Cross-reactivity and performance assessment of four microcystin immunoassays with detoxication products of the cyanobacterial toxin, microcystin-LR

J. S. Metcalf, K. A. Beattie, J. Ressler, S. Gerbersdorf, S. Pflugmacher and G. A. Codd

ABSTRACT

Three commercially available enzyme-linked immunosorbent assay (ELISA) kits and a non-commercial ELISA for the immunoassay of microcystins (cyanobacterial hepatotoxins) were tested with chemically produced conjugation products of microcystin-LR (MC-LR), a biologically derived MC-LR conjugate produced by a cytosolic extract of *Artemia salina* and authentic MC-LR. In all cases, the detoxified conjugation products were chromatographically distinguishable from the parent toxin in terms of retention time, but not in their UV absorption spectra, and were more immuno-reactive than MC-LR in the ELISAs. When initially dissolved in methanol, the highest cross-reactivity in the ELISAs was with the biologically derived glutathione MC-LR conjugate, followed by the chemically derived products in decreasing order of cross-reactivity: cysteine-glycine-MC-LR (cys-gly-MC-LR)>glutathione-MC-LR (GSH-MC-LR)>cysteine-MC-LR (cys-MC-LR). The authentic toxin (MC-LR) had the lowest cross-reactivity with all immunoassays. When the toxin conjugates were prepared and tested in water, the order of cross-reactivity was changed; the sequence was, in decreasing order: GSH-MC-LR>biologically derived glutathione conjugate>cys-MC-LR>cys-gly-MC-LR>MC-LR in all cases. No significant differences between cross-reactivity with microcystin products were found according to ELISA format or by the use of monoclonal versus polyclonal antibodies. The results are discussed in terms of the applicability of ELISAs for the detection and quantification of microcystins and their products in water bodies and organic matrices.

Key words | cyanobacteria, ELISA, immunoassays, microcystin

J. S. Metcalf
K. A. Beattie
S. Gerbersdorf
G. A. Codd (corresponding author)
Division of Environmental and Applied Biology,
School of Life Sciences,
University of Dundee,
Dundee DD1 4HN,
UK
Tel: +44-1382-344272
Fax: +44-1382-344275
E-mail: *g.a.codd@dundee.ac.uk*

J. Ressler
S. Pflugmacher
Leibniz Institute of Freshwater Ecology and Inland Fisheries,
Müggelseedamm 301,
12561 Berlin,
Germany

INTRODUCTION

Immunoassays, principally enzyme-linked immunosorbent assays (ELISA), are an increasingly common component of the repertoire of methods for the analysis of microcystins (cyanobacterial hepatotoxins), in raw and treated waters (Codd *et al*. 1999). In principle, this is due to the fact that immunoassays can provide highly sensitive, easy-to-use screens for the analysis of these potent toxins. At present, there are over 65 known variants of microcystin, with a large range in toxicity between individual microcystin variants (Codd *et al*. 1999). Differences also occur in the cross-reactivity of different microcystin variants in immunoassays based on antibodies that have been produced against one microcystin variant (microcystin-LR (MC-LR); e.g. Chu *et al*. 1989; Metcalf *et al*. 2000*a*). Immunoassays are also being increasingly used to analyse microcystins, and the related nodularin hepatotoxins, in more complex matrices. These include plankton and animals that have been exposed to the toxins in natural waters or in the laboratory; e.g. *Daphnia* (Throstrup and Christoffersen 1999), fish such as catfish

(Zimba *et al.* 2001), *Tilapia* (de Magalhães *et al.* 2001), flounder and cod (Sipia *et al.* 2001), and mouse livers (Kondo *et al.* 1996).

Recent work has shown that MC-LR, one of the most common and toxic of the microcystin variants, can be transformed to less toxic conjugation products in aquatic plants (Pflugmacher *et al.* 1998*a*), aquatic invertebrates (Pflugmacher *et al.* 1998*b*) and vertebrates (Kondo *et al.* 1992, 1996; Pflugmacher *et al.* 1998*b*). The widely occurring glutathione S-transferase system was subsequently found to be one of the mechanisms for microcystin detoxication. A proposed order of stepwise detoxication products is: MC-LR>glutathione-MC-LR (GSH-MC-LR)>cysteine-glycine-MC-LR (cys-gly-MC-LR)>cysteine-MC-LR (cys-MC-LR) (Pflugmacher *et al.* 2001). These chemically synthesised detoxication products of MC-LR have been found to cross-react with equal affinity in a commercially available ELISA kit for microcystin analysis (Metcalf *et al.* 2000*b*). These findings raise questions about the applicability of ELISAs for microcystin analysis in waters containing organic material of animal and plant origin, and in samples taken directly from animals and plants exposed to microcystins. The purpose of the present study was to prepare chemically and biologically formed detoxication products of microcystin and to examine the cross-reactivity of these products by all known, currently available, commercial microcystin ELISA kits and by a laboratory-prepared ELISA.

MATERIALS AND METHODS

Preparation of chemically derived conjugates of microcystin-LR

Microcystin-LR was purified from *Microcystis* PCC 7813 to greater than 99% by high performance liquid chromatography with photodiode array detection (HPLC-PDA) as described by Metcalf *et al.* (2000*a*). MC-LR conjugation products were prepared *in vitro* using reduced glutathione (GSH; Sigma Chemical Co., Poole, UK), L-cysteine-glycine (Sigma) and L-cysteine (Sigma). Reactions were allowed to proceed at 25°C for 24 hours according to Pflugmacher *et al.* (1998*b*). Products were analysed by HPLC-PDA and stored at −20°C, prior to further purification.

Preparation of a biologically derived conjugate of microcystin-LR

Cysts of the brine shrimp *Artemia salina* were obtained from Sciento (Manchester, UK). These were soaked in artificial seawater for 24 h at 27°C with continuous aeration and constant light. Microcystin-LR was incubated in the presence of an extract of the *A. salina* nauplii at 25°C for 24 hours as described in Pflugmacher *et al.* (1998*b*). Conjugation was confirmed by HPLC-PDA and the products stored at −20°C before purification.

Purification and quantification of microcystin-LR and microcystin-LR detoxication products

All MC-LR conjugation products were analysed by HPLC-PDA and peaks were noted with a retention time different from that of MC-LR, but with the characteristic UV spectrum with a maximum absorbance at 238 nm. These purified products were quantified, dried *in vacuo* and stored at −20°C for ELISA analysis.

ELISA analysis

Purified MC-LR conjugates were dissolved in either 100% (v/v) methanol or 100% (v/v) Milli-Q water and quantified by HPLC-PDA with reference to a gravimetric MC-LR standard prepared in 100% (v/v) methanol. Dilutions were performed using Milli-Q water for both methanol and water resuspensions to concentrations of 2.0, 1.5, 1.0 and 0.5 μg MC-LR equivalents l^{-1}. These dilutions were analysed using three commercially available microcystin ELISA kits and one non-commercial ELISA method. Commercial ELISA kits were purchased from SDI (Strategic Diagnostics Ltd, Hampshire, UK), EnviroLogix (Crop Enhancement Systems Ltd, Norfolk, UK) and Mitsubishi (Wako Chemicals GmbH, Neuss, Germany), and all were used according to the manufacturers'

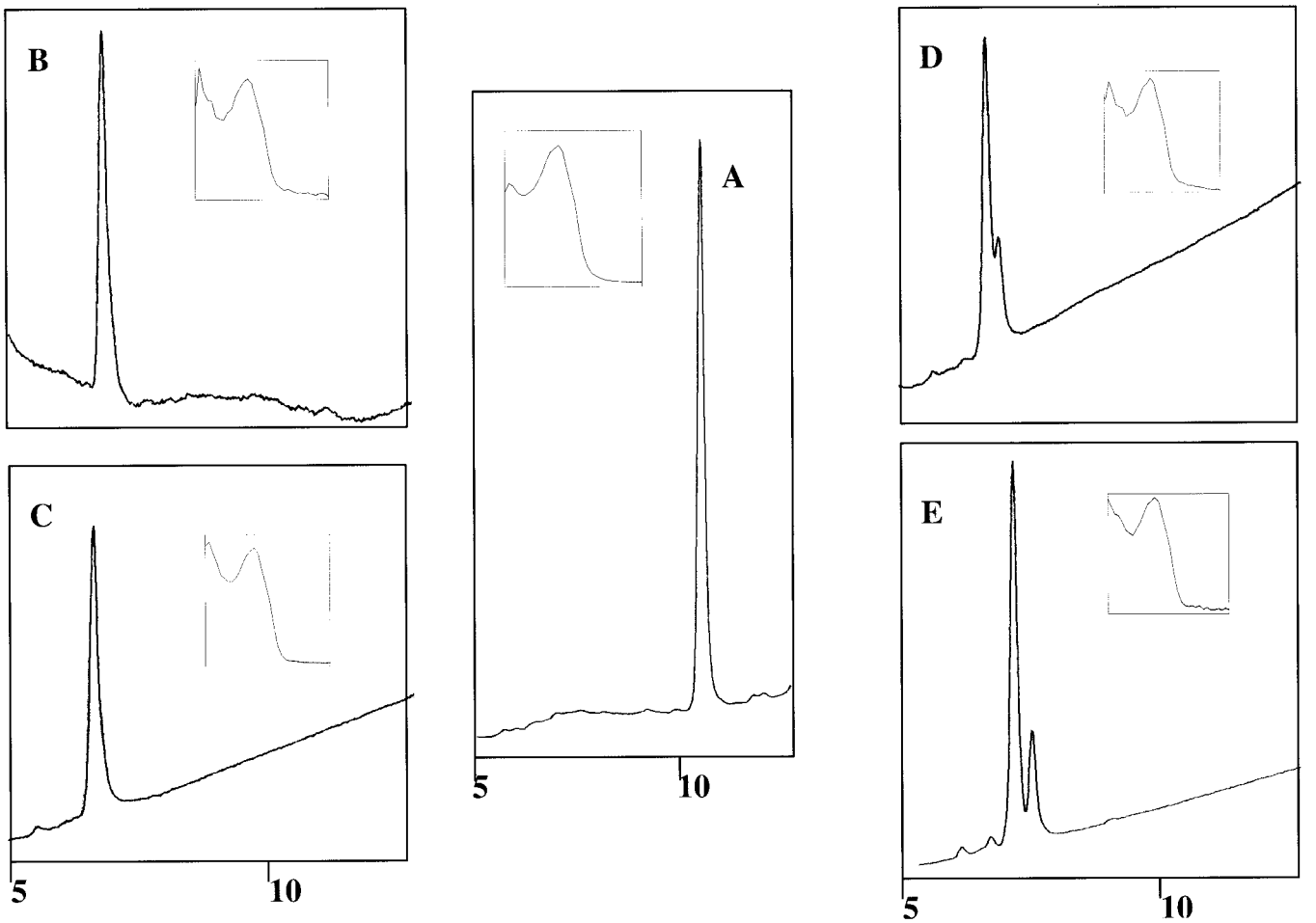

Figure 1 | HPLC-PDA chromatograms and UV absorption spectra of conjugates (detoxication products) of MC-LR in comparison with the parent toxin. Large plots, HPLC trace chromatograms for: (A) MC-LR; (B) biologically prepared GSH-MC-LR; (C) chemically prepared GSH-MC-LR; (D) cys-gly-MC-LR; and (E) cys-MC-LR. Vertical axes, absorbance at 238 nm; horizontal axes, retention time (minutes). Insets: corresponding photodiode array UV absorption spectra. Vertical axes, absorbance; horizontal axes, wavelength, 200–300 nm.

instructions. The fourth ELISA was based on antibodies raised against MC-LR according to Metcalf *et al.* (2000*a*).

RESULTS

Microcystin-LR conjugates were prepared both chemically and using the *A. salina* extract *in vitro* (Figure 1). In comparison with the retention time of the MC-LR standard (Figure 1A, 10.8 min), the retention times of the biologically derived GSH-MC-LR conjugate (Figure 1B), and the chemically derived GSH-MC-LR (Figure 1C), cys-gly-MC-LR (Figure 1D) and cys-MC-LR (Figure 1E) conjugates were all between 5 and 6 min. However, in all cases, a UV absorption spectrum characteristic of MC-LR (λ max 238 nm) was displayed (Figure 1B–E, insets). Furthermore, no breakdown of products to, or contamination by (residual) MC-LR was observed (Figure 1B–E). For both the cys-gly-MC-LR and the cys-MC-LR conjugates, two peaks were observed between 5 and 6 min, and both peaks had characteristic MC-LR spectra in each case. Differences may be due to the multiple locations for cys-gly- and cys-binding to the MC-LR molecule, and these may be further elucidated by NMR.

In order to assess potential responses of the ELISAs to the MC-LR products compared with the authentic toxin, previously purified conjugates were resuspended in both methanol and water, and analysed. Conjugates prepared in methanol were quantified by HPLC-PDA as MC-LR equivalents and diluted to concentrations of 0.5 and 1.0 μg MC-LR equivalents l^{-1}. In all cases, the MC-LR products gave positive reactions with the ELISAs, as did authentic MC-LR (Table 1). The ELISAs included formats based on both polyclonal (EnviroLogix, SDI, Dundee) and monoclonal (Mitsubishi) antibodies. The indicated concentrations of MC-LR equivalents obtained for all of the toxin conjugates, at both concentrations tested, were either approximately equal to, or more often greater than, the added concentrations of MC-LR equivalents. Furthermore, the indicated concentrations of MC-LR conjugates were at least equal to, and more often greater than the actual and indicated concentrations of authentic toxin (Table 1). Although the actual added concentrations of analytes (0.5 and 1.0 μg MC-LR equivalents l^{-1}) were within the linear ranges of detection for MC-LR for each ELISA, the indicated concentrations with the conjugation products at the higher concentration were above the maxima of the linear ranges of detection.

Analysis by EnviroLogix ELISA (Table 1) indicated that the biologically derived and chemically synthesised cys-gly-conjugates cross-reacted to the greatest degree with the MC-LR antibodies, followed by the GSH- and cys-conjugates, with indicated MC-LR equivalents of 2.39, 2.34, 2.10 and 2.06 μg l^{-1} when analysed at an added concentration of 1 μg MC-LR equivalents l^{-1}. In contrast, the addition of authentic MC-LR to the EnviroLogix format, to an actual added concentration of 1 μg l^{-1}, gave an indicated concentration of 1.38 μg l^{-1} (Table 1). Analysis of the same conjugation products by SDI ELISA kit at an added concentration of 1 μg MC-LR equivalents l^{-1} resulted in the biologically derived conjugate cross-reacting to the greatest degree, with an indicated MC-LR equivalent concentration of 2.19 μg l^{-1}. Cross-reactivity decreased to indicated concentrations of 1.87, 1.77, 1.45 and 1.32 μg l^{-1} for the cys-gly-, chemical GSH-, cys-conjugates and MC-LR, respectively. Similar relative responses were recorded with the Mitsubishi kit and with the ELISA based on antibodies to MC-LR produced in this laboratory (Table 1); i.e. all MC-LR conjugation products reacted positively, giving indicated concentrations above those added and above those of added MC-LR.

The same conjugates were also prepared in water, quantified by HPLC-PDA and diluted for immunoassay by the different ELISA kits. In all cases, in comparison with MC-LR, the detoxified products again reacted positively, giving indicated MC-LR equivalents in excess of those added (Table 1). The order of cross-reactivity was the same with all kits; the chemically produced GSH-conjugate was the most reactive, followed by the biological GSH-conjugate, the cys-conjugate, cys-gly-conjugate and finally the parent toxin. ELISA analysis of the parent toxin in water generally resulted in good agreement with the concentrations of analyte used, although the SDI kit gave higher positive values.

DISCUSSION

Microcystins can undergo several fates in aquatic environments, including glutathione-S-transferase-catalysed conjugation, a major route for microcystin detoxication (Pflugmacher *et al.* 2001). The detoxication products described here, produced chemically and biologically, using *A. salina* extracts, all exhibited characteristic MC-LR-like UV absorption spectra, with absorption maxima at 238 nm after HPLC-PDA analysis. These findings indicate that HPLC-PDA analysis does not distinguish between microcystins and conjugated, detoxified microcystin products in samples containing multiple microcystin variants with different retention times. The retention times of the products during separation by HPLC were between 4 and 6 min shorter than that of MC-LR (Figure 1), consistent with the more hydrophilic nature of the toxin products than the native toxin in the separation procedure used. Previous studies have investigated the distribution of radiolabelled microcystin to elucidate fates of this toxin in animals. Robinson *et al.* (1991*a*) investigated the distribution, excretion and hepatic metabolism of tritiated MC-LR in rats. Of the radiolabel detected, 43% was associated with an HPLC peak that was more hydrophilic than the parent toxin. After heat

Table 1 | Responses of four ELISAs to microcystin-LR and detoxication products prepared in methanol and water

		Indicated concentration			
Analyte	**Actual conc.**	**EnviroLogix**	**SDI**	**Mitsubishi**	**Dundee**
In methanol:					
MC-LR	0.5	0.46 ± 0.02	0.65 ± 0.01	0.45 ± 0.01	0.55 ± 0.05
	1.0	1.38 ± 0.19	1.32 ± 0.05	1.26 ± 0.09	1.10 ± 0.09
GSH-MC-LR (B)	0.5	1.42 ± 0.01	1.72 ± 0.06	2.08 ± 0.08	0.69 ± 0.03
	1.0	2.39 ± 0.04	2.19 ± 0.00	3.12 ± 0.03	3.98 ± 0.57
GSH-MC-LR (C)	0.5	1.01 ± 0.11	1.17 ± 0.01	1.37 ± 0.10	0.96 ± 0.10
	1.0	2.10 ± 0.06	1.77 ± 0.04	4.32 ± 1.65	1.62 ± 0.02
Cys-gly-MC-LR	0.5	1.29 ± 0.02	1.29 ± 0.06	1.15 ± 0.18	1.20 ± 0.10
	1.0	2.34 ± 0.10	1.87 ± 0.10	3.32 ± 0.20	2.33 ± 0.02
Cys-MC-LR	0.5	0.89 ± 0.09	0.75 ± 0.19	0.85 ± 0.05	0.81 ± 0.14
	1.0	2.06 ± 0.04	1.45 ± 0.02	2.00 ± 0.49	1.30 ± 0.00
In water:					
MC-LR	0.5	0.42 ± 0.02	0.97 ± 0.12	0.50 ± 0.01	0.45 ± 0.02
	1.0	1.10 ± 0.07	1.72 ± 0.05	1.02 ± 0.04	1.08 ± 0.11
GSH-MC-LR (B)	0.5	1.82 ± 0.01	2.18 ± 0.37	2.56 ± 0.06	1.70 ± 0.71
	1.0	2.50 ± 0.04	2.26 ± 0.01	3.18 ± 0.07	4.19 ± 0.81
GSH-MC-LR (C)	0.5	2.30 ± 0.16	1.88 ± 0.05	2.90 ± 0.06	3.37 ± 1.27
	1.0	2.80 ± 0.11	2.18 ± 0.04	3.46 ± 0.00	10.00 ± 0.00
Cys-gly-MC-LR	0.5	0.69 ± 0.01	0.93 ± 0.08	1.45 ± 0.12	0.80 ± 0.04
	1.0	1.45 ± 0.04	1.54 ± 0.08	2.18 ± 0.10	1.31 ± 0.03
Cys-MC-LR	0.5	1.42 ± 0.19	1.57 ± 0.05	2.20 ± 0.14	1.30 ± 0.19
	1.0	2.21 ± 0.11	2.05 ± 0.05	2.69 ± 0.22	2.35 ± 0.51

(B) biologically produced using *Artemia salina* extract containing glutathione S-transferases.
(C) chemically produced.
Actual conc., quantified as microcystin-LR equivalents ($\mu g\ l^{-1}$).
Indicated concentration, microcystin-LR equivalents ($\mu g\ l^{-1}$) according to each respective ELISA.
$n=2$, EnviroLogix and SDI; $n=3$, Mitsubishi and Dundee; ±=standard deviation (Mitsubishi and Dundee) or the range between individual observations (EnviroLogix and SDI).

denaturation and pronase digestion, extractions of liver that had been exposed to tritiated MC-LR released 80% of the bound radiolabel and, of this, 22% was the parent toxin and the remainder accounted for two biotransformation products (Robinson *et al.* 1991*b*). When compared with the parent toxin HPLC retention time of 10 min, the liver biotransformation products had retention times of 5.6 and 6.7 min and accounted for 52% and 13% of the radiolabel administered, respectively (Robinson *et al.* 1991*b*). Williams *et al.* (1997) have found that ^{14}C-MC-LR is metabolised to compounds more polar than the parent toxin when administered to salmon.

Antibody-based methods are being increasingly applied for the detection and analysis of microcystins in raw and treated waters (e.g. McDermott *et al.* 1995; Nagata *et al.* 1997) and in organic matrices from material exposed to the water-borne toxins. For example, using an immunoaffinity purification method, Kondo *et al.* (1996) administered purified microcystins to mice and recovered the parent toxin and its metabolites. At 3, 6 and 24 h after injection, a small percentage of the applied dose was associated with the livers and several metabolites were detected. Water fleas (*Daphnia magna*) exposed to microcystins have been analysed by ELISA to determine toxin uptake by this key member of many freshwater aquatic food webs (Throstrup and Christoffersen 1999); positive results have been obtained with single animals. Glutathione conjugates of MC-LR have already been identified in *D. magna*, the water plant *Ceratophyllum demersum*, the mussel *Dreissena polymorpha* and the roach, *Rutilus rutilus* (Pflugmacher *et al.* 1998*b*; Kamjunke *et al.*, 2002). Recent studies using catfish (Zimba *et al.* 2001) and *Tilapia* (de Magalhães *et al.* 2001) have investigated the accumulation of microcystins in fish flesh. However, in the light of recent studies (Metcalf *et al.* 2000*b*) and the present communication, it is inferred that ELISAs based on antibodies against MC-LR will give positive results with authentic microcystins and microcystin-derived conjugation products.

If the microcystin conjugation products were present in water samples or complex matrices, their detection and quantification by ELISA would result in an overestimation of both the quantity and toxicity of microcystin(s) in the samples. These overestimations would arise because: (a) the polyclonal antibody- and monoclonal antibody-based ELISAs gave positive results with all of the MC-LR products tested (Table 1); (b) the indicated concentrations of MC-LR equivalents (actually MC-LR conjugation products), according to ELISA, were higher than the added concentrations (Table 1); (c) the toxicities of the toxin conjugates are substantially lower than that of MC-LR according to protein phosphatase inhibition and mouse bioassays (Metcalf *et al.* 2000*b*).

A provisional guideline of 1 $\mu g\, l^{-1}$ has been specified by the World Health Organisation for the concentration of MC-LR in drinking water (WHO 1998). If conjugation products of MC-LR are present in waters and other matrices that are being screened by ELISAs based on MC-LR antibodies, the high cross-reactivity of the latter, indicating the presence of MC-LR equivalents, may trigger unnecessary water body interventions and restrictions if microcystin health guidelines were exceeded.

The overestimation of MC-LR equivalents in the case of ELISA of the microcystin conjugation products may be due to antibody recognition of steric changes on the MC-LR molecule during conjugation. The production of antibodies against the MC-LR hapten requires conjugation to other larger, more immunogenic proteins. The chemical production of these immunogens may result in changes to the conformation of the MC-LR molecule to more closely resemble the conformation(s) of the toxin conjugation products.

ELISAs have numerous applications in laboratory research into the production and fates of microcystins. In natural and man-made aquatic environments, they can serve as useful screens for the analysis of microcystins in raw and treated water and in clinical and environmental organic matrices. The methods are sensitive, and relatively quick and simple to use. However, in the event of positive results, confirmatory physico-chemical methods and/or toxicity-based methods are required.

CONCLUSIONS

Four conjugation (detoxication) products of the cyanobacterial hepatotoxin MC-LR cross-reacted with equal or greater affinity than MC-LR in four ELISA assays.

The toxin conjugation products included chemically and biologically formed examples. The ELISA formats included polyclonal and monoclonal antibodies. Confirmatory physico-chemical and/or toxicity-based methods are recommended for application to samples which are positive by ELISA for microcystins from sources where microcystin conjugation products are likely to occur.

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