Quantitative liquid chromatography–tandem mass spectrometry method for determination of microcystin-RR and its glutathione and cysteine conjugates in fish plasma and bile

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ABSTRACT

A rapid and sensitive liquid chromatography–mass spectrometry (LC–MS) method was developed and validated for the simultaneous determination of microcystin-RR (MC-RR) and its glutathione and cysteine conjugates (MC-RR-GSH and MC-RR-Cys, respectively) in fish plasma and bile. The analytes were extracted using methanol, followed by an Oasis mixed-mode cation-exchange polymeric sorbent. The separation was performed on a reversed-phase Waters XBridge C18 column with the gradient mobile phase, consisting of water and acetonitrile (both acidified with 0.5% formic acid). Mean recoveries of MC-RR, MC-RR-GSH and MC-RR-Cys ranged from 80.7 to 93.7%, 81.1 to 93.1% and 80.3 to 93.2%, respectively, at three concentrations (0.2, 1.0 and 5.0 μg mL⁻¹). Limits of detection (LODs) for MC-RR, MC-RR-GSH and MC-RR-Cys were 6.12 and 9 ng mL⁻¹, respectively. Limits of quantification (LOQs) were 15, 30 and 22.5 ng mL⁻¹ for MC-RR, MC-RR-GSH and MC-RR-Cys, respectively. This method makes it feasible for the identification and quantification of MC-RR, MC-RR-GSH and MC-RR-Cys in limited and complex biological fluid samples (such as plasma and bile, typically 50 μL), which were previously excluded or difficult to study due to the relatively large sample volumes.

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1. Introduction

Microcystins (MCs) are a family of cyanobacterial blooms in eutrophic fresh and brackish waters [1,2]. To date, more than 80 structural analogs of MCs have been identified, and microcystin-RR (MC-RR) is one of the dominant variants in cyanobacterial blooms in lakes of China [3,4]. MCs primarily act as hepatotoxins because the liver is the target organ in various animals. Exposure to MCs, such as drinking water contaminated with MCs or ingesting algae synthesizing the toxins, poses a health risk to aquatic organisms and terrestrial animals, as well as humans [5–7]. In 1996, an episode of human intoxications by acute exposure to MCs led to the death of 52 patients during renal dialysis treatment in Caruaru, Brazil [8]. Recently, hepatotoxic microcystins were identified in the serum (average 0.228 ng MC-LR eq mL⁻¹) of a chronically exposed human population together with indications of hepatocellular damage [9].

The poisoning incidents of animals and human exposed to MCs emphasize the importance to study the mechanism of this compound in the detoxification. Previous studies have shown that GSH plays an important role in the detoxification of MCs in aquatic animals. The microcystin-glutathione conjugate has been demonstrated to form via glutathione S-transferase, which appeared to be the first step of detoxification, and then degraded to the cysteine conjugate [10–13]. However, these studies focused only on qualitative analyses. Recently, LC–ESI–MS in selected reaction monitoring mode (SRM) was first applied to simultaneously quantify MC-LR and its glutathione conjugate (MC-LR-GSH) in fish tissues [14]. Wu et al. [15] also developed a novel method for the simultaneous determination of MC-RR and its metabolites (MC-RR-GSH and MC-RR-Cys) in fish liver by LC–ESI–MS/MS. Based on the analytical methods described above, there have been several studies to investigate MCs and the GSH pathway metabolites in various tissues of aquatic animals in both field and laboratory conditions [16–18]. However, for biological fluids (e.g. plasma and bile), analysis of environmental pollutants is challenging due to the matrices are very complex, the concentrations are low and the amount of sample is often limited which potentially excluded small volume samples or small species from opportunities to study. Developing a method...
to identify and quantify MC-RR and its GSH pathway metabolites is necessary for further studying the detoxification mechanism of MCs, as plasma and bile are both very important and basic body fluids.

In the present study, a simple, fast and sensitive method for simultaneous quantification of MC-RR, MC-RR-GSH and MC-RR-Cys in plasma and bile was first developed using LC–MS with ESI(+) and SRM. This method was fully validated according to FDA guidelines, and successfully applied to pharmacokinetic studies of MC-RR, MC-RR-GSH and MC-RR-Cys in bighead carp after intraperitoneally (i.p.) injected with MC-RR-GSH.

2. Materials and methods

2.1. Chemicals and reagents

MC-RR was extracted and purified from *Microcystis aeruginosa* collected from Lake Dianchi, China, by the method described in Ramanan et al. [19] and Dai et al. [14]. The purity of MC-RR (>95%) was determined with the standard MC-RR (Wako Pure Chemical Industries, Osaka, Japan) using HPLC (LC–20A, Shimadzu, Kyoto, Japan) and its identity was confirmed using LC–MS (Thermo Electron, Waltham, MA, USA). l-Glutathione and L-cysteine were purchased from Acros Organics (Geel, Belgium), and the purity of both was greater than 99%. MC-RR-GSH and MC-RR-Cys were formed using an improved method described in Wu et al. [15] and their purity was over 95% confirmed by HPLC and LC–MS. HPLC-grade acetonitrile and methanol were supplied by TEDIA company (Fairfield, USA). Water was purified in a Milli-Q water purification system (Millipore, MA, USA). All other reagents were of analytical grade.

2.2. Preparation samples

2.2.1. Materials

Samples of plasma and bile were obtained from healthy bighead carp (*Aristichthys nobilis*, 118 ± 9 g), purchased from a local fish hatchery in WuHan, China. No MC-RR, MC-RR-GSH and MC-RR-Cys was detected using the developed method in the present study.

Stock solutions (10 µg mL⁻¹) were prepared in pure water, consisting of MC-RR, MC-RR-GSH and MC-RR-Cys. The mixture was diluted with pure water to make a series of working standard solutions. Quality control (QC) samples were prepared from the processed extract of plasma and bile samples spiked with low, medium and high concentrations (0.2, 1.0 and 5.0 µg mL⁻¹). All solutions were stored at −80 °C before use.

2.2.2. The preparation of plasma samples

Typically samples of plasma (50 µL) were spiked with low, medium and high levels of MC-RR, MC-RR-GSH and MC-RR-Cys. The three concentrations consist of 0.02, 0.1 and 0.5 µg of analytes, respectively. The spiked samples were added with 0.15 mL of EDTA-Na₂ saturated aqueous solution and 0.8 mL of ice-cold methanol. In the mixture, the ratio of methanol to water was around 4:1 (v/v), and this solution was commonly used to extract MCs in various animal tissues [20–22]. Then the mixture was vortex-mixed, and sonicated for 1 min (30 °C to 30 °C), 60 W, 20 kHz, Branson Digital Sonifier, Danbury, CT, USA) at 0 °C. After precipitation, the samples were centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was transferred to another clean tube, and the residue was extracted with 1 mL methanol aqueous solution (2:1, v/v) for another time.

2.2.3. The preparation of bile samples

Typically bile samples (50 µL) were spiked with three concentrations of MC-RR, MC-RR-GSH and MC-RR-Cys. The low, medium and high levels consisted of 0.02, 0.1 and 0.5 µg of MC-RR, MC-RR-GSH and MC-RR-Cys, respectively. The spiked samples were vortexed for 1 min, extracted with 100 µL of methanol and sonicated for 1 min at 0 °C. Then the mixture described above was centrifuged at 12,000 rpm for 10 min at 4 °C. After supernatant collection, the extraction was repeated again.

2.2.4. Cleanup procedure

The cartridge, Oasis MCX (a mix-mode cation-exchanger containing sulfonic acid groups) was selected to extract MC-RR and its metabolites. For the solid-phase extraction (SPE) using strong cation exchanger, the extracts of plasma and bile samples were dried, redissolved in 0.2 mL solution of 0.01 M EDTA-Na₂–5% acetic acid and loaded onto a column, which was conditioned and equilibrated with methanol and pure water in sequence. After loading, the column was first washed with 2% aqueous formic acid and then with 90% methanol. Finally, MC-RR and its glutathione and cysteine conjugates were eluted using 10 mL of 15% aqueous ammonia in methanol. The solvent was removed by evaporation, and the dry residue was redissolved in 100 µL of the LC mobile phase. Ten microliter was injected into the LC–MS system for analysis.

2.3. Instrumentation

Qualitative and quantitative analyses were performed by a Surveyor HPLC system (Thermo Electron Corporation, San Jose, CA, USA) comprising pump, autosampler and photoelectric diode array detector (PDA) combined with a LCQ Advantage MAX ion trap mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA) equipped with electrospray ionization (ESI) interface.

The sample was separated on a Waters XBridge C18 column (2.1 mm × 100 mm, d overturned, Waters Corporation, USA). The mobile phase consisted of (A) water and (B) acetonitrile (both containing 0.05% formic acid). A gradient program was used, starting from 5% B to 95% B in 20 min using a flow rate of 0.2 mL/min⁻¹. Mobile phase B rapidly changed from 95% to 5% in 0.5 min. Equilibration at initial conditions was performed for 5 min before the next injection. The injection volume was typically 10 µL. The temperatures of the vial tray and column oven in the autosampler were set to 10 and 40 °C, respectively. The effluent was transferred on-line to ESI–MS system without splitting. The optimized ionization conditions of the MS/MS instrument were: collision gas, ultra-high purity helium (He); nebulizing gas, high purity nitrogen (N₂); sheath gas (N₂) flow rate, 20 units; the capillary temperature, 250 °C. The tube lens voltage was 55.5 V for MC-RR and 45.5 V for MC-RR-GSH and MC-RR-Cys. The collision energy was 32% for MC-RR, 34% for MC-RR-GSH and 30% for MC-RR-Cys. The instrument control, data processing, and analysis were conducted by using Xcalibur software.

2.4. Method validation

External calibration samples were prepared by adding the analytes to blank plasma and bile sample extracts cleaned up by SPE. The concentrations of MC-RR, MC-RR-GSH and MC-RR-Cys were confirmed to be 0.02, 0.1, 0.2, 1.0, 2.0 and 5.0 µg mL⁻¹. The recovery was calculated by comparing the peak area, obtained from blank samples spiked before extraction, with that of QC samples. The values were expressed as mean ± standard deviation (SD).

The limit of detection (LOD) is defined as “the smallest amount of an analyte that can be reliably shown to be present or measured under defined conditions; the smallest amount that is clearly distinguishable from background or “blank”” [23,24]. Method
2.5. Application

The LC–MS method was applied to study the concentration–time profiles of MC-RR, MC-RR-GSH and MC-RR-Cys in plasma and bile samples of bighead carp. These samples were collected from the experiment of Li et al. [27]. Briefly, the carp were divided into two groups at random, and fasted for 24 h before the initiation of the experiment. 0.55 μmol MC-RR-GSH kg⁻¹ body weight (BW) was i.p. injected in the treated group. The control group was injected with the same volume of vehicles, which was 0.9% (w/v) saline solution. And then plasma and bile samples were collected at 0, 1, 2, 6 and 24 h post-injection, respectively. Samples were extracted and cleaned up following the method described above.

3. Results and discussion

3.1. LC–MS/MS analysis of MC-RR, MC-RR-GSH and MC-RR-Cys

MC-RR, MC-RR-GSH and MC-RR-Cys were analyzed by ESI–MS/MS in a selective reaction monitoring mode (Figs. 1 and 2). Based on SRM chromatogram monitored ion at m/z 673.79, and the product ions at m/z 536.97, 608.91, the peak obtained at 10.11 min was derived from MC-RR-GSH. The product ions were abundant at m/z 513.36 and 519.95 of MC-RR-Cys, and they were formed from the parent ion of 580.62. The selected ion chromatogram showed ions at m/z 452.89 and 887.50 for MC-RR, and they were correspondent to the ion at m/z 520.08. The retention times of MC-RR-Cys and MC-RR were 10.29 and 11.12 min, respectively. With the help of high accuracy MS/MS, the data obtained from fragment ions was used for structural analysis and quantitative purposes of MC-RR, MC-RR-GSH and MC-RR-Cys.

3.2. Optimization of sample preparation

To avoid contaminating the ion source and enhance the responses of analytes, an efficient and clean extraction was very important in the process of sample preparation. The effect of different extraction solvents on the recovery of analytes in plasma and bile is shown in Fig. 3. Methanol and acetonitrile were the...
most widely used as extraction solvent in biological fluid samples [228–30]. In the plasma preparation, methanol was chosen for further development as it had better recoveries (93.7%, 91.1% and 85.8% for MC-RR, MC-RR-GSH and MC-RR-Cys, respectively), which were obviously higher than those of acetonitrile (78.6%, 75.0% and 78.2%) and EDTA-Na2 (0.01 M–5% acetic acid (35.6%, 32.9% and 32.8%). The quite lower recoveries for EDTA-Na2 (0.01 M–5% acetic acid might be due to high amounts of endogenous components, which caused some undesirable ion suppression to the analytes. For bile samples, experiments showed that methanol has yielded satisfied recovery of MC-RR, MC-RR-GSH and MC-RR-Cys (87.5%, 87.7% and 87.0%, respectively). This novel extraction approach has distinct advantages, including the use of smaller samples (typically 50 μL), which allows for its applicability for limited liquid samples. It is also user-friendly, as for most purposes it eliminates lyophilization requirements.

Oasis mix-mode ion-exchange cartridge (MCX) was chosen to obtain clean extract of analytes, due to MC-RR, MC-RR-GSH and MC-RR-Cys are more hydrophobic. Then the eluent was concentrated to meet the limit of determination by LC–MS.

### 3.3. Precision and LOD

The within day and between days precisions of MC-RR, MC-RR-GSH and MC-RR-Cys were summarized in Table 1. The results indicate that the assays have remarkable reproducibility and are under the acceptable level (<15%). The LOD values of MC-RR, MC-RR-GSH and MC-RR-Cys were 6, 12 and 9 ng mL\(^{-1}\), respectively, in both plasma and bile while the corresponding LOQs were 15, 30 and 22.5 ng mL\(^{-1}\), respectively.

### 3.4. Selectivity and linearity

Samples prepared from the blank plasma and bile spiked with three levels (low, medium and high) of analytes after extraction were used as positive controls, while the blank plasma and bile samples were used as negative controls. The representative TIC chromatogram of a blank plasma sample spiked with the medium concentration was shown in Fig. 1. The LC–MS analyses of the spiked extracts and the blank samples (Fig. S1) indicate that little inference is present at the known retention time of analytes.
Table 1

Precisions of the method expressed as RSD for the three analytes in plasma and bile of bighead carp.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Within-assay RSD (%; n = 5)</th>
<th>Between-assay RSD (%; 3 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Medium&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MC-RR (plasma)</td>
<td>2.2</td>
<td>4.8</td>
</tr>
<tr>
<td>MC-RR-GSH (plasma)</td>
<td>2.0</td>
<td>2.8</td>
</tr>
<tr>
<td>MC-RR-Cys (plasma)</td>
<td>2.1</td>
<td>6.4</td>
</tr>
<tr>
<td>MC-RR (bile)</td>
<td>10.8</td>
<td>8.1</td>
</tr>
<tr>
<td>MC-RR-GSH (bile)</td>
<td>3.3</td>
<td>4.1</td>
</tr>
<tr>
<td>MC-RR-Cys (bile)</td>
<td>7.9</td>
<td>8.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Low fortification: 0.2 μg mL<sup>−1</sup> for MC-RR, MC-RR-GSH and MC-RR-Cys.
<sup>b</sup> Medium fortification: 1.0 μg mL<sup>−1</sup> for MC-RR, MC-RR-GSH and MC-RR-Cys.
<sup>c</sup> High fortification: 5.0 μg mL<sup>−1</sup> for MC-RR, MC-RR-GSH and MC-RR-Cys.

Calibration curves were created for MC-RR, MC-RR-GSH and MC-RR-Cys in fish plasma and bile (Table 2). The correlation coefficient (r<sup>2</sup>) values showed good linearity for the quantification of analytes ranging from 0.02 to 5.0 μg mL<sup>−1</sup>.

3.5. Extraction recovery and stability

The recovery of an analyte is determined from the ratio of peak areas, obtained from the spiked biological matrices, with that of the QC sample. The extraction recoveries of analytes, which were evaluated at low, medium and high levels, ranged from 80.3% to 93.7% for plasma, and from 80.7% to 93.2% for bile. The data were shown in Table 3, and were within the acceptance criteria.

The stability for the spiked plasma and bile was tested at low, medium and high concentration in triplicate. Analytes of MC-RR, MC-RR-GSH and MC-RR-Cys were stable after three freeze/thaw cycles (−20 °C to room temperature) in 72 h (Table S1). The degradation of analyte was also evaluated in an autosampler post-extraction for 24 h at ambient temperature (10 °C). The results showed that the degradation was less than 10.2% (Table S2). Thus, MC-RR, MC-RR-GSH and MC-RR-Cys are considered to be stable in plasma and bile matrices under frozen storage and assay processing.

3.6. Method application

Recently, several studies have investigated MCs and the GSH pathway metabolites in tissues of aquatic animals in both field and laboratory conditions [16–18]. However, we still face challenging problems in detection and quantification of MCs and its GSH pathway metabolites in animal fluid samples (e.g. blood and bile): low concentration of target compounds, complex matrix, or limited amount of samples. In the present study, based on the established method, MC-RR-GSH, MC-RR-Cys and MC-RR were simultaneously determined in plasma and bile samples of bighead carp exposed to MC-RR-GSH. We aimed to directly trace the toxicokinetics of this glutathione conjugate in vivo and to further identify metabolic characteristics of the GSH pathway, which play an important role in the detoxification of MCs in various animals.

Table 2

Calibration curves for quantification of MC-RR, MC-RR-GSH and MC-RR Cys by LC–ESI-MS/MS (X, concentration μg mL<sup>−1</sup>; Y, peak area).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sample</th>
<th>Calibration curves</th>
<th>r&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC-RR</td>
<td>Plasma</td>
<td>Y = 8.27127e + 007 × X</td>
<td>0.9941</td>
</tr>
<tr>
<td></td>
<td>Bile</td>
<td>Y = 7.23128e + 007 × X</td>
<td>0.9916</td>
</tr>
<tr>
<td>MC-RR-GSH</td>
<td>Plasma</td>
<td>Y = 2.87446e + 008 × X</td>
<td>0.9921</td>
</tr>
<tr>
<td></td>
<td>Bile</td>
<td>Y = 1.33254e + 008 × X</td>
<td>0.9982</td>
</tr>
<tr>
<td>MC-RR-Cys</td>
<td>Plasma</td>
<td>Y = 7.67421e + 007 × X</td>
<td>0.9925</td>
</tr>
<tr>
<td></td>
<td>Bile</td>
<td>Y = 4.02492e + 007 × X</td>
<td>0.9942</td>
</tr>
</tbody>
</table>

Osmosis of MC-RR-GSH from the peritoneal cavity into the plasma occurred rapidly after exposure to MC-RR-GSH, with the highest concentration (2.464 ± 0.343 μg mL<sup>−1</sup>) at 2 h post-injection (Fig. 4A). However, a continuous decrease of MC-RR-GSH was observed after 2 h, with a 3.8-fold decrease until 24 h post-injection. These results indicated that MC-RR-GSH is effectively eliminated from the blood, and still not completely converted to its downstream metabolites MC-RR-Cys at 24 h post-injection. The MC-RR-Cys content increased from 0.081 ± 0.010 (1 h post-injection) to 1.291 ± 0.125 μg mL<sup>−1</sup> (24 h post-injection) in a time-dependent manner. Aside from MC-RR-Cys, free MC-RR was also determined.

Fig. 4. The concentration-time profiles for MC-RR, MC-RR-GSH and MC-RR-Cys in plasma (A) and bile (B) of bighead carp after intraperitoneal injection of MC-RR-GSH.
Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spiked level (μg·mL⁻¹)</th>
<th>MC-RR</th>
<th>Mean ± SD (%)</th>
<th>RSD (%)</th>
<th>MC-RR-GSH</th>
<th>Mean ± SD (%)</th>
<th>RSD (%)</th>
<th>MC-RR-Cys</th>
<th>Mean ± SD (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>0.2</td>
<td>83.6 ± 12.6</td>
<td>15.1</td>
<td></td>
<td>93.1 ± 7.6</td>
<td>8.2</td>
<td></td>
<td>80.3 ± 4.9</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>93.7 ± 3.8</td>
<td>4.1</td>
<td></td>
<td>91.1 ± 3.2</td>
<td>3.5</td>
<td></td>
<td>85.8 ± 2.0</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>90.9 ± 7.8</td>
<td>8.8</td>
<td></td>
<td>88.0 ± 2.7</td>
<td>3.2</td>
<td></td>
<td>83.1 ± 1.3</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Bile</td>
<td>0.2</td>
<td>80.7 ± 8.1</td>
<td>10.1</td>
<td></td>
<td>81.1 ± 2.8</td>
<td>3.7</td>
<td></td>
<td>87.0 ± 8.3</td>
<td>9.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>87.5 ± 7.9</td>
<td>9.0</td>
<td></td>
<td>87.7 ± 3.0</td>
<td>3.5</td>
<td></td>
<td>88.4 ± 11.4</td>
<td>12.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>86.3 ± 11.1</td>
<td>12.9</td>
<td></td>
<td>81.8 ± 5.5</td>
<td>6.7</td>
<td></td>
<td>93.2 ± 12.2</td>
<td>13.1</td>
<td></td>
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</tbody>
</table>

References


Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.jchromb.2014.05.057

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4. Conclusion

In this study, a simple and sensitive LC–MS/MS method was first developed and validated for simultaneous determination of MC-RR, MC-RR-GSH and MC-RR-Cys in fish plasma and bile. Mean recoveries of MC-RR, MC-RR-GSH and MC-RR-Cys ranged from 80.7% to 93.7%, from 81.1% to 91.1%, and from 80.3% to 93.2%, respectively, at three concentrations (low, medium and high). Validation data demonstrate that low concentrations of MC-RR, MC-RR-GSH and MC-RR-Cys can be detected in plasma and bile applying this method. Therefore, our novel method can be used to study the toxicokinetics of MC-RR and its metabolites in small biological fluid samples, and further to evaluate the glutathione pathway in the detoxification of MC-RR in fish.