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Ultrafiltration improves ELISA and Endopep MS analysis of botulinum neurotoxin type A in drinking water

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Abstract

The objective of this study was to adapt and evaluate two in vitro botulinum neurotoxin (BoNT) detection methods, including the Botulinum Toxin ELISA and the Endopep MS (a mass spectrometric-based endopeptidase method), for use with drinking water samples. The method detection limits (MDL) of the ELISA and Endopep MS were 260 pg/mL and 21 pg/mL of BoNT/A complex toxin, respectively. Since toxin could be present in water samples at highly dilute concentrations, large volume (100-L) samples of municipal tap water from five US municipalities having distinct water compositions were dechlorinated, spiked with 5 μg BoNT/A, and subjected to tangential-flow ultrafiltration (UF) using hollow fiber dialyzers. The recovery efficiency of BoNT/A using UF and quantified by ELISA ranged from 11% to 36% while efficiencies quantified by MS ranged from 26% to 55%. BoNT/A was shown to be stable in dechlorinated municipal tap water stored at 4 °C for up to four weeks. In addition, toxin present in UF-concentrated water samples was also shown to be stable at 4 °C for up to four weeks, allowing holding of samples prior to analysis. Finally, UF was used to concentrate a level of toxin (7 pg/mL) which is below the MDL for direct analysis by both ELISA and Endopep MS. Following UF, toxin was detectable in these samples using both in vitro analysis methods. These data demonstrate that UF-concentration of toxin from large volume water samples followed by use of existing analytical methods for detection of BoNT/A can be used in support of a monitoring program for contaminants in drinking water.

Keywords

Botulism; Detection; ELISA; Mass spectrometry; Toxin; Water quality

1. Introduction

Botulinum neurotoxin (BoNT) can produce lethal effects following ingestion of contaminated products. Although botulism has not been associated with the presence of BoNT in drinking water, intentional contamination of public drinking water systems

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represents a potential threat. BoNT has been suggested as a feasible agent for contamination primarily because of its suspected stability in untreated water (Khan et al., 2001; Meinhardt, 2005). In addition, BoNTs are extremely potent, and estimates of the human lethal dose for serotype A (BoNT/A) have been reported to be as low as 70 ng (Gill, 1982) and in the case of ingestion, as high as 70 μg (Arnon et al., 2001). The 90th percentile drinking water intake rate has been reported to be 2.3 L/day (U.S. EPA, 1997), therefore, a BoNT/A concentration of ~30 pg/mL would represent a potential human health hazard, assuming that the lowest estimated human lethal dose (i.e. 70 ng) is contained in daily water intake.

Various studies have demonstrated that BoNT is stable in untreated water but rapidly inactivated by free chlorine at levels typically used in municipal water systems (Brazis et al., 1959; Notermans and Havelaar, 1980). Nonetheless, several conditions could still present a risk to public health. Microbial contamination has been shown to increase free chlorine demand which could reduce disinfectant levels leaving insufficient free chlorine available for BoNT inactivation (Helbling and VanBriesen, 2007). Furthermore, chlorine residuals can vary greatly throughout a drinking water distribution system, with some areas offering reduced or minimal protection. Another widely used water system disinfectant, monochloramine, is significantly less effective at inactivating BoNT compared to free chlorine (Brazis et al., 1959). Finally, untreated bottled or well water provides no means of inactivation of BoNT.

Currently, there is no standardized in vitro method available to response laboratories for the detection of BoNT in drinking water samples. Therefore, the purpose of this study was to adapt and evaluate the Botulinum Toxin Enzyme Linked Immunosorbent Assay (ELISA) (Maslanka et al., 2011) and Endopep-MS (Barr et al., 2005; Parks et al., 2011) for use with drinking water samples. Both of these analytical methods have been used with various clinical and food specimens at CDC and have reported detection limits at or below 30 pg/mL (Barr et al., 2005; Maslanka et al., 2011; Parks et al., 2011). For ELISA analysis, the level of toxin in water samples was quantified by comparing the level of antibody binding with that of known concentrations of BoNT/A. For Endopep MS analysis, the endopeptidase activity of BoNT for specific peptide substrates was used to measure the level of peptide cleavage of a given sample compared with that of a known concentration of BoNT/A.

A further purpose of this study was to investigate the ability to concentrate toxin from large volumes of water (100-L) using ultrafiltration (UF) in order to analyze otherwise undetectable levels of toxin. The UF method uses hollow fiber filters to simultaneously concentrate viruses, bacteria, and parasites by size exclusion (Hill et al., 2007). This method can be used to recover a target analyte from water, for downstream detection, quantification, and analysis. The ability to detect and quantify BoNT/A was tested using both the Botulinum Toxin ELISA and Endopep MS on both UF-concentrated and non-concentrated drinking water samples from different municipal water sources.

2. Materials and methods

2.1. Toxins and water types used in this study

BoNT/A is produced by *Clostridium botulinum* in a progenitor form where the neurotoxin is associated with other non-toxic proteins. This complex form of BoNT/A was used in this study and purchased from Metabiologics (Madison, WI). A single lot of toxin was used throughout which contained a specific toxicity of 3.6×10^7 mouse LD_{50}/mg, as determined by the vendor.

Where indicated, deionized water (DI) and chlorine-demand-free (CDF) water were used. Briefly, CDF was prepared by adding 5.65–6% sodium hypochlorite (Fisher Scientific, Fair Lawn, NJ) to local municipal tap water to achieve a concentration > 10 mg/L free chlorine (Clesceri et al., 2005). The solution was held for 24 h and then exposed to UV light overnight to eliminate the chlorine residual. To assess the effect of oxidizing agents that might be present in water samples on the detection of BoNT/A, free chlorine (3 mg/L) or monochloramine (2 mg/L) was added to CDF followed by toxin addition. Dechlorination of either free chlorine or monochloramine was achieved by addition of sodium thiosulfate (50 mg/L). Free and total chlorine concentrations were measured using the Hach DPD Methods 8021 and 8167. Monochloramine was measured by the indophenol method using Hach Method 10171.

Municipal water samples were obtained from separate locations with known differences in their water compositions (e.g., specific conductance, total organic carbon). Tap water samples (water system A) were collected at CDC after flushing for 5 min to purge the system of water that may contain less than expected disinfectant levels and impurities derived from the building piping. The remaining water samples (water systems B, C, D, and E) were finished water obtained from municipal water treatment plants and shipped to CDC. These waters represent a range of water quality characteristics that might influence the analysis, and are listed in Table 2. Monthly average values for total organic carbon (TOC), pH, hardness, and chlorine and/or monochloramine concentration were provided by the municipal water suppliers.

Water samples were also analyzed at CDC for the following water quality parameters: specific conductance (using an Oakton CON 100 Conductivity/°C meter), pH (using an Accumet® Research AR25 Meter with an Accumet Standard Size Combination Electrode), turbidity (using a Hach Model 2100N Laboratory Turbidimeter), total hardness [using Hach Method 8213 with a Hach Hardness (Ca/Mg) Reagent Set (Hach Cat. No. 24480-00) and Hach Model 16900 digital titrator], alkalinity [using a Hach Alkalinity Test Kit, Model AL-DT, Digital Titrator (Product # 2063700)] and TOC (using Hach Method 10129 with a Hach Low Range Total Organic Carbon Reagent Set (Cat. No. 2760345) and the Hach DR/2400 Portable Spectrophotometer).

2.2. Botulinum Toxin ELISA

Type A Botulinum Toxin ELISA kits (CDC catalog number KT0064) were used in this study (Maslanka et al., 2011). The intended use of the ELISA is to determine presence or absence of toxin. In order to obtain quantitative results, a calibration curve of BoNT/A

complex (1000 pg/mL–15 pg/mL) was generated by diluting the toxin in gelatin buffered saline (GBS; 0.2% gelatin, 0.4% Na_2HPO_4, pH 6.2). In addition to the calibration curve, a separate quality control (QC) sample (500 pg/mL BoNT/A diluted in GBS) was tested on each plate to assess inter-assay variation. Where indicated, 0.5% Triton X-100 (Sigma, St. Louis, MO) was added to water samples. All samples were tested in triplicate wells to minimize the effects of well-to-well variation. The background subtracted absorbance value (BSAV) was calculated by subtracting the absorbance value read at 690 nm from the signal read at 450 nm. For each ELISA plate, a linear equation was calculated by plotting the average triplicate BSAV for the calibration curve samples against their known concentrations. Calibration curves were only accepted if the linear regression value (r^2) was ≥0.95. For unknown samples, the BSAV of triplicate wells was used in the linear equation derived from the calibration curve to calculate a toxin concentration.

2.3. Endopep MS

Magnetic beads (20 μL) coated with monoclonal antibodies to BoNT/A were incubated at room temperature with 0.5 mL of water sample and agitated using a KingFisher 96 instrument (ThermoFisher, Waltham, MA) at the very slow setting for 1 h. Beads were then washed twice in 1 mL of phosphate buffered saline containing Tween 20 (PBST) and finally in water. Beads were transferred into a 96-well plate and reconstituted in a 20-μL solution containing 0.05 M Hepes (pH 7.3), 25 mM dithiothreitol, 20 μM $ZnCl_2$, 1 mg/mL bovine serum albumin (BSA), and 50 pmol/μL of peptide substrate as described elsewhere (Parks et al., 2011). Samples were incubated at 37 °C for 4 h without agitation.

After the 4 h incubation, 6 μL of each reaction supernatant was combined with 2 μL of formic acid to halt the reaction and then with 2 μL of an internal standard mixture, as described previously (Parks et al., 2011). Peptides in each reaction mixture were first separated on a 1 mm C18 BEH column using a Nanoacquity Ultra High Pressure Liquid Chromatography instrument (Waters, Milford, MA) and then introduced into the ABI 4000 QTRAP mass spectrometer (Applied Biosystems, Carlsbad, CA) where a minimum of three ion transitions are monitored for each cleavage product and internal standard over a 10 min period.

A BoNT/A calibration curve was prepared in GBS and run simultaneously with the experimental samples to permit quantification. Peak height ratios of substrate cleavage products compared to the internal standard were calculated from triplicate injections of each sample and averaged to give final results. A QC sample containing BoNT/A (500 pg/mL) spiked in GBS was tested in parallel with each sample set to assess inter-assay variation.

2.4. Method detection limit determination

The method detection limit (MDL) represents the minimum concentration where there is 99% confidence that the target analyte (i.e. BoNT/A) is present while the quantitation limit (QL) represents the smallest detectable concentration of the analyte above the MDL where the intended level of precision is achieved (Code of Federal Regulations, 2011). These analyses were performed in DI water using an amount of toxin expected to be detectable for each method (2500 pg/mL for ELISA, 50 pg/mL for Endopep MS) and processed through

the entire analytical method including the addition of 50 mg/L sodium thiosulfate. For the ELISA analysis, samples were diluted 1:10 in kit-supplied dilution buffer treated with 0.5% Triton X-100. The MDL and QL were determined using seven independently spiked replicates (Code of Federal Regulations, 2011). The MDL was calculated by multiplying the standard deviation of the toxin concentrations in the replicate samples by the 3.14 (Student's t-statistic for seven replicates) while the QL was determined by multiplying the standard deviation of the replicate samples by 10.

2.5. Ultrafiltration of large volume water samples

Dechlorinated 100-L water samples were concentrated using hollow fiber ultrafilters that retained substances with a molecular weight greater than ~30 kDa according to the method of Hill et al. (2007). A total of 1 mL of BoNT/A diluted to the desired level in GBS was introduced into the UF apparatus using a syringe pump and delivered at a constant rate over the course of the experiment. Subsequently, 0.01% Tween 80 was back flushed through the system to recover any toxin remaining on the filter. For recovery efficiency experiments, a total of 5 μg BoNT/A was introduced into the UF apparatus. To assess the ability of UF to concentrate a low level of toxin, a total of 700 ng BoNT/A was introduced into the UF apparatus. Recovery efficiencies were calculated by dividing the amount of BoNT/A measured in UF concentrates by the amount of BoNT/A added to the 100-L water samples (and multiplying by 100).

2.6. Mouse bioassay

Where indicated, the mouse bioassay was performed as previously described (CDC, 1998) to detect BoNT/A in selected samples. Pairs of ICR/CD1 mice were injected interperitoneally with 0.5 mL of sample and monitored for symptoms of botulism (including ruffled fur, labored breathing, pinched waist) for up to four days according to a protocol approved by the CDC Institutional Animal Care and Use Committee.

2.7. Sample holding time studies

To simulate sample holding times after collection in the field, dechlorinated tap water samples from water system A were spiked with 5000 pg/mL BoNT/A and then held for 4 weeks at 4 °C. In parallel, 100-L of dechlorinated local tap water spiked with a total of 5 μg BoNT/A was concentrated by UF and held at 4 °C to assess toxin stability in UF-concentrated water and to discover any possible assay inhibition due to co-concentration of other matrix components. Samples were assayed using both ELISA and Endopep MS immediately after inoculation and at days 2, 7, 14, and 28.

3. Results

3.1. Quantification of BoNT/A using the Botulinum Toxin ELISA

Initially, the detection of BoNT/A in the calibration curve dilution matrix (GBS) was compared with toxin detection in a clean water matrix (DI water) to ensure accurate calculation of sample concentration. Thus, two-fold serial dilutions of BoNT/A representing a concentration range of 1000 pg/mL–15 pg/mL were prepared in GBS and compared to identical dilutions generated in DI water. Using the ELISA, a linear relationship between

the BSAV and BoNT/A concentration was observed (r^2>0.99) (Fig. 1A). However, when BoNT/A was diluted in DI water a reduced signal was observed compared to similarly spiked GBS samples. At the calibration curve midpoint (500 pg/mL BoNT/A), the difference in BSAV between samples diluted in DI water versus GBS was statistically significant ($p<0.01$).

To mitigate the sample bias discovered between GBS and DI water, 0.5% Triton X-100 was added to the DI water samples containing toxin. The resulting calibration curve overlapped that of the curve prepared in GBS (Fig. 1A). At the calibration curve midpoint (500 pg/mL BoNT/A), no significant difference in BSAV between samples diluted in DI water containing 0.5% Triton X-100 versus GBS was observed. As a result, all further water samples analyzed by ELISA were processed with 0.5% Triton X-100.

3.2. Quantification of BoNT/A using Endopep MS

BoNT/A diluted in either GBS or DI water yielded linear standard curves (r^2>0.99) using the Endopep MS detection method as shown in Fig. 1B. Similar to the findings with ELISA, the standard curve prepared in DI water demonstrated reduced signals (i.e. peak height ratios) compared to the standard curve prepared in GBS. Triton X-100 was not tested as an additive to limit signal inhibition in DI water because it has previously been shown to mask signals due to the ionization of the detergent in the mass spectrometer (S. Kalb, unpublished data). Since a QC sample containing 500 pg/mL BoNT/A diluted in GBS was used to assess inter-assay variation, the GBS standard curve was selected for all further studies.

3.3. Effect of dechlorinating agent on detection of BoNT/A

Both ELISA and Endopep MS failed to detect BoNT/A spiked at 5000 pg/mL in water samples containing 3 mg/L free chlorine (Fig. 2). Samples containing 2 mg/L monochloramine had no effect on toxin detection. Addition of sodium thiosulfate effectively prevented toxin inactivation by free chlorine and showed no inhibitory effect on toxin detection in CDF or samples containing monochloramine. Notably, the toxin level calculated using Endopep MS was consistently lower than that using the ELISA.

3.4. Method detection limit and quantitation limit determination

For MDL and QL determination using the ELISA, DI water samples containing 50 mg/L sodium thiosulfate and 0.5% Triton X-100 were spiked with 2500 pg/mL BoNT/A. The average BoNT/A concentration determined using these samples was 2500 pg/mL (range: 2400–2600 pg/mL). The MDL and QL were 260 pg/mL and 830 pg/mL respectively (Table 1). Further analysis demonstrated that toxin spiked at a level just below the QL (800 pg/mL) and analyzed by ELISA yielded calculated toxin concentrations that were ~85% of the spike level with low variability (CV<3%) between replicates (n=7) (data not shown).

For Endopep MS analysis, DI water samples containing 50 mg/L sodium thiosulfate were spiked with 50 pg/mL BoNT/A. The average BoNT/A concentration determined using Endopep MS was 43 pg/mL (range: 31–50 pg/mL). These data were used to calculate a MDL and QL of 21 pg/mL and 68 pg/mL respectively (Table 1).

3.5. Ultrafiltration of municipal drinking water samples

Large volume (100-L) tap water samples (water system A) were spiked with a total of 5 μg BoNT/A during the UF process, resulting in a BoNT/A concentration of 50 pg/mL (~2 mouse LD_{50}). The average recovered UF-concentrate volume was 523 mL (n=3), which represented an approximately 190-fold concentration (v/v). ELISA analysis yielded an average toxin concentration of 4000 pg/mL (standard deviation=0.2 pg/mL) which corresponded to a recovery efficiency of 35–46%. Using the Endopep MS, the average toxin concentration of these samples was 4300 pg/mL (standard deviation=1.9 pg/mL) resulting in a calculated recovery efficiency of 31–55%.

Additionally, 100-L municipal drinking water samples from different geographical locations (water systems B–E) were spiked with 5 μg/mL of BoNT/A and subjected to UF concentration. Recovery efficiencies for BoNT/A in these samples ranged from 11 to 36% when examined by ELISA and from 26 to 53% when examined by Endopep MS (Table 2).

In order to evaluate whether UF could be used to concentrate BoNT/A from a level below the MDL of either the ELISA or Endopep MS (Table 1) to a detectable level, 100-L of dechlorinated local tap water was spiked with a total of 700 ng BoNT/A, generating a final BoNT/A concentration of 7 pg/mL and processed using the UF method. In parallel, a 7 pg/mL BoNT/A sample was generated but not subjected to UF. BoNT/A in the UF-concentrated sample was detected at levels above the MDL and QL of the respective analytical method while the non-concentrated (7 pg/mL) sample was not detected by either analysis method (Table 3). Based on the specific activity of the toxin used in this study (3.6×10^7 mouse LD_{50}/mg), the level of toxin in the non-concentrated sample was spiked at a level expected to be less than a single mouse lethal dose (~0.25 mouse LD_{50}/mL). We used the mouse bioassay to confirm that this sample did not demonstrate toxicity and also that the UF-concentrated sample did show mouse toxicity within 24 h of injection (data not shown).

3.6. Evaluation of sample holding time prior to analysis

Toxin was spiked in non-concentrated local tap water (water system A) or concentrated by UF and stored at 4 °C prior to analysis. Non-concentrated samples were spiked with 5000 pg/mL BoNT/A. Simultaneously, 100-L water samples were spiked with 5 μg BoNT/A (i.e. 50 pg/mL) and subjected to UF, which increased the calculated BoNT/A concentration to a level of approximately 3700 pg/mL when tested by ELISA or 2700 pg/mL when tested by Endopep MS (Fig. 3A and B).

Using ELISA, the BoNT/A concentration remaining in non-concentrated and UF-concentrated water after 28 days storage at 4 °C was calculated to be 83% and 94% of the initial toxin level, respectively (Fig. 3A). Using the Endopep MS, the BoNT/A concentration remaining in non-concentrated and UF concentrated water was calculated to be 92% and 60% of the initial toxin level, respectively (Fig. 3B). Interestingly, the highest toxin concentrations calculated using Endopep MS (but not ELISA) were observed on day 21 for both UF concentrated and non-concentrated water samples.

4. Discussion

Laboratories have traditionally used the mouse bioassay to detect BoNT in foods and clinical samples (CDC, 1998). More recently, several in vitro detection methods have been developed and reviewed elsewhere (Cai et al., 2007; Lindström and Korkeala, 2006; Sharma and Whiting, 2005). In this study, we evaluated the use of two in vitro detection methods, namely the Botulinum Toxin ELISA and Endopep MS, to detect and quantify BoNT in drinking water.

Both of the in vitro assays evaluated in this study utilize antibodies to capture toxin but the level of toxin present in a sample is determined by two different processes. For ELISA, the level of toxin is a function of antibody binding whereas the level of toxin determined through the use of the Endopep MS is a function of both antibody binding and endopeptidase activity for specific substrates. Surprisingly, both assays demonstrated signal inhibition when BoNT/A complex was diluted in DI water. Although it is unknown why this matrix appears to be inhibitory, it is possible that changes in the osmolarity of water compared to that of buffer affected the conformation of the protein and therefore the exposure of epitopes for antibody binding.

Previous studies indicate that BoNTs degrade rapidly in the presence of free chlorine and somewhat less rapidly in the presence of monochloramine (Brazis et al., 1959; Notermans and Havelaar, 1980). Another report indicated that handheld immunoassay detection limits for staphylococcal enterotoxin B and ricin in water were increased when free chlorine was added to the samples (Wade et al., 2011). Accordingly, it is not surprising that both in vitro methods used in this study failed to detect BoNT/A (500 pg/mL) spiked in water containing free chlorine. BoNT/A spiked in water containing monochloramine was detectable using both in vitro methods. However, further studies are needed to determine if extended exposure to monochloramines affects BoNT stability.

Sodium thiosulfate is frequently used as a dechlorinating agent for drinking water microbiological and chemical methods and it is of particular importance that its addition does not inhibit BoNT/A detection. In this study, sodium thiosulfate was shown to be an effective dechlorinating agent and showed no effect on the ability to detect toxin in non-chlorinated water using either the Botulinum Toxin ELISA or Endopep MS.

Neither ELISA nor Endopep MS could quantify toxin at a level (7 pg/mL) less than the most conservative estimate of a human health hazard (~30 pg/mL) based on daily water intake of 2.3 L/day (U.S. EPA, 1997). Notably, mouse bioassay also failed to detect toxin at a level of 7 pg/mL. In this study, UF was used in combination with two in vitro BoNT detection assays adapted for toxin quantification in water. Because UF concentrates the level of toxin present in water, toxin was successfully detected using either in vitro assay as well as by the mouse bioassay. Analysis of ultrafiltration using multiple municipal water samples spiked at a concentration of 5 μg BoNT/A per 100-L revealed recovery efficiencies in the range of 11–35% when detected with ELISA and 26–55% when detected with Endopep MS. These recovery efficiencies for BoNT/A are comparable with that of bacterial and viral pathogens examined in other studies (Hill et al., 2007; Polaczyk et al., 2008).

Additional analysis of these data demonstrated that water quality characteristics may have an effect on method performance. Linear correlations between water quality parameters listed in Table 2 and toxin recovery measured by the assays were investigated. Statistically significant correlations (p-value of slope b0.05) at the 95% confidence level were noted between the specific conductance, alkalinity, and hardness for both the ELISA and Endopep MS assays. This corresponds to observations that the water (water system C) with the highest hardness, alkalinity, and specific conductance was associated with the lowest toxin recovery efficiency regardless of the analytical method used. The correlation between TOC concentration and BoNT/A recovery efficiency was not statistically significant for either ELISA or Endopep MS when all water samples were evaluated. However, when waters with TOC values less than 2.5 mg/L were considered (i.e. water system E was removed from the analysis), a significant correlation (pb0.05) between toxin recovery and TOC was observed for recoveries measured by Endopep MS but not ELISA. Due to the limited number of water samples examined in this study, further experiments are necessary to better understand these relationships.

BoNT/A was stable up to 28 days in dechlorinated UF-concentrated and non-concentrated tap water. Notably, the stability of toxin was significantly longer than that reported by Notermans and Havelaar (1980) where toxin in distilled water was inactivated by nearly 50% within 15 days and was completely inactivated in surface and drinking water within 12 days. Additional study is required to determine if the addition of a dechlorinating agent prevents degradation of low toxin levels, as this might also have utility during the forensic investigation aspect of an intentional contamination incident.

The Endopep MS assay demonstrated a higher level of variability in toxin quantification throughout the time course study compared to the Botulinum Toxin ELISA most likely due to independent extraction and quantification of toxin among water sample replicates. Nonetheless, both analytical methods demonstrated a remaining level of toxin in the range of 1000–5000 pg/mL throughout the time course study.

In summary, this study provides evidence that UF can be used to significantly increase the concentration of toxin present in drinking water samples for improved analysis by various in vitro detection methods, as well as the mouse bioassay. The use of UF appears to be required to detect the presence of toxin at the most conservative estimates for human toxicity. Moreover, these concentrated samples are stable and can be assayed for toxin presence even after extended storage as might be expected if a large number of samples had to be analyzed. Finally, these results suggest that UF may be used to concentrate other toxins in drinking water where the toxin is large enough to be retained by an ultrafilter.

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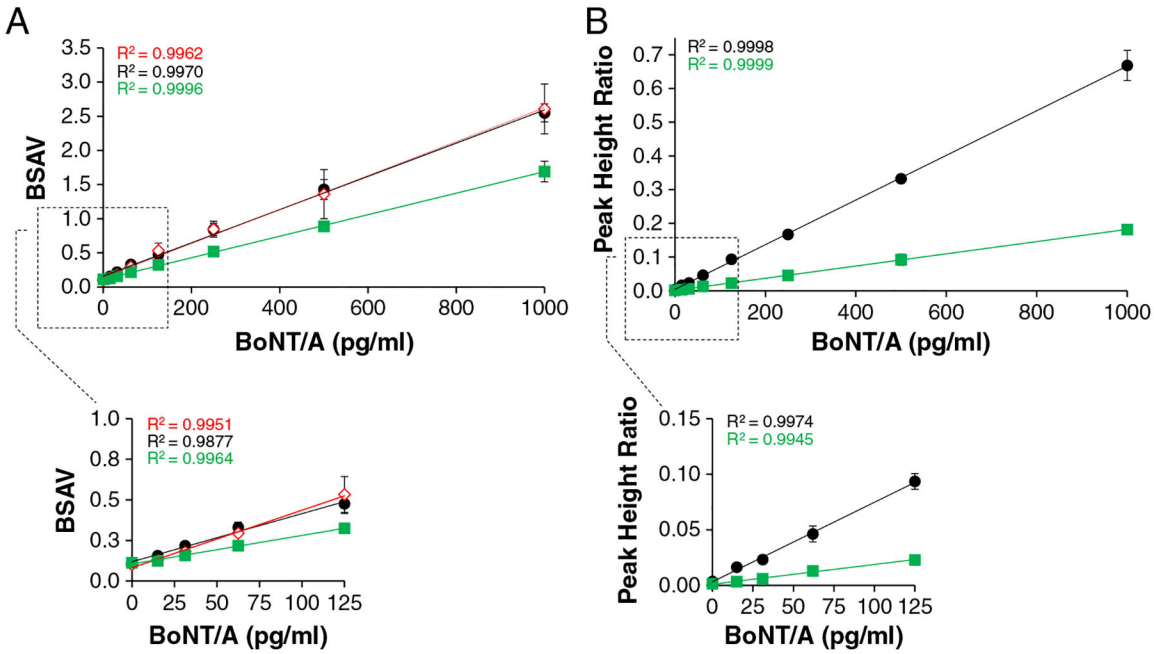

Fig. 1.

BoNT/A calibration curves. Shown are two-fold dilution series of BoNT/A complex (final concentration: 1000 pg/mL–0 pg/mL). The dilution series was performed in either GBS (black solid circles), DI water (green solid squares), or DI water containing 0.5% Triton X-100 (red open diamonds). Linear regression trend lines are shown and R^2 values are in colored font corresponding to the appropriate trend line. Panel A: Calibration curve tested using the Botulinum Toxin ELISA. BSAV = Background subtracted absorbance value. Panel B: Calibration curve tested using Endopep MS. Error bars represent standard deviation of three independently diluted samples. An enlarged view of the section of the calibration curves bounded by the dotted line is shown below each panel.

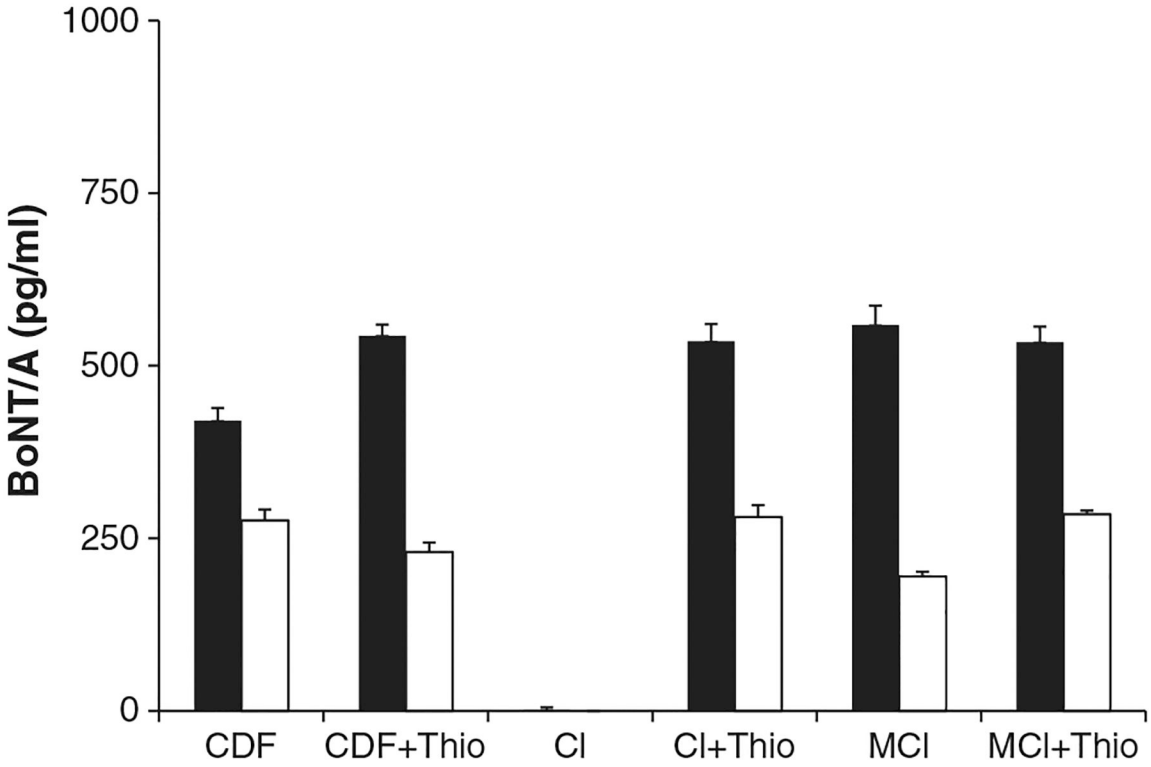

Fig. 2.

Effect of dechlorinating agent on detection of BoNT/A. Chlorine demand free (CDF) water was treated with 3 mg/L free chlorine (Cl) or 2 mg/L monochloramine (MCl). A dechlorinating agent (50 mg/L sodium thiosulfate) was added to CDF (CDF+Thio), Cl (Cl+Thio), and MCl (MCl+Thio) samples. All samples were spiked with a final concentration of 500 pg/mL BoNT/A. Shown is the average BoNT/A concentration of triplicate samples determined using the Botulinum Toxin ELISA (solid bars) or Endopep MS (open bars). Error bars represent standard deviations of the triplicate samples.

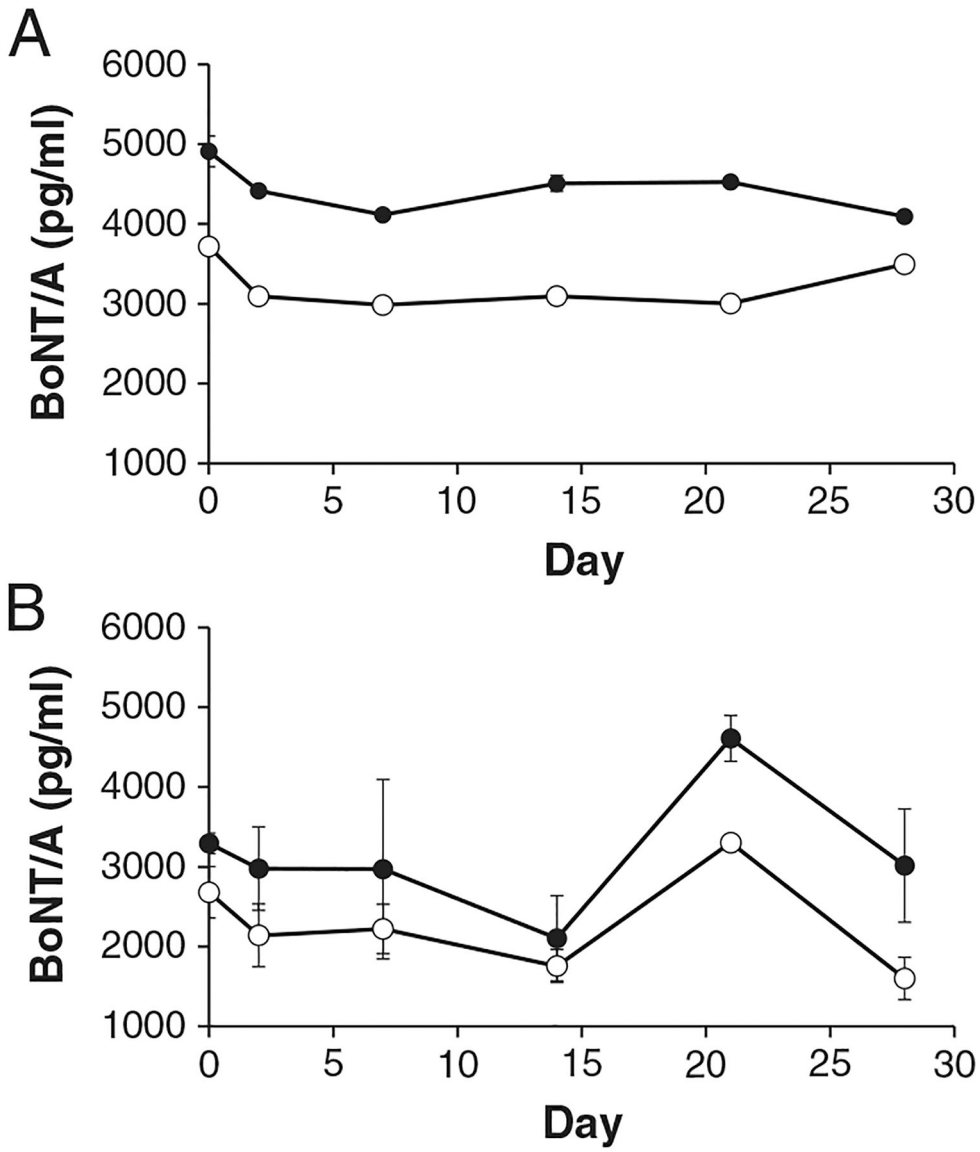

Fig. 3.

Effect of extended storage at 4 °C of municipal tap water samples. Shown is the average BoNT/A concentration determined using the Botulinum Toxin ELISA (Panel A) and Endopep MS (Panel B) at each time point. Samples include UF-concentrated water spiked with 5 μg BoNT/A per 100-L (open circle) and non-concentrated water spiked with 5000 pg/mL BoNT/A (solid circle). Error bars represent standard deviations of triplicate samples.

Table 1

Method detection limit (MDL) and quantification limit (QL) determination.

	BoNT/A (pg/mL)		
Analytical method	**Spiking level**	**MDL**	**QL**
Botulinum Toxin ELISA	2500	260	830
Endopep MS	50	21	68

Table 2

Ultrafiltration recovery efficiencies for BoNT/A (5 μg) spiked in various 100-L municipal tap water samples.

Water system	Water quality analysis							Recovery efficiency (%)	
	Turbidity (NTU)	pH	Specific conductance (μS/cm)[a]	Alkalinity (mg/L)[a]	Free chlorine (mg/L Cl_2)[b]	Total organic carbon (mg/L)	Total hardness (mg/L Ca)[a]	ELISA	Endopep MS
A	0.21	8.9	119	17	1.3	0.3	17	35	55
B	0.14	7.4	409	90	ND[c]	2.3	190	27	53
C	0.09	7.6	1090	264	0.29	ND[d]	350	11	26
D	0.06	8.5	339	79	1.3	1	130	36	42
E	0.11	9.2	366	86	ND[c]	7.6	65	26	49

[a] Water quality parameter is correlated with recovery efficiency (p-value of slope <0.05 at 95% confidence level).

[b] Free chlorine level determined prior to addition of dechlorinating agent.

[c] Free chlorine level not detected (ND) since this water system uses monochloramine. Monthly average monochloramine levels in water systems B and E were 3.4 mg/L and 2.4 mg/L, respectively.

[d] This water system has a ground water source for which the total organic carbon level is not detectable.

Table 3

Detection of a low BoNT/A level in UF-concentrated and non-concentrated municipal tap water (water system A).

	Non-concentrated water[a]	UF-concentrated water[b]	
Analytical method	**BoNT/A (pg/mL)**	**BoNT/A (pg/mL)**	**Recovery efficiency (%)**
Botulinum Toxin ELISA	<MDL	460	40
Endopep MS	<MDL	190	16

[a] Non-concentrated tap water was spiked with 7 pg/mL BoNT/A.

[b] 100-L of tap water was spiked with a total of 700 ng BoNT/A during the UF process as described in the Materials and methods section and concentrated to a final volume of 610 mL.