Serologic Evaluation of Human Microcystin Exposure

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ABSTRACT: Microcystins are among the most commonly detected toxins associated with cyanobacteria blooms worldwide. Two episodes of intravenous microcystin exposures occurred among kidney dialysis patients during 1996 and 2001. Analysis of serum samples collected during these episodes suggests that microcystins are detectable as free and bound forms in human serum. Our goal was to characterize the biochemical evidence for human exposure to microcystins, to identify uncertainties associated with interpretation of these observed results, and to identify research needs. We analyzed serum samples using enzyme-linked immunosorbent assay (ELISA) methods to detect free microcystins, and gas chromatography/mass spectrometry (GC/MS) to detect 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB). MMPB is derived from both free and protein-bound microcystins by chemical oxidation, and it appears to represent total microcystins present in serum. We found evidence of free microcystins in patient serum for more than 50 days after the last documented exposure. Serum concentrations of free microcystins were consistently lower than MMPB quantification of total microcystins: free microcystins as measured by ELISA were only 8–51% of total microcystin concentrations as detected by the GC/MS method. After intravenous exposure episodes, we found evidence of microcystins in human serum in free and protein-bound forms, though the nature of the protein-bound forms is uncertain. Free microcystins appear to be a small but variable subset of total microcystins present in human serum. Research is needed to elucidate the human toxicokinetics of microcystins, in part to determine how observed serum concentrations can be used to estimate previous microcystin exposure. Environ Toxicol 22: 459–463, 2007.*

Keywords: microcystins; human serum; toxicokinetics; MMPB; 2-methyl-3-methoxy-4-phenylbutyric acid

INTRODUCTION

Microcystins are among the most commonly detected toxins associated with cyanobacteria blooms worldwide. Microcystins are a group of cyclic polypeptides of varying potency (Rinehart et al., 1994). They are produced by at least six genera of cyanobacteria, and microcystin occurrence has been reported worldwide (de Figueiredo et al., 2004). Microcystins are characterized as hepatotoxins and the liver is the primary target organ, however, other tissues and organs may be adversely affected (Falconer et al., 1983; Milutinovic et al., 2003; Moreno et al., 2005; Maidana et al., 2006). A recent report identified organic ion transporting polypeptides as mediating uptake of microcystins across membranes (Fischer et al., 2005). The presence of functional transporters may be a major factor in determining tissue tropisms. Humans are at risk of exposure to microcystins via contaminated surface waters, kidney dialysate, and dietary supplements (Jochimsen et al., 1998; Gilroy et al., 2000; Rinta-Kanto et al., 2005).

Two well-documented episodes of human exposure to microcystins via contaminated dialysate occurred in Brazil during 1996 and 2001 (Jochimsen et al., 1998; Soares et al., 2006). During both events, dialysate was prepared from microcystin-contaminated drinking water derived from surface sources. During 1996, 131 patients were exposed. Of these, 100 developed acute liver failure and over 50 died after exposure to contaminated dialysate (Carmichael et al., 2001; Azevedo et al., 2002). During 2001, 44 patients were exposed (Soares et al., 2006).

Biological evidence of exposure can greatly improve health studies of human exposure episodes to microcystins by increasing the specificity of any observed associations. However, there are no standard methods to detect microcystins in human tissues. We have reported an enzyme-linked immunosorbent assay (ELISA) method to detect free microcystins in human serum (Hilborn et al., 2005). However, free microcystins may represent only a portion of microcystins present in human tissue; microcystins appear to be present in tissue in free and bound forms (MacKintosh et al., 1995; Williams et al., 1997a,b). Free and bound microcystins may be oxidized to yield *erythro*-2-methyl-3-methoxy-4-phenylbutyric acid (MMPB). MMPB is an approximation of total microcystins present in tissue (Ott and Carmichael, 2006).

We analyzed serum samples collected from patients exposed to microcystins during each of these exposure episodes. Our goal was to characterize the biochemical evidence for human exposure to microcystins, to identify uncertainties associated with the interpretation of results, and to identify research needed to further characterize the human toxicokinetics of microcystins.

MATERIALS AND METHODS

Biological Samples

Blood samples were collected from exposed patients during investigations of microcystin exposure events at dialysis clinics in Caruaru, Brazil, during 1996, and in Rio de Janeiro, Brazil, during 2001–2002 (Carmichael et al., 2001; Soares et al., 2006). Blood samples were collected as part of the public health response to the outbreak of toxicoses during the 1996 episode in Caruaru. During the Rio de Janeiro event, patient consent was obtained to collect repeated blood samples after approval of the study protocol by the ethical committee of the Federal University of Rio de Janeiro. Blood samples were processed; serum was separated from blood cells and was frozen at −70°C.

Archived serum samples were extracted with methanol and analyzed for free microcystins from Caruaru patients using ELISA plate kits (EnviroLogix, Portland, ME), as previously described (Hilborn et al., 2005). Serum samples from Rio de Janeiro patients and unexposed controls were analyzed for free microcystins by a previously described ELISA method (Carmichael and An, 1999). All reported values from ELISA analyses of serum represent free microcystins in microcystin-LR equivalents, and were compared to standard curves created from spiked samples.

To investigate the amount of total microcystins potentially present and recoverable in serum, a subset of serum samples collected from Caruaru patients with sufficient quantity for analysis were selected. Samples underwent Lemieux oxidization, solid phase extraction, and gas chromatography/mass spectrometry (GC/MS) to detect MMPB as previously described (Ott and Carmichael, 2006; Yuan et al., 2006).

We performed descriptive analyses of serum microcystin concentrations over time where dates of blood collection were available. During database development, free serum microcystin concentration values less than the limit of quantification, <0.16 ng/mL, were entered as zero. These data were then used to calculate measures of central tendency and for graphical display.

RESULTS

Blood samples were collected from 51 microcystin-exposed Caruaru patients and serum was analyzed for free microcystin concentrations. Among these samples, free microcystin concentrations ranged from <0.16–28.8 ng/mL (median = 1.53 ng/mL). Twenty-four of these patients had a recorded blood collection date. Among this subgroup, free serum microcystins ranged from <0.16–7.5 ng/mL (median = 1.98 ng/mL); blood samples from these patients were collected during March 22 – April 12, 1996.

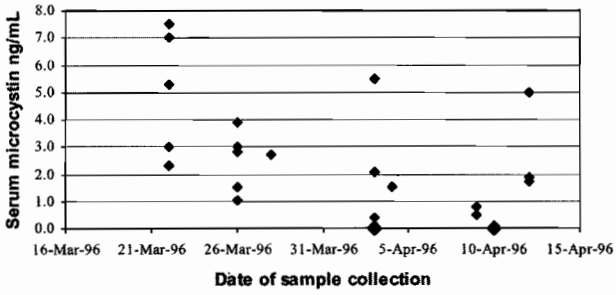

Fig. 1. Serum microcystin concentrations of 24 Caruaru, Brazil, patients. Microcystin exposures were documented during mid February, 1996. Note: Each small symbol indicates data from an individual. Two large symbols indicate serum concentrations of individuals whose blood collection dates and serum microcystin concentrations coincide.

Among the 44 microcystin-exposed Rio de Janeiro patients, a subset of 12 were followed over time (Soares et al., 2006). Five to 10 blood samples were collected from each of these 12 patients during November 2001 – February 2002. Free microcystin concentrations ranged from <0.16–0.96 ng/mL (median = 0.34 ng/mL).

Among the 24 patients from Caruaru and 12 patients from Rio de Janeiro with blood collection dates, we detected evidence of free microcystin LR equivalents in serum by the ELISA method for more than 50 days after the last date of documented microcystin exposure occurred (Figs. 1 and 2).

Sufficient quantities of serum sampled from six Caruaru patients were available for further analysis of total microcystins. Dates of collection were not available for these samples. ELISA analysis of this subset yielded free microcystin concentrations ranging from 6.78 to 26.30 ng/mL (median = 13.08 ng/mL). GC/MS analysis yielded MMPB concentrations that ranged from 45.71 to 112.93 ng/mL (median = 52.80 ng/mL). Therefore, serum concentrations of free microcystins as measured by ELISA were consistently lower, only 8–51% of total microcystin concentrations as detected by the GC/MS method for MMPB analysis (Fig. 3).

DISCUSSION

To our knowledge, these two episodes represent the only documented human exposure to microcystins where biological samples provide evidence of exposure. We report an unexpectedly long duration of detectable serum microcystin concentrations. We detected free microcystins in the serum of exposed dialysis patients for more than 50 days after the date of their last documented microcystin exposure. The time period during which total microcystins are detectable in human serum after exposure is unknown.

After the intravenous exposure episode in Caruaru, we found evidence of microcystins in human serum in free and protein-bound forms, though the nature of the protein-bound forms is uncertain. Free microcystins appear to be a small but variable subset of total microcystins present in human serum. As MMPB is derived from both free and protein-bound microcystins by chemical oxidation, it appears to represent total microcystins present in serum. However, the percent of microcystins oxidized to MMPB and the recovery of bound microcystins present in human serum is unknown. Additionally, the relationship between free and total microcystins in serum is unknown due to limited knowledge of distribution and clearance.

Human serum samples may be easily analyzed for the presence of free microcystins using a screening assay such as the ELISA. However, quantification of free serum microcystins does not accurately represent total toxin concentrations, although it appears that ELISA may be useful to detect free microcystins in serum samples for an extended period of time. An important applied outcome is to determine how measured serum concentrations of microcystins can be used to estimate the dose and timing of human microcystin exposure. However, currently, interpretation of the observed free serum microcystin concentrations in relation to total absorbed dose is not clear. Quantification of free and bound (total) toxin using a method such as MMPB may provide a better estimation of absorbed dose.

Rodent studies involving intravenous injections of microcystins show a rapid decline in serum concentrations as microcystins are bound in the liver (Falconer et al., 1986; Robinson et al., 1991). While microcystin was reported to persist in mouse liver for 6 days (Robinson et al., 1991), serum levels showed a biphasic decrease of less than an hour's duration with an initial half-life of 2 min or less and a second half-life of 7 or 42 min in mice and rats, respectively. The results of intravenous rodent exposures might suggest that serum microcystin levels in the exposed patients should be extremely low within hours or days depending upon the unknown human serum half-life. However, neither study made serum measurements over days that would allow comparison with the observed human data.

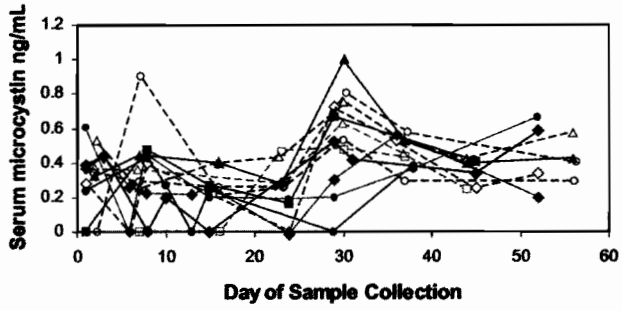

Fig. 2. Rio de Janeiro, Brazil, dialysis patients, $n = 12$. Each symbol is specific to an individual. Samples were collected for up to 57 days after exposure.

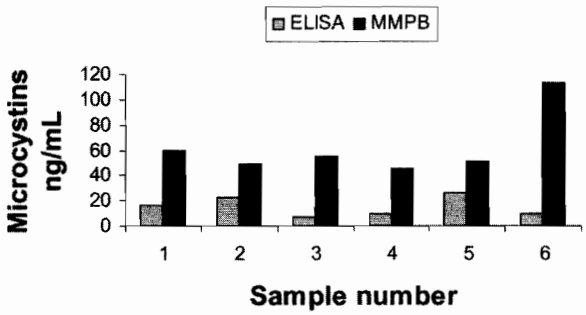

Fig. 3. Concentrations of free microcystins as determined by ELISA and total microcystins by the MMPB method in six serum samples.

Animal studies provide limited information with which to evaluate the observed persistence of measurable human serum microcystin concentrations. Following intraperitoneal injection in mice, serum concentrations rose as microcystins were absorbed, then a slow decline was observed with substantial concentrations remaining at 24 h (Lin and Chu, 1994). Lin and Chu reported that virtually identical levels were detectable in serum and liver as measured by ELISA and radiolabeled microcystin. This is in contrast to the disparity between free and total microcystin concentrations observed in exposed dialysis patients. This might reflect a species difference, but it may also reflect the form of the tritiated compound used in the mouse studies (which is unspecified). Introduction of tritium radiolabel using sodium borohydride reduces the methyldehydroalanine (MDha) residue of microcystin to the dihydro form, removing the double bond involved in the covalent adduction of the target protein phosphatases (MacKintosh et al., 1995; Craig et al., 1996). Loss of the ability of microcystins to covalently bond with protein phosphatases could potentially alter the observed distribution of the radiolabeled microcystin in these studies. Tritiated microcystin can be prepared by methods that do not reduce the MDha double bond, minimizing the potential for alterations in protein binding and tissue distribution (Robinson et al., 1989).

Limitations to our report include those of observational studies of naturally occurring human exposures. Information collected from the study of dialysis patients may not be generalizable to a larger population. Variability of exposure, patient characteristics, and metabolic response to microcystins were present but uncharacterized during this study. Although microcystins were implicated in these documented episodes of human exposure via dialysis, it is possible that patients experienced continued microcystin exposure by some route that was not measured during this study. The six serum samples from Caruaru patients that were analyzed for free and total microcystins appear to differ from the group of samples collected from the Caruaru exposure episode. The median free microcystin concentration of these six samples was 13.08 ng/mL versus a median of 1.53 ng/mL among other exposed patients. The relationship between free and total serum microcystin concentrations may differ among persons depending upon the magnitude of microcystin exposure.

All dialysis patients were exposed via the intravenous route. Serum concentrations of microcystins and duration of detectable free serum microcystins may vary after exposure by other routes. Limited animal data following oral exposure suggest that much less microcystin reaches the liver by comparison with intravenous or intraperitoneal exposures, though these results may differ from naturally occurring exposures due to the use of reduced, radiolabeled dihydromicrocystin (Nishiwaki et al., 1994).

We are now capable of detecting biological evidence of human microcystin exposure using simple tools such as ELISA, however, we are constrained by a lack of knowledge of important factors. Research needs include: (1) the characterization of bound serum microcystins; (2) knowledge of the distribution and clearance of microcystins over time is needed to interpret biological evidence of exposure; (3) blood sample collection during future episodes of documented human exposure to measure free and total microcystins over time. In particular, investigation into the nature of the bonds (noncovalent, covalent, chemistry of linkage, etc.) between microcystins and serum proteins, such as albumin, are needed, particularly in light of the known reactivity of cysteine-34 in human albumin.

Development of a physiologically based pharmacokinetic model for microcystins in animals and humans, in contrast to the existing classical compartmental analyses, would be valuable to integrate the pharmacokinetic data reported in the literature. These models capture explicit biological hypotheses and facilitate the evaluation of consistencies or inconsistencies across multiple datasets. Additional experimental studies in animals could then be targeted to answer specific questions. Care in the choice of methods will be important, however, due to differences in activity of the radiolabeled microcystins to carry out the full range of reactions occurring with the unlabeled compound. *In vitro* studies may provide information to assist in species comparison between rodents and humans.

Although we found detectable microcystin concentrations in human serum for 50 days after the last documented exposure, the interpretation of these findings is unclear. Additional information on the human toxicokinetics of microcystins is needed to determine the physiologic significance of microcystin concentrations in serum.

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