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High-performance liquid chromatographic resolution of dinophysistoxin-1 and free fatty acids as 9-anthrylmethyl esters

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Abstract

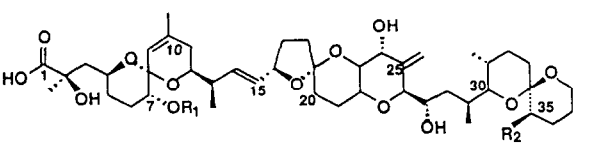
Resolution of a mixture of dinophysistoxin-1 (DTX1) and free fatty acids (FFAs) including eleven components which are the dominant FFAs in mussels and scallops was carried out by high-performance liquid chromatography (HPLC) with fluorimetric detection after their derivatization with 9-anthryldiazomethane (ADAM). Clear separation was obtained for a mixture of DTX1 and FFAs. As an application of the HPLC resolution of both DTX1 and FFAs, hydrolysis products of dinophysistoxin-3 (DTX3), a mixture of 7-O-acyl esters of DTX1, were separated as a replacement of direct HPLC analysis of DTX3. Determination of FFAs in scallops by HPLC–fluorometry is also described.

1. Introduction

Diarrhetic shellfish poisoning (DSP) was first described in Japan by Yasumoto et al. [1] as a new type of a seafood disease resulting from ingestion of shellfish infested with dinoflagellate toxins. In recent years, reports concerning DSP have been markedly increasing both in number and in geographical distribution with the general recognition of a new type of marine toxin [2].

Among DSP toxins, all being lipophilic compounds, the most important responsible for diarrheal symptoms are okadaic acid (OA) and its derivatives, dinophysistoxin-1 (35-methylokadaic acid; DTX1) and dinophysistoxin-3 (7-O-acyl-35-methylokadaic acid; DTX3) [3–5] (Fig. 1). The relative ratio of the individual toxins in shellfish shows significant annual and regional variations. In contrast to the predominant presence of OA in European mussels, with a few exceptions, the major toxin of Japanese mussels is DTX1, and scallops cultured in northern Japan occasionally contain DTX3 as a principal toxin [6–8].

Routine monitoring of shellfish for DSP toxins is generally done using rat or mouse bioassay [9], but instrumental methods are required for confirmation. The most common method for OA and DTX1 is high-performance liquid chromatography (HPLC) with precolumn derivatization with 9-anthryldiazomethane (ADAM) or 4-



okadaic acid (OA) : $R_1 = H$ $R_2 = H$

dinophysistoxin-1 (DTX1) : $R_1 = H$ $R_2 = CH_3$

dinophysistoxin-3 (DTX3) : R_1 = acyl $R_2 = CH_3$

Fig. 1. Structures of DSP toxins.

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bromomethyl-7-methoxycoumarin (Br-Mmc) [10,11]. On the other hand, confirmation of DTX3 by instrumental means has been carried out by detection of DTX1 obtained from DTX3 via alkaline hydrolysis, as DTX3 can be separated from other DSP toxins (OA and DTX1) by partitioning between 80% methanol (OA and DTX1) and *n*-hexane (DTX3) or isolated by column chromatography [12]. However, the biological activities of the toxins associated with DTX3 increase with increasing degree of unsaturation of the acyl chain [5], and therefore the true toxicity of a particular sample would depend on the distribution of acyl derivatives in the DTX3 mixture. Hence HPLC separation of free fatty acids (FFAs) derived from DTX3 coupled with the determination of DTX1 (from DTX3) is necessary to obtain more detailed information in analysis of DTX3.

In this study, resolution of a mixture of DTX1 and FFA standards was carried out by gradient HPLC after derivatization with ADAM to determine DTX3. As an application of the proposed HPLC separation, HPLC analyses of hydrolysis products obtained from DTX3 standard and FFAs which are regarded as interferences in mouse bioassays [13] are described.

2. Experimental

2.1. 9-Anthryldiazomethane

9-Anthryldiazomethane (ADAM) was obtained from Funakoshi Pharmacy (Tokyo, Japan).

2.2. FFA standards

Myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and arachidic acid (C20:0) were obtained from Wako (Osaka, Japan). Palmitoleic acid (C16:1), *cis*-6,9,12,15-octadecatetraenoic acid (C18:4) and arachidonic acid (C20:4) were purchased from Sigma (St. Louis, MO, USA). *cis*-5,8,11,14,17-Eicosapentaenoic acid (C20:5) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (C22:6) were supplied by Dr. Y. Itabashi of Hokkaido University (Hakodate, Japan).

2.3. DSP toxin standards

Authentic DTX1 and DTX3 obtained from midgut glands of scallops (*Patinopecten yessoensis*) collected at Mutsu Bay, Japan, were provided by Professor T. Yasumoto of Tohoku University (Sendai, Japan).

2.4. Sample material

Non-toxic scallops *Patinopecten yessoensis* were collected at Mutsu Bay, Japan, in September 1993. The specimens were kept frozen at −70°C until used.

2.5. Hydrolysis of DTX3 standard

DTX3 (1 μg) was hydrolysed by heating in 100 μl of 0.2 *M* NaOH–MeOH (10:90) at 75°C for 1 h in a 1-ml coloured vial. After removing the methanol from the reaction mixture under nitrogen, the aqueous layer was acidified with 200–300 μl of 0.1 *M* HCl and then extracted with diethyl ether (400 μl × 3). The combined ether layers which contain the DTX1 and FFAs were washed with 200 μl of water, then reacted with ADAM according to the previous method [10] after evaporating the solvent.

2.6. Derivatization and purification

Derivatization by labelling the carboxylic group of DTX1, FFAs and hydrolysis products of DTX3 with ADAM were performed essentially according to the method of Lee et al. [10].

DTX1 (1 μg), standard FFA mixture including 100 ng of the respective components and hydrolysis products from 1 μg of DTX3 were reacted with 100 μl of 0.1% ADAM–MeOH for 1 h in the dark at ambient temperature. A 200-μl volume of 0.1% ADAM–MeOH was used for derivatization of FFAs from scallops. For purification of the 9-anthrylmethyl (9-AM) ester of DTX1, the ADAM reaction mixture was placed on an ordinary Sep-Pak silica cartridge column

(Waters, Milford, MA, USA), then washed with 5 ml of *n*-hexane–dichloromethane (1:1, v/v) and subsequently eluted with 8 ml of pure dichloromethane. Finally, fractions of 9AM-DTX1 were obtained with 5 ml of dichloromethane–methanol (9:1, v/v) [10]. Purification of the 9AM esters of FFAs and the hydrolysis products of DTX3 was not carried out.

2.7. HPLC

HPLC separation was carried out with a Hitachi (Tokyo, Japan) L-6200/L-6000 gradient system equipped with a Develosil ODS-5 column (250 mm × 4.6 mm I.D.) (Nomura Chemical, Seto, Japan) with a stepwise gradient of solvent A (acetonitrile–methanol–water, 8:1:1, v/v/v) and solvent B (methanol) at ambient temperature and a flow-rate of 1.1 ml/min. The following linear gradient was used: segment 1, 100% A for 20 min, i.e., as reported for the resolution of OA and DTX1 by Lee et al. [10]; segment 2, initial conditions changed to 100% B over 30 min; and segment 3, 100% B for 50 min. The peaks of fluorescent derivatives were monitored with a Hitachi F-1050 spectrofluorimeter. The excitation and emission wavelengths were set at 365 and 412 nm, respectively. The FFA content in the midgut glands of scallop extracts was determined by comparing the peak areas of each of FFA with those of the standard.

3. Results and discussion

Fig. 2 shows the HPLC separation of a mixture of DTX1 and eleven FFAs, i.e., the available major FFAs of mussels and scallops [14], as their ADAM derivatives on the Develosil ODS-5 column. Injection was carried out by co-injection of DTX1 and a mixture of FFAs. 9AM-DTX1 and -FFAs were separated into eleven peaks within 80 min with a separation factor (α) from 1.01 to 2.06 (Table 1). 9AM esters of FFA were eluted in order of increasing carbon number and decreasing number of double bonds after elution of 9AM-DTX1. Linearity of peak area with increasing concentration of FFAs or DTX1 was

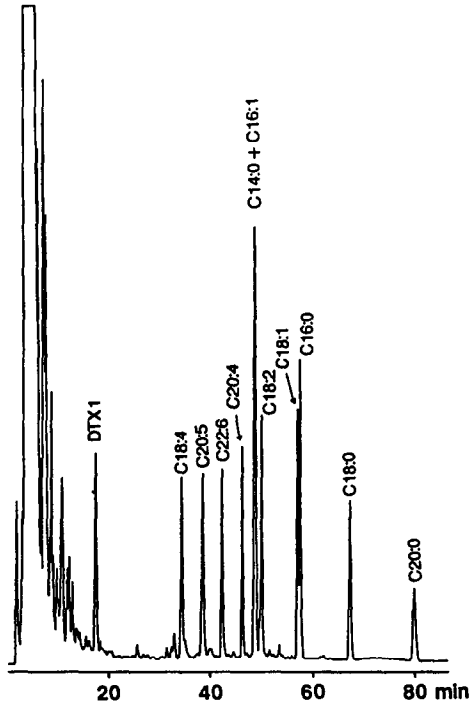

Fig. 2. HPLC separation of the 9AM esters of DTX1 and FFAs on the Develosil ODS-5 column. Amounts of 50 ng of DTX1 and 10 ng of each FFA were co-injected. For derivatization procedure and chromatographic conditions, see text.

confirmed over a wide range of concentrations, showing that the reaction of ADAM with FFAs or DTX1 was quantitative and reproducible.

Chromatographic data for the separation of a mixture of DTX1 and FFAs are given in Table 1. Because the retention times for the individual peaks were very sensitive to slight differences in the temperature and constitution of mobile phase, the relative retention times (RRTs) of respective peaks with respect to C18:0 were preferred for peak identification.

The FFA profile and DTX1 arising after hydrolysis of 1 μg of DTX3 standard is shown in Fig. 3. This demonstrates that the hydrolysis of DTX3 is connected with the release of FFAs and DTX1. The amount of DTX1 contained in the reaction mixture was 1020 ng, which was calculated from the HPLC data. This is slightly high compared with the theoretical amount (about 800 ng) estimated in the hydrolysis products resulting from 1 μg of DTX3 standard.

Table 1
Chromatographic data obtained on the separation of 9-anthrylmethyl esters of dinophysistoxin-1 and free fatty acids on a Develosil ODS-5 column

DTX1[a] and FFAs[b]	t_R[c]	k'[d]	α[e]
DTX1	0.26	13.0	2.06
C18:4	0.52	26.8	1.12
C20:5	0.58	30.1	1.10
C22:6	0.64	33.1	1.09
C20:4	0.69	36.2	1.05
C14:0 + C16:1	0.73	38.2	1.03
C18:2	0.75	39.1	1.14
C18:1	0.85	44.6	1.01
C16:0	0.86	45.1	1.17
C18:0	1.00	52.7	1.19
C20:0	1.19	62.9	

[a] DTX1 = dinophysistoxin-1.
[b] FFAs: free fatty acids are expressed as the number of carbon atoms: number of double bonds.
[c] Relative retention time with respect to C18:0.
[d] Capacity factor.
[e] Separation factor (the ratio of capacity factors).

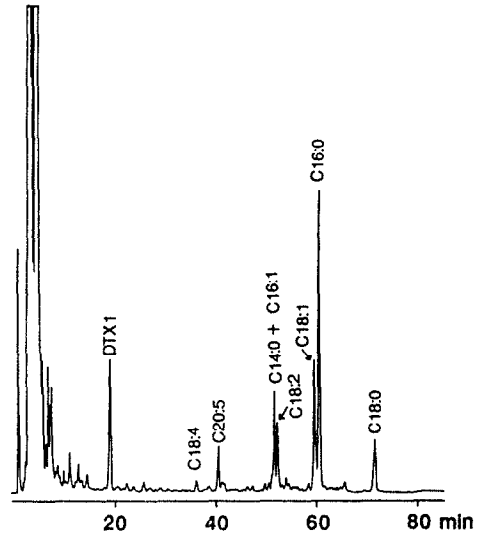

Fig. 3. HPLC separation of the 9AM esters of DTX1 and FFAs resulting from hydrolysis of DTX3 standard on the Develosil ODS-5 column. An aliquot (one tenth) of the hydrolysis products obtained from 1 μg of DTX3 standard was injected. The peak of DTX1 corresponds to 102 ng. For derivatization procedure and chromatographic conditions, see text.

The proportion of FFAs obtained from the DTX3 standard is given in Table 2. The most prominent FFA was C16:0. These results show directly the percentages of particular DTX3 homologues differing in acyl groups, and therefore the amount of each DTX3 homologue is calculable from the amount of DTX1 (from DTX3) and the percentage of each FFA obtained from DTX3. Although 1 μg of DTX3 standard was subjected to hydrolysis reaction, the total amount of DTX3 homologues calculated from the HPLC data was high (Table 2). This discrepancy may arise from the errors in subdivision of standard toxins.

Takagi et al. [13] pointed out a significant disadvantage of the official mouse bioassay test, that is, the false-positive results caused by the presence of large amounts of FFAs in samples not contaminated by DSP toxins: 3–14 mg of FFAs per gram of midgut glands showed positive results in the official mouse bioassay. They also indicated differences in interfering property of

Table 2
Proportion of free fatty acids and amount of particular dinophysistoxin-3 obtained from HPLC of hydrolysis products of 1 μg of dinophysistoxin-3 standard

FFA[a]	t_R[b]	Composition (mol%)	DTX3[c] (ng)
C18:4	0.53	1.6	22
C20:5	0.59	5.5	76
C22:6	–	–	–
C20:4	–	–	–
C14:0 + C16:1	0.74	6.8	88
C18:2	0.75	9.7	131
C18:1	0.85	20.5	277
C16:0	0.86	43.5	573
C18:0	1.00	12.4	168
C20:0	–	–	–
Total		100.0	1335

[a] FFAs: free fatty acids are expressed as the number of carbon atoms: number of double bonds.
[b] Relative retention time with respect to C18:0.
[c] DTX3 containing FFAa (mass) = DTX1 (mass, from DTX3) × mol% FFAa/100 × $[M_r(\text{DTX1}) + M_r(\text{FFAa}) - M_r(H_2O)]/M_r(\text{DTX1})$ where M_r indicates molecular mass.

FFAs for the mouse intraperitoneal (i.p.) test and suggested that polyunsaturated fatty acids (PUFAs), especially C20:4 and C20:5, are active agents as mouse lethal material.

Fig. 4 shows the FFA profile extracted from midgut glands of non-toxic scallops by the procedure described in the official method for mouse bioassay [9]. Whereas C14:0 and C16:1 were not separated, again a fair separation of the other principal FFAs was achieved. Table 3 gives the concentrations of the individual FFAs in the midgut glands of the scallops determined as 9AM esters by fluorescence detection after HPLC separation. The total amount of FFAs of 270 μg/g midgut glands found in the samples is close to that in mussels collected in the Gulf of Trieste [15]. This basic FFA content is not sufficient to interfere in the mouse bioassay.

It has been proposed that DTX3 may be the result of acylation of DTX1 in the midgut glands of scallops because DTX3 has not been detected in dinoflagellates [16], and similar FFA constituents in DTX3 and midgut glands in scallops have therefore been suggested. The DTX3 standard used in this study was obtained from midgut glands of scallops collected at Mutsu Bay. The FFA composition derived from the DTX3 standard under investigation was simpler than that obtained from midgut glands of scallops collected at the same location (Tables 2 and 3). The similarity of high percentages of C16:0 in FFAs from the DTX3 standard and the midgut gland of scallops is noteworthy. On the other hand, the percentages of PUFAs differed; the absence of C20:4 and C22:6 and the low proportion of C18:4 and C20:5 in the DTX3 standard (Table 2) were notably different from the results obtained for scallops (Table 3). The absence of C20:4 and C22:6 and the low level of C18:4 and C20:5 are due to degradation of the DTX3 esterified by these PUFAs during the extraction and purification of DTX3 standard from scallops owing to its instability [12].

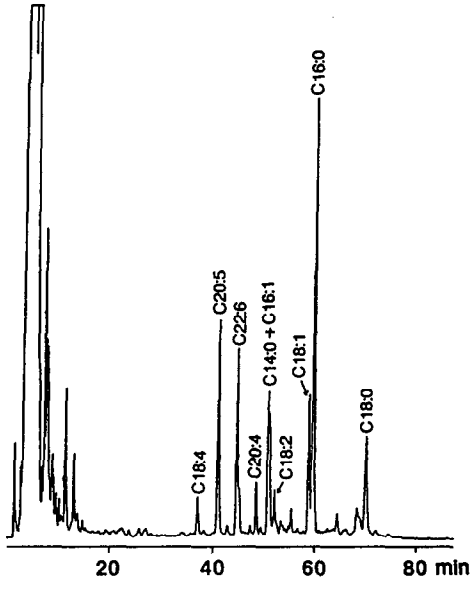

Fig. 4. HPLC separation of the 9AM esters of FFAs from midgut glands of scallops collected at Mutsu Bay, Japan, in September 1993, on the Develosil ODS-5 column. An aliquot (1/8000 of 0.91 g of midgut glands of scallops) of the extract was injected. The peak of C16:0 corresponds to 9.4 ng. For derivatization procedure and chromatographic conditions, see text.

Table 3
Individual free fatty acid contents of 1 g of midgut glands of scallops collected at Mutsu Bay in September 1993

FFA[a]	t_R[b]	Concentration (μg/g MG)[c]	Composition (mol%)
C18:4	0.53	9	3.5
C20:5	0.59	46	15.8
C22:6	0.64	36	11.3
C20:4	0.69	10	3.5
C14:0 + C16:1	0.73	23	9.9
C18:2	0.75	11	3.9
C18:1	0.85	32	11.5
C16:0	0.86	83	33.3
C18:0	1.00	20	7.3
C20:0	–	–	–
Total		270	100.0

[a] FFAs: free fatty acids are expressed as the number of carbon atoms: number of double bonds.
[b] Relative retention time with respect to C18:0.
[c] Midgut gland.

4. Conclusions

This study demonstrates that the clear elution profile for a mixture of DTX1 and FFAs obtained by HPLC with programmed gradient

elution and fluorometric detection is applicable to the analysis of the hydrolysis products of DTX3 standard. The most prominent component of DTX3 standard obtained from midgut glands of scallops collected at Mutsu Bay was that containing C16:0, which was the major FFA in midgut glands of scallops collected in the same location.

The concentration of FFAs obtained from midgut glands of scallops was also determined by HPLC–fluorometry. The FFA content found in scallops collected at Mutsu Bay in September 1993 was far below that reported to cause death in mice. The HPLC separation of FFAs described here will allow a more accurate interpretation of DSP because the determination of FFAs is very important in scallops from late spring to early summer, as they contain FFAs at levels high enough to give false-positive results. Although the peaks of C14:0 and C16:1 overlapped, they can be separated by subjecting of these overlapping peaks collected in the first HPLC run to a second HPLC run using a different column, if detailed percentages of C14:0 and C16:1 are required.

A problem with this HPLC method is interferences that prevent the sensitive and unambiguous analysis of a sample and instability of the 9AM ester [10,11,17]. We found that the 9AM esters of DTX1 and FFAs were stable for at least 1 month when stored at −40°C in methanol, showing no change in fluorescence intensity. Although clear detection was obtained for an aliquot (one tenth) of individual 9AM esters derived from hydrolysis products of 1 μg of DTX3 standard, clean-up of ADAM derivatives was required when the amount of DTX3 standard subjected to the hydrolysis reaction was below 200 ng. Clean-up of 9AM-DTX1 and -FFAs was investigated by using a Sep-Pak silica cartridge column to avoid interferences from ADAM reagent in peak detection, but satisfactory results were not obtained owing to the difference in the adsorption properties of DTX1 and FFAs on Sep-Pak silica. We are now examining the clean-up of 9AM-DTX1 and -FFAs and modification of the HPLC equipment [11] to achieve the detection of 9AM esters.

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