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Quantitatively evaluating detoxification of the hepatotoxic microcystins through the glutathione and cysteine pathway in the cyanobacteria-eating bighead carp

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ABSTRACT

Glutathione (GSH) and cysteine (Cys) conjugation have long been recognized to be important in the detoxification of microcystins (MCs) in animal organs, however, studies quantitatively estimating this process are rare, especially those simultaneously determining multiple toxins and their metabolites. This paper, for the first time, simultaneously quantified MC-LR (leucine arginine), MC-RR (arginine arginine), MCLR-GSH/Cys and MCRR-GSH/Cys in the liver, kidney, intestine and muscle of the cyanobacteria-eating bighead carp i.p. injected with two doses of MCs using liquid chromatography electrospray ionization mass spectrometry (LC–ESI-MS). MCLR-Cys and MCRR-Cys content were much higher in kidney than in liver, intestine and muscle, suggesting the organotropism to kidney, while MCLR-GSH and MCRR-GSH were always below the detection limit. Bighead carp effectively metabolized MC-LR and MC-RR into the cysteine conjugates in kidney, as the ratios of MCLR-Cys to MC-LR and MCRR-Cys to MC-RR reached as high as 9.04 and 19.10, respectively. MC-LR and MC-RR were excreted mostly in the form of MCLR/RR-Cys rather than MCLR/RR-GSH, while MCs-GSH might act as mid-metabolites and changed to the more stable MCs-Cys rapidly. Cysteine conjugation of MCs appears to be an important biochemical mechanism for the cyanobacteria-eating fish to resist toxic cyanobacteria. A comparison of such detoxification mechanisms between fish and mammals would be interesting in the future studies.

1. Introduction

Microcystins (MCs) are a family of potent hepatotoxins produced by cyanobacteria. To date, more than 90 analogues of MCs have been identified (Ufelmann et al., 2012), among which MC-LR (leucine arginine), and RR (arginine arginine) are the most common variants (Gupta et al., 2003). It is well known that MCs are tumor promoter (MacKintosh et al., 1990), due to their abilities of strong inhibition of protein phosphatase 1 and 2A (MacKintosh et al., 1990; Matsushima et al., 1990; Nishiwaki-Matsushima et al., 1992; Yoshizawa et al., 1990). The high incidence of primary liver cancer in eastern China is considered to be related to the presence of MCs in drinking water (Ueno et al., 1996). MCs can cause illnesses and even death in human (Azevedo et al., 2002). In Brazil, more than 50 patients died due to the use of MCs contaminated hemodialysis waters (Pouria et al., 1998). Recently, MCs were detected in the serum of fishermen surrounding Lake Chaohu (China) with dense toxic *Microcystis* blooms (Chen et al., 2009). A large amount of studies show that MC-RR and MC-LR are the two most important variants accumulating in aquatic animals, and MC-RR is often more abundant than MC-LR (Cazenave et al., 2005; Li et al., 2005; Pietsch et al., 2001; Xie et al., 2004).

Many studies have demonstrated that glutathione plays an important role in the detoxification of MCs in both mammals and aquatic organisms (Ito et al., 2002; Kondo et al., 1992, 1996; Pflugmacher et al., 1998; Zhang et al., 2009). For example, the glutathione and cysteine conjugates of MCs were identified by Frit-FAB LC/MS in liver samples of mouse and rat treated with MCs (Kondo et al., 1992, 1996). Meanwhile, the formation of MC-LR glutathione conjugate has been proved in various aquatic animals in vitro and is suggested to be the first step in detoxification of MCs in aquatic animals (Pflugmacher et al., 1998). In addition, distributions of MCLR-GSH and MCLR-Cys in intestine and kidney were further confirmed in mice by immune-staining methods (Ito et al., 2002). However, previous studies primarily focused on qualitative but not quantitative analysis, and only recently, LC–ESI-MS was used to quantify MC-LR and its glutathione conjugates in fish organs

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(Dai et al., 2008). Using this method, Zhang et al. (2009) described the seasonal changes of MC-LR and its glutathione and cysteine conjugates in three aquatic animals from Lake Taihu, and raised a hypothesis that, in detoxification of MC-LR, MCLR-Cys might play a more important role than MCLR-GSH in wild fish. More recently, Wu et al. (2010) developed a method for extraction, identification and quantification of MC-RR and its metabolites (MCRR-GSH and MCRR-Cys) in fish liver. Nevertheless, so far there is no literature on simultaneous quantification of MC-RR, MC-LR and their glutathione and cysteine conjugates in fish or other animal organs either under laboratory or field conditions. Hence, it is an imperative need to understand the biotransformation and detoxification mechanisms of these two toxin variants in animals.

The present study was therefore undertaken (1) to simultaneously examine the dynamics of MC-LR, MC-RR and their metabolites (MCLR-GSH/Cys, MCRR-GSH/Cys) in selected organ tissues of bighead carp (*Aristichthys nobilis*), after i.p. injections of different dosage of MCs, and (2) to identify the roles of glutathione and cysteine conjugates of MCs in detoxification process, so as to provide information regarding the metabolic mechanisms of bighead carp to resist MC stress.

2. Materials and methods

2.1. Materials

MC-LR and MC-RR (Fig. 1) were extracted and purified from surface blooms collected from Lake Dianchi in China with an improved Ramanan method (2000). The content of purified MC-LR and MC-RR was over 95% and its identity was confirmed by LC–MS (Thermo Electron, Waltham, MA, USA). MCLR-GSH, MCLR-Cys, MCRR-GSH and MCRR-Cys (Fig. 1) were prepared following the method of Dai et al. (2008) and Wu et al. (2010). L-Glutathione and L-Cysteine (>99% purity) were purchased from Acros Organics (Geel, Belgium). Aqueous ammonia (25%, AR) and formic acid (FA) were purchased from SCRC and Aldrich, respectively. Methanol and acetonitrile were of HPLC grade (Fisher Scientific International, Inc, USA). Ultrapure water was collected from a Milli-Q water purification system from Millipore (Bedford, MA, USA). Other reagents were all of analytical reagent grade.

2.2. Animal experiment and sample collection

Bighead carp, a freshwater cyanobacteria-eating fish, is very important to Chinese people because of its use as food fish and also for biological control of cyanobacterial blooms (Xie and Liu, 2001). Healthy bighead carps (*Aristichthys nobilis*) with mean weight of 250 ± 29 g and mean body length of 20.75 ± 1.07 cm were obtained from a local fish hatchery (Wuhan City, China). Fish were acclimated for two weeks in nine aquaria (150 L, $n = 18$ fish/aquarium) containing dechlorinated tap water and fed with commercial bighead carp food at a rate of 2% of body weight per day. Feeding was terminated 2 days before initiation of the experiment, and no food was supplied to fish during the experimental period. Water temperature was controlled at $20 \pm 1\,^{\circ}C$, and dissolved oxygen was 6.8 ± 0.7 mg/L. At the initiation of the experiment, the fish were divided at random into three groups with 54 animals in each group. In two groups, the fish were administered a single i.p. injection of approximately 1 mL suspension of extracted solution of MCs (contain MC-RR and -LR, purity > 95%) at a dose of either 50 or 200 μg MC-LR_{eq} kg^{-1} bw. The control group was injected the same volume of vehicle, 0.9%, w/v saline solution.

For each group, nine fish were sacrificed at 1, 3, 12, 24, 48 and 72 h post-injection, respectively. Sampled fish were anesthetized with 0.02% tricaine methane sulfonate and subsequently killed by a blow to the head. After dissected, liver, kidney, intestine (no contents were present) and muscle were collected and frozen at $-80\,^{\circ}C$ immediately.

2.3. Sample preparation

For the analyses of fish organs, resources (e.g. kidney) did not allow analysis of individual replicate fish. Therefore, we pooled, respectively, all liver, kidney, intestine and muscle of three dissected fish for each group and time point. Thus, each value represents an average amount of MCs or their conjugates in the organs of three individuals and triplicate composite samples per time point were analyzed for each group.

All samples were lyophilized by Christ@ Alpha 2–4 freeze dryer (Martin Christ, Osterode, Germany). 0.2 g lyophilized samples were extracted three times with 5 mL of 0.01 M EDTA-Na_2-5% acetic acid by ultrasonication for 3 min (30% amplitude, 60 W, 20 KHz, Sonics VC130 PB, Newtown, CT, USA) at $0\,^{\circ}C$ and then centrifuged at $15{,}000 \times g$ (BR4, Jouan, Winchester, VA, France) at $4\,^{\circ}C$. The supernatant was treated by the method of Dai et al. (2008) and Wu et al. (2010), respectively, for the extracting and enriching MC-LR/RR and their metabolites.

2.4. Analysis of MCs and their metabolites

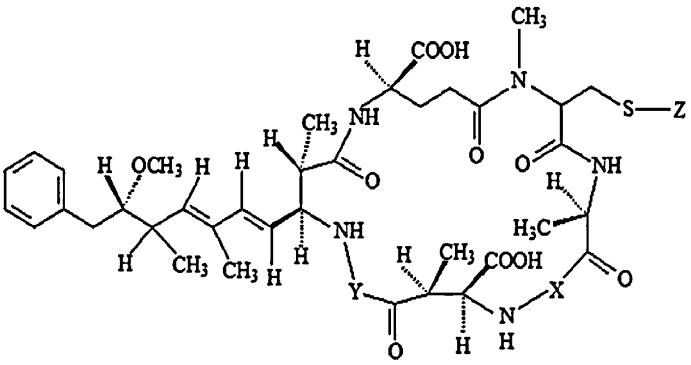
Qualitative and quantitative analyses were performed by a Finnigan LC–MS system comprising a Thermo Surveyor auto sampler, a Surveyor mass spectrum pump, a Surveyor photo diode array system (set at 238 nm), and a Finnigan LCQ-Advantage MAX ion trap mass spectrometer (Thermo Electron, Waltham, MA, USA) equipped with an atmospheric pressure ionization fitted with an electrospray ionization source (ESI) (Thermo Electron). A 2.1 mm × 100 mm (3.5 μm) Waters XBridge C18 column (Waters Corporation, USA) with a 2.1 mm × 10 mm (5 m) C18 guard column (Thermo Electron Corporation, USA) was used for separation. The instrument control, data processing, and data analysis were conducted by using Xcalibur software (Thermo Electron).

2.4.1. Chromatographic separation

The mobile phase consisted of solvent A [water + 0.05% (v/v) formic acid]/solvent B [acetonitrile + 0.05% formic acid]. The following linear gradient program: 0 min (75% A, 25% B), 8 min (45% A, 55% B), 13 min (40% A, 60% B), 14 min (30% A, 70% B), 15 min (75% A, 25% B), 20 min (75% A, 25% B), was applied to separate MC-LR, MCLR-GSH and MCLR-Cys. The gradient program: 0 min (95% A, 5% B), 1.0 min (65% A, 35% B), 17.0 min (55% A, 45% B), 17.5 min (30% A, 70% B), 18.0 min (5% A, 95% B), 20.0 min (5% A, 95% B), 20.01 min (95% A, 5% B), 25.0 min (95% A, 5% B), was used for separating MC-RR, MCRR-GSH and MCRR-Cys. The vial temperature was set at $10\,^{\circ}C$, and column temperature was set at $25\,^{\circ}C$ for MC-RR, MCRR-GSH and MCRR-Cys, and $40\,^{\circ}C$ for MC-LR, MCLR-GSH and MCLR-Cys. The total flow rate was set at 0.2 mL/min at analysis stage. After the analysis stage, the flow rate was increased to 0.3 mL/min for 5 min before the next injection to renew the initial condition rapidly. 10 μL sample solution was injected for each run.

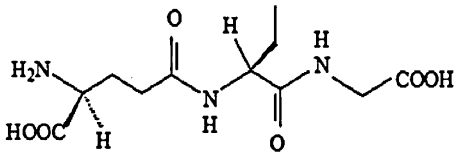
2.4.2. MS/MS parameters

Before quantitative analysis, syringe pump was applied for tuning the mass spectrometer and optimizing the ESI source. The MS analytical conditions were set as follows: The ESI spray voltage 4.5 kV; sheath gas flow rate 20 units; auxiliary gas 4.5 units; multiplier voltage −852 V; tube lens voltage, 55 V for MC-LR, 50 V for MCLR-GSH and MCLR-Cys, 55.5 V for MC-RR, 45.5 V for MCRR-GSH and MCRR-Cys; collision energy, 36% for MC-LR, 24% for MCLR-GSH, 34% for MCLR-Cys, 40% for MC-RR and MCRR-GSH, and 34%



	X	Y
Microcystin LR	Leu	Arg
Microcystin RR	Arg	Arg

GSH conjugate Z=



Cys conjugate Z=

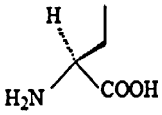


Fig. 1. Chemical structures of MC-LR/RR and their GSH and Cys conjugates. MC = microcystin, GSH = glutathione, Cys = cysteine.

for MCRR-Cys, automatic gain control (AGC) on maximum isolation time was 300 ms, and three microscans per scan were acquired.

The limit of detection (LOD) for MC-LR and its conjugates (MCLR-GSH and MCLR-Cys) was 0.007 $\mu g\,g^{-1}$ DW. For MC-RR, MCRR-GSH and MCRR-Cys, the LOD was 0.005 $\mu g\,g^{-1}$ DW.

2.5. Recovery experiment

Recovery experiments were carried out in quadruplicate spiking 200 mg of homogenized freeze-dried fish liver samples with MC-LR/RR, MCLR/RR-GSH and MCLR/RR-Cys solution at 0.5 $\mu g\,g^{-1}$ DW. The extraction and analysis were performed as described above, and the recovery and the relative standard deviation of the analytical method were calculated.

2.6. Statistics

Values were showed by means ± standard error (SE). Values below LOD were represented in the data set by half of LOD, while the values for the target compounds that were not detected in the samples were set by zero. Normal distribution for data was analyzed by Shapiro Willks test. One-way ANOVA was carried out to determine whether various organs of bighead carp were significantly different in the content of MC-LR, MC-RR and their metabolites. All statistical tests were performed using SPSS for Windows (Ver. 13.0; SPSS, Chicago, IL, USA). Statistical significance was set at $P < 0.05$.

3. Results

3.1. Characterization of MC-LR, MC-RR and their metabolites

The peak at 11.83 min was confirmed to be MC-LR according to the presence of daughter ions at *m/z* 599.20, 977.47 and the parent ion at *m/z* 995.50 (Fig. 2a, d). Similarly, the peak at 10.74 min with daughter ions at *m/z* 586.94, 1168.17 and the parent ion at *m/z* 652.00, was MCLR-GSH (Fig. 2a, b), and the peak at 10.90 min was from MCLR-Cys, with daughter ions at *m/z* 599.28, 1029.43 and parent ion at *m/z* 1116.50 (Fig. 2a, c).

The retention time for MC-RR, MCRR-GSH and MCRR-Cys were 10.67, 8.78 and 10.13 min, respectively (Fig. 2e). For MC-RR, the daughter ions were at *m/z* 452.85 and 887.42 from the parent ion at *m/z* 520.00 (Fig. 2h). For MCRR-GSH, the daughter ions were at *m/z* 520.08 and 608.38 from the parent ion at *m/z* 673.70 (Fig. 2f). And daughter ions for MCRR-Cys were at *m/z* 513.41and 1008.42 from precursor ion at *m/z* 580.00 (Fig. 2g).

3.2. Quantitative distribution and dynamics of MC-LR, MC-RR and their metabolites

For the control group, none of MC-LR, MCLR-GSH, MCLR-Cys, MC-RR, MCRR-GSH or MCRR-Cys was detected during the experiment.

3.2.1. High dose group

MC-LR (Fig. 3) was distributed in all analyzed organs, and the mean MC-LR content was in the order of intestine ($1.466 \pm 0.447\ \mu g\,g^{-1}$ DW) > kidney ($0.086 \pm 0.036\ \mu g\,g^{-1}$ DW) > liver ($0.038 \pm 0.019\ \mu g\,g^{-1}$ DW) > muscle ($0.015 \pm 0.011\ \mu g\,g^{-1}$ DW). MC-LR content were much higher in intestine than in other organs during the experiment, with the maximum value $2.202 \pm 0.185\ \mu g\,g^{-1}$ DW at 1 h post-injection. MC-LR concentration in liver ranged between 0.014 ± 0.006 and $0.103 \pm 0.015\ \mu g\,g^{-1}$ DW. In kidney, MC-LR increased from $0.012 \pm 0.003\ \mu g\,g^{-1}$ DW (at 1 h post-injection) to $0.175 \pm 0.011\ \mu g\,g^{-1}$ DW (at 72 h post-injection) in a time-dependent manner. However, MC-LR in muscle maintained stable, ranging between 0.008 ± 0.001 and $0.018 \pm 0.002\ \mu g\,g^{-1}$ DW.

MC-RR (Fig. 4) was mainly distributed in liver, kidney and intestine. In muscle, the concentration was below LOD during the study period. The mean content was in the order of kidney ($0.135 \pm 0.083\ \mu g\,g^{-1}$ DW) > liver ($0.107 \pm 0.035\ \mu g\,g^{-1}$ DW) > intestine ($0.077 \pm 0.352\ \mu g\,g^{-1}$ DW) > muscle ($0.002 \pm 0.003\ \mu g\,g^{-1}$ DW). At 1 h post-injection, similar to MC-LR, MC-RR was most abundant in intestine ($0.439 \pm 0.215\ \mu g\,g^{-1}$ DW), followed by liver ($0.255 \pm 0.032\ \mu g\,g^{-1}$ DW) and kidney ($0.179 \pm 0.003\ \mu g\,g^{-1}$ DW). MC-RR concentration in the kidney increased in a time-dependent manner.

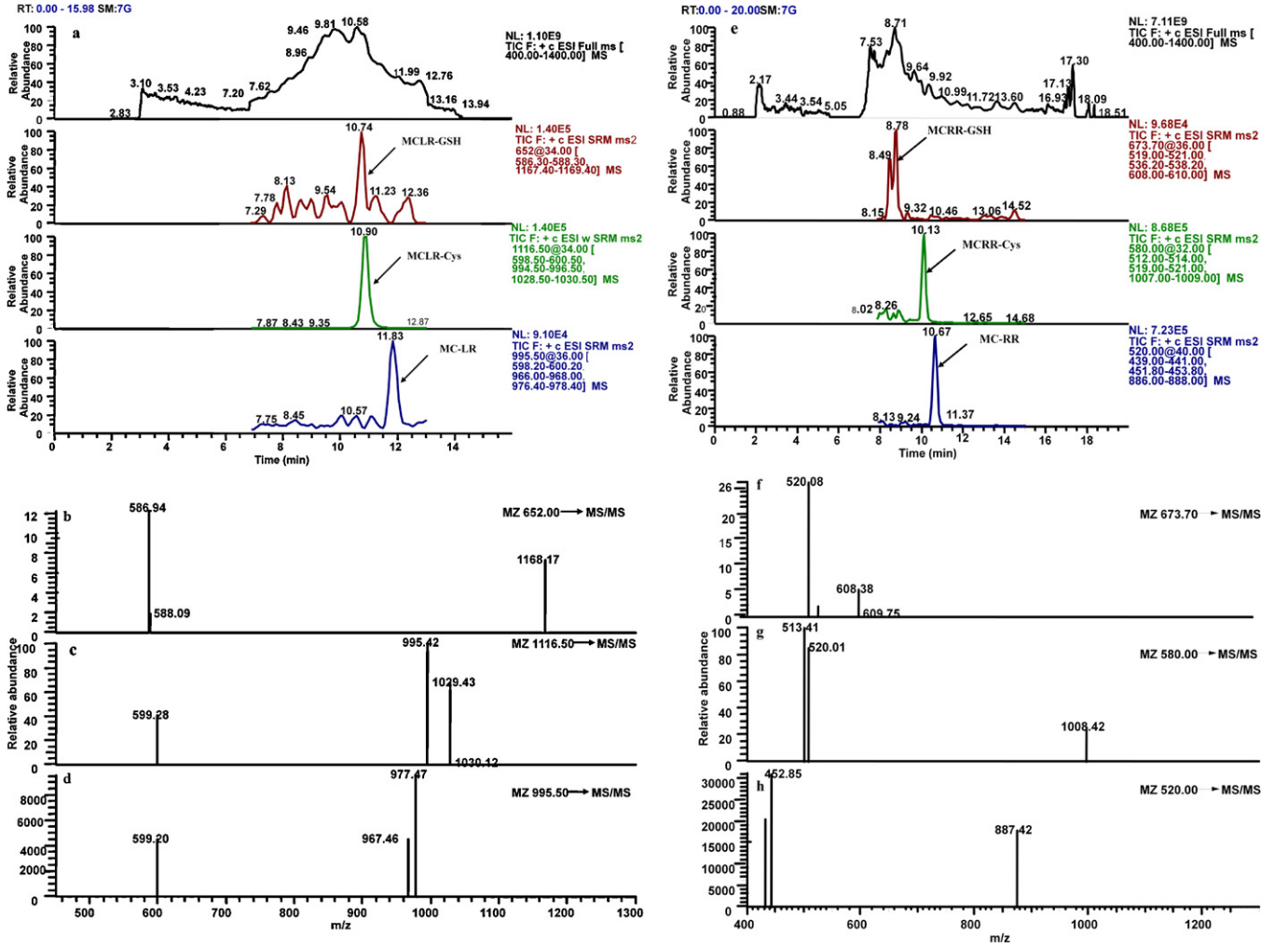


Fig. 2. TIC chromatography and daughter-ion mass spectrum of the bighead carp (*Aristichthys nobilis*) kidney at 200 μg MC-LR_{eq} kg^{-1} bw dosage after 48 h post-injection. Shown are total ion current and SRM chromatograms of (a) MCLR-GSH, MCLR-Cys and MC-LR, and (e) MCRR-GSH, MCRR-Cys and MC-RR; daughter-ion mass spectrum of (b) MCLR-GSH, (c) MCLR-Cys, (d) MC-LR, (f) MCRR-GSH, (g) MCRR-Cys, and (h) MC-RR.

During the study period, the MCLR-Cys content varied remarkably in different organs (Fig. 3). MCLR-Cys was most abundant in kidney, and after being detected at 12 h post-injection (0.003 ± 0.001 μg g^{-1} DW), it increased sharply, reaching as high as 0.122 ± 0.032 and 0.696 ± 0.036 μg g^{-1} DW at 48 and 72 h post-injection, respectively. However, MCLR-Cys was below LOD in other organs except for muscle at 3 h post-injection (0.008 ± 0.001 μg g^{-1} DW). Statistic analysis showed that the content of MCLR-Cys was significantly higher in kidney than in other organs ($F = 3.67$, df = 3, 67, $P = 0.005$).

MCRR-Cys (Fig. 4) was detected in all analyzed organs while the content was significantly higher in kidney than in other organs ($F = 6.908$, df = 3, 57, $P = 0.000$). In kidney, the content increased from 0.020 ± 0.003 μg g^{-1} DW at 1 h post-injection, reaching as high as 4.335 ± 0.009 μg g^{-1} DW at 72 h post-injection. The content of MCRR-Cys in liver, intestine and muscle were near LOD.

The glutathione conjugates of MC-LR and MC-RR, namely MCLR-GSH and MCRR-GSH, were occasionally detected and always below LOD during the experiment.

3.2.2. Low dose group

As some fish died during the experiment, there was lack of kidney samples at 72 h for MC-RR, MCRR-GSH and MCRR-Cys analysis in the 50-dosed group.

Similar to 200-dosed group, MC-LR was distributed in all analyzed organs in 50-dosed group, and the mean MC-LR content was in the order of kidney (0.030 ± 0.012 μg g^{-1} DW) > intestine (0.027 ± 0.033 μg g^{-1} DW) > liver (0.018 ± 0.012 μg g^{-1} DW) > muscle (0.006 ± 0.015 μg g^{-1} DW) (Fig. 3). At 1 h post-injection, the maximum MC-LR content was present in intestine (0.105 ± 0.019 μg g^{-1} DW), and decreased to a low level (0.025 ± 0.007 μg g^{-1} DW) as experiment progressed. However, other organs especially the kidney accumulated more MC-LR than intestine after 1 h post-injection. At 72 h post-injection, in terms of toxin burden, kidney ranked first (0.045 ± 0.005 μg g^{-1} DW), followed by muscle (0.024 ± 0.008 μg g^{-1} DW), whereas content of MC-LR in liver decreased to 0.007 ± 0.001 μg g^{-1} DW.

MC-RR (Fig. 4) was detected in all the four organs. Similar to MC-LR, MC-RR in intestine showed the highest peak at 1 h post-injection (0.205 ± 0.017 μg g^{-1} DW), and then decreased sharply. In liver, MC-RR concentration was near LOD in most time-points except at 72 h post-injection (0.036 ± 0.001 μg g^{-1} DW). In kidney, the maximum MC-RR value (0.060 ± 0.011 μg g^{-1} DW) was observed at 48 h post-injection. However, MC-RR in muscle was barely detectable at 1, 3, 48 h post-injection.

MCLR-Cys was not detected in liver, intestine and muscle, yet, in kidney, substantial MCLR-Cys was observed increasing from 0.008 ± 0.005(1 h post-injection) to 0.706 ± 0.009 μg g^{-1} DW at 72 h post-injection (Fig. 3). MCRR-Cys (Fig. 4) was detected

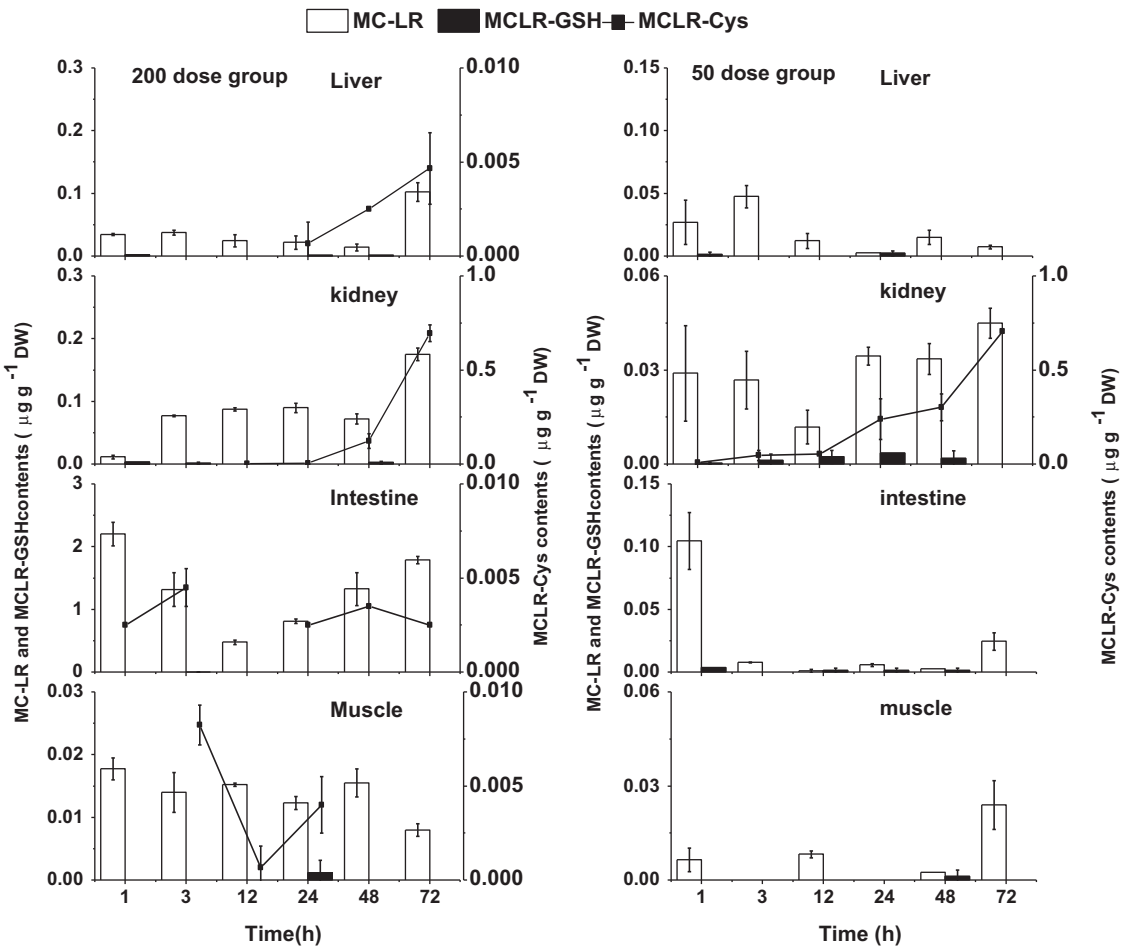


Fig. 3. MC-LR, MCLR-GSH and MCRR-Cys content in liver, kidney, intestine and muscle of bighead carp (*Aristichthys nobilis*) with microcystins equivalent to 200 and 50 μg MC-LR_{eq} kg^{-1} bw, respectively. Values are expressed as means ± standard errors for three replicates; DW = dry weight; LOD (limit of detection) = 0.005 μg g^{-1} DW.

in all analyzed organs. In kidney, MCRR-Cys showed sustained increase from 0.005 ± 0.003 μg g^{-1} DW at 1 h post-injection to 0.220 ± 0.050 μg g^{-1} DW at 48 h post-injection. MCRR-Cys in liver, intestine and muscle was always near LOD. Statistical analysis showed that MCRR-Cys was significantly higher in kidney than in other organs ($F = 6.612$, df = 3, 53, $P = 0.001$).

The glutathione conjugates of MC-LR and MC-RR were always below LOD during the experiment.

3.3. Biotransformation from MC-LR, MC-RR to their cysteine conjugates

In the high dose group, MCLR-GSH and MCLR-Cys content were much lower than free MC-LR not only in intestine, liver and muscle, but also in kidney before 48 h post-injection. Although MCLR-Cys in kidney reached as high as 0.696 ± 0.036 μg g^{-1} DW at 72 h post injection, it was still much less than free MC-LR in intestine. Namely, MC-LR was present mainly in the form of un-conjugated. Different from the biotransformation of MC-LR, MC-RR decreased in all analyzed organs with the progress of experiment except for kidney. MCRR-Cys was detected in all the four organs at most time-points especially in kidney, from 24 h post-injection where the MCRR-Cys content greatly exceeded MC-RR. The ratios of MCRR-Cys to MC-RR reached as high as 19.10 at 72 h post-injection, which was much higher than the ratio of MCLR-Cys to MC-LR (3.97). Apparently, the biotransformation from MC-RR to MCRR-Cys was more effective than that of MC-LR to MCLR-Cys at the high dosage.

In the low dose group, although MC-LR and MC-RR were present in all analyzed organs at most time-points, the concentrations were always low, and the cysteine conjugated MCs were always higher than un-conjugated MCs, especially in kidney. The ratio of MCLR-Cys to MC-LR and MCRR-Cys to MC-RR in kidney at 48 h post injection was 9.04 and 3.67, respectively. So, in the low dose group, MC-LR and MC-RR were biotransformed successfully to MCLR-Cys and MCRR-Cys, respectively.

3.4. Recoveries

The average recoveries from fish liver samples were 72% (range, 61–75%), 85% (range, 76–88%) and 87% (range, 81–94%) for MCLR-GSH, MCLR-Cys and MC-LR, respectively. The relative standards deviations (RSDs) of MCLR-GSH, MCLR-Cys and MC-LR were 9%, 8%, and 6%, respectively. For MCRR-GSH, MCRR-Cys and MC-RR, recoveries were 64% (range, 57–68%), 91% (range, 87–93%), and 95% (range, 92–97%), respectively. The RSDs of MCRR-GSH, MCRR-Cys and MC-RR were 6%, 3% and 3%, respectively.

4. Discussion

The present study, for the first time, simultaneously and quantitatively analyzed the dynamics of MC-LR, MC-RR and their glutathione and cysteine conjugates in selected organ tissues of bighead carp under laboratory conditions to better understand the roles of glutathione and cysteine conjugates of MCs in detoxification process.

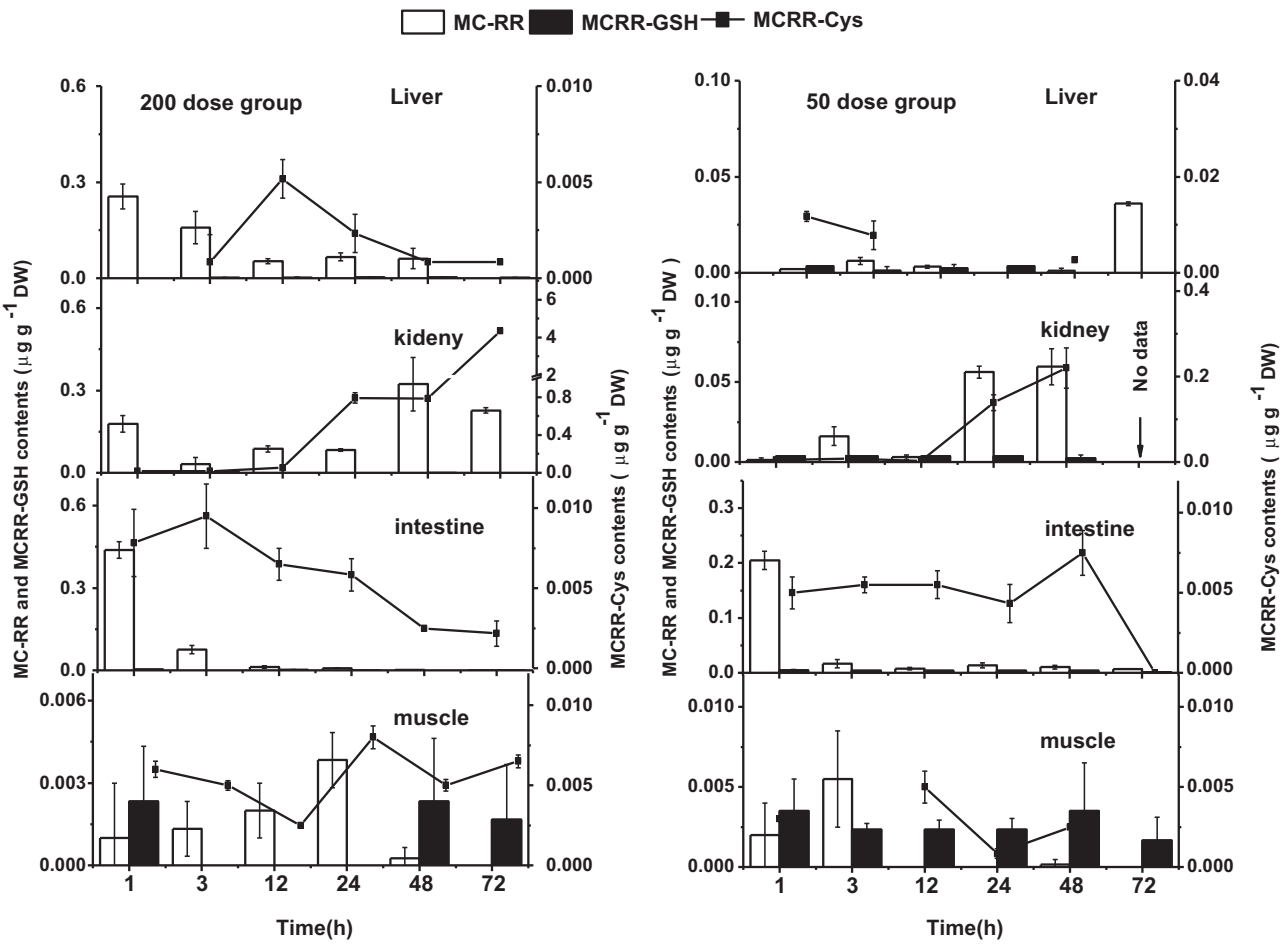


Fig. 4. MC-RR, MCRR-GSH and MCRR-Cys content in liver, kidney, intestine and muscle of bighead carp (*Aristichthys nobilis*) with microcystins equivalent to 200 and 50 μg MC-LR_{eq} kg^{-1} bw, respectively. Values are expressed as means ± standard errors for three replicates; DW = dry weight; LOD (limit of detection) = 0.005 μg g^{-1} DW; no data = not analyzed.

It is well known that GSH plays a significant role in the metabolism of xenobiotics, and its conjugation with xenobiotics is considered as an important metabolic pathway for detoxification (Ketterer et al., 1983). GSH can conjugate with MC-LR via glutathione-S-transferase in various aquatic macrophyte, invertebrates, fish eggs and fish, and subsequently degraded to MCLR-Cys (Carmichael, 1992; Dittmann and Wiegand, 2006; Pflugmacher et al., 1998). In the present work, although MCLR-GSH and MCRR-GSH could be qualitatively detected occasionally in liver, kidney, intestine and muscle, their content was always below LOD, while substantial amount of MCLR/RR-Cys was detected. In previous field study of Lake Taihu, GSH conjugates of MCs were almost undetectable in snail (*Bellamya aeruginosa*), shrimp (*Macrobrachium nipponensis*) and silver carp (*Hypophthalmichthys molitrix*) (Zhang et al., 2009). Meanwhile, microperfusion studies of single proximal rat kidney tubules showed that the S-substituted glutathione derivatives were very rapidly ($t_{1/2} \approx 3.5$ s) degraded (Anders, 1980). All above results indicated that the glutathione conjugate of MCs might act as mid-metabolites and change rapidly to the more stable metabolite MCLR/RR-Cys.

In the present study, it is interesting to note that, MCLR-Cys and MCRR-Cys greatly preferred kidney to liver at both doses, and that MCLR-Cys and MCRR-Cys were detected in kidney soon after i.p. injection, increasing sharply afterwards. Similarly, an immunostaining study by Ito et al. (2002) also showed that the injected MCLR-Cys was prominently observed in kidney of mice but not in liver. Therefore, kidney was the important excretory organ for MCLR-Cys and MCRR-Cys. In a field study, Chen et al. (2007) reported that MCLR-Cys was qualitatively detected in the kidney of bighead carp collected from Lake Taihu in most months. Zhang et al. (2009) also showed that MCLR-Cys content in the kidney of silver carp collected from Lake Taihu were relatively high, varying from 1.85 to 7.93 μg g^{-1} DW with an average of 4.01 μg g^{-1} DW, whereas no MCLR-GSH was detected in the kidney during the half-year study. All above reports together with the present results suggest that MCLR-Cys and MCRR-Cys may have a special affinity for kidney (namely organotropism) in both mammals and aquatic animals.

For the organotropism of MCLR-Cys and MCRR-Cys to kidney, there are several possible explanations. Firstly, some specific proteins or polypeptides, such as members of oatp/OATP superfamily locate in the cytomembrane of kidney (Fischer et al., 2005), and help in the transportation of MCLR-Cys and MCRR-Cys, produced by other organs (such as liver), into kidney. Secondly, MCLR/RR-GSH might be effectively converted to MCLR/RR-Cys in kidney. Since the activity of γ-glutamyltransferase that catalyze the conversion of MCLR-GSH to MCLR-γ-glutamylcysteine is highest in kidney (Anders, 1980; Dittmann and Wiegand, 2006), it is reasonable that MCs-Cys are effectively accumulated in kidney and that MCs-Cys are organotropic to kidney rather than to liver. In addition, after exposure to MCs, the GSH conjugation of MC-LR/RR in kidney is effectively catalyzed by increased glutathione S-transferase (GST) and subsequently degraded to MCLR/RR-Cys. Li et al. (2008) reported that when goldfish (*Carassius auratus* L.) were i.p. injected

with MC extracts, the transcription of GST alpha in two dose groups (50 and 200 $\mu g\ LR_{eq}\ kg^{-1}$ bw) was suppressed in both liver and intestine but induced in kidney, and that increased transcription of GST theta and GST pi in the high dose group were also detected in kidney.

Thirdly but also importantly, there is generally at odds with the accepted notion that the GSH derivative is convert to the Gly-Cys derivative, to the Cys derivative and finally to mercapturic acid. Hodgson (2004) in their book, mentioned that there is not a separate pathway for the two derivatives (MC-GSH and MC-Cys), but a difference in the toxicokinetics, and our study showed that the pathways also differ. Bagu et al. (1997) confirmed that the inhibition of protein phosphatases 1 and 2A for MCs is through binding covalently with the Cys-273 of the PP-1 and 2A. Based on the above comments together with the present results, we speculate that a different detoxification pathway exists in aquatic animals: MC-LR and MC-RR firstly conjugate with polypeptides or proteins (mainly PP-1 and 2A) containing Cys residues, subsequently, MCLR/RR-Cys is degraded from these polypeptide or protein, and finally is excreted in the form of MCLR/RR-Cys mainly from kidney.

In the present study, substantial MCLR-Cys and MCRR-Cys in kidney for both treatment groups were detected. The high ratios of MCLR-Cys to MC-LR and MCRR-Cys to MC-RR in kidney after 24 h post-injection indicate that bighead carp can effectively metabolize MC-LR and MC-RR into their cysteine conjugates. This biotransformation appears to be an important biochemical mechanism for the phytoplanktivorous fish, bighead carp, to resist MCs' toxicity during its grazing on toxic cyanobacteria.

Meanwhile, it should also be noted that in the high dose group, the biotransformation from MC-LR to MCLR-Cys was neither efficient as that in the low dose group, nor efficient as that of MC-RR biotransformation to MCRR-Cys. Considering that cysteine conjugates with MCs mainly act as excretion forms of MCs from kidney, the efficient biotransformation of MCs to MCs-Cys may be triggered to perform as an important protection mechanism to resist MCs at relatively low dose exposure. However, when fish was exposed to the high dosage of MC-LR, one of the most toxic microcystin variants, the balance between accumulation and detoxification was broken, i.e., the biotransformation of MC-LR to MCLR-Cys was negatively influenced, leading to substantial accumulation of MC-LR in various organs of the bighead carp. As MCLR-Cys is more water soluble than MC-LR and thus enhances the excretion of MC-LR (Dittmann and Wiegand, 2006), such a negative influence on biotransformation may also explain why MCLR-Cys content in the organs of bighead carp was lower at the high dose group in the present study.

5. Conclusions

(1) Content of MCLR-Cys and MCRR-Cys were much higher in kidney than in liver, intestine and muscle, suggesting their organotropism to kidney. As MCLR-GSH or MCRR-GSH was always below LOD, MCs-GSH might act as mid-metabolites and changed to the more stable MCs-Cys rapidly.
(2) Bighead carp can effectively metabolize MC-LR and MC-RR into their cysteine conjugates. MC-LR and MC-RR were excreted mostly in the form of MCLR/RR-Cys rather than MCLR/RR-GSH from kidney.
(3) Cysteine conjugation of MCs appears to be an important biochemical mechanism for phytoplanktivorous fish to resist toxic cyanobacteria.
(4) When exposed to high dosage of MCs, transformation from MC-LR to MCLR-Cys was negatively influenced, consequently leading to substantial accumulation of MC-LR.

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