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The extraction and analysis of cylindrospermopsin from human serum and urine

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ABSTRACT

The naturally derived cyanotoxin, cylindrospermopsin (CYN), has been detected in freshwater systems worldwide and poses a threat to human health. The methods for the extraction and detection of this toxin in source water are well documented, but methods for CYN determination in exposed individuals have not been investigated. In this study, the extraction and detection of CYN from two different matrices, serum and urine, was explored. Both serum and urine matrices inherently produce interference with analytical analyses and require extensive clean-up. Methods for extraction of CYN from both matrices were developed and validated using fortified samples. Serum extraction included homogenization followed by protein precipitation and solid phase extraction (SPE). Urine samples were processed using filtration, pH manipulation, and SPE. Analyses using a commercially available enzyme-linked immunosorbent assay (ELISA) and liquid chromatography coupled with mass spectrometry (LC/MS/MS) were assessed. Matrix effects inhibited ELISA's use as a quantitative tool for both matrices. LC/MS/MS was determined to be the most effective and reproducible means to detect and quantify CYN. The method detection limits determined in this study using LC/MS/MS were 0.25 and 0.50 ng mL^{-1} for serum and urine, respectively. This method can be used to test individuals exposed to blooms of cyanobacteria producing CYN.

1. Introduction

Cylindrospermopsin (CYN) is a hepatotoxic and genotoxic cyanobacterially derived metabolite detected in freshwater sources around the world (Runnegar et al., 1994; Humpage et al., 2000; Carmichael et al., 2001). CYN was first discovered following a poisoning event in 1979 when 148 people were hospitalized in Palm Island (Queensland, Australia) with hepatoenteritis (Byth, 1980; Bourke et al., 1983). *Cylindrospermopsis raciborskii* was implicated in the poisoning as one possible cause and it was later determined that the cyanobacterium produced the hepatotoxin CYN. Cyanobacteria that have been known to produce the toxin cylindrospermopsin include *Anabaena lapponica* (Spoof et al., 2006), *Anabaena bergii* (Schrembi et al., 2001), *Aphanizomenon ovalisporum* (Banker et al., 1997), *Aphanizomenon flos-aquae* (Preußel et al., 2005), *C. raciborskii* (Hawkins et al., 1997), *Lyngbya wollei* (Seifert et al., 2007), *Raphidiopsis curvata* (Li et al., 2001), and *Umezakia natans* (Harada et al., 1994). Although cylindrospermopsin is not detected as frequently in freshwater sources as other cyanotoxins (*e.g.* microcystin) in the United States, the species known to produce the toxin are commonly identified in many waterways (Kinnear, 2010).

Our knowledge of cylindrospermopsin toxicity is continually growing, which makes defining risk a challenge. Due to increasing evidence of its genotoxic and cytotoxic effects (Gutiérrez-Praena et al., 2012; Štraser et al., 2011; Žegura et al., 2011), there is considerable

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concern with both acute and chronic exposure and how this may affect human populations. A tolerable daily intake (TDI) level was calculated to be 0.02 μg/kg (bw) in a review by Duy et al. (2000), but this value was extrapolated from a no-observable-adverse-effect level (NOAEL) obtained from an acute intraperitoneal study with mice, which did not account for oral toxicity. Data reflecting more recent studies, such as that of Shaw et al. (2000) and Humpage and Falconer (2002), has since added to the Duy review. A NOAEL based on oral toxicity data and the observed effects was determined to be 30 μg/kg (bw) per day. A practical guideline value of 1 μg/L in drinking water is thus proposed (Humpage and Falconer, 2002). Individuals exposed to CYN at levels higher than the NOAEL may exhibit symptoms and detection within their blood and/or urine may be possible.

Presently, there is not a lot of data on CYN uptake and distribution within the human body. Much of what we know has been inferred from mouse bioassay and cell line assays. The target organ in CYN toxicity is the liver, but there is evidence showing that other organs are affected, such as the lungs, kidneys, intestinal tract, heart, and potentially the reproductive organs (Bazin et al., 2012; Terao et al., 1994; Young et al., 2008; Hawkins et al., 1985). This implies that systemically circulated CYN may be detectable after exposure. Additionally, the main excretion pathway determined by Norris et al. (2001) was through the urinary system, with only a fraction potentially protein bound. This indicates that some CYN remains unmetabolized when excreted and may be detectable in its native state in urine.

Due to the toxicity and risk CYN poses to human populations, the accurate detection and quantification of this toxin in complex matrices is an important goal. Currently, diagnosis of CYN intoxication for humans and other animals is a clinical analysis of exclusion, accompanied by evidence of exposure to CYN measured in sources of exposure (*e.g.* water, aerosol samples, food products). Blood and urine samples collected and analyzed from exposed populations would aid in verifying linkages to exposure and symptoms. Therefore, the development and evaluation of extraction and analysis techniques for cylindrospermopsin in serum and urine samples were explored. Preparation techniques evaluated included sonication, centrifugation, pH manipulation, protein precipitation, and solid phase extraction (SPE). Two analysis techniques, a commercially available CYN enzyme linked immunosorbent assay (ELISA) and high performance liquid chromatography coupled with mass spectrometry (LC/MS) were assessed.

2. Materials and methods

2.1. Standards and reagents

HPLC grade acetonitrile, methanol, acetone, sodium hydroxide, hydrochloric acid (certified ACS, >99%), formic acid, and ammonium formate were purchased from Thermo Fisher Scientific (Waltham MA). All mobile phases for HPLC were filtered through 0.45 μm PVDF Millipore filters (Thermo Fisher Scientific) before use. DI water (18 MΩ-cm) was provided in-house by a Pure Lab Ultra Filtration System (Siemens Water Technologies Corp. Warrendale, PA). Human serum (male AB sterilized pooled) was purchased from Thermo Fisher (Cellect Human Pooled Serum Cat. 2930149 Lot#R22711, Cellect Human Pooled Serum Cat. 2930149 Lot#7825J, Cellect Human Pooled Serum Cat. BP2657100 Lot#083144). CYN standards were acquired from the National Research Council Canada/Certified Reference Materials (CRM-CYN Ottawa, Ontario, CA) and GreenWater Laboratories (Palatka, FL). Carbograph (graphitized carbon) SPE cartridges (150, 300 and 500 mg) and bulk sorbent carbograph were purchased from Alltech Associates, Grace Davison Discovery Science (Deerfield, IL).

2.2. Extraction

2.2.1. Serum

Human serum was initially assessed neat (without extraction), but interference with the ELISA assay and the non-homogeneous nature of serum required some processing prior to analysis. Extraction techniques explored included sonication (probe and bath), protein precipitation (100% methanol, 100% acetone, 99% MeOH/1% formic acid, 99% acetonitrile/1% formic), and solid phase extraction (SPE). Evaluation of extraction and analysis methods was based on spike returns and matrix effects. A final extraction and analysis method was proposed (Fig. 1).

2.2.1.1. Homogenization. Two homogenization techniques, probe and bath sonication, were assessed on spiked and

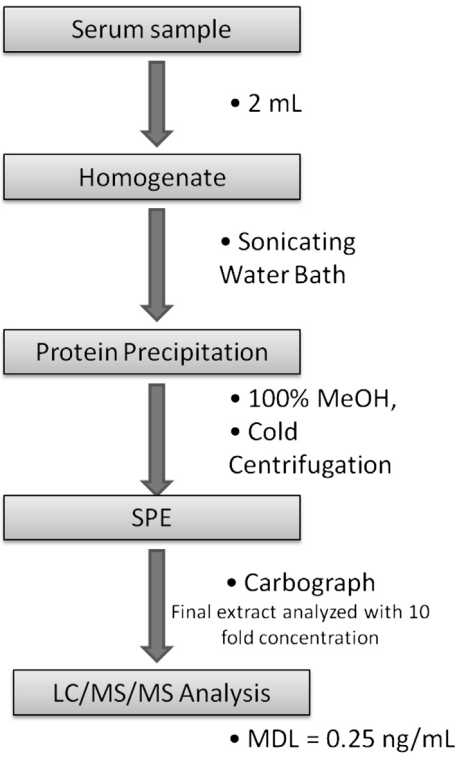

Fig. 1. Schematic showing suggested protocol for serum extraction and analysis.