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Fluorimetric determination of diarrhetic shellfish toxins in scallops and mussels by high-performance liquid chromatography

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Abstract

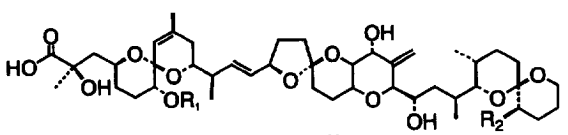
The fluorimetric determination of okadaic acid (OA) and dinophysistoxin-1 (DTX-1), the principal toxins of diarrhetic poisoning, is reported. The digestive glands of mussels or scallops were homogenized with 2-propanol. OA and DTX-1 were extracted from the homogenate, with hexane–ethyl acetate and labelled with 2,3-(anthracenedicarboximido)ethyl trifluoromethanesulfonate in dry acetonitrile. After cleaning up by passage through a short silica gel column, the fluorescent derivatives were determined by HPLC. The derivatives were at first separated on a Develosil Ph-5 column, and only the target fraction obtained was introduced into a Develosil ODS K-5 column by a valve-switching device. Both toxins were determined in the range 2.5–500 pg, and the detection limits were 0.8 pg (OA) and 1.3 pg (DTX-1) with a signal-to-noise ratio of 3.

Keywords: Diarrhetic shellfish poisoning; Okadaic acid; Dinophysistoxin-1; Toxins

1. Introduction

Diarrhetic shellfish poisoning (DSP) is a worldwide problem of public health and in the shellfish industry [1–4]. Up to the present, about ten fat-soluble polyethers are known to be DSP toxins [5–8]. Among these, okadaic acid (OA) and dinotphysistoxin-1 (35-methylokadaic acid) (DTX-1) are the principal toxins, with free carboxylic acid groups (Fig. 1).

A mouse bioassay method has been widely used for the assay of the toxins [9]. Recently, some HPLC methods have been proposed for determining these toxins individually [10–13]. These methods involved a highly sensitive and selective detection system as fluorimetry [10–12] with 9-anthroyldiazomethane and ionspray mass spectrometry [13], and made it possible to determine these toxins at nanogram levels. An immunoassay method has also been reported for



Okadaic acid : R1=R2=H
DTX-1 : R_1=H, R_2=-CH_3

Fig. 1. Structures of OA and DTX-1.

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the determination of total of OA and DTX-1 [14]. The detection limit of the method was 5 ng/ml.

We have developed 2,3-(anthracenedicarboximido)ethyl trifluoromethanesulfonate (AE-OTf) as a highly sensitive fluorescent labelling reagent for carboxylic acids [15]. Here, we report the determination of OA and DTX-1 after derivatization with AE-OTf followed by analysis with an LC–LC system.

2. Experimental

2.1. Materials

Standard OA and DTX-1 were prepared by the method of Lee et al. [10]. *n*-Hexane, dichloromethane, acetone, ethyl acetate and 2-propanol were used as received. Acetonitrile, which was distilled under P_2O_5 and stored over molecular sieves 4A, was used as the solvent for both AE-OTf and tetraethylammonium carbonate (TEAC) solutions. AE-OTf and TEAC were prepared by a previously reported method [15]. AE-OTf solution was stored below 0°C and used within 1 h after preparation. Methanol and water were of HPLC analysis grade. The solvents were purchased from Wako (Osaka, Japan) or Kanto Chemical (Tokyo, Japan). LiChrolute Si 60 (Merck, Darmstadt, Germany) was used in a silica gel column to clean up OA and DTX-1 derivatives before HPLC analysis.

Scallops were purchased from a food market in Sendai (Japan) and mussels were frozen product from New Zealand.

2.2. Extraction of OA and DTX-1 from digestive glands

The extraction procedure used was a slightly modified method of Pereira et al. [12]. One gram of the digestive gland of a scallop or a mussel was homogenized with 5 ml of 2-propanol. The homogenate was centrifuged at 6000 rpm for 5 min below 5°C and the precipitate was homogenized again with 5 ml of 2-propanol. The two supernatants were combined and an aliquot (0.5 ml) was transferred into a test-tube fitted with a screw-cap. To the test-tube, 0.75 ml of *n*-hexane, 1.0 ml of ethyl acetate and 1.5 ml of 0.32 *M* Na_2SO_4 solution were added and vortex mixed for 1 min, then the mixture was centrifuged at 2000 rpm for 5 min below 5°C. After the upper phase had been removed, 0.5 ml of ethyl acetate was added for extraction. After centrifugation, the two upper phases were combined and diluted to 5 ml with methanol.

2.3. Derivatization and clean-up procedure

An aliquot (0.5 ml) of the extract was placed in a test-tube and 75 μl of TEAC solution (1.5 m*M*) were added. The solvent was removed under reduced pressure followed by complete drying under a stream of nitrogen. To the residue, 100 μl of AE-OTf solution (2.25 m*M*) were added and vortex mixed for 30 s, and the mixture was reacted for more than 10 min at room temperature. After the reaction, the solvent was removed under reduced pressure and the residue was dissolved in 200 μl of dichloromethane. The solution was loaded on to a short column packed with LiChrolute Si 60 (100–120 mg), which was washed with 2 ml of dichloromethane before loading. After loading, the column was washed with 200 μl of dichloromethane followed by 4 ml of dichloromethane–acetone (975:25, v/v). The OA and DTX-1 derivatives were eluted with 2 ml of dichloromethane–acetone–methanol (95:5:10, v/v/v). After removal of the solvent under reduced pressure, the residue was dissolved in 200 μl of acetonitrile and an aliquot (2 μl) was injected into the HPLC system.

2.4. Separation of AE derivatives of OA and DTX-1

The system consisted of two PU-980 pumps (JASCO, Tokyo, Japan), a Rheodyne Model 7125 sample injector, an HV-992-01 valve-switching device and an FP-920 spectrofluorimeter (JASCO).

First, a sample was injected on to a Develosil Ph-5 (5 μm) column (50 mm × 4.6 mm I.D.) (Nomura Chemical, Aichi, Japan) and eluted with methanol–water (80:20, v/v) at 0.8 ml/min.

The eluate between 2 min 45 s and 4 min 5 s was introduced into a Develosil ODS K-5 (5 μm) column (150 mm × 4.6 mm I.D.) (Nomura Chemical), which was eluted with methanol–water (80:20, v/v) at 0.8 ml/min. During the separation, both columns were kept at 60°C in a water-bath. The fluorescence intensities of the OA and DTX-1 derivatives were monitored at 462 nm (excitation at 298 nm) and their peak areas were calculated with a Chromatocorder 12 (System Instrument, Tokyo, Japan).

2.5. Recovery test from scallop and mussel homogenate

Volumes of standard OA and DTX-1 solutions were placed in a test-tube so as to be equivalent to 50 or 500 ng of each toxin. After the solvent had been removed under reduced pressure, 0.5 ml of 2-propanol homogenate from a scallop or a mussel was added, followed by vortex mixing for more than 30 s. OA and DTX-1 were extracted from the sample with *n*-hexane–ethyl acetate followed by labelling with AE-OTf, clean-up and HPLC analysis as described above. The recoveries were estimated using the standard OA and DTX-1 derivatives, which were cleaned up with a silica gel column as described above.

3. Results and discussion

3.1. Extraction, derivatization and clean-up

The extraction procedure reported by Pereira et al. [12] was modified in its scale. With this procedure, both OA and DTX-1 were quantitatively extracted. The toxins were derivatized with AE-OTf in the presence of TEAC as a base. When OA and DTX-1 in a scallop or a mussel extract were labelled with AE-OTf, both toxins gave almost the same peak areas with the use of more than 2 m*M* AE-OTf, and the reactions were completed within 10 min in each case. OA and DTX-1 were added to the homogenate of a scallop or a mussel to give 100 ng/ml of each toxin. In the proposed method, both toxins in an extract were labelled with 2.25 m*M* AE-OTf for more than 10 min at room temperature. These conditions made it possible to label at least up to 10 μg/g of toxins in a scallop and a mussel.

Although the AE-OTf solution in dry acetonitrile gave almost constant peak areas even 6 h after preparation when it was kept below 0°C, it was used within 1 h.

After the reaction, the mixture was cleaned up by passage through a short silica gel column to remove interfering substances, such as fatty acid derivatives and decomposed products of the reagent. Table 1 shows the recoveries of both toxins in the eluting solvent [2 ml of dichloromethane–acetone–methanol (95:5:10, v/v/v)] after rinsing the column with 4 ml of dichloromethane–acetone. There were almost no significant differences in the removal of interfering substances when more than 100 mg of silica gel was packed in the column. As neither OA nor DTX-1 derivatives were eluted by dichloromethane, it was used as the solvent to wash the column and to load a sample. When the column was rinsed with 4 ml of dichloromethane–acetone (950:50 or 966:34, v/v), small amounts of both toxins were

Table 1
Effect of washing solvents on the recoveries of OA and DTX-1 derivatives from a short silica gel column

Washing solvent dichloromethane–acetone	Recovery (%)	
	OA derivative	DTX-1 derivative
950:50	83.8	69.6
966:34	87.1	84.2
975:25	88.0	89.1

Sample (200 μl) was injected on to the column as a dichloromethane solution and rinsed with 200 μl of dichloromethane followed by rinsing with 4 ml of washing solvent. The acid derivatives were eluted with 2 ml of dichloromethane–acetone–methanol (95:5:10).

eluted in the eluate, and their recoveries were lower. On the other hand, by rinsing with 4 ml of dichloromethane–acetone (975:25, v/v), neither toxin was eluted in the eluate, and almost 90% of the toxins were recovered in the eluting solvent.

3.2. HPLC separation

Although most interfering substances were removed by passage through a silica gel column, it was impossible to remove interferents completely because of the trace levels of OA and DTX-1 and the high sensitivity of the method. To remove these interfering substances, we employed a two-column system equipped with a valve-switching device. First, a sample was loaded on to a Develosil Ph-5 column, from which OA and DTX-1 derivatives were eluted very close together, and most peaks were eluted within 20 min (Fig. 2A, B and C). The target fraction 1 (fr. 1) which contained both toxin derivatives were cut out and loaded by valve switching on to a Develosil ODS K-5 column, which gave different separation patterns to those on the first column. Fig. 3 shows the effects of the timing of the valve switching on the peak areas of both toxin derivatives. On cutting out a wider range than 2 min 45 s and 4 min 5 s, their peak areas were almost constant.

Fig. 2 also shows typical chromatograms of a standard mixture of OA and DTX-1 (D) and toxin-free mussel extract (E) separated by the LC–LC system. Part (a) in Fig. 2E is magnified eightfold in sensitivity in Fig. 2F.

The interfering substances in the sample were effectively removed by the proposed system. As we used a two-column system which gave different separation patterns, this method made it possible to assign the toxins from their retention times with higher accuracy than using a single-column system.

3.3. Quantitativeness, sensitivities, reproducibilities and recoveries of OA and DTX-1

Table 2 gives the data for standard OA and DTX-1. With the proposed method, the calibration graphs for both toxins show good linearity at least between 2.5 and 500 pg on-column, and their detection limits were 0.8 pg (OA) and 1.3 pg (DTX-1) at a signal-to-noise ratio of 3. The determination ranges of the toxins in scallop and mussel were 0.05–10 μg/g and their detection limits were 20–30 ng/g. The sensitivities

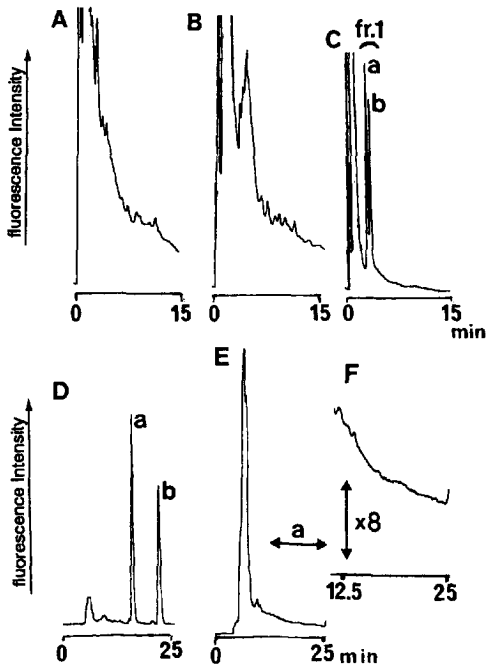

Fig. 2. Typical chromatograms obtained using a Develosil Ph-5 column (A, B and C) and an LC–LC system (D, E and F). Samples were the extracts of a scallop (A) and a mussel (B, E and F) and a standard mixture of OA and DTX-1 (C and D). Peaks a and b are derivatives of OA and DTX-1, respectively. Fr. 1 is a fraction that contained OA and DTX-1. Part (a) in E is magnified eightfold in sensitivity in F.

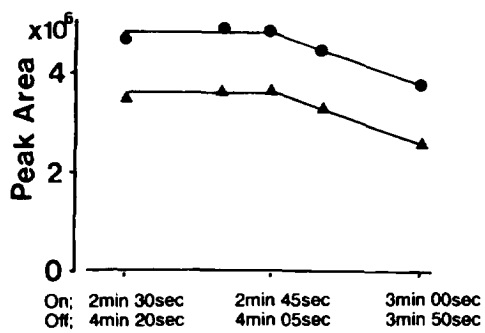

Fig. 3. Effect of valve switching timing on peak areas of (●) OA and (▲) DTX-1.

Table 2
Quantitativeness, sensitivities and reproducibilities for OA and DTX-1 derivatives

Parameter	OA derivative	DTX-1 derivative
r[a]	0.9997	0.9991
(range)	(2.5–500 pg)	(2.5–500 pg)
Detection limit (pg) ($S/N = 3$)	0.8	1.3
R.S.D. (%)[b] ($n = 5$)	5.0 (50 pg)	4.0 (50 pg)

[a] r = Correlation coefficient.
[b] R.S.D. = relative standard deviation of peak area

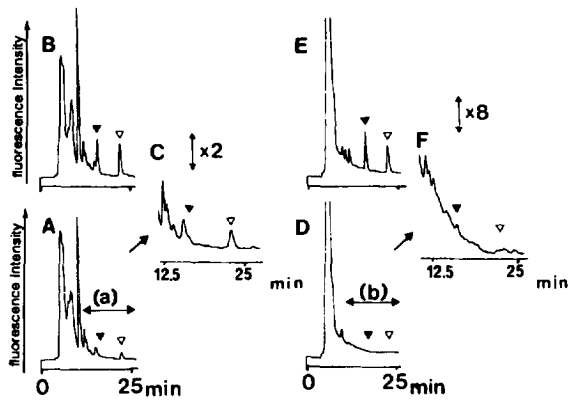

Fig. 4. Typical chromatograms for OA and DTX-1 in a scallop (A, B and C) and a mussel (D, E and F). A and D are chromatograms of extracts from a scallop and a mussel, respectively; B and E are chromatograms of extracts from a scallop and a mussel to which a mixture of OA and DTX-1 was added (10 ng/ml of each toxin), respectively; C and F are chromatograms with parts (a) and (b) magnified by two- and eightfold in sensitivity, respectively.

were more than ten times higher than those reported for HPLC methods [10–13].

Table 3 gives the recoveries of OA and DTX-1 which were added to a 2-propanol homogenate of a scallop or a mussel. The relative standard deviations for OA and DTX-1 were about 1% (10 μg/g) and 4% (1 μg/g), respectively. Using the proposed method, OA and DTX-1 were almost quantitatively recovered from a homogenate of a scallop and a mussel and labelled with AE-OTf.

3.4. Application of the method

The samples (five scallops and five mussels) were analysed by the proposed method. Fig. 4 shows typical chromatograms of a scallop (A) and a mussel (D). Parts (a) and (b) are magnified two- and eightfold in sensitivity in Fig. 4C and F, respectively. Fig. 4B and E are chromatograms for the 2-propanol homogenate of a scallop and a mussel to which both OA and DTX-1 were added to give 10 ng/ml of each toxin. As shown in Fig. 4A and C, a peak at 23.3 min had the same retention time as DTX-1, whereas no peak was detected at 16.3 min where OA should be eluted. On the other hand, a small peak at 16.3 min was detected in a mussel (Fig. 4D and E). The data are given in Table 3. Data for other samples are given in Table 4. Although no or trace peaks of OA were detected in all the samples analysed, some samples gave an appar-

Table 3
Recoveries of OA and DTX-1 from a homogenate of a scallop or a mussel

Homogenatecompound	Added (μg/g)				
	0	1.0		10	
	Found (μg/g)	Found (μg/g)	Recovery (%)	Found (μg/g)	Recovery (%)
ScallopOA	n.d.[a] ($n = 5$)	0.91 ± 0.06 ($n = 5$)	91	10.36 ± 0.12 ($n = 5$)	103.6
DTX-1	0.37 ± 0.03 ($n = 5$)	1.53 ± 0.07 ($n = 5$)	116	11.32 ± 0.13 ($n = 5$)	109.5
MusselOA	<0.05[b] ($n = 4$)	1.03 ± 0.04 ($n = 4$)	103	10.34 ± 0.10 ($n = 4$)	103.4
DTX-1	n.d. ($n = 4$)	1.15 ± 0.05 ($n = 4$)	115	10.91 ± 0.14 ($n = 4$)	109.1

[a] n.d. = Not detected.
[b] <0.05 = peak was detected.

Table 4
OA and DTX-1 determined in scallops and mussels

Sample	OA (μg/g)	DTX-1 (μg/g)
Scallop A	<0.05[a]	0.10
B	<0.05	0.07
C	n.d.[b]	<0.05
D	<0.05	0.06
Mussel E	n.d.	n.d.
F	n.d.	0.07
G	n.d.	<0.05
H	n.d.	n.d.

[a] n.d. = Not detected.
[b] <0.05 = peak was detected.

ent peak of DTX-1. The levels of OA and DTX-1 detected, however, were too low for other HPLC methods to detect them and below the limit defined by the law.

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