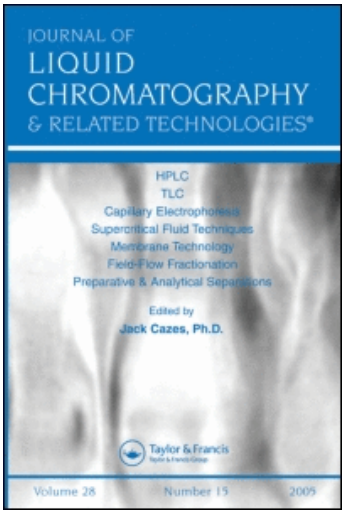

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Comparison of Fluorescence Immunochromatography and HPLC for the Trace Analysis of Algal Toxins

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ABSTRACT

New monoclonal antibodies (mAbs) against microcystin (MC), a cyclic peptide toxin of the freshwater cyanobacterium *Microcystis aeruginosa*, were prepared from cloned hybridoma cell lines. Using these mAbs, an immunochromatographic assay system was designed for the detection of cyanobacterial hepatotoxins, MCs in water. The immunochromatographic assay system is composed of anti-MCs-mAb, fluorescence conjugates in detection solution, an immunochromatographic assay strip, and a laser fluorescence scanner. The performance of the immunochromatographic assay system was compared with high performance liquid

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chromatography (HPLC) detection. As the detection limit of HPLC is several orders of magnitude higher than with immunochromatographic assay systems, attention was also given to preconcentration of samples with solid phase extraction cartridges.

Key Words: Microcystins; Monoclonal antibodies; Detection; Immunochromatography; HPLC.

INTRODUCTION

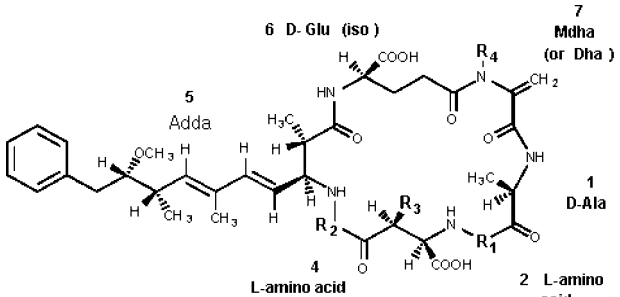
Water blooms of toxic cyanobacteria (blue-green algae) have been detected in freshwater lakes and reservoirs for drinking water all over the world. Cyanobacteria of the genera *Microcystis* produce a range of cyclic polypeptide toxins, known as microcystins (MCs).[1] Their basic structure is a cyclic heptapeptide and their structural variations give rise to more than 50 types of MCs known today (Fig. 1).[1] The most extensively studied form is MC-LR which contains L-leucine and L-arginine in the two main variant positions.

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

MCs are potent inhibitors of protein phosphatases 1 and 2A,[7–9] which are regulatory enzymes present in the cytosol of the mammalian cells. This action may explain the effects of MCs 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]

As MCs are potent hepatotoxins for humans and animals, the development of sensitive and reliable detection methods becomes 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, physicochemical techniques have been used as a sensitive method of analysis,[15–17] but this approach relies on the availability of



	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 MCs. A characteristic of MCs 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.

toxin standards for comparison and is, therefore, only applicable to known toxins. It is also a relatively slow technique and requires expensive equipment and appropriate training.

In this study, we used a new immunochromatographic assay system using anti-MCLR-monoclonal antibody (mAb). In this assay system, unknown samples containing MCs are 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 detection solution compete for binding to capture the antibody, which was 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 the 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 in the laser fluorescence scanner and converted into area value, and the concentration of MCs in unknown sample is calculated from the standard curve or the equation of the standard curve.

This article deals with the comparison of the fluorescence immunochromatographic assay method using mAb against the MC, and the high performance liquid chromatography (HPLC) detection of MCs in water using the solid phase extraction.

EXPERIMENTAL

Structures of MCs, and their derivatives used in these experiments, are shown in Fig. 1. *Microcystis aeruginosa* NIES 298, a cyanobacterium known to produce MCs (MCs), was provided by the National Institute of Environmental Research, Korea. One liter batch cultures of cells were grown in MA (*Microcystis aeruginosa*) medium. Cultures were maintained at 20–25°C under constant illumination by a white fluorescent light on the surface of the growth flask. Cells were harvested by centrifugation ($9000 \times g$, 5 min) and were lyophilized prior to storage at −20°C. MCLR was purified by several steps of open column chromatography and identified by HPLC 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 an 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 produce mAbs against MCLR, MCLR 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. BALB/c 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 mAbMC 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 fluorescence immunochromatography, 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 and 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 and ammonium sulfate precipitation, and finally purified using protein-G column.

For fluorescence immunochromatographic assay, an assay strip was in-house-fabricated to fit into the holder of a laser fluorescence scanner, which mainly consisted of a nitrocellulose membrane, a sample pad, an absorption pad, and a backing card. The backing polystyrene card is a support that the nitrocellulose membrane, sample, and absorption pad are laid on its adhesive side. The nitrocellulose membrane (Millipore HF 180) is the place where the detection zone is located, and the bottom side of membrane was coated with a plastic thin film. The control line on the detection zone was dispensed with streptavidin (2.5 mg/mL) for the internal standard and the test line was coated with anti-MCLR-mAb (350 μg/mL) or MCLR–BSA (80 μg/mL) conjugates for detection of MCLR in a sample. The width of the dispensing line was 1 mm and the dispensing volume was 1 μL/cm. The control and the test line were located 31 and 33.5 mm down from the sample pad, respectively (Fig. 2). Before being placed on the nitrocellulose membrane, the sample pad (S&S 903, $4 \times 25\ mm^2$) was completely soaked in PBS containing 1%

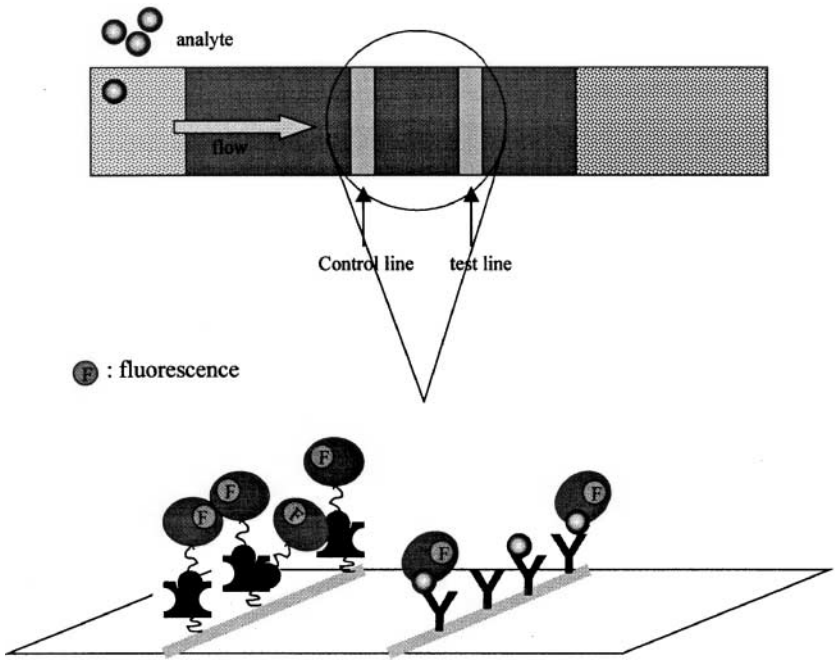


Figure 2. A schematic diagram of fluorescence immunochromatographic assay strip.

BSA and 0.05% Tween-20, and vacuum dried at 50°C for 1 hr. The absorption pad (S&S 470, $4 \times 20\,mm^2$) was set up on the nitrocellulose membrane along the detection zone to remove the postreaction solution, which passed by the detection zone. The assembled strip on a polystyrene card was placed into a plastic housing ($15 \times 90\,mm^2$), which was designed to fit into the holder of the laser fluorescence scanner. The oval window of the plastic housing for the scanning of the detection zone was 15 mm, and the diameter of the sample well for holding 100 μL of sample mixture was 5 mm. In the case of the test line on nitrocellulose membrane being dispensed with anti-MCLR–mAb, the detection solution was a mixture of MCLR-FL and biotin-FL. The concentrations of MCLR-FL and biotin-FL were 1.18 μg/mL and 92 ng/mL, respectively. When MCLR–BSA conjugate was coated at the test line, the concentration of mAb-FL in the detection solution was 4.6 μg/mL, and biotin-FL was diluted to 46 ng/mL. Various different concentrations (of 80 μL) of MCLR in D.W. and 20 μL of detection solution were mixed well, loaded onto the well of sample pad on the cartridge, waited for 15 min, and scanned in the laser fluorescence scanner. The raw data of scanning were displayed and the relative intensity of the fluorescence peak of the test, and the control line was converted into the area value (test: A_T, control: A_C) by using a Visual Basic terminal software program. The ratio of A_T/A_C was plotted against time (sec) (or step number), and the equation for the standard curve and correlation value was obtained from Microsoft Excel program.

RESULTS AND DISCUSSION

Production and Purification of Monoclonal Antibody

KLH-conjugated MCLR was used as an immunogen for the production of mouse mAb. Immunization, cell fusion, and screening of hybridoma cells producing anti-MCLR antibodies were conducted according to a standard method.[18] In brief, BALB/c mice of 6–8 weeks old were immunized with 100 μg of MCLR–KLH conjugate mixed with complete Freund's adjuvant. The first injection was followed by three or four booster injections of the same amount of immunogen mixed with incomplete Freund's adjuvant at 3–4 week intervals. Serum was taken from the tail of a mouse and tested for antibody titer, usually after the 3rd injection. For the fusion, spleen cells from immunized mouse were combined with SP2/0–Ag-14 myeloma cells, and 1 mL of 50% polyethylene glycol (PEG) 1500 in DME medium was added, drop by drop, for over 60 sec. Fused cells were selected in hypoxantinaminoperterin thymidine (HAT) medium for 2 weeks and culture

supernatants of hybridoma cells were then collected and screened by ELISA assay, with the BSA-conjugated MCLR as an antigen. The positive clones were frozen first and screened further by two successive limited dilutions after thawing. Ascites fluids were produced for a large-scale production of the mAbs by induction of a tumor with prisitine, prior to injection of 1×10^7 hybridoma cells into the peritoneal cavity of the mouse.

The mAb was purified from the supernatant of hybridoma cells that were cultured in serum-free DEME medium through a series of purification steps such as membrane ultrafiltration, ammonium sulfate precipitation, and then protein-G column. The eluted mAb with 100 mM glycine–HCl (pH 2.5) was neutralized with 0.1 vol. of 1 M Tris (pH 8.0), the concentration measured, and stored at -70°C until use. When ascites fluids were used, they were directly applied onto a protein–G column after clearing by centrifugation. The antibodies were eluted with 100 mM glycine–HCl (pH 2.5), eneutralized, and dialyzed against phosphate buffered saline solution (PBS).

Detection of MCs Using Fluorescence Immunochromatography

When a water sample is applied to our fluorescence immunochromatographic strip, two chromatographic lines of fluorescence intensity curves always appear. The fluorescence intensity of the first line (also called the test line) is inversely proportional to the concentration of MC in water 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 MC in the water sample. This phenomenon results from the way the fluorescence immunochromatographic strip was made. 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 on the detection zone, respectively. The sample mixture (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 detection 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 were made using standard solutions of different concentrations of MCLR. In this experiment, the area value of the test line (A_T) was derived 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). From Fig. 3, we could observe that the linear dynamic range of the fluorescence immunochromatographic strip is 50–1400 pg/mL, and the minimum detection level of the strip would be about 50 pg/mL.

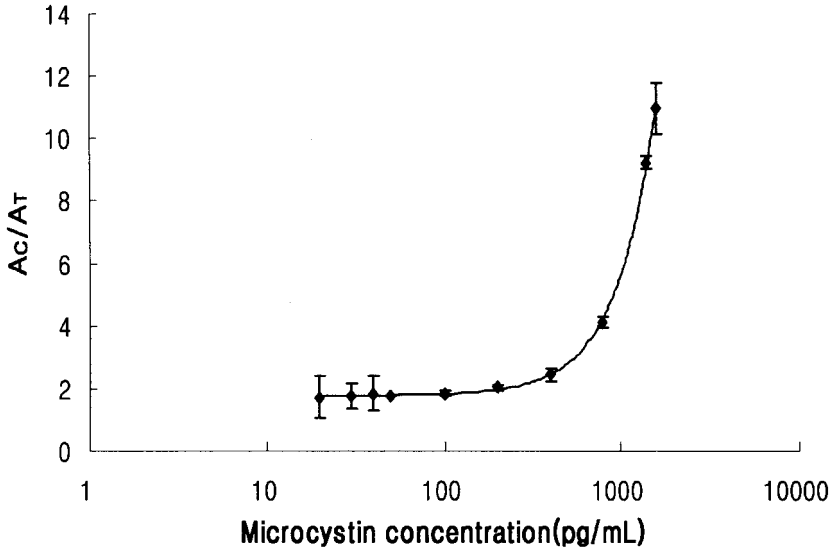


Figure 3. A_T/A_C was plotted against different concentrations of MC. Each point on the graph represented the mean values, and error bars represented standard deviation values of three independent experiments. Ten different concentrations (1600, 1400, 800, 400, 200, 100, 50, 40, 30, and 20 pg/mL) of free MC samples were used.

By using fluorescence immunochromatography, we analyzed the content of MCs in cultured samples. Ten samples (sampling on every 2 days) were used to observe the variation of MC contents in cultured sample.

The results of fluorescence immunochromatography for cultured samples are shown in Table 1 and Fig. 4. From Fig. 4, the amount of MC in cultured sample increases during the growth phase, being highest in the 15th day.

HPLC Detection of MCs

In order to concentrate the MCs effectively in water samples, a new concentrating apparatus utilizing C_{18} solid phase extraction cartridges was designed (Fig. 5). The concentrating apparatus consisted of four separate Baker J. C_{18} cartridges (500 mg) in parallel. The flow in the whole system was controlled by a peristaltic pump. Properly rinsed by passing 10–15 mL of methanol and conditioned, the C_{18} cartridges can be used to analyze a large volume of water samples. The flow rate at which water samples were passed through the cartridges can be dictated by the type and capacity

Table 1. Quantitative analysis of MCs for cultured samples using fluorescence immunochromatography.

Culture period (days)	Total MC concentration (pg/mL)
1	356.438
3	536.664
5	643.701
7	783.357
9	844.837
11	818.082
13	1272.552
15	1342.588
17	1146.045
19	685.991

of stationary phase involved. In this study, the flow rate was changed from 2.0 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

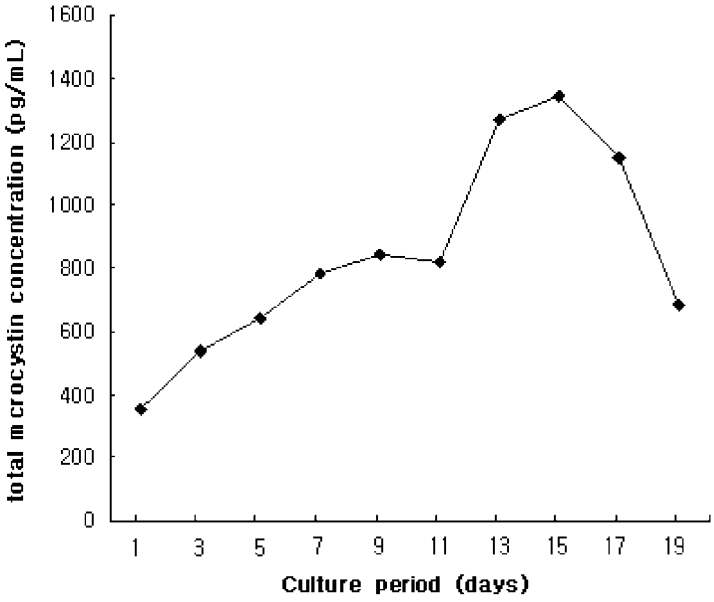


Figure 4. Daily variation of total MC in cultured samples.

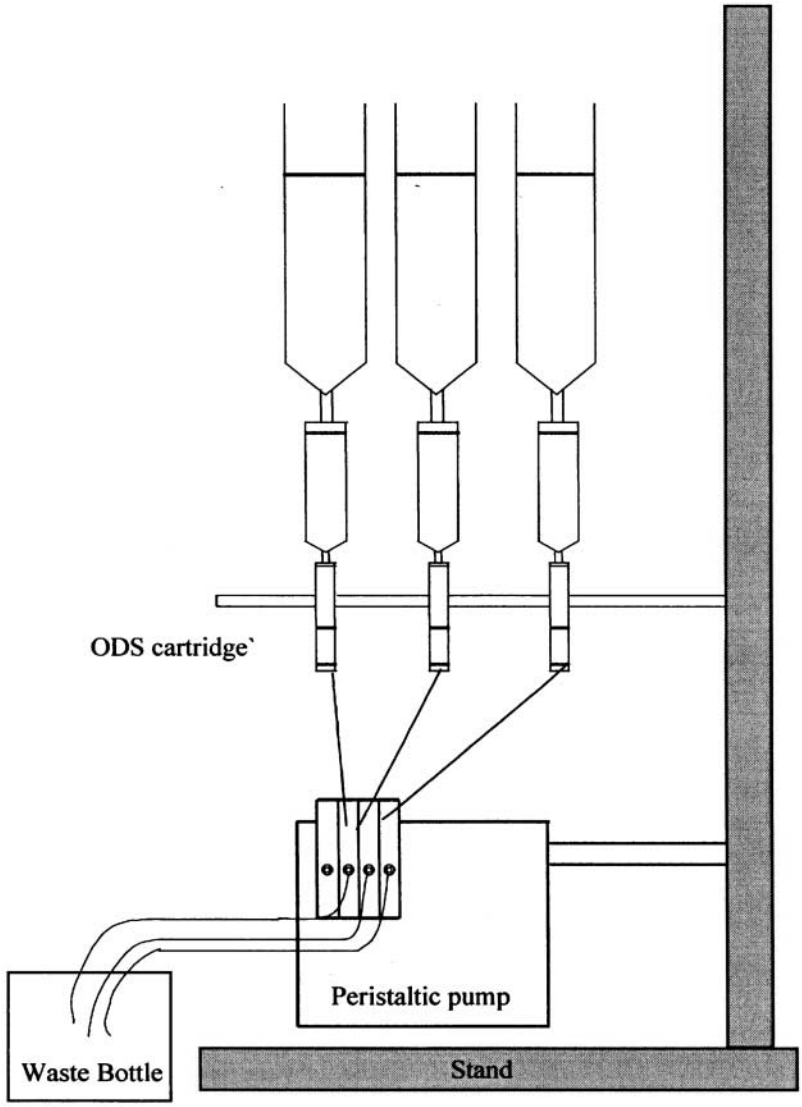


Figure 5. The concentrating apparatus utilizing C_{18} solid phase extraction cartridges.

rates above 4.0 mL/min, even with samples filtered through membranes of 0.45 μm pore size. The recovery experiments with different flow rates were made. The best recovery was obtained at the flow rate of 2.5 mL/min. When the flow rate was 3.0 or 3.5 mL/min, the amount of MCs adsorbed

on C_{18} cartridges decreased rapidly resulting from too short sample-stationary phase contact times.

In order to load MCs 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 working pH the C_{18} sorbent ranged from 4 to 7, best recovery result is when the pH of the water sample is 7.

After the concentration step using solid phase C_{18} cartridges, quantitative analysis of MCs in water samples was performed by using an HPLC with UV detection. With HPLC, individual MCs can be separated and recognized on the basis of their retention times and characteristic UV absorption spectra. In Fig. 6, a typical HPLC chromatogram is shown. MCRR elutes in 11.52 min and MCLR elutes in 18.55 min. To compare quantitation of MCs with fluorescence immunochromatography and HPLC, both results are tabulated in Table 2. The fluorescence immunochromatography and HPLC results correlated very well with the correlation coefficient (R^2) of 0.9871 (Fig. 7).

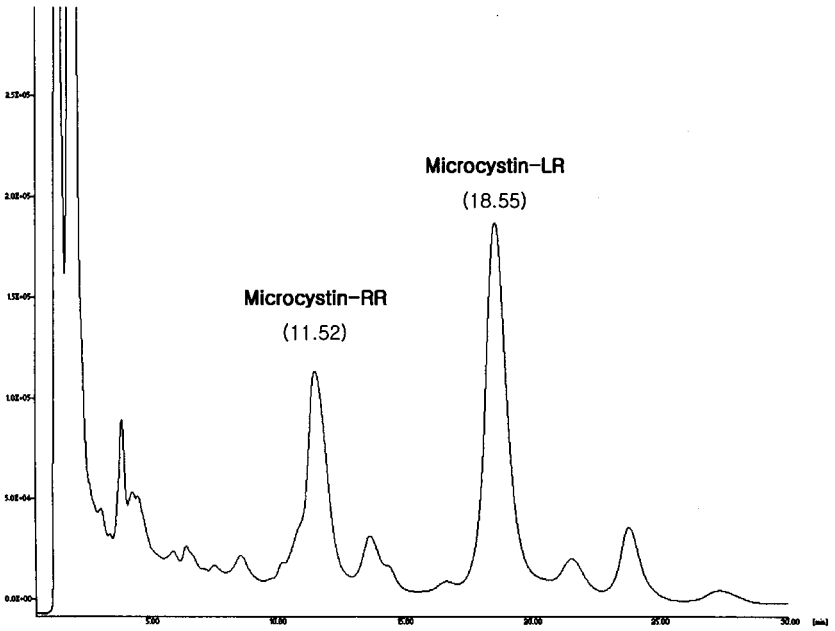


Figure 6. Typical HPLC chromatogram for the cultured samples. (column: Waters spherisorb S5 ODS2, $4.6 \times 150\,mm^2$; mobile phase: [methanol, acetonitrile = 50 : 50] : 0.025 M phosphate buffer = 44 : 56; flow rate: 1.0 mL/min, detection: UV 238 nm).

Table 2. Comparison of HPLC and fluorescence immunochromatography for the analysis of MCs in cultured samples.

Culture period (days)	HPLC (pg/mL) MCRR	MCLR	Total MC	Fluorescence immunochromatography (pg/mL)
1	216.918	142.349	359.267	356.438
3	342.287	197.609	539.896	536.664
5	369.191	275.599	644.79	643.701
7	330.145	396.024	726.187	783.357
9	374.178	501.249	875.427	844.837
11	311.558	568.257	879.815	818.082
13	266.357	997.016	1263.373	1272.552
15	248.198	1143.113	1391.311	1342.588
17	250.134	983.149	1233.283	1146.045

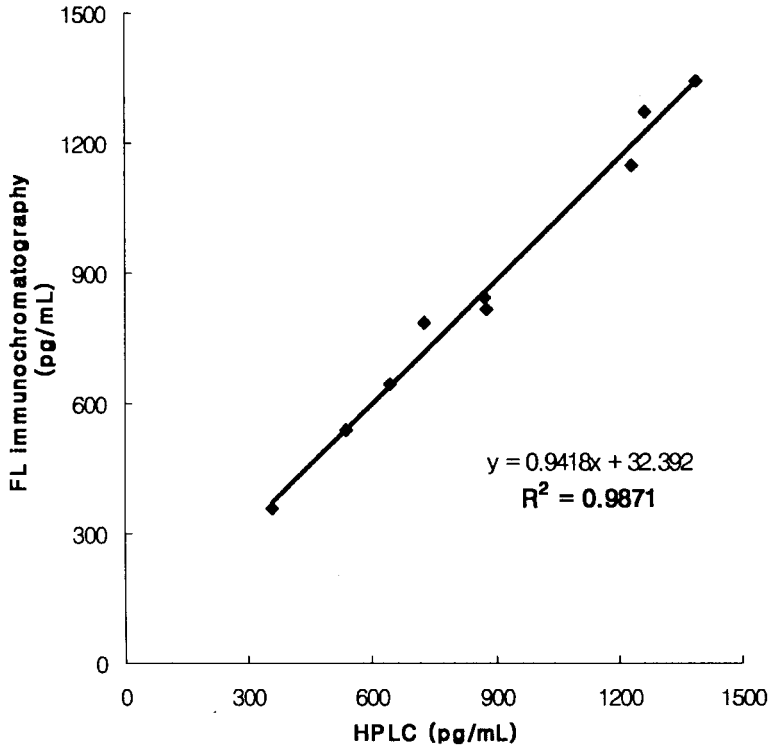


Figure 7. Correlation between fluorescence immunochromatography and HPLC.

The aim of this study was an application of fluorescence immunochromatography with our mAbs that is sensitive enough for monitoring MCs in drinking water supplies. The performance of the fluorescence immunochromatography with our mAbs was satisfactory. As fluorescence immunochromatography assay is easy to perform and its quantitative range is within MC concentrations in water samples, it shows potential for routine use in monitoring MCs in drinking water supplies.

ACKNOWLEDGMENT

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