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**CHARACTERIZATION OF BOTULINUM NEUROTOXIN TYPE A
SUBTYPES BY IMMUNOCAPTURE ENRICHMENT AND LIQUID
CHROMATOGRAPHY TANDEM-MASS SPECTROMETRY**

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ABSTRACT

Botulinum neurotoxins (BoNT) are divided into 7 toxinotypes based on their immunological properties and each toxinotype contains several subtypes according to their amino acid sequences. Here, we designed a spectrometry method able to identify BoNT/A subtypes in complex matrices including crude culture supernatants, food and environmental samples. Peptides from BoNT light chain (L) specific of the subtypes BoNT/A1 to A3 and BoNT/A5 to A8 were identified. The method consists of an immunocapture step with antibodies specific of BoNT/A L chains followed by liquid chromatography/triple quadrupole tandem mass spectrometry (LC-QqQ-MS). BoNT/A subtypes were correctly identified in culture supernatants and in tape water or orange juice samples with a limit of detection of 20 to 150 mouse lethal doses (MLD) and to a lower extent in serum samples.

INTRODUCTION

Botulinum neurotoxins (BoNTs) are the most potent toxins and are responsible for a severe paralytic disease, botulism, in man and animals. Most often, botulism occurs subsequently to the ingestion of food contaminated with *Clostridium botulinum* and containing preformed BoNT. The toxin is absorbed from the intestine, and reaches the neuromuscular junction via the blood or lymph circulation and the interstitial fluid. BoNTs act at the neuromuscular junction in inhibiting the evoked release of acetylcholine, thus leading to paralytic symptoms including blurred vision, ptosis, dry mouth, difficulty in swallowing, then descending flaccid paralysis of voluntary muscles and respiratory insufficiency [1-3].

BoNTs are divided into 7 toxinotypes (A to G) based on their immunological properties using the mouse biological test and specific neutralizing antisera [4,5]. A new subtype, called H, has been reported and waits for further characterization [6]. BoNT genes have been sequenced from a large number of strains and sequence comparison has permitted to identify sequence variations in each toxinotype. Thereby, botulinum toxinotypes are divided into subtypes [7]. Five BoNT/A subtypes (A1 – A5) have been identified and recently three additional subtypes (A6 to A8) are under characterization [8,9] (GenBank ACW83608, JQ954969, KF667385, respectively). Signification of sequence diversity in each toxinotype is not yet well known, but could be important in diagnostic tests and development of therapeutic agents such as those based on immunotherapy. Indeed, BoNT/A1 and BoNT/A2, which differs by 10% at the amino acid sequence level, show large differences in monoclonal antibody-binding affinity. Among six monoclonal antibodies, which bind to BoNT/A1 with

high affinity, three show a marked decrease in binding affinity (500 to more than 1000 fold) to BoNT/A2. Only combinations of monoclonal antibodies, which tightly bind to toxin subtype, potentially neutralize the corresponding toxin *in vivo*. Association of the three monoclonal antibodies with high affinity binding to subtypes A1 and A2, completely neutralizes A1 or A2 toxin, while replacement of two from three monoclonal antibodies by two having a low binding affinity to BoNT/A2 induces a decrease in BoNT/A2 neutralization (50 fold less) [10]. Sequence variation among subtypes might also impact the toxin activity and subsequently the course of the disease. The enzymatic site of BoNT/A3 and BoNT/A4 light (L) chain is conserved, but non-conservative mutations are observed in domains involved in substrate (SNAP-25) recognition [11]. Therefore, L chains from subtypes A3 and A4 show different catalytic properties with the substrate SNAP25 compared to L chain from subtypes A1 and A2, which show the same catalytic activity, although all L chain isoforms bind SNAP25 with similar affinity. L chain from subtype A4 and to a lower extent from subtype A3, cleaves less efficiently SNAP25 than L chain subtype A1 (2 and 23 fold less, respectively) [12,13]. BoNT/A1 to A5 exhibit distinct *in vitro* catalytic activity as well as different biological activity in neuronal cell and mouse models [14]. BoNT/A2 enters neuronal cells faster than BoNT/A1 and it is more potent [15]. In addition, BoNT/A2 is a more potent inhibitor of neurotransmission at the neuromuscular junction [16]. However, both toxins have comparable duration of action [17].

Subtype identification is usually performed via PCR amplification of *bont* genes and subsequent DNA sequencing [7,18]. This method requires the presence of the toxigenic strain in the samples and optimally strain isolation and DNA extraction. However, certain samples might contain BoNT in the absence of the bacterium yielding impossible the use of traditional DNA-based methods. Mass spectrometry (MS) methods have been developed to detect active BoNTs through peptide identification from substrate cleavage (Endopep-MS) (review in [19]). Endopep-MS is a sensitive method to detect BoNT types, but it cannot identify the toxin subtypes, since all the subtypes of each type recognize the same cleavage site. Tryptic digestion of the toxin itself and analysis of tryptic peptides by MS might allow subtype determination. Thereby, BoNT/A1 and BoNT/A2 have been identified in spiked milk samples based on differential peptides between the two subtypes [20]. A similar approach has been described to differentiate the subtypes of BoNT/B [21]. A multiple enzyme and sequential in-gel digestion approach (MESID) has been proposed for BoNT subtyping. The multiple proteolytic enzymes used in this method facilitate near-complete sequence coverage of all the seven BoNT toxinotypes [22].

Here, we report a liquid chromatography/triple quadrupole tandem mass spectrometry with multiple reaction monitoring method (LC-QqQ-MS) for subtyping BoNT/A in culture supernatants or biological and food samples.

EXPERIMENTAL

Safety considerations

BoNTs have been handled with strict respect to safety rules for the handling of toxic substances.

Materials and chemicals

C. botulinum strains used in this study are listed in Table 1. *C. botulinum* strains were grown in trypticase-glucose-yeast extract medium in anaerobic conditions for 4 days. Culture supernatants were stored at -80°C until use. The presence of toxin in the supernatant was first detected by immune-chromatographic test (BTA Gold Assay, NBC-Sys, France) and the toxin activity ($\text{MDL}_{100} \cdot \text{ml}^{-1}$) was then tested by mouse bioassay as previously described [23]. All the peptides used were provided by Proteogenix (France) with purity greater than 95%. The lyophilized peptides were suspended in H_2O /HPLC-grade acetonitrile (ACN) 50/50 at a concentration of $1 \text{ mg} \cdot \text{ml}^{-1}$ and stored at -20°C. A rabbit anti-peptides polyclonal antibody (anti-peptides PAb) was specifically generated by Proteogenix for the immune-magnetic separation. Two peptides: Q67-K84 (QVPVSYDSTYLSTYSTDNEK) and D49-K66 (DTFTNPEEGDLNPPPEAK) from light chain (Lc) were selected for the immunization. The peptide Q67-K84 is common to the subtypes A1, A2, A3, A5, A6, A7 and A8. The peptide D49-K66 is present in all subtypes but shows one mutation in the A7 subtype (I50 to T) and in the A8 subtype (K55 to E). Despite these mutations, the two peptides were recognized by the anti-peptides PAb. The reactivity of the anti-peptides PAb with the BoNT/A1, /A2, /A3, /A5, /A7 and /A8 was checked by Elisa procedure (data not shown). The absence of cross-reactivity against BoNT/A heavy chain (Hc), BoNT/B and BoNT/E was tested by Western-Blot (data not shown).

The food and environmental samples were from local source (local tap water), local grocery (orange juice), and French Defense Health Service (human serum).

Animals and ethics statement

Approval of Ethic Committee of DGA Maîtrise NRBC was obtained. BALB/C mice (female, 4 weeks old) were purchased from Charles River. The animals were fed with

standard diet and water ad libidum. The mice severely injured were euthanized. Determination of mouse lethal dose (MLD₁₀₀) was performed as previously described [24]. Briefly, 0.5 ml of serial 10- fold and then 2-fold dilutions of samples in phosphate buffer (50 mM, pH 6.3) containing 0.2% gelatin were injected intraperitoneally (3 mice per dilution). The mice were observed up to 4 days.

Preparation of anti-peptides PAb coated magnetic beads

Protein G-conjugated magnetic beads (Dynabeads Protein G, Novex) (250 µl) were transferred in a 2 ml Eppendorf low binding micro centrifuge tube and were washed three times with 500 µl of PBST (Phosphate Buffer Salt, 0.1 % Tween 20). After removing the final solution wash, the beads were suspended in 500 µl of PBST containing 100 µg. ml⁻¹ anti-peptides PAb. The mixture was incubated for 30 min with rotation at laboratory temperature. The beads were washed three times with 1 ml of PBS containing 0.15 M NaCl. Antibodies were cross-linked to the beads by incubating, for 30 min with rotation at laboratory temperature, in 1250 µl of PBS containing 0.15 M NaCl and 5 mM BS₃ (crosslinking reagent). 65 µl of Tris/HCl 1 M pH 7.5 were added for quenching the cross-linking reaction. After incubation for 15 min at laboratory temperature with rotation, the beads were washed three times with 1 ml of PBST and resuspended in 250 µl of PBST. They were stored at 4°C for as long as 3 weeks.

In-gel digestion

Each culture supernatant of distinct *C. botulinum* strains from BoNT subtype was precipitated with 60 % of ammonium sulfate. After precipitation, the pellet was dissolved in 50 mM sodium citrate buffer pH 5.5 and dialyzed overnight at 4°C against the same buffer. The dialyzed sample was fractionated via molecular size chromatography [25] using an AKTÄ system and an S300 column (HiPrep 26/60 Sephacryl S-300 HR - GE Healthcare Bio-Sciences). For each subtype, the relevant fractions were pooled and considered as pre-purified complexes. The amount of proteins on pre-purified complexes was determined by the Bio-Rad protein assay with Bovine IgG as standard protein. 1 to 2 µg of each pre-purified complex for each BoNT subtype were run on a SDS-PAGE. After migration, gels were fixed and stained with Coomassie staining solution (Bio-Rad, Marnes la Coquette, France). Based on protein size, gel regions containing protein to identify were excised and cut into small pieces. Gel pieces were destained by washing cycles in water and acetonitrile for 10 min. After destaining, gel pieces were dried by vacuum centrifugation and reduced with DTT

10mM in 50 mM ammonium carbonate buffer and alkylated with iodoacetamide 55mM in 50 mM ammonium carbonate buffer. Gel pieces were washed again with 50 mM ammonium carbonate buffer and dehydrated in 100% acetonitrile (ACN) before trypsin digestion (15 $\mu\text{g}.\text{ml}^{-1}$) at 4°C during 45 min and 2 hours at 37°C. Tryptic peptides were extracted from the gel in three successive steps (15 min each), using 2 cycles of 5% formic acid, and then 100% ACN extraction solutions. After drying by vacuum centrifugation, tryptic peptides were resuspended in 20 μl of 1% TFA before LC-ESI-MS/MS analysis.

Immunocapture enrichment and trypsin digestion

Culture supernatants or samples, crude or diluted in PBS, in a volume of 500 μl were treated with beta-mercaptoethanol (35 μl of 14 M stock solution) for 10 min at 95 °C in a dry heating block. After cooling at room temperature, each sample was mixed with 100 μl of PAb-coated magnetic beads in an Eppendorf low binding microcentrifuge tube. The mixture was incubated for 120 min at laboratory temperature with rotation. The beads were then recovered using a magnetic particle concentrator and washed three times with 1 ml of Hepes buffer (pH 7.6, 50 mM). The beads were then suspended in 100 μl of 100 mM ammonium carbonate buffer (pH 8.5) and incubated for 10 min at 95°C. 15 μl of 100 $\mu\text{g}.\text{ml}^{-1}$ aqueous trypsin solution (sequencing grade modified trypsin, Promega) was added and the enzymatic digestion was carried out at 38°C for 120 min. After digestion, the magnetic beads were removed and 2 μl of analytical grade formic acid (FA) was added. The solution was vacuum-dried and resuspended in 50 μl of H₂O/ ACN/FA 93/5/2 before the LC-QqQ-MS analysis..

LC-ESI-MS/MS analysis

ESI-LC-MS/MS analysis was performed on an Esquire 6000 mass spectrometer (Bruker Daltonics, Bremen, Germany) connected with an Ultimate 3000 high performance liquid chromatograph (Dionex Corp., Sunnyvale, CA). The protein digest (10 μL) was injected on a 0.3 \times 150 mm PepMap100 capillary column, particle size 5 μm , 100 Å pore size (LC Packings, Amsterdam, The Netherlands). Samples were eluted using a gradient starting with a linear increase from 5 to 10% acetonitrile over 4 min, followed by an increase from 10 to 80% acetonitrile over 42 min with 0.2% acid formic throughout as additives. The data acquisition was performed using the data-dependent mode where the three highest-intensity precursors in an MS1 survey scan were selected for collision-induced dissociation. The resulting MS/MS data were searched for protein candidates with a database search against NCBI database using MASCOT software (Matrix version 2.2.07, Sciences, London, U.K.).

The mass tolerance of precursor ions and fragment ions was 0.5 Da. Resulting peptides were filtered with a significance threshold of $p < 0.05$.

LC-QqQ-MS analysis

The HPLC system used for this analysis was an Ultimate 3000 RSLC equipped with a degasser, a binary pump, an auto-injector and a column oven (Thermo Scientific) coupled to a TSQ Quantiva electrospray ionization triple quadrupole mass spectrometer (Thermo Scientific). Chromatographic separations were performed on Zorbax SB-C18 2.1×150 mm, $3.5 \mu\text{m}$, 80\AA pore size (Agilent) using 0.2% (v/v) formic acid as eluent A and acetonitrile with 0.2% formic acid as eluent B with a flow rate of $200 \mu\text{L}/\text{min}$ at 25°C . Samples ($10 \mu\text{l}$) were analyzed in a gradient mode: 5% eluent B (0–2 min) and 50% B (30 min). The mass spectrometer ESI source was operated in positive ionization mode. The following conditions were applied: spray voltage, 4000 V; sheath gas, 50 Arb; aux gas, 10 Arb; sweep gas, 5 Arb; ion transfer tube temp, 325°C ; vaporizer temp, 275°C ; CID gas, argon, 2 mTorr.

Food and biological samples

Food and human serum samples were artificially spiked with culture supernatant of each *C. botulinum* A subtype, and 0.5 ml of each sample were treated as above described for the culture supernatants except that the serum samples were not heated. Serial 10 fold dilutions of each sample were tested by mouse bioassay and by immunocapture/ LC-QqQ-MS method.

RESULTS

Selection of specific peptides for botulinum toxin A subtypes detection

MS-based analysis can distinguish proteins on the basis of amino acid sequence. BoNT/A1 to BoNT/A8 subtypes share 84.5 to 97.2 % identity at the amino acid level (Table 2). We first identified the tryptic cleavage sites on the sequences of the L chain (amino acids 1 to 449) of each BoNT/A subtype and we then analyzed all the subsequent peptides for their specificity versus the other BoNT/A subtypes by Blast. Thereby, 26 peptides of each BoNT/A subtypes were analyzed *in silico*. Two to 12 unique peptides for each BoNT/A subtype were synthesized and analyzed in MS (Table 3). For each subtype, two specific peptides yielding the highest MS signals were selected. One peptide was common to both BoNT/A2 and

BoNT/A8, and two other to BoNT/A1 and BoNT/A6. Moreover, two additional peptides which were conserved in all BoNT/A subtypes were also selected (Table 4).

To validate the selection of these peptides for the identification of BoNT/A subtypes, BoNT preparations of each subtype (1 to 2 μg) were run on a SDS-PAGE and the bands containing the L chain were cut out of the gel. After in gel trypsin digestion, the peptides were extracted and analyzed with LC-ESI-MS/MS (data not shown). BoNT/A4 and BoNT/A6 were not included in this study due to the difficulty to obtain BoNT/A4 preparations containing sufficient amounts of toxin and to the fact that BoNT/A6 (strain CDC41370) was not available in our collection.

Identification of BoNT/A subtype in culture supernatants

To identify BoNT/A subtype in a complex environment like culture supernatant, we used an immunocapture step. For that purpose, magnetic beads were coated with antibodies raised against two synthetic peptides conserved in BoNT/A light chain subtypes as described in the Experimental part. Culture supernatant samples (500 μl) were reduced by addition of betamercaptoethanol to allow the separation of L and H chains and were then incubated with coated magnetic beads. After washing, the bound material was digested by trypsin *in-situ*, dried, and analyzed by LC-MS/MS. An example of conserved peptide detection by LC-MS/MS in culture supernatants of representative strains of each BoNT/A subtype is shown in Fig. 1. *Clostridium sporogenes* which is closely related to *C. botulinum* A strains showed no detectable peptide corresponding to L chain fragment. Fig. 2 shows identification of BoNT/A2 through detection of the specific subtype peptide (DVASTLNK) detection by LC-MS/MS in *C. botulinum* culture supernatants. The global results of peptide detection in *C. botulinum* culture supernatants are listed in Table 5. The selected conserved peptides were detected in all the *C. botulinum* culture supernatants tested, whereas the selected specific subtype peptides were only identified in the corresponding *C. botulinum* subtype culture supernatants. However, the I376-K387 peptide was detected in both *C. botulinum* A2 and A8 subtype culture supernatants, in contrast to the D292-K299 peptide which was only found in *C. botulinum* A2 strains (Table 5).

Sensitivity

The limit of detection (LOD) of the LC-QqQ-MS method was estimated by using serial dilutions of *C. botulinum* culture supernatants. LOD corresponded to the highest dilution giving a signal with a signal-to-noise ratio of 3. The results with representative *C.*

botulinum culture supernatant subtypes and expressed in equivalent MLD100 which have been determined by the mouse bioassay, are shown in Table 6. The MS methodology could detect BoNT ranging from 20 to 140 MLD/0.5 ml according to the subtype.

Identification of BoNT/A subtype in different matrices

In order to validate the LC-QqQ-MS methodology with biological, food or environmental samples, LOD was determined in various representative matrices (tap water, orange juice, human serum) spiked with the distinct BoNT/A subtypes. As shown in Table 7, the BoNT/A subtypes A1 to A8 were successfully identified in all the different samples. The highest LOD values were obtained with tap water and orange juice and were comparable to those obtained with culture supernatants (Table 6), whereas the detection was slightly lower (4 to 10 fold less) in human serum.

DISCUSSION

Detection of BoNT in biological samples and food is critical for early diagnosis of botulism and to apply an efficient treatment of the disease. Investigation of BoNT in food or environmental samples is also essential for identifying the source of contamination and preventing outbreak extension of botulism. Typing and subtyping are important to estimate the severity of the disease and to adapt the appropriate treatment notably the specificity of the antisera. MS is a rapid and powerful method to identify proteins. Here, we show that a LC-QqQ-MS method coupled to an immunopurification step allows identification of BoNT/A type and subtypes from complex samples. The method based on L chain peptides specific of BoNT/A subtypes A1 to A8 yielded unambiguous identification of the subtypes. A previous MS approach has been designed to differentiate BoNT/A1 and BoNT/A2 [20]. Identification of BoNT types A1 to G was achieved by using multiple enzyme digestion in gel and subsequent LC-QqQ-MS analysis. This method is predicted to identify the subtypes but has not yet been validated [22]. Our procedure efficiently discriminated the distinct BoNT/A subtypes. One important advantage is that complex samples can be used. Subtyping was efficiently performed with crude culture supernatants. An additional advantage is that this method of subtyping is performed in one step without the need of further techniques or reagents to address the subtyping. However, this method would be tested with complex and naturally contaminated samples like food or stools. LOD of the proposed method (20 to 140 equivalents MLD in 0.5 ml) is in the range of the minimal toxin concentrations found in food. The sensitivity of the method could be increased by using a greater sample volume. Using

artificially spiked samples representative of food, environmental and biological samples, the LC-QqQ-MS method allowed the identification of all the BoNT/A subtypes tested. LOD varied with the matrix, with the lowest values in serum samples. Albeit the proposed method is not sensitive enough to detect limited BoNT amounts, it is appropriate for contaminated environmental or food samples which usually contain significant BoNT concentrations (100 MLD/g and more). The advantage of the LC-QqQ-MS approach is to allow subtype identification in the absence of viable neurotoxin producing bacteria or available DNA in samples to perform a genetic subtyping. Further developments are still required for application with biological samples. It is noteworthy that this methodology might be updated to identify new subtypes that will be evidenced, by selecting additional specific peptides on light and/or heavy chains.

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LEGENDS TO FIGURES

Figure 1. Example of detection of a conserved peptide in culture supernatants from representative *C. botulinum* A subtypes. Ion chromatograms of SRM transition 565.8/684.4 (LYGIAINPNR peptide) from BoNT/A1 (strain Hall), BoNT/A2 (strain 9336), BoNT/A3 (strain Loch Maree), BoNT/A5 (strain 126.07), BoNT/A7 (strain 148.08), and BoNT/A8 (strain 217.12) supernatants. *Clostridium sporogenes* (strain 261.05) supernatant was used as negative control. The expected retention time is 15.7 min.

Figure 2. Example of detection BoNT/A2 in culture supernatants from representative *C. botulinum* A subtypes. Ion chromatograms of SRM transition 424.2/215.1 (DVASTLNK peptide) from BoNT/A1 (strain Hall), BoNT/A2 (strain 9336), BoNT/A3 (strain Loch Maree), BoNT/A5 (strain 126.07), BoNT/A7 (strain 148.08), and BoNT/A8 (strain 217.12) supernatants. *Clostridium sporogenes* (strain 261.05) supernatant was used as negative control. The expected retention time is 10.1 min.

Neurotoxin gene cluster	Strain	Lethal activity (MLD100/ml)
<i>ha-bont/A1</i>	Hall	$2 \cdot 10^4$
<i>ha-bont/A1</i>	Legroux	$1 \cdot 10^5$
<i>orfX-bont/A1(B)</i>	NCTC2916	$1 \cdot 10^5$
<i>orfX-bont/A1</i>	200.04	$2 \cdot 10^5$
<i>orfX-bont/A2</i>	136.06	$2 \cdot 10^4$
	133.06	$2 \cdot 10^4$
	9336	$2 \cdot 10^5$
	181.02	$2 \cdot 10^5$
	1618	$2 \cdot 10^5$
<i>orfX-bont/A3</i>	Loch Maree	$2 \cdot 10^3$
<i>ha-bont/A5</i>	126.07	$2 \cdot 10^4$
<i>orfX-bont/A7</i>	148.08	$2 \cdot 10^4$
<i>orfX-bont/A8</i>	217.12	$1.6 \cdot 10^4$

Table 1. List of *C. botulinum* strains used in this study, their neurotoxin gene cluster, and mouse lethal activity of culture supernatant as tested with mouse bioassay.

	A2	A3	A4	A5	A6	A7	A8
A1	90.0	84.9	89.4	97.2	95.7	93.8	93.4
A2		93.4	88.4	90.5	91.7	89.8	93.5
A3			84.5	85.4	86.5	85.1	88.0
A4				87.6	87.9	86.9	89.1
A5					95.9	94.4	93.7
A6						93.0	93.1
A7							91.4

Table 2. Amino acid sequence identity (%) of BoNT/A subtypes. Amino acid sequences from representative strains are: BoNT/A1 from strain Hall (YP_001386738.1); BoNT/A2 from strain Kyoto (CAA51824.1); BoNT/A3 from strain Loch Maree (YP_0017157303.1); BoNT/A4 from strain BA857 (YP_002860313.1); BoNT/A5 from strain IBCA94-0216 (ACT33194.1); BoNT/A6 from strain CDC 41370 (ACW83608.1); BoNT/A7 from strain 148.08 (AFV13854.1); BoNT/A8 from strain 217.12 (AHA83316.1).

Specificity	Tryptic digest fragment	peptide sequence
Conserved ^a	I24-K34	IPNAGQMGPVK
Conserved ^a	Q67-K84	QVPVSYDSTYLSTDNEK
Conserved ^a	G114-K128	GIPFWGGSTIDTELK
Conserved ^b	N178-R187	NGYGSTQYIR
Conserved ^a	L232-R241	LYGIAINPNR
Conserved ^b	N418-K427, N414-K423 for BoNT/A3	NFTGLFEFYK
BoNT/A1 and A6 ^c	S167-R177	SFGHEVLNLTR
BoNT/A1 and A6 ^c	V382-R393	VNYTIYDGFNLR
BoNT/A2 and A8 ^d	F213-R231	FATDPAVTLAHELIHAEHR
BoNT/A2	D292-K299	DVASTLNK
BoNT/A2 and A7 ^e	S302-K314	SIIGTTASLQYMK
BoNT/A2	M344-K359	MLTEIYTEDNFVNFFK
BoNT/A2 and A8 ^c	I376-K387	INIVPDENYTIK
BoNT/A2	G394-R411	GANLSTNFNGQNTEINSR
BoNT/A3	I38-R48	IHEGVWVIPER
BoNT/A3	I98-R105	IYSTGLGR
BoNT/A3	M106-K113	MLLSFIVK
BoNT/A3	V129-R145	VIDTNCINVIPGGSYR
BoNT/A3	S146-K166	SEELNLVITGPSADIIQFECK
BoNT/A3	S167-R177	SFGHDFVNLTR
BoNT/A3	T247-R264	TNAYYEMSGLEVSFEELR
BoNT/A3	T265-R280	TFGGNDTNFIDSLWQK
BoNT/A3	D285-R295	DAYDNLQNIAR
BoNT/A3	T302-K314	TIVGTTTPLQYMK
BoNT/A3	Y321-K330	YFLSEDASGK
BoNT/A3	G348-K359	GFTELEFVNPFK
BoNT/A5	I98-R105	IYSELGR
BoNT/A5	T265-K272	TFGEHDAK
BoNT/A5	I376-R393	INIVPEVNYTIYDGFNLR
BoNT/A6	N394-K415	NTNLAANFNGQNTEINNMNFAK
BoNT/A7	D49-K66	DIFTNPEEGDLNPPPEAK
BoNT/A7	S146-K166	SEELNLVIIGPSADIINFECK
BoNT/A7	F213-R231	FAIDPAVTLAHELIHAGHR
BoNT/A7	E292-K299	EVASILNK
BoNT/A7	M376-R393	MNIVPEVNYTIYDGFNLR
BoNT/A8	D12-K23	DTVNGVDIAYIK
BoNT/A8	D49-K55	DTFTNPK
BoNT/A8	E56-K66	EGDLNPPPEAK
BoNT/A8	T265-K272	TFGGHNAK
BoNT/A8	N394-R411	NTNLAANFNGQNTEINSR

Table 3. Proteolytic peptides (peptide size between 7 and 25 amino acids) of BoNT/A1 to A8 light chains.

^a Peptides conserved in BoNT/A1-/A3 and BoNT/A5-/A8 light chains.

^b Peptides conserved in BoNT/A1 to A8 light chains

^c Peptides common to BoNT/A2 and BoNT/A6 light chains

^d Peptides common to BoNT/A2 and BoNT/A8 light chains

^e Peptides common to BoNT/A2 and BoNT/A7 light chains

Specificity	Tryptic digest fragment	Proteotypic peptide sequence	SRM transitions		Retention time (min.)
			Precursor (m/z)	Product (m/z)	
Conserved	G114-K128	GIPFWGGSTIDTELK	810.9	601.3	20.5
				725.9	
				1020.5	
Conserved	L232-R241	LYGIAINPNR	565.8	334.2	15.7
				684.4	
				854.5	
BoNT/A1	S167-R177	SFGHEVLNLTR	424.9	389.3	15.3
				503.3	
				657.3	
BoNT/A1	V382-R393	VNYTIYDGFNLR	737.9	214.1	19.0
				884.4	
				997.5	
BoNT/A2	D292-K299	DVASTLNK	424.2	187.1	10.1
				215.1	
				633.4	
BoNT/A2 and A8	I376-K387	INIVPDENYTIK	709.9	228.1	16.3
				341.2	
				979.5	
BoNT/A3	S167-R177	SFGHDVFNLTR	431.6	276.2	16.4
				389.3	
				503.3	
BoNT/A3	G348-K359	GFTELEFVNPFK	714.4	391.2	22.0
				505.3	
				880.5	
BoNTA5	I98-R105	IYSELGR	469.8	277.2	12.1
				662.4	
				825.4	
BoNTA5	I376-R393	INIVPEVNYTIYDGFNLR	714.0	440.3	22.6
				721.4	
				884.4	
BoNTA7	E292-K299	EVASILNK	437.3	487.3	13.1
				574.4	
				645.4	
BoNTA7	M376-R393	MNIVPEVNYTIYDGFNLR	720.0	359.17	22.0
				458.2	
				884.4	
BoNTA8	E56-K66	EGDLNPPPEAK	583.8	541.3	10.5
				638.4	
				752.4	

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Table 4. MS analysis of BoNT/A light chain peptides. Results of MS analysis of synthetic peptides specific of each BoNT/A subtype. 3 transitions for each analysis, n=3.

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Strain \ Peptide	L232-R241 (conserved)	G114-K128 (conserved)	S167- R177 (BoNT/A1)	V382- R393 (BoNT/A1)	D292- K299 (BoNT/A2)	I376-K387 (BoNT/A2- /A8)	S167- R177 (BoNT/A3)	G348- K359 (BoNT/A3)	I98-R105 (BoNT/A5)	I376-R393 (BoNT/A5)	E292-K299 (BoNT/A7)	M376- R393 (BoNT/A7)	E56-K66 (BoNT/A8)
Hall - BoNT/A1	+	+	+	+	-	-	-	-	-	-	-	-	-
Legroux - BoNT/A1	+	+	+	+	-	-	-	-	-	-	-	-	-
NCTC2916 - BoNT/A1	+	+	+	+	-	-	-	-	-	-	-	-	-
200.04 - BoNT/A1	+	+	+	+	-	-	-	-	-	-	-	-	-
136.06 - BoNT/A2	+	+	-	-	+	+	-	-	-	-	-	-	-
133.06 - BoNT/A2	+	+	-	-	+	+	-	-	-	-	-	-	-
9336- BoNT/A2	+	+	-	-	+	+	-	-	-	-	-	-	-
181.02- BoNT/A2	+	+	-	-	+	+	-	-	-	-	-	-	-
1618- BoNT/A2	+	+	-	-	+	+	-	-	-	-	-	-	-
Loch Maree- BoNT/A3	+	+	-	-	-	-	+	+	-	-	-	-	-
126.07- BoNT/A5	+	+	-	-	-	-	-	-	+	+	-	-	-
148.08- BoNT/A7	+	+	-	-	-	-	-	-	-	-	+	+	-
217.12- BoNT/A8	+	+	-	-	-	+	-	-	-	-	-	-	+

Table 5. Results of peptide detection by LC-MS/MS in *C. botulinum* culture supernatants. n=3.

<i>C. botulinum</i> A subtype	Detected peptide	LOD (MLD100)
Hall (A1)	S167-R177	110 ± 16
9336 (A2)	D292-K299	30 ± 4.5
Loch Maree (A3)	S167-R177	50 ± 7.5
126.07 (A5)	I98-R105	20 ± 3
148.08 (A7)	M376-R393	140 ± 21
271.12 (A8)	E56-K66	50 ± 7

Table 6. Estimated limit of detection (LOD). LOD is expressed in equivalent MLD₁₀₀/0.5 ml. Signal to noise ratio of 3. Results are mean values ± SD. n=3.

Matrices	Hall (A1)	9336 (A2)	Loch Maree (A3)	126.07 (A5)	148.08 (A7)	271.12 (A8)
Tape Water	70	20	50	15	110	50
Orange juice	110	50	70	20	150	60
Human serum	300	200	250	100	500	300

Table 7. Estimated limit of detection (LOD expressed in equivalent $MLD_{100}/0.5$ ml) in various samples. Signal to noise ratio of 3. $n=3$.

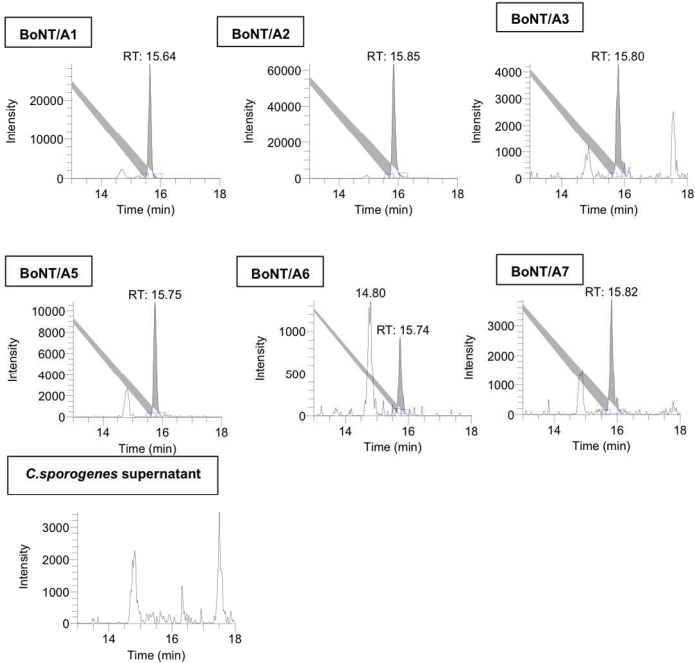


Figure 1

Example of detection of a conserved peptide in culture supernatants from representative *C. botulinum* A subtypes. Ion chromatograms of SRM transition 565.8/684.4 (LYGIAINPNR peptide) from BoNT/A1 (strain Hall), BoNT/A2 (strain 9336), BoNT/A3 (strain Loch Maree), BoNT/A5 (strain 126.07), BoNT/A7 (strain 148.08), and BoNT/A8 (strain 217.12) supernatants. *Clostridium sporogenes* (strain 261.05) supernatant was used as negative control. The expected retention time is 15.7 min.

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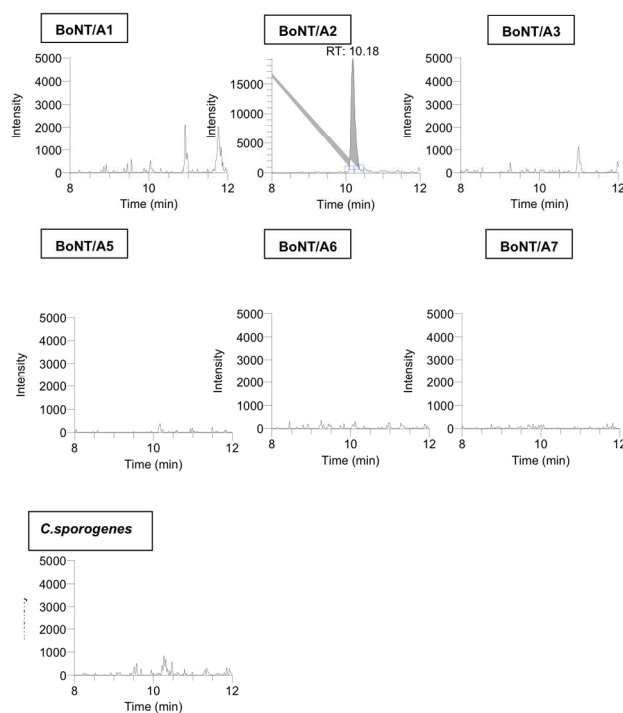


Figure 2

Example of detection BoNT/A2 in culture supernatants from representative *C. botulinum* A subtypes. Ion chromatograms of SRM transition 424.2/215.1 (DVA_{ST}L_{NK} peptide) from BoNT/A1 (strain Hall), BoNT/A2 (strain 9336), BoNT/A3 (strain Loch Maree), BoNT/A5 (strain 126.07), BoNT/A7 (strain 148.08), and BoNT/A8 (strain 217.12) supernatants. *Clostridium sporogenes* (strain 261.05) supernatant was used as negative control. The expected retention time is 10.1 min.

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