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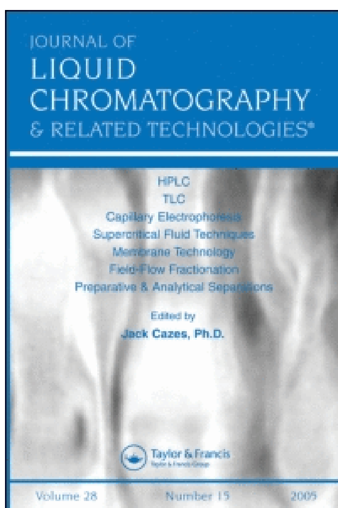

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Method for Quantifying Microcystins in Fish Using Immunoaffinity Purification

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Abstract: Immunoaffinity purification coupled with high performance liquid chromatography with photodiode array detection was used to quantify microcystins in fish samples. The immunoaffinity purification method consisted of heat denaturation, pronase digestion, and Sep-Pak C18 extraction together with subsequent purification using an immunoaffinity column. The immunoaffinity purification allowed precise analysis of microcystins-RR, -YR, and -LR in fish samples by eliminating co-extracted substances. An internal standard of [D-Asp^3]microcystin-LR was used to improve precision and for the recovery corrections. The calibration curves for all 3 microcystins showed linear relationship at concentrations from 40 to 200 ng/g.

Keywords: Microcystin, Immunoaffinity purification, Fish, HPLC, Quantification, Internal standard

INTRODUCTION

Toxic cyanobacteria blooms occur in eutrophic lakes and drinking water supplies throughout the world.[1] Microcystins are the most commonly

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found toxins, and are produced by freshwater cyanobacteria *Microcystis, Oscillatoria, Anabaena* and *Nostoc.*[2] The presence of microcystins in aquatic environments is increasingly being acknowledged as a public health hazard because acute exposure to high concentrations of microcystins is responsible for the deaths of wildlife and domestic animals, and chronic exposure may promote tumorigenesis in the liver.[3] A disease referred to as netpen liver disease, has been observed in Atlantic salmon reared in net-pens in the coastal waters of Canada and USA, and microcystin-LR was found in livers of salmon.[4] To prevent the risk posed by microcystins, it is important to monitor levels of microcystins in fish and mussels, as well as in water reservoirs where cyanobacterial blooms occur.

Microcystins exist in free and protein-bound forms in biological samples such as samples of fish, mussels, and experimentally dosed mouse and rat livers.[5,6] Magalhaes et al. reported the accumulation of microcystins in fish tissues, mentioning that their results might be underestimated because they only measured the free form of microcystins.[7] Protein-bound microcystins need to be released from binding proteins when measuring the total microcystin concentration. Williams et al. measured total microcystin concentrations in the livers of Atlantic salmon using Lemieux oxidation and subsequent gas chromatography/mass spectrometric analysis of the reaction product.[5] In spite of the successful application of the method, they could not detect individual microcystins.

We have established a two step clean up method for a high performance liquid chromatography (HPLC) based analysis of microcystins in lake water, which consisted of a solid-phase extraction on an ODS silica gel cartridge followed by a clean-up step on silica gel.[8] The method was successfully applied to the identification of microcystins in freshwater mussels.[9] However, it was unable to eliminate co-extracted substances in the fish samples (data not shown). We have reported a method of immunoaffinity purification for the determination of microcystins in lake waters.[10,11] We have also reported that the method enabled us to identify microcystins and their metabolites formed in vivo in mouse and rat livers by HPLC and liquid chromatography/mass spectrometry, by removing co-extracted substances in the hepatic cytosols.[12–14] In the present study, we describe a method of quantifying microcystins in fish using immunoaffinity purification.

EXPERIMENTAL

Materials

Microcystins -LR, -YR, and -RR were purchased from Kanto Chemicals Co. (Tokyo, Japan). [D-Asp^3]microcystin-LR was purified from a natural bloom collected in Lake Suwa in Japan as described previously.[15] Rainbow trout

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was purchased at a local fish market. All reagents were of HPLC grade or analytical grade.

Preparation of Immunoaffinity Column

Immunoaffinity gel was prepared by coupling M8H5 anti-microcystin-LR monoclonal antibodies to Affi-Gel 10 (Bio-Rad, Hercules, CA, USA) as described previously.[14,16,17] The gel obtained was suspended in phosphate-buffered saline (PBS) containing 0.1% sodium azide and stored at 4°C. The gel mixture (0.5 mL) was transferred to a polypropylene cartridge (Muromac column; Muromachi Kagaku Kogyo Ltd., Tokyo, Japan) when used.

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Synthesis and Purification of Dihydromicrocytin-LR, Dihydromicrocystin-RR, and Microcystin-LR 6-Monomethyl Ester

Three modified microcystins were produced as candidates for an internal standard. Microcystin-LR (2.2 mg) and sodium borohydride (0.4 mg) were dissolved in 0.2 mL of 2-propanol. The reaction was allowed to proceed with continuous stirring at room temperature for 1.5 hr. The reaction was quenched with the addition of 0.1 mL of 10% acetic acid and the products were evaporated to dryness under reduced pressure at 35°C. The isolation of dihydromicrocystin-LR was carried out by loading the reaction products in water (5 mL) on a preconditioned Sep-Pak C_{18} cartridge (500 mg, Waters Co., Milford, MA, USA). The cartridge was then rinsed with water (5 mL) and 10% methanol/water (8 mL), and the products were eluted with 90% methanol/water (15 mL). Final purification was accomplished by using TOYOPEARL HW-40F gel chromatography (11 × 950 mm, Tosoh, Tokyo, Japan; flow rate 1 mL/min; detection, UV 238 nm) with 80% methanol/water as the mobile phase, to yield 1.7 mg of the purified dihydromicrocystin-LR.

The reaction of microcystin-RR (2.0 mg) with borohydride (0.4 mg) yielded 1.5 mg of the purified dihydromicrocystin-RR.

Microcystin-LR (5.7 mg) was dissolved in 1 mL of 5% hydrochloric acid/methanol. The reaction was allowed to stand at room temperature for 1 hr. The reaction was quenched with the addition of silver(I) carbonate. After filtration through absorbent cotton, the products were evaporated to dryness under reduced pressure at 35°C. Final purification was accomplished by preparative HPLC separation using a Nucleosil 5C18 column (4.6 × 150 mm, Chemco Scientific Co., Osaka, Japan; flow rate 1 mL/min; detection, UV 238 nm) with 0.05% trifluoroacetic acid/water as the mobile phase to yield 5.0 mg of the purified microcystin-LR 6-monomethyl ester.

Extraction and Purification

A 5 g portion of fish sample (liver or tissue) was minced in 100 mM Tris-HCl buffer (pH 7.2, 5 mL) and then homogenized. An internal standard of [D-Asp^3]microcystin-LR (500 ng) was spiked after homogenization. The homogenate was centrifuged at 10,000 g for 10 min. The operation was repeated and the resulting supernatant was combined and then centrifuged at 100,000 g for 60 min. The supernatant was heat denatured for 30 min at 90°C after addition of an equal volume of 100 mM potassium phosphate buffer (pH 7.5). Then the solution was treated with pronase (20 mg, Boehringer Mannheim Biochemicals, Indianapolis, IN, USA) and incubated for 60 min at 37°C. After three cycles of heat denaturation and pronase digestion, the resulting solution was centrifuged at 10,000 g for 10 min, and the supernatant was extracted with a Sep-Pak C_{18} cartridge (2 g). The cartridge was washed with water (40 mL) and the eluate obtained from the cartridge with 90% aqueous methanol (40 mL) was evaporated to dryness under reduced pressure at 35°C. The resulting residue was dissolved in PBS containing 0.1% bovine serum albumin (BSA) (5 mL), and the solution was subjected to further purification with an immunoaffinity column, which was preconditioned with PBS (5 mL), methanol (5 mL), distilled water (5 mL), and PBS containing 0.1% BSA (5 mL). After washing with PBS (5 mL) and distilled water (5 mL), the microcystin fraction was eluted with 100% methanol (10 mL). The eluate was evaporated to dryness under reduced pressure at 35°C and then subjected to HPLC.

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HPLC

The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-10AD pump coupled to a SPD-10AM UV-photodiode array detector set at 200–300 nm and a CLASS-10 integrating system. Separation was accomplished under reversed phase isocratic conditions with a TSK-GEL ODS-80Ts column (150 × 4.6 mm, Tosoh, Tokyo, Japan) and a mobile phase of acetonitrile:0.05 M phosphate buffer (pH 3.0) (3 : 7). The flow rate was 1.0 mL/min.

RESULTS AND DISCUSSION

We first examined whether our previously reported method of immunoaffinity purification could remove co-extracted substances in fish samples. Rainbow trout livers spiked with microcystins-RR, -YR, and -LR at a concentration of 100 ng/g were extracted with Tris-HCl buffer and then heat denatured, digested with pronase, treated with Sep-Pak C_{18}, and purified with the immunoaffinity column. When an extract was prepared without immunoaffinity

purification, the added microcystins could not be analyzed due to peaks of co-extracted substances in the liver (Fig. 1a). When this extract was purified further with the immunoaffinity column, the peaks of all of the added microcystins were clearly detected by eliminating co-extracted substances (Fig. 1b).

We then examined the recovery from rainbow trout livers spiked with microcystins-RR, -YR, and -LR at 100 ng/g. The overall recovery and C.V. values of the 5 repeated measurements were 40.0% and 7.8% for microcystin-RR, 38.6% and 4.8% for microcystin-YR, and 33.8% and 4.6% for microcystin-LR, respectively. The heat denaturation and pronase digestion were responsible for the decrease in the recovery. In fact, when a mixture

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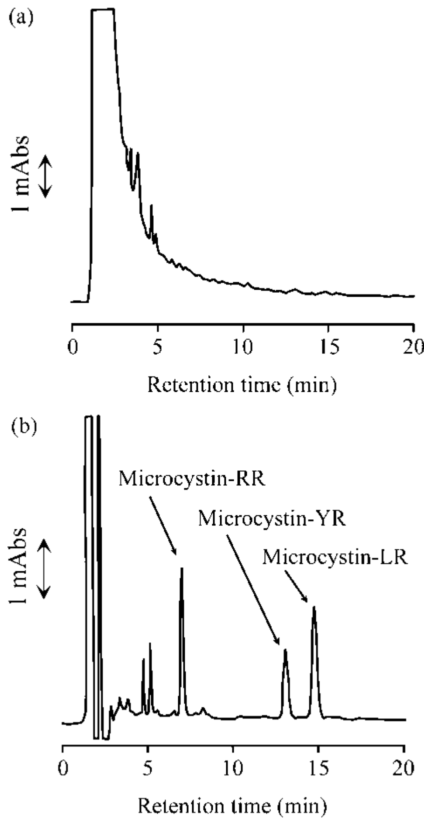

Figure 1. Chromatograms of rainbow trout liver extracts before (a) and after (b) immunoaffinity purification. A mixture containing 1000 ng each of microcystins-RR, -YR, and -LR was added to 5 g of rainbow trout liver and subjected to immunoaffinity purification. The peaks of co-extracted substances in liver were effectively eliminated by the purification process.

of 3 microcystins (5 μg each) was subjected to heat denaturation and pronase digestion, 15 to 30% of the microcystins were lost. The heat denaturation and pronase digestion were, nevertheless, indispensable because protein bound microcystins need to be released from binding proteins to measure the total microcystins in fish samples. We therefore, used an internal standard to correct the low levels of recovery of microcystins.

Three chemically prepared microcystin derivatives, dihydromicrocystin-LR, dihydromicrocystin-RR, and microcystin-LR 6-monomethyl ester, and [D-Asp^3]microcystin-LR purified from a natural bloom were examined as candidates for an internal standard as follows: 500 ng of each test compound dissolved in 0.1% BSA-PBS (5 mL) was applied to an immunoaffinity column. The column was washed with PBS, followed by distilled water, and the desired fraction was eluted with 100% methanol. The immunoaffinity column recovered (3 repeated measurements) 84% of [D-Asp^3]microcystin-LR, 79% of dihydromicrocystin-LR, 78% of dihydromicrocystin-RR, and 30% of microcystin-LR 6-monomethyl ester, showing that the latter can not be used. HPLC conditions that can resolve the three microcystins and an internal standard were then examined. Only [D-Asp^3]microcystin-LR was separated from the three microcystins. Although [D-Asp^3]microcystin-LR is purified from naturally occurring water blooms, we selected [D-Asp^3]-microcystin-LR as the internal standard because its detection rate is very low.[2]

To examine the applicability of the new method to the quantification of microcystins in fish, rainbow trout livers, spiked with a series of microcystins at concentrations of 40, 80, 120, and 200 ng/g, and the internal standard [D-Asp^3]microcystin-LR at a set concentration of 100 ng/g, were subjected to the immunoaffinity purification and HPLC. The recovery fluctuated; 27.1–44.9% for microcystin-RR, 26.7–38.4% for microcystin-YR, 27.8–45.0% for microcystin-LR, and 38.7–53.4% for [D-Asp^3]microcystin-LR. Due to these fluctuations, the calibration curves showed poor linearity without correction using an internal standard ($r = 0.970–0.982$) (Fig. 2a). In contrast, the calibration curves with the correction showed a linear relationship from 40 to 200 ng/g of all 3 microcystins ($r = 0.992–0.995$) (Fig. 2b). These results indicate that the use of an internal standard allows for the correction of an incomplete recovery and improves the reproducibility of the calibration curves. The time required to perform the entire procedure from the extraction of toxin to the measurement of HPLC was, at most, one day.

The proposed method was applied to tissue samples of rainbow trout. The co-extracted substances in the tissue were effectively eliminated and the peak of the internal standard [D-Asp^3]microcystin-LR was clearly detected (Fig. 3). The recovery of the added internal standard [D-Asp^3]microcystin-LR ranged 37.2–54.6% (4 repeated measurements), which was nearly equal to that in liver samples. These results indicate that the new method can also be applied to tissue samples.

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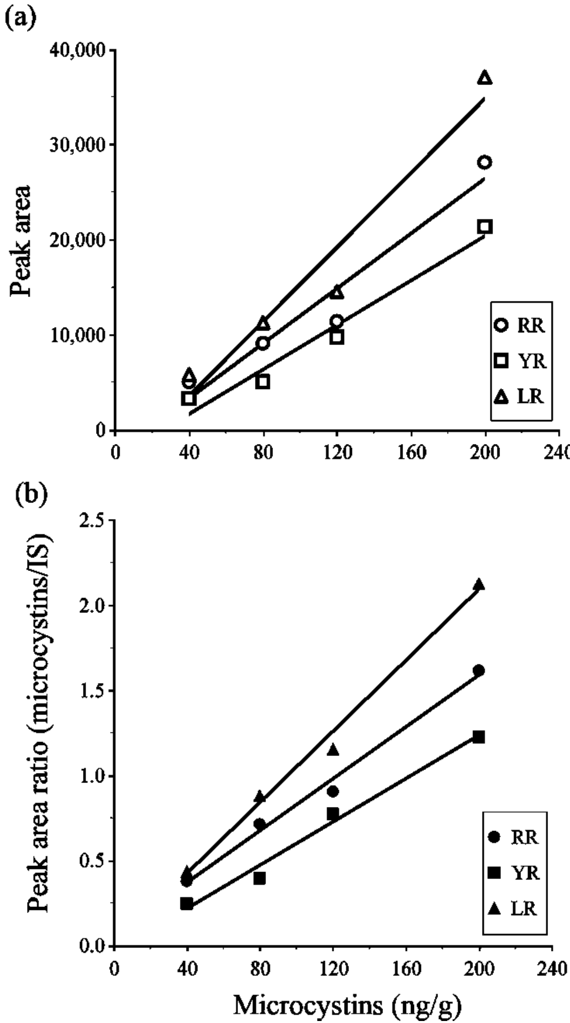

Figure 2. Calibration curves for microcystins-RR, -YR, and -LR (a) without and (b) with the correction using the internal standard. The calibration curves for all 3 microcystins showed a linear relationship from 40 to 200 ng/g with the correction.

We developed a method of quantifying microcystins in fish using immunoaffinity purification. The use of an internal standard, [D-Asp^3]microcystin-LR, permitted corrections for incomplete recovery and improved the reproducibility of the calibration curves. Accordingly, we recommend using the method described here for monitoring total (free and protein-bound form) microcystin levels in fish.

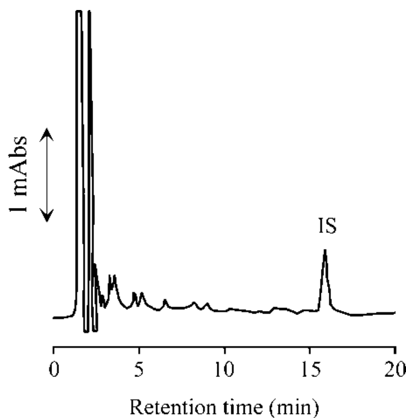

Figure 3. Chromatogram of a rainbow trout tissue extract after immunoaffinity purification. The internal standard of [D-Asp^3]microcystin-LR (500 ng) was added to 5 g of rainbow trout tissue and subjected to immunoaffinity purification. The co-extracted substances in the tissue were effectively eliminated and the peak of the added internal standard [D-Asp^3]microcystin-LR was clearly detectable.

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