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Development of high sensitivity and separation detection method for ciguatoxin analogues by LC/MS/MS

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1. Overview

We've developed the new high sensitivity and selectivity method to detect ciguatoxin analogues (CTXs) using $[M+Li]^+$ or $[M+Na]^+$ by LC/MS/MS.

2. Introduction

Ciguatera Fish Poisoning is the world's most prevalent source of food poisoning, and it is caused by ciguatoxins (CTXs), thermostable polyether toxins produced by benthic dinoflagellates. More than 20 variants of the CTX1B and CTX3C series have been identified. CTXs are found in natural samples in trace amounts, and they pose neurotoxic effects at concentrations as low as 0.2 µg/kg. The US FDA and EFSA recommends a control level of 0.01 µg CTX1B equivalent/kg. Previously published work (Yogi, K. et. al.; *Anal. Chem.* 2011, 83, 8886-8891)[1)] using a LC/MS/MS instrument achieved the control levels monitoring the sodium adducts of the targets of interest ($[M+Na]^+$ > $[M+Na]^+$). Initial efforts to replicate the work with a Shimadzu's LC/MS/MS were unsuccessful. In this study, we optimized a highly sensitive method for the detection of CTXs using the sodium and lithium adducts, $[M+Na]^+$ and $[M+Li]^+$ ions, by adding very small amounts of alkali metals, such as Na^+ and Li^+ to the mobile phase. This work demonstrates that CTXs can be successfully detected at the low concentrations recommended by FDA with good chromatographic separation by LC/MS/MS. We report in this poster the new analytical conditions and accuracy of the method using $[M+Li]^+$.

3. Materials and Methods

3-1. CTXs reference materials

1) qNMR quantified reference materials of five CTX analogs were provided by JFRL (Japan Food Analysis Center) [2)].
2) CTX mix standard solution containing nine CTX analogs was prepared at NIHS (National Institute of Health Science).

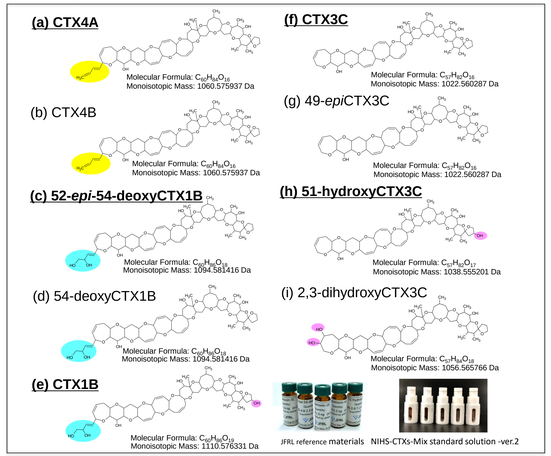

Figure 1. The structure of the nine compounds.
JFRL reference materials : (a) CTX4A, (c) 52-*epi*-54-deoxyCTX1B, (e) CTX1B, (f) CTX3C, (h) 51-hydroxyCTX3C

3-2. Analytical conditions

The analysis was performed with the LCMS-8060NX coupled with a Nexera™ X3 UHPLC system (Shimadzu Corporation, Kyoto, Japan), following conditions summarized in Table 1.

Table 1 Analytical conditions of UHPLC and MS

■**UHPLC conditions**

Equipment	: Nexera X3 system (Shimadzu Corporation)
Column	: Shim-pack Velox™ C18 (50 mm × 2.1 mm I.D., 1.8 µm)
Mobile phase A	: water/formic acid (1000 : 1, v : v)
Mobile phase B	for Na Adduct: acetonitrile/formic acid/0.05 M sodium hydroxide aqueous solution (1000 : 1 : 1, v : v : v)
Mobile phase B	for Li Adduct: acetonitrile/formic acid/0.1 M lithium hydroxide monohydrate solution (1000 : 1 : 1, v : v : v)
Flow rate	: 0.4 mL/min (0.6 mL/min from12.01 to17.0 min)
Time program	: B Conc. 40 % (0.0 – 2.5 min) → 85 % (12.0 min) → 100 % (12.01 - 17.0 min) → 40 % (17.01 - 20 min)
Column temperature	: 40 °C
Injection volume	: 5 µL

The flow was loaded into the MS detector between 2.5 to 12.5 min using a flow switching valve.

■**MS conditions**

Equipment	: LCMS-8060NX
Ionization	: ESI, Positive MRM mode
IF/Ion focus voltage	: +1 / +4 KV
IF/DL/HB temperature	: 350 °C/300 °C/400 °C
Nebulizer gas	: 3 L /min
Heating gas	: 15 L/min
Drying gas	: 5 L/min
CID gas pressure	: 270 Kpa
Collision Energy	: -40 V : CTX1B, 2,3-dihydroxyCTX3C, 52-*epi*-54-deoxyCTX1B, 54-deoxyCTX1B, 51-hydroxyCTX3C -30 V : 49-*epi*CTX3C, CTX3C, CTX4A, CTX4B

MRM transition for Na Adduct: $[M+Na]^+$> $[M+Na]^+$
m/z 1133.60: CTX1B
m/z 1079.60: 2,3-dihydroxyCTX3C
m/z 1117.60: 52-*epi*-54-deoxyCTX1B & 54-deoxyCTX1B
m/z 1061.60: 51-hydroxyCTX3C
m/z 1045.60: 49-*epi*CTX3C & CTX3C
m/z 1083.60: CTX4A & CTX4B

MRM transition for Li Adduct: $[M+Li]^+$> $[M+Li]^+$
m/z 1117.60: CTX1B,
m/z 1063.60: 2,3-dihydroxyCTX3C
m/z 1101.60: 52-*epi*-54-deoxyCTX1B & 54-deoxyCTX1B
m/z 1045.60: 51-hydroxyCTX3C
m/z 1029.60: 49-*epi*CTX3C & CTX3C
m/z 1067.60: CTX4A & CTX4B

4. Results

4-1. Mass spectra obtained with conditions previously published

Detected ions of CTX1B using methanol (Fig. 2 (a)) and acetonitrile (Fig. 2 (B)) with a method previously reported[1) 3)] were confirmed analyzing CTXs standards by Scan mode. Only $[M+Na]^+$ ion was detected with high sensitivity in previously reports, but multi-ionic species such as dehydration, ammonia addition, sodium addition and potassium addition were produced using these mobile phases. Method conditions were evaluated for improving the sensitivity of these species as alternative for quantifying CTXs in natural samples.

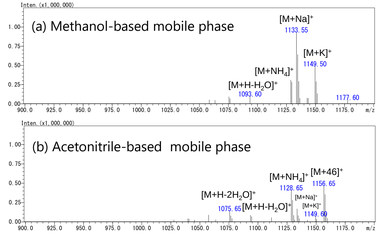

Figure 2. Mass spectra of CTX1B using different mobile phases (Scan mode)
(a)mobile phase : A: 5 mM ammonium formate + 0.1% formic acid water, B: Methanol
(b)mobile phase : A: 2 mM ammonium formate water, B: Acetonitrile

4-2. Formation of $[M+Na]^+$ or $[M+Li]^+$ ion and Optimization of Collision energy (CE)

The addition of Na^+ or Li^+ to the mobile phase is known to increase the formation of sodium or lithium adducts through cataionization[4)] . A trace amount of alkali metal was added to the mobile phase in order to obtain the desired ions, either $[M+Na]^+$ or $[M+Li]^+$ (Table 1). The specific ions from CTX1B are shown in Fig. 3 (SIM spectra) as an example. Same results were obtained with the other target compounds analyzed in this study. When Li^+ was added to the mobile phase, $[M+Na]^+$ was not detected. These results suggested that enhanced sensitivity could be achieved using alternative ions, such as $[M+Li]^+$.

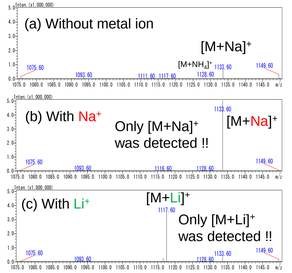

Figure 3 Multi spectra of CTX1B by different mobile phases (SIM).

Unfortunately, $[M+Na]^+$ and $[M+Li]^+$ precursor ions did not provide any useful product ions. Thus, we optimized the CE to provide the highest S/N when monitor $[M+Na]^+$ > $[M+Na]^+$ or $[M+Li]^+$ > $[M+Li]^+$ on MRM analysis mode. Optimized CE for CTX1B are shown in Table 1. The best CE of the remaining 8 analogs were optimized in similar way.

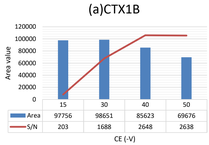

Figure 4 The relationship between the CE and the peak area or S/N.

4-3. Improved peak separation and sensitivity

In Fig. 5 ((a), (b), (c)), separation of the target compounds using previously reported conditions ((a); Oshiro, N.et. al., *J. Mar. Sci. Eng.* 2023, 11, 242)[5)] , Na-added mobile phase (b), and Li-added mobile phase (c) is compared. 2,3-dihydroxyCTX3C and 51-hydroxyCTX3, 49-*epi*-CTX3C and CTX3C, CTX4A and CTX4B showed dramatically better separation under new conditions (Fig.5 (b)(c)). 6 analogs other than 2,3-dihydroxyCTX3C and 51-hydroxyCTX3 and CTX3C had good sensitivity in Na-added mobile phase. It was confirmed that the sensitivity of the 9 analogs of interest was comparable when using Li-added mobile phases.

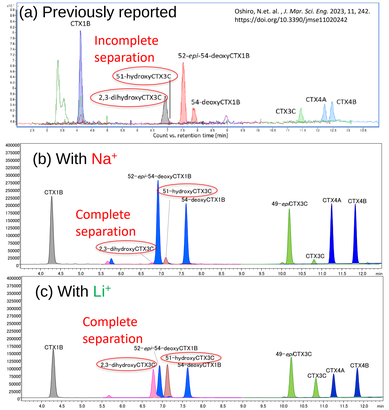

Figure 5 MRM chromatograms of 9 analogues (1 ng/mL NIHS-CTX-Mix ver.2)

4-4. Evaluation of the limit of quantification(LOQ)

Calibration curves were generated using JFRL reference materials to confirm the sensitivity of $[M+Li]^+$> $[M+Li]^+$. Each chromatogram at the lowest point of the calibration curve is shown in Figure 6. The range of the calibration curve was determined so that the average Accuracy in 3 replicate analyses of each calibration point was 100±5%, and the lowest point was designated as the LOQ. When 1 mL of the assay solution was prepared from 5 g of fish meat, the LOQs of the five standard substances adopted as the lowest point of the calibration curve in this method ranged from 0.0004 to 0.0012 µg/kg (Table 2).

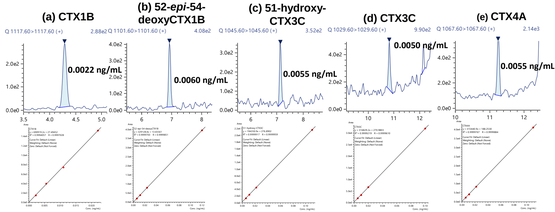

Figure 6 MRM chromatograms of the lowest point of the calibration curve and the calibration curve (JFRL Reference Materials)

Table 2 LOQ, reproducibility and accuracy of each analogue (n=3)

	LOQ (ng/mL)	LOQ (µg/kg)	Retention time (min)	%RSD (Area)	Accuracy (%)	S/N
CTX1B	0.0022	0.0004	4.289	11.02	98	81
52-*epi*-54-deoxyCTX1B	0.0060	0.0012	6.931	5.38	105	64
51-hydroxy-CTX3C	0.0055	0.0011	7.121	8.76	103	22
CTX3C	0.0050	0.0010	10.815	12.32	102	22
CTX4A	0.0055	0.0011	11.250	17.28	105	36

S/N was calculated by rms method with 0.5 min near the peak as noise.

5. Conclusions

- ✓ The new optimized LC/MS/MS conditions enable sensitivity exceeding the FDA tolerance level for CTXs.
- ✓ These results suggest that a general-purpose LC-MS/MS from any manufacturer can be used for highly sensitive analysis of CTXs.

6. Acknowledgements

We are grateful to Dr. Takeshi Yasumoto for providing the standard of CTXs.

7. Reference

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4. Klijnstra, Mirjam D., and Arjen Gerssen. ; A Sensitive Lc-Ms/Ms Method for Palytoxin Using Lithium Cationization, *Toxins* 10, no. 12 (2018): 537
5. Oshiro, N.et. al. ; Analytical Studies on Ciguateric Fish in Okinawa, Japan (II):The Grouper Variola albimarginata. *J. Mar. Sci. Eng.* 2023, 11, 242.

8. Patents

This analytical method is patent pending. PCT/JP2022/045132, 2022-089617(JP)