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Short Communication

Comparison of high-performance liquid chromatography with radioimmunoassay for the determination of domoic acid in biological samples

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ABSTRACT

A reversed-phase liquid chromatographic method employing UV absorption detection at 242 nm was compared to a radioimmunoassay technique for the determination of the marine toxin, domoic acid, in several types of seafood and biological samples. Agreement between the two methods for spiked samples of mussels and rat serum was very good over a range of concentrations of 0.15–7.3 μg/g domoic acid. Also, a very good correlation was observed between the two methods for naturally incurred residues of domoic acid in razor clams, anchovies and crab meat over a concentration range of 0.6–43 μg/g domoic acid.

INTRODUCTION

Domoic acid is a marine toxin (produced by the phytoplankton species, *Nitzschia pungens*), that has been identified in various types of shellfish on the Atlantic and Pacific coasts of the USA and Canada [1]. Methods most commonly employed for determining the compound in contaminated samples involve high-performance liquid chromatography (HPLC) with a variety of sample extraction techniques [2–8]. The procedure most commonly used at present employs methanol–water extraction [6] with or without a cleanup step involving disposable solid-phase extraction (SPE) cartridges filled with strong anion-exchange (SAX) resin.

Only one report has appeared in the literature describing the application of immunoassay techniques for the determination of domoic acid [9]. This work compared enzyme-linked immunosorbent assay (ELISA) with radioimmunoassay (RIA) for the determination of the toxin in urine and serum of experimental animals (monkeys, rats). The methods were found to be very sensitive enabling the detection of domoic acid at low ng/ml levels in the samples. Immunoassay methods are particularly advantageous because they are capable of rapidly screening many samples at a time, at a relatively low cost. This approach can be particularly useful for screening samples for toxic chemicals for regulatory purposes. This prompted us to evaluate the approach for the determination of domoic acid in shellfish and to compare it to HPLC in terms of accuracy and ease of analysis. The comparison to HPLC is important since immunoassay techniques are

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susceptible to giving false positives or false negative results due to the presence of coextractives which may bind to enzymes or to matrix effects which may inhibit binding of the domoic acid. HPLC in this regard can act as an extremely useful confirmation technique for samples found to be positive by immunoassay. The comparison of the two techniques was also applied to rat urine, serum and feces in animal feeding studies for which the RIA method was initially developed. Confirmation by HPLC of the values found by RIA adds important information to the metabolism of domoic acid in animals.

EXPERIMENTAL

Reagents

Standard solutions of domoic acid (DACS-1, National Research Council of Canada, Halifax, Canada) were prepared in doubly deionized water. All solvents and chemicals were HPLC- or analytical-grade materials. All solutions of standards and samples were refrigerated when not in use. [^{3}H]Domoic acid (specific activity 165 GBq/nmol) was obtained from Amersham Labs., UK. Prior to use the [^{3}H]domoic acid was purified by HPLC.

Sample extraction

The extraction procedure was based on the methanol–water extraction method described elsewhere [6,7] with modifications. For shellfish, 10 g homogenized tissue were mixed with 10 ml water in a 50-ml centrifuge tube for 1 min using a vortex mixer. Following this, 20 ml methanol were added and the contents mixed again for 1 min. The mixture was centrifuged and the clear supernatant decanted into a clean tube. A 10-ml volume of methanol was added to the residue and the contents mixed and centrifuged as above. The clear supernatant was combined with the first and the total volume adjusted to 50.0 ml. A 5-ml aliquot of this was used for SPE cleanup and HPLC while a 1.0-ml aliquot was diluted with phosphate-buffered saline (PBS) for RIA.

Serum and urine samples were diluted ten times with methanol–water (50:50, v/v) before SPE cleanup and HPLC. A 2-ml volume of diluted serum and 2.5 ml of diluted urine were used for SPE cleanup. For RIA, 0.1 ml sample was diluted with PBS. Feces samples (2 g) were extracted with methanol–water (50:50, v/v) as described above for the shellfish samples. A 2.0-ml aliquot (0.1 g equivalent feces) of the supernatant was used for SPE cleanup.

Solid-phase extraction cleanup

All HPLC determinations were performed after SPE cleanup either with a SAX resin [6] for shellfish and serum samples or with a strong cation-exchange (SCX) cartridge followed by a C_{18} (reversed-phase) cartridge [8] for urine and feces samples.

For SPE-SAX cleanup an aliquot of sample extract was passed though a 3-ml Supelclean LC-SAX cartridge (Supelco, USA) preconditioned with 6 ml methanol, 3 ml water and 3 ml methanol–water (50:50, v/v). The effluent was discarded and the cartridge washed with 5 ml acetonitrile–water (10:90, v/v) which was discarded. Domoic acid was eluted with 3 ml acetonitrile–formic acid–water (10:2:88, v/v/v). A 20-μl aliquot was analysed by HPLC.

The SPE-SCX cleanup was carried out by passing 2.5 ml of diluted urine or feces extract (adjusted to pH 3–4) through a 3-ml Bond Elut SCX cartridge (Baker, USA) preconditioned with 6 ml methanol and 6 ml 0.1 *M* HCl. The effluent was discarded and the cartridge washed with 3 ml water which was also discarded. Domoic acid was eluted with 6 ml 0.7 *M* HCl directly onto a 3 ml SPE-C_{18} cartridge (Baker) preconditioned with 6 ml methanol and 6 ml 0.7 *M* HCl. The effluent was discarded and the cartridge washed with 3 ml water which was also discarded. Domoic acid was eluted with 4 ml acetonitrile–acetic acid–water (20:1:79, v/v/v). A 20-μl aliquot was analysed by HPLC.

High-performance liquid chromatography

The HPLC system consisted of a ternary low-pressure gradient pump (Eldex, Model 9600) connected to a rotary loop injector (Rheodyne, Model 8125) with a 20-μl sample loop and a reversed-phase C_{18} column (Supelco LC-18, 150×2.1 mm I.D., 5 μm). The column effluent was monitored with a diode array UV absorb-

ance detector (Hewlett-Packard, Model 1040A) set to 242 nm. The mobile phase was 0.2% (v/v) formic acid plus 12% (v/v) acetonitrile in water (pH 3.0). The flow-rate was set to 0.5 ml/min.

Radioimmunoassay

RIA was carried out exactly as described earlier using polyclonal antibodies generated from rabbits [9]. Briefly, domoic acid standards were prepared at concentrations of 1.0–8.0 ng/ml in PBS, pH 7.0. Samples were diluted with PBS to fall within the same domoic acid concentration range. To 200-μl aliquots of standards or diluted samples in small glass test tubes were added 100-μl volumes of antiserum in PBS. The tubes were mixed using a vortex mixer and then incubated overnight at 4°C. A 500-μl volume of[^{3}H]domoic acid (ice cold) in PBS was added to each tube and mixed and allowed to equilibrate for 30 min at 4°C. Free and bound domoic acid were separated by adding 500 μl of a suspension consisting of 10 mg/ml charcoal and 1 mg/ml dextran in PBS. After mixing, the tubes were allowed equilibrate for 10 min at 4°C and then centrifuged. The supernatant was removed to a scintillation vial, mixed with scintillator (Aquasol) and placed in a liquid scintillation counter (LKB) for tritium quantitation. Non-specific binding was determined by substituting PBS for the antibody. A zero point on the standard curve was determined by substituting PBS for the standard.

RESULTS AND DISCUSSION

The HPLC system functioned well for all analyses. The narrow-bore (2.1 mm I.D.) column was selected for this work because of the very good mass detection limits obtained and the low flow-rates employed. The methanol–water extraction procedure was found to be satisfactory for the seafood samples and the serum and urine samples. However, for rat feces only 2.0 g of material could be extracted with the volumes of solvents employed. Also, only 0.1 g equivalent feces could be applied to the SPE cartridges without causing overloading and poor cleanup efficiency.

The SPE-SAX cartridges provided very good cleanup of the seafood samples. Fig. 1 shows typical results obtained for mussels, razor clams, crabmeat and anchovies at a variety of domoic acid concentrations. Recoveries of domoic acid through this extraction and cleanup procedure were usually $>90\%$ ($n=6$) over the range of 0.2–40 μg/g domoic acid with good repeatability similar to that described earlier [6]. The detection limits were estimated to be about 0.1 μg/g (3:1, signal-to-noise) under the conditions employed. The same cleanup procedure was also found to be very satisfactory for the monkey and rat serum samples. The resulting chromatograms were very clean and good recoveries ($>90\%$) and repeatability (11% relative standard deviation, R.S.D., $n=5$) obtained from spiked samples over the concentration range of 0.2–5 μg/g. Detection limits were *ca.* 0.05 μg/g under the conditions employed in the experimental.

The urine and feces samples were found to be more difficult to purify. The SPE-SAX cleanup was not effective in removing interfering coextractives from the samples, making the determination of domoic acid at low μg/g levels difficult. However, the SPE-SCX-C_{18} combination cleanup produced cleaner chromatograms and permitted the detection of domoic acid at levels of about 0.1 μg/g. Fig. 2 shows chromatograms obtained for rat urine spiked with domoic acid using the SPE-SCX-C_{18} cleanup. The recoveries averaged 81% from 0.2–1.0 μg/g spiking levels with a R.S.D. of 13% ($n=3$). For feces, recoveries at a spiking level of 1 μg/g were 82 and 90% for duplicate samples.

Table I compares results obtained by the HPLC procedure to those obtained by RIA. As can be seen there is a good correlation between the two methods for the seafood samples and for spiked rat serum over a range of about 0.15–43 μg/g domoic acid. The correlation coefficient was calculated to be 0.9897 with a slope of 1.014, indicating a very good quantitative agreement between the two methods. Under the conditions of the analysis the two methods produced similar detection limits (approximately 0.05–0.1 μg/g) in the samples analysed. However, the RIA method has the potential for detecting lower amounts by reducing the dilution of the samples before analysis. An additional advantage of the

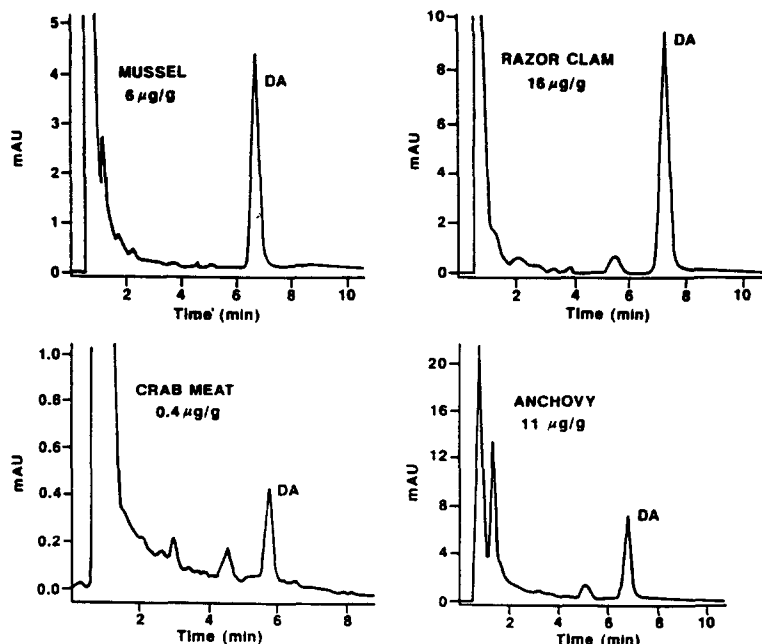

Fig. 1. Chromatograms of seafood samples containing domoic acid. Cleaned up using SPE-SAX. Razor clam, (16 μg/g domoic acid), anchovy (11 μg/g), mussel (6 μg/g, spiked) and crabmeat (0.4 μg/g). DA = Domoic acid. Chromatograms obtained on different days with slight changes in domoic acid retention time.

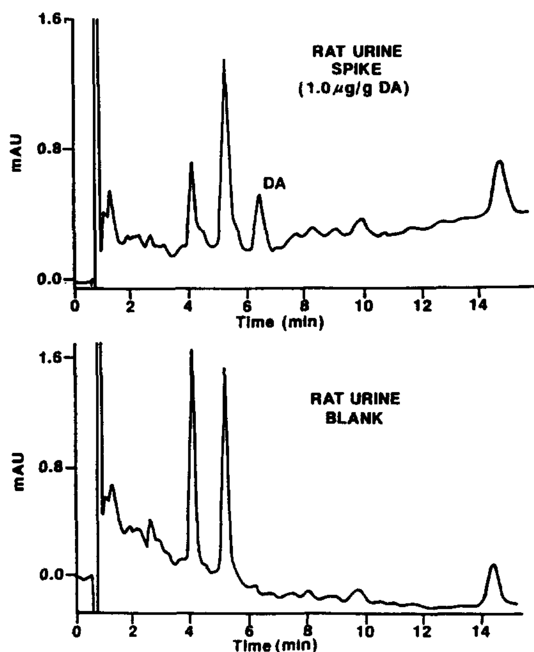

Fig. 2. Chromatograms of blank and spiked (1.0 μg/g domoic acid) extracts of rat urine cleaned up using SPE-SCX-C_{18}.

TABLE I

COMPARISON OF RESULTS BY HPLC AND RIA

Sample[d]	Domoic acid concentration (μg/g)	
	HPLC[a]	RIA
Razor clam (1)	43	38, 49[b]
Razor clam (2)	15	16
Anchovy	11	11
Crab meat	0.60	0.73
Mussel (1) blank	nd[c]	nd
Mussel (1) spiked	7.3	6.5
Mussel (2) blank	nd	nd
Mussel (2) spiked	5.3	4.8, 5.3[b]
Rat serum blank	nd	nd
Rat serum spiked	0.15	0.14, 0.17[b]
Rat serum spiked	0.39	0.39
Rat serum spiked	7.1	7.9

[a] Corrected for recovery. Values in the text.
[b] Duplicate determinations.
[c] Not detected (<0.1 μg/g, HPLC; <0.05 μg/g, RIA).
[d] (1) and (2) indicate different samples.

RIA method for the samples analysed was that the SPE cleanup procedure was not required. However, the linear range of the immunoassay technique is rather limited (about a 10-fold range) which requires re-analysis of unknown samples, if the domoic acid concentration falls outside the linear range.

There is always the possibility that cross-reactivity with other chemicals can lead to false results in RIA and other immunoassay methods. In the present case, no false positives (greater than 0.05 μg/g) were found in any of the samples. The good agreement between the HPLC and RIA methods for both spiked and naturally incurred domoic acid shellfish samples clearly indicates that cross-reactivity and matrix effects are not significant in the immunoassay method.

In a further comparison, the HPLC method was applied to the determination of domoic acid in serum and urine samples from rats and monkeys that had received domoic acid either orally or by intravenous injection. The samples had been frozen for about a year. The overall results correlated well although the HPLC values were only 64% (average of 11 samples), of the RIA values (0.5–11 μg/g) obtained at the time of the study. (The RIA analysis was not repeated at the time of the HPLC analysis.) It is likely that this difference is due to the long time between RIA and HPLC analyses. Domoic acid is known to be unstable in biological extracts even when frozen. We have observed that domoic acid degrades substantially (by 50% or more) in extracts or tissue homogenates of shellfish which had been stored frozen for 6 months. Domoic acid is not metabolized by the rat or monkey [10] ruling out the possibility that the RIA method detected a domoic acid metabolite which might account for the difference.

This study has shown that HPLC and RIA provide similar results for domoic acid in seafood and biological fluids. It indicates the potential of immunoassay for rapid screening and quantitation of domoic acid with confirmation by HPLC.

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