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Study of a toxin–alkaline phosphatase conjugate for the development of an immunosensor for tetrodotoxin determination

Abstract This paper describes a direct competitive immunoenzymatic spectrophotometric assay (ELISA) for tetrodotoxin (TTX) determination and the adaptation of this method for use in an electrochemical assay format. The novelty of this work involves the use of the antigen labelled with alkaline phosphatase (AP); this conjugate was prepared in our laboratory as there is no commercially available conjugate of any kind for TTX. The new conjugate was characterized in terms of its affinity for the specific antibody as well as the residual concentration and the residual activity of the enzyme (AP) incorporated as label. The proposed method based on the new conjugate showed satisfactory results for TTX determination: for the spectrophotometric method the dynamic range was 4–15 ng mL^{-1} with a limit of detection (LOD) of 2 ng mL^{-1} (R=0.9247), whereas for the electrochemical protocol the dynamic range was 2–50 ng mL^{-1} and the LOD was 1 ng mL^{-1}.

Keywords Immunoassay/ELISA · Bioanalytical methods · Electroanalytical methods · Toxin–alkaline phosphatase conjugate · Seafood toxin

Introduction

Every year a large number of people fall victim to some sort of poisoning induced after ingestion of low molecular weight marine toxins. Several families of organisms are responsible for this poisoning and the symptoms after ingestion of contaminated shellfish are varied and dependent largely on the concentration of toxin in the shellfish and the amount consumed [1]. Given this situation,

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screening of raw materials is desirable in order to ascertain the quality of products.

One of the most lethal of the seafood toxins is tetrodotoxin (TTX) which is fatal to humans and other mammals, birds, most fish and other marine creatures. It is produced by the fish family of *Tetradontidae* (such as *Sphoeroides rubripes*, the Japanese puffer fish known as "fugu") where it is primarily concentrated in the skin, gonads, liver and intestines, although the flesh may be hazardously toxic. Outbreaks of TTX food poisoning have been reported in various countries, particularly Japan, where puffer fish is considered a delicacy [2–5]; other reported cases of poisonings, including fatalities, have involved puffer fish from the Atlantic Ocean, Gulf of Mexico and Gulf of California. The level of toxicity is seasonal, and, in Japan for example, fugu is served only from October through March. TTX is a stable toxin unaffected by cooking or freezing [6]. Its metabolic precursor is uncertain; no algal source has been identified and until recently TTX was assumed to be a metabolic product of the host.

Tetrodotoxin is an especially potent non-protein toxin (Fig. 1) which specifically blocks voltage-gated sodium channels on the surface of the nerve membranes causing blood vessels to relax and leading to a sudden drop in blood pressure and shock. The LD$_{50}$ of TTX in mammals is 2–10 µg kg^{-1} i.v. and 10–14 µg kg^{-1} subcutaneously [7].

Methods for the analysis of shellfish toxins can be grouped into five main categories: chromatographic analysis, immunological analysis, receptor binding assays, cytotoxicity/cell culture assays and algal monitoring. Each strategy has its merits and drawbacks. While laborious and expensive mouse assay methods (the bioassay) can be used to estimate the toxicity of the fish [8, 9], this method is not particularly sensitive nor is it specific. HPLC and electrospray-ionization mass spectrometry are more sensitive, but require expensive instrumentation, chemical clean-up and skilled operators [7, 8, 10–12, 14, 15].

Detection methods for tetrodotoxin include the bioassay, first utilised in 1941, and more recently enzyme immunoassay [9, 16]. As with most seafood toxins the bioassay was
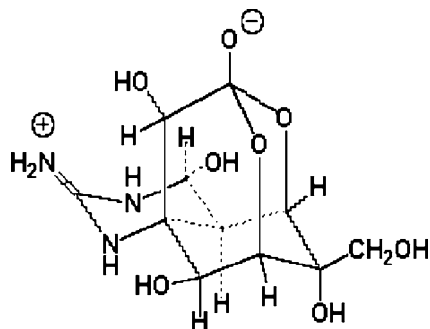
Fig. 1 Structure of tetrodotoxin

used for detection of tetrodotoxin but sensitivity was a problem. A competitive TTX ELISA , developed by Kawatsu et al. [16], was able to measure linearly 2–100 ng mL^{-1} TTX and 1–30 ng mL^{-1} , respectively, using the system of Rivera et al. [9] and this was in line with the system of Kreuzer et al. [13] for both ELISA and immunosensor (0.1–100 ppb). Low levels, which could not be detected by the mouse bioassay, could be determined by these competitive EIA systems or by HPLC [17].

In this paper, we propose two protocols for TTX determination developing a spectrophotometric assay and an electrochemical immunosensor using a newly synthesized TTX conjugate. The preparation of a conjugate in which an antigen (ligand) is coupled to an enzyme suitable for assay read-out is often a necessary step in the development of an enzyme-linked immunosorbent assay (ELISA). In the particular case of TTX, this was obligatory because there was no commercially available conjugate. While many techniques are available [18], one of the most commonly used methods to accomplish the conjugation is through the coupling of an amine with a carboxylic functional group in the presence of an activator, such as carbodiimide [19].

This paper reports the 1) synthesis and characterisation of the TTX–AP conjugate and its assessment in terms of stability, enzyme residual activity and antigen residual affinity; 2) development and optimisation of the two immunochemical assay systems (spectrophotometric and electrochemical) for determination of TTX.

Materials and method

Materials

Plastic microtitre plates, Nunc-Immuno Plate Maxi Sorp Surface, were purchased from Nalge Nunc International (Denmark). The BCA Protein Assay Kit for protein assay using bicinchoninic acid was purchased from Pierce (Rockford, IL, USA). Tetrodotoxin (citrate-free) was purchased from Alexis Biochemicals (Italy). TXO1 anti-tetrodotoxin monoclonal antibody was obtained from Hawaii Biotech (USA).

Tween 20 (polyoxyethylene-sorbitanmonolaureate), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), Sephadex G-25, 4-nitrophe-

nyl phosphate (4-NPP) disodium salt hexahydrate, 1-naphthyl phosphate disodium salt (1-NPP), phosphatase alkaline (AP) from bovine intestinal mucosa (3,650 U mg^{-1} protein; 7.9 mg mL^{-1}) were purchased from Sigma–Aldrich (USA). Anti-mouse IgG (H+L) from horse was purchased from Vector Laboratories Inc. (Burlingame, USA).

Screen-printed electrodes were produced in the Biosensor Laboratory of the University of Florence (Italy). Electrodes were printed with a 245 DEK (Weymouth, UK) screen-printing machine using different inks obtained from Acheson Italiana (Milan, Italy). Graphite-based ink (Elettrodag 421), silver ink (Electrodag 477 SS RFU) and insulating ink (Elettrodag 6018 SS) were used. The substrate was a polyester flexible film (Autostat HT5) obtained from Autotype Italia (Milan, Italy). The printing procedure has been described in previous papers [20, 21]. The electrodes were produced in foils of 20 sensors. Each sensor consists of three printed electrodes, a carbon working and two silver electrodes, acting as counter and pseudoreference, respectively. The diameter of the working electrode was 0.3 cm, resulting in an apparent geometric area of 0.07 cm^2. The resistance of these electrodes was in the range of 0.2–0.3 W.

Buffer solutions

A 0.01 M PAS buffer (Na$_2$HPO$_4$ 0.1 M + NaH$_2$PO$_4$ 0.1 M + NaCl 0.1 M + 0.1% NaN$_3$), pH 7.0, was used as eluting buffer for gel filtration chromatography of the conjugate on a Sephadex G-25 column.

1 M diethanolamine buffer (DEA), pH 9.8, containing 1 mM MgCl$_2$ and 0.15 M KCl, was used to solubilise the enzymatic substrates (4-NPP for spectrophotometric and 1-NPP for electrochemical procedure) .

Poly(vinyl alcohol) (PVA) solution 1% (w/v) in 50 mM carbonate buffer (CB) was used as blocking reagent and phosphate-buffered saline (15 mM PBS pH 7.4: Na$_2$HPO$_4$ 0.015 M + KH$_2$PO$_4$ 15 mM + NaCl 0.1 M) was used for the competition step. The washing solutions (PBS-T), used after each assay step, were prepared by adding 0.05% Tween 20 (v/v) to the phosphate buffer for the spectrophotometric method and 0.01% Tween 20 (v/v) for the electrochemical method.

Apparatus

A Bio-Rad Model 550 microplate reader was used to read the absorbance. Incubations at elevated temperatures were carried out in a thermostatic oven (High Performance Oven, Model 2100). UV spectra of TTX, AP and the conjugate, TTX–AP, were obtained with an UV/VIS UNICAM 8625 spectrophotometer in the range 200–600 nm.

The screen-printed electrodes were characterized with differential pulse voltammetry (DPV) performed with an AUTOLAB electrochemical system (Eco Chemie, Utrecht,

The Netherlands) equipped with a PGSTAT-12 and GPES software (Eco Chemie, Utrecht, The Netherlands).

Preparation of TTX–AP conjugate

Tetrodotoxin (TTX) was coupled to AP by the succinimide ester reaction [22, 23] via carbodiimide using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). A 1 mg mL^{-1} solution of TTX was added to EDC and NHS (50 mg mL^{-1} each) to reach a final volume of 1 mL, and the reaction mixture was stirred for 1 h at room temperature. All solutions were prepared in 0.1 M PBS pH 7.4, except that TTX was solubilised in a small volume of diluted H$_2$SO$_4$ (2 M) due to its limited solubility. A 50-μL aliquot of AP (10,000 UI) was added and the mixture was stirred for 24 h at 4 °C. The mixture was dialysed against PAS buffer overnight at 4 °C. Finally the resulting solution was passed through a Sephadex G-25 column, which was eluted with PAS buffer.

The concentration of enzyme in the conjugate was determined spectrophotometrically at 405 nm and the residual enzymatic activity of conjugated AP was determined using the Boehringer assay with OD read at λ=507 nm (20 μg mL^{-1} and 56 U mL^{-1}, respectively). The analysed fractions were stocked and stored at −20 °C. The conjugation ratios (mol toxin bound per mol AP) could not be determined because the TTX showed no significant UV absorption.

As a final step, the conjugate was concentrated using a so-called speed vac concentrator and then stored at −30 °C.

Immnunoassay procedures

Spectrophotometric ELISA procedure

The microtitre plates were coated with 10 μg mL^{-1} anti-mouse IgG (H+L) in 50 mM carbonate buffer (pH 9.5) overnight at 4 °C, as a preactivation step of the plastic support. The preactivation consists of a preimmobilisation of anti-IgG (mouse) antibodies for improvement of the amount and orientation of the antibodies specific (anti-TTX) for the analyte to be analysed.

After blocking with 1% poly(vinyl alcohol) (PVA) in the same buffer solution used for the pre-coating step for 1 h at 37 °C, the plates were coated with monoclonal antibody (MAb) in phosphate buffer solution (pH 7.4) for 2 h at 37 °C. The competition step was carried out for 2 h at room temperature in the working range 3–50 ng mL^{-1} of TTX with a fixed dilution of labelled toxin (TTX–AP) and also a fixed concentration of antibody. As substrate, *p*-nitrophenyl phosphate (5 mg mL^{-1}) in DEA buffer pH 9.8 was used. It is hydrolysed to *p*-nitrophenol by AP and detected spectrophotometrically at 405 nm. The concentration of the resulting enzymatic product is inversely proportional to the amount of analyte.

Microtitre plates were washed once with PBS containing 0.05% Tween 20 and twice with PBS after each step.

Electrochemical immunosensor procedure

The screen-printed electrodes (SPE) were used to assemble the electrochemical immunosensors. The working electrode (SPE) was coated with 8 μL of 10 μg mL^{-1} Anti-IgG (mouse) in 50 mM carbonate buffer pH 9.6 overnight at 4 °C. After rinsing with 50 μL of PBS-T (PBS+0.01% Tween 20), the SPE was incubated with 8 μL of blocking solution (1% PVA in PBS) for 1 h at 37 °C. After another washing step, 6 μL of primary antibody (2 μg mL^{-1} of monoclonal anti-TTX in PBS) was added to the working electrode and left to react for 2 h at 37 °C. For the competition step, different TTX standard solutions (from 1 to 100 ng mL^{-1}) were added followed immediately by a fixed concentration of TTX–AP conjugate (1:4 v/v) to reach a final volume of 6 μL on each electrode surface. The competition reaction was allowed to proceed to dryness at room temperature in the dark for 2 h. The electrodes were then rinsed with PBS-T and PBS and finally the substrate solution, 1-naphthyl phosphate 2 mg mL^{-1} in DEA buffer pH 9.8, was added and left to react at room temperature for 2 min. Electrochemical detection of the α-phenol formed was carried out using differential pulse voltammetry (DPV) performed in the potential range between 0 and 600 mV at scan rate of 100 mV s^{-1}.

Calibration plots and interpretation of results

Spectrophotometric and electrochemical calibration curves (3–50 ng mL^{-1} TTX and 1–100 ng mL^{-1}, respectively) were fitted using a non-linear 4-parameter logistic calibration plot [24] described by the following function:

$$f(x)=\left\{[1-a]\big/[1+(x/c)b]\right\}+d \qquad (1)$$

where parameters a and d are the asymptotic maximum and minimum values, respectively; c is the value at the inflection point (IC$_{50}$), and b is the slope.

The absorbance values were converted into their corresponding test inhibition values (%A/A_0) as follows:

$$\%A/A_0 = 100(A - A_{sat})\big/(A_0 - A_{sat}) \qquad (2)$$

where A is the absorbance value of competitors, A_{sat} and A_0 are the absorbance values corresponding to the saturating and the non-competition analyte, respectively (as evaluated by the four parameter logistic function).

The detection limit (LOD) was defined as the concentration of toxin equivalent to three times the value of the standard deviation (σ), measured in the absence of toxin (A_0, no competition point).

The working range was evaluated as the toxin concentration that gives test inhibition values of 90% and 10% of A/A_0 [25]. The data obtained for each curve were plotted and fitted using Sigma Plot software (SPSS).
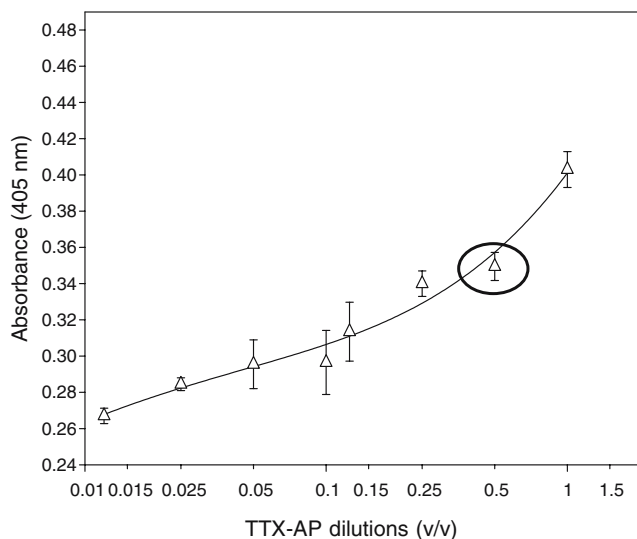
Fig. 2 Optimisation of the conjugate dilutions expressed as binding capacity of the monoclonal antibody versus TTX–AP; antibody concentration 5 μg mL^{-1} and TTX–AP working range from undiluted to 1:80 (v/v). The chosen TTX–AP dilution was 1:2 (v/v) for the spectrophotometric method

Results and discussion

Characterisation of TTX–AP conjugate

Prior to optimising the synthesis of the TTX–AP conjugate, different parameters like solvent, reaction time, temperature and choice of separation column were studied. The first step was to find a solvent for TTX that would not negatively affect the carbodiimide-mediated coupling. Solvents like acetic acid and sulfuric acid at different dilutions in distilled water were used to test the solubility of TTX prior to the conjugation procedure. Acetic acid (which is in fact recommended by the supplier) was tested first, resulting in very good TTX solubility; however, it was
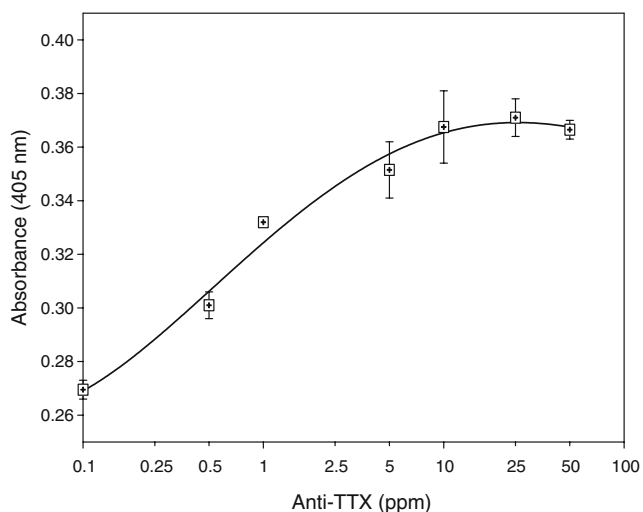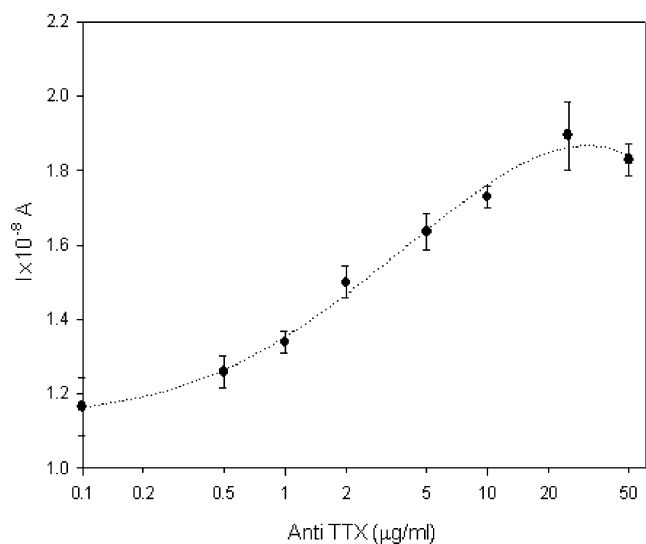

Fig. 3 Optimisation of the antibody concentration using a dilution of 1:2 (v/v) TTX–AP. A concentration of 3 μg mL^{-1} was chosen for the competition step
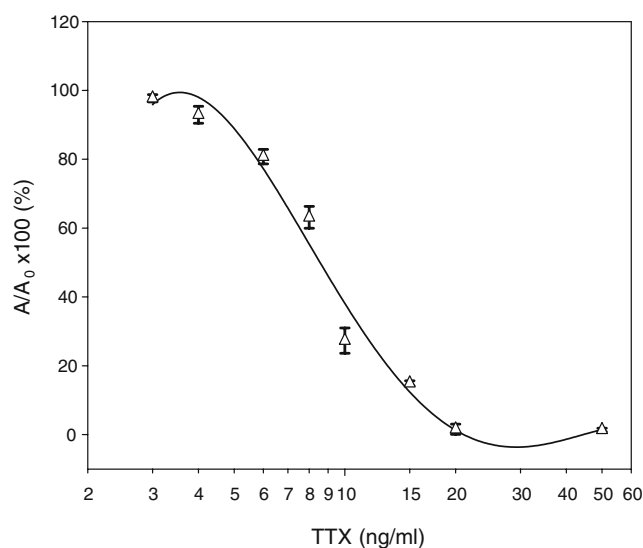
Fig. 4 Calibration graph for tetrodotoxin determination using the spectrophotometric method. TTX dynamic range 4–15 ng mL^{-1}, antibody concentration 3 μg mL^{-1}, TTX–AP dilution 1:2 (v/v)

unsuitable for the chosen type of conjugation, because this method (EDC with NHS) needs to be carried out in an acidic medium (pH 4–6) free of -NH$_2$ and -COOH groups. Different dilutions of H$_2$SO$_4$(0.4, 1 and 2 M) in distilled water were used for TTX solubilisation and then a dilution of 1:10 (v/v) was chosen for the subsequent conjugation step. When the reactions, first between the toxin with EDC and NHS and then the coupling reaction with enzyme, were completed the resulting solution was dialysed and then passed through a Sephadex G-25 gel filtration column for purification. The resulting fractions (containing TTX–AP, free TTX and free AP, respectively) were collected and analysed by UV spectrophotometry (λ=280 nm) and compared with the spectra for TTX and AP. The desired fractions were first analysed for protein concentration by using a BCA kit (λ=570 nm) to obtain information regarding the residual concentration of enzyme (total concentration including free and bound enzyme), which was about 20 μg mL^{-1}. Then, information regarding the residual enzyme activity was obtained using the Boehringer Mannheim quality control assay (λ=405 nm). This activity corresponded to 56 U mL^{-1} against 100 U mL^{-1} of initial activity.

An evaluation of affinity in terms of the residual capacity of the conjugated antigen (TTX–AP) to be recognised by its own antibody (MAb) has been carried out using a spectrophotometric binding ELISA. As can be seen from Fig. 2, the conjugate shows a satisfactory binding capacity, which is a function (directly proportional) of its dilution (sigmoid behaviour). This study demonstrated that the conjugation reaction did not destroy the capacity of the toxin, bound to the enzyme, to recognise his specific antibody.

Fig. 5 Optimisation of the antibody concentration of electrochemical procedure using a dilution of 1:4 (v/v) TTX–AP. A concentration of 2 μg mL^{-1} was chosen for competition step

Optimization of spectrophotometric ELISA

The resulting conjugate was successfully used to determine the toxin by a direct competitive ELISA method, in which the competition takes place between free and labelled toxin for the binding sites of the antibody. The ELISA parameters were studied to evaluate the specificity and sensitivity of the assay using the prepared conjugate. Firstly, the pH and ionic strength of the buffers, incubation time and temperature were studied. Then optimisation of the competitive parameters (such as TTX–AP dilution, antibody and toxin concentrations and substrate concentration) was carried out.
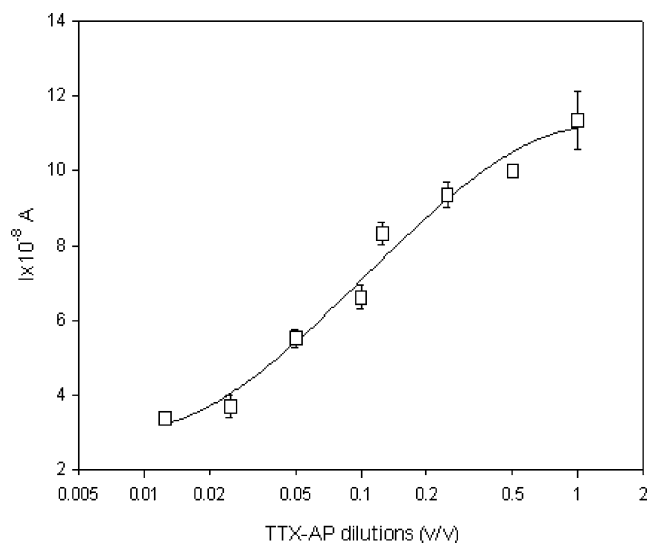
Fig. 6 Optimisation of the conjugate dilutions expressed as binding capacity of the monoclonal antibody versus TTX–AP; antibody concentration 2 μg mL^{-1} and TTX–AP working range from undiluted to 1:80 (v/v). The chosen dilution was 1:4 (v/v) TTX–AP for the electrochemical method
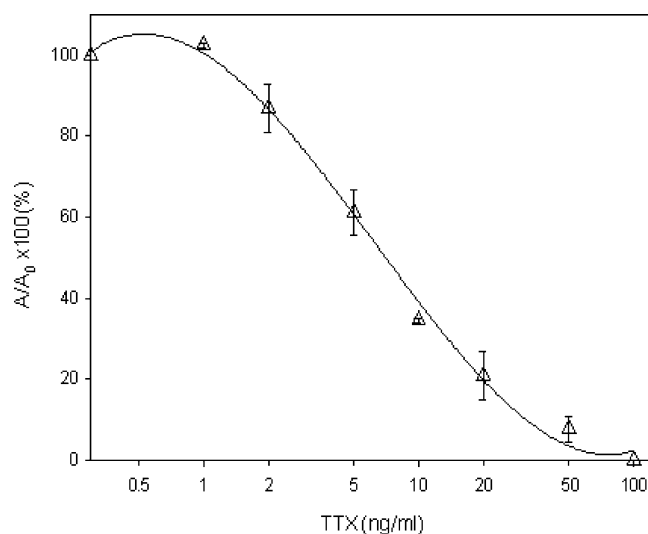
Fig. 7 Calibration graph for TTX determination using the electrochemical method (TTX dynamic range 2–50 ng mL^{-1}, antibody concentration 2 μg mL^{-1}, TTX–AP dilution 1:4 (v/v))

Different dilutions of conjugate, from 0 to 1:80 v/v dilution (Fig. 2), were tested. The chosen working dilution of TTX–AP (1:2 v/v) was defined as the conjugate dilution that gave 80% of the maximum response. This dilution is a compromise between the high dilutions that are required to achieve a low detection limit and the need to produce a sufficiently high signal. Prior to the competition step, an antibody concentration of 3 μg mL^{-1} was finally chosen, from the working range 0–50 μg mL^{-1} (Fig. 3), which normally corresponds to a value that gives 30–70% tracer binding [25]. Using the optimised parameters, a competitive ELISA was performed by incubating toxin standards in PBS in the range between 3 and 50 ng mL^{-1} with fixed dilution of TTX–AP conjugate in PBS (1:2 v/v) (Fig. 4). The dynamic working range was between 4 and 15 ng mL^{-1} with a detection limit of 2 ng mL^{-1}.

Optimization of electrochemical ELISA parameters

For the detection of tetrodotoxin, an amperometric immunosensor was assembled using screen-printed electrodes (SPE$_s$) coated with a monoclonal antibody in a direct

Table 1 Stability study expressed as residual enzymatic activity using the Boeringer method

Days	Residual enzymatic activity (U mL^{-1})[a]
0	56
10	52.6
21	51.3
30	48.9
70	4.7

[a]Against 100 U mL^{-1} of native activity

Fig. 8 Stability study of the conjugate expressed as binding capacity (ELISA measurements for a certain dilution of conjugate with an immobilised specific antibody on microplates)
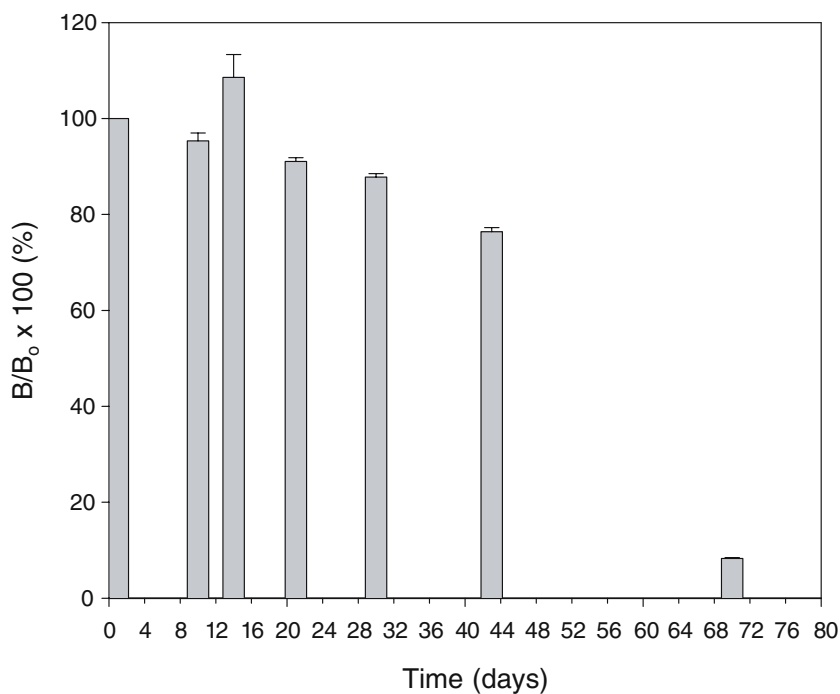
competitive assay format similar to that developed for the spectrophotometric procedure. A significant advantage of the electrochemical format method is the fact that the reagents could be added on the working electrode surfaces in very small volumes.

DPV was the electrochemical technique chosen for the detection of the enzymatic product and performed in the potential range between 0 and 600 mV at scan speed of 100 mV s^{-1} using 1-naphthyl phosphate as substrate.

As for the spectrophotometric study, immunoassay parameters such as the amount of antibody, antigen conjugate, time and incubation temperature of each step were evaluated and optimised. Figures 5 and 6 show the results obtained from the coating and binding studies. The optimised concentration of the specific antibody for TTX and for the TTX–AP conjugate were 2 μg mL^{-1} for the antibody anti-TTX and 1:4 v/v for TTX–AP, respectively. A standard curve (Fig. 7) was obtained using TTX standard solutions (1–100 ng mL^{-1}) prepared in PBS and was fitted using the non-linear 4-parameter logistic calibration plots. For this format the dynamic range was found to be between 2 and 50 ng mL^{-1} with a detection limit of 1 ng mL^{-1}.

TTX–AP stability study

To evaluate the stability of the TTX–AP conjugate, residual activity analysis was carried out from time to time using the Boehringer assay kit in order to established residual enzymatic activity (Table 1) for up to 70 days. The residual binding capacity (Fig. 8) was determined on a microplate and the results were calculated as triplicate measurements with the formula $B/B_{o} \times 100$ (%), where B_{o} is the absorbance value (at $\lambda=405$ nm) which corresponds to that for the conjugate in the first day of preparation and B is

the absorbance after a certain number of days of lifetime. As can be seen from both studies, the conjugate was relatively stable for approximately 1 month, with a decrease of the binding capacity/enzymatic activity of about 10%.

Conclusions

This work reports the preparation of a new tetrodotoxin (TTX)–alkaline phosphate (AP) conjugate and its use in the development of an immunosensor for tetrodotoxin detection. A spectrophotometric ELISA was also developed to check the functionality of the reagents.

The format for both spectrophotometric and electrochemical ELISA using labelled TTX–AP was a direct competitive immunoassay. The conjugation method, based on water-soluble carbodiimide, occurred between free amino groups of enzyme and the carboxylic group of the toxin. Results were satisfactory (20 μg mL^{-1} and 56 U mL^{-1} of the residual concentration and activity of the enzyme). The working range obtained were 4–15 ng mL^{-1} (spectrophotometric) and 2–50 ng mL^{-1} (electrochemical), respectively. The results are in any case in agreement with other works [9, 16]. The lifetime of the conjugate as about one month, but this aspect could be improved.

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