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Rapid extraction combined with LC-tandem mass spectrometry (CREM-LC/MS/MS) for the determination of ciguatoxins in ciguateric fish flesh

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ABSTRACT

Ciguatera is a significant food borne disease caused by potent polyether toxins known as ciguatoxins, which accumulate in the flesh of ciguateric fish at risk levels above 0.1 ppb. The management of ciguatera has been hindered by the lack of analytical methods to detect and quantify clinically relevant levels of ciguatoxin in easily prepared crude extracts of fish. Here we report a ciguatoxin rapid extraction method (CREM) that allows the rapid preparation of fish flesh extracts for the detection and quantification of ciguatoxin by gradient reversed-phase liquid chromatography-tandem mass spectrometry (LC/MS/MS). CREM-LC/MS/MS delivers a linear response to P-CTX-1 spiked into fish prior to extraction. A similar response was obtained for P-CTX-1 spiked after extraction, indicating >95% extraction efficiency was achieved overall and 85% at the limit of quantification (0.1 ppb). Using this approach, levels ≥0.1 ppb P-CTX-1 could be detected and quantified from an extract of 2 g fish flesh, making it suitable as a confirmatory assay for suspect ciguateric carnivorous fish in the Pacific Ocean. The approach is designed to simplify the extraction and analysis of multiple samples per day.

1. Introduction

Ciguatera (fish poisoning) is a major economic and social problem throughout tropical and sub-tropical waters, with ~ 25,000 persons poisoned annually (Lewis, 2001). The disease is characterised by neurological and gastrointestinal disorders, which typically appear from 1 to 24 h following the consumption of contaminated fish and can last for months or longer (Gillespie et al., 1986). The toxins involved are potent sodium channel activator toxins known as ciguatoxins (Lewis et al., 2000) that are produced by the benthic dinoflagellate *Gambierdiscus* spp. (Lewis and Holmes, 1993; Holmes and Lewis, 2002). The ciguatoxins and structurally related brevetoxins compete at site 5 on voltage sensitive sodium channel (Lombet et al., 1987). Two related families of Pacific ciguatoxins (P-CTX) have been identified in ciguateric Pacific Ocean fish (Murata et al., 1990; Lewis et al., 1991, 1993; Satake et al., 1993). A third family of Caribbean ciguatoxins (C-CTX) contaminate ciguateric fish of the Caribbean Sea (Lewis et al., 1998), with a fourth family of Indian Ocean ciguatoxins contaminating fish of the Indian Ocean (Hamilton et al., 2002a,b). All ciguatoxins identified to date are heat stable polyether toxins of 1023–1157 Da. P-CTX-1 remains the most potent ciguatoxin characterised (Lewis et al., 1991), often contributing ~90% of the total lethality of carnivorous ciguateric fish capture in the western Pacific Ocean (Lewis and Sellin, 1992), and posing a health risk at levels ≥0.1 ppb (Lewis, 2001).

The traditional method of detecting the presence of ciguatoxins in fish involves testing lipid extracts by the mouse bioassay (Lewis and Sellin, 1993). More recently,

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cytotoxicity (Manger et al., 1995), radioligand binding (Poli et al., 1997) and antibody-based sandwich (Oguri et al., 2003) assays have been shown to have potential as cost-effective screens for the detection of ciguateric fish. Analytical liquid chromatography-tandem mass spectrometry (LC/MS/MS) procedures have also been developed for determining ciguatoxins in fish extracts (Lewis et al., 1999). However, lack of a rapid extraction procedure to simplify ciguatoxin analysis limits the usefulness of this approach. In this report, we describe a ciguatoxin rapid extraction method (CREM) combined with gradient reversed-phase liquid chromatography-tandem mass spectrometry (CREM-LC/MS/MS) approach for the detection and quantification of clinically relevant levels of P-CTX-1 in fish flesh.

2. Materials and methods

2.1. Extraction of fish

Coral trout (*Plectropomus maculatus*), a species implicated in ciguatera outbreaks in Queensland, was obtained from commercial outlets in Queensland, diced and kept frozen at −20 °C until use. Two gram portions of the fish were either spiked with P-CTX-1 (Lewis et al., 1991) or left unspiked before being cooked at 70 °C for 20 min in capped 50 ml Falcon tubes. Samples were then cooled before homogenisation (IKA Ultra Turrax T25, setting 5) with 8 ml methanol/hexane (3:1) until no lumps of fish remained (2 × 30 s). The homogenate was centrifuged at 4000 rpm for 20 min at RT, the upper hexane phase carefully removed using a pasture pipette and discarded, and the lower aqueous methanol phase (∼5.5 ml) transferred into a single use syringe and filtered through a 0.45-μm Millipore aqueous membrane filter (Millex®-HA) into a glass vial. The extraction procedure is designed for use on fish flesh with normal water content and variations in water content may influence extraction efficiency.

2.2. Solid phase extraction (SPE) of the crude extract

The crude extract was adjusted to 50–55% aqueous methanol by the addition of 2 ml H_2O, before cleanup through a C18 SPE cartridge. A number of reverse-phase C18 cartridge types and elution conditions were trialled. The optimised procedure used a 900 mg C18 SPE cartridge (Alltech Prevail Maxi-Clean) pre-conditioned with 4 ml of water before loading the ∼7.5 ml sample and a 0.5 ml 65% methanol rinse of the vial. Initial studies showed that spiked P-CTX-1 started eluting during the wash step when methanol was ≥70%, while no P-CTX-1 was detected in ≤65% aqueous methanol washes. To maximise cleanup without the loss of analyte, the cartridge was washed with 6 ml 65% methanol (discarded) before P-CTX-1 was eluted with 8 ml of 80% aqueous methanol.

To reduce matrix interference and the lengthy drying step associated with removal of water from the C18 cleanup step, an additional orthogonal normal phase SPE cleanup step was developed. To prepare the sample for this step, the 80% methanol fraction was collected into a 50 ml Falcon tube and made more polar by the addition of 4.2 ml of 1 M NaCl, before extraction with 6.7 ml chloroform with vigorous shaking. The resulting two phases were separated by centrifugation (∼2000 rpm for 4 min on a bench-top centrifuge), the upper aqueous methanol phase discarded, and the lower chloroform phase transferred to a scintillation vial and dried under N_2 and low heat to remove any remaining methanol and water, which affect normal phase SPE cleanup. After comparing a number of commercially available normal phase SPE cartridges, we selected a silica SPE cartridge (Silica Plus, Waters), pre-conditioned with 4 ml chloroform and loaded the sample in 4 ml chloroform and a 0.5 ml chloroform rinse of the vial. The cartridge was then washed with 4 ml chloroform before P-CTX-1 was eluted with 8 ml chloroform/methanol (9:1). For both SPE cleanup steps, syringe assisted positive displacement was used to shorten the conditioning, sample loading, washing and final elution steps.

To validate the method, unspiked fish flesh samples were either spiked with P-CTX-1 just prior to the final N_2 drying step, or left unspiked as negative controls. All samples were evaporated under N_2 and low heat before being redissolved in 200 μl of 50% aqueous methanol prior to LC/MS/MS analysis. To confirm its suitability for naturally contaminated fish, a ciguateric giant queenfish (*Scomberoides commersonnianus*) implicated in human ciguatera poisoning in Queensland was also extracted and analysed by CREM-LC/MS/MS.

2.3. LC/MS/MS

The LC system comprised a C18 column (5 μm Phenomenex Luna, 2.1 × 250 mm) fitted with a pre-column (Phenomenex C18, 4 × 2.1 mm, 5 μm). Solvent A was aqueous 2 mM ammonium formate and 0.1% formic acid, and Solvent B was 95% acetonitrile with 2 mM ammonium formate and 0.1% formic acid. The column was eluted at 400 μl/min with a linear gradient from 35% B to 100% B over 5 min. 100% B was held for 2 min before returning to 35% B at 7.1 min. The column was then equilibrated for 5 min with 35% B prior to the next run, allowing a 12 min turn-around between analyses.

Two triple-quadrupole mass spectrometers with Turbo-Ion-Spray ionization (AB Sciex Instruments) were used for the detection of P-CTX-1 in this study. Both mass spectrometers detected positive ions using multiple reactant monitoring (MRM) with resolution for both Q1 and Q3 set at low. The API 2000 was employed for the initial development work optimising the reversed-phase C18 SPE cleanup step. However, the API 4000 (QTRAP), with its greater sensitivity due in part to its orthogonal spray source and design of the collision cell, was used for subsequent analyses. MS/MS conditions were established using pure P-CTX-1 ($[M + NH_4]^+$ *m/z* 1128.7) dissolved in 50% B and injected directly into the mass spectrometer at 10 μL/min. MS/MS signals were optimised for the dominant product ions originating from the $[M + NH_4]^+$ ion. For the API 4000, a declustering potential of 110 V and a collision energy of 32 eV were used (CUR 25, TEM 300, EP 10 and CXP 15). As found previously using an API-III triple-quadrupole MS (Lewis et al., 1999), three dominant fragment ions could readily be generated from the $[M + NH_4]+$ ion of P-CTX-1 (*m/z* 1128.7 → 1093.7; 1128.7 → 1075.7; 1128.7 → 1057.7)