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# The use of Endopep-MS to detect multiple subtypes of botulinum neurotoxins A, B, E, and F☆

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ABSTRACT

Botulinum neurotoxins (BoNTs) cause the disease botulism, which can be lethal if untreated. Rapid determination of exposure to BoNT is an important public health goal. Previous work in our laboratory focused on the development of Endopep-MS, a mass spectrometry-based endopeptidase method for detecting and differentiating BoNT A–G in buffer and BoNT A, B, E, and F in clinical samples. We introduce here the use of Endopep-MS to detect non-commercial subtypes of BoNT A, B, E, and F which have been associated with botulism outbreaks. We have now tested and successfully detected 15 of the 17 known subtypes of BoNT A, B, E, and F by Endopep-MS. Extraction of BoNT A and B from a complex mixture prior to analysis is accomplished by using monoclonal antibodies specific for the catalytically inactive heavy chain of the toxin. These antibodies have high-binding affinities and do not interfere with the catalytic activity of the light chain resulting in a lower limit of detection for BoNT A and B than previously reported. We also report for the first time limits of detection for BoNT A2, A3, B2, and bivalent B using Endopep-MS.

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## 1. Introduction

Botulinum neurotoxins (BoNTs) are protein toxins produced by some species of the genus *Clostridium*, in particular, *Clostridium botulinum*, *C. butyricum*, *C. baratii*, and *C. argentinense*. BoNTs cause the disease known as botulism, which may be contracted by ingestion of food containing the toxin [1,2], colonization of the bacteria in the gastrointestinal tract of infants or immunocompromised individuals, inhalation of the toxin, or contact of the bacterium with a wound [1]. Due to its high toxicity, availability, and ease of preparation, it is considered a likely agent for bioterrorism [3]. Treatment of botulism involves administration of therapeutic immunoglobulin product and is most effective when administered within 24 h of exposure [1]. The antitoxins are serotype-specific, so rapid determination of exposure to BoNT and rapid serotyping of the toxin involved are important for efficient treatment.

Our laboratory has previously reported on the development of an assay for BoNT detection termed the Endopep-MS method [5–8]. This method detects all seven BoNT toxin types (BoNT A–G) and involves incubation of BoNT with a peptide substrate that mimics BoNT's natural target, either synaptosome-associated protein (SNAP-25) or vesicle-associated membrane protein 2 (VAMP-2, also called synaptobrevin 2) [9,10]. Each BoNT cleaves the peptide substrate in a specific, toxin-dependent location which is different for each of the BoNT toxin types [2,5–8]. The reaction mixture then is introduced into a mass spectrometer, which detects and accurately reports the mass of any peptides within the mixture. Detection of the peptide cleavage products corresponding to their specific toxin-dependent location indicates the presence of a particular BoNT toxin type, A–G. If the peptide substrate either remains intact or is cleaved in a location other than the toxin-specific site, then that BoNT toxin type is not present at detectable levels. Previous publications [5–7] have demonstrated that this method can detect BoNT at levels comparable with or lower than levels detected with mouse bioassays, the current standard as described in Ref. [11].

As previously reported, Endopep-MS is quite effective in identification of BoNT A, B, E, and F in clinical samples by using an

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antibody affinity concentration/purification step prior to reaction with the substrate [7,8]. Polyclonal antibodies to BoNT A, B, E, and F are available commercially and were found to be successful for concentration and purification of BoNT from a complex matrix. However, because polyclonal antibodies consist of a heterogeneous mixture of antibodies, they recognize various portions of the BoNT antigen molecule, including the enzymatically active light chain of the toxin. In fact, binding of polyclonal anti-BoNTA was reported to interfere with the activity of the light chain of BoNT A as measured by Endopep-MS [7]. Because Endopep-MS detects the presence of BoNT by measuring the activity of the light chain, inhibition of activity can unfortunately raise the limit of detection of BoNT.

By contrast, monoclonal antibodies recognize specific protein epitopes, ensuring that monoclonal antibodies recognize a single antigenic site. Monoclonal antibodies have recently been produced to BoNT A [12–20] and some of these antibodies are known to bind the heavy chain portion of BoNT [15,19,21], which is not enzymatically active. It was suspected that the use of monoclonal antibodies which recognize only the heavy chain of the toxin would therefore lower the limit of detection of at least some of the toxin types.

BoNT toxin types A–F are known to exhibit genetic and amino acid variance within each toxin type, or serotype. As currently defined, BoNT A consists of A1–A4 subtypes [22]. This variability between the BoNT A subtypes consists of 16% or less amino acid variance [22] and has been reported to affect binding of the toxin to monoclonal antibodies against BoNT A [21]. BoNT B is currently divided into B1, B2, nonproteolytic (np) B, and bivalent (bv) B subtypes, with an amino acid variance of 7% or less [19]. E1, E2, E3, E4 (Italian *butyricum*), and E5 (Chinese *butyricum*) subtypes currently comprise BoNT E. The latter two subtypes were isolated from BoNT E-producing *C. butyricum* strains. BoNT F is divided into proteolytic F, npF, bvF and BoNT F-producing *C. baratii* subtypes. The BoNT E subtypes exhibit 5% or less amino acid variance, and the F subtypes have 32% or less variance [22].

Because the amino acid variance can result in antigenic differences, it was uncertain if the amino acid variance would also result in enzymatic differences in terms of substrate and cleavage location. BoNT A1, A2, B1, E3, and proteolytic F are commercially available and have been successfully detected using the Endopep-MS method [5–8]. BoNT A3, A4, B2, npB, bvB, E1, E2, E4, E5, npF, bvF, and F *baratii* strains have been associated with outbreaks of botulism [22–34] and could be associated with future outbreaks of botulism. Therefore, it was a natural expansion of previous work that we examine the ability of Endopep-MS to detect these non-commercial subtypes of BoNT A, B, E, and F.

Fifteen subtypes of BoNT A, B, E, and F were available in sufficient quantities for testing and here we report an improved Endopep-MS method which can now successfully detect 15 of the 15 subtypes of BoNT A, B, E, and F tested. We were unable to obtain BoNT subtype E4 (Chinese *butyricum*) and work is still in progress on the measurement of BoNT F toxin from *C. baratii*. Improved limits of detection are reported for BoNT A1 and B1 through the use of monoclonal antibodies specific for the heavy chain of BoNT for extraction of these toxins, and we report for the first time limits of detection for BoNT A2, A3, B2, and bv B.

## 2. Materials and methods

### 2.1. Materials

Botulinum neurotoxin is very toxic and therefore requires appropriate safety measures. All neurotoxins were handled in a level 2 biosafety cabinet equipped with HEPA filters. BoNT A, B, E, and F crude culture supernatants were produced by incubating subcultures of each strain (Table 1) for 5 days at 35 °C. BoNT A2 was supplied by USAMRIID. After centrifugation, supernatants were removed and filtered through 0.22 μm filters. The filtered supernatants were tested for upper limits of toxicity, which indicated that the toxins were all present at concentrations of ≤10 μg/mL.

**Table 1**
Strain and source identification of subtypes utilized in this work

| Subtype | Genomic ID [22] | Strain |
|---|---|---|
| A1 | A150 | Hall |
| A2, bvB | Ab149 | CDC 1436 |
| A3 | A254 | Loch Maree |
| A4, bvB | Ba207 | Strain 657 |
| B1 | B155 | Okra |
| B2 | B162 | 213B, CDC 7949 |
| npB | B257 | 17B, CDC 25765 |
| E1 | E542 | Beluga |
| E2 | E544 | CDC 5247 |
| E3 | E185 | Alaska E43 |
| E4 | E543 | BL5262 |
| Proteolytic F | F188 | Langeland |
| npF | F550 | ATCC 23387 |
| bvB and bvF | Bf258 | An436 |

Bivalent subtypes include information on both subtypes.

Several supernatants were titered using mouse intraperitoneal (i.p.) endpoint assays to determine specific activity of the toxins. Initial assays using dilutions of $1 \times 10^{-4}$, $5 \times 10^{-4}$, $1 \times 10^{-5}$, and $5 \times 10^{-5}$ were done to determine a starting value for the toxin titrations. Groups of five mice were used for the initial assays. All toxins had initial titers ranging from $5 \times 10^4$ $LD_{50}$/mL to $10^5$ $LD_{50}$/mL. The final titrations involved eight twofold dilutions ranging from 200 to 1.56 $LD_{50}$/mL (20, 10, 5, 2.5, 1.25, 0.625, 0.312, and 0.156 $LD_{50}$/mouse), based on the above initial titers. Groups of 10 mice were used for the final titration.

For all procedures, toxins were diluted in gelatin-phosphate buffer (0.2% gelatin, 0.4% sodium phosphate), pH 6.2, and female Crl:CD-1 mice, 16–22 g on receipt (Charles River Laboratories, Raleigh, NC) were used. Animals were injected i.p. with total volumes of 0.1 mL diluted toxin. Mice were observed for 5 days, survivors were tallied, and the results were analyzed using probit analysis (SPSS, Chicago, IL). The final $LD_{50}$/mL of the preparation was calculated by dividing the initial dilution used in the assays by the probit result. Titrations were done in duplicate and averaged to obtain the working titers for each toxin. Duplicate titrations were also done on commercially obtained toxins (Metabiologics, Madison, WI; Wako Biologicals, Richmond, VA) using their stated toxicity as the initial dilution, and the titers were averaged to obtain their working titer.

Animal studies were conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhere to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Polyclonal rabbit-specific IgGs were provided by Metabiologics (Madison, WI) in 150 mM potassium phosphate, pH 7.4. Monoclonal antibody RAZ1 is a high-affinity human IgG1 antibody which binds all four BoNT/A subtypes with high affinity [17]. CR2 is a humanized IgG1 monoclonal antibody which binds all four subtypes of BoNT/A with high affinity [13]. 1B18 and B12.1 are fully human IgG1 monoclonal antibodies isolated via yeast display from human volunteers immunized with pentavalent BoNT toxoid. Both 1B18 and B12.1 bind all four BoNT/B subtypes with high affinity (I.N. Geren, manuscript in preparation). Monoclonal antibodies were expressed from stable CHO cell lines, purified by protein G affinity chromatog-

**Table 2**
Amino acid sequence of peptides utilized in and produced by the Endopep-MS method

| Peptide | Sequence | *m*/*z* observed |
|---|---|---|
| BoNT A substrate | Biotin-KGSNRTRIDQGN**QR**ATRXLGGK-Biotin | 2878.7 |
| BoNT A NT product | Biotin-KGSNRTRIDQGNQ | 1699.9 |
| BoNT A CT product | RATRXLGGK-Biotin | 1197.8 |
| BoNT A internal standard | R(A+7)TRXLGGK-Biotin | 1204.8 |
| BoNT B substrate | LSELDDRADALQAGAS**QF**ETSAAKLKRKYWWKNLK | 4026.8 |
| BoNT B NT product | LSELDDRADALQAGASQ | 1759.9 |
| BoNT B CT product | FETSAAKLKRKYWWKNLK | 2283.4 |
| BoNT B internal standard | LSELDDR(A+7)DALQAGASQ | 1766.4 |
| BoNT E substrate | IIGNLRHMALDMGNEIDTQNRQID**RI**MEKADSNKT | 4042.6 |
| BoNT E NT product | IIGNLRHMALDMGNEIDTQNRQIDR | 2922.8 |
| BoNT E CT product | IMEKADSNKT | 1136.6 |
| BoNT F substrate | LQQTQAQVDEVVDIMRVNVDKVLERD**QK**LSELDDRADAL | 4497.1 |
| BoNT F NT product | LQQTQAQVDEVVDIMRVNVDKVLERDQ | 3168.9 |
| BoNT F CT product | KLSELDDRADAL | 1345.8 |

The observed *m*/*z* of each peptide is also included. Residues in bold and underlined indicate the location of cleavage by BoNT.

raphy, and buffer exchanged into phosphate buffered saline (PBS) at a concentration of 1 mg/mL, exactly as previously described [15]. Dynabeads® Protein G were purchased from Dynal (Lake Success, NY) at 1.3 g/cm$^3$ in phosphate buffered saline (PBS), pH 7.4, containing 0.1% Tween®-20 and 0.02% sodium azide. All chemicals were from Sigma–Aldrich (St. Louis, MO) except where indicated. Peptide substrates were synthesized by Los Alamos National Laboratory (Los Alamos, NM, Table 2).

### 2.2. BoNT extraction

The IgG was immobilized and cross-linked to the Dynabeads® Protein G as described in the manufacturer's protocol using 40 μg of IgG for one toxin type diluted into 500 μL of PBS for every 100 μL of Dynabeads® Protein G. Cross-linked IgG-coated Dynabeads® were stored in PBS-Tween buffer (PBS with 0.05% Tween®-20) at 4 °C for up to 12 weeks. An aliquot of 20 μL of antibody-coated beads was mixed for 1 h with a solution of 5–100 μL of each culture supernatant mixed with 495 μL of phosphate buffered saline with 0.01% Tween (PBST) buffer. After mixing for 1 h with constant agitation at room temperature, the beads were washed twice in 1 mL each of PBST and then washed once in 100 μL of water. For limit of detection work, BoNT at varying levels was spiked into an aliquot of 500 μL of human serum. Negative controls consisted of human serum with no spiked toxin. The remainder of the extraction protocol was as above.

### 2.3. Endopep-MS reaction

The reaction was performed as previously described [4,5] with a few modifications. In all cases, the final reaction volume was 20 μL; the final concentration of the reaction buffer was 0.05 M Hepes (pH 7.3), 25 mM dithiothreitol, 20 μM $ZnCl_2$, and 1 mg/mL bovine serum albumin; the final concentration of the peptide substrate was 50 pmol/μL. All samples then were incubated at 37 °C for 4 h.

### 2.4. MS detection

For qualitative work, 2 μL of each reaction supernatant was mixed with 18 μL of matrix solution consisting of alpha-cyano-4-hydroxy cinnamic acid (CHCA) at 5 mg/mL in 50% acetonitrile, 0.1% TFA, and 1 mM ammonium citrate. For quantitative work, a master mix was created consisting of nine parts matrix solution and one part internal standard peptide (Table 2) in water at 1 μM for the BoNT A internal standard and 10 μM for the BoNT B internal standard. We pipetted 0.5 μL of this mixture onto each spot of a 192-spot matrix-assisted laser desorption/ionization (MALDI) plate (Applied Biosystems, Framingham, MA). Mass spectra of each spot were obtained by scanning from 1100 to 4800 *m*/*z* in MS-positive ion reflector mode on an Applied Biosystems 4800 Proteomics Analyzer (Framingham, MA). The instrument uses a nitrogen laser at 337 nm, and each spectrum is an average of 2400 laser shots.

## 3. Results

### 3.1. Qualitative detection of BoNT A1–A4 subtypes

BoNT A can be classified into four subtypes: A1–A4 [22]. We previously reported the ability of BoNT A1 to cleave its peptide substrate, derived from the *in vivo* target of SNAP-25 [5–8]. In the presence of BoNT A1, the substrate at *m*/*z* 2878.7 is cleaved into an N-terminal cleavage product at *m*/*z* 1699.9 and a C-terminal cleavage product at *m*/*z* 1197.8. Both products and the substrate are easily detectable by mass spectrometry. Following extraction of BoNT A1–A4 from culture supernatants, those toxins were subjected to Endopep-MS and the reaction supernatant was analyzed via MALDI-MS. Fig. 1A is the spectrum from the BoNT A1 reaction, consisting of intact substrate at *m*/*z* 2878.7, N-terminal cleavage product at *m*/*z* 1699.9, and C-terminal cleavage product at *m*/*z* 1197.8. This spectrum demonstrates that BoNT A1 cleaves its peptide substrate between the asparagine and the arginine (Table 2), as only cleavage at this location can produce cleavage products at *m*/*z* 1699.9 and 1197.8.

Fig. 1B is the spectrum from the BoNT A2 reaction. The results are very similar to the BoNT A1 results with intact substrate at *m*/*z* 2878.7, N-terminal cleavage product at *m*/*z* 1699.9, and C-terminal cleavage product at *m*/*z* 1197.8. The presence of peaks at *m*/*z* 1699.9 and 1197.8 indicate that BoNT A2 cleaves its peptide substrate at the same location as A1, namely between the asparagine and arginine.

Fig. 1C is the spectrum from the BoNT A3 reaction. Again, the results show intact substrate at *m*/*z* 2878.7, N-terminal cleavage product at *m*/*z* 1699.9, and C-terminal cleavage product at *m*/*z* 1197.8. These peaks verify that BoNT A3 cleaves its peptide substrate at the same location as BoNT A1 and A2. Fig. 1D is the spectrum from the BoNT A4 reaction. The intact substrate at *m*/*z* 2878.7, N-terminal cleavage product at *m*/*z* 1699.9, and C-terminal cleavage product at *m*/*z* 1197.8 verify that BoNT A4, as with BoNT A1–A3, all cleave the same peptide substrate at the same location. These data are the first known proof that BoNT A1–A4 all cleave the SNAP-25 peptide substrate at the same location. These data also demonstrate that Endopep-MS can be used to detect all known BoNT A subtypes.

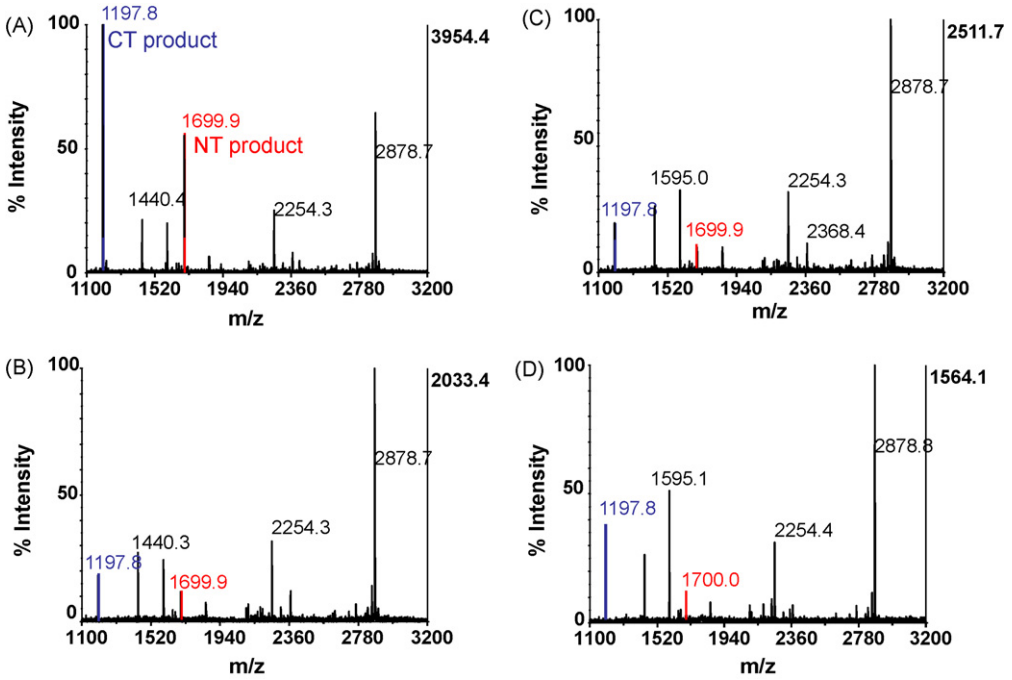


**Fig. 1.** Mass spectra for the reaction of BoNT A1 (A), BoNT A2 (B), BoNT A3 (C), and BoNT A4 (D) with the peptide substrate. The substrate is present at *m*/*z* 2878.7, the N-terminal cleavage product in red at *m*/*z* 1699.9, and the C-terminal cleavage product in blue at *m*/*z* 1197.8.

### 3.2. Qualitative detection of BoNT B1, B2, npB, and bvB subtypes

BoNT B can be classified into four subtypes: B1, B2, npB, and bvB [22]. We previously reported the ability of BoNT B1 to cleave its peptide substrate derived from the *in vivo* target of VAMP-2 [5–7]. In the presence of any one of the four subtypes of B, the substrate at *m*/*z* 4026.8 is cleaved into an N-terminal cleavage product at *m*/*z* 1759.9 and a C-terminal cleavage product at *m*/*z* 2283.4 as seen in Fig. 2. These data are the first known data demonstrating that BoNT B2, npB, and bvB all cleave their peptide substrate at the same location as BoNT B1 and demonstrate that Endopep-MS can be used to detect all BoNT B subtypes.

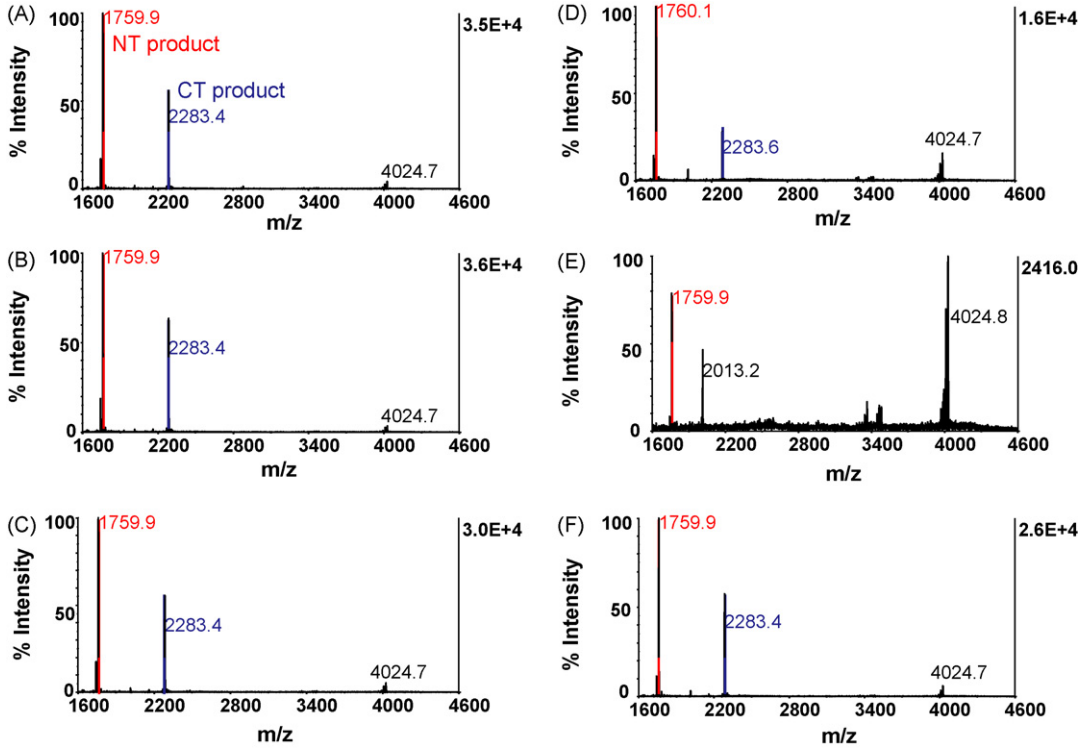


**Fig. 2.** Mass spectra for the reaction of BoNT B1 (A), BoNT B2 (B), BoNT Ba4 (C), BoNT Bf (D), and nonproteolytic B without (E) and with (F) trypsin activation prior to analysis. The substrate is present at *m*/*z* 4026.8, the N-terminal cleavage product in red at *m*/*z* 1759.9, and the C-terminal cleavage product in blue at *m*/*z* 2283.4.

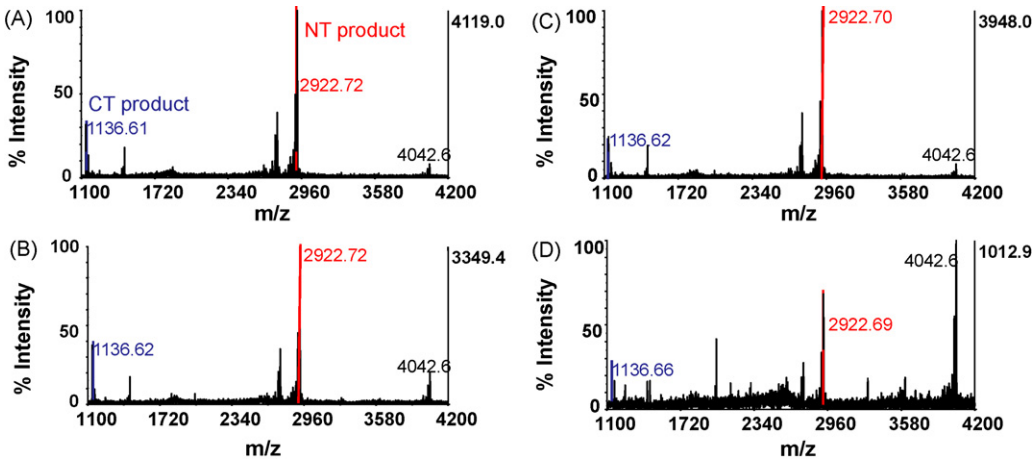


**Fig. 3.** Mass spectra for the reaction of BoNT E1 (A), BoNT E2 (B), BoNT E3 (C), and BoNT E4 (D) with the peptide substrate. The substrate is present at *m*/*z* 4042.6, the N-terminal cleavage product in red at *m*/*z* 2922.8, and the C-terminal cleavage product in blue at *m*/*z* 1136.6.

All BoNT are produced as a single chain protein and must be cleaved into a heavy chain and a light chain in order to be enzymatically active. Proteolytic strains of *Clostridia* produce proteases such as trypsin which cleave the neurotoxin into an active form. Nonproteolytic (np) B requires the addition of trypsin or another protease to cleave the toxin into the more toxic dichain configuration. Incubation with trypsin cleaves the single chain into its dichain form, which increases toxicity by approximately two orders of magnitude. Thus, npB is typically trypsinized prior to detection via the mouse bioassay. We investigated the role of trypsin with npB by analyzing two npB samples in parallel—one sample was tryptically activated prior to toxin extraction and the other sample was not tryptically activated. Fig. 2E is the spectrum obtained without trypsin activation and Fig. 2 is the spectrum obtained with trypsin activation. Although the N-terminal cleavage products are present in both spectra at *m*/*z* 1759.9, it is apparent that there is substantially more substrate (*m*/*z* 4026.8) present in the sample without trypsin activation. This is a result of the decreased ability of npB without trypsin activation to cleave its peptide substrate. Therefore, it is apparent that npB toxin requires trypsin activation for optimal detection via Endopep-MS. However, if a substantial amount of npB is present, it is detectable without trypsin activation.

### 3.3. Qualitative detection of multiple BoNT E and F subtypes

BoNT E can be classified into five subtypes: E1–E5 [22]. We previously reported the ability of BoNT E3 to cleave its peptide substrate derived from the *in vivo* target of SNAP-25 [5–7]. In the presence of BoNT E1, E2, E3, or E4, the substrate at *m*/*z* 4042.6 is cleaved into an N-terminal cleavage product at *m*/*z* 2922.8 and a C-terminal cleavage product at *m*/*z* 1136.6 (Fig. 3). An E Chinese *butyricum* (BoNT E5) toxin sample was unavailable, so we could not test the ability of Endopep-MS to detect that subtype. However, these data demonstrate the ability of Endopep-MS to detect four of the five subtypes of BoNT E, and are the first known proof that BoNT E1, E2, and E4 all cleave their peptide substrate at the same location as BoNT E3.

BoNT F can be classified into four subtypes: proteolytic F, npF, bvF and F *baratii* [22]. We previously reported the ability of BoNT proteolytic F to cleave its peptide substrate derived from the *in vivo* target of SNAP-25 [5–7]. In the presence of proteolytic F, npF, or bvF, the substrate at *m*/*z* 4497.1 is cleaved into an N-terminal cleavage product at *m*/*z* 3168.9 (not visible in all cases) and a C-terminal cleavage product at *m*/*z* 1345.8 (Fig. 4). Cultures of F *baratii* are very rare and many only produce toxin at very low levels. We were unable to obtain samples of BoNT F toxin from *C. baratii* in sufficient amounts for testing, so work is still in progress on the measurement of BoNT F toxin from *C. baratii*. However, these data demonstrate the ability of Endopep-MS to detect three of the four subtypes of BoNT F, and are the first known proof that BoNT proteolytic F, npF, and bvF cleave their peptide substrate at the same location.

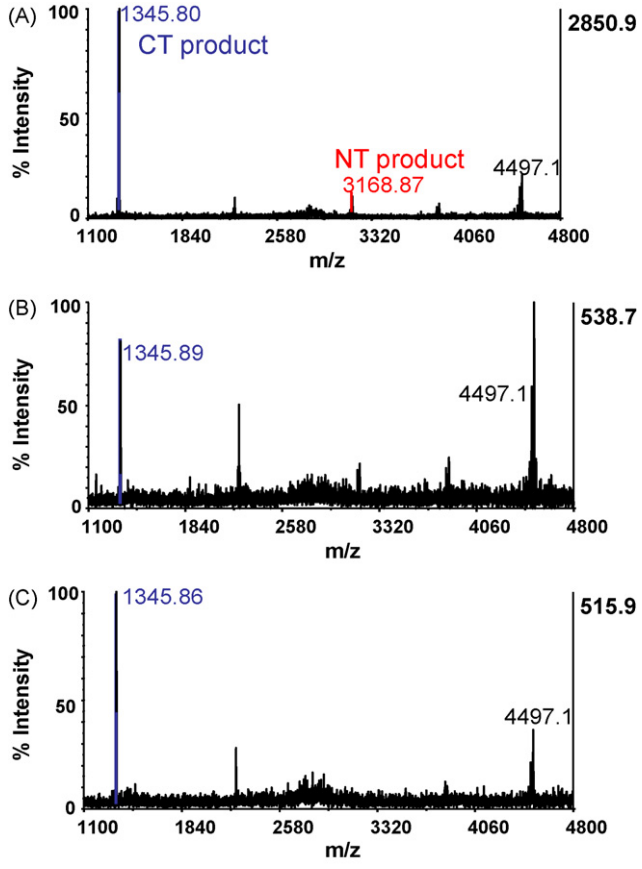


**Fig. 4.** . Mass spectra for the reaction of BoNT protF (A), BoNT npF (B), and bivalent F (C), with the peptide substrate. The substrate is present in red at *m*/*z* 4497.1, the N-terminal cleavage product in blue at *m*/*z* 3168.9, and the C-terminal cleavage product at *m*/*z* 1345.8.

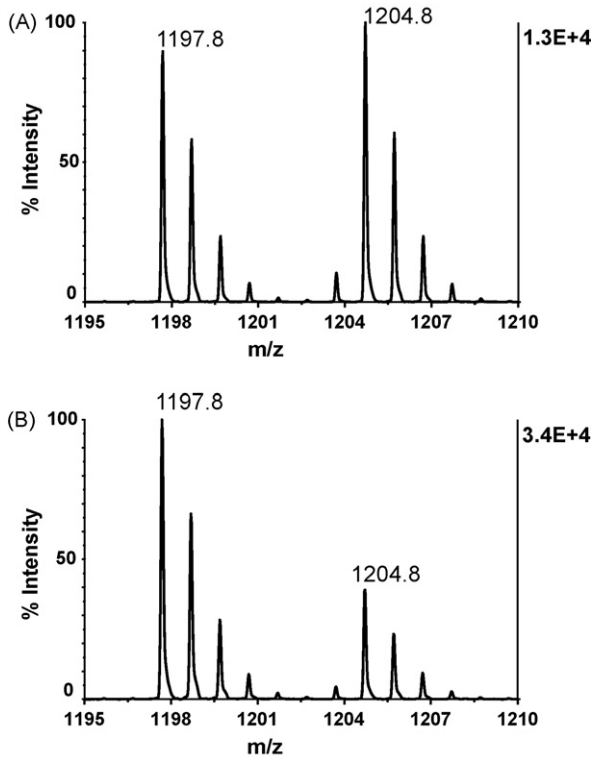


**Fig. 5.** Mass spectra for the reaction of BoNT A1 with polyclonal (A) antibody extraction and monoclonal (B) antibody extraction. Both samples contain the same level of internal standard at *m*/*z* 1204.8, but each sample contains a different level of C-terminal cleavage product at *m*/*z* 1197.8.

### 3.4. Comparison of polyclonal and monoclonal antibodies for detection of BoNT A

Our laboratory previously reported the extraction and concentration of BoNT prior to its detection via Endopep-MS [7]. After examining many possibilities for BoNT extraction from a clinical or food sample, we determined that the use of immunoaffinity improved purification. We used polyclonal antibodies to BoNT A, B, E, and F to extract those toxin types from clinical samples. However, we suspected that the polyclonal antibodies may be interfering with the toxin's activity in some cases by binding the enzymatically active light chain of the toxin and inhibiting its interaction with the peptide substrate. Therefore, we wanted to explore the possibility that monoclonal antibodies targeted against the catalytically inactive heavy chain of the neurotoxin might produce more sensitive results, since they should not interfere with the catalytic activity of the light chain.

Monoclonal antibodies RAZ1 and CR2 have been shown to bind the heavy chain of A1–A4 with high affinity [13,17]. Fig. 5A is the spectrum obtained from the Endopep-MS reaction of BoNT A1 utilizing polyclonal antibodies and Fig. 5B is the spectrum obtained from the Endopep-MS reaction of BoNT A1 utilizing a monoclonal antibody mixture of RAZ1 and CR2. Both spectra contain a peak corresponding to the intact substrate at *m*/*z* 2878.7 (not shown) as well as peaks corresponding to the N-terminal cleavage product at *m*/*z* 1699.9 (not shown) and the C-terminal cleavage product at *m*/*z* 1197.8.

Both spectra also contain the internal standard at *m*/*z* 1204.8. The internal standard is an isotopically labeled version of the C-terminal cleavage product in which the alanine in position two has a +7 mass difference than that of a naturally occurring alanine. Because the internal standard has the same peptide sequence as the C-terminal cleavage product, it can be used to compare the amount of C-terminal cleavage product in two or more samples. The peak areas of the C-terminal cleavage product and its internal standard and the ratio of those peak areas are listed in Table 3. Monoclonal antibodies were also utilized to compare BoNT A2–A4, and those results are also listed in Table 3. In all BoNT A cases, extraction with monoclonal antibodies resulted in an increase in the formation of the C-terminal cleavage product. The average increase in the C-terminal cleavage product was twofold.

### 3.5. Comparison of polyclonal and monoclonal antibodies for detection of BoNT B

Monoclonal antibodies B12.1 and 1B18 bind the heavy chain of BoNT B1, B2, npB, and bvB with high affinity (I.N. Geren, manuscript in preparation). Indeed, when a mixture of monoclonal antibodies were used to extract BoNT B1, B2, npB, and bvB, the N-terminal and C-terminal cleavage products could be detected in all cases (Fig. 2). Extraction of BoNT B1, B2, npB, and bvB with the polyclonal antibodies was compared to extraction with a monoclonal antibody mixture. As with BoNT A, an internal standard was utilized.

The internal standard for BoNT B is an isotopically labeled version of the N-terminal cleavage product, and in the case of this internal standard, the alanine in position eight has a +7 mass difference than that of a naturally occurring alanine. The peak areas of the N-terminal cleavage product and its internal standard for BoNT B subtypes and the ratio of those peak areas are also listed in Table 3. It is apparent that the sample with monoclonal antibody extraction contains more N-terminal cleavage product than the sample with polyclonal antibody extraction. On average for BoNT B, there was a sixfold increase in the N-terminal cleavage product when using monoclonal antibodies.

### 3.6. Limits of detection of BoNT A and B subtypes utilizing monoclonal antibodies

Because it was apparent that the ability to detect BoNT A and B subtypes was improved using monoclonal antibodies compared to polyclonal antibodies, we wanted to explore the limits of detection of the BoNT A and B subtypes using monoclonal antibodies for toxin extraction from serum. Varying levels of BoNT A1–A3, B1, B2, and bvB were spiked into serum and following extraction, were subjected to Endopep-MS for detection.

Following this protocol, levels of BoNT A1 and A2 as low as 0.5 mouse $LD_{50}$ could be detected in serum (supplemental data). Levels of BoNT A3 as low as 0.05 mouse $LD_{50}$ could be detected. It is unclear at this point if the lower detection limit of BoNT A3 compared to BoNT A1 and A2 is due to an increased concentration of the neurotoxin in the BoNT A3 supernatant or due to an increase in specific activity of BoNT A3. We did not titer the BoNT Ba4 toxin sample for activity, so we were unable to obtain a limit of detection for BoNT A4. BoNT A4 toxin is produced in conjunction with bivalent BoNT B, and the BoNT B level is significantly greater than that of the BoNT A4 toxin level, so that only minor amounts of BoNT A4 are produced, and titration of the BoNT A toxin component of the sample would be difficult.

Levels of BoNT B1 as low as 0.1 mouse $LD_{50}$ could be detected in serum (supplemental data) utilizing monoclonal antibodies for toxin extraction. Levels of BoNT B2 as low as 0.005 mouse $LD_{50}$ could be detected in serum (supplemental data). Levels of BoNT bvB from BoNT Ba4 as low as 0.05 mouse $LD_{50}$ could be detected (supplemental data). Again, it is unclear if the variance in detection limits for the BoNT B subtypes is due to a difference in concentration of the toxin, or due to a difference in specific activity of the toxin. We were unable to obtain a limit of detection for BoNT npB

**Table 3**
Peak areas and peak area ratios of cleavage products and internal standards (ISTD) for BoNT A and B obtained with polyclonal and monoclonal antibodies

| Toxin type | Antibody | Peak area of product | Peak area of ISTD | Peak area ratio of product/ISTD |
|---|---|---|---|---|
| BoNT A1 | Polyclonal | 170,000 | 167,000 | 1.02 |
| BoNT A1 | Monoclonal | 263,000 | 172,000 | 1.53 |
| BoNT A2 | Polyclonal | 93,600 | 66,800 | 1.40 |
| BoNT A2 | Monoclonal | 185,000 | 91,000 | 2.03 |
| BoNT A3 | Polyclonal | 195,000 | 110,000 | 1.78 |
| BoNT A3 | Monoclonal | 290,000 | 98,300 | 2.96 |
| BoNT A4 | Polyclonal | 446 | 7,440 | 0.06 |
| BoNT A4 | Monoclonal | 1,390 | 6,700 | 0.21 |
| BoNT B1 | Polyclonal | 46,000 | 100,000 | 0.46 |
| BoNT B1 | Monoclonal | 288,000 | 93,900 | 3.08 |
| BoNT B2 | Polyclonal | 120,000 | 113,000 | 1.06 |
| BoNT B2 | Monoclonal | 326,000 | 105,000 | 3.09 |
| BoNT npB | Polyclonal | 11,700 | 120,000 | 0.1 |
| BoNT npB | Monoclonal | 91,700 | 111,000 | 0.83 |
| BoNT bvB | Polyclonal | 46,600 | 98,700 | 0.47 |
| BoNT bvB | Monoclonal | 356,000 | 117,000 | 3.04 |

Larger ratios indicate higher amounts of cleavage.

as this sample was not titered for specific activity using the mouse bioassay.

## 4. Discussion

Previous work in our laboratory established the use of Endopep-MS for the detection of commercial types of BoNT A, B, E, and F. However, it was unknown if other, non-commercial subtypes of BoNT A, B, E, and F would cleave the peptide substrate and if so, cleave it in the same location. These experiments determined that multiple BoNT A, B, E, and F subtypes cleave their respective substrates at identical sites, despite having large genetic differences in their neurotoxins. These data imply that the non-commercial subtypes of BoNT A, B, E, and F have the ability to cleave the natural, *in vivo* substrate, SNAP-25 or VAMP-2, in the same location as the commercial subtypes of BoNT A, B, E, and F.

These data also demonstrate the utility of Endopep-MS for detecting the presence of multiple, diverse BoNT A, B, E, and F; in some cases, at levels well below that of the current standard, the mouse bioassay. Mouse bioassay detection of some BoNT subtypes; in particular, BoNT A4 and BoNT bvB, can be challenging due to the presence of multiple toxin types. BoNT A4 is currently only known to be produced as a minor component of strain 657 where the major component is bivalent BoNT B [22]. Bivalent B is found in combination with BoNT A or F, as BoNT Ab, Ba, or Bf. Regardless of the partnering toxin, the BoNT B sequence for this subtype is similar. BoNT E5 and BoNT F *baratii* were currently unavailable in sufficient amounts for analysis. However, toxins representing the other 15 of the 17 BoNT A, B, E, and F subtypes were available and were compared in this study.

Previously, we noted that the activity of BoNT A in particular seemed to be inhibited in the presence of polyclonal antibodies [7]. The use of monoclonal antibodies offered an opportunity to extract BoNT without inhibiting its enzymatic activity, provided that the antibodies were directed against the heavy chain, which is catalytically inactive. The BoNT A monoclonal antibodies utilized in these experiments were known to bind the heavy chain of all four subtypes of BoNT A with high affinity [13,17]. The combination of high affinity and binding to nonenzymatic epitopes of these antibodies resulted in a decrease in the limit of detection of BoNT A1 in serum to a level comparable or better than that of the mouse bioassay. Furthermore, limits of detection of BoNT A3 were established and were found to be approximately one order of magnitude lower than that of the mouse bioassay, the current standard for detection of BoNT.

High-affinity antibodies for BoNT B that are directed against the heavy chain of the toxin were also obtained. Through the use of these antibodies, limits of detection of BoNT B1 in serum decreased by a factor of 5, to levels below that of the mouse bioassay. Furthermore, limits of detection of BoNT B2 and bvB were established and were found to be approximately two orders of magnitude lower than that of the mouse bioassay. The production of monoclonal antibodies for BoNT E and F are ongoing, and we hope to evaluate these monoclonal antibodies for the extraction BoNT E and F and subsequent testing by Endopep-MS.

One very interesting aspect of this work is that the limits of detection of some toxin subtypes varied by as much as two orders of magnitude when compared against *in vivo* toxicity (mouse $LD_{50}$). It is unclear if this phenomenon is due to concentrations of toxin which may vary by two orders of magnitude. It is possible that this variance is due to heavy chain activity *in vivo* which plays a role in the mouse bioassay but is not measured with Endopep-MS. It is also possible that this variance is due to different specific activities of the toxin; i.e., a certain amount of BoNT A3 may be significantly more active than the same amount of BoNT A1. This question can be answered through quantitation of the amount of BoNT A1, A2, A3, or A4 which is responsible for a quantified activity level. These experiments are ongoing in our laboratory, and we hope that the results of those experiments help to shed some light on this interesting observation.

In conclusion, we have demonstrated that Endopep-MS can be used to detect non-commercial subtypes of BoNT A3, A4, B2, npB, bvB, E1–E3, npF, and bvF in addition to the previously reported detection of commercial subtypes BoNT A1, A2, B1, E3, and proteolytic F. Through the use of monoclonal antibodies directed against the catalytically inactive heavy chain for extraction of BoNT A and B, we have demonstrated that the limits of detection of BoNT A and B spiked into serum can be significantly lowered when compared with polyclonal antibodies [7]. These experiments demonstrate the utility of Endopep-MS to detect a wide range of BoNT A, B, E, and F subtypes.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2008.04.004.

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