Analytical Methods

PAPER

View Article Online
View Journal | View Issue

Cite this: *Anal. Methods*, 2017, **9**, 6430

Quantitative and rapid detection of microcystin-LR using time-resolved fluorescence immunochromatographic assay based on europium nanospheres

Yi Zhang,[a] Xin-Liang Ding,[b] Ming-Ming Guo,[a] Ting-Ting Han,[a] Zi-Jian Huang,[c] Hong-Tao Shang*[d] and Biao Huang*[a]

In the present study, a novel time-resolved fluorescence immunochromatographic assay was established for the rapid quantitative detection of microcystin-leucine arginine (MC-LR). In this method, the europium nanoshpere labelled with anti-MC-LR antibodies was used as the luminescent tracer, dissolved in the running buffer and then added with the sample solution on the pad. MC-LR-BSA and goat anti-mouse antibody were dispensed on the nitrocellulose membrane for the test and the control line, respectively. The optimal parameters were 0.05 g L^{-1} MC-LR-BSA, 1 : 100 colloidal europium–antibody conjugate, and 10 min reaction time. The linear working range for MC-LR was 0.1–5 μg L^{-1} with an IC50 of 0.78 μg L^{-1} and a sensitivity of 0.035 μg L^{-1}. The low cross-reactivity was observed with MC-YR and MC-LF. The assay accuracy was confirmed by the HPLC method with a correlation coefficient of 0.99. When the variable coefficients were 4.4% and 5.4%, the average recoveries of tap and lake water were 94.6% and 102.8%, respectively. The time-resolved fluorescence immunochromatographic assay provides a sensitive, simple, and speedy performance for MC-LR quantitative determination and has a potential use for water sample screening.

Received 24th August 2017
Accepted 24th October 2017

DOI: 10.1039/c7ay02036h

rsc.li/methods

Introduction

Surface water pollution is a growing problem due to the fast development of agriculture and industry. During the past few years, cyanobacterial blooms have already occurred frequently in some lakes, which have raised a great concern around the world. They not only can change the water qualities, but can also produce a wide range of toxins, which definitely will be harmful to aquatic organisms and even human beings.[1–3] As one of the toxins produced by cyanobacteria, microcystin has more than 90 isoforms, among which microcystin-leucine arginine (MC-LR) is the most widespread and hazardous toxin.[4–6] As is known, its inhibition to the serine/threonine protein phosphatase type 2A (PP2A) and type 1 (PP1A) is a potential threat to the gastrointestinal tract, reproductive cells, and many organs such as liver, kidney, and even brain.[5,7–13] Therefore, in 1998, the World Health Organization (WHO) has set up a provisional guideline value of 1 μg L^{-1} for MC-LR in drinking water. Soon afterwards, in 2006, China modified the national standard, adding MC-LR into the toxicological index (GB 5749-2006) for drinking water quality and added as one of toxins required inspection. Thus, it is extremely important to develop a sensitive method to quantitate the value of MC-LR in drinking water.

To date, numerous methods, such as serine–threonine protein phosphatase inhibition assay (PPIA),[14] high-performance liquid chromatography (HPLC) (ISO, 2005), and enzyme-linked immunosorbent assays (ELISA),[15] have been applied for MC-LR analysis in water quality monitoring. HPLC is a relative golden-standard method for the component confirmation, but it relies on the time-consuming sample preparation, expensive facilities, and well-trained personnel. PPIA and ELISA are both colorimetric and sensitive technologies, but they require costly reagents and special operation circumstance. Furthermore, the PPIA method can only recognize the total MCs and cannot identify a specific isoform alone. Lei *et al.* reported an ultrasensitive MC-LR detection method named time-resolved fluorescence immunoassay (TRFIA),[16] which was based on the europium (Eu)-labelled technology. It provided a sensitivity of 0.1 μg L^{-1} with a broad dynamic range from 0.01 to 20 μg L^{-1}. Although its 96-well design was quite

[a]*Key Laboratory of Nuclear Medicine, Ministry of Health, Jiangsu Key Laboratory of Molecular Nuclear Medicine, Jiangsu Institute of Nuclear Medicine, Wuxi, Jiangsu 214063, China. E-mail: huangbiao@jsinm.org; Tel: +86-510-85520770*

[b]*Department of Public Health, Wuxi Center for Disease Control and Prevention, Wuxi, 214023, China*

[c]*Department of Biological Sciences, Xi'an Jiaotong-Liverpool University, Suzhou 215123, China*

[d]*Physical Examination Center, Jiangsu Province Hospital of Traditional Chinese Medicine, Nanjing 210029, China. E-mail: hongtao3626@163.com; Tel: +86-25-86618472*

suitable for high throughput screening, it was not suitable for a rapid performance and also lacks portability. Thus, a simple, sensitive, and portable method for quantified low-concentration MC-LR detection should be a better choice for the environmental monitoring of MC-LR pollution and health risk.

With the development of biosensor technology in recent decades, some quick and convenient requirements, such as obtaining the results in 5–10 min without a complicated sample pre-treatment or a standard series, have been fulfilled by immunochromatographic assay. Moreover, utilizing a colloidal gold as a tracer, this assay can provide determination of results with naked eyes instead of a large apparatus, which is quite suitable for field operation.[17–19] Although this assay can provide any quantitative data, it is still unable to figure out the weak positive samples distinctly.[20,21] Recently, this weakness has been completely solved using a novel time-resolved fluorescence immunochromatographic assay with europium nanospheres.[22,23] This assay provides a rapid detection of a substance at low concentrations in a sensitive and quantitative way. With the equipment being miniaturized, some even palm-sized, this method may definitely have a broad prospect for environmental inspection.

In the present study, a time-resolved fluorescence immunochromatographic assay for the MC-LR quantification has been first established. The proposed method could offer a simple and sensitive strategy with fluorescence on the strip and even be applied to test the MC-LR in the water samples with satisfactory results.

Experimental

Reagents and materials

MC-LR-bovine serum albumin (BSA) and monoclonal anti-MC-LR antibody were provided by Bio-Sensor Food Safety Technology Co., Ltd. (China). Goat anti-mouse polyclonal antibody was purchased from Jackson ImmunoResearch (USA). The carboxyl nanobeads containing Eu fluorescent (200 nm), also called Eu nanospheres, were obtained from Seebio Biotech Co., Ltd. (China). The sample pad, nitrocellulose membrane, absorbent pad, and packing cards were purchased from Jieyi Biotech (China). *N*-Hydroxysuccinimide (NHS), 2-[*N*-morpholino]ethanesulfonic acid (MES), 1-ethyl-3-(3-dimethyllaminopropyl)-carbodiimide hydrochloride (EDC), and BSA were supplied by Sigma-Aldrich (USA). All other reagents were of analytical grade and provided by domestic manufacturers.

Instruments

A portable fluorescence reader HG-98 was obtained from Huguo Scientific Instrument (Shanghai, China). The dispenser and the cutting system were purchased from Safecare Biotech Co., Ltd. (Hangzhou, China). A mode of 5417R centrifuge was provided by Eppendorf (USA). The ultrasonic apparatus CD-2000 was obtained from Jeken Co. (Shenzhen, China).

Preparation of anti-MC-LR antibody-labelled Eu nanospheres

At first, 1 mg of carboxyl beads was diluted in 500 μL MES solution (pH = 5.0, 0.1 mol L^{-1}), dispersed by an ultrasonic apparatus, and centrifuged at 15 000*g* for 20 min. The supernatant was discarded carefully. Nanospheres were washed twice by repeating the previous steps. After this, 1 mg NHS and 1 mg EDC were added to the nanospheres, and the reaction volume was complemented to 500 μL with MES buffer. The activation was maintained for half an hour in a dark place under continuous shaking. Then, the beads were washed 3 times. After this, a certain amount of MC-LR antibody and the activated beads were added to a tube and then incubated in the dark place for 2 h at room temperature. Later, the reaction was suspended by adding 10 μL of 0.05 mol L^{-1} phosphate buffer (pH = 7.2) containing 10% BSA, and the mixture was shaken for 0.5 h in the dark place. Distilled water was used to wash the antibody-labelled Eu nanospheres, and the excess antibody was removed by the abovementioned method. Finally, the MC-LR antibody-conjugated beads were suspended in a 0.05 mol L^{-1} phosphate buffer (pH = 7.2), containing 1% BSA and 0.1% Tween-20 to a concentration of 0.02 g L^{-1}. The buffer containing anti-MC-LR antibody-nanospheres was used as a running buffer in this study.

Fabrication of the immunochromatographic strip

The time-resolved fluorescence immunochromatographic system consisted of four parts: a sample pad, a nitrocellulose membrane, an absorbent pad, and a backing board, as shown in Fig. 1. At first, the nitrocellulose membrane was adhered to the middle of the backing board. The MC-LR-BSA and goat anti-mouse antibody (0.75 g L^{-1}) were dispensed as the test line and the control line, respectively, on the nitrocellulose membrane with an interval of 5 mm width. Then, the membrane was dried at 30 °C for 3 h to immobilize the above mentioned proteins. The sample pad and the absorbent pad were pasted separately at two ends of the nitrocellulose membrane, on which was sprinkled the test and control line, respectively, with a 2 mm overlap. After assembling, the immunochromatographic system was cut into a 4 mm-wide strip. The strip was packaged in the plastic card and sealed in the aluminum film bag for the following use. These procedures were finished under the following condition: the humidity was under 35% and the temperature was about 20–25 °C.

Preparation of the sample

The water samples used for MC-LR analysis included tap water from our laboratory and lake water from Taihu Lake, Wuxi, China. The lake water samples were passed through a 0.22 μm cellulose ester membrane before the analysis, and the MC-LR concentrations in these samples were determined by HPLC.

Fig. 1 The structure diagram of time-resolved fluorescence immunochromatographic assay for MC-LR. (A) Side view, (B) top view; (a) sample pad, (b) test line, (c) control line, (d) absorbent pad, (e) nitrocellulose membrane, and (f) backing board.

The MC-LR-free samples were used to spike with various concentrations of MC-LR for the recoveries.

Procedure of the assay

The strips and agents were placed at room temperature for more than 15 min before the assay. Then, 20 μL of the standard or the sample solution was added to the sample pad with a 50 μL running buffer. After several minutes, the cassette containing the immunochromatographic strip was inserted into the reader HG-98. The fluorescence signals of the test and control lines were obtained in the machine, and the analyte content was quantified by calculating the T/C ratio.

Statistical analysis

Each standard and sample were measured three times, and all data available have been presented as mean or mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant. The software Origin 8.0 (OriginLab, USA) was used to evaluate the data. The comparison test was performed, and the correlation coefficients were stated as the correlation of two methods.

Results and discussion

Assay principle

A direct competitive mode was used as the assay principle of the immunochromatographic assay, which is shown in Fig. 2. The assay particularity was based on Eu embraced to the nanospheres. With 340 nm of excitation light, Eu nanospheres would emit the light at 613 nm, which would be obtained by the apparatus.[23] Different from a normal light, these two lights could provide low backgrounds and high fluorescence, which made the method unique and critical to quantitation.

In this assay, the solution containing free MC-LR in the sample and running buffer was titrated on the sample pad. Under the action of capillary chromatography, the mixture of MC-LR antigen and antibody beads migrated along the membrane. When they moved into the test line, the MC-LR-unbound nanospheres would combine with the immobilized MC-LR-BSA. Later, the excess antibody nanospheres were captured by the second antibody on the control line. Finally, the immune mixture flowed to the absorbent pad at the end of the strip. According to the assay principle, the fluorescence intensity of the test line had a negative correlation with the MC-LR concentration. This means the more the MC-LR in the sample, the lower the fluorescence signal on the test line. Moreover, since there was no MC-LR in the buffer, the Eu fluorescence would reach the peak. The monoclonal antibody beads captured by the control line would emit a special signal and certify the validity of the strip. The signals of the Eu beads from the test and control lines were obtained, respectively, in the reader, and the MC-LR concentration could be further calculated.

Parameter optimization

Several parameters have been optimized to obtain the best sensitivity. The optimal concentration of MC-LR-BSA was chosen on the test line. As shown in Fig. 3, a series of concentrations of artificial antigen on the test line was estimated in the range from 0.02 to 5 g L^{-1} when 20 μL of the blank sample and 50 μL of the running buffer were loaded on each sample pad. Since 30–50% of antigen–antibody binding rate was more suitable for the competitive assay, 0.05 g L^{-1} was chosen for the antigen spread on the test line.

The anti-MC-LR antibodies were confirmed to be successfully conjugated to the nanospheres by the immunochromatographic assay with high fluorescence on the test and control line when added with the blank standard and diluted in the running buffer. Moreover, the amount of anti-MC-LR antibody conjugated to the nanospheres could be estimated. Since WHO has demanded that the concentration of MC-LR in the drinking water should be below 1 μg L^{-1}, the fluorescence counts of 1 μg L^{-1} and the blank MC-LR standard (B_1/B_0) were assessed. The rate of B_1/B_0 could present the assay competitive efficiency and the standard curve slope. The lower the rate, the more sensitivity the method reflects. Moreover, the CV, obtained from SD/mean, needs to be considered. When it was below 10%, the assay was supposed to be accurate. The different weight ratios of Eu and antibody were assessed and plotted as 10, 20, 50, 100, 200, and 500. From the result shown in Fig. 4, the optimal conjugate rate of anti-MC-LR monoclonal antibody and Eu nanospheres was 1 : 100 (wt : wt).

Then, the reaction time was optimized. Due to the immunochromatographic character, the assay could be finished within 15 min at room temperature.[22,23] In this study, 5, 10, and 15 min were considered as the chromatographic time. The standard curves for MC-LR in a series of reaction times are

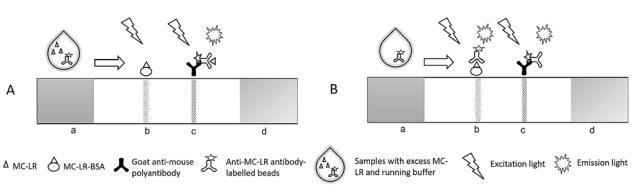

Fig. 2 Schematic of the principle for the immunochromatographic detection (A) with excess MC-LR and (B) without MC-LR.

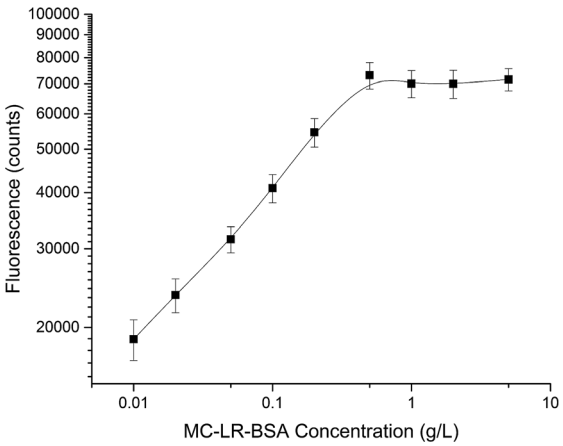

Fig. 3 The effect of different concentrations of MC-LR-BSA.

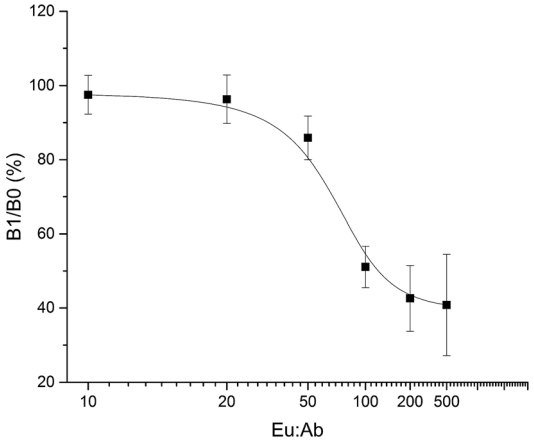

Fig. 4 The effect of conjugate rates of Eu nanospheres and anti-MC-LR monoclonal antibody.

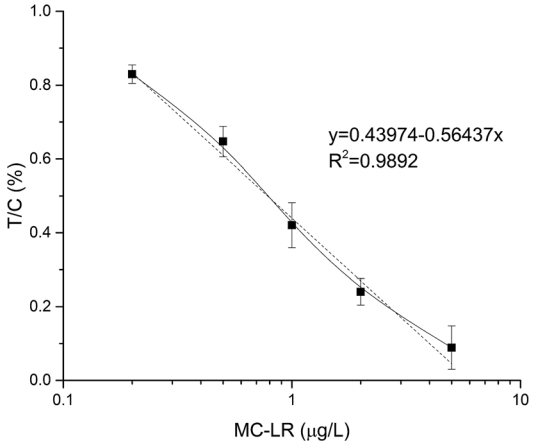

Fig. 5 The calibration curve of MC-LR time-resolved immunochromatographic assay.

listed in Table 1. After reaction for 10 min, the antigen–antibody conjugation had reached the saturation on the test line. Taking into account the adjusted R-square, intercept, slope, inter-CV, and fluorescence yield of B_0, 10 min was selected as the optimized chromatographic time for this assay.

Analytical performance

The ratio of the time-resolved fluorescence signal of the test line and the control line was inversely proportional to the concentrations of MC-LR in the solution, as plotted in Fig. 5. After the data were transformed logarithmically, the linear equation of the standard curve was $Y = 0.43974 - 0.56437X$, with the MC-LR concentrations ranging from 0.1 to 5 μg L^{-1}. The median inhibitory concentration (IC50) of the assay was 0.78 μg L^{-1}. Since WHO and the national standard have set up a guideline value of 1.0 μg L^{-1} for MC-LR in the drinking water, the IC50 value lower than 1 μg L^{-1} is quite important to detect the toxin. Thus, it should be a suitable choice for water sample analysis.

Evaluation of the assay

The sensitivity of the time-resolved immunochromatographic assay, calculated from the mean fluorescence of the blank standard plus 2SD, was 0.035 μg L^{-1} for MC-LR. The effective working range was from 0.035 to 5 μg L^{-1}. After the standard series was measured three times, the average dose of IC20, IC50, and IC80 was 0.15, 0.78, and 2.62 μg L^{-1}, respectively. The coefficient of variation (CV), standing for the drift rate of the method, was defined as the level of SD/mean in the MC-LR standards from the three-time determination. The inter-CV was 10.7%, which indicated that the drift rate was low and the assay was stable for further use because MC included various isomers. In this study, we used 5 μg L^{-1} of both MC-YR and MC-LF as the samples to test the concentration of MC-LR by the time-resolved immunochromatographic assay. The results indicated that the cross-reactivity rate was 9.1% and 7.9%, and more importantly, this antibody had specificity to MC-LR.

A total of 32 collected water samples were quantitatively detected by two methods: HPLC and time-resolved immunochromatographic assay. The correlation between these assays was quite good such that the linear equation was $y = 0.9228x + 0.0034$ and $R = 0.99$. The result of each method was analysed by the paired sample *T*-test. The *P* value was above 0.05, estimating no significant difference between two assays. The MC-LR level determined by this novel-developed method was dependable for the real sample analysis. Thus, this time-resolved immunochromatographic method was technically appropriate to detect MC-LR in the water samples.

Detection of MC-LR in water samples

To verify the capacity of this time-resolved immunochromatographic assay to determine MC-LR in several solutions, the MC-LR-free samples (tap water and lake water) determined by HPLC

Table 1 The characterization of the MC-LR standard curve during different assay times

Time (min)	5	10	15
Adj. R-square	0.99423	0.98921	0.98128
Intercept	0.50789	0.43974	0.46686
Slope	−0.37918	−0.56437	−0.46363
Inter-CV (%)	7.54	4.34	8.16
B_0 (cps)	59 070	73 082	72 966

Table 2 The recoveries of MC-LR by the time-resolved immunochromatographic assay

Sample	Added (μg L^{-1})	Observed (μg L^{-1})	Recovery (%)	Intra-CV (%)
Tap water	0.2	0.186 ± 0.011	93.0	5.9
	1	0.965 ± 0.032	96.5	3.3
	2	1.883 ± 0.075	94.2	4.0
	Average		94.6	4.4
Lake water	0.2	0.205 ± 0.011	102.5	5.4
	1	0.976 ± 0.074	97.6	7.6
	2	2.165 ± 0.068	108.3	3.1
	Average		102.8	5.4

were obtained and spiked with different concentrations of MC-LR. The samples were diluted to the levels of 0.2, 1, and 2 μg L^{-1}. For each sample, three determinations were performed by the proposed method. The results are shown in Table 2. Since the average recoveries were 94.6% and 102.8%, respectively, the observed amounts in both tap and lake water had a little bias when the average intra-CV was 4.4% and 5.4%, respectively. These data indicated that the newly developed method could be useful in the routine detection of waterworks.

Conclusions

A novel immunochromatographic assay was proposed by utilizing the time-resolved fluorescence technique. By this method, MC-LR could be quantitated rapidly in the water samples. After the working conditions were optimized, this assay could offer a sensitivity of 0.035 μg L^{-1} and the IC50 of 0.78 μg L^{-1} with a linear range from 0.1 to 5 μg L^{-1}. Thus, this monitoring method has a series of features such as a good precision, a low analytical sensitivity, a high spiked recovery in the water samples, and a suitable working range. The results of MC-LR detection in the water samples were well consistent with those obtained with the HPLC method and the developed method; this demonstrated the accuracy of the assay. Since the detecting equipment is miniaturized, the fluorescence immunochromatographic method based on Eu nanospheres is simple, sensitive, and economical for the MC-LR quantitative determination.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was supported by Preventive Medicine Research Project of Jiangsu provincial Commission of Health and Family Planning (Y2015072) and National Natural Science Foundation of China (No. 81502831).

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