Bull. Environ. Contam. Toxicol. (1991) 47:329–334

Environmental Contamination and Toxicology

Determination of Domoic Acid in Serum and Urine by Immunochemical Analysis

H. Newsome, J. Truelove, L. Hierlihy, and P. Collins

Food Directorate, Bureau of Chemical Safety, Health Protection Branch, Health and Welfare Canada, Ottawa, K1A OL2, Canada

An outbreak of food poisoning originating from the consumption of cultured blue mussels that occurred in Canada in 1987 was attributed to the potent neuroexcitatory amino acid, domoic acid. (Wright et al. 1988, Iverson et al. 1989, Quilliam and Wright 1989). The original method used for detection of this toxin was the AOAC mouse bioassay for paralytic shellfish poison (PSP) (AOAC 1984). The PSP method, involving intraperitoneal (IP) injection of mice with an acidic extract of whole mussels, produced, as a positive effect, a very characteristic syndrome in mice that was distinct from PSP and produced consistent dose-response curves (Iverson et al. 1989). This method, although of inestimable value during the initial investigation of the problem, can be replaced with liquid chromatographic methods which have greater sensitivity and do not require animal facilities (Lawrence et al. 1989, Pocklington et al. 1989). However, in spite of the improvement over the original mouse bioassay procedure, the present chromatographic methods do not have sufficient sensitivity and/or specificity for the assay of domoic acid in serum or urine of experimental animals dosed at low levels. The present paper describes a method used for the development of a specific antibody against domoic acid in rabbits and its subsequent use in an enzyme-linked immunosorbant assay (ELISA) and a radioimmunoassay (RIA).

MATERIALS AND METHODS

RIA-grade bovine serum albumin (BSA), human serum albumin (crystallized), ovalbumin (fraction V), goat anti-rabbit IgG peroxidase conjugate (enzyme conjugate), Tween 20, o-phenylenediamine dihydrochloride, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from the Sigma Chemical Co., St. Louis, MO. Tri-n-butylamine and iso-butyl chloroformate were purchased from the Aldrich Chemical Co., Milwaukee, WI. Charcoal (Norit A) and dextran (low fraction) were purchased from J.T. Baker, Toronto, Ont. Microtitration plates were purchased from Dynatech Inc., Chantilly, VA. Domoic acid was a gift from Dr. J. Wright, National Research Council, Halifax, NS, Canada, while ^{3}H-domoic acid (specific activity, 165 GBq/mmol) was purchased from Amersham Laboratories, Buckinghamshire, England. Prior to use, the ^{3}H-domoic acid was purified using liquid chromatography. The system used was a Waters 990 diode array detector monitoring 242 nm (wavelength maximum for domoic acid) and a Waters C18 μBondapak column. The mobile phase was 8% acetonitrile in water acidified with 0.2% glacial acetic acid and

Send reprint requests to J. Truelove at the above address.

degassed. The flow rate was 1.5 mL/min. Forty microliters of the original tritiated domoic acid solution (37 MBq/mL) was injected onto the column, and the fraction absorbing at 242 nm was collected. Under the above conditions, domoic acid eluted in 6.5 min. The volume of the collected fraction was reduced to near dryness at 30°C using a rotary evaporator. The residue was redissolved in approximately 4 mL of 0.1 N HCl. A C18 Sep-pak (Waters) column was conditioned with 5 mL of methanol followed by 5 mL of distilled water. The sample was applied, and then the column was rinsed with 5 mL of 0.02 N HCl followed by 0.5 mL of distilled water. Domoic acid was eluted from the column with 4.0 ml of 40% methanol in water. The volume was reduced to near dryness, and the residue was redissolved in buffer to the required activity. The specific activity following the cleanup procedure was 72 GBq/mmol. Phosphate-buffered saline used for the RIA method (PBS2) contained 10 mmol NaH_2PO_4:H_2O and 140 mmol NaCl at pH 7.0 plus 0.1 % NaN_3 and 0.1 % BSA, whereas phosphate-buffered saline used for the ELISA method (PBS1) contained 20 mmol of $NaH_2PO_4 \cdot 2H_2O$ and 140 mmol of NaCl adjusted to pH 7.2.

The immunogen for antiserum production was prepared by adding 100 mg of EDC to 12.4 mg (40 µmol) of domoic acid in 2 mL of PBS1, then adding 28 mg of human serum albumin in 1 mL of PBS1. After mixing, the solution was incubated at 4°C for 19 h, then dialyzed against 4 L of distilled water for 3 days with twice daily changes of water. The protein solution was diluted to 10 mL with distilled water and stored frozen in 1 mL aliquots. For immunization, the solution was thawed, 0.5 mL of 0.42 M NaCl added, and an emulsion prepared by adding 1.5 mL of adjuvant and mixing. New Zealand White rabbits (3) were immunized by subcutaneous injection of 0.5 mL of the emulsified immunogen at 5 sites using 0.1 mL per site. The initial immunization was performed using complete Freund's adjuvant whereas incomplete adjuvant was used for subsequent boosts made at the rate of once every 28 days. Useful antibody titers were obtained 4 months after the initial injection. Final serum collection was made after 9 months.

The plate coating protein for ELISA was prepared by coupling domoic acid to ovalbumin via a mixed anhydride. Eight microlitres of isobutyl chloroformate and 15 µL of tri-n-butylamine were added to 16.2 mg (52 mmol) of domoic acid in 0.5 mL of dioxane. After 30 min at room temperature, the solution was added dropwise to a stirred solution of 44 mg ovalbumin in 3 mL of 0.2 M $NaHCO_3$, pH 9.3. After incubation overnight at 4° C, the conjugate was dialysed against distilled water as described for the immunogen and stored frozen in 200 µL aliquots at -20°C.

Microtitration plates were washed, unless otherwise indicated, with 0.1 % Tween 20 in PBS1 (Tween solution) using a Titertek Microplate Washer model 120 (Flow laboratories, Mississauga, Ont.) set for 5 wash cycles and 3 soak periods of 30 s each.

Plates were coated by adding 180 µL of a solution of 0.88 µg/mL of coating protein in 0.2 M Na_2CO_3, pH 9.6 containing 10 µg/mL ovalbumin to each well and incubating overnight at 4°C. They were then washed three times with Tween solution, twice with distilled water and stored in a sealed plastic bag at -20° C. Coated plates were routinely treated with 200 µL of a 1 % solution of BSA in distilled water for 30 min at room temperature prior to use, to reduce non-specific binding (NSB).

For ELISA, standards of domoic acid were prepared by dissolution of the crystalline material in distilled water and serial dilution to give solutions of 0.16-10.5 ng/mL in 0.1% BSA/distilled water. Samples were diluted as indicated below with 0.1 % BSA in distilled water. Antiserum and enzyme conjugate were diluted in 0.1% BSA in PBS1. The ELISA was conducted by adding 100 uL of antiserum diluted 1:1000 to a 1.0-mL aliquot of sample or standard in a 12 x 75 mm glass test tube and incubating for 30 min at 4° C. Triplicate 200-µL aliquots were then added to a sensitized plate, and the plate was incubated for 30 min at 4° C. The wells were emptied and washed, and 210 µL of a 1:1000 dilution of enzyme conjugate was added. After 1 h at room temperature, the wells were again emptied, washed, and reacted for 20 min with 210 µL of substrate consisting of 35 mg of **o**-phenylenediamine hydrochloride and 20 µL H_2O_2 in 50 mL of citrate buffer, pH 5.0 (Voller et al. 1976). The reaction was terminated with 50 µL of 2.5 M H_2SO_4 and the optical densities recorded using a Titertek Multiscan MCC with the sample filter set at 492 nm and reference filter at 620 nm. Samples were quantitated by reference to a least squares plot of the log of the concentration verses the logit of the optical density of a series of standards run concurrently. Cross reactivity with glutamic, aspartic and kainic acid was evaluated using 10, 50 and 100 ng/mL of the test compound.

For RIA, domoic acid standards were prepared at concentrations ranging from 1.0 to 8.0 ng/mL in PBS2, and samples were diluted with PBS2 as indicated below. The assay was conducted by adding 200-µL aliquots of standard or diluted sample in duplicate to 12 x 75 mm glass test tubes. One hundred microliters of a 1:25 dilution of antiserum in PBS2 was added, and the tubes were mixed using a vortex mixer (approx. 3 s) and then incubated overnight at 4° C. Five hundred microliters of ice cold ^{3}H-domoic acid in PBS2 containing 865 Bq were added, vortexed 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 PBS2. The charcoal-dextran suspension was stirred in an ice bath for 1 h before use and continuously during use. After mixing, the tubes were allowed to sit for 10 min at 4° C and were then centrifuged at 1000 x g for 4 min. The supernatant was decanted into glass scintillation vials, 10 mL Aquasol was added, and the vials were placed in an LKB liquid scintillation counter for tritium quantitation. NSB was determined by substituting PBS2 for antibody, and a zero point on the standard curve was determined by substituting PBS2 for standard. Cross reactivity with glutamic and aspartic acid was measured at 0.1 and 100 µg/mL and with kainic acid at 0.1 to 10 µg/mL.

Pooled samples of serum or urine from rats and monkeys (Macaca fascicularis) were used for recovery experiments.

RESULTS AND DISCUSSION

All 3 rabbits produced antisera with useable titers measured at four months after the initial immunization. The antiserum producing an inhibition curve with the greatest sensitivity using the ELISA method was chosen for optimization with respect to incubation times and plate coating protein concentration. Although it was necessary to use the antiserum at higher concentrations in the RIA, the serum producing the standard curve with the greatest sensitivity in the ELISA also produced the optimum curves in the RIA. No cross reactivity with glutamic, aspartic or kainic acid was found using either method.

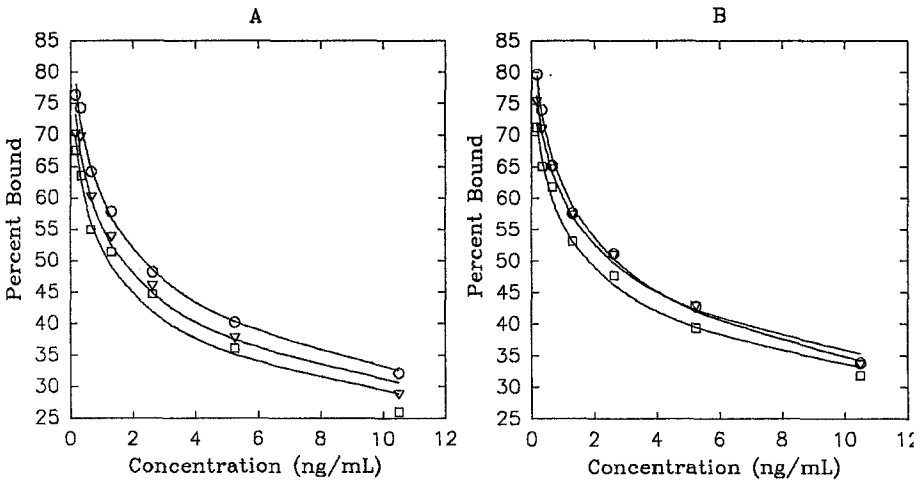

Figure 1. A, ELISA competitive inhibition curves for BSA (O), serum diluted 1:100 (Δ) and serum diluted 1:50 (□) and B, for BSA (O), urine diluted 1:250 (Δ) and urine diluted 1:1000 (□). Solid lines are the least squares regression of the logit transformation of the percent binding verses the log transformation of the concentration.

Using ELISA, serum diluted 1:50 depressed the binding of antiserum to the microtitration plate, resulting in overestimation of the amount of domoic acid present. This effect was less pronounced at a dilution of 1:100, where the range of domoic acid concentration in the undiluted serum was from 0.016 to 1.05 µg/mL (Fig. 1, A). The magnitude of the deviation between standard curves in PBS1 and those in diluted serum was inconsistent from assay to assay such that a suitable correction factor could not be applied. ELISA was more applicable to the determination of domoic acid in urine, as shown by the data in Figure 1, B. Dilutions of 1:250 gave accurate quantitation at levels corresponding to 40 ng/mL to 2.6 µg/mL in urine. The coefficient of variation (CV) for triplicate wells averaged 3.0 % for the standards and 2.8 % for the diluted urine. The ELISA method with urine diluted at 1:100 produced erroneously high results.

To determine whether the ELISA method accurately reflected the amount of domoic acid in non-spiked samples, urine was collected from a rat that was injected IP with 2 mg/kg domoic acid plus 0.37 MBq tritiated domoic acid. Urine samples were collected at approximately 76 and 116 min post-injection. Analysis by scintillation counting and by thin-layer chromatography followed by radioactivity scanning indicated that the urine concentration was 146 and 47 µg/mL. ELISA conducted on $1:1.6x10^3$ dilutions indicated the presence of 144 and 40 µg/mL respectively.
The RIA competitive inhibition curve for domoic acid in serum diluted 1:50 was coincident with that in PBS2 and resulted in accurate determinations in serum equivalent to concentrations of 50 to 400 ng/mL in undiluted serum (Fig. 2, A). Increasing the concentration of serum in the assay to a 1:10 dilution produced a positive deviation, resulting in an underestimation of the amount of domoic acid present

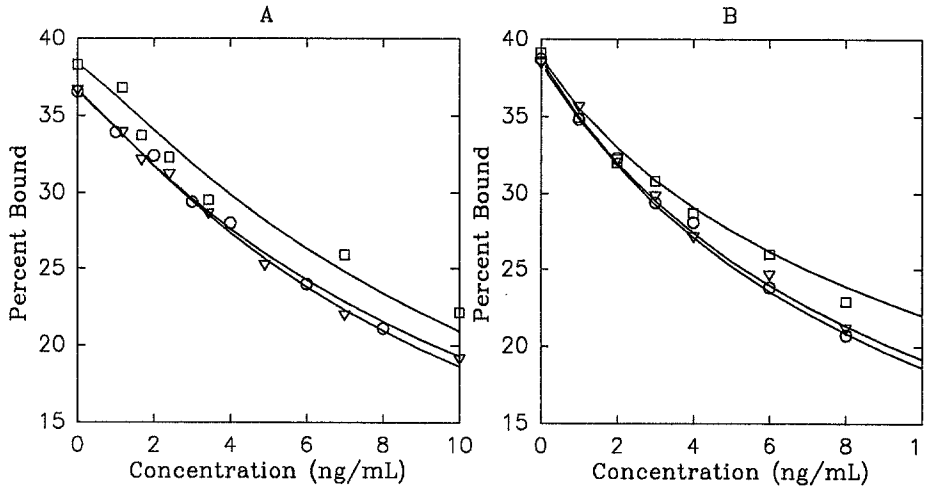

Figure 2. A, RIA competitive inhibition curves for PBS2 (O), serum diluted 1:50 (Δ) and serum diluted 1:10 (□) and B, for PBS2 (O), urine diluted 1:150 (Δ) and urine diluted 1:50 (□). Solid lines are the least squares regression of the logit transformation of the percent binding verses the log transformation of the concentration.

(Fig. 2, A). RIA was also suitable for the determination of domoic acid in urine at a minimum dilution of 1:150 (useful range equivalent to 150 to 1200 ng/mL in undiluted urine) while a 1:50 dilution deviated from the standard curve in PBS2 (Fig. 2, B). The CV for triplicate determinations in PBS2, serum or urine was consistently less than 3 %. NSB for urine at 1:150 dilution (16 % of total activity) was consistently higher than that for PBS2 (13.7 % of total activity) and therefore in order to use a reference curve prepared in PBS2, urine samples at 1:150 dilution were corrected for NSB of urine samples at the same dilution. This correction was not necessary at urine dilutions of 1:400 or greater.

Of the two methods evaluated, ELISA was the most sensitive method for the determination of domoic acid in either serum or urine, but gave inconsistent results with various serum dilutions. Accurate results were attainable in serum or urine using RIA.

Acknowledgments. The authors appreciate the technical assistance of Mrs. Cheryl Armstrong and Mr. Leigh Martin.

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Received December 1, 1990; accepted April 5, 1991.