Journal of Chromatography, 462 (1989) 419–425
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 056

Note

Confirmation of domoic acid in molluscan shellfish by chemical derivatization and reversed-phase liquid chromatography

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(Received October 4th, 1988)

Domoic acid (Fig. 1), a neurotoxic amino acid originally isolated from the red alga, *Chondria armata*[1,2] was found to be the toxic substance in contaminated mussels from Prince Edward Island in eastern Canada[3].

Analytical methodology employed for its determination at low μg/g levels has involved either hot acid or water extraction of the sample followed by dilution, filtration and chromatographic analysis using reversed-phase liquid chromatography (LC)[4,5]. Detection of the substance was by UV absorption at 242 nm, the absorbance maximum of the compound. Under the conditions employed, less than 1 μg/g of domoic acid could be detected in mussels, clams and oysters. However, confirmation of positive results are often necessary particularly if the levels are high enough to be a health concern. To date the only means of confirming domoic acid is either to collect the isolated substance by LC and perform mass spectrometric (MS) analysis or to use a diode array detector to obtain a complete UV spectrum of the eluting peak. The latter does not provide an unequivocal identification since many compounds have absorption maxima near 242 nm. Also, few laboratories are equipped with this type of detector.

An alternative approach is to prepare a chemical derivative and reanalyse the sample with comparison to a known standard carried through the same reaction procedure. The present work evaluates two derivatization techniques for domoic acid and applies them to mussel tissue. One involves the formation of the phenyl isothiocyanate (PITC) derivative of the amino moiety and the other, an esterification of the three carboxylic acid groups.

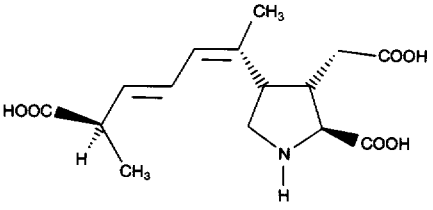

Fig. 1. Structure of domoic acid.

0021-9673/89/$03.50

EXPERIMENTAL

Reagents

Domoic acid was obtained from the National Research Council's Atlantic Research Laboratory (Halifax, Canada). Solutions were prepared in twice deionized water (Milli-Q, Millipore, Bedford, MA, U.S.A.). Phenyl isothiocyanate was obtained from Pierce. Triethylamine was obtained from Aldrich (99+%, Gold Label). Acetonitrile was HPLC-grade. All other solvents and chemicals were analytical reagent grade materials. All standard and sample solutions were refrigerated when not in use. Acetyl chloride (Gold Label) was obtained from Aldrich.

Preparation of derivatization reagent solutions

The PITC reagent was prepared fresh daily as for normal amino acid analysis by mixing 200 μl of methanol, 50 μl triethylamine, 50 μl water and 20 μl PITC.

The esterification reagent was prepared as described earlier[6] by adding 10 ml of isopropanol into a 30-ml headspace vial which was then sealed with a silicone septum. A disposable syringe needle was inserted through the septum and left as a vent. The contents were cooled in an ice bath, then 3 ml acetyl chloride added slowly using a 5-ml glass-bodied syringe, swirling the mixture during addition. The vent, seal and septum were removed and immediately loosely replaced with a new seal and septum. The headspace was briefly flushed with nitrogen and the container quickly sealed. This solution was used for the derivatization.

Liquid chromatography

The direct LC determination of domoic acid was carried out isocratically exactly as described earlier[5]. The LC system consisted of a Model 110B pump (Beckman) a 20-μl loop injector (Beckman), a Supelcosil LC-18 column (15 cm × 4.6 mm I.D., 5 μm), a variable-wavelength UV detector (Micrometrics) set to 242 nm and an integrating recorder (Varian). The mobile phase was acetonitrile–water (12:88, v/v) adjusted to pH 2.5 with 2% (v/v) orthophosphoric acid, degassed and filtered before use. The flow-rate was 1.0 ml/min.

The same system was employed for the PITC derivatives but using a mobile phase of acetonitrile–water (32.5:67.5) at pH 2.5. Reagent peaks were eluted from the column by washing the column using a step gradient of 99.8% acetonitrile from 10 to 16 min, then reequilibrating for 5 min before the next analysis. For the ester derivatives, gradient elution was used with a Supelcosil LC-18 column (as above) with an initial mobile phase of 0.02 *M* phosphate buffer, pH 3, containing 0.02% triethylamine changing linearly to 100% acetonitrile from 0 to 15 min.

Sample extraction

Mussel tissue was extracted by boiling the homogenized sample with an equal weight of 0.1 *M* hydrochloric acid for 5 min. The mixture was quickly cooled and centrifuged. An aliquot of the clear supernatant was diluted with water for direct LC analysis. Details of this procedure are published elsewhere[5].

Clean-up procedure for derivatization

Both derivatization procedures required additional sample clean-up before car-

rying out the reactions. The reason for this was to remove the abundance of coextracted amino acid and other proteinaceous material in the initial sample extracts. These substances interfered in the determinations of the derivatives by producing an intractable tar, consuming reagent and/or yielding interfering peaks in the resulting chromatograms.

The clean-up was carried out by passing 1 ml of undiluted clear supernatant from the extraction above, through a 1-ml phenylsulfonic acid strong cation-exchange solid phase extraction cartridge (Baker). (The cartridge was preconditioned with 6 ml of methanol followed by 6 ml of 0.1 *M* hydrochloric acid.) The sample effluent was discarded and the cartridge rinsed with 3 ml of water which was also discarded. The cartridge was then dried by aspiration. The domoic acid was eluted with two 3-ml volumes of 0.5 *M* hydrochloric acid, the first 3 ml being allowed to remain in the cartridge for 5 min before elution. The last drops of hydrochloric acid were forced from the cartridge by pushing air through it. The 0.5 *M* hydrochloric acid was collected and further cleaned up as follows.

A 1-ml octadecyl (3 ml reservoir) cartridge (Baker) was conditioned with 6 ml of methanol followed by 6 ml of water and finally 6 ml of 0.5 *M* hydrochloric acid ensuring that the cartridge did not run dry after the last wash. The combined acid fraction from the phenylsulfonic acid cartridge was transferred to the octadecyl cartridge and passed through at *ca.* 2 ml/min. The effluent was discarded. The cartridge was then washed with 3 ml of water which was discarded and all remaining water forced from the column with air. The domoic acid was eluted with 2 ml of 20% acetonitrile in water (1% acetic acid) after allowing it to remain in the cartridge for 1 min. After this another 2 ml were passed through the cartridge and collected, forcing out the last few drops with air. These fractions were combined and used for derivatization.

Phenyl isothiocyanate derivatization

A 0.2–1.0 ml aliquot of the cleaned up extract (enough to yield 1–10 μg domoic acid) was evaporated to dryness in a 2-ml culture tube under nitrogen, in a water bath at 50°C. After this, 50 μl of PITC derivatization solution were added and the tube swirled to ensure that the reagent wets the residue. The contents were permitted to react at room temperature for 20 min, swirling the tube after 10 min. The contents were then evaporated to dryness in a water bath at 40°C under nitrogen for about 20 min to ensure that excess reagent was evaporated. The residue was dissolved in 1 ml of water by strirring on a vortex mixer for 1 min. The solution was filtered (0.45 μm) before injection into the LC system.

Esterification

A 1-ml aliquot of the cleaned up extract was evaporated to dryness in a 15-ml graduated centrifuge tube, under nitrogen, at 50°C. A 1.0-ml volume of methylene chloride was added and evaporated to dryness to remove the last traces of water. After this, 0.6 ml of esterification solution was added using a syringe. The tube was stoppered tightly with a plastic cap, vortexed for 1 min to dissolve or suspend the residue and heated for 30 min at 100°C in an oil bath with the liquid level of the bath at that of the tube contents. The tube was then cooled to room temperature and the contents evaporated just to dryness under nitrogen at 35°C (higher temperatures and

unnecessarily long evaporation times led to derivative losses). The residue was dissolved in 1 ml of acetonitrile–water (1:1), filtered (0.45 μm) and analysed by LC.

RESULTS AND DISCUSSION

The PITC derivatization was found to work well for domoic acid. Although structural confirmation by MS was not carried out it is most probable that the PITC reacts with domoic acid in an analogous fashion to other amino acids[7]. However, under the conditions employed, several large reagent peaks were always present in the chromatograms and eluted after the domoic acid derivative.

Fig. 2 compares results obtained for a cleaned up mussel extract, before and after PITC derivatization, employing gradient elution to enable the detection of both free and derivatized domoic acid on the same chromatogram. (Conditions, 10–64% acetonitrile in water, pH 3, from 0 to 12 min; 64–99.8% acetonitrile in water from 12 to 16 min.) It can be seen that the original domoic acid peak (6 min) has disappeared and the derivative peak is observed at *ca.* 10.5 min. Quantitatively, the conversion to the derivative was always greater than 90% under the reaction conditions used. For routine confirmation of domoic acid in mussel tissue, an isocratic system was employed as described in the experimental section. Fig. 3 shows chromatograms obtained under isocratic conditions for a reagent blank, a mussel blank and a mussel tissue spiked with 19 ppm domoic acid, all after derivatization. The derivatized compound can be clearly observed at this level. The confirmation limit was estimated to be 2–5 μg/g domoic acid in mussel tissue depending upon quantity of extract injected. The absolute sensitivity of the detector to the derivative is the same as for domoic acid itself since the chromophoric group responsible for the absorption at 242 nm is unchanged.

During the investigation of the esterification reaction; a series of alcohols were evaluated. Fig. 4 shows a chromatogram of the separation of a mixture of the trialkyl-

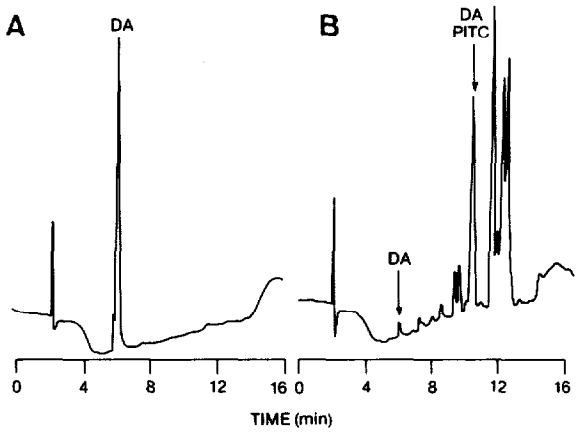

Fig. 2. Chromatograms of domoic acid in contaminated mussel tissue (380 μg/g) before (A) and after (B) PITC derivatization. Gradient conditions as described in the text. Detector, 242 nm, 0.16 absorbance units full scale. Chromatogram A, 126 ng domoic acid injected. Chromatogram B, 95 ng equivalent domoic acid injected.

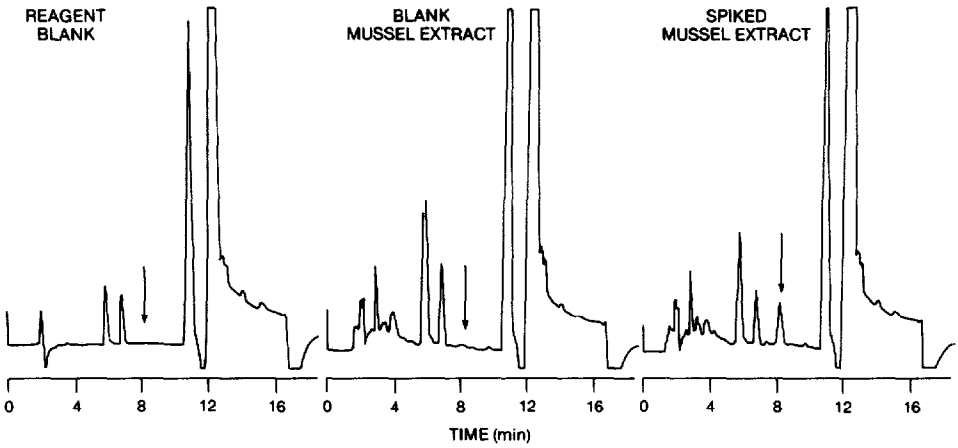

Fig. 3. Isocratic chromatograms of a reagent blank, a control mussel extract and the same extract spiked at 19 μg/g domoic acid, after PITC derivatization. The arrow indicates retention time of domoic acid. Mobile phase as described in the text. Other conditions as in Fig. 2.

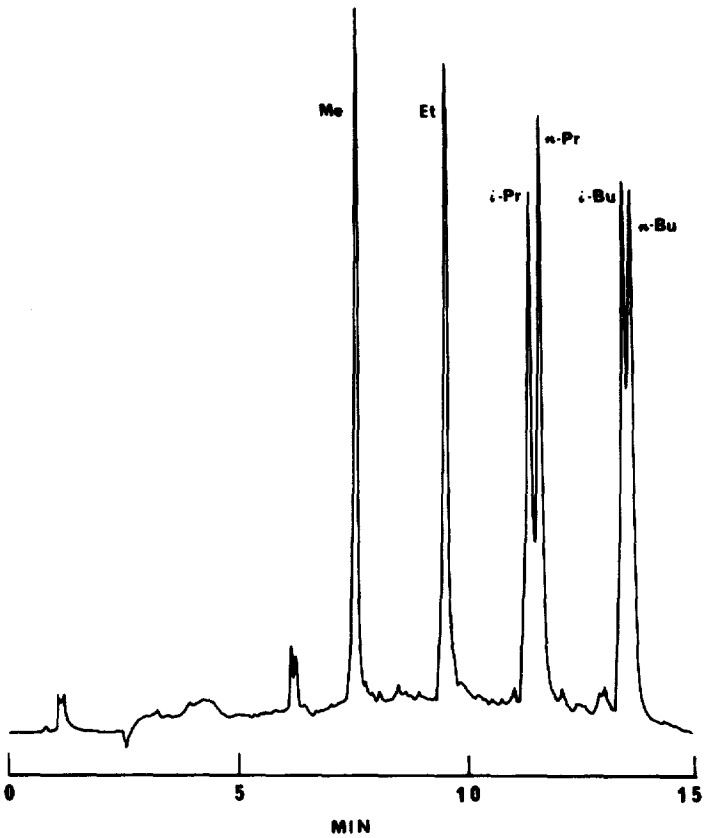

Fig. 4. Chromatogram of a mixture of trialkyl ester derivatives of domoic acid. Methyl (Me), ethyl (Et), isopropyl (i-Pr), *n*-propyl (*n*-Pr), isobutyl (i-Bu) and *n*-butyl (*n*-Bu) derivatives. Conditions as described in the text. Detector, 242 nm, underivatized domoic acid elutes at 4.5 min.

esters obtained using six different alcohols. The trialkyl derivatives were confirmed by gas chromatography–MS after formation of the N-trifluoracetyl derivative[6]. For routine work, the isopropyl triester was employed although the others would also be suitable.

Fig. 5 shows the application of the esterification to the confirmation of domoic acid in mussel tissue at 308 μg/g after the solid phase extraction clean-up. The triisopropyl ester is easily observed at this concentration. A small amount of triethylamine was required in the mobile phase to produce reproducible chromatography. Once the carboxylic acid groups are esterified the molecule becomes basic, thus necessitating the amine to prevent tailing. The detection limit under these conditions was estimated to be in the low μg/g range, similar to underivatized domoic acid. The absolute detector response of the derivative was similar to the parent domoic acid both in terms of molar absorbtivity and UV spectra. All trialkyl derivatives had spectra virtually identical to the undervatized domoic acid. (Determined using an LKB diode array detector under identical chromatography conditions.) This is ex-

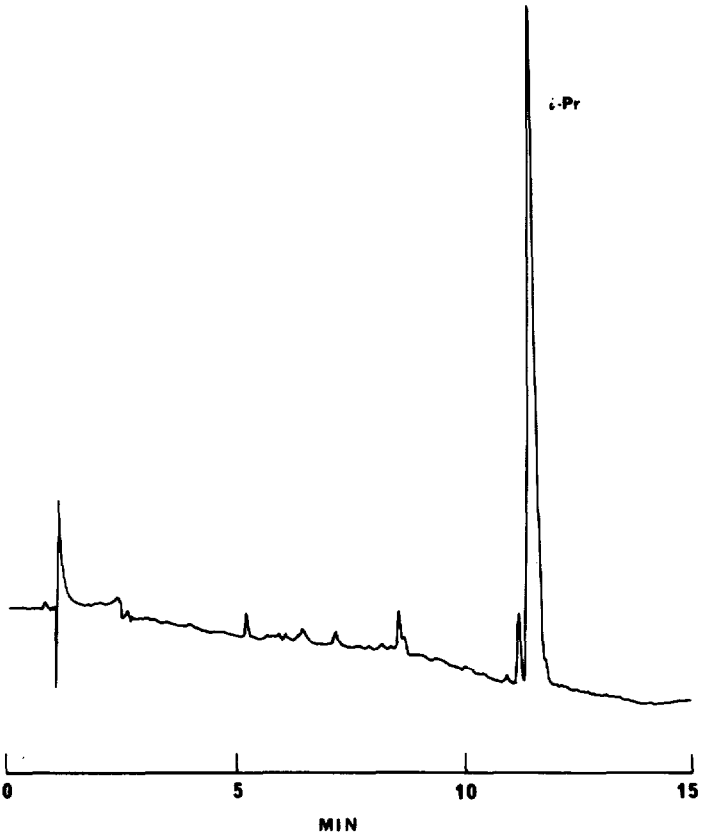

Fig. 5. Chromatogram of an extract of contaminated mussel (308 μg/g) after clean-up and esterification hydrochloric acid–isopropanol. Conditions as described in the text. i-Pr = Isopropyl.

pected since the chromophoric group is not appreciably affected by the esterification. Thus the actual detection limits should be similar to the parent domoic acid.

The above described derivatization procedures offer two independent means (derivatives at the –N or –COOH moiety) of confirming domoic acid residues in mussel extracts. The procedure should be applicable to other molluscan shellfish after solid phase extraction clean-up of the extracts.

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