect the analytical column. Other chromatographic conditions, especially the composition of mobile phase, were optimized through several trails to obtain good resolution, symmetric peak shapes and a short run time. It was found that use of a mixture of methanol and water with 0.05% formic acid could

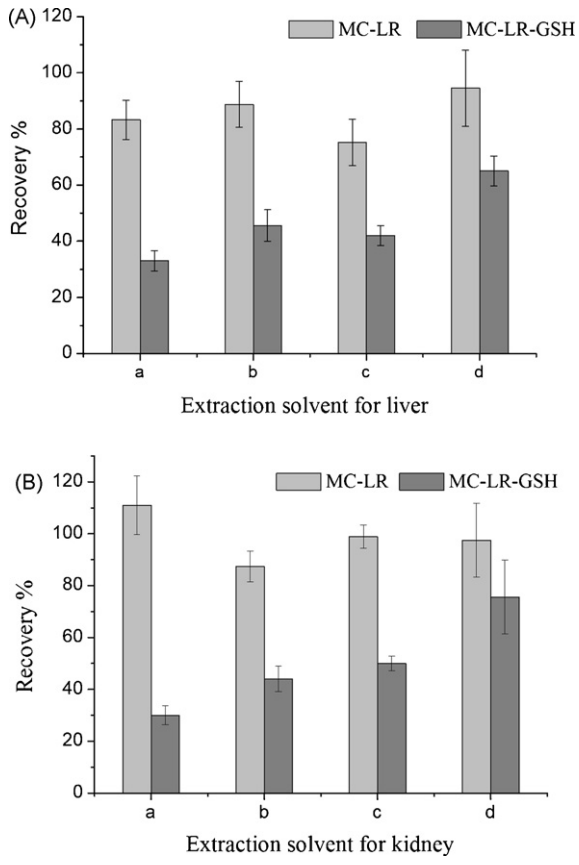

Fig. 3. Recoveries of MC-LR and MC-LR-GSH spiked in liver (A) and kidney (B) by different extraction solvent systems: a, acidified methanol (0.05% TFA, v/v); b, EDTA–Na_2 (0.01 M)–acidified methanol (0.05% TFA, v/v); c, 5% acetic acid; d, EDTA–Na_2 (0.01 M)–5% acetic acid.

achieve these goals and was finally adopted as the mobile phase for the chromatographic separation.

For the quantification of MC-LR and MC-LR-GSH in fish liver and kidney, some parameters related to mass spectrometric detection were investigated. The capillary temperature, vaporizer temperature, and flow rate were optimized to obtain protonated molecules of the analytes. The fragment energy was optimized to achieve maximum response of the fragment ion peaks. Selected reaction monitoring (SRM) using tandem mass spectrometer (MS/MS) refers to the dedicated use of a mass spectrometer to acquire and record ion current at only selected mass-to-charge ratio (*m*/*z*) values, resulting in higher analytical sensitivity and selectivity in complex matrices. Considering the interference from many ion of the same *m*/*z* ratio in the tissue samples and higher signal-noise ratio, SRM mode was chosen in the MS/MS experiments. The protonated precursor molecular ions $[M+H]^+$ of MC-LR (*m*/*z* 995.5) and the protonated precursor molecular ions $[M+2H]^{2+}$ of MC-LR-GSH (*m*/*z* 652.0) were selected and fragmented by helium gas collision in the ion trap at a relative collision energy of 24%. For MC-LR, the most abundant product ions were at *m*/*z* 599.2 ($[Arg–Adda–Glu+H]^+$) and 977.4 ($[M+H–H_2O]^+$) and for MC-LR-GSH at *m*/*z* 587.3 ($[M+2H–MeAsp]^{2+}$) and 1168.4 ($[M+H–Adda]^+$). These product ions were extracted for quantification (Fig. 4).

3.3. Selectivity

LC–MS analysis of extracts of fish liver and kidney indicated little interference from fish tissue matrix compounds as no interfering peaks were present at the known retention time and mass-to-charge ratios of MC-LR and MC-LR-GSH. The

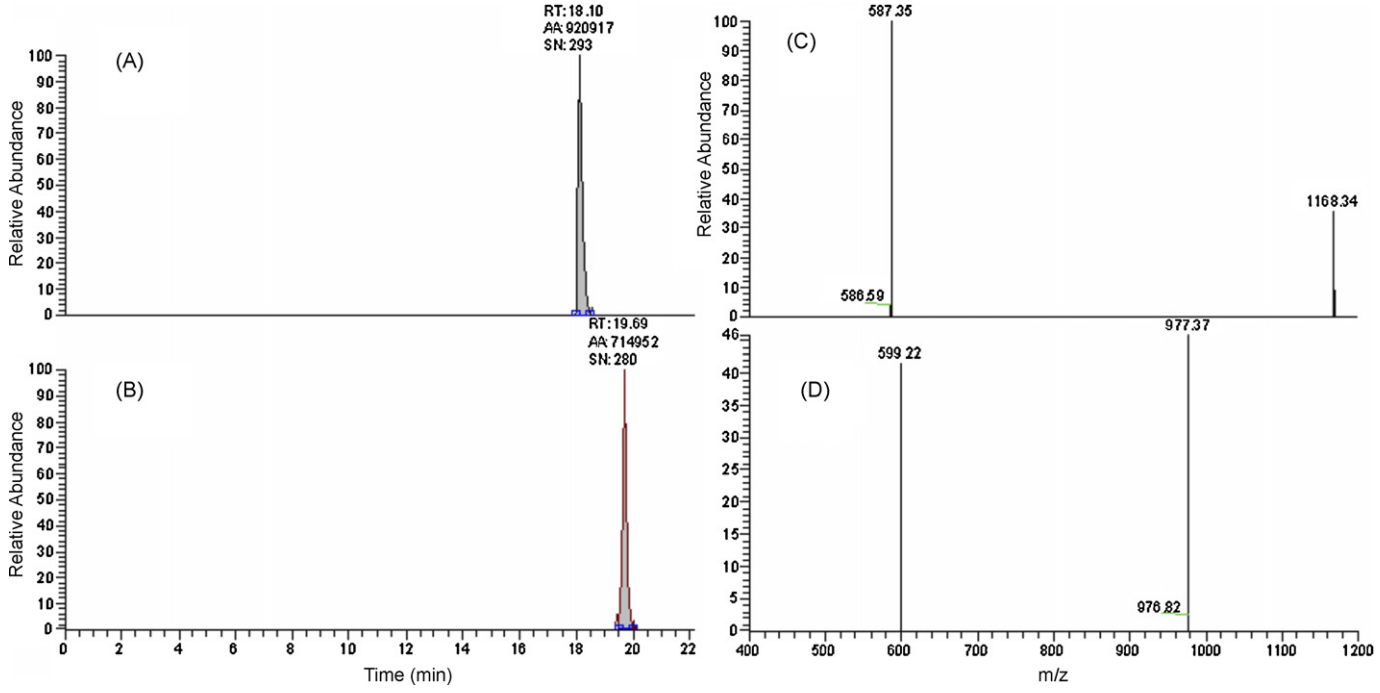

Fig. 4. Selected reaction monitoring (SRM) chromatogram and product ion mass spectrum of the studied MC-LR-GSH (A, C) and MC-LR (B, D). Spiking concentrations of MC-LR and MC-LR-GSH were 0.5 μg mL^{-1}.

Table 3
Calibration equations for the determination of MC-LR and MC-LR-GSH by liquid chromatography ion-trap tandem mass spectrometry (X, concentration $\mu g\,mL^{-1}$; Y, peak area)

Compound	Sample	Regression equations	r^2
MC-LR	Liver	$Y = -47031.8 + 1.84172e+006 \times X$	0.9943
	Kidney	$Y = -35664.4 + 1.91313e+006 \times X$	0.9968
MC-LR-GSH	Liver	$Y = 22553.4 + 1.76444e+006 \times X$	0.9940
	Kidney	$Y = -3152.94 + 1.70044e+006 \times X$	0.9953

retention times were 19.6 min for MC-LR and 18.1 min for MC-LR-GSH. The product ions for quantitative determination of analyte were at *m/z* 599.2, 977.4 for MC-LR and *m/z* 587.3, 1168.4 for MC-LR-GSH.

3.4. Linearity

To evaluate the linearity of the LC/MS/MS method, the calibration curves were created for MC-LR and MC-LR-GSH both in liver and kidney matrices (Table 3). The correlation coefficient (r^2) values for the calibration curves were both >99%. The assay was proved to be linear and acceptable. Good linearity was observed over the concentration ranges of 0.02–5.0 $\mu g\,g^{-1}$ DW for liver and kidney.

3.5. Precision and detection limits

The precision of the method was assessed in both liver and kidney by performing replicated analyses of spiked samples against calibration standards. The procedure was repeated on the same day and for different days on the same spiked standard series. The within-day and between-day precisions of the method are presented in Table 4. The data indicate that the precision of the method are acceptable. LOD and LOQ were calculated using QC samples with low concentration of MC-LR and MC-LR-GSH. The LOD and LOQ values were 0.02 $\mu g\,g^{-1}$DW and 0.007 $\mu g\,g^{-1}$DW, respectively, in both fish liver and kidney.

3.6. Recovery

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrices compared to the detector response obtained for the true concentration of the pure authentic standard. The recovery was determined for fish liver and kidney spiked with low, medium, and high concentrations of MC-LR and MC-LR-GSH with three replicates, respectively. The results are summarized in Table 5. The average recovery of MC-LR in liver and kidney spiked with low, medium, and high concentrations were all >90% and of MC-LR-GSH >70% except the liver sample spiked with low concentration, which indicated that the recovery of MC-LR and MC-LR-GSH from fish liver and kidney was concentration-independent in the concentration range evaluated.

3.7. Stability

The freeze and thaw stability of the analytes was tested. Three aliquots at each of the low and high concentrations in liver and kidney were kept at −20 °C for 24 h and thawed unassisted at room temperature. After a complete thaw, the samples were refrozen for 24 h under the same conditions. The freeze–thaw cycle was repeated three times before analysis. The results

Table 4
Precisions of the method expressed as RSD for the different analytes in liver and kidney

Analyte	Inter-assay RSD (%, $n = 5$)			Intra-assay RSD (%, 3 days)		
	Low[a]	Medium[b]	High[c]	Low	Medium	High
MC-LR (liver)	11.2	7.5	2.5	11.4	12.8	4.8
MC-LR-GSH (liver)	2.3	8.8	8.8	12.9	8.6	8.3
MC-LR (kidney)	4.5	6.9	2.1	7.1	4.9	2.9
MC-LR-GSH (kidney)	5.6	4.7	3.0	11.1	6.4	5.3

[a] Low fortification: 0.2 $\mu g\,g^{-1}$ for MC-LR and MC-LR-GSH.
[b] Medium fortification: 1.0 $\mu g\,g^{-1}$ for MC-LR and MC-LR-GSH.
[c] High fortification: 5.0 $\mu g\,g^{-1}$ for MC-LR and MC-LR-GSH.

Table 5
Recoveries of MC-LR and MC-LR-GSH in fish liver and kidney (mean ± SD, $n = 3$)

Treatment	Spiked ($\mu g\,g^{-1}$)	MC-LR		MC-LR-GSH	
		Mean ± SD (%)	RSD (%)	Mean ± SD (%)	RSD (%)
Liver	0. 2	94.5 ± 13.5	14.2	65.0 ± 5.2	8.1
	1.0	91.0 ± 4.3	4.82	72.7 ± 2.2	3.1
	5.0	95.8 ± 18.1	18.8	75.3 ± 2.03	2.7
Kidney	0.2	97.5 ± 14.3	14.6	75.6 ± 14.2	18.7
	1.0	97.8 ± 9.7	9.9	74.2 ± 2.5	3.3
	5.0	103.6 ± 11.8	11.3	72.8 ± 5.4	7.5

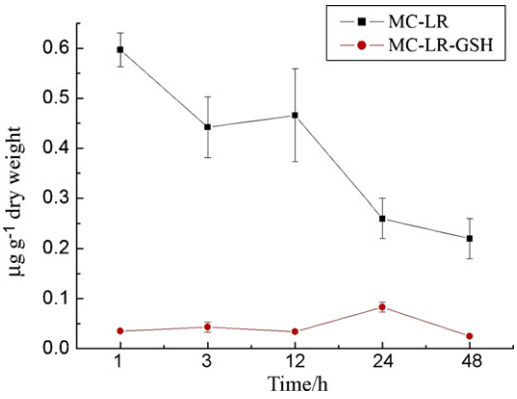

Fig. 5. The mean ($n = 3$) liver concentration–time profiles for MC-LR and MC-LR-GSH after the intraperitoneal injection of single dose of MC-LR (100 μg kg^{-1} BW) to crucian carps.

showed that MC-LR and MC-LR-GSH were stable after three freeze–thaw cycles in 72 h. The short-term temperature stability experiments showed that MC-LR and MC-LR-GSH at low and high concentrations in liver and kidney 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 liver and kidney were more than 90%, which suggested that MC-LR and MC-LR-GSH could remain at 4 °C for at least 24 h. Therefore, MC-LR and MC-LR-GSH could be considered to be stable in both liver and kidney matrices under frozen storage and assay processing.

3.8. Application

The method was applied to analyze the liver and kidney samples obtained from crucian carp after the intraperitoneal injection of single dose of MC-LR. The mean ($n = 3$) liver concentration–time profiles for MC-LR and MC-LR-GSH were shown in Fig. 5. In the liver, the maximum concentration of MC-LR was 0.59 μg g^{-1} DW at 1 h and the maximum concentration of MC-LR-GSH was 0.083 μg g^{-1} DW at 24 h. In the kidney, no MC-LR and MC-LR-GSH was detected.

4. Conclusion

For the first time, the LC/MS/MS method was developed and validated for the simultaneous determination of MC-LR and its glutathione conjugate in fish tissues. 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 of 91–103% for MC-LR and 65.0–75.7% for MC-LR-GSH. Validation results demonstrated that this method can be applied for determining low concentrations of MC-LR and its glutathione conjugate in complex biological matrices such as fish liver and kidney in pharmacokinetic studies. Following a slight modification, this method can be also used to determine MC-LR and its glutathione conjugate in various tissues of other aquatic organisms. It is currently being used to further pharmacokinetic studies.

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