Harmful Algae 80 (2018) 80–87

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Development of an LC–MS/MS method to simultaneously monitor maitotoxins and selected ciguatoxins in algal cultures and P-CTX-1B in fish

J. Sam Murray, Michael J. Boundy, Andrew I. Selwood, D. Tim Harwood*

Cawthron Institute, Private Bag 2, Nelson 7010, New Zealand[1]

ARTICLE INFO

Keywords:
Ciguatera fish poisoning
Gambierdiscus
Ciguatoxin
Maitotoxin
LC–MS/MS

ABSTRACT

Ciguatera fish poisoning is a serious human health issue that is highly localized to tropical and sub-tropical coastal areas, affecting many of the indigenous island communities intrinsically linked to reef systems for sustenance and trade. It is caused by the consumption of reef fish contaminated with ciguatoxins and is reported as the most common cause of non-bacterial food poisoning. The causative toxins bioaccumulate up the food web, from small herbivorous fish that graze on microalgae of the genus *Gambierdiscus* into the higher trophic level omnivorous and carnivorous fish predating on them. The number of *Gambierdiscus* species being described is increasing rapidly and the role of other toxins produced by this microalgal genus in ciguatera intoxications, such as maitotoxin, remains unclear. Ciguatoxins and maitotoxin are among the most potent marine toxins known and there are currently no methods of analysis that can simultaneously monitor these toxins with a high degree of specificity. To meet this need a rapid and selective ultra-performance liquid chromatography tandem mass spectrometry method has been developed to rapidly screen *Gambierdiscus* cultures and environmental sample device extracts for ciguatoxins and maitotoxins. A fast sample preparation method has also been developed to allow sensitive quantification of the potent ciguatoxin fish metabolite P-CTX-1B from fish extracts, and this method has been subjected to a small validation study. Novel aspects of this approach include the use of alkaline mobile phase for chromatographic separation and specific monitoring of the various toxins. This method has good potential to help evaluate ciguatera risk associated with *Gambierdiscus* and related microalgal species, and to help promote method development activities for this important and analytically challenging toxin class.

1. Introduction

Ciguatera fish poisoning (CFP) is one of the most common food-borne illnesses related to finfish consumption and is prevalent in circumtropical regions of the world, including areas of the Pacific Ocean, Indian Ocean, Caribbean Sea, and Gulf of Mexico (Bagnis et al., 1980; Botana, 2014). It has been known for centuries and is caused by the consumption of ciguatoxin-contaminated tropical and sub-tropical reef fish. Its true incidence is not known, but is estimated that 10,000–50,000 people per year suffer from this poisoning syndrome with more than 400 species of fish known to be vectors (FAO, 2014). Ciguatoxins (CTXs) are extremely lipophilic (fat-soluble) ladder-shaped polyether marine toxins produced by *Gambierdiscus* spp. that are odourless, tasteless and heat-stable (Lewis, 2001; Friedman et al., 2008). They represent a structurally diverse toxin class with more than 20 congeners identified from the Pacific region (P-CTXs; (Yogi et al., 2011)), two from the Caribbean (CCTXs; (Vernoux and Lewis, 1997)) and others detected in fish caught in the Indian Ocean (Hamilton et al., 2002), although the molecular structure of these congeners have not yet been characterized.

Gambierdiscus spp. are epibenthic dinoflagellates, well known in tropical reef areas although their presence in temperate environments is increasing (Llewellyn, 2010; Rhodes et al., 2014a). Currently, 15 different *Gambierdiscus* species have been described (Rhodes et al., 2017), although this list will undoubtedly continue to increase as three have been described within the past year (Fraga et al., 2016; Smith et al., 2016; Kretzschmar et al., 2017). A complex array of polyether toxins are produced by *Gambierdiscus* spp. including CTXs, maitotoxin (MTX) (Yasumoto et al., 1977; Holmes et al., 1990), a sulphated polyether compound putatively assigned MTX-3 (Holmes et al., 1990), gambieric acids (Nagai et al., 1992), gambierol (Satake et al., 1993), gambieroxide (Watanabe et al., 2013), and gambierone (Rodríguez et al., 2015). Very few species have been demonstrated to produce CTXs and only a handful of laboratories have them in culture (Chinain et al., 2010; Rhodes et al., 2014b). Many factors affect the growth and toxin

* Corresponding author.
E-mail address: tim.harwood@cawthron.org.nz (D.T. Harwood).
[1] www.cawthron.org.nz

https://doi.org/10.1016/j.hal.2018.09.001
Received 12 March 2018; Received in revised form 4 September 2018; Accepted 5 September 2018

production, with the presence of bacteria having been demonstrated to influence the growth and toxicity of laboratory cultures (Sakami et al., 1999). The current dogma for human CFP intoxication is that CTXs bioaccumulate and bio-magnify up the food chain as small herbivorous reef fish graze on the toxic micro-algae, these fish are then predated upon by larger omnivorous and carnivorous fish. During bio-magnification bio-transformation occurs creating more toxic forms and generating complex suites of congeners to monitor. For example, it has been shown that the Pacific algal congener P-CTX-4 A undergoes acid-catalysed spiroisomerization and oxidation to form P-CTX-1B (Ikehara et al., 2017).

There is a clear global need for new analytical tools to help identify toxin-producing *Gambierdiscus* spp. and toxic fish as the uptake, tissue distribution, metabolism and toxicity of CTXs in fish remains poorly understood. The US FDA have proposed an initial guidance limit of 0.01 μg/kg (ppb) for Pacific CTX-1B equivalents in fish samples (USFDA, 2011). In addition, the CODEX Committee on Contaminants in Foods (CCCF) is considering the establishment of maximum limits for C-CTX-1 and P-CTX-1 (*sic*), and/or the development of risk management guidelines. To help promote research activities on CFP, in 2015 UNESCO formulated an explicit global strategy through its Intergovernmental Panel on Harmful Algal Blooms (http://hab.ioc-unesco.org). One of the three elements of the strategy involves improved analytical methods for toxin detection in microalgal cells and fish. Development of this type of methodology will help understand the fundamental drivers of this poisoning syndrome that affects many indigenous island communities who are dependent on reef fish for sustenance and trade.

Traditionally, the analysis of CTXs has employed a mouse bioassay that was first detailed in the late 1960s (Scheuer et al., 1967). Other *in vivo* assays have been developed using a variety of animal species, including mongooses (Banner et al., 1960); rats, frogs and, chickens (Hashimoto and Yasumoto, 1965); cats (Hashimoto et al., 1969); and guinea pigs (Kimura et al., 1982), with each showing varying levels of success. As with other animal bioassays used for marine toxins these are nonspecific, making it impossible to determine the identity of the toxin (s) responsible for the observed toxicity. To discern the chemical nature of the toxins responsible for CFP, it has been necessary to develop more specific and selective methods of analysis for CTXs, such as liquid chromatography-mass spectrometry (Lewis et al., 2009; Oshiro et al., 2010; Wu et al., 2011; Yogi et al., 2011), various cellbased assays (Dechraoui et al., 2005; Abraham et al., 2012; Lewis et al., 2016; Pisapia et al., 2017) and a ciguatoxin receptor binding assay (Darius et al., 2007; Hardison et al., 2016). These methods require efficient and efficacious sample-extract preparation techniques, to optimise extraction and concentration of toxins, and to remove matrix-derived co-extractives that negatively impact sample analysis. The net result of these requirements is that the sample preparation methods involve multiple steps, rendering the overall process cumbersome and time-consuming, often necessitating a full day for the preparation of just a few samples. A number of these methods have been compared in the following reviews (Caillaud et al., 2010; Harwood et al., 2017a).

There are currently no chemical methods that have the required sensitivity and specificity to rapidly monitor both MTXs and CTXs simultaneously, which is necessary to enable screening of microalgal culture extracts to identify toxic species. In addition, there are no accredited methods of analysis for CTXs from fish samples. From an analytical perspective this is due to the complexity of the sample matrix, the very low levels of the various CTXs observed, and the lack of reference toxin material. In this study we aimed to develop a method that allowed rapid screening of both MTXs and CTXs in *Gambierdiscus* spp. extracts, and to also devise a simplified sample preparation procedure for accurate quantitation of P-CTX-1B in fish samples. The screening method represents an extension of the previously described method of analysis for just MTX in algal extracts (Selwood et al., 2014). Progress has been made but new knowledge and ongoing analytical improvements are needed.

2. Materials and methods

2.1. Chemicals and reagents

High purity methanol (MeOH) and acetonitrile (MeCN) was obtained from Thermo-Fisher (Fisher-Optima). HiPerSolv Chromanorm dichloromethane (DCM) was from VWR International. Purified water (18.2 MΩ) was produced with a Milli-Q system (Millipore, Canada). Ammonium hydroxide (≥25%) was from Honeywell Research Chemicals. Ciguatoxin (CTX) reference material; P-CTX-1B; P-CTX-3B; P-CTX-3C; P-CTX-4 A; P-CTX-4B was generated from natural sources and provided by Dr Mireille Chinain from the Institut Louis Malardé (Pape'ete, French Polynesia). Certified P-CTX reference material was generated and provided by Professor Takeshi Yasumoto at the Japan Food Research Laboratories (Tokyo, Japan); P-CTX-1B 43.3 ± 1.3 ng; P-CTX-2 38.4 ± 2.5 ng (52-*epi*-54-deoxyCTX-1B); P-CTX-3C 38.5 ± 2.6 ng; P-CTX-4 A 55.1 ± 5.2 ng. Purified maitotoxin (MTX) 10 μg was also gifted from Prof Yasumoto. Mixed stock solutions of the various CTXs were prepared in MeOH ranging from 0.1 to 10 ng mL^{-1}.

2.2. Microalgal sample preparation

Micro-algal culture isolates of *Gambierdiscus* spp. (n = 89) used for analysis were held within the Cawthron Institute Culture Collection of Microalgae and sourced from five different locations in the South Pacific Ocean; Australia, French Polynesia, the Cook Islands, Tonga and the Kermadec Islands. The species analysed were *G. australes* (n = 54), *G polynesiensis* (n = 2), *G. pacificus* (n = 6), *G. capenteri* (n = 15), *G. honu* (n = 8), *G. cheloniae* (n = 2) and *G. lapillus* (n = 2). Cultures were harvested in the stationary phase and contained at least 1×10^6 cells. To form a pellet the cells were centrifuged at 3200 × *g* for 10 min. The supernatant was discarded, and the cell pellet was extracted with MeOH at approximately 2×10^5 cells/mL (5 mL for 1×10^6 cells) using ultrasonication for 10 min in a 59 kHz water bath (model 160 H T, Soniclean Pty, Australia), followed by centrifuging at 3200 × *g* for 5 min at 4 °C to pellet remaining intact cells and other insoluble debris. A second identical extraction was performed and the resulting supernatant was pooled to give a final extract that contained approximately 1×10^5 cells mL^{-1}. An aliquot of the pooled sample was transferred into a 2 mL glass autosampler vial for analysis by liquid chromatography tandem-mass spectrometry (LC–MS/MS). Samples with P-CTX or MTX levels above the calibration range were diluted into range with MeOH. To assess extraction efficiency, four replicate extractions (10 mL) of a *G. polynesiensis* (CAWD212) cell pellet (2×10^6 cells) was performed and each extract analysed by LC—MS/MS for the presence of P-CTXs. For a summary of the microalgal extraction protocol refer to Table 1a.

2.3. SPATT bag sample preparation

Solid phase adsorption toxin tracking (SPATT) bags (2.5–10 g Diaion® HP20, Supelco, USA) have been used for *in vitro* exposure and field deployment as described (Roue et al., 2018). The nylon mesh bags were rinsed thoroughly with MilliQ water, to remove possible epiphytes or cell debris adhering to the bags, and then carefully opened to retrieve the HP20 resin. Using a fritted funnel under vacuum, the resin was washed thoroughly with MilliQ water to eliminate salts. The HP20 resin from each SPATT bag was then extracted four times with 25 mL of 100% MeOH for toxin desorption. The four extracts were combined (total volume of 100 mL) and dried under vacuum. For SPATT bags deployed in the field, the resulting dried extracts were further partitioned between 50 mL of DCM and 25 mL of 60% aqueous MeOH. The resulting liposoluble (DCM) and hydrosoluble (aq. MeOH) fractions likely to contain P-CTXs and MTXs, respectively, were dried under vacuum and stored at + 4 °C until analysis by LC-MS/MS.