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Development of quantitative NMR method with internal standard for the standard solutions of paralytic shellfish toxins and characterisation of gonyautoxin-5 and gonyautoxin-6

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ABSTRACT

The chemical analysis of paralytic shellfish toxins (PSTs) requires standard solutions with accurate concentration. The mouse toxicity in each toxin is also essential knowledge for the introduction of chemical analysis as an alternative method to mouse bioassay (MBA) in routine monitoring of shellfish. In this study, we developed the quantitative analysis of PSTs by nuclear magnetic resonance (NMR), using *tert*-butanol as an internal standard. Only proton signals with longitudinal relaxation time (T_1) of less than 2.5 s, including the internal standard, were used for quantitation of toxins. Our method showed good precision (<3%) and accuracy (slope: 1.0038, R^2: 1.0000). The limit of quantitation (LOQ) at 5% relative standard deviation (RSD) was calculated to be 0.16 mM, which corresponded to 67 μg/mL as Saxitoxin (STX) diacetate form, while the limit of detection (LOD) was 0.04 mM. Gonyautoxin-5 (GTX5) and gonyautoxin-6 (GTX6) isolated from mussels were quantified by our method, and the toxicities of GTX5 and GTX6 were obtained by the MBA in which mice were standardized by STX provided from FDA. The specific toxicities of GTX5 and GTX6 newly calculated by the MBA were 120 MU/μmol (29 μg STX equiv./μmol) and 105 MU/μmol (25 μg STX equiv./μmol), respectively. These results are useful to convert the amount of GTX5 and GTX6 into the mouse toxicity, especially in the areas where the dinoflagellate *Gymnodinium catenatum* predominantly produces both toxins.

1. Introduction

Paralytic shellfish toxins (PSTs, Fig. 1A) produced by toxigenic dinoflagellates (Negri et al., 2003; Shimizu et al., 1975), cyanobacteria (Molica et al., 2002; Onodera et al., 1997; Pomati et al., 2000) and bacteria (Kodama et al., 1990) are a group of potent neurotoxins. The toxins are transferred to predatory organisms such as shellfish and crabs through food chain. Some PSTs are chemically or enzymatically converted from parent toxins in the predatory organisms (Arakawa et al., 1995; Lin et al., 2004; Oshima, 1995a; Sullivan et al., 1983). Paralytic shellfish poisoning (PSP) is a human illness caused by consumption of shellfish and crabs contaminated with PSTs.

The mouse bioassay (MBA), one of biological methods for PSTs, has been accepted as an official testing method that can quantify the total toxicity of various toxin analogues in shellfish. On the other hand, high performance liquid chromatography (HPLC) equipped with fluorometric monitor (Oshima, 1995b; Sullivan, 1990) or mass spectrometry (Aversano et al., 2005) can selectively identify and quantify individual toxins. These methods are superior to the MBA in sensitivity, selectivity and reproducibility. In such chemical analyses, it is essential to prepare calibrants with individual toxin to obtain accurate concentrations. Additionally, the relative toxicity of each

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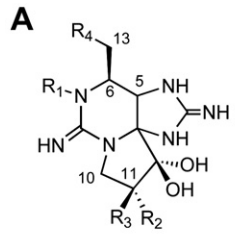

R_1	R_2	R_3	R_4: H_2N	$-O_3S$	HO
H	H	H	STX	GTX5	dcSTX
OH	H	H	neoSTX	GTX6	dcneoSTX
OH	OSO_3^-	H	GTX1	C3	dcGTX1
H	OSO_3^-	H	GTX2	C1	dcGTX2
H	H	OSO_3^-	GTX3	C2	dcGTX3
OH	H	OSO_3^-	GTX4	C4	dcGTX4

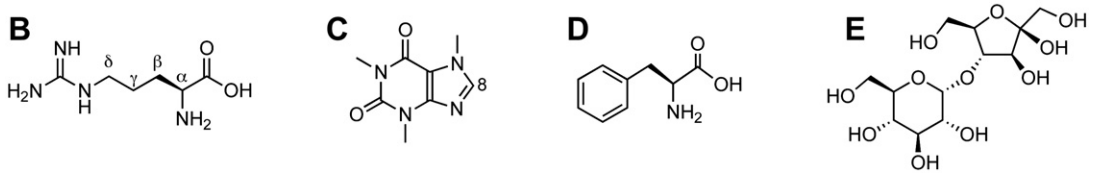

Fig. 1. Structures of a group of paralytic shellfish toxins (A), and compounds (B–E) used in the experiment. Compounds labeled by B–E correspond to L-arginine, caffeine, L-phenylalanine and sucrose, respectively.

analogue must be known by intraperitoneal injection in mice to use such methods as an alternative method to the MBA. One of the major problems in the preparation of PST standards is that a lyophilized powder of toxin acetate is difficult to weigh (Laycock et al., 1994). In our previous study, the toxin standard solutions were prepared and the concentrations were estimated from nitrogen contents of the toxin by elemental analysis (Oshima, 1995b). Other groups also prepared the toxin standard solution by using elemental analysis (Genenah and Shimizu, 1981) and ^{1}H NMR spectroscopy (Laycock et al., 1994).

^{1}H NMR spectroscopy has advantages of being a non-destructive method and of being able to directly quantify in deuterated solution with one referential material (Dagnino and Schripsema, 2005; Larive et al., 1997). Moreover, an NMR signal (integral value) is proportional to a number of nuclei under a specific condition (Holzgrabe et al., 2005; Rizzo and Pinciroli, 2005) regardless of any compounds while spectroscopic responses such as UV absorbance and fluorescence by other analytical instruments are different in respective compounds.

Previously, ^{1}H NMR spectroscopy has been applied to quantify marine toxins, including PSTs (Burton et al., 2005), using external calibration. In the present study we describe a method based on internal calibration allowing both for quantitation and certification of purity. The quality of the NMR quantitation with an internal standard was evaluated by using L-arginine as a model compound of PSTs because L-arginine has a guanidino group in its structure. Although the standard solutions of ten major toxins were prepared in our laboratory, characterizing both the concentration estimated by elemental analysis (Harada et al., 1982) and the relative toxicity of toxin analogues, the accurate concentration of GTX6 had not been obtained due to the low quantity of toxin obtained at the time. GTX6 is often a dominant toxin in bivalves contaminated with PSTs by the toxic dinoflagellate *Gymnodinium catenatum* which produces predominantly C1, C2, GTX5 and GTX6 (Miyamura et al., 2007; Oshima et al., 1993). Thus, the relative toxicity and certified calibrants of GTX6 have been required worldwide. In our present study, GTX6 isolated from mussels was quantified by our NMR method and the specific toxicity relative to STX was calculated. In addition, GTX5 was also quantified and the relative toxicity was re-calculated and compared with previous data.

2. Materials and methods

2.1. Reagents

The contaminated mussels *Mytilus edulis* were collected at Inokushi bay, Oita, in spring 2003. Acetic acid (analytical grade) for the final purification, L-arginine, L-phenylalanine (>99%), sucrose, caffeine (>98.5%) and deuterium oxide (99.9 atom% D) were purchased from Wako Pure Chemicals (Tokyo, Japan). Acetic-d_3 acid-d (99.5 atom% D), *tert*-butanol (≥99.5%, HPLC grade), *N*, *N*-dimethylformamide (DMF, ≥99.9%, HPLC grade) and propionic acid (99.5+%) were from Sigma-Aldrich. NMR tubes (Kusano Science Corporation, Japan) were used for standard grade (ø 5 mm). Electronic force balance was used DU-550 (Advantec, Japan) with 0.01 mg precision.

2.2. Saxitoxin

Saxitoxin dihydrochloride salt (STX·2 HCl, Lot No. 085-114) was provided by Food and Drug Administration (FDA) in USA.

2.3. Isolation of GTX5 and GTX6

GTX5 and GTX6 were isolated from 930 g of digestive glands of mussels *M. edulis* at Inokushi Bay, Oita, Japan in spring 2003. The digestive glands were homogenized and then suspended with an equal volume of 0.1 M HCl. The suspension was heated for 10 min at 75 °C, and then was centrifuged for 10 min at 8000 rpm at 4 °C to separate the supernatant from the pellet. After decanting the supernatant, the pellet was re-suspended with 50 mM HCl, and the