Chapter 6

Detection, Differentiation and Subtyping of Botulinum Neurotoxins in Clinical Samples with Mass Spectrometry

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Botulinum neurotoxins (BoNTs) are the most toxic substances known to man and are the etiologic agents responsible for the deadly disease botulism. To provide a means of assessing exposure to BoNTs, we have developed the Endopep-MS method, which can detect and differentiate BoNTs in clinical and food samples. The method involves extracting the toxin with high affinity antibodies that are selective for only one of the BoNT serotypes; cleavage of synthetic peptides according to the specific enzymatic activity for each of the BoNT serotypes; and selectively detecting the toxin-dependent peptides by using high resolution matrix-assisted laser–desorption ionization time-of-flight mass spectrometry. We can achieve limits of detection lower than that of the mouse bioassay, the historic standard, in clinical samples with BoNT/A, /B, /E, and /F, the four serotypes typically associated with human cases of botulism. We discuss the application of the method with two examples: an outbreak of botulism from hot dog chili sauce and botulism in Mississipi catfish. We have also added proteomic analysis to identify BoNT subtypes and strains allowing epidemiologists to quickly assess possible similarities and differences between concurrent botulism outbreaks.

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Introduction

Botulinum neurotoxins (BoNT) are the most toxic substances known (*1*). They are produced under anaerobic conditions by strains of *Clostridium botulinum*, *C. butyricum*, *C. baratii*, and *C. argentinese* (*1*, *2*). Intoxication with one of the seven distinct serotypes of BoNT (A-G) causes the deadly disease known as botulism, which is characterized by a flaccid paralysis (*2*). Four serotypes of BoNT, /A, /B, /E, and /F, are known to cause botulism in humans (*2*). Typically, human cases of botulism are from ingestion of foods that contain the toxins or colonization of toxin producing species of *Clostridia* in the gastrointestinal tracts of infants or immunocompromised adults or in wounds (*2*). In 2008, the CDC reported 153 cases of botulism in the US (*3*). The 2008 botulism cases included 12% foodborne, 73% infant, 15% wound and 1% of unknown etiology. The 18 foodborne botulism cases reported in 2008 were caused by type A toxin (56%) and type E toxin (33%) with 11% unknown toxin type. The infant botulism cases were caused by type A toxin (45%), type B toxin (54%), type F toxin (1%) and a dual toxin producing (Bf) *C. botulinum* species (1%). The 15 wound botulism cases were all from type A toxin (*3*).

BoNTs are zinc metalloproteases that cleave and inactivate specific cellular proteins that are essential for the release of the neurotransmitter acetylcholine (Figure 1). Each of the BoNT toxins are composed of a heavy chain which is about 100,000 daltons and a light chain of about 50,000 daltons (*1*). The heavy chain is responsible for receptor binding and delivering the catalytic light chain into the area of target neurons (*4*, *5*). The light chain is the zinc metalloprotease portion of the toxin that selectively cleaves neuronal proteins required for normal exocytosis (*1*). Although the light chain is responsible for the specific toxicity, the heavy chain is required to produce the *in vivo* toxicity.

Each BoNT serotype has a specific toxin dependent cleavage site. BoNT A, C and E cleave SNAP-25. BoNT A cleaves SNAP-25 between glutamine-196 and arginine-197 (*6–9*), BoNT C cleaves SNAP-25 at the adjacent residue between arginine-197 and alanine-198 (*10*, *11*), while BoNT E cleaves SNAP-25 between arginine-180 and isoleucine-181 (*7–9*). BoNT B, D, F, G all cleave synaptobrevin 2 (also called VAMP 2). The cleavage of synaptobrevin 2 by BoNT B, D, F, and G are at glutamaine-75 (*12*), lysine-58 (*8*, *13*), glutamine-57 (*13*), and alanine-61 (*14*, *15*) respectively. Of the serotypes mentioned, only BoNT C cleaves more than one site on a specific protein. In addition to cleaving SNAP-25, BoNT C also cleaves syntaxin between lysine-253 and alanine-254 (*16*, *17*).

In addition to the 7 serotypes of BoNT there are subtypes that at have similar serological properties. Within serotype A there are 5 known subtypes, A1-A5 (*18*, *19*). These currently known subtypes have between 2% to 16% differences in amino acid sequence. Within a subtype, there are different strains that can differ by as little as one amino acid difference. BoNT/B is currently divided into B1, B2, B3, nonproteolytic (np) B (B4), bivalent (bv) B (B5), and B6 subtypes (*18*, *20*), with an amino acid variance of 7% or less. E1, E2, E3, E4 (Italian *butyricum*), E5 (Chinese *butyricum*), and E6 subtypes currently comprise BoNT/E (*18*, *21*, *22*). Two E 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*

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subtypes (*18*). The BoNT E subtypes exhibit 5% or less amino acid variance while the known F subtypes have up to 32% variance.

It should be noted that botulinum neurotoxins are very toxic and must be handled using care and appropriate safety measures. All neurotoxins require handling in a level 2 biosafety cabinet equipped with HEPA filters.

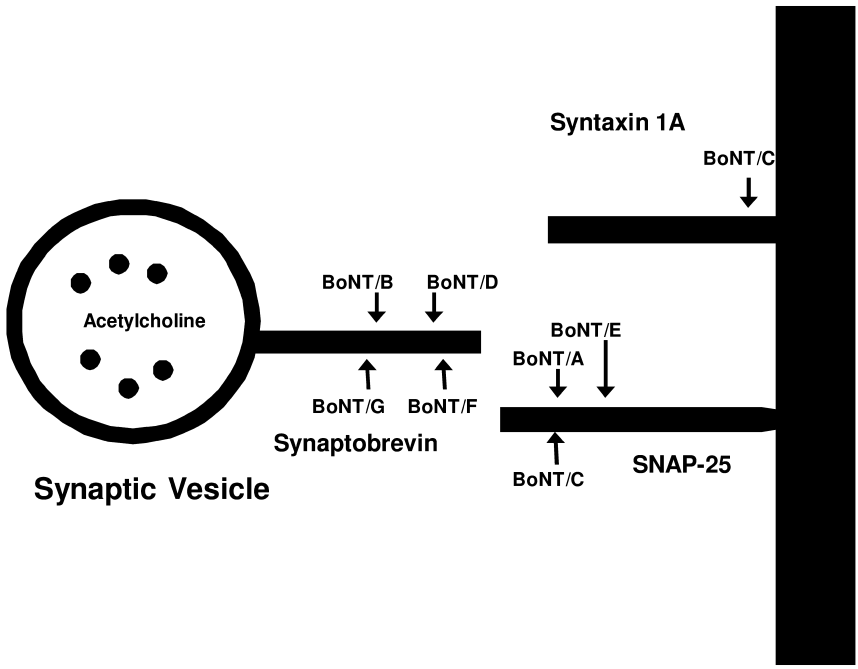

Figure 1. Schematic of the soluble N-ethylmaleimide sensitive fusion protein attachment receptor (SNARE) complex. All proteins must be intact for acetylcholine release. The cleavage sites of the proteins by BoNT are depicted.

Detection and Serotype Differentiation of Botulinum Neurotoxins

Historically, the mouse bioassay has been the most commonly used and accepted method to detect BoNT and confirm a diagnosis of botulism (*2*, *23*). The mouse bioassay employs mixtures of neutralizing antibodies given to mice in conjunction with the sample in question to differentiate the toxin serotype. Mice receiving the appropriate anti-BoNT serotype antibody with the toxic sample survive, while mice treated with the other serotype antibodies do not survive. The mouse bioassay is very sensitive detecting 1 mouse $LD_{50,}$ which is thought to contain approximately 10 pg of active toxin for BoNT/A (*24*). However, the mouse bioassay is slow (taking up to 4 days) for final results, requires several grams of sample and requires a great deal of live animal use. Detection limits

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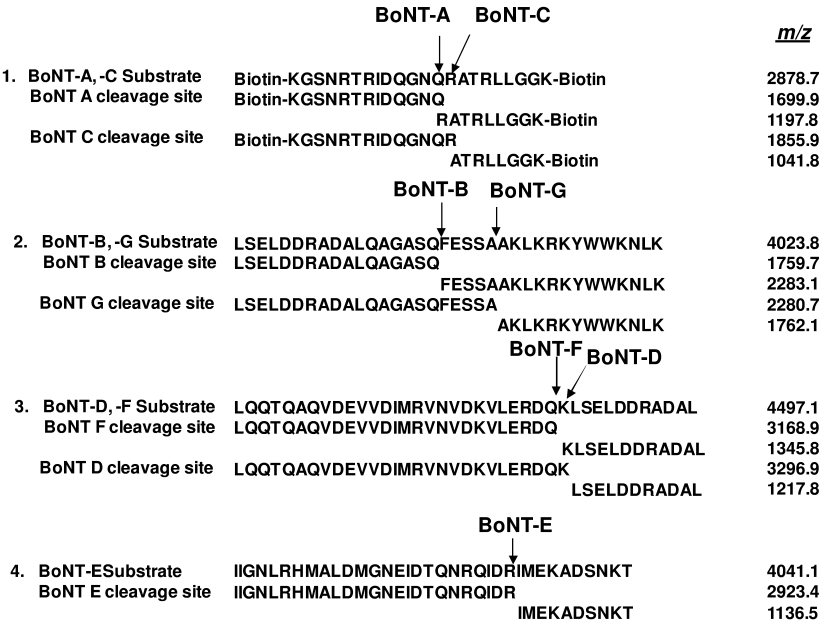

Figure 2. Amino acid sequences of peptide substrates used in the Endopep-MS assay for detection and differentiation of botulinum neurotoxins (BoNT). Cleavage products of each serotype of BoNT and their observed m/z are also present. Peptide 1 is a mimic of the natural substrate for BoNT/A and /C whose natural target is SNAP-25. Peptide 2 is the substrate for BoNT/B and /G and is a mimic for VAMP 2. Peptide 3 is the substrate for BoNT/D and /F and is a mimic for VAMP-2, and peptide 4 is the substrate for BoNT/E and is a mimic for SNAP-25.

for *in vitro* methods and purified BoNT standards are generally reported as the number of mouse LD_{50} and therefore in this chapter, we will hold to the same convention.

To improve the detection of BoNT, a mass spectrometry based method called Endopep-MS has been developed (*25–30*). The Endopep-MS method detects BoNT and determines the serotype at the same time. It is similar in concept to previous non-mass spectrometry based *in vitro* methods for BoNT/A and /B (*31–34*) . The Endopep-MS method is based on extraction with antibodies specific to the toxin serotype (*27*). A reaction of the toxin with synthetic peptides that mimic the natural targets of the toxin produces unique cleavage products that can be differentiated by mass spectrometry (Figure 2) (*25–30*). Since each BoNT serotype has a different cleavage site, the product peptides not only detect the toxin activity but also differentiate the serotype.

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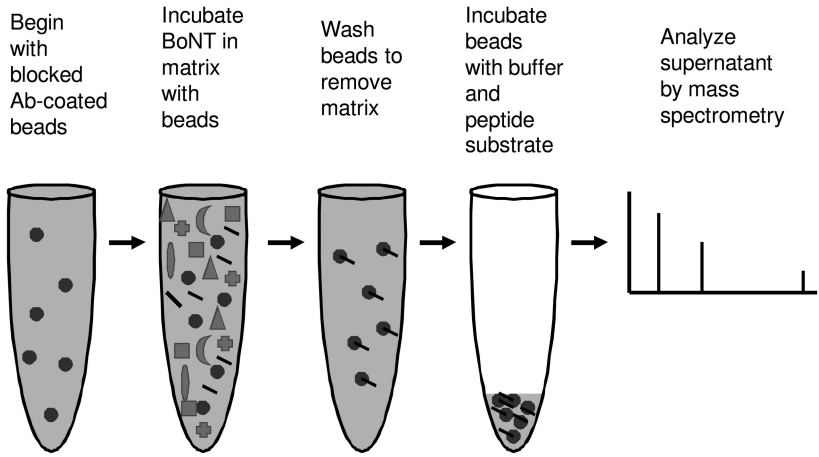

Figure 3. Protocol for sample preparation for Endopep-MS method to detect and differentiate botulinum neurotoxins (BoNT). Magnetic beads are coated with serotype specific antibodies and then incubated with the sample to extract BoNT. The beads are washed to remove nonspecific binding compounds and then incubated with peptide mimics to the natural substrate of the BoNT. The cleavage of the peptide mimic is then detected by matrix assisted laser desorption mass spectrometry.

Extraction of BoNT from Clinical, Food and Culture Supernatants

The selective extraction of the toxin from culture supernatants, serum, stool, and food samples is necessary for detection of the enzymatic activity of BoNT. Clinical samples and foods contain high levels of endogenous proteases that can cleave the peptide substrates or cleave the product peptides before they can be detected by the mass spectrometer (*27*). The selective extraction of very low levels of BoNT from a complex matrix is essential to the Endopep-MS method. Antibodies specific for each BoNT serotype are an efficient way of selectively extracting the toxin from cultures, clinical and food samples (*27*). Antibodies are bound and cross-linked to magnetic beads and added to the culture, serum or a stool extract. The toxin binds to the antibodies that are attached to the magnetic beads. The beads containing antibodies and toxin are removed from the samples and washed to remove materials that were nonspecifically bound (Figure 3). Polyclonal antibodies produced in animals such as rabbits tended to yield higher detection limits for BoNT especially BoNT/A and /B (*27*). We therefore use mixtures of high-affinity monoclonal antibodies from yeast displays that bind selectively to specific epitopes on the BoNT (*28*, *35*). These antibodies were discovered using a yeast library produced from individuals immunized against BoNTs. The panels of antibodies discovered from the yeast displays were then tested in the Endopep-MS method and multiple antibodies for each serotype that yielded the best sensitivity and selectivity were incorporated into the method.

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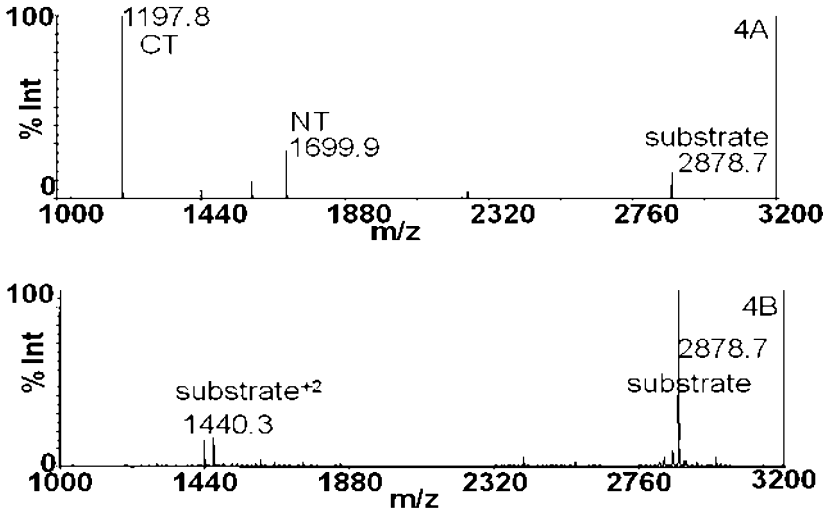

Figure 4. Mass spectrometry detection of the cleavage products for botulinum neurotoxin A (BoNT A). Panel A shows the substrate (Biotin-KGSNRTRIDQGNQRATRLLGGK-Biotin, MH^+ = 2878.7) is cleaved by BoNT A at a toxin dependent site to form two product peptides. The N-terminal (NT) product peptide is Biotin-KGSNRTRIDQGNQ (MH^+ = 1699.9 and the C-terminal (CT) product peptide is RATRLLGGK-Biotin (MH^+ = 1197.8). Panel B is a negative control and shows the singularly and doubly charged substrate ions at m/z 2878.7 and 1140.3 respectively.

Endopep-MS Peptide Cleavage Reactions and Mass Spectrometry Detection

BoNTs are zinc metalloproteases that cleave and inactivate specific cellular proteins essential for the release of the neurotransmitter acetylcholine (Figure 1). Each BoNT serotype recognizes and cleaves a unique site on either SNAP-25 or VAMP-2. We have synthesized peptides that mimic the specific portions of SNAP-25 and VAMP-2 that are substrates for all seven BoNT serotypes (Figure 2) (*26*). The endopeptidase activity is used to detect and differentiate the serotype by allowing the toxin to cleave its specific peptide substrate and detect the cleavage products by mass spectrometry (*25*, *26*). In the presence of active BoNT, the peptide substrate is cleaved in a unique location that is characteristic for each serotype. The reaction mixture is then analyzed by mass spectrometry, which detects the peptides present and accurately reports the mass of each peptide. The presence of the peptide cleavage products corresponding to their toxin-dependent location indicates the presence of a particular serotype of BoNT (*25–30*). If the peptide substrate either remains intact or is cleaved in a location other than the toxin-specific site, that serotype of BoNT is not present above the detection limit

of the method. There is no cross-reactivity between the toxin serotypes (*25*, *26*). Each BoNT serotype cleaves only its peptide substrate, and only in the site that is specific for the serotype. Thus, the mass spectral determination of the enzymatic activity differentiates the toxin serotype (*25–30*).

There are advantages to using the specific enzymatic activity to detect and differentiate BoNT. The first major advantage is that the Endopep-MS is an activity-based method that measures enzymatically active toxin only. Protein toxin can be inactivated in several ways, including heat and chemicals, but only active toxins are a threat to human health. Methods such as ELISA tend to measure both active and inactive toxins. The Endopep-MS measures only BoNTs that have their enzymatic activity. The second major advantage is sensitivity. BoNTs are toxic at very low levels because they are efficient enzymes. Measuring the enzymatic activity is an enhancement in the amount of product peptide that is detected. If each BoNT cleaves the substrate peptide 1000 times, then there are three orders of magnitude more product peptide than toxin, yielding much lower limits of detection than could be obtained by detecting the toxin directly. The third major advantage is the specificity of the enzymatic reaction, which allows toxin serotypes to be determined in a single experiment.

Mass Spectrometry Detection of Cleavage Products

The BoNT dependent cleavage reactions are diluted in matrix solution and then deposited on a MALDI plate. After drying, the MALDI plate is placed in the instrument, and spectra of the BoNT dependent cleavage reactions are acquired using a MALDI-TOF mass spectrometer in positive ion, reflector mode. Data is acquired in a mass range of *m/z* 1100 to 4800 to obtain data on the smallest of the cleavage products at *m/z* 1198 up through the largest of the intact substrate peptides at *m/z* 4497. The presence of peaks which correspond to cleavage products indicates the presence of a particular serotype of BoNT. The absence of those peaks indicates the absence of BoNT or the presence of BoNT below the limit of detection. Data acquisition on a MALDI-TOF instrument allows for rapid analysis of each sample, with data acquisition requiring only 15 seconds per sample.

Figure 4A shows the mass spectrum of toxin-dependent cleavage products of the peptide substrate for BoNT/A. Peaks at *m/z* 1198 and 1700 indicate the presence of BoNT/A. These peaks are absent in Figure 4B as this is a negative control. The dominant peak in the negative control is the substrate at *m/z* 2879 as it remains uncleaved.

Figure 5 depicts mass spectra for the reactions of BoNT/B (5A), /E (5B), and /F (5C) with their peptide substrates. Peaks at *m/z* 1760 and 2283 indicate the presence of BoNT/B; 1137 and 2923 are markers for BoNT/E; and 1346 and 3169 indicate BoNT/F.

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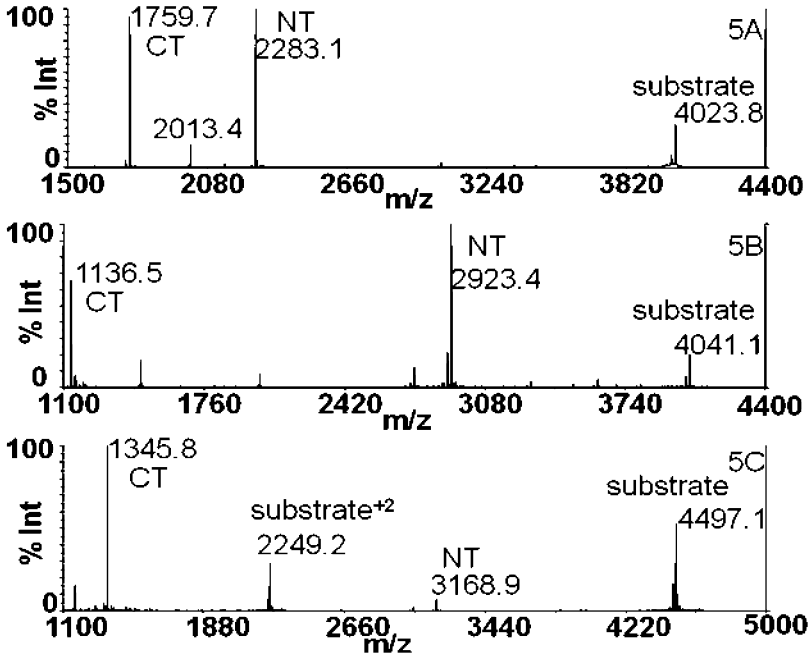

Figure 5. MALDI-TOF mass spectra of the Endopep-MS reaction of BoNT/B (5A), /E (5B), or /F (5C). Peaks at m/z 4024, 4041, and 4497 indicate the presence of the intact peptide substrates for BoNT/B, /E, and /F respectively; cleavage products indicative of BoNT/B are present at m/z 1760 and 2283; 1137 and 2923 are markers for BoNT/E, and peaks at m/z 1346 and 3169 indicate BoNT/F.

Workflow of BoNT Analysis by Endopep-MS

Testing for the presence or absence for BoNT by Endopep-MS is typically accomplished in less than 6 hours as shown in Figure 6, which is a shorter time frame than sample analysis by mouse bioassay which can take 1-4 days. First, samples are aliquoted into 96 well plates, along with all reagents required for the toxin extraction portion of the assay. Next, antibody-coated beads are mixed with the sample for 1 hour. Beads are then washed to reduce non-specific binding, and then the beads are reconstituted in a mixture of the reaction buffer with peptide substrate. This mixture is incubated at 37°C for 4 hours. A portion of the reaction supernatant is then added to matrix solution and spotted on a MALDI plate. As discussed above, the speed of the mass spectrometer allows for rapid analysis of each reaction. Typically, less than 8 samples are analyzed for botulinum neurotoxin at the same time. If the sample size is between 8 and 96 samples, testing time is lengthened by approximately 1 hour.

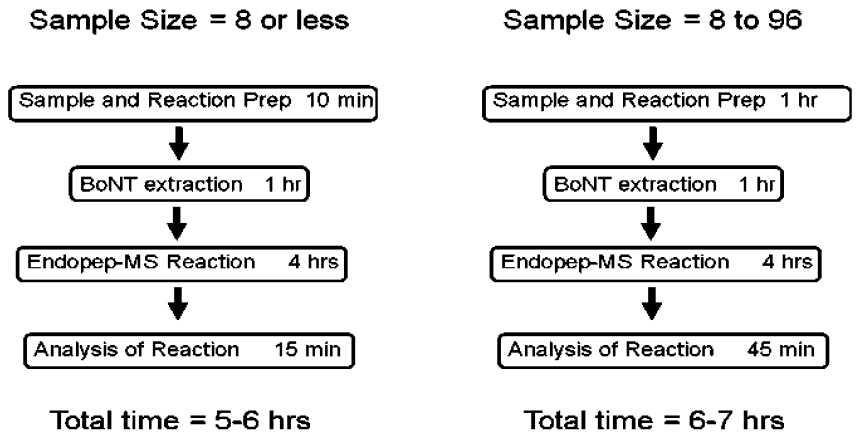

Figure 6. The typical sample workflow for BoNT analysis by Endopep-MS.

Limits of Detection of BoNT in Clinical Samples

As discussed above, BoNT analysis by Endopep-MS is advantageous in terms of speed of the assay. However, speed is not the only advantage of the Endopep-MS method. The limit of detection of toxin with the mouse bioassay is defined as 1 mouse LD_{50} which is thought to be about 10 pg or 67 attomole for BoNT A (*2*). We can achieve limits of detection lower than that of the mouse in most clinical samples. Our work has primarily focused on BoNT/A, /B, /E, and /F as these four serotypes are typically associated with human cases of botulism. Currently, the Endopep-MS method has limits of detection of 0.5, 0.1, 0.1, and 0.1 mouse LD_{50} respectively in human serum. The limits of detection in stool are slightly higher than serum because proteases are more abundant in stool than serum; nonetheless, the current limits of detection of BoNT/A, /B, /E, and /F in stool are 0.5, 0.1, 5, and 0.1 mouse LD_{50} respectively.

Applications of Endopep-MS Assay—Example #1

The limits of detection reported above were obtained with BoNT spiked into matrices. It is important to demonstrate the utility of this method on samples involved in botulism outbreaks in addition to spiked samples. In the summer of 2007, a botulism outbreak was reported in commercially-canned hot dog chili sauce (*36*). We obtained some of the chili sauce extract and tested 0.5 mL of it for BoNT/A, /B, /E, and /F by Endopep-MS. Within 6 hours, we determined that the sample was positive for BoNT/A as evidenced by the presence of peaks at *m/z* 1198 and 1700 in Figure 7C corresponding to cleavage of the peptide substrate by BoNT/A. Figure 7A and 7B are the positive and negative controls. The positive control (Figure 7A) was an extract from a control chili sauce spiked with 1000 mLD_{50} of BoNT A and the negative control is extract of chili sauce with no toxin. The sample was either negative for BoNT/B, /E, and /F or those BoNTs were not present above the limits of detection.

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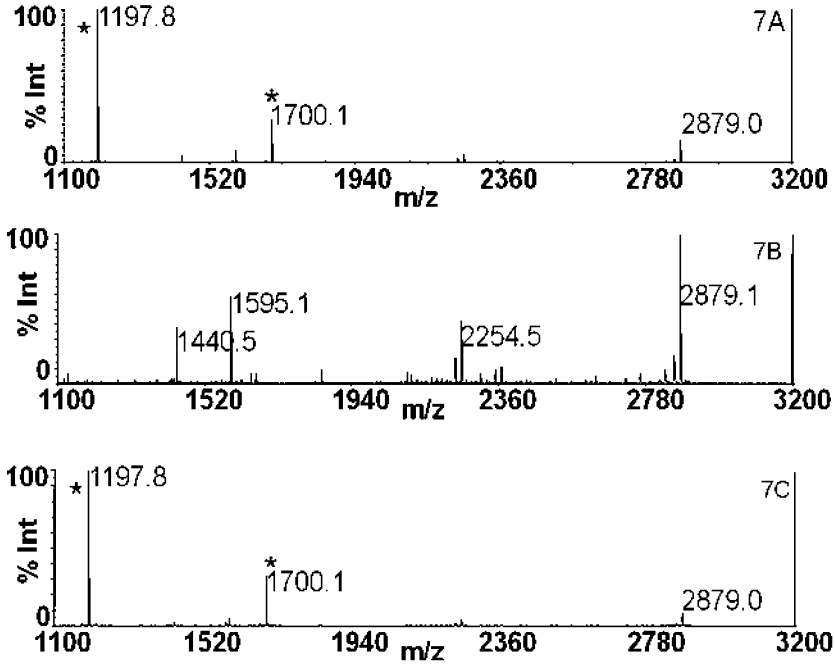

Figure 7. MALDI-TOF mass spectra corresponding to the reaction of chili extract spiked with 1000 mouse LD_{50} of BoNT/A (7A), unspiked chili extract (7B), or chili extract (7C) suspected in a botulism outbreak. Peaks at m/z 1198 and 1700 indicate the presence of BoNT/A in a sample.

Application of Endopep-MS Assay—Example #2

In the spring of 2007, botulism was suspected in catfish in Mississippi (*37*). Serum from the fish was tested by mouse bioassay for the presence of BoNT, and the results were negative. Catfish serum (0.5 mL) was tested for BoNT/A, /B, /E, and /F by Endopep-MS. Within 6 hours the sample was positive for BoNT/E as evidenced by the presence of peaks at *m/z* 1137 and 2923 in Figure 8C corresponding to cleavage of the peptide substrate by BoNT/E. The sample was either negative for BoNT/A, /B, and /F or those BoNTs were present below limits of detection. Although our method as described here does not permit accurate quantification of toxin present in a sample, we estimate that the level of toxin present in the catfish serum to be between 0.01 and 0.5 mouse LD_{50}. This low level of toxin might explain the negative results by mouse bioassay. Figure 8A is the positive control of serum spiked with 0.5 mouse LD_{50} of BoNT/E and Figure 8B is a negative control (blank serum).

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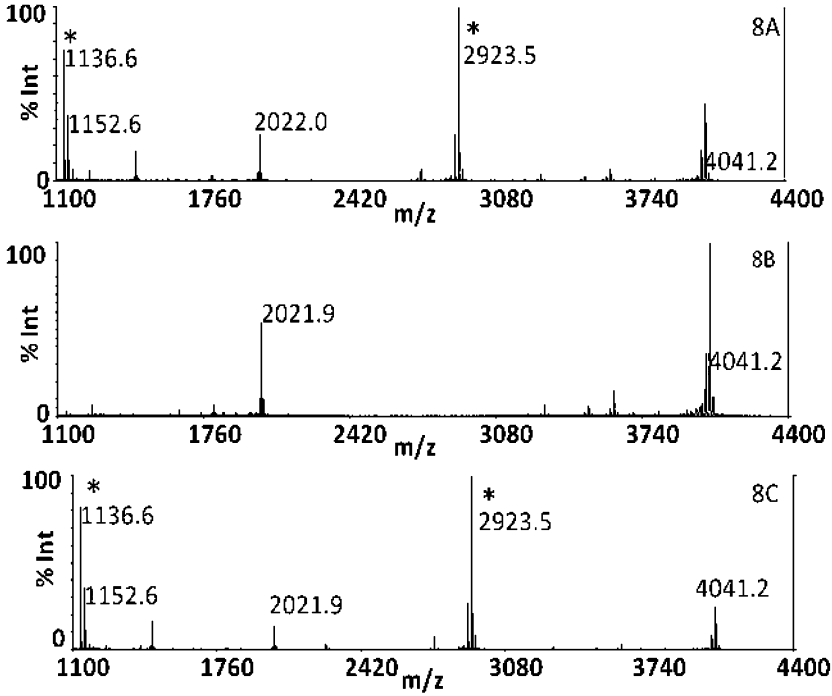

Figure 8. MALDI-TOF mass spectra corresponding to the reaction of serum spiked with 0.5 mouse LD_{50} of BoNT/E (8A), unspiked serum (8B), and catfish serum suspected in a botulism outbreak (8C). Peaks at m/z 1137 and 2923 indicate the presence of BoNT/E in a sample.

Subtyping of BoNT/A

Most of the serotypes of BoNT can further be classified into subtypes, which account for ≥2% amino acid variation between BoNTs of the same serotype. Identification of the subtype can provide useful information for epidemiologists or forensics experts as one can assess the relationship, if any, between concurrent botulism outbreaks. There are currently five recognized subtypes of BoNT/A, known as /A1, /A2, /A3, /A4, and /A5 (*24*, *25*). BoNT/A1 through /A4 have been tested and the toxins have been detected by Endopep-MS (*28*). BoNT/A5 has only recently been discovered and is not yet unavailable for testing. However, the subtypes of BoNT/A cannot be distinguished through Endopep-MS alone as all subtypes of BoNT/A cleave the peptide substrate in the same location, yielding the same data for all subtypes. Therefore, the Endopep-MS method was extended to identify the subtype of BoNT.

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PFVNKQFNYKDPVNGVDIAYIKIPNVGQMQPVKAFKIHNKIWVIPERDTFTNPEEGDLNP
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STQYIRFSPDFTFGFEESLEVDTNPLLGAGKFATDPAVTLAHELIHAGHRLYGIAINPNR
VFKVNTNAYYEMSGLEVSFEELRTFGGHDAKFIDSLQENEFRLYYYNKFKDIASTLNKAK
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SVMTTNIYLNSSLYRGTKFIIKKYASGNKDNIVRNNDRVYINVVVKNKEYRLATNASQAG
VEKILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQDNNGNDIGF
FIGFHQFNNIAKLVASNWYNRQIERSSRTLGCSWEFIPVDDGWGERPL

Figure 9. Sequence of botulinum neurotoxin A1 (BoNT/A1 or Hall strain). Underlined residues are mutated in BoNT/A2.

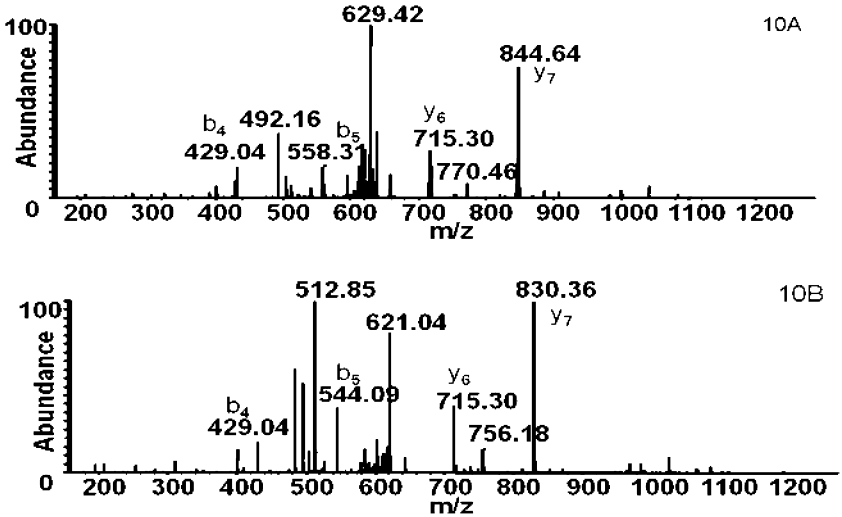

Figure 10. Mass spectra of MS/MS fragmentation of tryptic peptides from BoNT/A1 (10A) and /A2 (10B). The peptide sequence in BoNT/A1 is SFGHEVLNLTR, and the E is mutated to a D in BoNT/A2. This mutation is shown by the altered b_5 and y_7 ions in the spectra.

Following the Endopep-MS reaction, the BoNT attached to the antibody-coated beads is tryptically digested. The resultant tryptic peptides are analyzed by LC-MS/MS. Data are searched against a protein database containing protein sequences of all known subtypes of BoNT. Using this procedure, all the subtypes

and strains tested can be differentiated. An example is the differentiation of BoNT/A1 from /A2 (*29*). BoNT/A1 is approximately 90% homologous to /A2; however, one can take advantage of the 10% difference to positively identify BoNT as /A1 or /A2. The amino acid sequence for BoNT A (Hall strain) is shown in Figure 9 with the residues that are different between BoNT/A1 and BoNT/A2 underlined. Through this process, we acquired mass spectrometric evidence for 76 of the known 131 amino acid differences between BoNT/A1 and /A2. An example of one of these 76 amino acid mutations is depicted in Figure 10. Figure 10A represents the MS/MS fragmentation of a tryptic peptide from BoNT/A1 (S^{166}-R^{176}) with the sequence SFGHEVLNLTR. This peptide has a single amino acid mutation in BoNT/A2 where glutamic acid-170 is mutated to an aspartic acid to yield the tryptic peptide from /A2 SFGHDVLNLTR. This amino acid mutation results in a different molecular weight and MS/MS fragmentation pattern, as depicted in Figure 10B. These data indicate that mass spectrometry can be used to distinguish the subtype of BoNT/A. It is important to note that BoNT/A2 is the only known subtype that has the peptide SFGHDVLNLTR.

Conclusions

Diagnosis, treatment and prevention of disease are the central goals of public health. Rapidly identifying botulinum neurotoxins to confirm a diagnosis or discover a source to prevent additional cases of botulism is critical. The Endopep-MS assay is a rapid, sensitive and select method to detect and differentiate BoNT. The method has three levels of selectivity including extraction of the toxin with selective high affinity antibodies, the selective enzymatic activity of the toxin and the use of high resolution MALDI-TOF/MS to specifically identify the cleavage products. The method obtains attomole/mL detection limits because of a toxin dependent amplification of the product peptides due to the BoNT enzymatic activity. Additional information can then be obtained by the proteomic analysis of the tryptic digestion of the extracted toxin. This approach can yield information on the subtype of toxin strain which can help epidemiologists identify similarities or differences in concurrent outbreaks.

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