Simultaneous quantitative determination of microcystin-LR and its glutathione metabolites in rat liver by liquid chromatography–tandem mass spectrometry

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Abstract

The roles of glutathione (GSH) and cysteine (Cys) in the detoxification of Microcystin-LR (MC-LR) have recently become a popular area of research. However, lacking analysis methods for MC-LR-GSH and MC-LR-Cys (two main GSH pathway metabolites) in mammals, elucidation of the detoxification mechanism and metabolic pathway of MC-LR in mammals is difficult. In this study, a novel method for the simultaneous quantitative analysis of MC-LR, MC-LR-GSH and MC-LR-Cys in rat liver was developed and validated. The analytes were simultaneously extracted from rat liver using 3 M sodium chloride solution containing 0.01 M EDTA-Na2–5% acetic acid, followed by solid-phase extraction (SPE) on Oasis HLB and silica cartridges and determination by liquid chromatography–electrospray ionization mass spectrometry (LC–ESI–MS/MS). Under the optimized pretreatment conditions and instrument parameters, good recoveries of MC-LR, MC-LR-GSH and MC-LR-Cys were obtained at three concentrations (0.2, 1.0 and 2.5 μg g⁻¹ dry weight (DW)) with values ranging from 97.7 ± 4.2 to 98.7 ± 5.1%, 70.1 ± 4.8 to 71.1 ± 4.1% and 79.8 ± 3.5 to 81.4 ± 4.0%, respectively. The relative standard deviations (RSDs) of these compounds at 0.2, 1.0 and 2.5 μg g⁻¹ DW were between 4.3% and 6.9%. The limits of detection (LODs) were 0.005, 0.007 and 0.006 μg g⁻¹ DW and the limits of quantification (LOQs) were 0.017, 0.023 and 0.020 μg g⁻¹ DW for MC-LR, MC-LR-GSH and MC-LR-Cys, respectively. Furthermore, this method was successfully applied to both time- and dosage-effect studies of MC-LR, MC-LR-GSH and MC-LR-Cys in vivo.

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1. Introduction

As cyanobacterial blooms frequently occur in freshwaters throughout the world, microcystin-LR, which is one of the most common toxins produced by the toxigenic cyanobacteria [1], has raised global concerns in environmental science [2,3], toxicology [4,5], and epidemiology [6] due to its hepatotoxicity and potential carcinogenic activity [7–11]. Extensive studies have reported that MC-LR can be absorbed, transported and accumulated in organs, causing the poisoning or death of aquatic animals and mammals [12–20], and posing potential threats to human health [21–26]. MC-LR is demonstrated to conjugate with GSH via glutathione S-transferase (GST) and further degrades to MC-LR-Cys [27], thereby increasing the water solubility, enhancing the excretion and reducing the toxicity of MC-LR [28,29].

MC-LR and its glutathione and cysteine conjugates (MC-LR-GSH and MC-LR-Cys) were firstly identified in rat liver using Frit-FAB LC/MS [29,30]. However, the high-speed centrifugation, heat denaturation and pronase digestion in the analyte extraction process makes this method complicated and time-consuming and affects the accuracy of the analytical results. The distribution of MC-LR, MC-LR-GSH and MC-LR-Cys in mice organs was also studied using an immune-staining method [27,31], which is convenient but susceptible to interference, preventing the accurate identification of analytes. Most important of all, in these studies, the two metabolites were only qualitatively analyzed, which could not effectively explain the detoxification mechanism and metabolic pathways of MC-LR in organisms.

In recent years, some analytical methods [32,33] have been established for the quantitative determination of MCs and their metabolites in fish tissues based on LC–ESI–MS/MS, providing...
technical support for the study of the metabolism of MCs in aquatic animals. However, there were also some drawbacks. For example, the recoveries and intra-assay precision of MC-LR-GSH were not very good; the recovery at 0.2 μg g⁻¹ DW was only 65.0%, and the precision was 12.9%. Furthermore, MC-LR-Cys, another important metabolite in MCs detoxification, could not be detected in fish tissues in the previous study [32]. More regrettably, the existing studies only focused on aquatic animals and were thus limited and incomplete regarding risk evaluation and clinical diagnosis. Since mammal metabolism and physiological function are closer to human beings, a quantitative study of the bioaccumulation, distribution and metabolism of MC in mammals may allow a more representative prediction of the potential threat to humans. Furthermore, it could also underpin the development of the diagnosis and treatment of MC-LR intoxication. So far, however, there have been no reports of the simultaneous quantitative analysis of MCs and their metabolites in mammals. To address this problem, we have tried to use our previously established method [32] to analyze these three target compounds in rat liver. However, the recoveries of MC-LR-GSH and MC-LR-Cys were only 35.0% and 55.4%, respectively, and the RSD of MC-LR-GSH was as high as 13.7%. Therefore, a suitable method for the determination of MC-LR and its two main GSH pathway metabolites in mammals is imperative.

In the present study, a sensitive and effective analytical method for the simultaneous qualitative and quantitative determination of MC-LR, MC-LR-GSH and MC-LR-Cys in rat liver based on LC–MS/MS was developed and validated for the first time. The extraction solution, SPE cartridge, cleanup and enrichment procedures were optimized, making it possible to extract and concentrate the target analytes from the rat liver efficiently. The LC and MS/MS conditions were also investigated to detect these compounds with low limits of quantification (LOQs), good reproducibility and high accuracy. It is obvious that this method allows the extensive study of the metabolic detoxification mechanism of MC-LR in mammalian organs and further application to clinical treatment of human diseases induced by MCs intoxication.

2. Experimental

2.1. Materials and chemicals

Considering the quantity of the toxin needed and the experimental cost, the cyanobacterial toxin MC-LR was extracted and purified from freeze-dried surface blooms according to Ramanan et al. [34]. The purity of MC-LR was over 95%, with the remaining 5% of the substance being mainly comprised of pigment, as determined by high-performance liquid chromatography (HPLC, LC–20A, Shimadze, Kyoto, Japan). The identity of MC-LR was confirmed using LC–MS (Thermo Electron, Waltham, MA, USA).

L-Glutathione and L-cysteine were purchased from Acros Organics (purity >99%, Geel, Belgium, GR). MC-LR-GSH and MC-LR-Cys were formed using the method of Dai et al. [32] and Zhang et al. [35]. The purity of the two conjugates was over 95% and fulfilled our criteria. As confirmed by HPLC and LC–MS, the impurity (<5%) was not the parent MC-LR but instead some unknown compounds that do not affect the qualitative and quantitative analysis of the target compounds.

Formic acid (FA) and acetic acid (AR) were purchased from Aldrich (Sigma, USA) and Sinopharm Chemical Reagents (Shanghai, China), respectively. Methanol (Merck, Germany) and acetonitrile (Merck, Germany) were of HPLC grade, and ultrapure water was prepared by a Milli-Q water purification system (Millipore, Bedford, MA, USA). The other reagents included in this study were all of analytical reagent grade. The SPE cartridges used for the sample clean-up were Oasis HLB cartridge (200 mg/6 mL, Waters, Milford, MA, USA) and Sep-Pak silica gel cartridge (2 g/12 mL, Waters, Milford, MA, USA). The mobile phases were composed of solvent A (water: FA = 95: 5 (v/v)), solvent B (acetonitrile: FA = 95: 5 (v/v)) and solvent C (methanol: FA = 95: 5 (v/v)), all of which were well mixed and ultrasonically oscillated for 1 h.

2.2. Standard solutions

Stock solutions were prepared by dissolving purified MC-LR, MC-LR-GSH and MC-LR-Cys in HPLC water. The final concentration of each analyte was 10 μg mL⁻¹. A sequence of standard solutions ranging from 0.01 to 2.5 μg mL⁻¹ (level = 6) was prepared by diluting the stock solutions with HPLC water. All standard solutions were stored at −80 °C and prepared before use.

2.3. Instrumentation

Qualitative analyses of MC-LR, MC-LR-GSH and MC-LR-Cys were performed using an LC–MS/MS system (Finnigan, USA), consisting of a Surveyor HPLC system and an LCQ Advantage MAX ion trap mass spectrometer equipped with an ESI (+) probe. Separation was conducted using a Waters XBridge C18 column (2.1 mm × 100 mm × 3.5 μm, Waters Corporation, USA) with a C18 guard column (2.1 mm × 10 mm × 5 μm, Thermo Electron Corporation, USA).

2.3.1. Chromatography

The linear gradient program was as follows: (min/%B) 0/25, 7/25, 8/55, 13/60, 14/70, 15/25, 20/25. The total flow rate was 0.2 mL min⁻¹, which was increased to 0.3 mL min⁻¹ for the last 6 min. The temperatures of column oven and vial tray were 25 °C and 10 °C, respectively. Lastly, 10 μL of sample solution were injected for each run.

2.3.2. MS/MS parameters

The MS analytical conditions were as follows. Nitrogen (purity ≥99.995%) set to 20 and 5 units (a scale of arbitrary units in the 0–100 range defined in the LCQ system), was used as the sheath and auxiliary gas, respectively. The ESI spray voltage was set at 4.54 kV, and the capillary temperature was set at 250 °C, with the multiplier voltage set at −1336.28 V. The tube lens voltage was 55 V for MC-LR and 50 V for MC-LR-GSH and MC-LR-Cys. The collision energies of MC-LR, MC-LR-GSH and MC-LR-Cys were 36%, 24% and 34%, respectively. The isolation widths for the three compounds were both m/z 2.0. Quantification was performed in selective reaction-monitoring mode (SRM), monitoring the transition of the precursor ion at m/z 995.50 to the m/z 599.20 and 977.47 product ions for MC-LR, the precursor ion at m/z 652.0 to the m/z 587.3 and 1168.3 product ions for MC-LR-GSH and the precursor ion at m/z 1116.5 to the m/z 599, 995 and 1029 product ions for MC-LR-Cys.

2.4. Sample preparation

The liver samples obtained from healthy male SD rats (purchased from Laboratory Animal Center of Wuhan University) were used for the development of the method after freezing and lyophilization by a freeze dryer at −80 °C (Alpha 2–4, Martin Christ, Germany). No analyte (MC-LR, MC-LR-GSH or MC-LR-Cys) was detected in these samples using the developed method.

The lyophilized blank liver samples (50 ± 0.3 mg) were spiked with 50 μL standard solutions at concentration of 0.2, 1.0 and 2.5 μg mL⁻¹, which were equivalent to 0.2, 1.0 and 2.5 μg g⁻¹ DW of the analytes, respectively. The spiked liver samples were well homogenized and extracted three times with 5 mL of extraction solvent (water containing 0.01 M EDTA-Na₂, 5% acetic acid and 3 M NaCl) by ultrasonication at 0 °C for 3 min (30% amplitude, 60 W,
The supernatant was evaporated to dryness by a rotary evaporator (EYELA, Japan), and then 10 mL of methanol was used to separate the analytes from salt (EDTA-Na₂, NaCl in extraction solvent) and hydrophilic endogenous impurities. Lastly, the analytes were dried by a rotary evaporator, dissolved in 5 mL of HPLC water and stored at 4 °C before use.

2.5. Cleanup procedure

The supernatant liver samples prepared above were loaded onto an Oasis HLB cartridge that had been conditioned and equilibrated with 10 mL of methanol and 10 mL of HPLC water in sequence. The cartridge was washed with 30 mL of HPLC water and 20 mL of 20% (v/v) aqueous methanol, respectively. Analytes were eluted with 25 mL of methanol. The eluent was evaporated to dryness using a rotary evaporator (EYELA, Japan). The residue was reconstituted with 2 mL of methanol and subsequently loaded onto a Sep-Pak silica gel cartridge that had been preconditioned with 10 mL of methanol. The silica gel cartridge was washed with 10 mL of methanol and then eluted with 25 mL of 70% methanol solution (70 mL methanol: 30 mL HPLC water). This eluent was then evaporated to dryness, and the residue was reconstituted with 1.5 mL of methanol. Lastly, the extract was evaporated to dryness using a centrifuge enrichment apparatus and reconstituted with 100 μL of LC mobile phase. 10 μL of the aliquots were injected into the LC–MS system for quantitative analysis.

2.6. Method validation

2.6.1. Identification of MC-LR, MC-LR-GSH and MC-LR-Cys

To define the qualitative and quantitative ions, the three compounds were identified by ESI LC–MS/MS in scan mode. The maximum deviation of the retention time between the sample and the reference standard was 2.5%. The ion ratios for every compound were obtained from the ratio of the abundance of different ions for the same compound and were within the specified tolerances accepted in the European Union decision [36].

2.6.2. Calibration curve, LOD and LOQ

Calibration samples were prepared by adding standard solution to blank liver sample extracts after pretreatment, with the concentrations in the series being 0.01, 0.05, 0.1, 0.5, 1.0 and 2.5 μg g⁻¹ DW. The limit of detection (LOD) and limit of quantification (LOQ) were calculated in a matrix solution (spiked with 0.2 μg g⁻¹ DW analytes) as 3 and 10 times the average background noise, respectively.

2.6.3. Recovery and precision assays

The recoveries and precisions were evaluated at three levels (0.2, 1.0 and 2.5 μg g⁻¹ DW) by comparing the peak areas of samples spiked prior to pretreatment to the calibration samples with the corresponding concentration.

The precisions are defined as the coefficient of variation (CV), indicating the consistency between measured values. The assays described above were repeated five times within the same day to obtain the intraday precision and five times over three different days to obtain the interday precision. All values given in the tables are based on triplicate analyses.

2.7. In vivo experiments

To demonstrate the reliability and applicability of the developed method, it was applied to quantify MC-LR, MC-LR-GSH and MC-LR-Cys in infected rat liver samples. Male SD rats from the Laboratory Animal Center of Wuhan University were divided into two groups at random for both time- and dosage-effect studies of MC-LR, MC-LR-GSH and MC-LR-Cys in vivo.

The time-effect study was conducted in the liver of male SD rats treated with a single intra-peritoneal (i.p.) injection of MC-LR at a dose of 11 μg mL⁻¹ body weight. Liver samples were collected at 2 h, 8 h, 1 d, 3 d, 5 d and 7 d post-injection. The rats for the dosage-effect study were administered with i.p. injections of MC-LR at doses of 11, 20 and 36 μg mL⁻¹. Liver samples were collected at 24 h post-injection. The control rats for the two groups were synchronously injected with the same volume of 0.9% (w/v) saline solution. All of the liver samples were stored at −80 °C before analysis and pretreated following the process described in Sections 2.4 and 2.5.

3. Results and discussion


MC-LR, MC-LR-GSH and MC-LR-Cys were identified by ESI LC–MS/MS in scan mode. The chromatograms and mass spectra in Fig. 1 show that the peaks at 10.42, 10.57 and 11.94 min correspond to MC-LR-GSH, MC-LR-Cys and MC-LR, respectively. The precursor ion [M+H⁺]⁺ present at m/z 995.50 with the corresponding product ions abundant at m/z 599.20 and 977.47 was MC-LR. Similarly, the abundant precursor ion [M+2H⁺]²⁺ at m/z 652.0 with product ions at m/z 587.3 and 1168.3 was confirmed to be MC-LR-GSH, and the precursor ion [M+H⁺]⁺ at m/z 1116.5 with product ions at m/z 599, 995 and 1029 was MC-LR-Cys.

3.2. Method optimization

In our verification test, the method proposed by Dai et al. [32] was used to analyze MC-LR, MC-LR-GSH and MC-LR-Cys in rat liver. However, the results were not ideal: the recoveries of MC-LR-GSH and MC-LR-Cys were only 35.0% and 55.4%, respectively, and the RSD of MC-LR-GSH was as high as 13.7%. Therefore, a suitable method for the analysis of these compounds in mammals is imperative, and in the following, a series of parameters and conditions were investigated. All experiments were performed in triplicate.

3.2.1. Optimization of sample preparation

The unacceptable results obtained using Dai’s method might be caused by the complicated proteins and endogenous compounds in rat liver. The proteins may combine with MC-LR (MC-LR-GSH and MC-LR-Cys) through the synergistic effect of many forces [37,38]. As a result, the target compounds may not adhere to the SPE column, being directly lost. Hence, to change the ionic strength and diminish protein–analyte interactions, sodium chloride was included in the extraction solvent [39]. The removal of proteins could not only prevent protein foam and reduce the formation of emulsion during the extraction process but also protect the instrument performance.

Different salinities have disparate dissociation efficiencies. To achieve the maximum efficiencies for the target compounds, different extraction solvents with NaCl concentrations ranging from 0 M to 3.5 M (level = 7) were compared. According to Fig. 2, MC-LR, MC-LR-GSH and MC-LR-Cys had the maximum response when using an extraction solvent with 3 M and 3.5 M of NaCl. Considering that 3.5 M NaCl was already a supersaturation of the extraction solvent, the EDTA-Na₂ (0.01 M)-NaCl (3 M)-5% acetic acid system was chosen and tested as an efficient solvent.

3.2.2. Selection of SPE cartridges

In recent years, SPE has been widely used for the enrichment and purification of MCs [40–42]. As there were significant differences...
in the physicochemical properties (structure, polarity, solubility, acid-base property, etc.) of the impurities and considering the complexity of the matrix in mammalian liver, it was difficult to remove all of the impurities with a single SPE cartridge.

To remove a substantial amount of the impurities that exist in mammalian liver, special attention was paid into the optimization and selection of the SPE cartridge. Based on the polarity and electrical properties of our target analytes, ion-exchange and reverse-phase cartridges were considered suitable due to their prominent selectivity and high precision. In this approach, a variety of available commercial cartridges, including WCX (60 mg/3 mL, Waters, Milford, MA, USA), MCX (60 mg/3 mL, Waters, Milford, MA, USA), C18 and HLB, were compared in terms of the enrichment and extraction efficiencies for the three target compounds in the liver matrix. All of the cartridges were operated under their optimum elution conditions and a sufficient elution volume as suggested by the operating manual. As shown in Fig. 3, HLB had the maximum response for MC-LR, MC-LR-GSH and MC-LR-Cys, which was attributed to the copolymer in HLB cartridge being a hydrophilic-lipophilic balanced sorbent that provides higher and more reproducible recoveries for the target compounds relative to other sorbents.

To achieve accurate quantification of the analytes, a second extraction was applied as an effective auxiliary procedure to remove the remaining interfering impurities. The silica gel cartridge was chosen for the additional extraction in our study due to its excellent adsorption and separating capacity for the target analytes. In addition, it is a commonly used and economical cartridge for biological sample analysis. The effects of the silica gel cartridge on the response values of MC-LR and its metabolites were shown in Fig. 4. The results indicated that, relative the use of an HLB cartridge alone, the combination of the HLB and silica gel cartridges promoted the sample clean-up, thereby minimizing the matrix effects and decreasing the amount of the matrix injected...
onto the column. Moreover, there were no significant differences in the peak areas of the target analytes between the HLB cartridge alone and the HLB and silica gel cartridges, indicating that no additional loss was induced. Thus, the HLB coupled with a silica gel cartridge was chosen for further development.

Fig. 2. Effect of different NaCl concentrations in the extraction solvent.

3.2.3. Optimization of elution conditions

The eluent strength and elution volume were two key factors in the elution and collection of the analytes during the SPE procedure. First, the effects of various elution solvents on the elution of the analytes compounds from the SPE cartridge vary greatly, and only the optimal eluent strength could elute the analytes completely while allowing the impurities with stronger absorbent ability to adhere to the SPE column. Second, regarding the elution volume, insufficient eluent could not adequately elute the analytes. In contrast, superfluous eluent might introduce undesired impurities, leading to an increase in the interference for LC/MS analysis. Moreover, it can cause loss of the analytes and waste of time during the evaporation concentration step.

Based on our previous works, pure methanol was demonstrated to be optimal for eluting the target compounds from the HLB cartridge and was selected in our study (data not shown). To achieve optimal elution of the solvents toward the target analytes, the eluent volume was investigated from 5 mL to 35 mL. The results, shown in Fig. S1 in the supplementary material, revealed that 25 mL of pure methanol for the HLB cartridge yielded the best results for MC-LR, MC-LR-GSH and MC-LR-Cys.

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2014.05.046.

Fig. 3. Effect of the solid-phase extraction cartridges on the response values of MC-LR and its metabolites.

Fig. 4. Effect of the silica gel cartridge on the response values of MC-LR and its metabolites (A: HLB cartridge, B: HLB + silica gel cartridge).
Table 1
The linearity, precision, limit of detection, limit of quantification and stability of the method.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Linearity (R²)</th>
<th>Intra-assay RSD (%; n=5)</th>
<th>Inter-assay RSD (%; 3 d)</th>
<th>LOD⁺ (µg g⁻¹)</th>
<th>LOQ⁺ (µg g⁻¹)</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC-LR</td>
<td>0.9909</td>
<td>6.1</td>
<td>4.4</td>
<td>2.1</td>
<td>1.0 µg g⁻¹</td>
<td>2.5 µg g⁻¹</td>
</tr>
<tr>
<td>MC-LR-GSH</td>
<td>0.9901</td>
<td>5.5</td>
<td>6.5</td>
<td>6.6</td>
<td>1.0 µg g⁻¹</td>
<td>2.5 µg g⁻¹</td>
</tr>
<tr>
<td>MC-LR-Cys</td>
<td>0.9921</td>
<td>8.2</td>
<td>8.1</td>
<td>7.1</td>
<td>1.0 µg g⁻¹</td>
<td>2.5 µg g⁻¹</td>
</tr>
</tbody>
</table>

a Limit of detection was calculated on the basis of S/N=3.
b Limit of quantification was calculated on the basis of S/N=10.

3.3. Method validation

The optimized method has been validated in terms of selectivity, linearity, precision, detection limit, recovery and repeatability.

3.3.1. Selectivity and linearity

To assess the selectivity of this method, extracts obtained from the spiked liver samples (0.2 µg g⁻¹ DW) (Fig. 1) were subjected to the pretreatment and analysis procedure. LC–MS/MS analysis showed that the three compounds were clearly detected at 10.41, 10.54 and 11.84 min, and no significant interfering peak was detected at the known retention time. To check the linearity of the method, calibration curves were established for MC-LR, MC-LR-GSH and MC-LR-Cys in liver matrices at six concentration levels ranging from 0.01 to 2.5 µg g⁻¹ DW. The calibration curves demonstrated good linearity, with R² values of 0.9909, 0.9901 and 0.9921 for MC-LR, MC-LR-GSH and MC-LR-Cys, respectively (Table 1). These results confirmed that the selectivity and linearity of the approach were satisfactory.

3.3.2. Precision, LOD and stability

Precision assays were carried out at three different levels (0.2, 1.0, and 2.5 µg g⁻¹ DW) by analyzing spiked samples against calibration standards (n=3), and the results were shown in Table 1. The intraday precision and the interday precision were below 8.2%, and 9.0%, respectively, both of which were within the acceptable level (<15%). The three compounds displayed satisfactory responses to LC–MS detection (Table 1); the LODs of MC-LR, MC-LR-GSH and MC-LR-Cys were calculated in the matrix solution as 3 times the average background noise, with values of 0.005, 0.007 and 0.006 µg g⁻¹ DW, respectively. The LOQs were calculated as 10 times the average background noise, with values of 0.017, 0.023 and 0.020 µg g⁻¹ DW for MC-LR, MC-LR-GSH and MC-LR-Cys, respectively.

The target compounds were thought to undergo a certain degree of degradation during the sample pretreatment process. Thus, verification of the stability was required to ensure the accuracy of the results. To evaluate the stability of the three compounds in liver matrices, blank samples were spiked with 0.2 and 2.5 µg g⁻¹ of the analytes and processed via a freeze-thaw (−20 °C to room temperature) day-cycle (24 h) three times before analysis. The results were compared to those samples analyzed immediately after pretreatment, revealing that MC-LR, MC-LR-GSH and MC-LR-Cys were stable for three freeze-thaw day-cycles (72 h). The degradations of two different spiked levels (0.2 and 1.0 µg g⁻¹ DW) at room temperature for 24 h were less than 9.0% (Table 1). Consequently, these three compounds were considered to be stable in rat liver matrices during assay processing and frozen storage.

3.3.3. Recovery and matrix effects

The recoveries were studied using blank liver samples spiked with MC-LR, MC-LR-GSH and MC-LR-Cys at 0.2, 1.0 and 2.5 µg g⁻¹ DW, with three replicates. The average recoveries in spiked rat liver were in the range of 97.7–98.7% for MC-LR, 70.1–71.1% for MC-LR-GSH and 79.8–80.3% for MC-LR-Cys (Table 2). The recoveries above were all acceptable according to the guidelines (>70%).

Fig. 5. Proportions of methanol in water in the elution step for the silica gel cartridge (elution volume: 35 mL.).

Considering that proportions of methanol in water (v/v) from 55% to 90% were suitable for the elution for the silica gel cartridge, a gradient experiment was conducted to ascertain the optimum proportion. As could be seen in Fig. 5, 70% methanol (v/v) was sufficient to obtain the best response for all of the analytes from the silica gel cartridge. Similar to the HLB cartridge, the volume of eluent for the silica gel cartridge was also investigated from 5 mL to 35 mL, and the optimal responses for the three compounds were obtained at 25 mL (Fig. S2). In this way, we conclude that the optimal volume of the eluent was 25 mL.

Supplementary Fig. S2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2014.05.046.

Based on the results above, an optimal operation procedure is schematically proposed in Fig. S3.

Supplementary Fig. S3 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2014.05.046.
effects were evaluated by comparing the peak areas of spiked liver samples (0.2 µg g⁻¹ DW) to those of the standard solvent. The results indicated that the matrix effects were minimized to less than 18.8%, and the optimized method efficiently decreased the amount of the matrix injected onto the chromatographic column.

### 3.4. In vivo experiments

The optimized method was applied to analyze liver samples of male SD rats, i.e., injected with MC-LR. Both the time and dosage effects of MC-LR, MC-LR-GSH and MC-LR-Cys were studied.

The mean (n=3) liver concentration-time profiles from the time-effect study (Fig. 6) showed that both MC-LR and MC-LR-GSH have the highest peaks at 1 d post-injection, with concentrations of 0.021 ± 0.004 µg g⁻¹ DW and 0.008 ± 0.0005 µg g⁻¹ DW, respectively. The contents then decreased to a low level as the experiment progressed. In contrast, the MC-LR-Cys content increased rapidly, peaking at 0.055 ± 0.005 µg g⁻¹ DW at 5 d post-injection, and then decreased as the experiment progressed. The concentration of MC-LR-GSH remained close to the LOD, whereas the cysteine conjugates were abundant throughout the experiment. Similar results were reported in previous field and laboratory studies [35,43,44]. It was speculated that Cys conjugation might play a more significant role in MC-LR detoxification. Recently, a MC-GSH direct exposure study showed that MC-GSH could be rapidly converted into MC-Cys within 0.5 h post-injection [45]. The above results suggest that MC-LR-GSH might act as a highly reactive intermediate that could be rapidly changed to MC-LR-Cys. Aside from the GSH pathway, MC-LR-Cys may also be degraded from conjugates of MC-LR that directly interacted with polypeptide or proteins (mainly PP-1 and 2A) containing Cys residues in animals exposed to MC-LR [43,44]. On this basis, a dosage-effect study was conducted at 24 h post-injection. The result in Fig. 7 showed that the three target compounds were all detected in the low-(11 µg mL⁻¹), medium-(20 µg mL⁻¹) and high-dose (36 µg mL⁻¹) groups, and the contents, especially for MC-LR and MC-LR-Cys, display a significant dose-response relationship. The ratios of MC-LR to MC-LR-GSH in the low- and medium-dose groups were 2.72 and 2.79, respectively. However, the ratio of MC-LR to MC-LR-GSH was 7.54 in the high-dose group, indicating that the detoxification efficiency was significantly reduced (P<0.001) under high MC-LR exposure. The time- and dose-response regularities first reported in rats were in agreement with previously published studies in aquatic animals to a certain extent [35,43,44], and more in-depth studies on the metabolic detoxification mechanism between different types of animals would be interesting in the future.

It should be noted that the histopathological changes, response of the antioxidant defense system and relationships between GSH pathway related antitodal genes (GST, GPx, GR and γ-GCS) at the transcriptional level in both time- and dosage-effect studies were also investigated in the livers of the MC-LR-treated rats (another part of the same experiment). The results demonstrated that MC-LR induced obvious time- and dose-dependent response in hepatic lipid peroxidation, depletion of GSH levels and activities of GSH pathway related enzymes (unpublished data). All of the results mentioned above suggest the important role of the GSH pathway in the detoxification of MC-LR-induced toxicity.

### 4. Conclusions

In the present study, we established a sensitive and accurate stable analytical method based on LC–ESI-MS/MS for the simultaneous quantification of MC-LR, MC-LR-GSH and MC-LR-Cys in rat liver. This is the first assay for the analysis of MC-LR and its two important GSH pathway metabolites in mammals.

In this method, a series of parameters and conditions, such as the efficiency of the combined SPE column, SPE adsorbent, eluent strength, elution volume and salt concentration of the extraction solvent, were optimized and selected. The developed method was shown to be efficient in extraction and concentration from complex biological matrices (rat liver) and validated to yield good results, e.g. satisfactory recoveries, linearity, repeatability (RSD) and LOD. Furthermore, it was successfully applied to the rat liver samples for both time- and dosage-effect studies of MC-LR, MC-LR-GSH.
and MC-LR-Cys. This method would be a beneficial contribution to the intensive study of the metabolic pathway and detoxification mechanism of MC-LR. In addition, it could also provide more reliable information for further pharmacokinetic and clinical studies, thereby predicting the potential threat to humans accurately and providing powerful tools for obtaining insights into MC-LR-induced toxicity risk assessment and management.

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