Microcystin Detection Characteristics of Fluorescence Immunochromatography and High Performance Liquid Chromatography

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Different detection characteristics of fluorescence immunochromatography method and high performance liquid chromatography (HPLC) method for the analysis of cyanobacterial toxins were studied. In particular, low and high limits of detection, detection time and reproducibility and detectable microcystin species were compared when fluorescence immunochromatography method and high performance liquid chromatography method were applied for the detection of microcystin (MC), a cyclic peptide toxin of the freshwater cyanobacterium *Microcystis aeruginosa*. A Fluorescence immunochromatography assay system has the unique advantages of short detection time and low detection limit, and high performance liquid chromatography detection method has the strong advantage of individual quantifications of several species of microcystins.

Key Words : Microcystins, Detection characteristics, Immunochromatography, High performance liquid chromatography (HPLC)

Introduction

Microcystins are a family of cyclic polypeptides produced by different species of cyanobacteria (blue-green algae), which can form blooms in lakes and water reservoirs.[1] Their basic structure is a cyclic heptapeptide and their structural variations give rise to more than 50 types of microcystins known today (Fig. 1).[1] The most extensively studied form is microcystin-LR (MCLR), which contains L-leucine and L-arginine in the two main variant positions.

Microcystins and related polypeptides are potent hepatotoxins in fish, birds, and mammals.[2] The consequence of an acute poisoning by these compounds is the rapid disorganization of the hepatic architecture,[2,3] leading to massive intrahepatic hemorrhage, often followed by death from hypovolemic shock or hepatic insufficiency.[4] Matsushima *et al.*[5] report that microcystins penetrate with difficulty the epithelial cells, which reflects tissue specificity, and their target cell is the hepatocyte. This cellular specificity and organotropism of microcystins is due to the selective transport system, the multispecific bile acid transport system, present only in hepatocytes.[3,6]

Microcystins are potent inhibitors of protein phosphatases 1 and 2A,[7-9] which are regulatory enzymes present in the cytosol of mammalian cells. This action may explain the effects of microcystins as cancer promoters[10,11] and the promotion of primary liver cancer in humans exposed to long-term low doses of these cyclic peptide toxins through drinking water[12-14] as well as the cytoskeletal disruption and formation of plasma membrane blebs (blebbing) in hepatocytes.[3]

Since microcystins are potent hepatotoxins for humans and animals, the development of sensitive and reliable detection methods is of great importance. The efforts have been aimed at developing more sensitive screening methods to replace the nonspecific mouse bioassay, traditionally used for the identification of toxic strains of *Microcystis*.

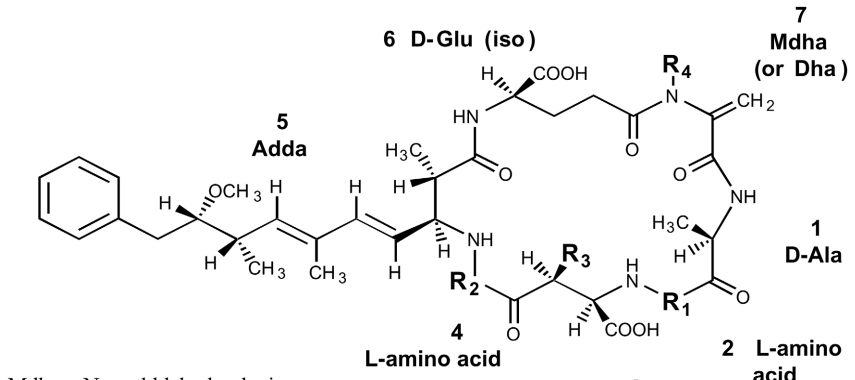
Thus far, HPLC techniques have been used as a sensitive method of analysis,[15-17] but this approach relies on the availability of toxin standards for comparison and is therefore only applicable to known toxins. HPLC is also a relatively slow technique and requires expensive equipment and appropriate training.

The development of biological methods was first focused on enzyme-linked immunosorbent assays (ELISA).[18,19] However, ELISA also has rather long analysis time and require appropriate training. In the present study, we used a new fluorescence immunochromatography assay system, employing monoclonal antibodies of microcystin LR. This immunochromatographic assay system was rapid and sensitive in the detection of microcystins in water samples.

This study deals with different detection characteristics of fluorescence immunochromatography method and HPLC for the analysis of cyanobacterial toxins.

Experimental Section

Structures of microcystins and their derivatives used in our experiments are shown in Figure 1. Several kinds of *Microcystis aeruginosa* (MA), cyanobacteria known to produce MCs, were used. One liter batch cultures of cells were grown in MA medium. Cultures were maintained at 20-25 °C under constant illumination by white fluorescent light incident on the surface of the growth flask. Cells were harvested by centrifugation ($9000 \times g$, 5 min) and were lyophilized before storage at −20 °C. Microcystins were identified by high performance liquid chromatography using Beckman equipment. The equipment included a 116 pump (SYSTEM GOLD Programmable Solvent Module 126), 126 Detector(SYSTEM GOLD Programmable Detector Module 166) and a multisolvent delivery system. Chromatograms were monitored at UV 238 nm. The column was a



Mdha : N-methldehydroalanine
Dha : dehydroalanine
Adda : (2S,3S,8S,9S)-3-amino-9-methoxy-10-phenyl-2,6,8-trimethyl-deca-4,6-dienoic acid

	R_1	R_2	R_3	R_4	MW
Microcystin - LR	Leu	Arg	CH_3	CH_3	994
Microcystin - YR	Tyr	Arg	CH_3	CH_3	1044
Microcystin - RR	Arg	Arg	CH_3	CH_3	1037

Figure 1. Structure of microcystins. A characterisitic of microcystins and related cyanobacterial toxins is the hydrophobic amino acid Adda which contains in position 5 two conjugated double bonds. Numbers represent the positions of the corresponding amino acid.

Ultrasphere 5 μm ODS (Beckman 4.6 mm × 25 cm). Methanol/0.02 M Na_2SO_4 (55 : 45) was used as a mobile phase at a flow rate of 2 mL/min.

To concentrate the microcystins effectively in water samples, a new concentrating apparatus utilizing C_{18} solid phase extraction cartridges was designed. The concentrating apparatus consisted of four separate Baker J. C_{18} cartridges (500 mg) in parallel. A peristaltic pump controlled the flow in the whole system. Properly rinsed by passing 10-15 mL of methanol and conditioned, the C_{18} cartridges could, be used to analyze a large volume of water samples (6 L).

To produce monoclonal antibodies (mAb) against microcystin-LR, microcystin-LR was conjugated to bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) in the presence of 1-ethyl-3,3'-dimethyl-aminopropyl-carbodiimide (EDAC). Hybridomas producing anti-MCLR Mabmcs were prepared by a standard method for immunization and cell fusion Six-week-old mice were immunized with MCLR-KLH. The initial injection used 0.2 mL of the conjugate solution and 0.2 mL of complete Freund's adjuvant. Booster injections used conjugate solution and incomplete Freund's adjuvant. The mAb was produced in BALB/c mice by the hybridoma cell line, SP_2/O-Ag14.

Two weeks after fusion, the hybridomas were screened for the production of anti-MCLR antibodies by an indirect ELISA in which the MCLR-protein conjugates were coated onto plates. Hybridomas were estimated as positive for the generation of specific antibodies in case they were positive for MCLR-BSA, MCLR-KLH. The positive hybridomas were cloned several times by a limiting dilution method. Each of the established hybridoma cells producing the antibody was grown in medium supplemented with HT. Large quantities of antibodies were prepared from serum-free cultured supernatants of hybridomas by membrane ultrafiltration, ammonium sulfate precipitation, with a final purification using a protein G column.

In the fluorescence immunochromatographic assay system, an unknown sample containing MCs is simply mixed with the detection solution containing fluorescence-conjugated MCs (or fluorescence-conjugated mAb) and fluorescence-conjugated biotin as an internal standard. MCs in the sample and fluorescence conjugated-MCs in the detection solution competes for binding to capture antibodies, which are coated at the test line on the detection zone as they flow laterally from the sample pad to absorption pad. The fluorescence-conjugated biotin in sample mixture is captured by the streptavidin that was dispensed at the control line on the detection zone. The intensity of captured fluorescence conjugates on the detection zone is scanned by a Laser Fluorescence Scanner (Boditech, Chunchon, South Korea) and converted into area value. The concentration of MCs in the unknown sample is calculated from the standard curve or the equation of the standard curve. Figure 2 shows the schematic diagrams of the fluorescence immunochromatographic assay strip.

Results and Discussion

Low and High Limits of Detection. When a water sample is placed on our fluorescence immunochromatographic strip, two chromatographic lines of fluorescence intensity curves always appear. The fluorescence intensity of the first line (which is also called the test line) is inversely proportional to the concentration of microcystin in water

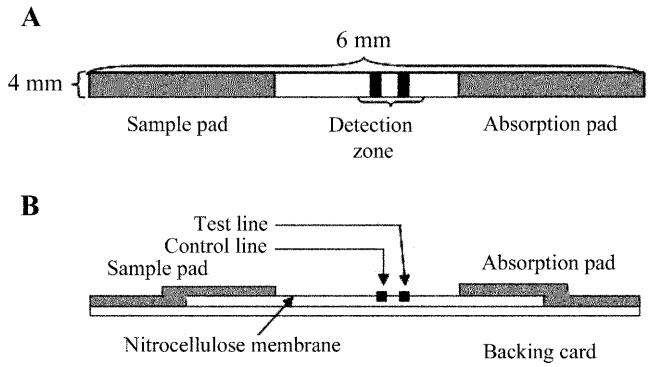


Figure 2. Schematic diagrams of fluorescence immunochromatographic assay strip. A and B represent the upper and the side view of the strip, respectively.

sample. The second line of fluorescence intensity curves (which is called the control line) is related to the mass transport of the sample and should show a constant value regardless of the concentration of microcystin in the water sample. This phenomena results from the method of making the fluorescence immunochromatographic strip. Anti-MCLR-mAb (350 μg/mL) and streptavidin (2.5 mg/mL) were dispensed at the test line and control line of an internal standard in the detection zone, respectively. A sample mixture of 100 μL containing 80 μL of sample and 20 μL of the detection solution was loaded onto the sample pad of the immunochromatographic assay strip. The detector solution contained the MCLR-FL (1.18 μg/mL) and the biotin-FL (92 ng/mL). To evaluate the performance of the fluorescence immunochromatographic strip, a series of experiments was performed using standard solutions of different concentrations of MCLR. In this experiment, the area value of the test line (A_T) was derided by the area value of the control line (A_C), and the ratios of A_T/A_C were plotted against different concentrations of MCLR (Fig. 3).

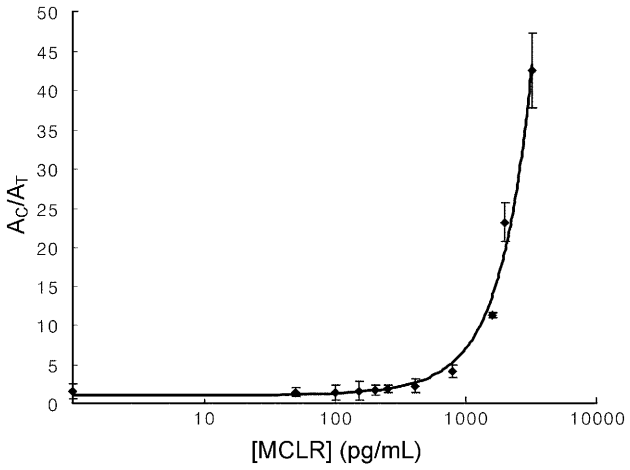


Figure 3. A_C/A_T were plotted against different concentrations of microcystin. Each points on graph represented the mean values and error bars represented standard deviation values of three independent experiments. Eleven different concentrations (3200, 2000, 1600, 800, 400, 250, 200, 150, 100, 50, 0 pg/mL) of free microcystin samples were used.

We set the area value of the fluorescence peak at the control line (A_C) as constant as possible by applying the same amount of fluorescence labeled biotin. In the meantime, the area value of the fluorescence peak at the test line (A_T) was inversely proportional to the concentration of microcystin in water. Thus, the ratio (A_C/A_T) increases as the concentration of microxystin in the water sample increases. In Figure 3, only the data points in the range of 200 pg/mL to 1600 pg/mL show small error bars, which mean small standard deviation values. From Figure 3, we conclude that the low and high detection limits of the fluorescence immunochromatographic strip are 200 pg/mL and 1600 pg/mL.

For the high performance liquid chromatographic system to detect microcystins in water samples, the low detection limit was about 0.5 μg/mL (Fig. 4) and the high detection limit for HPLC method was about 100 mg/mL. Above that concentration, the peak shape became very so broad and showed severe peak tailing.

Detection Time and Reproducibility. Fluorescence immunochromatography is a fast method that requires neither a complicated extraction system nor trained, qualified personnel. Furthermore, the detection time of the fluorescence immunochromatography is less than 20 min to complete the measurement of microcystin in a sample. HPLC usually takes more than 60 min for a sample, which include time to stabilize a detector, run a sample and wash the column.

As far as reproducibility is concerned, both techniques, *i.e.* fluorescence immunochromatography and HPLC, showed similar results (Table 1, 2). In Table 1 and Table 2, the relative standard deviations of quantitative measurements using these two techniques, ranged from about 3.0 to 10.0, except that the relative standard deviations of the HPLC peak area increased as the concentration of microcystin LR in water increases.

Detectible Microcystin Species. To determine how many microcystin species can be detected, HPLC was first employed. To use HPLC for the detection of trace amounts of microcystins in a water sample a concentration step is necessary, because the detection limit of HPLC is about 0.5

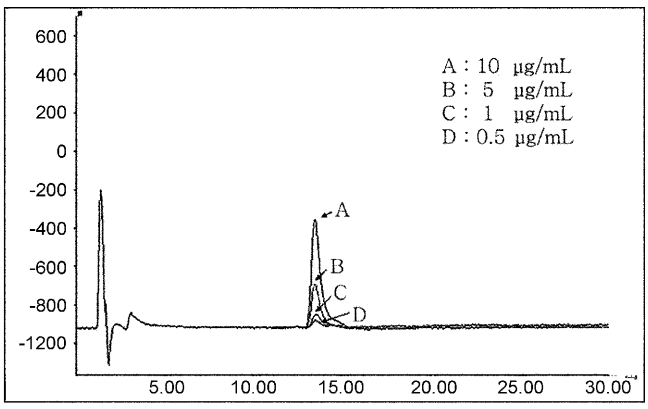


Figure 4. HPLC chromatograms with different concentrations of microcystin in water samples.

Table 1. Reproducibility of detection data of microcystins in water using HPLC

	MCRR 100 μg/mL		MCLR 10 μg/mL		MCLR 1 μg/mL	
	RT (min)	Peak Area	RT (min)	Peak Area	RT (min)	Peak Area
1	13.425	211752.73	19.683	129150.64	19.867	25929.56
2	12.117	209788.42	17.652	104476.85	18.025	17313.46
3	12.417	203292.6	17.158	86719.82	17.708	9565.09
4	12.308	170353.04	18.058	80121.53	18.133	7644.47
5	12.458	200366.25	18.192	80866.97	19.883	8719.86
Average	12.545	199110.61	18.149	96267.16	18.723	13834.49
SD	0.46	14965.98	0.85	18642.54	0.95	6944.03
RSD (%)	3.67	7.52	4.68	19.37	5.07	50.19

Table 2. Reproducibility of detection data of microcystins in water using fluorescence immunochromatography. The standard deviation values were calculated from five independent experiments

Microcystin LR (pg/mL)	A_C/A_T	Average concentration	S D	RSD (%)
1600	11.22	1574.53 pg/mL	0.387	3.45
800	4.09	829.94 pg/mL	0.315	7.70
400	2.30	409.24 pg/mL	0.202	8.78
250	1.90	269.87 pg/mL	0.100	5.26
200	1.79	227.03 pg/mL	0.114	6.37

μg/mL. To concentrate the microcystins effectively in water samples, a C_{18} solid phase extraction cartridges was used. The flow rate at which water samples were passed through the cartridges can be dictated by the type and capacity of the stationary phase involved. In the present study, the flow rate was changed from 2.0 mL/min to 3.5 mL/min. The use of flow rates above 4.0 mL/min increased its resistance to passage of water samples. This prevented the use of flow rates above 4.0 mL/min even with samples filtered through membranes of 0.45 μm pore size. The best recovery was obtained at a flow rate of 2.5 mL/min. When the flow rate was 3.0 mL/min or 3.5 mL/min, the amount of microcystins adsorbed on C_{18} cartridges was decreased rapidly, resulting from too short sample-stationary phase contact times.

To load microcystins to the reversed phase C_{18} cartridge, some considerations in relation to the pH of the water sample have to be taken into account. Even though the recommended pH of working for the C_{18} sorbent ranges from 4 to 7, we observed the best recovery result when the pH of the water sample is 7.

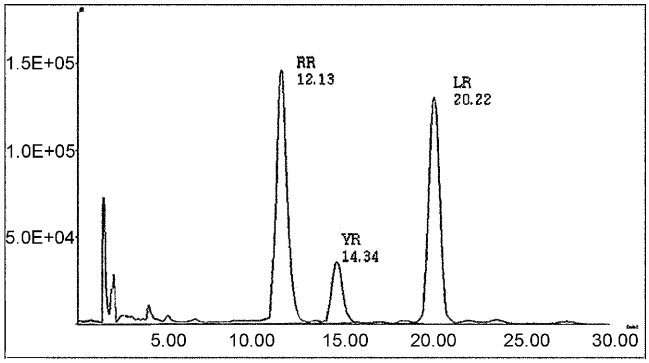


Figure 5. Typical HPLC chromatogram for the cultured samples. (Column; Waters spherisorb S5 ODS2, 4.6 × 150 mm, Mobile phase; [methanol : acetonitrile = 50 : 50] : 0.025 M phosphate buffer = 44 : 56, Flow rate; 1.0 mL/min, Detection; UV 238 nm).

After a concentration step using solid phase C_{18} cartridges, quantitative analysis of microcystins in water samples was performed using a HPLC with UV-Vis detection. With HPLC, individual microcystins can be separated and recognized on the basis of their retention times and characteristic UV absorption spectra. In Figure 5 a typical HPLC chromatogram is shown. Microcystin RR elutes in 12.14 min, microcystin YR elutes 14.34 min and microcystin LR elutes in 20.22 min. When six cultured samples composed of different microcystis species were analyzed, three different microcystins, *i.e.* microcystin LR, RR, YR could be separated and identified (Table 3).

On the other hand, to estimate the epitope of the monoclonal antibodies which is used for fluorescence immunochromatography, microcystin LR, RR, YR were subjected to indirect competitive enzyme-linked immunosorbent assay (ELISA). Microcystin RR and microcystin YR as well as microcystin LR showed a fairly good binding ability against our monoclonal antibody (Fig. 6). This result suggests that the epitope recognized by the monoclonal antibody is located around the Adda portion in the structure of microcystins. Microcystin LR, RR, YR have a unique common structural feature of hydrophobic β-amino acid abbreviated

Table 3. Comparison of HPLC and fluorescence immunochromatography for the analysis of microcystins in cultured microcystis samples. ND means 'not detected'

Cultured Microcystis	HPLC (pg/mL)				Fluorescence Immunochromatography (pg/mL)
	Microcystin LR	Microcystin RR	Microcystin YR	Total Microcystin	
1	365.4	276.2	43.2	684.8	672.1
2	244.5	211.4	ND	455.9	446.3
3	315.3	213.2	32.4	560.9	537.7
4	232.7	205.1	ND	437.8	450.4
5	311.4	241.8	ND	553.2	559.5
6	472.8	321.3	31.1	825.2	812.1

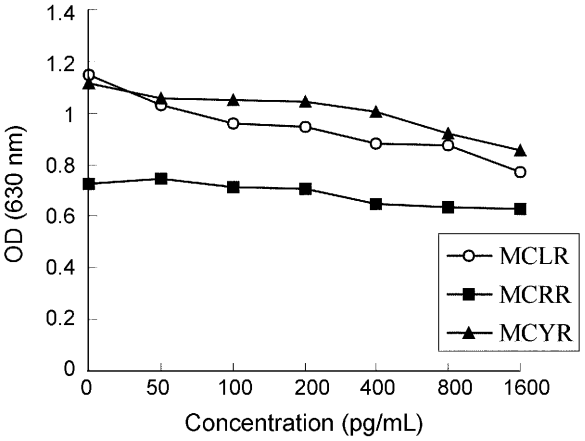


Figure 6. Specificity of monoclonal antibodies to different MC species. Known concentrations of different MCs were used in the competitive ELISA.

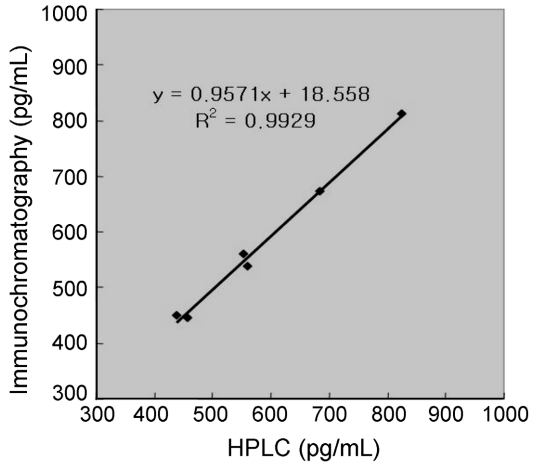


Figure 7. Correlation between fluorescence immunochromatography and HPLC.

to Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid). For these reasons, fluorescence immunochromatography could not detect individual microcystins, but did detect the total amout of microcystins in water samples. To compare the quantitation of microcystins for fluorescence immunochromatography and HPLC, both results are listed in Table 3. Fluorescence immunochromatography and HPLC results correlated very well with the correlation coefficient (R^2) of 0.9929 (Fig. 7).

This study compares the detection characteristics of fluorescence immunochromatography and high performance liquid chromatography for the analysis of cyanobacterial toxins. The fluorescence immunochromatographic system used in this study has the unique advantages of short detection time and low detection limit, whereas the high performance liquid chromatography detection method has the strong advantage of individual quantifications of several species of microcystins. Therefore, these two techniques supplement each other. In particular, the performance of the fluorescence immunochromatographic system with our monoclonal antibodies was very satisfactory. Since a fluorescence immunochromatographic assay is easy to perform and its quantitative range is within microcystin concentrations in natural waters, the technique shows potential for routine use in monitoring of microcystins in water.

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