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Quantification of Botulinum Neurotoxin Serotypes A and B from Serum Using Mass Spectrometry

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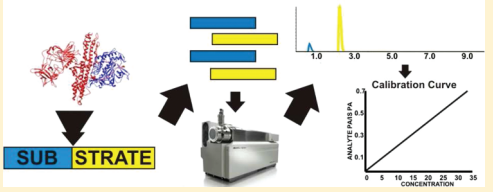
Supporting Information

ABSTRACT: Botulinum neurotoxins (BoNT) are the deadliest agents known. Previously, we reported an endopeptidase activity based method (Endopep-MS) that detects and differentiates BoNT serotypes A−G. This method uses serotype specific monoclonal antibodies and the specific enzymatic activity of BoNT against peptide substrates which mimic the toxin's natural target. Cleavage products from the reaction are detected by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. We have now developed a multiple reaction monitoring method to quantify the biological activity of BoNT serotypes A (BoNT/A) and B (BoNT/B) present in 0.5 mL of serum using electrospray mass spectrometry. The limit of quantification for each serotype is 1 mouse intraperitoneal lethal dose ($MIPLD_{50}$) corresponding to 31 pg of BoNT/A and 15 pg of BoNT/B in this study. This method was applied to serum from rhesus macaques with inhalational botulism following exposure to BoNT/B, showing a maximum activity of 6.0 $MIPLD_{50}$/mL in surviving animals and 653.6 $MIPLD_{50}$/mL in animals that died in the study. The method detects BoNT/B in serum 2−5 h after exposure and up to 14 days. This is the first report of a quantitative method with sufficient sensitivity, selectivity, and low sample size requirements to measure circulating BoNT activity at multiple times during the course of botulism.



Botulinum neurotoxins (BoNTs) are the causative agents of botulism and are the most toxic substances known.[1] Seven serotypes (A−G) of the toxin can be produced from the various strains of the *C. botulinum*, *C. butyricum*, *C. baratii*, and *C. argentinense*[2,3] with human cases being derived from intoxication with serotypes A, B, E, and F. Left untreated, botulism can cause paralysis and death from respiratory failure. In vivo targets for BoNT include the SNARE (soluble NSF-attachment protein receptor) family of proteins responsible for neurotransmitter release.[4] Each BoNT serotype has a unique and specific cleavage site on SNAP-25, VAMP-2, or syntaxin. Lethal amounts are estimated at 10 ng/kg for human beings via inhalation.[5] In the United States, infant botulism is the most frequent form of the disease followed by foodborne and wound botulism.[6] Due to these factors, BoNT has attracted attention as a possible agent of bioterrorism and remains a public health threat.

The quantification of toxemia in other diseases such as anthrax has led to enhanced knowledge of the course of disease, effectiveness of treatments, and clinical outcomes.[7,8] Quantification of BoNT activity in clinical samples is rarely performed but doing so in serum could enhance the understanding of disease progression and treatment. More importantly, the correlation between BoNT levels and clinical outcome is still largely unknown. Activity quantification could identify when antitoxins and other medical countermeasures would be the most effective as one aspect of the botulism pharmacokinetic profile.[9] Serum based analysis also provides an advantage as stool collection may be problematic from botulism induced constipation.[5]

The mouse bioassay (MBA) has historically been the most common method used to confirm clinical botulism and contains two distinct parts. The mouse lethality assay is very sensitive detecting BoNT at the level of 1 mouse intraperitoneal LD_{50} ($MIPLD_{50}$) per injected volume with the capability to identify serotype in 1−4 days.[10] The quantitative portion of the assay is primarily used for BoNT potency in pharmaceutical product testing.[11] For quantification, the MBA has several shortcomings. The $MIPLD_{50}$ is not a standardized unit of measure.[12] The number of animals needed to produce statistically useful quantitative data is large. In some cases, 48 mice were required to assay one sample.[12] Given the 0.4−1 mL sample requirements per mouse for MBA,[13] volume requirements could easily outpace what is available from the patient if quantitative information is desired. There is also significant concern that quantitative results from the mouse bioassay cannot be normalized between laboratories.[14] In fact, there are reports where quantitative MBA results used for commercially produced BoNT serotype A (BoNT/A) toxin can vary two-fold between manufacturers.[15] Possible sources of this disparity are related to carrier buffer

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differences, nonuniform standards, and the strain of mouse used for the test.

To improve the detection of BoNT in clinical and food samples, several in vitro assays have been developed. The major strategies for these methods include ELISA, PCR, and exploitation of the BoNT specific endopeptidase activity.[16−19] One of the most promising in vitro assays is the Endopep-MS method.[20] Briefly, the method employs mixtures of high affinity monoclonal antibodies to extract BoNT from a clinical sample (serum or stool), food extract, or culture supernatant. The extraction step is performed on antibody coated magnetic beads that are carefully washed to remove interfering substances and proteases from the sample to purify and concentrate the toxin. The extracted BoNT is then treated with a peptide substrate that mimics the natural targets of BoNT. Cleavage products are then accurately recorded by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). At present, the detection limits are lower than the mouse bioassay for BoNT/A, /B, /E, and /F.[21,22] Previously, no analytical methods have reported sufficient sensitivity and selectivity to accurately and precisely quantify BoNT in clinical samples. We therefore have added isotope dilution liquid chromatography tandem mass spectrometry (IDLC-MS/MS) to the Endopep-MS method to provide accurate and precise quantification of BoNT activity. Furthermore, we have applied this new quantification step to rhesus macaque serum samples with inhalational botulism and include the first reported serum BoNT levels over a 30 day time course study.

METHODS

Materials. Botulinum neurotoxins are very toxic and therefore require appropriate safety measures. All neurotoxins were handled in a level 2 biosafety cabinet equipped with HEPA filters. BoNT complex was purchased from Metabiologics (Madison, WI) and provided in a matrix of 200 mM NaCl. The BoNT/A (Hall strain) stock solutions had a concentration of 8.5×10^5 $MIPLD_{50}$/mL. The BoNT/B1 (Okra strain) had a concentration of 3×10^6 $MIPLD_{50}$/mL. All standard solutions of BoNT complex were titered by quantitative mouse bioassay. To ensure that results were comparable between the animal study and mass spectrometry (MS), BoNT aliquots from the animal challenge stock were provided for MS standard curves. Monoclonal antibodies for BoNT/A (RAZ1 and CR2) and for BoNT/B (1.B12 and 1.B18)[23] used in BoNT extraction were obtained from Dr. Jim Marks at the University of California at San Francisco. Dynabeads (M-280/Streptavidin) were purchased from Invitrogen (Carlsbad, CA) 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 purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated. Peptide substrates were synthesized at Los Alamos National Laboratory (Los Alamos, NM). Two peptide cleavage products and their corresponding internal standards were monitored for each BoNT serotype. For BoNT/A, the peptide substrate had a sequence of (Biotin)-KGSNRTRIDQGNQRATRXLGGK-(Biotin) with an average molecular weight of 2879.3 Da. The amino terminal (NT) cleavage product had a sequence of (Biotin)-KGSNRTRIDQGNQ with an average molecular weight of 1699.9 Da. The carboxyl terminal (CT) cleavage product had a sequence of RATRXLGGK-(Biotin) with an average molecular weight of 1197.5 Da. For BoNT/B, the peptide substrate had a sequence of LSELDDRADALQAGASQFESSAAKLKRKYWWKNLK with an average molecular weight of 4025.7 Da. The NT cleavage product had a sequence of LSELDDRADALQAGASQ with an average molecular weight of 1759.8 Da. The CT cleavage product had a sequence of FESSAAKLKRKYWWKNLK with an average molecular weight of 2283.7 Da.

The labeled internal standard for the BoNT/A NT product had a sequence of (Biotin)-KGSNRTR(I+7)DQGNQ with an average molecular weight of 1706.9 Da. For the CT product, the internal standard had a sequence of R(A+7)TRXLGGK-(Biotin) with an average molecular weight of 1204.5 Da. For BoNT/B, the internal standard for the NT product had a sequence of LSELDDR-(A+7)DALQAGASQ with an average molecular weight of 1766.8 Da. The internal standard for the CT product had a sequence of FESS(A+7)AKLKRKWWKNLK with an average molecular weight of 4025.5 Da.

Preparation of Antibody Coated Beads, Standards, and Quality Control Materials. Monoclonal antibody was bound to streptavidin Dynabeads after rinsing three times with HBS-EP buffer (GE Healthcare; Piscataway, NJ). A 2 μg aliquot of antibody was used per 100 μL of beads. An orbital shaker was used to bind antibody onto the beads for 1.5 h. For BoNT/A, quality control samples were prepared at final concentrations of 0.5, 45, and 90 $MIPLD_{50}$. BoNT/B quality control samples were prepared at final concentrations of 0.5, 16, and 32 $MIPLD_{50}$. Toxin dilutions were made in a reaction buffer containing 25 mM dithiothreitol, 20 μM $ZnCl_2$, and 1 mg/mL bovine serum albumin. To prepare standard spiking solutions, each toxin stock was diluted from 204.8 $MIPLD_{50}/\mu$L to 0.05 $MIPLD_{50}/\mu$L. Five microliters of each spiking solution was added to 500 μL of blank human serum (Interstate Blood Bank; Memphis, TN) to mimic the unknown sample matrix. The standard calibration series for BoNT/A contained final toxin concentrations of 0, 0.25, 0.50, 1.0, 2.0, 4.0, 8.0, 16, 24, 32, 45, 64, and 90 $MIPLD_{50}$. The standard calibration series for BoNT/B contained final concentrations of 0, 0.25, 0.50, 1.0, 2.0, 4.0, 8.0, 16, 24, and 32 $MIPLD_{50}$. Five hundred microliters of each sample (calibrant, quality control, and unknowns) was transferred to a 96 position deep well plate. Fifty microliters of 10× PBST and 20 μL of magnetic streptavidin beads (Invitrogen; Carlsbad, CA) were then added to each well. Endogenous toxin was bound onto the antibody coated beads for 1 h using an orbital shaker.

Toxin Extraction and Endopepeptidase Activity Assay. Toxin bound beads were recovered using a Kingfisher96 automated robot (ThermoScientific, USA). Toxin bound beads were washed twice with 2 M NaCl and then with HBS-EP buffer and DDI water.[24] The beads were reconstituted to a final volume of 20 μL in reaction buffer containing peptide substrate (50 pmol/μL) and incubated at 37 °C for 4 h. Supernatants were quenched with 2 μL of concentrated formic acid after the incubation. Two microliters of labeled internal standard was then added for each cleavage product produced during the incubation. All samples were transferred to autosampler vials for analysis.

IDLC-MS/MS Analysis and Quantification. All mass spectrometry measurements took place using a 4000 QTRAP (AB Sciex; Foster City, CA) fitted with a TurboV source. The interface temperature was set at 180 °C. The transitions used for the multiple reaction monitoring (MRM) runs were first determined through direct infusion of synthesized cleavage products, substrates, and internal standards using a syringe pump. A review of MRM concepts can be found in Elliot et al.[25] The most intense fragment ions were chosen to complete each transition which is

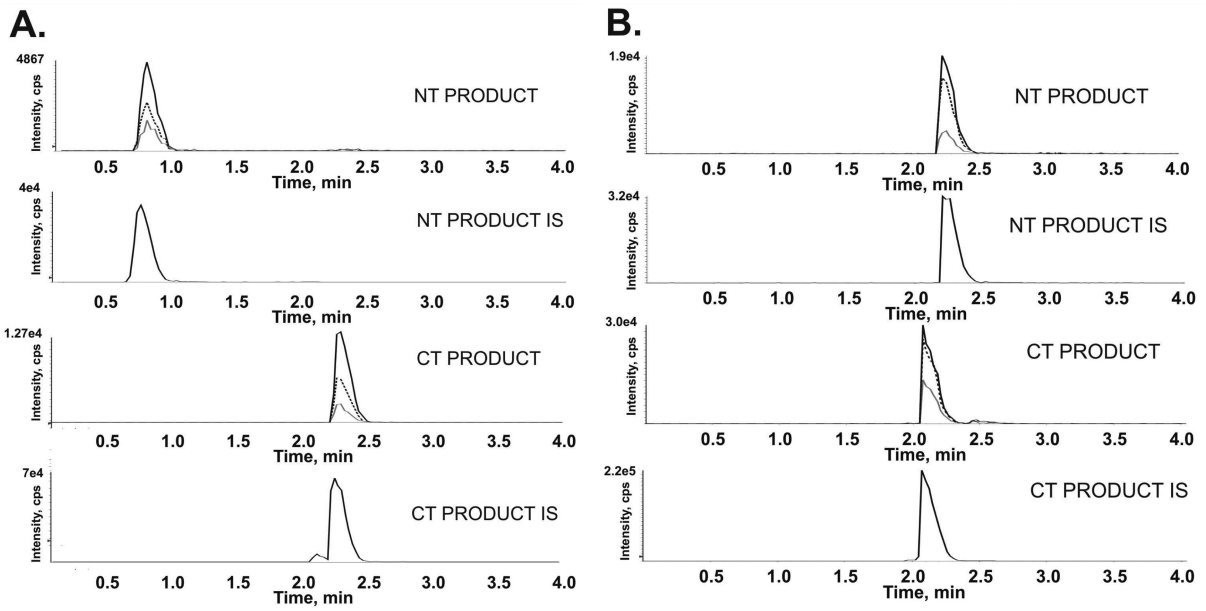

Figure 1. Selected ion chromatograms for BoNT/A (panel A) and BoNT/B (panel B). In panel A, the nt-product consist of the transitions 568/777.3 (quantitative), 568/720.3 (confirmation), and 568/412.2 (confirmation). The ct-product consists of the transitions 400/815.5 (quantitative), 400/744.4 (confirmation), and 400/643.4 (confirmation). The internal standards for the nt and ct products were 569/780.8 and 402/396.2, respectively. In panel B, the nt-product consist of the transitions 880/1526.7 (quantitative), 880/1455.7 (confirmation), and 880/1398.6 (confirmation). The ct-product consisted of three transitions: 458/640.7 (quantitative), 458/688.4 (confirmation), and 458/374.2 (confirmation). The internal standards for the nt and ct products were 884/1334.6 and 573/672.3, respectively. The smaller peak on the ct-product internal standard (panel A) is due to an impurity from the synthesis process.

composed of a precursor m/z and a unique product m/z ion pair. A total of eight transitions were monitored for each serotype. All separations took place using a 1 mm ID C18 BEH column (Waters; Beverly, MA) fitted to an Acquity UHPLC. Solvents for channel A and B were 100% water and 100% acetonitrile with a flow rate of 180 μL/min. Each solvent contained 0.1% formic acid as a modifier. A linear gradient of 90% to 5% solvent A was executed to separate cleavage products over the run. Briefly, solvent A was decreased to 10% over 6 min. After holding this solvent ratio for 1 min, the system was re-equilibrated to initial conditions for another minute and held constant for the remainder of the run. Each gradient run lasted 10 min. Samples were analyzed in triplicate using 5 μL per injection.

Method Validation. Figure 1 shows MRM transitions associated with BoNT/A (panel A) and BoNT/B (panel B) with their respective internal standards. Data from one transition (native and internal standard) was used to complete quantification using the Analyst software (version 1.4.2). Peak areas were manually inspected for consistency after initial integration by the software. Calibration curves were prepared using JMP 8 (SAS; Cary, NC) after averaging peak areas from triplicate injections in Excel (Microsoft, USA). R^2 values were $\geq$0.995 using a linear fit. Each calibration curve plotted toxin concentration versus the ratio of analyte to internal standard peak area. Calibration curves for BoNT/A contained 13 points. Calibration curves for BoNT/B contained 10 points. All results were archived in Excel. Three quality control (QC) samples were used to monitor calibration curve integrity and long-term instrument stability. Concentration values from each QC sample were plotted in control charts.[26] A run was deemed out of control if a majority of QC samples (2 of 3) registered toxin activities outside $\pm 2\sigma$. Out of control calibration curves were reprepared before analyzing unknowns. No adverse effects were seen in reanalyzing diluted samples that initially gave toxin activity data outside of the calibration range. The dilution factor in this situation was 1:4. Toxin activities for diluted samples were calculated per unit of sample volume extracted and scaled to standard units of $MIPLD_{50}$/mL.

Animal Study and Mouse Bioassay Information. Full details for the animal study and MBA testing can be found in the work by Sanford.[27] Briefly, serum samples were acquired from rhesus macaques exposed to aerosols containing BoNT/A1 or BoNT/B1. Estimates of the inhaled dosages were based on the atmospheric BoNT concentration in the exposure chamber, the total exposure time, and the total amount of air inspired during exposure measured using a real-time plethysmograph.

Circulating concentrations of BoNT/A1and BoNT/B1 were assessed using the MBA at 2, 5, and 7 h and 1, 2, 7, and 14 days after exposure. At each time point, four mice were injected with 0.5 mL of serum per mouse, for a total of 2 mL for each time point. Samples were only considered positive for BoNT if at least 3 mice died within 96 h of injection. The limit of detection in MBA was considered to be 2 $MIPLD_{50}$/mL. Studies were performed under an approved IACUC protocol in compliance with the Animal Welfare Act and other federal statutes and regulation relating to animals and experiments involving animals and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996.

RESULTS AND DISCUSSION

Establishing Linear Range. Establishing a linear range involved defining curvilinear behavior within the calibration region. To fully characterize this effect, standards were prepared from 0 to 1024 $MIPLD_{50}$ for each serotype. Figure 2 shows the results

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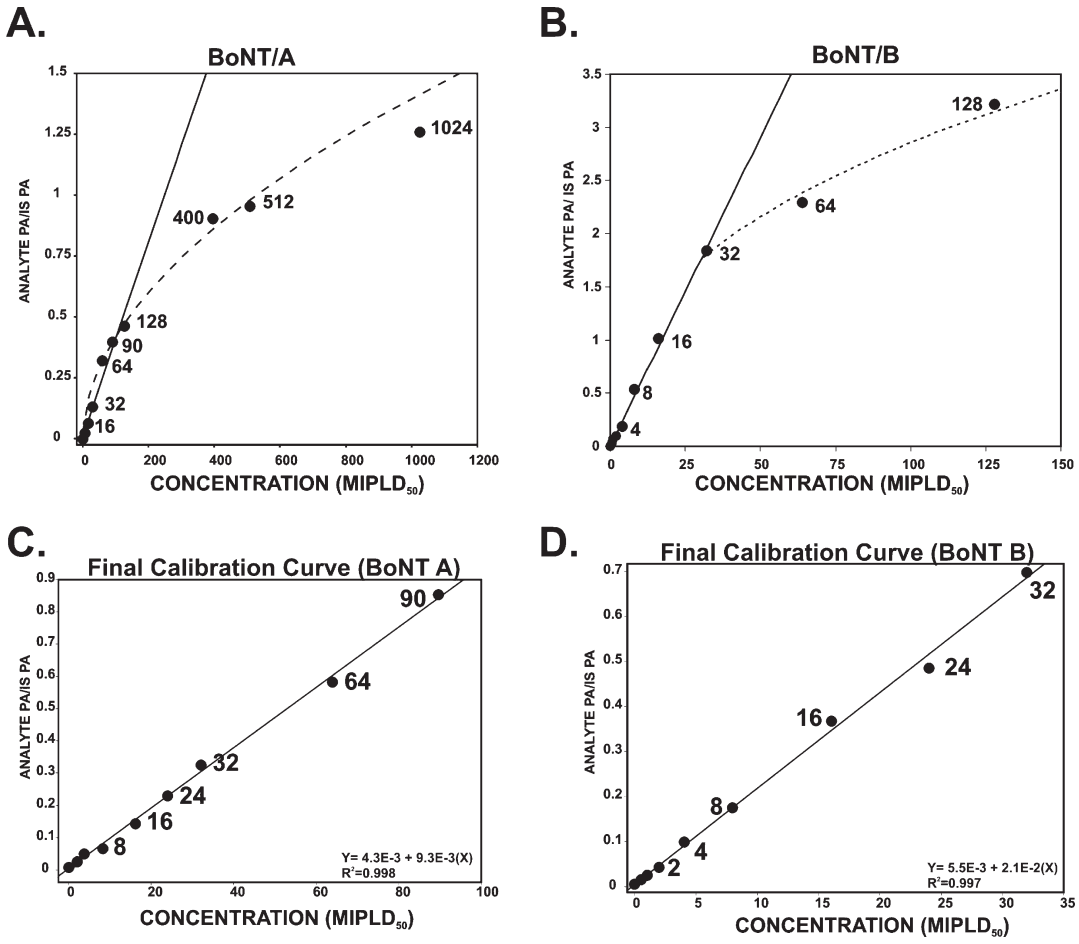

Figure 2. Linear region determinations for BoNT/A (panel A) and/B (panel B) with curvilinear regions denoted by dashed lines. Final calibration curves are also shown for each serotype (panels C,D).

from BoNT/A (Figure 2A) and/B (Figure 2B). Figure 2C,D displays final calibration curves for each serotype. Figure 2A,B shows curvilinear behavior at 90 $MIPLD_{50}$ for BoNT/A and 32 $MIPLD_{50}$ for BoNT/B. This behavior is primarily due to Michaelis−Menten reaction kinetics associated with available substrate to react with extracted BoNT from each sample. In the Endopep-MS reaction, a fixed amount of substrate is present. From the Michaelis−Menten equation ($V_o = [V_{max}{}^*[S_o]]/[[S_o] + K_m]$), a linear response for cleavage products is expected from the interaction of a peptide substrate and extracted BoNT. Here, V_{max} is the maximum velocity of the reaction and is directly proportional to the toxin concentration, S_o is the concentration of the substrate, and K_m is a specific constant unique to the substrate. When the substrate concentration is high and relatively constant ($[S] \gg K_m$), the rate of cleavage product formation (V) has a linear correlation with the BoNT concentration (V_{max}). As the reaction progresses, the substrate concentration decreases and the effect of K_m becomes more substantial.

Thus, the cleavage product formation no longer has a linear correlation with the BoNT concentration, and the calibration curves are expected to have a smaller range of linearity. The heterogeneous nature of having the BoNT bound to magnetic beads can also affect the cleavage reaction kinetics. The substrate has to diffuse through free solution and must partition into the solvent layer near the liquid−solid surface boundary in order to react with the BoNT and form the cleavage products. This requires the application of a modified Michaelis−Menten model which is $V = [S_0]/\{(1/k_L) + (1/[V_{max}/K_m])\}$.[28] The rate of BoNT substrate cleavage in the microenvironment is determined not only by the substrate concentration in the free solution ($[S_0]$) but also by the substrate diffusivity into the microenvironment (k_L) and the BoNT concentration (V_{max}/K_m). If the surface concentration of BoNT is too high ($1/k_L \gg 1/[V_{max}/K_m]$), then the reaction rate will be less dependent on the BoNT concentration ($1/[V_{max}/K_m]$) and is limited by $[S_0]$ and $1/k_L$. The linear nature of the response ratio vs BoNT concentration is therefore limited as shown in Figure 2A,B. In most clinical cases, serum levels of BoNT are expected to be low due to the high toxicity of the BoNT and therefore are within the linear response regions shown in Figure 2C,D. Clinical samples with higher BoNT levels can easily be diluted to fit within the linear range of the method.

Limit of Detection and Limit of Quantitation Calculations. For this method, a limit of detection (LOD) was found to be 0.18 $MIPLD_{50}/500\,\mu L$ for BoNT/A and /B. This value was calculated by the equation $LOD = [mean_B + 1.645(S_B + I_L)]/[1 - 1.645(S_L)]$[26,29] where $mean_B$ is the mean of the blank and S_B is the standard deviation of the blank. I_L and S_L refer to the intercept and slope of the line formed from the lowest five calibration points excluding the blank. Using BoNT/B as an example, response values ($n = 15$) from the 0.25, 0.50, 1.0, 2.0, and 4.0 $MIPLD_{50}$ calibrant points were used. Replicate data

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values at each calibrant level were averaged together and subjected to a linear curvefit to produce S_L and the I_L. Defining a limit of quantitation (LOQ) at there times the LOD yields a value of 0.5 $MIPLD_{50}$/500 μL or 1 $MIPLD_{50}$/mL.

Quality Control. Blind QC analysis was performed to gauge method accuracy. Three bench QCs were prepared at the LOQ, middle, and high end of each calibration range. In parallel, blind QCs were prepared and submitted to the analyst with no markings to indicate concentrations. Experimentally determined toxin activity from blind QCs was accepted if the concentration was within 20% of the theoretical value. The results of these analyses are shown in Table 1. Control charts were used to characterize bench QCs ($n \geq 15$) for each serotype and generate coefficient of variance (%CV) values. The %CVs for BoNT/A QCs were 19%, 11%, and 9% at 0.5, 45, and 90 $MIPLD_{50}$. The % CVs for BoNT/B QCs were 19%, 9%, and 15% at 0.5, 16, and 32 $MIPLD_{50}$.

Table 1. Bench and Blind QC Results for Activity Quantification[a]

	Sample Name	Theoretical Concentration ($MIPLD_{50}$)	Experimental Concentration ($MIPLD_{50}$)
BONT/A	Bench QC 1	0.5	0.6
	Bench QC 2	45	50.4
	Bench QC 3	90	101.8
	Blind QC 1	1	1.1
	Blind QC 2	85	84.8
	Blind QC 3	30	33.1
BONT/B	Bench QC 1	0.5	0.4
	Bench QC 2	16	17.1
	Bench QC 3	32	29.5
	Blind QC 1	1	0.9
	Blind QC 2	30	27.2
	Blind QC 3	15	17.2

[a] While bench QCs were prepared by the analyst, blind QCs were prepared by alternate staff where concentrations were unknown before analysis.

Application of the Method to BoNT/B Detection in Animal Sera. Qualitative testing for BoNT was performed by the MBA while quantitative analysis was done by the MS based method. As only serum from BoNT/B exposed animals gave positive results using MBA, all mass spectrometry efforts focused on this sample set to compare the two techniques. Table 2 shows MS and MBA qualitative data from 22 rhesus macaques exposed to BoNT/B1. A total of 116 samples were tested with MBA and MS. From this total, 26 were found to be positive by MBA with 66 positive samples via mass spectrometry. With MS, a sample was deemed positive if the BoNT activity was $\geq$1 $MIPLD_{50}$/mL. From this table, interesting trends can be drawn. First, all samples that showed positive results by MBA were also positive with MS. Second, with animal sera that had positive results at 2 h (e.g., RQ6574), MS alone was able to detect BoNT for 6/11 animals. An added advantage of the MS approach is the generation of quantitative information. Table 3 shows activity quantification data from MS superimposed on data from Table 2. From the 66 positive samples with MS, 15 samples had activity concentrations between 1 and 2 $MIPLD_{50}$/mL. Twenty samples had activity concentrations between 2 and 4 $MIPLD_{50}$/mL. Ten samples had activity concentrations between 4 and 10 $MIPLD_{50}$/mL. Activity concentrations for 21 samples were greater than 10 $MIPLD_{50}$/mL.

All animals that had positive samples by MS showed symptoms of botulism, and all animals that had no positive results for BoNT in their serum by MS showed no clinical signs of the disease. With the information in Tables 2 and 3, the data follows a logical progression when considering the estimated inhaled dosages. Animals with lower dosages tended to produce samples with lower BoNT activity. In contrast, animals receiving higher dosages produce samples with higher activities overall. Figure 3 shows graphs of BoNT activity for animals over the entire study.

Table 2. Qualitative IDLC-MS/MS and MBA Results from 22 Animals Challenged with BoNT/B[a]

Challenge Serotype	Monkey ID	Estimated Inhaled Dose $MIPLD_{50}$/kg	Survivor	Time to Death (hours)	Baseline	2 hour	5 hour	7hour	Day 1	Day 2	Day 7	Day 14
BoNT/B1	RQ6532	10,662	N	148	X						X	X
	RQ6540	19,301	N	93				X			X	X
	RQ6660	29,666	N	69				X	X		X	X
	RQ6665	32,140	N	74							X	X
	RQ6646	50,396	N	71			X		X	X	X	X
	RQ6574	51,123	N	24	X					X	X	X
	RQ6577	82,106	N	95	X						X	X
	RQ6558	93,932	N	29						X	X	X
	RQ6579	120,436	N	35			X	X		X	X	X
	RQ6626	126,777	N	32						X	X	X
	RQ6670	146,024	N	41			X	X		X	X	X
	RQ6538	322,956	N	20					X	X	X	X
	RQ6695	494,554	N	29						X	X	X
	RQ6661	676	Y	NA	X							X
	RQ6543	1,349	Y	NA	X							X
	RQ6650	1,673	Y	NA	X							X
	RQ6668	2,014	Y	NA	X							X
	RQ6662	19,212	Y	NA	X							X
	RQ6536	25,418	Y	NA				X				
	RQ6664	31,553	Y	NA			X	X				
	RQ6582	68,365	Y	NA								
	RQ6572	74,069	Y	NA								

[a] Items marked with "X" denote samples that were not accessible to MS. Light gray blocks denote positive results (BoNT activity $\geq$1 $MIPLD_{50}$/mL) for MS only. Dark gray blocks denote positive results for IDLC-MS/MS and MBA. White blocks indicate samples where BoNT was not detected by either technique.

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Table 3. Activity Quantification Data by IDLC-MS/MS Superimposed on Figure 3[a]

Challenge Serotype	Monkey ID	Estimated Inhaled Dose $MIPLD_{50}$/kg	Survivor	Time to Death (hours)	Baseline	2 hour	5 hour	7hour	Day 1	Day 2	Day 7	Day 14
BoNT/B1	RQ6532	10,662	N	148	X	<LOQ	<LOQ	<LOQ	2.8	3.7	X	X
	RQ6540	19,301	N	93	<LOQ	<LOQ	1.0	X	3.2	1.7	X	X
	RQ6660	29,666	N	69	<LOQ	<LOQ	1.5	X	X	1.4	X	X
	RQ6665	32,140	N	74	<LOQ	1.0	2.2	3.8	5.3	4.6	X	X
	RQ6646	50,396	N	71	<LOQ	2.5	X	9.6	X	X	X	X
	RQ6574	51,123	N	24	X	3.9	8.4	653.6	199.0	X	X	X
	RQ6577	82,106	N	95	X	1.9	8.7	15.4	14.6	11.3	X	X
	RQ6558	93,932	N	29	<LOQ	10.0	38.6	47.1	62.8	X	X	X
	RQ6579	120,436	N	35	<LOQ	1.5	X	X	29.3	X	X	X
	RQ6626	126,777	N	32	<LOQ	11.7	49.1	74.3	52.5	X	X	X
	RQ6670	146,024	N	41	<LOQ	1.5	X	X	28.0	X	X	X
	RQ6538	322,956	N	20	<LOQ	18.2	120.6	160.5	X	X	X	X
	RQ6695	494,554	N	29	<LOQ	11.1	69.0	87.0	79.1	X	X	X
	RQ6661	676	Y	NA	X	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	X
	RQ6543	1,349	Y	NA	X	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	X
	RQ6650	1,673	Y	NA	X	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	X
	RQ6668	2,014	Y	NA	X	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	X
	RQ6662	19,212	Y	NA	X	<LOQ	1.7	2.5	3.3	3.5	2.3	X
	RQ6536	25,418	Y	NA	<LOQ	<LOQ	2.6	X	3.9	2.4	1.9	<LOQ
	RQ6664	31,553	Y	NA	<LOQ	<LOQ	X	X	3.0	2.1	1.1	<LOQ
	RQ6582	68,365	Y	NA	<LOQ	<LOQ	1.2	2.0	2.3	2.2	1.9	<LOQ
	RQ6572	74,069	Y	NA	<LOQ	3.3	1.2	5.0	6.0	5.0	4.4	1.6

[a] Items marked with "X" denote samples that were not accessible to MS. As with Table 2, light gray blocks denote positive results (BoNT activity ≥ 1 $MIPLD_{50}$/mL) for MS only. Dark gray blocks denote positive results for IDLC-MS/MS and MBA. Time points marked as "<LOQ" were below the limit of quantitation.

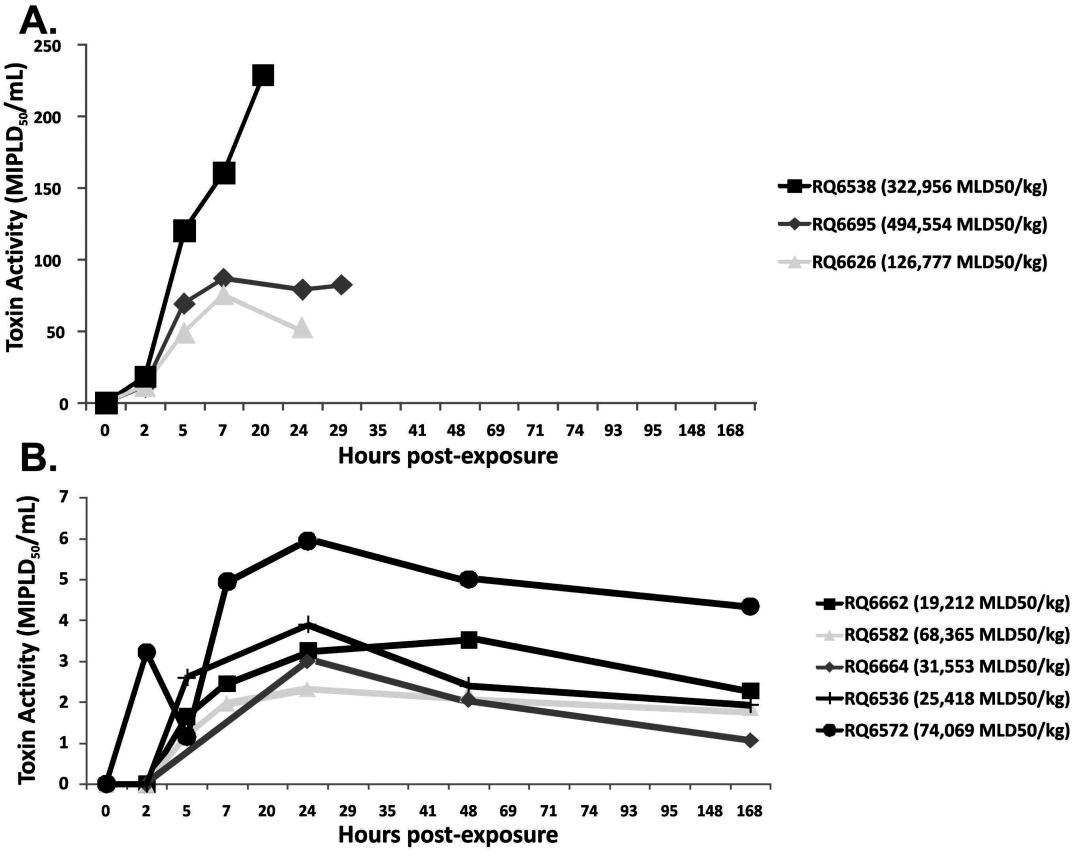

Figure 3. Activity data for select expired (A) and all surviving (B) animals exposed to BoNT/B. Figure S-1 (Supporting Information) summarizes activity data from expired animals not listed in panel A.

Selected data from expired animal serum (Figure 3A) shows a dramatic increase in BoNT activity between 2 and 7 h postchallenge. In that same time frame, data from all surviving animals (Figure 3B) shows a slight increase followed by reduced levels over the

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remainder of the study. Although it would be premature to infer biological significance, a clear trend in BoNT levels is visible between animals that survived the study versus expired cases. However, not all trends were this clear. Figure S-1 (Supporting Information) shows activity data of the ten remaining animals that expired during the study. RQ6574 showed an unusually high activity of 653.6 $MIPLD_{50}$/mL at 7 h postchallenge. This result could be explained by variability in individual animal physiology or amount of toxin inhaled by each animal.

CONCLUSIONS

In this report, we have demonstrated a mass spectrometry based method using a standard triple quadrupole mass spectrometer to quantify BoNT activity of serotypes A and B in serum. Linear ranges of these methods were established at 0−90 $MIPLD_{50}$ for BoNT/A and 0−32 $MIPLD_{50}$ for BoNT/B. A direct comparison of qualitative data was made between MBA and MS detecting BoNT/B in 66/116 samples with activities ≥1 $MIPLD_{50}$/mL with %CV values ≤20%. In this same study, MBA detected BoNT/B in 26/116 samples. These results indicate the ability of MS to give both qualitative and quantitative information from a single aliquot of 0.5 mL of serum. This increases the amount of information available for clinical sample reporting while keeping analysis time at 24−48 h.

These are the first results that show BoNT levels at different stages of clinical botulism in a nonhuman primate and the relationship between serum BoNT levels and clinical outcome. The trends in the rhesus macaque study are clear. Animals with the highest BoNT levels died at earlier time points than those with lower BoNT levels. Several macaques with lower BoNT levels recovered without treatment. Routine quantification of serum BoNT levels in animal studies and clinical cases may be able to establish a firm understanding of toxemia, clinical outcomes, and the effectiveness of various treatments for botulism. Mass spectrometry continues to grow as an important technique for biological and clinical applications. The use of MS based techniques is becoming routine in many clinical laboratories. As time passes, the integration of traditional biological techniques and mass spectrometry will have a major impact on the diagnosis, treatment, and prevention of disease.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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