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Short communication

Nano liquid chromatography with hybrid quadrupole time-of-flight mass spectrometry for the determination of yessotoxin in marine phytoplankton

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Abstract

Studies of yessotoxin involving confirmation of fragmentation processes using a high-resolution orthogonal hybrid quadrupole time-of-flight (QqTOF) mass spectrometer and nanoLC hybrid quadrupole TOF MS have been undertaken. The fragmentation of YTX was studied in negative mode using nano electrospray (nanoESI) QqTOF mass spectrometry. Three major molecule-related ions were observed, $[M - 2Na + H]^-$, $[M - Na]^-$ and $[M - 2Na]^{2-}$, and fragmentation of the latter was studied in detail. This showed that product ions were formed as a consequence of charge-remote fragmentation processes that included a strong directional cleavage of the polyether rings of YTX. NanoLC coupled with QqTOF MS was used to determine YTX in small samples of the phytoplankton, *Protoceratium reticulatum*, by monitoring the $[M - 2Na]^{2-}$ ion at *m/z* 570. A PepMap C_{18} nanoLC column (75 μm × 10 cm, 100 Å, 3 μm, LC Packings) was used and the solvent was acetonitrile/water (90:10 (v/v)) containing 1 mM ammonium acetate, at a flow rate of 400 nl/min, for 30 min. Calibrations obtained with YTX standard solutions were linear over four orders of magnitude, 0.75–250 ng/ml; r^2 = 0.9947–0.9998. Phytoplankton cells (ca. 100–300) were picked, extracted with methanol/water (40:60), and the YTX concentration was determined over the range 0.011–0.020 ng/cell. The detection limit (3 × S/N) of this method was ca. 0.5 pg YTX on-column.

Keywords: Yessotoxin; Phytoplankton

1. Introduction

Yessotoxin (YTX) is a cardiotoxin, that was previously included as in the diarrhetic shellfish poisoning class due to its structure that includes 11 fused polyether rings [1,2]. Bivalve shellfish accumulate YTX through filter-feeding on toxic phytoplankton [3]. The main structural characteristic of yessotoxins (YTXs) consists of a ladder-shaped polycyclic ether skeleton, a terminal side-chain and two sulphate groups (Fig. 1). Structural variations in analogues occur mainly in the side-chains and include 45-hydroxy-YTX, the main contaminant in mussels [1], and homoyessotoxin [4], which has an additional methylene in a sulphate side-chain. A fluorimetric LC method [5,6] was developed which involved derivatisation of YTX with the dienophile, DMEQ-TAD, but this method is not robust and requires the use of aggressive buffers. LC–MS and LC–MS/MS methods are rapidly becoming the methods of choice for the determination of toxins in shellfish and phytoplankton [6,7]. Previously published LC–MS methods for the analysis of YTX and its analogues include LC–MS/MS with selected reaction monitoring (SRM) [8], LC–MS [9] and LC–MS^3 (using an ion trap instrument) [10,11]. To develop a more accurate picture of the dynamics of harmful algal blooms and subsequent shellfish intoxications, the seasonal variation of toxin production and the toxin profiles of phytoplankton species need to be examined. However, such toxinological studies on marine phytoplankton requires the determination of extremely low concentrations of analytes (sub ppb) in very small samples (<30 individual cells). In

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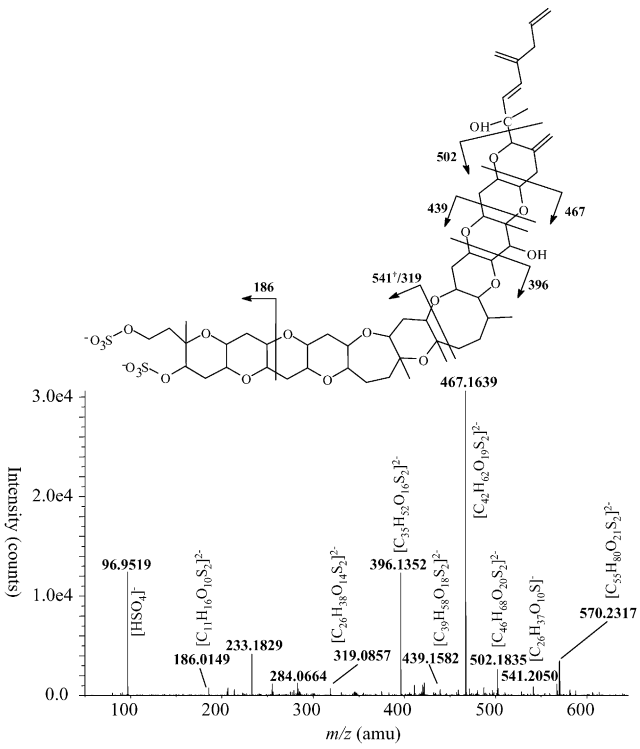

Fig. 1. Negative CID MS/MS spectrum from the $[M - 2Na]^{2-}$ ion, *m/z* 570, of yessotoxin and the proposed fragmentation processes.

some cases, collection of cells from microscope slides for analysis is the only option, as it is not possible to culture some marine phytoplankton, especially *Dinophysis* spp. [12]. One of the aims of this study was to develop a nanoLC–QqTOF MS method that could achieve sufficiently low limits of detection to enable the analysis of toxins in a small number of cells and to obtain high mass accuracy data to enable toxin confirmation. One of the main benefits of using nanoLC is that there is a higher concentration of analyte in a small solvent volume, which is almost completely evaporated at the MS interface; this is in contrast to the higher solvent flow rates used with conventional LC. The other important feature of nanoLC systems is the way in which samples are applied to the analytical column. The approach can essentially be described as on-line solid phase micro-extraction, which allows samples to be both pre-concentrated and partially cleaned up. The determination of YTX in *Protoceratium reticulatum* [13] was selected as a model for these studies, and this methodology will be expanded to include a range of marine phytoplankton that are difficult or impossible to culture.

2. Materials and methods

2.1. Chemicals and toxin standards

General purpose grade solvents were used during sample extraction and preparation, and were purchased from Labscan (Dublin, Ireland). Acetonitrile of ultra purity grade purchased from Romil (Cambridge, UK) and HPLC grade water purchased from Aldrich (Gillingham, UK) were used during nanoLC analysis. YTX standards are commercially unavailable and were prepared from contaminated mussels harvested from Norway using procedures that have been described previously [4]. A stock solution of YTX in methanol (ca. 5 μg/ml) was used and standards (0.75–250 ng/ml) were prepared immediately before use.

2.2. Culture of Protoceratium reticulatum

A clonal yessotoxin-producing strain of *P. reticulatum*, strain CAWD40, originating from New Zealand, was cultured. *P. reticulatum* was maintained at a temperature of 20 ± 1 °C, under 14:10 h light/dark cycle (ca. 50 mmol m^{-2} s^{-1} from a cool white lamp). Subculturing was undertaken approximately every three weeks by transferring a 10% inoculum into fresh medium. Culture media consisted of natural seawater (collected from the Kinsale harbour, Ireland) with added nutrients (nitrates and phosphates), vitamins (B_{12}, biotin and thiamine), trace metals (Na_2EDTA, $FeCl_3$, H_3BO_3, $MnCl_2$, $ZnCl_2$ and $CoCl_2$), selenium (Na_2SeO_3), and soil extract.

2.3. Picked phytoplankton cell preparation

P. reticulatum cells (100–300) were hand-picked from a microscope slide using a capillary for each analysis. Each sample was stored at −80 °C. Cell samples (100–300) were brought to 40% methanol, sonicated for 5 min, and extracted twice with equal volumes of hexane. The methanol extract was filtered (0.45 μm), evaporated under nitrogen and reconstituted in methanol/water (50:50 (v/v), 200 μl) prior to analysis.

2.4. Charge-remote fragmentation of YTX

A QqTOF mass spectrometer (QSTAR, Applied Biosystems, Warrington, UK) with a nanoESI (ES023C, Proxeon, Denmark) ion source was used in negative mode. YTX standard dissolved in methanol (0.01 μg/10 μl) and 2.0 M ammonium acetate (5 μl) were mixed and used to produce CID spectra from the molecule-related ion, *m/z* 570.2, $[M - 2Na]^{2-}$. The range, *m/z* 50–1200 was scanned (scan time of 1 s) and the optimized voltages were: declustering potential (DP) = −60 V; focusing potential (FP) = −245 V; declustering potential-2 (DP2) = −15 V; collision gas (CAD), 6 (arbitrary units); and collision energy of 44 V.

2.5. NanoLC–MS methodology

The nanoLC system consisted of an Ultimate micropump, Switchos microcolumn switching device, and a Famos carousel autosampler (LC Packings, Amsterdam, The Netherlands). A PepMap C_{18} nanoLC column (75 μm × 15 cm, 100 Å, 3 μm, LC Packings) was used with acetonitrile/water (90:10 (v/v)) containing 1 mM ammonium acetate,

at a flow rate of 400 nl/min, for 30 min. Solvents were degassed with high purity helium for 15 min prior to use. The wash solvent used with the Famos autosampler was acetonitrile with 1 mM ammonium acetate, and the optimal loading solvent for YTX when using the Switchos system was acetonitrile/water (70:30 (v/v)) with 1 mM ammonium acetate at a flow rate of 30 nl/min. Only glass syringes and containers were used for solvent and sample preparation to limit organic contamination. The nanoLC was interfaced to the QqTOF mass spectrometer using a liquid junction (ES241, Proxeon, Denmark) mounted on the nanoESI source head. YTX standards, in methanol, were prepared from a stock standard (ca. 5 μg/ml). TOF MS data were obtained whilst scanning in the range, *m/z* 500–1200 (scan time of 1 s), and the optimised voltages were: declustering potential = −90 V; focusing potential = −245 V; declustering potential-2 = −15 V; collision gas, 3 (arbitrary units); and ionspray voltage (IS) = −1800 V.

3. Results and discussion

3.1. Nano ESI hybrid quadrupole time-of-flight (QqTOF) MS of yessotoxin

The full scan mass spectrum of YTX, in negative mode, using a QqTOF instrument, revealed the presence of three molecule-related ions, $[M - 2Na + H]^-$, $[M - 2Na]^{2-}$, and $[M - Na]^-$. The main MS/MS fragmentation processes of YTX, generated under FAB conditions, were first proposed and discussed by Naoki et al. [14]. The product ion spectrum (Fig. 1) was obtained from the fragmentation of the $[M - 2Na]^{2-}$ precursor ion (*m/z* 570) of YTX. CID spectra were dominated by product ions from ring fragmentation processes and produced both single and double-charged species. Moderate collision energies (<40 V) favour the production of ring fragmentations from the double-charged ion, $[M - 2Na]^{2-}$ (Table 1). The CID spectrum of YTX showed that product ions were formed as a consequence of strong directional charge-remote fragmentation processes toward the negative charge residing on the sulphate(s). A series of singly charged ions, produced by the loss of HSO_4^-, were also observed. For example, the ion at *m/z* 541, $[C_{26}H_{37}O_{10}S]^-$, corresponded to the loss of HSO_4^- from the ion at *m/z* 319, $[C_{26}H_{38}O_{14}S_2]^{2-}$.

Table 1
Quadrupole TOF MS data for the product ions formed from the fragmentation of the double-charged precursor ion, $[M - 2Na]^{2-}$, of yessotoxin

Ion formula	Theoretical *m/z*	Observed *m/z*	Error (ppm)
$C_{55}H_{80}O_{21}S_2{}^{2-}$	570.2317	570.2317	–
$C_{26}H_{37}O_{10}S^-$	541.2107	541.2050	−11
$C_{46}H_{68}O_{20}S_2{}^{2-}$	502.1873	502.1835	7.6
$C_{42}H_{62}O_{19}S_2{}^{2-}$	467.1664	467.1639	5.4
$C_{39}H_{58}O_{18}S_2{}^{2-}$	439.1533	439.1582	−11
$C_{35}H_{52}O_{16}S_2{}^{2-}$	396.1349	396.1352	−0.8
$C_{26}H_{38}O_{14}S_2{}^{2-}$	319.0852	319.0857	−1.6
$C_{23}H_{32}O_{13}S_2{}^{2-}$	284.0642	284.0664	−7.7
$C_{11}H_{16}O_{10}S_2{}^{2-}$	186.0093	186.0149	−30

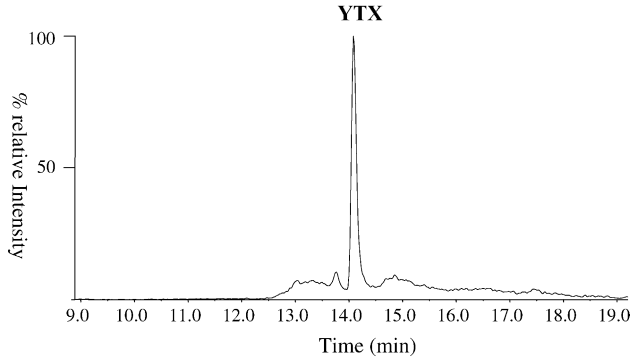

Fig. 2. NanoLC–QqTOF MS chromatogram of an extract (100 μl) from 100 cells of *P. reticulatum* containing yessotoxin (YTX) shown by the mass selection of the ion at *m/z* 570.2.

3.2. NanoLC–QqTOF MS analysis of yessotoxin

During nanoLC, selection of the loading solvent is important because a low organic solvent composition led to analyte (and matrix) being sorbed onto the Peek tubing connecting the autosampler and the switching valve causing large carryover signals. Conversely, if the percentage of organic solvent was too large, the analyte was displaced from the precolumn during the loading/washing phase of the analysis. For YTX, the optimised loading solvent composition was acetonitrile/water (70:30 (v/v)) with 1 mM ammonium acetate at a flow rate of 30 nl/min.

Isocratic nanoLC was performed using acetonitrile/water (90:10 (v/v)) containing 1 mM ammonium acetate, at a flow rate of 400 nl/min, for 30 min. MS signal was not acquired until 10 min after sample injection. Poor repeatability and prohibitively long retention times were obtained for YTX when lower flow rates were used. Calibration data were obtained with YTX standard solutions (0.75–250 ng/ml) with good linearity over four orders of magnitude; r^2 = 0.9947–0.9998. CID Cells (100–300) of *P. reticulatum* were individually picked from microscope slides, extracted and analysed for YTX. A typical chromatogram is shown in Fig. 2 and this was obtained from a sample extract containing 100 cells. The low level of interfering signals is indicative of the high selectivity of the technique; this chromatogram was obtained by an injection equivalent to a single cell (1 μl injection of an extract of 100 cells dissolved in 200 μl methanol). The limit of detection (3 × signal/noise) was approximately 0.5 pg YTX on-column. The content of YTX in the *P. reticulatum* culture was in the range, 0.011–0.020 ng/cell.

4. Conclusions

The first application of nanoLC hybrid quadrupole TOF MS to the determination of marine toxins has been demon-

strated. This method has a very high sensitivity and selectivity that permits its application to toxinological studies of marine phytoplankton. The detection limits of this method, ca. 0.5 pg, will allow single cell analysis and this could readily be used to determine the toxin profiles of phytoplankton species that cannot be readily cultured. In addition, the ability to obtain high mass accuracy data from a very small quantity of sample (ca. 10 ng) renders nanospray QqTOF MS a valuable analytical tool for the identification of marine toxins that are only available in sub microgram quantities.

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References

[1] T. Yasumoto, in: L.M. Botana (Ed.), Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection, Marcell Dekker, New York, 2000, p. 1.
[2] M. Murata, M. Kumagai, J.S. Lee, T. Yasumoto, Tetrahedron Lett. 28 (1987) 5869.
[3] M. Satake, K. Terasawa, Y. Kadowaki, T. Yasumoto, Tetrahedron Lett. 37 (1996) 5955.
[4] M. Satake, A. Tubaro, J.S. Lee, T. Yasumoto, Nat. Toxins 5 (1997) 107.
[5] T. Yasumoto, A. Takizawa, Biosci. Biotechnol. Biochem. 61 (1997) 1775.
[6] M.A. Quilliam, in: D. Barcelo (Ed.), Applications of LC–MS in Environmental Chemistry, Elsevier, Amsterdam, 1996, p. 415.
[7] R. Draisci, L. Palleschi, L. Giannetti, L. Lucentini, K.J. James, A.G. Bishop, M. Satake, T. Yasumoto, J. Chromatogr. A 847 (1999) 213.
[8] R. Draisci, L. Giannetti, L. Lucentini, E. Ferreti, L. Palleschi, C. Marchiafava, Rapid Commun. Mass Spectrom. 12 (1998) 1291.
[9] H. Goto, T. Igarashi, M. Yamamoto, M. Yasuda, R. Sekiguchi, R. Featherstone, J Chromatogr. A 907 (2001) 181.
[10] M. Fernández Amandi, A. Furey, M. Lehane, H. Ramstad, K.J. James, J. Chromatogr. A 976 (2002) 329.
[11] P. Ciminiello, C. Dell'Aversano, E. Fattorusso, M. Forino, S. Magno, R. Poletti, J. Chromatogr. A 968 (2002) 61.
[12] K.J. James, A.G. Bishop, M. Gillmann, S.S. Kelly, C. Roden, R. Draisci, L. Lucentini, L. Giannetti, P. Boria, J. Chromatogr. A 777 (1997) 213.
[13] M. Satake, L. MacKenzie, T. Yasumoto, Nat. Toxins 5 (1997) 164.
[14] H. Naoki, M. Murata, T. Yasumoto, Rapid Commun. Mass Spectrom. 7 (1993) 179.