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A Competitive Displacement Assay to Detect

Saxitoxin and Tetrodotoxin¹

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Running title: 'ssay for Saxitoxin and Tetrodotoxin Subject category: Special Techniques or Membranes and Receptors Key words: Saxitoxin, tetrodotoxin, seafood toxins, neurotoxins, binding assay, competitive displacement assay

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FOOTNOTES

¹The views of the authors do not purport to reflect the positions of the Department of th. Army or the Department of Defense.

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³Abbreviations used: STX, saxitoxin; TTX, tetrodotoxin; HEPES, N-2hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EDTA,

ethylenediaminetetraacetic acid; CPDA-1 plasma, citrate-phosphate-dextroseadenine-1 human plasma.

ABSTRACT

An assay is described which detects saxitoxin (STX) and tetrodotoxin (TTK) by their competitive displacement of $[^{3}H]$ -saxitoxin from its receptor in rat brain membranes. The assay has a sensitivity of .15 ng STX/ml and .3 ng TTX/ml for buffer samples. The assay was also applied to detection of these toxims in unextracted human plasma and found to have a sensitivity of .5 ng STX/ml and .6 ng TTX/ml. The competitive displacement assay appears to be the most sensitive procedure yet for detection of STX and TTX.

INTRODUCTION

Saxitoxin $(STX)^3$ and tetrodotoxin (TTX) are among the most lethal nonprotein toxins known $(LD_{50s} \cap 10 \pm g/kg$, i.p., mouse; ref 1). STX is one of the "paralytic shellfish poisons" produced by dinoflagellates of the genus <u>Gonyaulax</u>. These organisms can contaminate shellfish during periods of mass proliferation ("blooms"). Between 1971 and 1977, some 170 human cases of paralytic shellfish poisoning (none fatal) were reported in the U.S. and Europe (2,3). Thus, STX is a worldwide concern both to the public health community (4) and the shellfish industry (5). TTX, found in the ovaries, eggs, and liver of the puffer fish, is a public health concern only in Japan where puffer fish is regarded as a delicacy (6). TTX poisoning kills about 100 percons annually in Japan (6).

STX and TTX have the same mechanism of action; both bind to a common receptor associated with sodium channels in nerve and muscle membranes (7). This binding prevents action potential propagation which is central to nerve impulse conduction and muscle contraction (6).

This paper describes a simple and sensitive method to detect these toxins based on their ability to competitively displace $[^{3}H]$ -labeled saxitoxin from its receptor in rat brain membranes. The assay is easily capable of detecting ng/ml levels of STX and TTX in isotonic buffer and in unextracted human plasma, making it the most sensitive method yet for detection of these toxins. This assay may have applications in the screening of potentially toxic shellfish, in pharmacokinetic studies of STX and TTX blood concentrations in laboratory animals, and as a diagnostic or forensic tool in suspected cases of STX or TTX poisoning in humans.

MATERIALS AND METHODS

Saxitoxin used to develop standard curves was obtained as a generous gift from Dr. Edward Schantz, University of Wisconsin. Saxitoxin for $[^{3}H]$ -labeling was obtained from the Edgewood Arsenal, Aberdeen Proving Ground, MD 21010. Tetrodotoxin was purchased from Calbiochem. N-2-hydroxyethylpiperazine-N'-2ethanesulfonic acid (HEPES) was purchased from Sigma. Citrate-phosphatedextrose-adenine-1 (CPDA-1) human plasma was obtained from the Walter Reed Army Medical Center blood bank, Washington, DC. EDTA-anticoagulated human plasma was obtained from the U. S. Army Medical Research Institute of Infectious Diseases clinical laboratory, Frederick, MD 21701.

Rat Brain Membranes. Ten male Sprague-Dawley rats (250-300 g) were individually killed by CO_2 asphyxiation. The brains were removed, suspended each in 20 ml of .32 M sucrose, 5 mM sodium phosphate, pH 7.4 (buffer A), and homogenized with 10 strokes of a motor-driven Teflon glass homogenizer running at 900 rpm. The homogenate was centrifuged at 1000 x g for 10 min. The supernatant was saved while the pellet was resuspended in 20 ml of buffer A per brain and recentrifuged at 1000 x g for 10 min. The supernatants were pooled and centrifuged at 12,000 x g for 20 min. The pellet was retained being careful to exclude the dark, densely-packed material at the bottom (mitochondria). The total pellet from 10 brains was suspended in 250 ml of buffer A and centrifuged at 12,000 x g for 20 min. The pellet was suspended in approximately 5 times its volume of buffer A and was separated into 1 ml aliquots in 2 ml disposable Eppendorf centrifuge tubes. Approximately fifty 1 ml aliquots were obtained from the homogenization of ten brains. These

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samples were quick-frozen in liquid N_2 and stored for several months at -70°C. The concentration of protein per ml for each batch of membranes was determined using the procedure of Lowry, et al. (8). Membrane concentrations were typically 8-10 mg protein/ml, depending on the batch of membranes.

 13μ -Saxitoxin. STX was tritium-labeled by New England Nuclear Corporation and subsequently purified by high voltage paper electrophoresis according to the procedure of Ritchie, <u>et al.</u> (9). Fractions eluted from the paper electrophoretogram were analyzed for [3H]-STX using the fluorimetric procedure of Bates and Rapoport (10). Fractions containing [3H]-STX were pooled and the final concentration of STX was again determined using the Bates and Rapoport procedure. The radiochemical purity of the [3H]-STX was determined to be 33% using the procedure of Catterall and Morrow (11). The specific activity of the [3H]-STX was found to be approximately 3.3 Ci/mmol. [3H]-STX was stored at -20°C for up to 6 months.

Standardization of 3H_STX and Membrane Concentrations for the Competitive Displacement Assay. The concentration of 3H_STX to be used with a particular batch of membranes was standardized based on the 3H_STX specific binding curve, obtained as follows. Rat brain membranes were suspended at .17 mg protein/ml in 1.5 ml of 20 mM HEPES, 140 mM NaC1, 6 mM EDTA, pH 7.5 (buffer B) containing 3H_STX ranging from .11 to 10.6 nM. Parallel samples were prepared containing an additional 5 u M unlabeled STX. All samples were allowed to equilibrate at 4° C for 1 h. Samples were then filtered through Whatman GF/F filters and the filters rapidly washed in 10 ml ice-cold isotonic HEPES buffer. The membrane-bound 3H_STX trapped on each filter was quantitated by immersing the filter in 10 ml scintillation fluid and counting. The specifically-bound 3H_STX was determined by subtracting non-specifically bound $\begin{bmatrix} 3H \end{bmatrix}$ -STX (measured in the presence of 5 μ M STX) from the total bound $\begin{bmatrix} 3H \end{bmatrix}$ -STX (measured in the absence of 5 μ M STX) according to published procedures (11,12). The concentration of $\begin{bmatrix} 3H \end{bmatrix}$ -STX used in the

competitive displacement assay was that which saturated two-thirds of the specific saxitoxin receptors when membranes were suspended at .17 mg protein/ml. For the membranes used to produce the data in this paper, this concentration of $[^{3}\text{H}]$ -STX was 1.45 nM.

Procedure for the Competitive Displacement Assay. Samples of isotonic buffer containing STX or TTX were analyzed as follows. One-half ml of buffer sample was combined with .5 ml $|_{3H}|_{-STX}$ (at a concentration determined by the above standardization procedure) followed by .5 ml of rat brain membranes (.5 mg $|^{3}H|$ -STX and membranes were suspended in buffer B. protein/ml); Assay mixtures were incubated at 4° C for 1 h (enough time to reach binding equilibrium). One ml of each mixture was then filtered through a Whatman GF/F filter and washed rapidly with 10 ml ice-cold isotonic buffer B. The filter was added to 10 ml scintillation fluid, allowed to equilibrate at room temperature for 1 h, and counted for 2 min to determine the amount of membrane-bound [3H]-STX. All buffer samples were analyzed in duplicate. The amounts of membrane-bound 3H STX for both determinations were averaged and expressed as a fraction of $[3_{\rm H}]$ -STX bound in the presence of buffer containing no unlabeled toxins; i.e., B/B°. B° was determined as the average of six determinations with blank buffer. B/B^O values for each sample were compared to a standard displacement curve obtained by measuring $\begin{bmatrix} 3 \\ H \end{bmatrix}$ -STX binding in the presence of standard solutions of STX or TTX in buffer.

Samples of human plasma containing STX or TTX were analyzed as above with the following exceptions. Prior to being assayed all plasma samples were filtered through Whatman GF/F filters to remove cells and other insoluble materials. Plasma samples for random analysis were prepared in individual samples of EDTA-anticoagulated human plasma. B^{O} was determined using a pooled sample of EDTA-anticoagulated plasma containing no toxin. Standard plasma samples were prepared using a pooled sample of CPDA-1 human plasma.

RESULTS

Detection of STX and TTX in Isotonic Buffer. Figure 1a illustrates the displacement of $[^{3}H]$ -STX from rat brain membranes by unlabeled STX in buffer B. Each assay sample consists of .5 ml buffer containing the indicated concentration of unlabeled toxin + .5 ml $[^{3}H]$ -STX (a concentration which saturated 2/3 of the STX receptors) + .5 ml membranes (.5 mg protein/ml). Membrane-bound $[^{3}H]$ -STX was measured using a rapid filtration procedure described in Methods. The error bars in Figure 1a depict the mea. +/- 2 standard deviations on 6 determinations. The bars extend +/- 5% from the mean indicating good precision in our $[^{3}H]$ -STX binding measurements. The useful range of this displacement curve for detection of STX is between .15 and 10 mg/ml corresponding to B/B^o values of .9 and .2.

Figure 1b shows the displacement of $\begin{bmatrix} 3 \\ H \end{bmatrix}$ -STX from rat brain membranes by TTX in isotonic HEPES buffer. Displacement of $\begin{bmatrix} 3 \\ H \end{bmatrix}$ -STX by TTX occurs at slightly higher concentrations of TTX and over a broader concentration range than observed for STX. Nevertheless, the TTX standard curve still has a useful range of .8-70 ng TTX/ml.

The accuracy of the assay for buffer samples was determined by analyzing random samples containing STX or TTX. The STX samples ranged from .70 to 9.50 ng/ml; TTX samples ranged from 1.1 to 54.8 ng/ml. In representative experiments the mean ratio of $STX_{apparent}$ to STX_{actual} was 1.16 +/- .14 (1 SD; n = 10) and the mean ratio of $TTX_{apparent}$ to TTX_{actual} was 1.08 +/- .10 (1 SD; n = 9).

<u>Detection of STX and TTX in Human Plasma</u>. To be a useful technique, the competitive displacement assay must be capable of detecting STX and TTX in complex mixtures. We decided, therefore, to apply the assay to detection of

these toxins in human plasma. Figures 2a and 2b show the displacement of $\begin{bmatrix} 3\\ H \end{bmatrix}$ -STX from rat brain membranes by STX and TTX in CPDA-1 human plasma. The only clean-up performed on the toxin-spiked plasma samples prior to being assayed was filtration through Whatman GF/F filters to remove cells and other inscluble material. As Figure 2 indicates, the competitive displacement assay is extremely sensitive at detection of STX and TTX in human plasma, even with the minimal clean-up of samples. The useful range for detection of STX (Figure 2a) is .5 to 40 ng STX/ml plasma. The useful range for detection of TTX (Figure 2b) is .6 to 110 ng TTX/ml plasma.

The accuracy of the assay in plasma samples was determined by analyzing random samples of EDTA-anticoagulated plasma containing STX or TTX. Each sample consisted of plasma taken from a different individual which had been spiked with a known amount of toxin. STX samples were within the concentration range .70 to 9.50 ng STX/ml; TTX camples were within the range 1.1 to 54.8 ng/ml. In representative experiments the mean ratio of STX_{apparent} to STX_{actual} was 1.10 +/- .10 (1 SD; n = 9) and the mean ratio of TTX_{apparent} to TTX_{actual} was .85 +/- .06 (1 SD; n = 8).

DISCUSSION

The competitive displacement assay is the most sensitive procedure yet for the detection of STX or TTX (Table 1). This assay is able to accurately quantitate ng/ml levels of these toxins in isotonic buffer and in human plasma. It is a simple procedure requiring a liquid scintillation counter as the only major item of equipment. Thus, the competitive displacement assay is a technique for detecting STX and TTX which should be within the capabilities of many laboratories.

The competitive displacement assay detects STX and TTX by their specific binding to the sodium channel. The procedure is analogous to an immunoassay which detects compounds by their specific binding to the IgG molecule. The displacement assay, however, has two advantages over an immunoassay: 1) it does not require the tedious and sometimes difficult development of toxinspecific antibodies, 2) it detects these toxins not on molecular antigenicity tut on the basis of binding to the sodium channel, a characteristic more directly related to toxicity.

The primary limitation of this assay is that it cannot distinguish one sodium channel blocker from another; i.e., STX from TTX. Furthermore, this assay cannot distinguish STX from the other "STX-like" paralytic shellfish poisons produced by dinoflagellates of the genus <u>Gonyaulax</u> (13). Another limitation of the assay is imposed because monovalent cations affect the binding of STX to its receptor (14), making it more difficult to analyze samples of variable salt concentration; for example, urine.

The competitive displacement assay has several possible applications. This assay should be applicable to screening shellfish for paralytic shellfish poisons. The U. S. Food and Drug Administration has stipulated that the maximum allowable concentration of STX in shellfish is 80 u g/100 g shellfish meat. Under the extraction conditions of Schantz, <u>et al.</u> (15), this level of toxicity would result in .4 u g STX/ml extract. The competitive displacement assay is capable of detecting 1/1000th of this concentration which means that shellfish extracts could be diluted 100- to 1000-fold in isotonic HEPES buffer and assayed by the same approach used to analyze buffer samples. As evident from the above paragraph, the results of assaying shellfish could be expressed as "STX-equivalent units" with the understanding that paralytic shellfish poisons in general are being detected and not just STX.

The competitive displacement assay may also have applications in pharmacokinetic studies of STX or TTX concentrations in the blood of intoxicated laboratory animals. Finally, the displacement assay may have applications as a diagnostic or forensic tool in suspected cases of human STX or TTX poisoning.

ACKNOWLEDGEMENTS

The authors thank SP4 Roger P. Pickering for his outstanding technical assistance, Ms. Marian Bloxton for typing the manuscript, and Drs. John Hewetson and John O'Brien for their review of the manuscript.

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FIGURE LEGENDS

FIG. 1. [3H] -STX displacement from rat brain membranes by unlabeled STX (A) and unlabeled TTX (B) in isotonic buffer, pH 7.5. One-half ml of buffer containing the indicated concentration of toxin, .5 ml [3H] -STX (4.35 nM), and .5 ml rat brain membranes (.5 mg protein/ml) were combined and incubated at 4° C for 1 h. The buffer for all three components was 20 mM HEPES, 140 mM NaC1, 6 mM EDTA, pH 7.5. After the 1 h incubation, [3H] -STX binding to membranes was determined as described in Methods. B_{buffer} = membrane-bound[3H]-STX in the presence of buffer containing toxin; B^o_{buffer} = membrane-bound[3H]-STX in the presence of buffer without toxin. Circles without error bars indicate single determinations. Circles within error bars are the mean of six determinations. The error bars indicate +/- 2 standard deviations.

FIG. 2. 38 -STX displacement from rat brain membranes by unlabeled STX (A) and unlabeled TTX (B) in human plasma. Unlabeled STX and TTX were added to aliquots of CPDA-1 human plasma. Each sample of toxin-spiked plasma was filtered though a Whatman GF/F filter to remove cells and other insoluble material. One-half ml of plasma containing the indicated concentration of toxin, .5 ml $\begin{bmatrix} 3H \end{bmatrix}$ -STX (4.35 nH), and .5 ml rat brain membranes (.5 mg 3H STX and rat protein/ml) were combined and incubated at 4°C for 1 h. brain membranes were suspended in 20 mM HEPES, 140 mM NaC1, 6 mM EDTA, pH 7.5. After the 1 h incubation, $\begin{bmatrix} 3H \end{bmatrix}$ -STX binding to membranes was determined as described in Methods. $B_{plasma} = membrane-bound \begin{bmatrix} 3H \end{bmatrix}$ -STX in the presence of plasma containing toxin; $B_{plasma}^{o} = membrane-bound \begin{bmatrix} 34 \end{bmatrix}$ -STX in the presence of plasma without toxin. Circles without error bars indicate single Circles within error bars are the mean of six determinations. determinations. The error bars indicate +/- 2 standard deviations.



[Unlabeled TTK], ng/ml

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TABLE 1

Assay	Minimum detectable concentration of saxitoxin	Minimum detectable concentration of tetrodotoxin
Competitive displacement assay (this paper)	.15 ng/ml ^a	.8 ng/11 <u>a</u>
Sullivan and Iwaoka (16)	.5 ng <u>b</u> , <u>e</u>	
Bates and Rapoport (10)	1.5 ng/ml ^b	·
Shoptaugh <u>et al</u> . (19)	20 ng <u>c,d</u>	
Gershey <u>et al</u> . (17)	200 ng/mlb	
Schantz <u>et al</u> . (15) (mouse bioassay)	200 ng/ml ^a	200 ng/ml ^{<u>a</u>}
McFarren <u>et al</u> . (18)	500 ng/ml ^b	
Nunez <u>et al</u> . (20)		64.2 ng/ml ^a

SENSITIVITY OF ASSAYS FOR SAXITOXIN AND TETRODOTOXIN

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 $\frac{a}{b}$ Measured in buffer. $\frac{b}{c}$ Measured in seafood extract. $\frac{c}{c}$ Sensitivity not given in terms of concentration. $\frac{d}{d}$ Measured on a TLC plate.