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

Brian H. Raphael [a,*], Matthew Lautenschlager [a], Amy Kahler [a], Suresh Pai [a], Bryan A. Parks [a], Suzanne R. Kalb [a], Susan E. Maslanka [a], Sanjiv Shah [b], Matthew Magnuson [b], Vincent R. Hill [a]

[a] Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30333, USA
[b] National Homeland Security Research Center, U.S. Environmental Protection Agency, 26 West Martin Luther King Drive, Cincinnati, OH 45268, USA

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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.

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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 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

* Corresponding author at: Centers for Disease Control and Prevention, 1600 Clifton Road, MS G-29, Atlanta, GA 30333, USA. Tel.: +1 404 639 4292; fax: +1 404 639 4290.
E-mail address: BRaphael@cdc.gov (B.H. Raphael).

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-