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Microsphere-Based Immunoassay for the Detection of Azaspiracids

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Abstract

Azaspiracids (AZAs) are a group of lipophilic toxins discovered in mussels from Ireland in 1995 following a human poisoning incident. Nowadays the regulatory limit for AZAs in many countries is set at 160 Fg of azaspiracid equivalents per kg of shellfish meat. In this work a microsphere-based immunoassay has been developed for the detection of AZAs using a Luminex system. This method is based on the competition between AZA-2 immobilized onto the surface of microspheres and free AZAs for the interaction with a monoclonal anti-azaspiracid antibody (mAb 8F4). In this inhibition immunoassay the amount of mAb 8F4 bound to AZA-2-microspheres was quantified using a phycoerythrin-labeled anti-mouse antibody, and the fluorescence was measured with a Luminex analyzer. Simple acetate/methanol or methanol extractions yielded final extracts with no matrix interferences and adequate recovery rates of 86.5% and 75.8%, respectively. In summary, this work presents, a sensitive and easily performed screening method capable of detecting AZAs at concentrations below the range of the European regulatory limit using a microsphere/flow cytometry system.

Keywords

Azaspiracid poisoning; anti-azaspiracid antibody; flow fluorimetry system

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Introduction

Azspiracids (AZAs) are a class of polyether toxins discovered in 1995 due to a human intoxication episode reported in The Netherlands after the consumption of contaminated mussels (*Mytilus edulis*) harvested from Killary Harbour, Ireland [1,2]. These toxins are produced by the photosynthetic thecate dinoflagellate *Azadinium spinosum* [3].

The main toxin found in contaminated samples, azaspiracid-1 (AZA-1) was isolated in 1998 [2], and its structure was later fully elucidated through synthetic studies [4–10]. To date, more than 30 analogues have been described [11], and AZA toxins have been reported from many locations around the world [2,12–17].

The symptoms observed during azaspiracid poisoning (AZP) in humans are similar to signs of diarrhetic shellfish poisoning (DSP), including nausea, vomiting, severe diarrhea and stomach cramps [1]. These toxins have been responsible for several incidents of shellfish poisoning in Europe [18], and, as a consequence, a maximum level of 160 Fg of AZA equivalents per kg of shellfish meat destined for human consumption has been established in many countries for AZA-1, azaspiracid-2 (AZA-2) and azaspiracid-3 (AZA-3) [19] (Fig. 1), the better-known compounds of the group.

Azaspiracids have been described to induce multiple system damage in rodents and cause, occasionally, the appearance of lung tumors [20]. Several *in vitro* studies have demonstrated the high cytotoxicity of AZA-1, affecting different cell lines [21]; however, the mechanism of action of these toxins remains to be determined. Mouse and rat bioassays have been used for the detection of AZAs [1]. Nevertheless, mammalian bioassays have several disadvantages due to their lack of sensitivity and specificity and ethical considerations. Analytical methods using mass spectrometry (MS) have been developed for the detection of marine toxins, in order to reduce the number of laboratory animals sacrificed every year for this purpose. Recently, LC-MS/MS has been established as the official reference method to detect AZAs in Europe [22]. MS-based methods have some drawbacks due to the use of large amounts of solvents as well as requiring highly qualified personnel and expensive instrumentation. Moreover, these methods need certified standards of all analogues of a toxin group for accurate estimation of toxicity [23], and the presence of new possibly toxic compounds would be missed with these techniques. The development of alternative assays suitable for screening high numbers of samples for marine biotoxins is needed to reduce the number of analyses by more expensive and time consuming methods.

In the last few years, multiplexed assays that enable the rapid analyses of a large number of samples by combining the use of microspheres with flow fluorimetry have been described for the analysis of several contaminants in food [24,25]. The Luminex system is based on laboratory multi-analyte profiling (LabMAP™) technology that combines the use of fluorescently dyed 5.6 micron polystyrene microspheres with a Luminex analyzer. One hundred classes of microspheres are differentiated by their internal fluorescence. The surface carboxyl groups of each microsphere are used for coating with an analyte specific detection molecule, and a reporter molecule labeled with phycoerythrin (PE) enables quantification of analyte-related signal. Therefore, Luminex technology allows the simultaneous detection of multiple analytes within a single sample by combining different analyte-specific microsphere classes.

The aim of this work was to develop a flow fluorimetry-based immunoassay for AZAs detection using a specific monoclonal antibody, known as mAb 8F4, and a Luminex system.

Materials and methods

Materials

Certified reference standard materials of azaspiracid-1 (AZA-1), 8-methylazaspiracid (AZA-2) and 22-demethylazaspiracid (AZA-3) were obtained from the Institute for Marine Biosciences, National Research Council (Halifax, Canada). AZA-2, used for immobilization on the microsphere surface, was synthesized by Nicolaou and co-workers as previously described [5,7,9,26,27]. *N*-hydroxysuccinimide (NHS), boric acid, bovine serum albumin (BSA), Tween-20, dimethyl sulfoxide (DMSO) and sodium tetraborate decahydrate were obtained from Sigma-Aldrich (Madrid, Spain), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Pierce (Rockford, Illinois). Ethylenediamine and sodium azide were obtained from Fluka (Steinheim, Germany). Phycoerythrin (PE) Goat Anti-Mouse IgG was purchased from Invitrogen (Eugene, Oregon), and the anti-azaspiracid monoclonal antibody (mAb) 8F4 was produced as previously described [28]. Sodium chloride, acetic acid, methanol, sodium acetate anhydrous and sodium phosphates were obtained from Panreac (Barcelona, Spain). Ethanolamine was supplied by BiaCore AB (Uppsala, Sweden). Mussels (*Mytilus galloprovincialis*), scallops (*Pecten maximus*), clams (*Ruditapes philippinarum*) and cockles (*Cerastoderma edule*) were purchased from the market (Lugo, Spain). Calibration and performance verification kits for Luminex 200, carboxylated microspheres (LC10077-01) and sheath fluid were purchased from Luminex Corporation (Austin, Texas). Multiscreen 96-well filter plates (Durapore membrane), 33 mm Millex filter with 0.22 µm pore size and 0.45 µm pore size Ultrafree-MC centrifugal filters (Durapore membrane) were purchased from Millipore (Madrid, Spain).

Phosphate-buffered saline solution (PBS) was 130 mM NaCl, 1.5 mM NaH₂PO₄, and 8.5 mM Na₂HPO₄, pH 7.4. PBS-BT solution was PBS supplemented with 0.1% w/v BSA and 0.1% v/v Tween-20. Buffer solutions were filtered through a 0.22 µm pore size filter before use.

Coupling of azaspiracid to carboxylated microspheres

AZA-2 was immobilized on the surface of carboxylated microspheres using EDC/NHS. The coupling protocol was performed in an opaque 1.2 µm filter microplate where 150 µL of a mixture of equal volumes of EDC and NHS, at concentrations of 75 mg/mL and 11.5 mg/mL, respectively (both reagents dissolved in water), were added to a well-microplate containing 1.2×10^6 prewashed microspheres (LC10077-01). After 30 min of incubation the EDC/NHS mixture was removed by filtration and then 150 µL of 1 M ethylenediamine (dissolved in 50 mM borate buffer solution, pH 8.5) were added to the well and let to react for 1h. Then ethylenediamine was removed and 150 µL of 1 M ethanolamine were added. The ethanolamine was filtered off after 20 min. Finally, 50 µg of AZA-2 dissolved in 10 µL of DMSO, 30 µL of acetate buffer solution pH 4.5 and 10 µL of a mixture of equal volumes of EDC (54 mg/mL) and NHS (24 mg/mL) were added to the preactivated microspheres and allowed to couple for 4h. Once the coupling reaction period was completed, the microspheres were washed three times with 200 µL of PBS-BT and stored in PBS-BT supplemented with 0.01% w/v sodium azide at 4 °C in the dark until use. All the incubations were performed with constant shaking (700 rpm) at room temperature in the dark. All washes consisted of the addition of 200 µL PBS-BT, and solutions were removed using a vacuum manifold without exceeding 5 mm Hg of pressure.

Microsphere-based immunoassay for the detection of AZAs

An inhibition immunoassay for the detection of AZAs was designed using the competition between AZA-2 immobilized on the microsphere surface and free AZAs for the interaction with a monoclonal anti-AZA antibody (mAb 8F4). The first step was the incubation of 60

μL of sample or calibration solution with 60 μL of a 1:50000 dilution of the mAb 8F4 (2.8 mg/mL, stock solution) in 1.5 mL microtubes for 30 min. Then 100 μL of this mixture were added to a well of an opaque, 1.2 μm filter plate containing 2×10^3 prewashed AZA-2-coupled-microspheres. The AZA-2-microspheres were incubated with the sample-mAb 8F4 mixture overnight (15 h) at 4 °C with constant shaking. After three washing steps with PBS-BT, 100 μL of a 1:2000 dilution of PE-labeled anti-mouse antibody (1 mg/mL, stock solution) were added to the microspheres for 30 min, followed by three additional washes. Finally, the microspheres were resuspended in 100 μL of PBS-BT.

Quantification of the binding signal

PE fluorescence intensity bound to the AZA-2-microspheres was measured with a Luminex 200 analyzer (LuminexCorp, Austin, Texas). The Luminex analyzer detects individual beads by a flow fluorimetry system using a 635 nm laser to classify microspheres. PE (analyte-related) fluorescence is quantified after excitation with a 532 nm green laser. Default values of 7500–13500 were used for doublet discriminator gating of microspheres. The acquisition volume was 75 μL and minimum bead count was 100.

The mAb 8F4 binding response for each condition was expressed as percent of maximum binding and calculated as follows: $\% R = \{(S_i - S_{\min}) / (S_{\max} - S_{\min})\} \times 100$, where % R is the percentage of mAb 8F4 binding response, S_i is the signal obtained for a given condition, $S_{\max}$ is the signal obtained in control wells containing AZA-2-microspheres and mAb 8F4, and $S_{\min}$ is the signal obtained in control wells containing AZA-2-microspheres and no mAb 8F4.

Shellfish extraction procedure

Shellfish meat (whole body of mussel, cockle or clam, and muscle plus gonad of scallop) was homogenized with a blender, divided into aliquots and stored at –20 °C until use. The extraction procedure consisted of the addition of 5 mL of the extraction solution (70% methanol in 0.2 M sodium acetate buffer, pH 5.0, MeOH/NaOAc) to 1 g of sample homogenate. The mixture was vortexed for 3 min and then centrifuged at 3600 g during 8 min at room temperature. The supernatant was saved and the pellet was re-extracted with 3 mL of the extraction solution, as described. The supernatants were combined and the volume was made up to 10 mL with MeOH/NaOAc. This extract was then diluted 1:10 (v/v) in PBS-BT and filtered through a 0.45 μm filter. Mussels, scallops, clams and cockles used in this work did not contain detectable amounts of AZAs when tested by LC-MS/MS [29].

Safety

Azaspiracids are toxins with high, acute intraperitoneal toxicity in mice. Although no human intoxication has been reported, standard solutions should be handled with gloves and eye protection should be worn at all times. Appropriate disposal methods should also be utilized.

Data analysis

The results are expressed as mean $\pm$ SEM (standard error of the mean). For every experiment all conditions were tested in duplicate. The calibration curves were fitted using a four-parameter logistic equation obtained by a nonlinear regression fitting procedure (GraphPad Prism 5.0). The four-parameter fit equation was: $y = \{ \text{Min} + (\text{Max} - \text{Min}) / (1 + 10^{\exp((\log \text{EC}_{50} - x) \eta_H)}) \}$, where Min is the bottom or the response at infinite concentration, Max is the top or the response at 0 concentration, $\log \text{EC}_{50}$ is the concentration when the response is halfway between Min and Max and η_H is the Hill Slope. The limit of detection (LoD) was calculated using three times the standard deviation (SD) of raw binding signals of the highest point of the calibration curve, corresponding to the lower toxin concentration,

and interpolating this value in the calibration curve. The Student's t-test for unpaired data was used for statistical analysis, except for multiple comparisons, which were performed using ANOVA ($p < 0.05$).

Results and Discussion

This study presents for the first time a flow fluorimetry-based competition immunoassay to detect AZAs using a specific antibody. The assay design consists of the competition of free AZA toxins and AZA-2 immobilized on the surface of microspheres for binding to monoclonal antibody mAb 8F4. Finally, the mAb 8F4 antibody bound to AZA-2-microspheres is quantified using a PE-labeled anti-mouse antibody. AZA-2 was initially selected for immobilization due to its higher reported toxicity [2,30]. The use of synthetic AZA-2 for immobilization would warrant a source of toxin for assay production independent from cultures and natural toxic blooms.

The assay was optimized for maximum sensitivity by varying antibody dilutions and incubation times. In order to evaluate sensitivity, AZA-1 calibration curves were obtained for every condition. Serial dilutions of AZA-1 at several concentrations, ranging from 0.1 to 100 nM or 0.01 to 100 nM, depending on expected sensitivity, were prepared in buffer (PBS-BT/10% MeOH) and assayed with the microsphere-based detection method. At first, 1:2000 and 1:5000 dilutions of the mAb 8F4 stock (2.8 Fg/mL) were tested with short incubation times (30 min for Ab + sample incubation, and 30 min for Ab + sample + microsphere incubation). These conditions provided calibration curves with IC_{50} values for AZA-1 of 7.4 ± 1.3 nM and 3.6 ± 0.1 nM, for 1:2000 and 1:5000 dilutions, respectively (Fig. 2, Table 1). In order to increase the sensitivity of the method, a 1:50000 dilution of the mAb 8F4 stock was also tested in combination with overnight incubation. Therefore the anti-AZA antibody was added to the calibration solution or sample for 30 min, and then this mixture was incubated with AZA-2-microspheres overnight at 4 °C with constant shaking. These conditions provided calibration curves with an average IC_{50} value of 1.4 ± 0.2 nM (Table 1). The overnight assay also showed a wider dynamic range (Fig. 2) and a lower LoD than shorter protocols (Table 1). Moreover, this inhibition immunoassay has a high sensitivity compared to the sensitivity of previously published immunoassays for AZAs or AZA fragments [28,31].

The cross-reactivity of this AZA-detection method was also studied by comparing IC_{50} values of the calibration curves obtained for AZA-1, AZA-2 and AZA-3 within the same assay. Dilutions of 0.01, 0.1, 1, 3, 10, 30 and 100 nM of the three toxins were prepared in buffer and assayed using the overnight protocol. The IC_{50} values obtained in these matched experiments were 1.7 ± 0.4 , 4.2 ± 0.5 and 1.4 ± 0.5 nM for AZA-1, AZA-2 and AZA-3, respectively (mean $\pm$ SEM; $n = 3$) (Figs. 3A and 3B). The % cross-reactivity (% CR) was calculated as follows (AZA-1 was considered the reference toxin with 100% cross-reactivity): $\% CR = (IC_{50} \text{ of AZA-1} / IC_{50} \text{ of AZA}) \times 100$. These results demonstrate that the microsphere-based immunoassay is capable of detecting AZA-1, AZA-2 and AZA-3. The assay seems to be more sensitive for AZA-1 and AZA-3 and less efficient for AZA-2 (Figs. 3A and 3B). The cross-reactivity of this immunoassay did not show a good correlation with the reported relative toxicity of these molecules (LD_{50} values of 200 Fg/kg, 110 Fg/kg and 140 Fg/kg, for AZA-1, AZA-2 and AZA-3, respectively, intraperitoneal, mouse). [2,30]. However, the studies of AZA-1, 2 and 3 toxicity were performed before certified standards of these toxins were produced, and therefore the correlation of assay cross-reactivity with relative toxicity should be reevaluated when more toxicity data are available.

The performance of the microsphere-based assay with shellfish extracts was evaluated using two extraction solvents and different shellfish matrixes. Mussel, clam, cockle and scallop extracts were prepared using a simple extraction with MeOH/NaOAc, and analyzed in duplicate following the overnight assay protocol. The extraction procedure was also carried out using methanol instead of MeOH/NaOAc, and mussels as shellfish extracts. Maximum and minimum binding signals in buffer and in shellfish extracts were compared in order to analyze the matrix effect; and no statistically significant differences were observed between the data obtained in buffer and shellfish extracts in any condition (Table 2).

Moreover, the matrix effect on the AZA-detection method was evaluated by comparing calibration curves of AZA-1 in buffer and in mussel extract using the MeOH/NaOAc extraction procedure. Serial dilutions of AZA-1 at concentrations of 0.01, 0.1, 1, 3, 10, 30 and 100 nM were prepared in buffer or extract, and assayed following the overnight microsphere-based inhibition assay. Both calibration curves were very similar (Fig. 4A). The dynamic range (IC_{20} – IC_{80}), estimated LoD and IC_{50} values for AZA-1 obtained in shellfish extract were very close to the values obtained in buffer (Fig. 4B). The lack of matrix interference suggests that the quantification of the AZA content in natural samples could be performed using a calibration curve in buffer.

Considering sample dilution during the MeOH/NaOAc extraction protocol, the dynamic range of the assay in extract and the LoD (Fig. 4B), the microsphere-based immunoassay is able to detect AZA-1 in the range of 15–280 $\mu\text{g/kg}$ in shellfish meat. The EU has set a regulatory limit for AZAs of 160 $\mu\text{g/kg}$ [19], which is within the range of our assay.

The recovery of AZA-1 with this sample extraction protocol was also evaluated using methanol or MeOH/NaOAc as extraction solutions. For this purpose, an aliquot (1 g) of blank mussel homogenate was spiked with 84 ng of AZA-1. The spiked blank materials were extracted following the procedure described above and analyzed with the overnight microsphere-based immunoassay. The toxin content was quantified using calibration curves in MeOH/NaOAc or methanol extract depending on the solvent used for sample extraction, and the recovery rate was calculated as follows: % Recovery = (measured content / fortification level) $\times$ 100. The amount of AZA-1 detected by the microsphere-based assay in 1 g of mussel was 72.7 ng in the MeOH/NaOAc extract and 63.7 ng in the methanol extract, resulting in recovery efficiencies of 86.5% and 75.8%, respectively. These results indicate that both methanol and MeOH/NaOAc extracts are adequate in terms of recovery and matrix effects for shellfish sample screening. The extraction with MeOH is used for AZA detection by LC-MS/MS [29], however, for a multiplexed assay using Luminex technology with the capability of multidetection of hydrophilic and lipophilic toxins, we have been testing a combination of MeOH and acetate buffer to favor extraction of some hydrophilic toxins. The inclusion of acetate buffer in the extraction solution seems to improve AZA-1 recovery; and, additionally, it will facilitate inclusion of this single assay in a multiplexed marine toxin assay [32]. In summary, this paper reports, for the first time, a new sensitive screening assay to detect AZAs in the range of the European regulatory limit using a microsphere/flow fluorimetry-based immunoassay. This detection method could be included in the future in a multiplexed assay to allow the simultaneous detection of several groups of toxins in the same sample.

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Fig. 1.
Chemical structure of AZA-1, AZA-2 and AZA-3.

Fig. 2.

Calibration curves of AZA-1 in buffer using the microsphere-based inhibition assay with several assay conditions. Calibration curves were obtained using the inhibition assay with mAb 8F4 dilutions of 1:2000 and 1:5000 combined with 30 min incubations, and 1:50000 combined with an overnight incubation with AZA-2-coated microspheres. Data are expressed as percentage of maximum mAb 8F4 binding. The three calibration curves belong to non-matched experiments (mean $\pm$ SEM; $n = 4$ for 1:2000 dilution, $n = 3$ for 1:5000 dilution and $n = 7$ for 1:50000 dilution).

Fig. 3.

Cross-reactivity profile of AZA-1, AZA-2 and AZA-3 using the overnight microsphere-based inhibition assay. (A) Calibration curves of AZA-1, AZA-2 and AZA-3. Calibration solutions were prepared at concentrations ranging from 0.01 to 100 nM in buffer for the three toxins and assayed using the inhibition assay. The data are expressed as a percentage of maximum mAb 8F4 binding response. The calibration curves were obtained with a four-parameter fit. (B) IC₅₀, dynamic range, LoD and % cross-reactivity (% CR) values for AZA-1, AZA-2 and AZA-3. Data obtained from the curves in (A) (mean ± SEM; n = 3).

Fig. 4.

Effect of mussel matrix on the detection of AZA-1 using the microsphere-based Luminex system. (A) Calibration curves of AZA-1 in buffer and shellfish extract using the overnight microsphere-based immunoassay and MeOH/NaOAc as extraction solvent. AZA-1 standard was diluted at concentrations ranging from 0.01–100 nM in buffer and mussel extract. The data are expressed as a percentage of maximum mAb 8F4 binding response. The calibration curves were obtained with a four-parameter fit (mean ± SEM of two replicates). (B) IC₅₀, dynamic range (IC₂₀ – IC₈₀) and LoD values obtained from the curves in (A).

Table 1

IC₅₀, dynamic range (IC₂₀ – IC₈₀), limit of detection (LoD) and maximum and minimum binding signal of the microsphere-based assay for AZA-1 in buffer with several assay conditions. The IC₅₀, IC₂₀, IC₈₀, LoD and binding signals values were obtained from the experiments that generated Fig. 2 using a four-parameter fit equation (mean ± SEM, n = 4 for 1:2000 mAb 8F4 dilution, n = 3 for 1:5000 mAb 8F4 dilution and n = 7 for 1:50000 mAb 8F4 dilution).

Incubation time	30 min		Overnight
mAb 8F4 dilution	1:2000	1:5000	1:50000
IC ₅₀ (nM) <i>IC₅₀ (ng/mL)</i>	7.4 ± 1.3 <i>6.2 ± 1.1</i>	3.6 ± 0.1 <i>3.0 ± 0.1</i>	1.4 ± 0.2 <i>1.2 ± 0.2</i>
IC ₂₀ (nM) <i>IC₂₀ (ng/mL)</i>	4.3 ± 0.4 <i>3.6 ± 0.3</i>	1.9 ± 0.0 <i>0.7 ± 0.0</i>	0.4 ± 0.1 <i>0.3 ± 0.1</i>
IC ₈₀ (nM) <i>IC₈₀ (ng/mL)</i>	18.8 ± 0.7 <i>15.8 ± 0.6</i>	6.8 ± 0.0 <i>5.7 ± 0.0</i>	5.6 ± 1.2 <i>4.7 ± 1.0</i>
LoD (nM) <i>LoD (ng/mL)</i>	2.6 ± 0.4 <i>2.2 ± 0.3</i>	1.5 ± 0.1 <i>1.3 ± 0.1</i>	1.0 ± 0.6 <i>0.8 ± 0.5</i>
Maximum Binding Signal (RU)	2109 ± 180	1052 ± 78	617 ± 159
Minimum Binding Signal (RU)	2.6 ± 0.2	2.3 ± 0.2	3.2 ± 0.1

Table 2

Matrix effect of MeOH/NaOAc or methanol shellfish extracts on maximum and minimum binding signals using the overnight microsphere-based inhibition assay (mean $\pm$ SEM; n = 3).

MeOH/NaOAc		
	<i>Maximum Binding Signal (RU)</i>	<i>Minimum Binding Signal (RU)</i>
Mussel	1519 $\pm$ 292	1.8 $\pm$ 0.2
Scallop	1613 $\pm$ 193	1.7 $\pm$ 0.2
Clam	1637 $\pm$ 139	2.4 $\pm$ 0.1
Cockle	1680 $\pm$ 207	2.0 $\pm$ 0.3
Buffer	1644 $\pm$ 398	2.0 $\pm$ 0.0
Methanol		
	<i>Maximum Binding Signal (RU)</i>	<i>Minimum Binding Signal (RU)</i>
Mussel	971 $\pm$ 313	3.8 $\pm$ 1.3
Buffer	1109 $\pm$ 409	3.7 $\pm$ 0.9