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Research Article

Tetrodotoxin Detection by a Surface Plasmon Resonance Sensor in Pufferfish Matrices and Urine

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Tetrodotoxin (TTX) poisoning is most commonly associated with consumption of pufferfish. TTX is a low molecular weight (~319 Da) neurotoxin that selectively blocks voltage-sensitive Na^+-gated ion channels. The standard method accepted worldwide for monitoring TTX toxicity in food matrices is the mouse bioassay. Ethical concerns from live animal testing, low sample throughput, and analytical inaccuracies have led to the need for an alternative method. We have previously established that surface plasmon resonance (SPR) sensors can quantify TTX in aqueous buffer samples by an antibody-based inhibition assay. In this paper, we report the extension of the assay for the detection of TTX in both clinical- and food-relevant matrices. The assay was optimized for application to three relevant complex matrices: pufferfish liver extract, pufferfish muscle extract, and human urine. Matrix effects are discussed and calibration curves are presented. Naturally contaminated pufferfish liver and muscle extracts were analyzed by the SPR method, and the data is compared to liquid-chromatography electrospray-ionization multiple reactions monitoring mass spectrometry (LC/ESI/MRM/MS) data. Ten samples, including three from a poisoning incident, two control monkfish samples, and five toxic pufferfish samples, were analyzed using this method, and the data is compared to LC/ESI/MRM/MS analysis of the samples.

1. Introduction

Tetrodotoxin (TTX) poisoning is most commonly associated with consumption of pufferfish. TTX is a low molecular weight (~319 Da) neurotoxin that has been discovered in several animal species including pufferfish, newts, toads, octopus, arrow worms, and xanthid crabs [1]. Several analogues of the base molecule have also been identified, all of which exhibit the toxic effect of selectively blocking voltage-sensitive Na^+-gated ion channels [2]. Symptoms of TTX poisoning are manifested in humans as perioral numbness and paresthesia, distal limb numbness and paresthesia, ataxia, dizziness, and muscle weakness. In severe cases respiratory muscle paralysis, coma, hypotension and cardiac dysrhythmias occur with fatal consequences [3]. The lethal dose of TTX for mammals that causes death in 50% of a group of test animals (LD_{50}) is 2–10 μg/kg intravenously and 10–14 μg/kg subcutaneously [4].

The most common source of TTX poisoning is the Japanese pufferfish (*Takifugu* spp.) which preferentially accumulates TTX in high concentrations in the skin, liver, ovary, and intestines [5]. However, the muscle can also contain lethal concentrations [6]. The muscle is often consumed as a delicacy in Japan and requires special processing from trained and licensed individuals [3]. In recent years, several cases of TTX poisoning in the United States and Mexico have occurred from the consumption of pufferfish from the Atlantic Ocean, Gulf of Mexico, and Gulf of California [7].

For diagnosis of poisoning incidents, the suspected source (i.e., meal remnant) is often not available for analysis. Thus, it is necessary to also be able to analyze clinical samples collected from the patient. Two independent studies have been published, reporting concentrations of TTX in the urine and blood of patients from poisoning incidents using chromatography-based methods [8, 9]. A study by Kawatsu et al. quantified TTX in urine by immunoaffinity chromatography and high performance liquid chromatography (HPLC) in twelve samples from six poisoned patients collected from 1989 to 1996. They reported concentrations

of TTX in urine ranged from 6 to 100 ng/mL and required a sample volume of 100 mL for analysis [8]. In another study, Tsia et al. quantified TTX in urine and blood by liquid chromatography-mass spectrometry (LC-MS) from six poisoned fishermen. The concentration of TTX in blood ranged from 1.4 to 13 ng/mL and from 15 to 110 ng/mL in urine. The samples were collected approximately 10 hours after ingestion of the toxic fish and one patient died shortly after eating the toxic fish. The patient that died had TTX levels of 104 ng/mL in urine and 13 ng/mL in blood. The study showed that the concentration of TTX in urine is substantially higher than in blood 10 hours after ingestion indicating that TTX is easily metabolized and excreted in urine [9]. Therefore, urine is the preferable clinical matrix for testing for TTX poisoning.

The standard method accepted worldwide for determining TTX toxicity in food matrices is the mouse bioassay [10]. Ethical concerns from live animal testing [11] and the cost and labor, low sample throughput, and analytical inaccuracies associated with variances from live animals and sample matrices [12] have led to the need for an alternative method to the mouse bioassay. Biological methods for determining TTX toxicity such as receptor binding assays [13], cytotoxicity tests [14], and electrophysiological assays [15] have been demonstrated. Also, analytical methods such as HPLC and mass spectrometry can identify and quantify TTX and its naturally occurring congeners [16, 17]. Immunological methods, such as enzyme-linked immunosorbant assay (ELISA), have become popular in recent years because they are inexpensive, sensitive, and selective. An inhibition-type ELISA has been previously reported for quantification of TTX using a commercially available antibody [5, 18, 19]. Also, we have previously reported an inhibition immunoassay for quantification of TTX using a surface plasmon resonance (SPR) sensor [20]. SPR sensors can perform faster detections with high sensitivity and specificity. The advantages of SPR sensors as compared to other biosensor technologies is their ability to provide label-free and real-time detections for direct and continuous monitoring of biomolecular interactions [21, 22]. In our previous study, a surface was developed that specifically bound TTX antibody and resisted nonspecific adsorption from nonspecific antibodies and bovine serum albumen (BSA) [20]. The optimized inhibition assay demonstrated a detection limit of ~0.3 ng/mL in buffer, and the biochip was reproducibly regenerated for at least ten sample detection cycles.

In this work, we report the extension of the inhibition-based immunoassay by SPR sensor to the detection of TTX in both clinical and food relevant matrices. The assay was optimized for application to three complex matrices: pufferfish liver, muscle extracts, and human urine. The effect of each unique matrix on the assay and the surface is discussed. Calibration curves based on samples spiked with TTX standard are presented for each matrix. Samples of naturally contaminated pufferfish liver and muscle extracts were analyzed by the SPR method and compared to analysis from liquid-chromatography electrospray-ionization multiple reactions monitoring mass spectrometry (LC/ESI/MRM/MS). Ten samples, including three from a poisoning incident, two control monkfish samples, and five toxic pufferfish samples were also analyzed and compared to LC/ESI/MRM/MS analysis of the samples.

2. Materials and Methods

2.1. Materials. TTX was produced by Sankyo Co, Ltd. (Tokyo, Japan). Monoclonal rabbit antibody for tetrodotoxin (anti-TTX) was purchased from Hawaii Biotech, Inc. (Aiea, HI, USA) [5]. Lyophilized human urine, bovine serum albumen (BSA), phosphate buffered saline (PBS) (0.01 M phosphate, 0.138 M sodium chloride, 0.0027 M potassium chloride, pH 7.3), sodium acetate, hydrochloric acid, acetic acid, sodium hydroxide, and sodium chloride were from Sigma-Aldrich (St. Louis, MO, USA). Hydroxyl-terminated oligoethylene glycol(OEG)-alkanethiol (HS-$(CH_2)_{11}$-$(O(CH_2)_2)_4$-OH) and amine-terminated OEG-alkanethiol (HS-$(CH_2)_{11}$-$(O(CH_2)_2)_6NH_2$) were purchased from ProChimia (Gdansk, Poland). Formaldehyde (37%) was purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Naturally contaminated pufferfish muscle and liver extracts as well as samples collected during a toxic pufferfish outbreak case were provided by the FDA Center for Food Safety and Applied Nutrition (CFSAN).

2.2. Antibody-Based Inhibition Assay for Quantification of TTX by SPR Sensor. The methods and instrumentation used in this study were detailed in a previous publication that presents the development of an antibody-based inhibition assay for detection of TTX by SPR sensor [20]. The SPR sensor used in this work was a custom-built instrument based on the Kretschmann geometry of the attenuated total reflection (ATR) method and wavelength interrogation. For this SPR sensor, a 1 nm SPR wavelength shift at 750 nm to 751 nm represents a surface coverage of ~161 pg/mm^2 for proteins. The SPR biochips that exhibit specific binding of anti-TTX and resist protein fouling were coated with a self-assembled monolayer (SAM) consisting of amine terminated OEG alkanethiol and a hydroxyl terminated OEG alkanethiol. TTX molecules were then covalently linked to the exposed amine terminal group in the mixed SAM surface in an orientation that favors anti-TTX binding. The ratio of amine to hydroxyl terminated OEG alkanethiols and TTX chemistry were previously optimized to maximize specific anti-TTX binding, while minimizing nonspecific protein binding. An inhibition assay was used with the SPR sensor and functional SPR biochip to quantitatively detect TTX. The inhibition assay was performed by mixing a fixed concentration of anti-TTX with samples containing unknown TTX concentrations or known standards for determination of calibration curves. During the incubation period, TTX in the sample will bind to the anti-TTX paratope, reducing the number of antibodies with antigen-free binding sites. The samples were then flowed over the SPR biochip, and the SPR sensor detects the amount of antigen-free anti-TTX that binds to the surface. The SPR sensor response was directly related to the amount of antigen-free anti-TTX in the sample and therefore inversely related to the amount of TTX. Thus the maximum sensor response corresponds

to zero TTX in the sample, and a zero sensor response corresponds to total inhibition of anti-TTX binding, therefore a concentration of TTX outside of the quantifiable range of the method. Calibration curves for each detection matrix were calculated by normalizing the response from standards containing 10 pg/mL to 10 μg/mL TTX incubated with a fixed concentration of anti-TTX to the maximum response of the anti-TTX at the same concentration. Matrix effects on the anti-TTX binding were taken into account and appropriate standards were developed for each matrix and are discussed in this paper. The unit for normalized data was percent inhibition, which refers to the percentage of total response divided by the maximum response from only the antibody. Detection limit and detection range are commonly expressed as percent inhibitory concentration, which refers to the concentration of inhibitory molecule (TTX) needed to lower the maximum response to a certain percentage. For the work, the detection limit was 20% inhibitory concentration (IC_{20}) and the quantifiable range was IC_{20} to 80% inhibitory concentration (IC_{80}).

2.3. Pufferfish Tissue Extraction Method. Either whole organs (if <5 g) or 5 g subsamples of homogenized tissues were extracted twice with 10 mL of 1% acetic acid in methanol. Samples were homogenized using a motorized tissue homogenizer (Polytron PT 10–35 with a 12 mm generator, Kinematica AG, Switzerland), centrifuged, and combined supernatants were concentrated to <1 mL under vacuum. Samples were redissolved in 5 mL of 1% (v/v) acetic acid in High Performance Liquid Chromatography (HPLC) grade water, then defatted with chloroform. To do this, 5 mL of chloroform was added to samples, vortexed to mix, then separated by centrifugation. The top aqueous layer was saved, 5 mL of additional acidified water was added to the lower chloroform layer, and the process was repeated. The supernatants were combined giving an extract equivalent to 0.5 g tissue/mL. According to Chen and Chou [23], this method provided >90% extraction efficiency. In previous experiments, we spiked homogenized puffer fish muscle ($n = 3$), liver ($n = 2$), and phosphate buffered saline ($n = 2$) with 10 μg/mL TTX and achieved an average of 86.4 ± 18.9% recovery (data not shown).

2.4. Analysis of Samples by LC/ESI/MRM/MS. Initial toxin separations were performed according to Negri et al. [24]. An aliquot of each extract was filtered using a 0.22-μm cellulose acetate syringe filter, adjusted to a final concentration of 25% acetonitrile, and 10 μl was injected into an Agilent 1100 HPLC system equipped with a 250 mm × 2 mm inner diameter column packed with 5-μm TosoHaas TSK-GEL Amide-80 material. Toxins were eluted with 0.3 mL·min^{-1} of acetonitrile : HPLC grade water (70 : 30, v/v) with 5 mM ammonium formate and 26.5 mM formic acid. Mass spectrometry was performed with an API5000 (Applied Biosystems/MDS Sciex, Framingham, MA) equipped with a turbospray ionization source and operated in positive ion mode. The following instrument parameters were used: source temperature, 300°C; curtain gas (CUR), 45L N2/hr; nebulizer gas (GS1), 40L N^2/hr; turbo heater gas (GS2), 35L N^2/hr; spray voltage, 3200 V. Multiple reaction monitoring (MRM), in which the parent ion for each toxin is fragmented and monitored for the appearance of specific fragments characteristic of that compound, was used for measurement of the toxins. The MRM data acquisition method was separated into two periods, monitoring three fragmentation channels (MRM's) each. The three reactions monitored for TTX were from the decomposition of the protonated TTX molecule $[M+H]^+$ at *m/z* 320 fragmenting to ions at *m/z* 302, 256, and 162. A similar approach was used successfully to quantify TTX by Shoji et al. [17]. Dwell time for each reaction was 200 msec and entrance potential (EP) was 10 V for each. Declustering potential (DP), collision energy (CE), and exit potential (CXP) were independently optimized for each reaction using Analyst software (v 1.4.2) (Applied Biosystems/MDS Sciex, Framingham, MA). TTX was quantified by linear regression of the sum of the three fragment ions, using standards of the following concentrations: 1, 10, 100, 1,000 and 10,000 ng/mL TTX. Standards were diluted in 1% aqueous acetic acid with 25% acetonitrile.

3. Results and Discussion

3.1. Assay Development for Complex Media. Both the hydroxyl-terminated OEG-alkanethiol and amine-terminated OEG-alkanethiol components of the mixed SAM contain ethylene glycol units, which have been shown to minimize nonspecific binding of proteins [25]. While significant progress has been made in creating sensor surfaces resistant to protein adsorption, it is still difficult to produce surfaces that completely resist adsorption from real-world complex media [26]. Several nonfouling surface platforms have been investigated such as poly(ethylene glycol-) (PEG-) based [27, 28] or zwitterionic materials [29, 30]. The TTX immobilized sensing surface has previously been shown to be nonfouling to 1 mg/mL BSA and 10 μg/mL anti-hCG, using standard PBS buffer solution at pH 7.4. Using the previously established assay conditions the sensing surface was tested for nonspecific binding from complex media including urine, pufferfish liver extract, and pufferfish muscle extract. The samples matrices exhibited minimal nonspecific binding.

For detection in complex matrices, 200 μg/mL BSA was added to the assay running buffer and sample diluent. BSA is routinely added to immunoassay running buffers to reduce nonspecific binding and protect protein conformation to reserve their biological activity. However, when urine samples were diluted using 200 μg/mL BSA in PBS at pH 7.4, the 10% urine sample produced an unusual response (Figure 1), and the baseline did not restabilize for at least 20 minutes after injection. Injection of only BSA or 10% urine in PBS at pH 7.4 produced a step response corresponding to a bulk refractive index change. Also, 10% pufferfish liver and muscle extract, diluted using 200 μg/mL BSA in PBS, produced responses similar to the response from samples diluted in only buffer. Testing the pH of the 10% pufferfish extract diluted in PBS revealed that the final pH of the diluted sample was actually ~4-5. The pufferfish extract was 1% v/v acetic acid, which was used for dissociating TTX from tissue

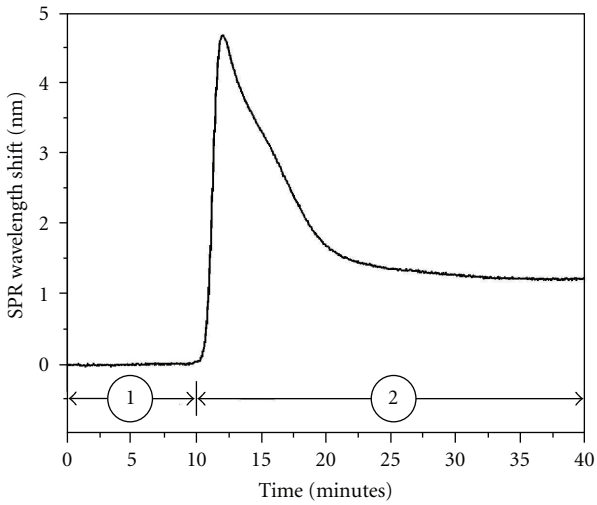

Figure 1: SPR sensorgram showing sensor response to (1) PBS at pH 7.4 with 200 μg/mL BSA for 10 minutes, followed by (2) 10% urine in PBS at pH 7.4 with 200 μg/mL BSA for 30 minutes. The spike in the data at 10 minutes is an unexpected response and does not stabilize until 20 minutes after injection. A 1 nm SPR wavelength shift at 750 nm to 751 nm represents a protein surface coverage of ~161 pg/mm^2.

and provides stable pH for the TTX molecules stored in the extract. Upon further testing, the nontypical response from the urine samples spiked with TTX and diluted with BSA was found to be associated with the pH of the running buffer. Changing the buffer from PBS at pH 7.4 to sodium acetate (SA) buffer at pH 4.5 eliminated the nontypical response caused by the combination of TTX immobilized surface, BSA, and urine (Figure 2(c)). It is hypothesized that TTX will associate strongly with proteins at neutral pH because of the strong charge associated with the guanidinium group. It is well known that in order to extract TTX from tissue, it requires solutions at low pHs [23]. The last modification to running buffer was increasing the buffer capacity, since the 10% pufferfish extract samples were outside of the buffer capacity of the PBS. The PBS was 10 mM buffer components, so the buffer concentration was increased to 100 mM in order to have enough buffering capacity for 10% samples of pufferfish extract. The optimized running buffer for complex media was 100 mM SA buffer at pH 4.5 with 50 mM NaCl and 200 μg/mL BSA (SAB). The optimized assay conditions for complex media resulted in surfaces that remain nonfouling to 10%, 1%, and 0.1% solutions of each matrix (Figure 2). Thus, the assay conditions were suitable for direct detection of antibody binding to the TTX-immobilized sensing surface. The response seen at 10 minutes and 25 minutes was a bulk refractive index shift caused by the difference in refractive index of the running buffer and diluted complex media.

3.2. Detection of TTX in 100 mM Sodium Acetate Buffer at pH 4.5. Changing the pH has the most significant effect on the calibration curve, because of the antibody-antigen binding dependence on pH. Typically antibodies have the highest response at pHs close to physiological pH of 7.4 in solution and are less active at higher or lower pH, however, immobilization of antibodies on surfaces can affect optimum protein activity. In the case of the anti-TTX, the overall response from 2 μg/mL anti-TTX in PBS at pH 7.4 was ~7.5 nm and in SA buffer at pH 4.5 was reduced to ~ 1.6 nm of SPR wavelength shift. The response of anti-TTX at the lower pH is sufficient for an antibody inhibition assay. However, lower antibody response will affect the calibration curve. Figure 3(a) shows averaged normalized SPR sensorgrams corresponding to the detection of various concentrations of TTX in SAB. Figure 4 shows the calibration curve for normalized wavelength shift versus concentration of TTX in sample with error bars. From the data shown in Figure 4, the IC_{80}, IC_{50}, and IC_{20} for samples incubated with 2 μg/mL anti-TTX are 226 ng/mL, 38 ng/mL, and 10 ng/mL, respectively. Thus, the detection limit corresponds to the IC_{20} of 10 ng/mL. The IC_{80}, IC_{50} and $IC_{20}$for detection TTX in phosphate buffered saline (PBS) at pH 7.4 with 2 μg/mL anti-TTX was 74, 13, and 2 ng/mL [20]. The IC_{80}, IC_{50}, and IC_{20} for detection in PBS at pH 7.4 with 1 μg/mL anti-TTX was 50, 6, 0.3 ng/mL and the maximum response from the 1 μg/mL anti-TTX reference in PBS was 3.74 nm [20]. In the previous work, an inhibition assay with 1 μg/mL anti-TTX provided a detection limit approximately one order of magnitude better than 2 μg/mL anti-TTX. The optimized assay for the complex matrices was run at pH 4.5. Thus, the higher concentration of antibody was chosen because of the lowered antibody response due to antibody-antigen binding dependence on pH.

3.3. Detection of TTX in Complex Media. Figure 3 shows the averaged normalized SPR sensorgrams corresponding to the detection of various concentrations of TTX in buffer and complex media. For each of the complex media, the data was normalized to the response from 2 μg/mL anti-TTX in SAB. While it is important to determine calibration curves for unique complex matrices, it is also important to normalize the data to a reproducible standard. For a unique detection matrix, it may not be possible to have a toxin-free sample, so normalizing data to a buffer standard is necessary. In Figures 3(b), 3(c), and 3(d), the sensorgrams show a substantial reponse at 10 minutes and 25 minutes, which does not correspond to the antibody response. This response is a bulk refractive index shift caused by the change in refractive index of the 10% sample compared to the running buffer. The bulk refractive index shift has no residual effect on antibody binding on the sensing surface. In Figures 3(b), 3(c), and 3(d) there is one sensorgram that does not exhibit the bulk refractive index response that corresponds to the 10% sample matrix, these sensorgrams are the antibody reference in buffer with no complex matrix.

Figure 4 is the calibration curves showing normalized wavelength shift versus concentration of TTX in buffer and complex media. The IC_{80}, IC_{50}, and IC_{20} should correspond to normalized values of 0.2, 0.5, and 0.8, respectively. From the data shown in Figure 4, the IC_{80}, IC_{50}, and IC_{20}

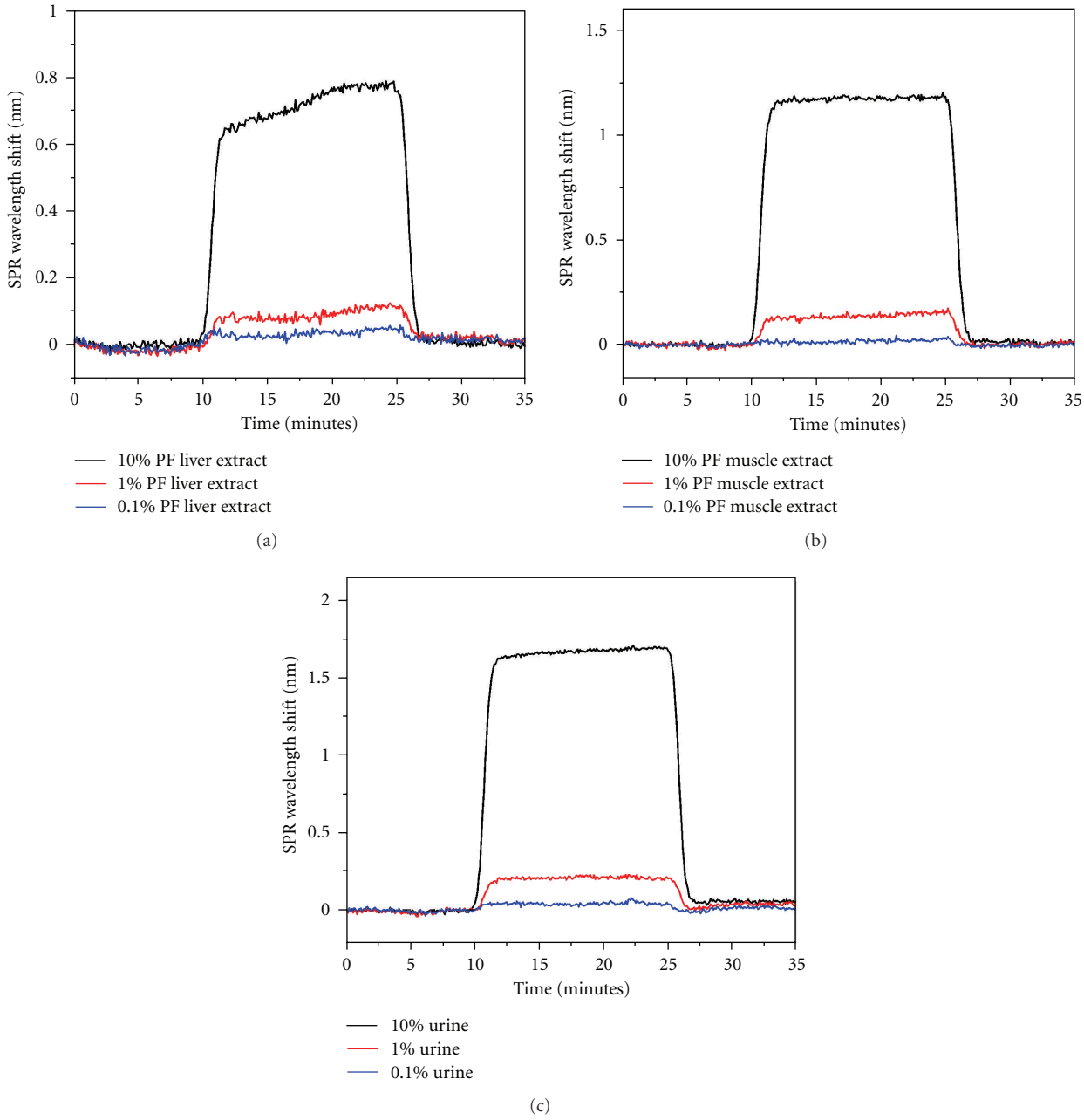

Figure 2: Control experiments demonstrating that the sensor surfaces are relatively nonfouling to the 10%, 1%, and 0.1% dilutions of complex media: (a) pufferfish liver extract, (b) pufferfish muscle extract, and (c) urine. The complex media were diluted in 100 mM sodium acetate buffer at pH 4.5 with 50 mM NaCl and 200 μg/mL BSA (SAB). A 1 nm SPR wavelength shift at 750 nm to 751 nm represents a protein surface coverage of ~161 pg/mm^2.

for 10% pufferfish liver extract samples incubated with 2 μg/mL anti-TTX were 95 ng/mL, 22 ng/mL, and 1 ng/mL, respectively. So the detection limit corresponds to the IC_{20} of 1 ng/mL. The IC_{80}, IC_{50}, and IC_{20} for 10% pufferfish muscle extract samples incubated with 2 μg/mL anti-TTX were 200 ng/mL, 32 ng/mL, and 6 ng/mL, respectively. So the detection limit corresponds to the IC_{20} of 6 ng/mL. The IC_{80}, IC_{50} and IC_{20} for 10% urine samples incubated with 2 μg/mL anti-TTX were 640 ng/mL, 53 ng/mL, and 17 ng/mL, respectively. Thus, the detection limit corresponds to the $IC_{20}$of 17 ng/mL.

In the 10% urine matrix, a higher antibody response was seen which results in a calibration curve that was shifted above the normalized value of one (maximum reference antibody response), when the detections were referenced to antibody response in buffer. Figure 2(c) shows that there was no significant nonspecific binding from the 10% urine matrix. Also, 10% urine in SAB does not cause the pH to deviate

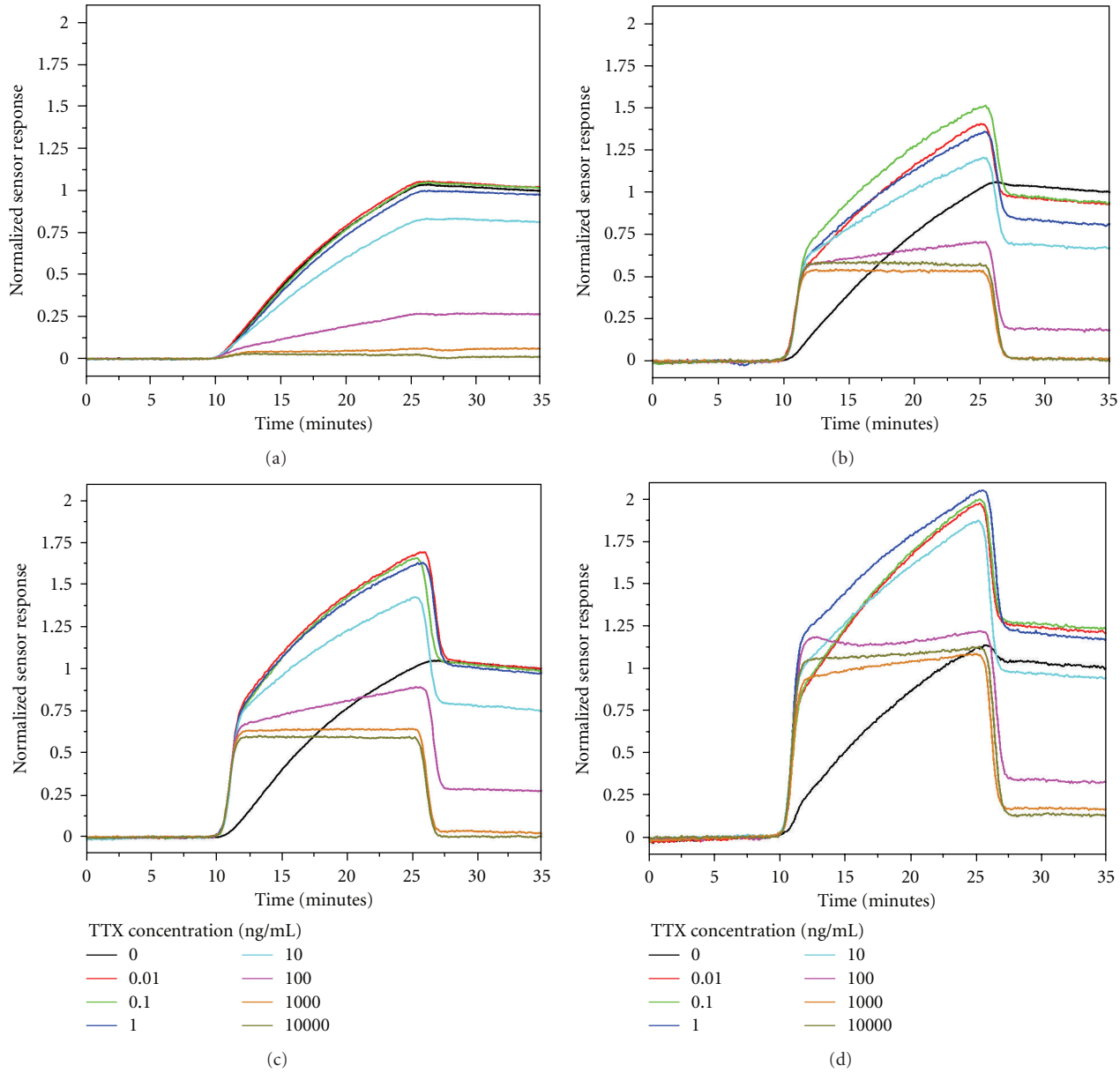

Figure 3: Normalized reference compensated SPR sensorgrams showing sensor responses for 2 μg/mL of anti-TTX antibody incubated with various concentration of TTX in (a) 100 mM sodium acetate buffer at pH 4.5 with 50 mM NaCl and 200 μg/mL BSA (SAB), (b) SAB at pH 4.5 with 10% pufferfish liver extract, (c) SAB at pH 4.5 with 10% pufferfish muscle extract, and (d) SAB at pH 4.5 with 10% urine. Samples containing complex media were normalized to an antibody reference in buffer. The data shown is the average from three detection cycles.

from the buffer pH of 4.5. But as shown in Figure 5(a), the 2 μg/mL anti-TTX response in 10% urine diluted in SAB was 1.75 nm, while the response in 10% water in the same buffer was 1.38. If the calibration curve is normalized to anti-TTX reference with 10% urine (Figure 5(b)), the calibration curves normalizes with maximum close to 1 and corresponds well with the detection curve for SAB; however, the calibration curve normalized to reference antibody in 10% water has an approximately 25% higher maximum response. From the data shown in Figure 5(b), the IC_{80}, IC_{50} and IC_{20} for samples incubated with 2 μg/mL anti-TTX and normalized to a reference with 10% urine were 263 ng/mL, 27 ng/mL, and 3/mL, respectively, compared to 640 ng/mL, 53 ng/mL, and 17 ng/mL, respectively, for the same data normalized to an antibody reference in SAB. However, for the data normalized to antibody reference in SAB, the maximum is ~1.2 instead of 1, so the IC_{80}, IC_{50}, and IC_{20}should correspond to a value of 0.24, 0.6, and 0.96 instead of 0.2, 0.5, and 0.8. If the values are recalculated using the actual maximum of 1.2 instead of 1, then the values are the same as for data referenced to antibody in 10% urine. Since it may be difficult to obtain toxin-free sample, the reference antibody needs to be measured in a standard system (buffer). However, it is necessary to determine quantification from calibration

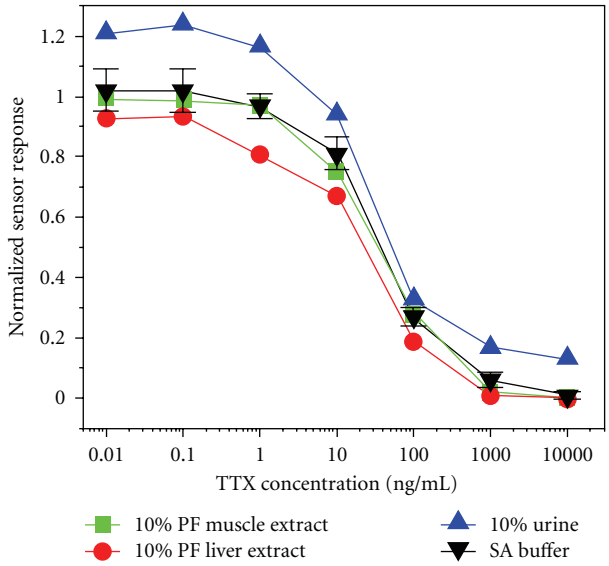

Figure 4: Calibration curves for the detection of TTX in SAB at pH 4.5 and pufferfish liver extract, pufferfish muscle extract, and urine diluted to 10% in SAB at pH 4.5. Normalized SPR resonant wavelength shift versus the concentration of TTX incubated with 2 μg/mL anti-TTX for 15 minutes by an inhibition assay. The data from the sensorgram was normalized to the maximum anti-TTX response in SAB.

curves for a specific matrix as opposed to calibration curves for standards detected in buffer. In the case of detection of TTX in urine, the urine matrix affects the antibody response and it is necessary to determine the IC_{80}, IC_{50}, and IC_{20} from the actual maximum and minimum values instead of using 1 and 0 as the maximum and minimum value.

3.4. Analysis of Naturally Contaminated Pufferfish Samples. Two samples of naturally contaminated pufferfish extract were prepared and analyzed by LC/ESI/MRM/MS at the US FDA CFSAN and were analyzed by inhibition assay with SPR sensor at the University of Washington laboratory. The samples were prepared from the liver and muscle from the same species of naturally toxic (with TTX) pufferfish. The pufferfish typically accumulates much higher concentration of TTX in the liver than in the muscle [1]; however, the muscle in often consumed as food. The relevant concentrations of TTX in the samples can be much higher than the quantifiable region of the developed TTX assay, so the sample is run at three dilution factors (10%, 1%, and 0.1%). By testing three dilutions of the sample, the assay can cover three to four orders of magnitude for concentrations of TTX. Figure 6(a) shows the SPR response for 10%, 1%, and 0.1% naturally contaminated pufferfish liver, and the corresponding calibration curve for detection in pufferfish liver. The 0.1% dilution of pufferfish liver had a detection that fell between the IC_{20} and IC_{80} (the quantifiable range). The assay showed that the concentration in the sample was 25 ng/mL ± 8 ng/mL, which corresponds to a concentration in the undiluted sample of 25 μg/mL ± 8 μg/mL. This sample had an extraction dilution factor of 2.04, so 2.04 mL of extract is equivalent to 1 g of tissue; therefore, the detected value is 5100 μg TTX/100 g of tissue. This value corresponds well with the value for this sample determined by LC/ESI/MRM/MS, which is 4803 μg/100 g of tissue. The other dilutions had responses more that 80% inhibition. Thus, the concentration was too high to reliably quantify. Figure 6(b) shows the SPR response for 10%, 1%, and 0.1% naturally contaminated pufferfish muscle and the corresponding calibration curve for detection of TTX in pufferfish muscle. The 1% dilution of pufferfish muscle had a detection that fell between the IC_{20} and IC_{80}. The concentration in the sample was 63 ng/mL ± 14/mL, which corresponds to a concentration in the undiluted sample of 6.3 μg/mL ± 1.4 μg/mL. This sample had an extraction dilution factor of 2.14, so 2.14 mL of extract is from 1 g of tissue; therefore, detected value is 1285 μg/100 g of tissue. This value is approximately double the value for this sample determined by LC/ESI/MRM/MS, which is 582 μg/100 g of tissue. The 10% dilution had a response that is more than 80% inhibition and the 0.1% dilution had a response with less than 20% inhibition, so the concentrations were outside of the quantifiable range.

3.5. Analysis of Poisoning Incident Samples by SPR Sensor and Compared with LC/ESI/MRM/MS. Ten samples of fish muscle tissue extract were analyzed by the SPR sensor assay in the laboratory at the University of Washington and by LC/ESI/MRM/MS at the US FDA CFSAN laboratory. These samples were collected as part of an outbreak of puffer fish poisoning (PFP) that occurred in 2007. The samples were run at 10%, 1%, and 0.1% dilutions in order to assure that one of the dilutions would fall within the quantification range of the calibration curve. The toxin concentration was calculated by normalizing the binding response from the antibody-sample mixture to the response from only the antibody in buffer. Then normalized signal was fit to the quantifiable range of the calibration curve that was collected in pufferfish muscle extract. The results for quantification of TTX by the SPR sensor assay compared to quantification by LC/ESI/MRM/MS are shown in Table 1. By testing three sample dilutions, the detection range for these tests was from 0.1 to 200 μg/mL or assuming an extraction dilution factor of 2, 20 to 40,000 μg/100 g of tissue. If the normalized response was greater than the IC_{20} for a 10% sample dilution, then the concentration of TTX in the sample was less than 0.1 μg/mL and was characterized in Table 1 as "<LOD." Assuming an extraction dilution factor of 2, then the sample had less than 20 μg/100 g of tissue. The detection limit for this matrix and antibody concentration of 20 μg TTX/100 g of tissue was comparable to the mouse bioassay which has a detection limit of 0.2 μg/mL or 40 μg TTX/100 g [31]. While there is no action level for TTX provided by the US FDA, the action level for saxitoxin (STX), responsible for paralytic shellfish poisoning and similar in pharmacology and potency to TTX [32], is 80 μg/100 g of tissue and can be used for comparison. The data from the SPR sensor assay corresponded to quantification determined by LC/ESI/MRM/MS by average within ~29% for 10% sample

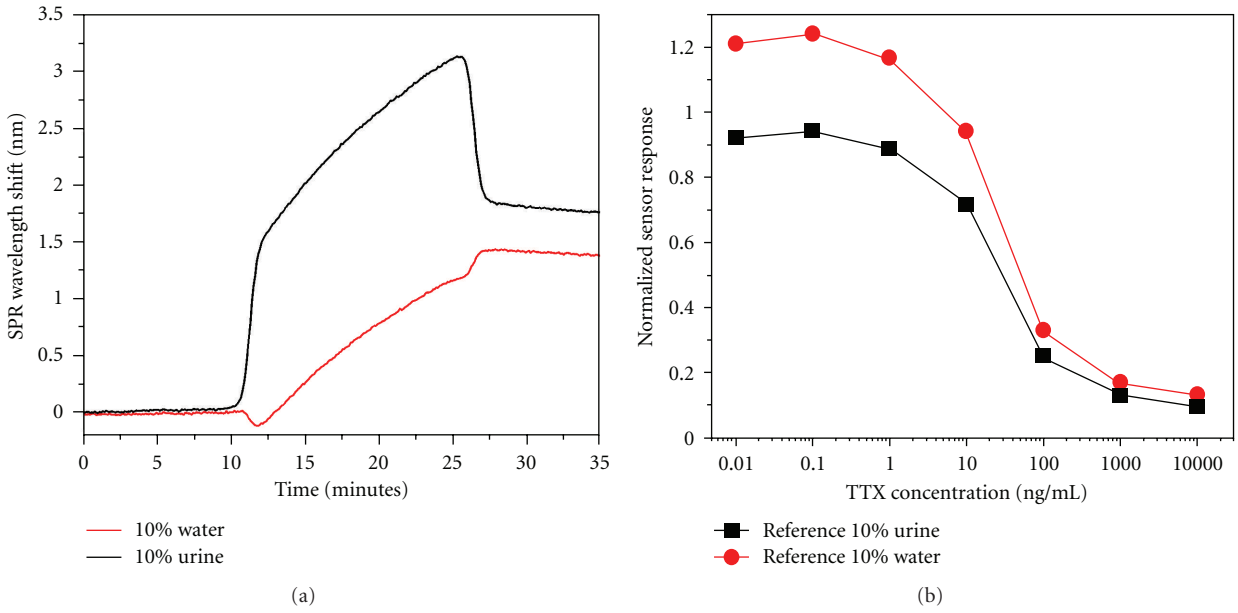

Figure 5: The effect of urine on antibody response. (a) SPR sensorgrams showing sensor responses for 2 μg/mL of anti-TTX in SAB at pH 4.5 with 10% urine or 10% water. (b) Calibration curves showing normalized wavelength shift versus concentration of TTX in sample for one set of data normalized to either reference antibody response with 10% urine or 10% water. The reference antibody response in urine was ~25% higher than in buffer without urine. A 1 nm SPR wavelength shift at 750 nm to 751 nm represents a protein surface coverage of ~161 pg/mm^2.

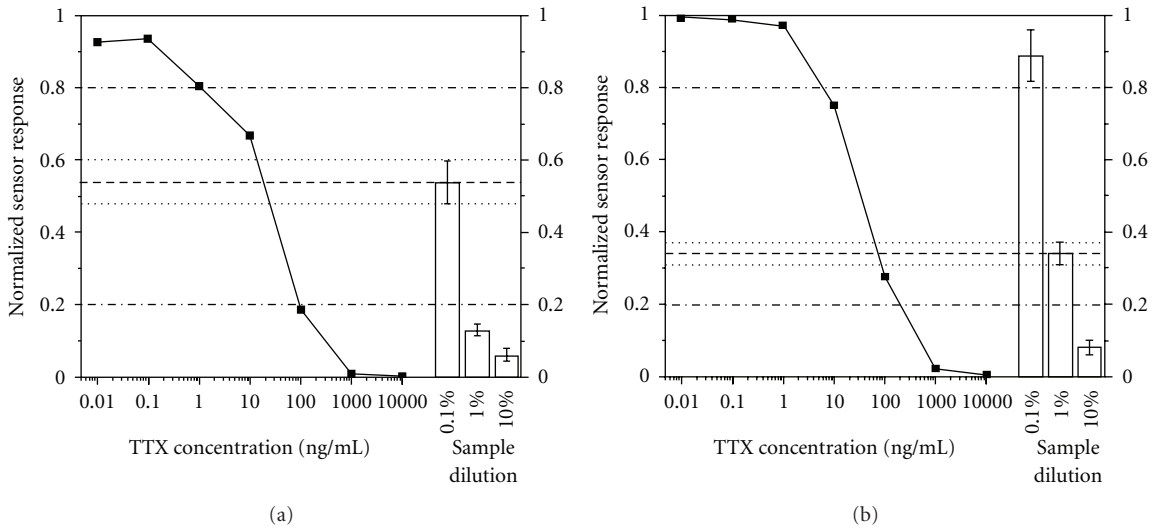

Figure 6: Analysis of (a) naturally contaminated puffer fish liver sample and (b) naturally contaminated puffer fish muscle sample.

dilutions and ~57% for 1% sample dilutions. For all but one sample that provided a detectable concentration of TTX, the SPR method predicted a concentration lower than determined by LC/ESI/MRM/MS. For samples that were quantified with 10% and 1% sample dilutions, the 10% sample always predicted a concentration higher than 1% sample by an average of ~23%. The lower predicted values for the 1% samples compared to 10% samples may be caused by a matrix effect, since the 1% samples were quantified by comparison to the calibration curve produced from 10% pufferfish liver in SAB. The two samples that had the highest concentration of TTX, "Raw Puffer 3" and "Raw Puffer 4," had values for the 10% dilution that were below the IC_{80}, but still above the IC_{90}, the predicted values from this data still corresponded well with the values from the 1% dilution and have good predictive value.

Table 1: Results of TTX in ten fish tissue extract samples measured by an SPR biosensor assay compared with LC/ESI/MRM/MS data.

Source	Sample dilution	SPR (μg/mL extract)	Extraction dilution	SPR (μg/100 g sample)	LC/ESI/MRM/MS (μg/100 g sample)
Cooked Fish Flesh 1	10%	1.6 ± 0.4	2.01	322 ± 80	374
	1%	1.2 ± 0.4		241 ± 80	
Cooked Fish Flesh 2	10%	2.5 ± 1.2	1.95	488 ± 234	655
	1%	1.8 ± 0.3		351 ± 59	
Soup Broth	10%	1.2 ± 0.4	1.97	236 ± 79	361
	1%	0.8 ± 0.2		158 ± 39	
Raw Puffer 1	10%	<LOD	1.89	ND	10
Raw Puffer 2	10%	2.4 ± 0.6	1.89	454 ± 113	214
	1%	1.8 ± 0.4		340 ± 76	
Raw Puffer 3	10%*	4.5 ± 0.9	1.86	837 ± 167	876
	1%	4.4 ± 1.4		818 ± 260	
Raw Puffer 4	10%*	4.2 ± 0.3	2.01	844 ± 60	961
	1%	3.8 ± 0.5		764 ± 101	
Raw Puffer 5	10%	<LOD	2.02	<LOD	10
Monkfish 1	10%	<LOD	1.78	<LOD	ND
Monkfish 2	10%	<LOD	1.98	<LOD	ND

* Below IC_{80}, above IC_{90}, <LOD: below limit of detection, ND: not detected.

4. Conclusions

The optimized assay was successfully used to detect TTX in naturally contaminated samples of pufferfish liver and pufferfish muscle. The data corresponded well with the concentration of TTX in the samples determined by LC/ESI/MRM/MS, demonstrating the ability of the TTX inhibition assay by SPR sensor to detect and quantify TTX in real samples.

The detection of TTX in the complex matrices of human urine, pufferfish liver extract, and pufferfish muscle extract required optimization of the running buffer. The optimized buffer conditions for these complex media were determined to be 100 mM sodium acetate buffer at pH 4.5 with 50 mM NaCl and 200 μg/mL BSA. Changing the pH of the assay from 7.4 to 4.5 caused the overall antibody response to be lowered from ~7.5 nm to ~1.6 nm of SPR wavelength shift for 2 μg/mL anti-TTX. The calibration curves for pufferfish liver extract and pufferfish muscle extract corresponded well with the calibration curves for detection in buffer. However, the calibration curve for urine normalized to antibody response in buffer had a ~25% higher maximum response than the calibration curve in buffer. If the calibration curve for detection in urine was normalized to the antibody response in 10% urine then the calibration curve corresponds well with that for detection in buffer. Control experiments show that there in no significant nonspecific response from any of the complex media using the optimized assay buffer, and the pH of the 10% urine sample is the same as the buffer. Thus, the higher antibody response was caused by the urine matrix, resulting in the shifted calibration curve for data normalized to a reference antibody in buffer. The optimized inhibition immunoassay for detection of TTX in complex matrices using an SPR sensor correlated well with analysis by LC/ESI/MRM/MS. The developed method provides a robust and regenerable assay for the sensitive and quantitative detection of TTX in complex matrices.

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