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Journal of Chromatography A, 848 (1999) 179–184

JOURNAL OF CHROMATOGRAPHY A

On-line trace enrichment for the simultaneous determination of microcystins in aqueous samples using high-performance liquid chromatography with diode-array detection

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Received 4 February 1999; received in revised form 6 April 1999; accepted 8 April 1999

Abstract

The need for a rapid, sensitive and reliable analytical method for cyanobacterial toxins, microcystins, has been emphasized by the awareness of toxic cyanobacteria as a human-health risk through drinking water. A new high-performance liquid chromatographic method with column switching was developed for the determination of microcystin-LR, -RR and -YR from water samples without pre-purification. The filtered water sample was passed through a Zorbax CN precolumn at a flow-rate of 3 ml/min for on-line trace enrichment. After valve switching, concentrated analytes were eluted in back-flush mode and separated on a Luna C_{18} column with a gradient of acetonitrile –20 m*M* phosphate buffer (pH 2.5). The method showed excellent precision, accuracy and speed with detection limits of 0.02 μg/ml from 100 ml of surface water. The total analysis time per sample was about 90 min. This method improves reliability, sensitivity and sample throughput, and shortens the analysis time compared to analysis methods using off-line solid-phase extraction.

Keywords: Water analysis; Column switching; Microcystins; Toxins

1. Introduction

Microcystins are strongly hepatotoxic cyclic heptapeptides produced by cyanobacteria such as *Microcystis*, *Anabaena* or *Nostoc* and more than 50 analogs have been identified [1–4]. The structures of these microcystins consist of D-alanine; ***X***; D-erythro-β-methylaspartic acid (MeAsp); ***Z***; 3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid (Adda); D-glutamic acid; *N*-methyldehydroalanine (Mdha), where ***X*** and ***Z*** represent variable amino acids and give the name to the molecule: for example, leucine and arginine (LR), tyrosine and arginine (YR), two arginines (RR), and tyrosine and methionine (YM).

The toxins inhibit protein phosphatase 1 and 2A [5–7] and have a tumor promoting activity [8]. They would threaten human health as liver tumor promot-

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00499-9

ers. Since toxic cyanobacterial blooms occur in eutrophic lakes, ponds and rivers all over the world, microcystins continue to represent a health risk to humans through drinking water. It is recommended that guideline level be 0.1 μg/l in drinking water for a long term exposure or 1 μg/l for a short term exposure on the basis of toxicity tests on mice and pigs [9].

The analytical methods including bioassay, chemical and biochemical methods as well as the overall analytical strategy have been reviewed [10,11]. Bioassays using mouse [12] or plant (*Sinapis alba* L.) [13] have been used in the screening but were found to be non-specific or more time-consuming (8 days). Biochemical methods include enzyme-linked immunosorbent assay (ELISA) [14,15] and protein phosphatase inhibition assay (PPIA) [15,16], which are advantageous as screening methods due to high sensitivity and the quick treatment of a large number of samples despite the poor identification ability and the potential for false-positives. Reversed-phase high-performance liquid chromatography (HPLC) with diode array detection [17–22] or electrochemical detection [23], LC–mass spectrometry (LC–MS) [24–26], capillary electrophoresis (CE) [26], and CE–MS [26] have been used as chemical methods for the identification and quantification of microcystins.

Sample clean-up is a necessary step for the trace level determination of microcystins in water and biological samples and has been performed by solid-phase extraction (SPE) using octadecyl silica (ODS) cartridges [13,17–22,24–26]. This SPE method require large samples (500–1000 ml water) and are more time-consuming, tedious and laborious.

The need for reliable, rapid and sensitive method for the identification and quantification of microcystins in the environment has been prompted. The objective of the present study was the development of a on-line sample clean-up in replacement of the off-line SPE for the determination of microcystins from water samples. Reversed-phase HPLC method for on-line trace enrichment was carried out by means of column switching technique [27–31]. This method offers practical advantages over off-line SPE with respect to time, effort, recovery, and sample volume.

2. Experimental

2.1. Chemicals

Microcystin-LR, -RR and -YR were purchased from Calbiochem (La Jolla, CA, USA). All reagents were of HPLC grade or analytical grade. HPLC-grade acetonitrile and methanol were obtained from Burdick and Jackson (Muskegon, MI, USA). Raw water samples were obtained from Daecheong Dam located in Taejeon serving as water supply reservoir. Drinking water sample was taken from Taejeon.

2.2. HPLC conditions

Column-switching system was employed for the on-line trace enrichment of microcystins from water samples and the instrumental arrangement was shown in Fig. 1. The HPLC system consisted of a Spectra System P4000 pump [Thermo Separation Products (TSP), CA, USA], a TSP high-pressure metering pump, a Spectra System UV 3000 multi scanning detector, a Rheodyne 7725i injector (Cotati, CA, USA), a Rheodyne LabPro PR 750–100 switching valve and data handling using a PC1000 software (TSP).

The analytical column was a Luna 2 C_{18} (250× 4.6 mm I.D., 5 μm, Phenomenex, CA, USA) protected by a Novapak C_{18} guard insert (4×10 mm I.D., Waters Co.) and the precolumn for on-line concentration of microcystins from water samples was a Zorbax CN cartridge (10×4.6 mm I.D.). The mobile phase consisted of a mixture of solvent A [acetonitrile–phosphate buffer (10 m*M*, pH 2.5) (25:75, v/v)] and solvent B (acetonitrile) as follows: 0% of B at 0 min, 20% of B at 38 min, 60% of B at 42 min to 50 min. The flow-rate was 1 ml/min and all separations were carried out at room temperature. The quantification wavelength was 238 nm and UV spectra of microcystins were obtained in the range of 200–300 nm for the identification.

2.3. Aqueous sample analysis using column switching technique

Water samples were filtered through a 47 mm A/E glass fiber filter (1.0 μm, Gelman Science). To

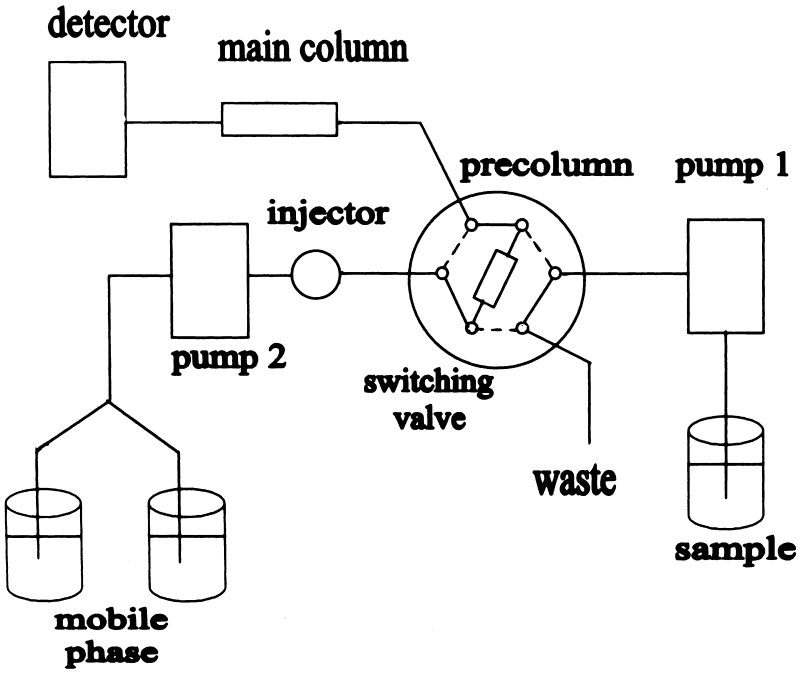

Fig. 1. Schematic diagram of a column switching system. --------: load: inject.

concentrate microcystins from water samples, filtered sample (100 ml) was passed through a Zorbax CN cartridge column at a flow-rate of 3 ml/min and the precolumn was washed with water (3 ml). On the while, the analytical column was equilibrated with the starting eluent under gradient elution of mobile phase. After on-line concentration of water samples on the precolumn, the switching valve was turned to inject position and the analytes enriched on the precolumn were eluted into the analytical column and separated by gradient elution immediately after valve switching. The valve was switched to the load position after 4 min when microcystin-LR, -RR and -YR were eluted completely from a precolumn to the analytical column. The precolumn was washed with methanol and re-equilibrated with water at a flow-rate of 3 ml/min for 10 min in order to concentrate another sample.

2.4. Evaluation of the method

Surface water samples were spiked with microcystin-LR, -RR and -YR at four concentrations to assess the recovery of the proposed method. The correlation of peak areas with the concentrations of microcystin-RR, -YR and -LR was examined in the range of 0.02–2 μg/l. The precision [relative standard deviation (RSD) of the results in replicate measurements] and accuracy (agreement between added and found values) of the method were evaluated. The limits of detection (LODs) for microcystin-LR, -RR and -YR were determined by a signal-to-noise ratio of greater than 3:1.

3. Results and Discussion

3.1. Optimization of on-line trace enrichment

Microcystins are cyclic heptapeptides of different hydrophobicity that can be readily separated by reversed-phase HPLC [17–26]. A gradient elution on an Luna 2 C_{18} column was necessary for the separation of microcystins from interference peaks in water samples. Retention times of microcystin-RR, -YR and -LR were highly reproducible and 15.5,

26.5, and 28.0 min, respectively (Figs. 2 and 3). The purity of microcystin peaks could be determined by using peak purity analysis of UV spectra in PC1000 software.

In off-line SPE for the sample clean-up of microcystins from water and biological samples [13,17–22,24–26], ODS cartridge has been widely used as the adsorbent and the complex clean-up steps should be included to eliminate the organic interferents which can be retained by non-selective strong adsorptivity of ODS.

For on-line trace enrichment of microcystins from water samples, it is necessary to choose precolumn packing and pH of the sample in such a way that microcystins are selectively adsorbed and the interfering compounds in water samples are not adsorbed on the precolumn.

The adsorptivities of LiChroprep RP-8 (91–99%) and Zorbax CN (93–102%) for microcystin-LR, -YR and RR were better than μBondapak phenyl (50–105%). Surface waters contain a large amount of organic matters, which can be co-extracted on the

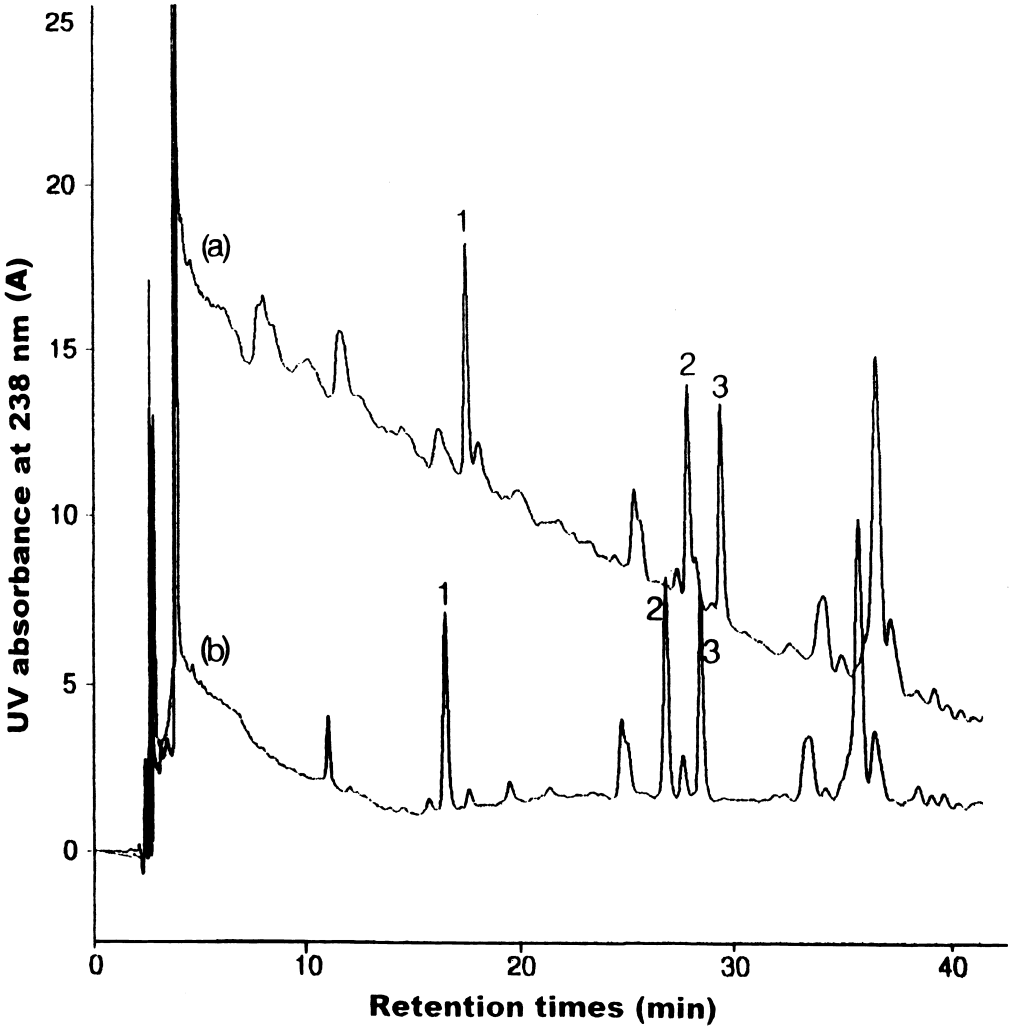

Fig. 2. Effect of the precolumn packing in on-line trace enrichment for the analysis of 100 ml of surface water sample spiked with 0.5 μg/l using (a) LiChroprep RP-8 precolumn (20×3.9 mm I.D, 25–40 μm) and (b) Zorbax CN precolumn (10×4 mm I.D.). Peaks: 1, microcystin-RR; 2, microcystin-YR; 3, microcystin-LR.

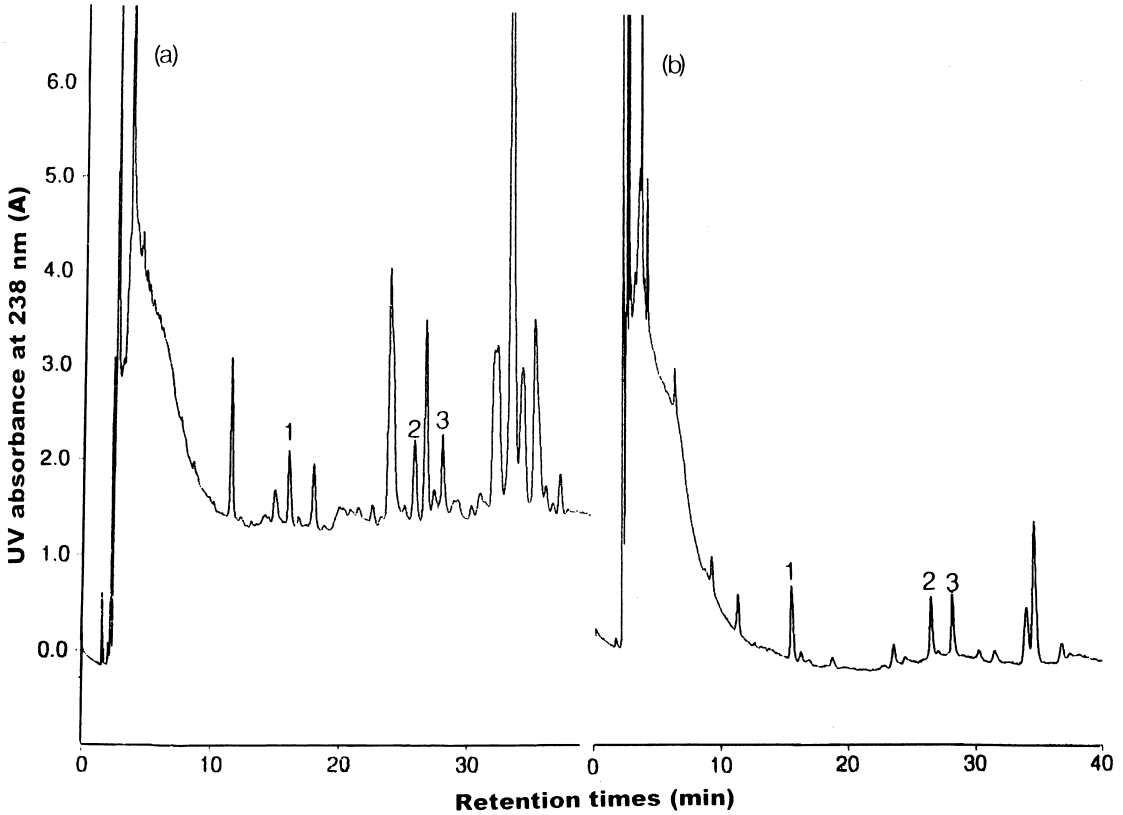

Fig. 3. Analysis of 100 ml of (a) surface water and (b) drinking water sample spiked with 0.1 μg/l of microcystins using column switching HPLC. Peaks: 1, microcystin-RR; 2, microcystin-YR; 3, microcystin-LR.

precolumn and result in the hump of interference peaks in the chromatogram. The effectiveness of the precolumn in removing the organic interfering matters from water sample is shown in Fig. 2; Zorbax CN precolumns have the lower adsorptivity for polar organic interference compounds in water samples than LiChroprep RP-8. Zorbax CN was chosen as precolumn packing because of its best recovery for microcystins and lower adsorptivity for organic interferents.

The selection of pH of the sample was important to obtain the best recovery and more clean chromatogram. The pK_a values of microcystin-LR, -RR and -YR are close to 3.5 [21], and thus, the capacity factors of microcystins decrease in a log–linear manner with increase from pH 2.5 to pH 7.0 on Zorbax CN precolumn. Acidification of water samples will result in the co-extraction of humic substances from water samples as well as higher capacity factors of microcystins. At neutral pH, humic substances were not extracted on ODS cartridge from water samples [21]. The pH of water sample was adjusted to pH 7.0 in which the retention of microcystins on a Zorbax CN remains sufficient and the majority of humic substances is not selectively adsorbed.

No breakthrough was observed after percolating 100 ml of distilled water spiked with microcystins onto the precolumn because of the high hydrophobicity of microcystins. After breakthrough plots, the mobile phase was passed through the precolumn to elute the adsorbed microcystins. The eluted peaks of microcystins exhibited nearly the same shapes of those of the direct mobile phase, and thus, microcystins were strongly retained in a narrow band on the top of the precolumn. The mobile phase (4 ml) seems to be sufficient for quantitative elution of microcystins from the precolumn.

Fig. 3 shows the typical chromatograms obtained from 100 ml surface water (3a) and drinking water (3b) spiked with microcystins at a concentration of 0.1 μg/l which is the suggested limit in drinking water for a long term exposure [9].

The Zorbax CN precolumn was exchanged after the analysis of 30 water samples (equivalent to 3 l water) and the analytical column showed no decrease in efficiency after analyzing 140 water samples.

3.2. Evaluation of the method

The calibration curves for microcystin-RR, -YR and -LR were linear in the range of 0.05–2 μg/l with correlation coefficients of 0.99. LODs of three microcystins were respectively 0.02 μg/l after enrichment of 100 ml surface water and these values meet the suggested limits of 0.1 μg/l in drinking water for a long term exposure. Mean recoveries of microcystin-RR, -YR and -LR were 99.7±7.5%, 96.3±6.8%, and 94.2±6.9%, respectively. RSDs in intra- and inter-assay for microcystin-RR, -YR and -LR varied from 3.1% to 7.8% of the found amounts in the spiked surface water samples. The inaccuracy for microcystin-RR, -YR and -LR were less than 7.0% compared to the added concentration.

Microcystins were not detected in the surface water and drinking water samples used in this study.

4. Conclusion

For the rapid, sensitive and reproducible determination of microcystins from water samples, a column switching HPLC method with direct percolation of water samples (100 ml) on Zorbax CN precolumn was developed. This method offers distinct practical advantages over the conventional methods using solid-phase extraction methods in terms of the speed, sample throughput, recovery and sample volume required. Total analysis time per a sample was about 90 min.

Acknowledgements

This study was supported by Wonkwang University in 1999. We thank Mr. Seok-Sang Jo in Insung Chromatech Co. for technical assistance.

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