ポストカラムHPLCを基本としたゴニオトキシン群の新しい分析法

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A New Analytical Method for Gonyautoxins Based on Postcolumn HPLC

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A new ion-pairing high-performance liquid chromatography (HPLC) method on a C_{30} column with a volatile mobile phase was developed to separate the gonyautoxin group (GTXs) from contaminants, allowing the utilization of liquid chromatography/mass spectrometry (LC/MS) with higher performance. A mobile phase consisting of 5 mmol/L heptafluorobutyric acid and 2% acetonitrile in 10 mmol/L ammonium acetate was adopted for separation of GTXs because the C_{30} column strongly retains GTXs under acidic conditions. The newly adopted method could efficiently separate GTXs from contaminants, especially in the toxic short-necked clam, whereas the routine HPLC so far used has poor resolution to separate GTXs from unknown interfering substances. In our method, GTXs were eluted in the order of GTX5, GTX3, GTX4, GTX2 and GTX1 from the C_{30} column, and were successfully determined by sonic spray ionization mass spectrometry (SSI-MS) with high sensitivity. This method is characterized by the combination of HPLC using a fluorescence detection system for PSP, and SSI-MS for measurement of the mass number.

Key words: paralytic shellfish poison (PSP); HPLC; gonyautoxin (GTX); Develosil C_{30} column; LC/SSI-MS

Introduction

The toxification of bivalves due to paralytic shellfish poison (PSP), which occurs in all coastal regions of Japan, is caused mainly by four species of toxic dinoflagellates, *Alexandrium tamarense*, *A. catenella*, *A. tamiyavanichii* and *Gymnodinium catenatum*. When a bloom of a toxic dinoflagellate appears, bivalves such as oyster, scallop, mussel and short-necked clam are contaminated with PSP, usually for a long period. The PSP infestation of bivalves poses a great problem to the fishery industry and to food hygiene.

Ion-pairing HPLC on a silica-based reversed-phase column with a postcolumn fluorescence detection system has already been established as a general method for analysis of PSP[1)-4)]. We have so far performed PSP analysis by using a C_8 column and phosphate buffer as a mobile phase. In analysis of GTXs in the short-necked clam by the above method, unknown contaminants disturbed the quantitative analysis of GTX1 due to insufficient separation, even after treatment with a Sep-Pak C_{18} cartridge column and ultrafiltration. Hence, to determine GTXs, it is required to establish an effective method to separate them from the contaminants.

This paper describes a new method to analyze GTXs, which consists of a new ion-pairing HPLC method with a C_{30} column using a volatile mobile phase and SSI-MS.

Materials and Methods

Reagents

Acetonitrile was of HPLC grade. All other reagents were of analytical grade. Heptafluorobutyric acid, orthoperiodic acid, and sodium 1-heptanesulfonic acid were purchased from Wako Pure Chemicals (Osaka). Standard PSP toxin (GTXs) was supplied by Dr. Y. Oshima, Tohoku University, Japan.

Extraction of PSP toxin from shellfish

Non-toxic short-necked clams (*Tapes japonica*) were collected from Hiketa Bay in 1999. After having been acclimated to filtered seawater for six days, they were fed on *Alexandrium tamarense*(HKTH97 15-09) which had been cultured in an enriched seawater Okaichi medium[5)] at 20℃ and at an illumination intensity of 80 μmol photon $m^{-2}\,sec^{-1}$ (12 hr light and 12 hr dark cycle). The short-necked clams consumed about 10^5 cells/day/clam over an eight-day period. Toxicity of the cultured *A. tamarense* was 18,300 cells/MU. After the eight-day feeding, the short-necked clams were toxified due to ingestion of *A. tamarense* plankton, resulting in the toxicity of 1.8 MU/g of the hepatopancreas.

Five hepatopancreas specimens were homogenized with an equal volume of 0.5 mol/L acetic acid. The homogenate was centrifuged at 2,000×*g* for 5 min and the supernatant obtained was passed through a Sep-Pak vac 1cc C_{18} cartridge column (Waters), followed by ultrafiltration using a 10,000-dalton cut-off filter of Ultrafree MC (Millipore). A 10 μL aliquot of the extract was used for the analysis.

HPLC- postcolumn fluorescence derivatization for analysis of GTXs

The HPLC system used was composed of a high-pressure pump (Hitachi L-7100), a silica-based reversed phase C_8 column (Inertsil C_8-5, GL Science, 250×4.6 mm i.d., 5 μm) or a C_{30} column (Develosil C_{30}, Nomura Chemical, 250×4.6 mm i.d., 5 μm), an auto sample injector (Hitachi L-7200), two reaction pumps (Hitachi L-6020) for delivering both oxidizing reagent and acid, a Teflon tubing coil (0.5 mm i.d., 10 m length), a spectrometric fluorescence detector (Hitachi L-7480) and a recorder (Macintosh Integrator, Tokyo Rika Kikai).

The mobile phase, consisting of 2 mmol/L sodium 1-heptanesulfonic acid in 10 mmol/L ammonium phosphate (pH 7.1) for the C_8 column or 5 mmol/L heptafluorobutyric acid and 2% acetonitrile in 10 mmol/L ammonium acetate (pH 3.8) for the C_{30} column, was pumped at a flow rate of 0.6 mL/min. The eluate from the column was continuously mixed with 7 mmol/L orthoperiodic acid in 50 mmol/L potassium phosphate buffer adjusted to pH 9.0 (flow rate 0.5 mL/min), heated at 65℃ and then acidified with 0.5 mol/L acetic acid (flow rate 0.5 mL/min)[3]. The fluorescence generated was monitored at 410 nm with excitation at 340 nm.

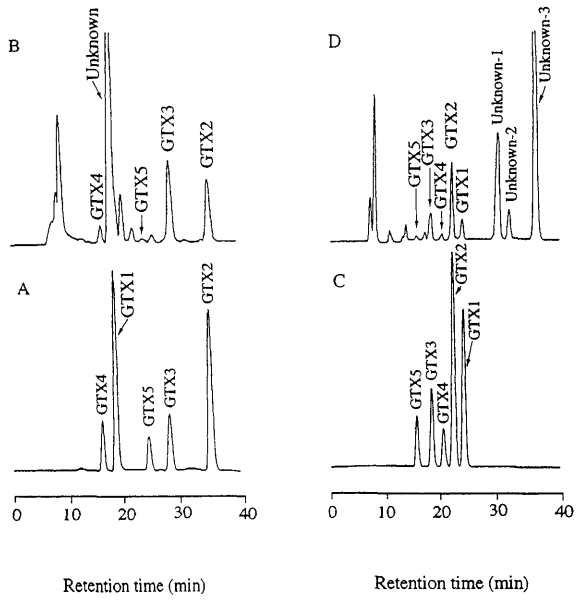

Fig. 1. Postcolumn HPLC chromatograms of GTX standards (A and C) and the extract from short-necked clam (B and D)
Columns: (A and B) Inertosil C_8 (250 mm×4.6mm i.d., 5 μm), (C and D) Develosil C_{30} (250 mm×4.6 mm i.d., 5 ìm); mobile phases: (A and B) 2 mmol/L 1-heptanesulfonic acid in 10 mmol/L ammonium phosphate, pH 7.1; flow rate: 0.6 mL/min, (C and D) 5 mmol/L heptafluorobutyric acid and 2% acetonitrile in 10 mmol/L ammonium acetate, pH 3.8, flow rate: 0.6 mL/min; fluorescence detector: 340 nm excitation, 410 nm emission; column temperature: 25℃; injection volume: 10 μL

LC/SSI-MS for analysis of GTXs

A sonic spray ionization (SSI) interface (Hitachi), with an M-8000 liquid chromatograph/three-dimensional quadrupole mass spectrometer (LC/3DQMS), was employed for the detection of GTXs. The protonated ions of GTXs formed by SSI were introduced into an ion-trap for mass spectrometry under the following conditions: drift voltage, +40 V; porous plate temperature, 250℃; sampling orifice temperature, 150℃. Full-scan spectra were measured in the positive ion mode over the mass range of *m/z* 200–600.

Results and Discussion

HPLC

The chromatograms obtained by routine HPLC on

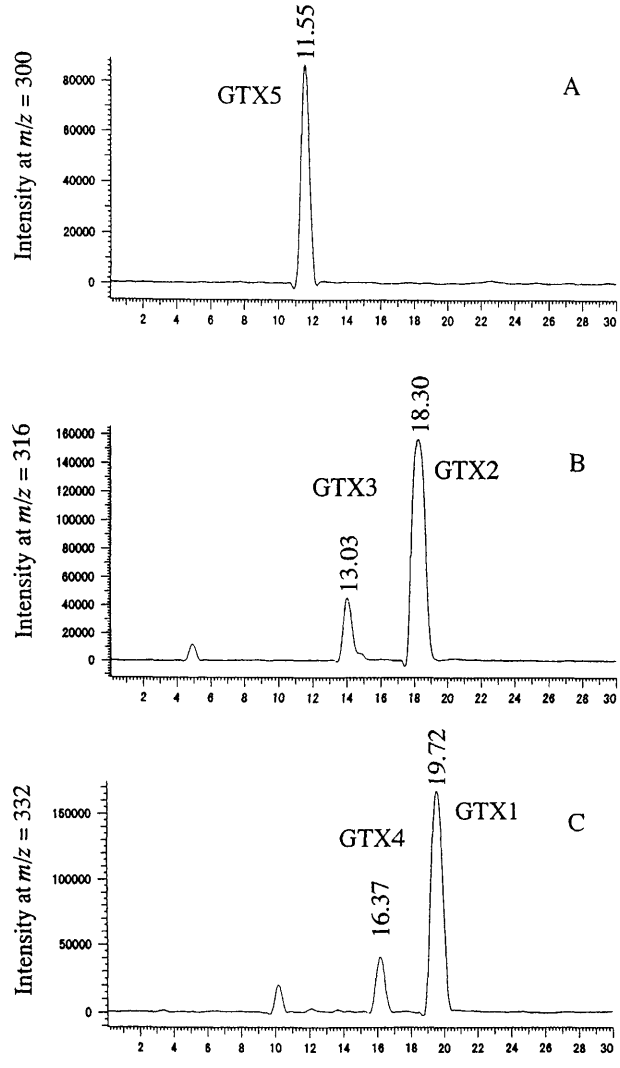

Fig. 2. MS chromatograms of standard GTX1–5
A; *m/z*=300 for GTX5, B; *m/z*=316 for GTX2 and GTX3, and C; *m/z*=332 for GTX1 and GTX4

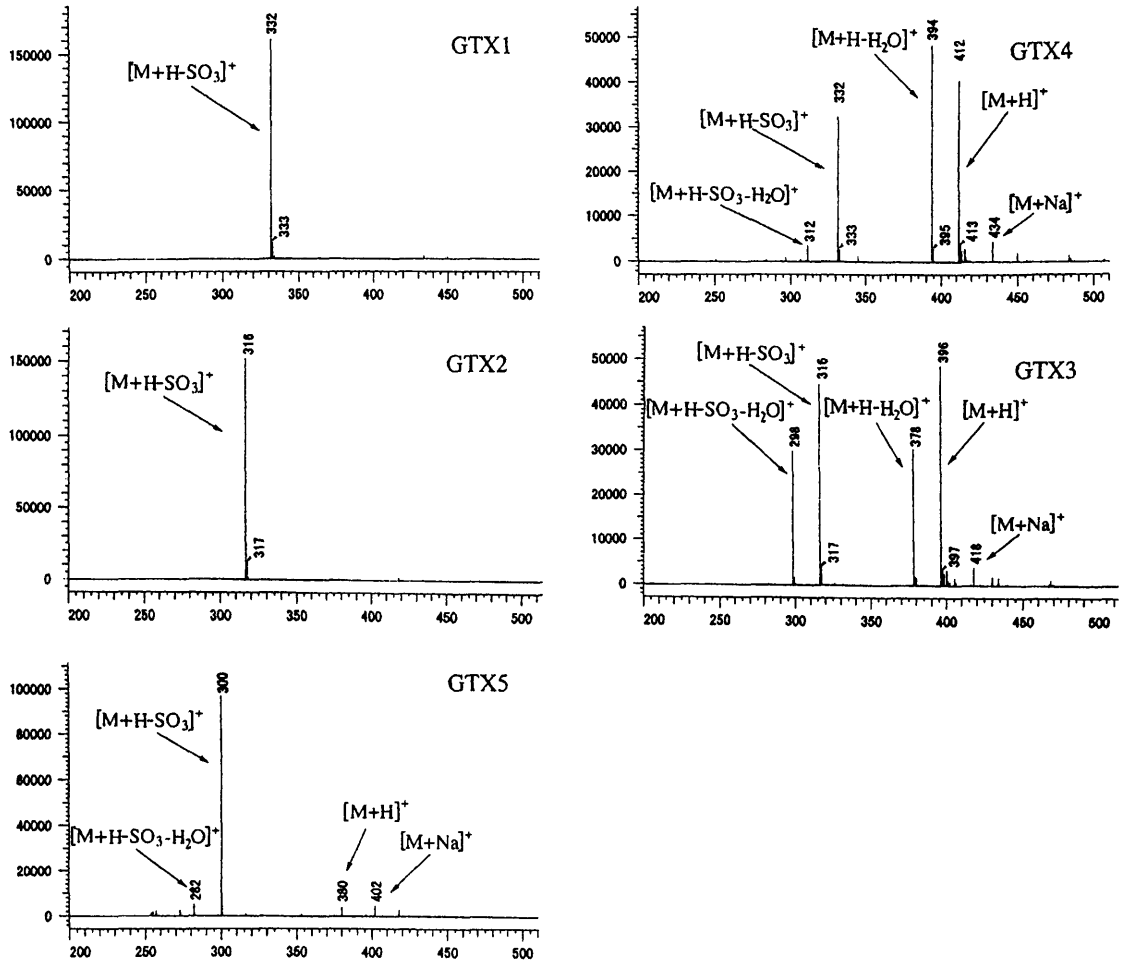

Fig. 3. Mass spectra of GTX1–5 by LC/SSI-MS

the C_8 column used so far for GTXs analysis are shown in Fig. 1A and Fig. 1B. In the analysis of the short-necked clam extract, a large peak which overlapped the GTX1 peak made quantitative analysis of GTX1 difficult. The separation was not improved by the use of HPLC on an ODS column[6] after preliminary purification of the extract. With the new HPLC analysis method on a C_{30} column with 5 mmol/L heptafluorobutyric acid and 2% acetonitrile in 10 mmol/L ammonium acetate (pH 3.8) as a volatile mobile phase, the contaminants eluted around 28–38 min were successfully separated from GTXs (Fig. 1C and Fig. 1D).

SSI-MS

Recently, SSI has been developed as the ionization method using neither heating nor high voltage[7]. Because it is a very useful tool for the PSP ionization procedure in MS, SSI[8] was adopted for analysis of GTXs instead of FAB-MS[9,10] or ESI-MS[11,12]. The GTX components eluted from the C_{30} column were identified from the results of ion monitoring (Fig. 2) and from mass spectra (Fig. 3). The GTXs readily formed a desulfonated fragment ion $[M+H-SO_3]^+$ on measurement of their LC/SSI-MS. The mass numbers of the desulfonated and protonated ions of GTX5, GTX2 and GTX3, and GTX1 and GTX4 were calculated as *m/z* 300, 316 and 332, respectively. Figure 2 shows the mass chromatograms of GTXs with these mass numbers. Retention times of GTX5, GTX3, GTX4, GTX2 and GTX1 were 11.55, 13.03, 16.37, 18.30 and 19.72 min, respectively.

Both GTX3 (11-β-hydroxysaxitoxin sulfonate) and GTX4 (11-β-hydroxyneosaxitoxin sulfonate) were detected as protonated molecular ions $[M+H]^+$ at *m/z* 396 and 412, respectively. It was found that the β form of GTXs (GTX3 and GTX4) is apt to generate fragment ions derived from dehydration $[M+H-H_2O]^+$, desulfonation $[M+H-SO_3]^+$ and dehydrate-desulfonation $[M+H-SO_3-H_2O]^+$. On the other hand, GTX2 (11-α-hydroxysaxitoxin sulfonate) and GTX1 (11-α-hydroxyneosaxitoxin sulfonate) gave desulfonate ions ($[M+H-SO_3]^+$) at *m/z* 316 and 332, respectively. The protonated molecular ion from the α form of GTXs (GTX1 and GTX2) was hard to detect even with the softest ionization method. The same tendency was also observed in PX1-2 (C1-2)*4. In the case of GTX5, the protonated ion ($[M+H]^+$) at *m/z* 380 showed a lower peak than the desulfonate ion ($[M+H-SO_3]^+$) at *m/z* 300. GTK5,

*4 Yoshioka, S., Analysis of paralytic shellfish poison by sonic spray ionization. Hitachi technical data, MS No. 78-1 and No. 78-2, 1999.

GTX3 and GTX4 gave rise to small sodium adduct peaks, $[M+Na]^+$.

The mass spectra of three unknown peaks between 28 and 38 min in Fig. 1D were measured by the same method as those of GTXs, and showed peak at *m/z* 198, 166 and 180, respectively, due to the protonated molecular ions. Since these ions did not coincide with those of GTX fragments such as dehydrated $[M+H-H_2O]^+$ and desulfonated $[M+H-SO_3]^+$ compounds, they were concluded to be due to contaminants in short-necked clams.

Detection limits of GTX1–5 in our HPLC method on the C_{30} column were 162 fmol/10 μL for GTX1, 26 fmol/10 μL for GTX2, 15 fmol/10 μL for GTX3, 26 fmol/10 μL for GTX4 and 53 fmol/10 μL for GTX5. These values are almost the same as those obtained with the routine HPLC on the C_8 column[3].

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