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Determination of Domoic Acid by Two Different Versions of a Competitive Enzyme-Linked Immunosorbent Assay (ELISA)

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Domoic acid has become well known as the causative agent of a potent neuroexcitation (amnesic shellfish poisoning, ASP) since the intoxication episode arising (in 1987) from the consumption of cultured blue mussels which originated in Prince Edward Island and contained large concentrations of domoic acid (Wright *et al.* 1989; Todd 1993). Methods for determination of domoic acid have been required to monitor food and the environment and to conduct fundamental studies in the laboratory and the field. Determinations by biological assays (Iverson *et al.* 1989; Wright *et al.* 1989), liquid chromatography (LC), and high performance liquid chromatography (HPLC) (Quilliam *et al.* 1989; Rao *et al.* 1988; Wright *et al.* 1989; Lawrence *et al.* 1989; Pocklington *et al.* 1990) have contributed much to the surveys for and research on domoic acid.

To add to the methods available, an enzyme-linked immunosorbent assay (ELISA) for domoic acid was developed by Newsome *et al.* (1991). They applied it to urine samples and blood sera and described the advantages of high specificity and quantitation inherent in this technique. The method, however, involved several steps and lacked the sensitivity we desired. This method, as originally described, depended on the physical immobilization of domoic acid on the microplate *via* a carrier protein. Recently, however, CovaLink NH microplates have been developed in which a projecting secondary amino group has been applied in ELISAs for coupling peptides, steroids, oligonucleotides, and DNA to the microplate well surface, directly and chemically (Søndergård-Andersen *et al.* 1990; Rasmussen 1990; Rasmussen *et al.* 1991; Yonezawa *et al.* 1993; Chevrier *et al.* 1993). The current study was undertaken to simplify and improve on the previous domoic acid ELISA method and has resulted in two different versions of the ELISA; these are based on a physical (Method-1) and a chemical (Method-2) immobilization of domoic acid.

MATERIALS AND METHODS

In Method-1, the microplates were coated with ovalbumin conjugated domoic acid prepared according to Newsome *et al.* (1991), (which depends on a physical

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adsorption of domoic acid *via* the coupled protein), and allowed to stand at 4 °C overnight. These plates were then washed with 20 m*M* phosphate buffer (pH 7.2) containing 140 m*M* NaCl and 0.1 % Tween 20 (PBS-Tween), followed by washing with distilled water. Coated plates were blocked with 1 % bovine serum albumin (BSA) in distilled water at room temperature for 1 hour and washed with distilled water.

The standards of authentic domoic acid (Diagnostic Chemical Ltd., Canada) from 1 pg to 10 μg/mL were prepared by a 5-fold serial dilution made with PBS containing 0.1 % BSA (PBS-BSA). An amount of 182 μL of each standard was dispensed into every well and 18 μL of rabbit anti-domoic acid serum, diluted 1000-fold with PBS-BSA, was added. After incubation at room temperature for 1 hour, the microplates were washed with 20 m*M* high ionic strength phosphate buffer (HISPB, pH 7.2) which contains, in addition to phosphate, 2.14 *M* NaCl, 40 m*M* $MgSO_4$ and 0.1 % Tween 20, and then 210 μL of horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG antibody (Sigma, U.S.A.), diluted 1000-fold with PBS-BSA, was added. The plates were incubated at room temperature for 1 hour and then washed again with HISPB. The concentration of the HRP remaining was determined by adding 210 μL of substrate solution containing 35 mg *o*-phenylendiamine (Sigma, U.S.A.) and 20 μL of 30 % H_2O_2 (Sigma, U.S.A.) in 50 mL of 200 m*M* citrate buffer (pH 5.0), and incubating for 10 min at room temperature; the reaction was terminated by adding 50 μL of 2.5 *N* H_2SO_4. The absorbance value for each well was determined by the microplate reader (THERMO max, Molecular Devices, USA) at 490 nm sample wavelength and 620 nm reference wavelength.

In Method-2, domoic acid was coupled covalently to the CovaLink NH microplate (Nunc, Denmark) using N-hydroxysuccinimide (NHS, Sigma, U.S.A.) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC, Sigma, U.S.A.). Formation of amide bonds between carboxylic acids of hapten and amines of CovaLink NH is generated by EDC in the presence of NHS to obtain an enhanced yield. Each volume of NHS and EDC, diluted with distilled water (final concentration of each volume was between 0 and 50 m*M*), was added to 18-fold volumes of domoic acid dissolved in distilled water in test tube. Aliquots (100 μL) of each activated domoic acid solution were dispensed into the appropriate well. Microplates were allowed to stand at 4 °C overnight; this was followed by washing with distilled water. A volume (200 μL) of HISPB was dispensed into each well and allowed to soak at room temperature for 10 min. After washing the wells with distilled water, 300 μL of 1 % BSA in distilled water was dispensed into each well for blocking and allowed to stand at room temperature for 1 hour. The plates blocked with BSA were washed once more with distilled water.

The preparation of standards of domoic acid, the reaction and treatment were carried out identically to that described for Method-1 except for the reaction time, *i.e.* the domoic acid standards and anti-domoic acid rabbit serum placed in each well were incubated at room temperature for 2 hours; for the reaction of HRP conjugated anti-rabbit IgG against the residual anti-domoic acid antibody the plates were incubated also at room temperature for 2 hours. In this experiment, the effect of various concentrations of NHS and EDC and several concentrations of domoic acid for coupling to the secondary amino group on the surface of the wells was tested and the optimal reaction times for absorption of anti-domoic acid rabbit serum and subsequent HRP conjugated anti-rabbit IgG were investigated. The results of the effects of NHS and EDC were analyzed by using SYSTAT ver. 5.1 (SYSTAT Inc.).

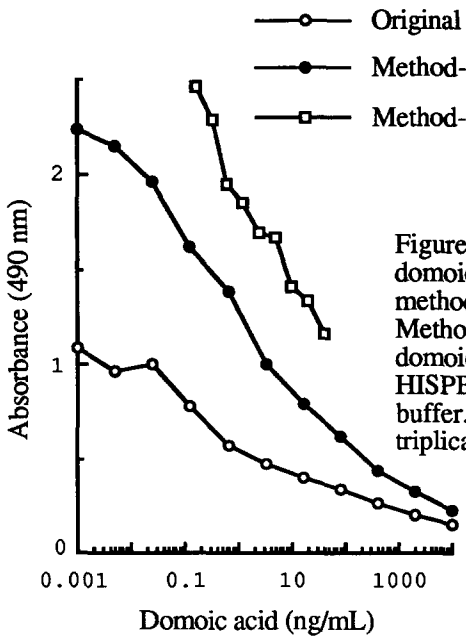

Figure 1. ELISA inhibition curves for domoic acid standards using the original method (Newsome *et al.* 1991) and Method-1 with physical adsorption of domoic acid on the microplate. HISPB, high ionic strength phosphate buffer. Each value is the mean of triplicate assays.

In both methods, cross reactivity with kainic acid, glutamic acid and proline was evaluated using the same molarity range as used for domoic acid standards. The coefficients of variation within the standard series by both methods was investigated and both versions of the modified ELISA were compared with determinations made in parallel using the original procedure described by Newsome *et al.* (1991).

RESULTS AND DISCUSSION

Three sets of inhibition curves were obtained by using (1) the original method reported by Newsome *et al.* (1991), (2) the Method-1 with PBS-Tween as a washing solution and (3) Method-1 with HISPB as a washing solution (Fig. 1). The inhibition curve for the original method showed the least slope and inaccurate quantitation at concentrations below 0.05 ng/mL was apparent. It was assumed that the competition between free domoic acid of standards and immobilized domoic acid (solid phase) for the anti-domoic acid rabbit serum would be incomplete under these conditions (4 °C for 30 min in a tube followed by a further 30 min at 4 °C in the microplate). The direct dispensation of the domoic acid standard and the anti-domoic acid rabbit serum into the wells (Method-1 with PBS), followed by incubation at room temperature for 1 hour to achieve completeness of the competitive reaction resulted in steeper inhibition curves but, also, a high background absorbance. An increase in the ionic strength of the washing solution through replacement of PBS with HISPB, was capable of reducing this background absorbance without any loss of sensitivity. No cross reactivity with kainic acid, glutamic acid and proline was observed in Method-1 and a high specificity of the rabbit anti serum against domoic acid was apparent in this ELISA system.

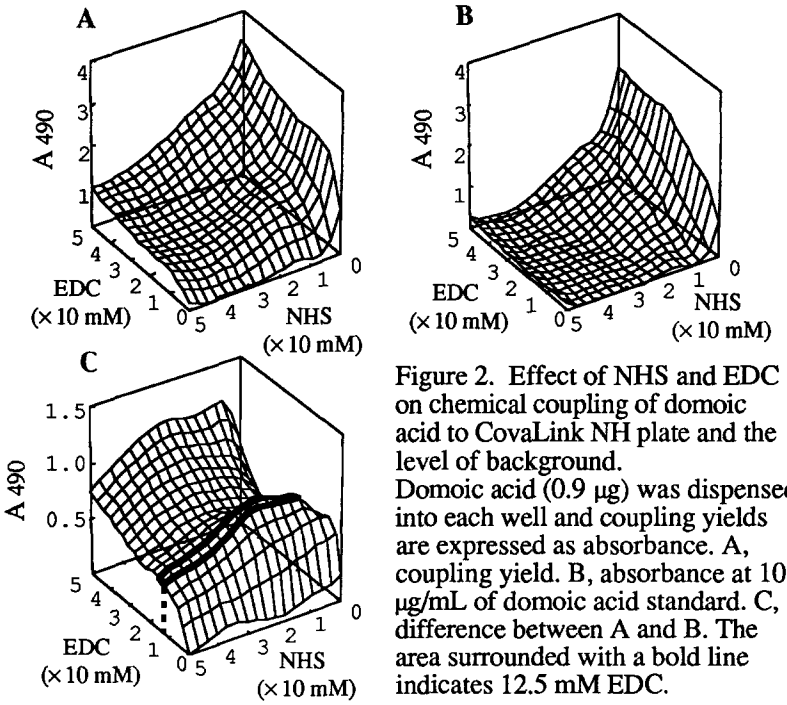

Figure 2. Effect of NHS and EDC on chemical coupling of domoic acid to CovaLink NH plate and the level of background.
Domoic acid (0.9 μg) was dispensed into each well and coupling yields are expressed as absorbance. A, coupling yield. B, absorbance at 10 μg/mL of domoic acid standard. C, difference between A and B. The area surrounded with a bold line indicates 12.5 mM EDC.

Diluting the anti-domoic acid rabbit serum reduced the high background values but a steeper inhibition curve was not obtained compared with that obtained with the original method (data not shown). A different approach was required to obtain the steeper inhibition curve necessary to ensure precision and quantitation. Method-1 in which every reaction was performed in the wells at room temperature and the microplate was washed with HISPB each time resulted in a considerable reduction in the background values and an increased steepness of inhibition curve, compared to the original method. Accordingly, Method-1 should provide a simplified and accurate determination of domoic acid with a high degree of sensitivity.

Method-1 depended on an adsorption of domoic acid *via* ovalbumin as the carrier molecule. As this physical adsorption would be affected by the physical properties of the plate this might increase the variation between ELISA systems (Yonezawa *et al.* 1993). To avoid this possibility Method-2 was based on a covalent conjugation of domoic acid onto the plate. This has obvious advantages since the coupling is conducted in a more controlled manner (Søndergård-Andersen *et al.* 1990). The coupling yield for domoic acid was estimated as the absorbance value for HRP reactions for plates following the reaction of 1000-fold diluted anti-domoic acid rabbit serum and PBS without domoic acid (0 μg/mL of standard domoic acid) (Fig. 2A). The amount of coupled domoic acid tended to be higher with increasing concentrations of EDC and, in contrast, to be lower with increased concentrations of NHS. Absorbance values for HRP reactions at 10 μg/mL levels of standard domoic acid showed a reduced value resulting from competition with the domoic acid on the well; however, the relationship between this absorbance and the concentrations of EDC and NHS was similar to the maximum yield observed at 0

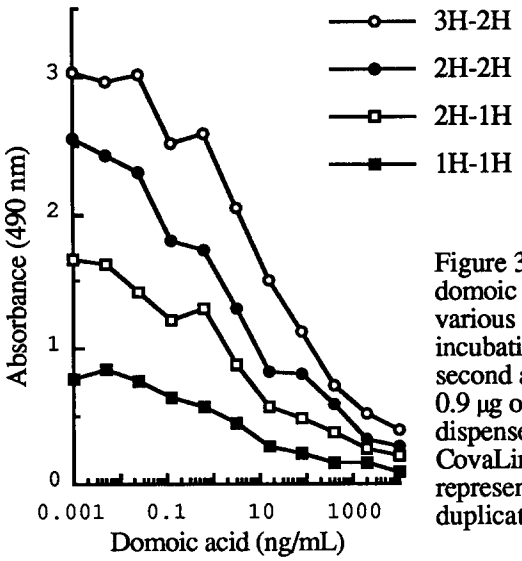

Figure 3. Inhibition curve of domoic acid standards in the various combinations of incubation times for the first and second antibodies. 0.9 µg of domoic acid was dispensed into each well of CovaLink NH plate. Each value represents the mean of duplicate assays.

µg/mL of domoic acid (Fig. 2B). In short the absorbance values were higher with increasing concentrations of EDC and lower with increased concentrations of NHS. Excess of EDC resulted in an increased background resulting from nonspecific binding of antibody to the plate and an excess of NHS gave a lower yield of coupled domoic acid as would be predicted by the work of Søndergård-Andersen *et al.* (1990). The differences between absorbance at 0 and 10 µg/mL of standard domoic acid under the varying concentrations of EDC and NHS were investigated (Fig. 2C) because the optimal concentrations of EDC and NHS which gave a stronger absorbance at 0 µg/mL and weaker absorbance at 10 µg/mL would provide the required steep inhibition curve. This figure shows that the largest differences were observed at concentrations of 12.5 and 50 m*M* EDC. The data indicated that the background absorbance values were lower at 12.5 m*M* EDC than at 50 m*M* and that the most effective coupling of domoic acid was achieved with 12.5 m*M* EDC and 15.0 m*M* NHS.

Various incubation times were investigated using the covalently coupled plates. The slope of the inhibition curve depended directly on the incubation time for both the anti-domoic acid rabbit serum and the HRP conjugated anti-rabbit IgG. The values obtained by the incubation of anti-domoic acid rabbit serum for 3 hours and for HRP conjugated anti-rabbit IgG for 2 hours were the highest; determinations for domoic acid concentrations below 25 pg/mL was not possible with this incubation regime (Fig. 3). Accordingly the incubation of each antibody for 2 hours was recommended.

These results, shown in Fig.3, were obtained by using the plates in which 0.9 µg of domoic acid was dispensed into each well for covalent binding. Larger amounts of domoic acid (9 µg/well) resulted in a slight increase in the steepness of the inhibition curve and a high background value and thus required the reduction of the incubation time (Fig. 4). The plate in which 0.09 µg of domoic acid was dispensed into each well showed results similar to those obtained with 9 and 0.9 µg/well and

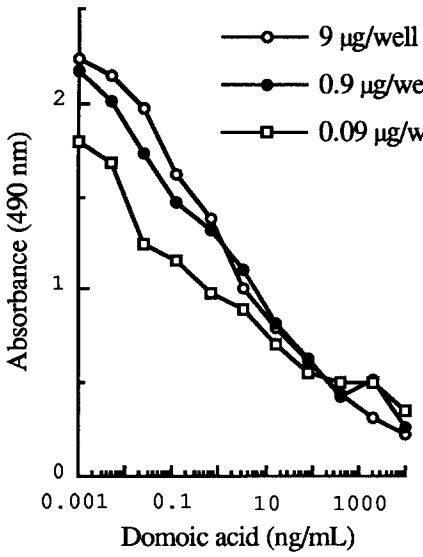

Figure 4. Comparison of inhibition curves in the various amounts of domoic acid dispensed into each well for coupling to the CovaLink NH plates.
Incubation for both the first and second antibody was performed at room temperature for one hr for 9 µg/well or two hr for 0.9 and 0.09 µg/well. Each value is the mean of triplicate assays.

exhibited a sensitivity similar to Method-1. Thus Method-2 as recommended consists of the dispensation of 0.09 µg of domoic acid with 12.5 m*M* EDC and 15.0 m*M* NHS into the individual wells for coupling and for analytical determinations the incubation of each anti-domoic acid rabbit serum and HRP conjugated anti-rabbit IgG for 2 hours at room temperature. As with Method-1, based on a physical immobilization of domoic acid and utilizing HISPB, there was no cross reactivity with kainic acid, glutamic acid and proline.

The precision of two these new versions has been demonstrated. The coefficients of variation within the standard series ranging from 1 pg/mL to 10 µg/mL, in triplicate, were plotted against a range of concentrations of the domoic acid standards (Fig. 5). The coefficients of variation for Method-1, based on a physical adsorption, ranged between 0.4 and 7.0 % for whole series. On the other hand, those for Method-2 based on covalent coupling of domoic acid (0.09 µg dispensed into each well) ranged between 0.9 and 11.0 %, except for those at 2 and 10 µg/mL.

In conclusion, Method-1 based on the physical adsorption of domoic acid on the plate utilizing ovalbumin resulted in a simplification of the original procedure (Newsome *et al.* 1991) and heightened sensitivity through use of a high ionic strength solution for the reduction of background absorbance. Method-2 based on covalent coupling of domoic acid has several advantages. Firstly, the binding of domoic acid onto the well surfaces would be expected to be stronger than in Method-1 where it is based upon physical adsorption *via* a carrier protein. In Method-2 the covalent link arises from the carboxyl groups of domoic acid actually forming an amide bond with the secondary amino group on the solid phase. Secondarily, the variability of the ELISA system, caused each time by preparation of protein-conjugates of domoic acid and immobilization of domoic acid *via* a protein molecule to the microplates can be eliminated since the chemical coupling procedure can be fully controlled. These improvements provide the capability for

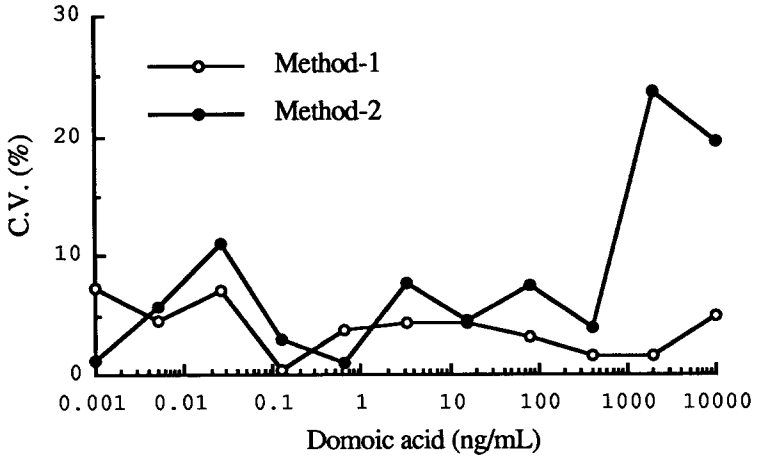

Figure 5. Coefficients of variation in Method-1 and 2 within the whole standard series.
Method-1; plate was physically sensitized with ovalbumin conjugated domoic acid. Method-2; plate was chemically sensitized with domoic acid.
Each value indicates the mean of triplicate assays.

determining lower levels of domoic acid (< 0.1 ng/mL) through increased sensitivity and widened the detectable range of domoic acid.

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