# ULTRA SENSITIVE HPLC DETECTION ASSAY FOR BOTULINUM NEUROTOXIN TYPE A

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## ABSTRACT

The botulinum neurotoxins are among the most potent toxins in nature. They are synthesized as single 150 kDa polypeptide chains which are subsequently cleaved to produce a 100 kDa heavy chain and a 50 kDa light chain linked by a disulfide bond. Four of the seven immunologically distinct serotypes, A, B, E, and F, cause botulism in humans. The 50 kDa light chain of each serotype is a zinc endoprotease that cleaves a single target protein which is essential for synaptic vesicle membrane fusion. This inhibits neurotransmitter release which leads to muscular paralysis.

These secreted toxins are regarded as major biological warfare threats. Due to their extreme potency and lethality, detection of these toxins requires a highly sensitive and reliable assay. Measurement of proteolytic activity provides a potentially sensitive and direct means for detecting these potent toxins. Substantial signal amplification can be expected as a result of catalytic turnover of the substrate. Each serotype of the neurotoxin selectively cleaves one membrane fusion protein at a specific site, creating an unique cleaved product, providing the basis for an inherently specific assay.

A sensitive assay focused on the enzymatic property of botulinum toxin type A (BoNT/A) has been developed. The sensitivity is significantly improved by the use of high affinity monoclonal antibodies, one specific for the heavy chain and one for the light chain, to capture and concentrate the toxin prior to addition of the substrate. The FRET peptide SNAPtide®, designed at List Biological Laboratories, was used as the substrate for BoNT/A. Generation of cleaved peptide was monitored using reverse phase HPLC with a fluorescence detector. In an initial study, cleavage of 20 µM SNAPtide® peptide substrate by 5 pg/ml BoNT/A at room temperature was detected in an overnight digestion. This study demonstrates that this method can be used to achieve the prerequisite sensitivity for BoNT/A which is 20 pg/ml, comparable to the amount detected using the gold standard mouse bioassay.

## INTRODUCTION

**Botulinum toxins** are synthesized as single 150 kDa polypeptide chains which are subsequently cleaved to produce a heavy chain and a light chain linked by a disulfide bond. There are three functionally distinct domains that mediate either binding to, translocation into, or cleavage of specific proteins in neuronal cells. This is illustrated in the crystal structure of botulinum toxin type A (BoNT/A) **Figure 1**.

The green portion represents the translocation domain which together with the blue and red binding domains define the 100 kDa heavy chain. The 50 kDa endoprotease domain is shown in aqua.

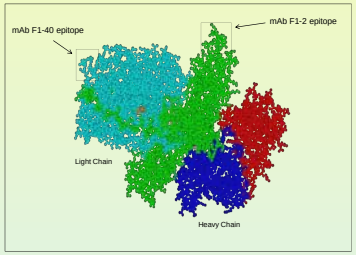



**Figure 1**: Crystal structure of botulinum neurotoxin type A. Lacy, DB *et al., Nat. Struct. Biol.* 1998, 10:898-902.

The zinc dependent N-terminal light chain is the catalytic subunit which, depending on the serotype of the neurotoxin, selectively cleaves one of the SNARE membrane fusion proteins. The type A neurotoxin cleaves the 25 kDa synaptosomal protein, SNAP-25, exclusively between residues $Gln^{197}$-$Arg^{198}$. The primary sequence of the C-terminal end is given below. The minimum effective BoNT/A substrate is 13 amino acids consisting of residues 190-202 of SNAP-25 (Schmidt JJ and Bostian KA, J. Protein Chem. 1997, 16:16-26). The blue arrow indicates the BoNT/A cleavage site.

---$L^{165}$-D-M-G-N-$E^{170}$-I-D-T-Q-$N^{175}$-R-Q-I-D-$R^{180}$-I-M-E-K-$A^{185}$

-D-S-N-K-**$T^{190}$-R-I-D-E-$A^{195}$-N-Q-R-A-$T^{200}$-K-M**-L-G-$S^{205}$-G

**SNAPtide®** is used as the substrate in these studies. It is based on the 13-amino acid sequence shown above and contains the FRET pair, ortho amino benzoic acid (o-Abz) on the N-terminal and a 2,4 dinitrophenyl (Dnp) on a lysine close to the C-terminal amino acid.

Two high affinity **anti-BoNT/A monoclonal antibodies** (mAb), F1-2 and F1-40, attached to magnetic beads were used in this analysis to capture and concentrate the BoNT/A (Stanker, LH et al. J. Immunol. Methods 2009, 77:4305-4313). The binding site for the F1-2 mAb is conformational and has been mapped to the translocation domain of the heavy chain. The epitope recognized by the F1-40 mAb has been mapped to the light chain. It binds a loop in the N-terminal half of the enzyme while the catalytic site is at the C-terminal region. The enzymatic activity of the BoNT/A is still observed in the presence of the F1-40 monoclonal antibody. Stanker et al. have developed highly sensitive sandwich ELISAs capable of detecting 2 pg/ml using the F1-2 to capture and biotinylated F1-40 mAb to detect.

SNAPtide® containing the FRET pair fluorescein-thiocarbamoyl (FITC), and 4[[4'(dimethyl-amino)phenyl]azo]benzoic acid (DABCYL) was used successfully in conjunction with the F1-2 mAb to detect BoNT/A in liquid food samples (Rasooly, R and Do, PM. Appl. Environ. Microbiol. 2008, 74:4309-4313). The mAb not only serves to concentrate the toxin but it also minimizes potential complex matrix effects including endogenous proteases and protease inhibitors. The increase in fluorescence intensity as the SNAPtide® was cleaved by BoNT/A in carrot juice was measured. The sensitivity of the assay was ~10 pg/ml BoNT/A. The dose dependent response started above 500 pg/ml.

The study presented here demonstrates significant increase in the level of detection using HPLC in combination with fluorescence detection. The peak from the o-Abz fluorescently labeled cleaved fragment generated by BoNT/A captured by the mAbs F1-2 or F1-40 is analyzed. Results obtained in buffer and in undiluted skim milk are presented.

## MATERIALS and METHODS

SNAPtide® substrate (Product #520) and Botulinum neurotoxin type A (Product #130A ) are products of List Biological Laboratories, Inc. The monoclonal anti-BoNT/A, F1-2 and F1-40 antibodies, were kindly supplied by Dr. Larry Stanker under an MTA with the USDA ARS. The immuno-magnetic beads were made using MyOne™ tosylactivated beads (Invitrogen, Carlsbad, CA). The MyOne™ beads are 1.08 µm in diameter, contain 8 $m^2$/g of surface area and 1 x $10^{12}$ beads/ml.

**Sample preparation:** Stock solutions (5 mM) of the **SNAPtide®** (Product #520) FRET peptide were made in dimethyl sulfoxide (DMSO). The SNAPtide® is diluted to 20 µM in 20 mM HEPES, pH 8.0 containing 1.0 mM $ZnCl_2$, 5 mM dithiothreitol (DTT), and 0.1 % Tween-20 (reaction buffer). The **BoNT/A** is prepared as a 100 ng/µl solution in 20 mM HEPES, pH 8.0 containing 0.2% Tween-20. Appropriate dilutions were made in 20 mM HEPES, pH 8.0. Details are given in the figure legends.

**HPLC:** For BoNT/A detection studies, the HPLC was performed using a Zorbax Eclipse Plus C18 reverse phase column, 4.6 x 150 mm (Agilent, Santa Clara, CA) attached to a Varian ProStar HPLC system (Varian, Walnut Creek, CA). Solvent A was 0.1% TFA and solvent B was 100% acetonitrile containing 0.1% TFA. The column gradient was as follows: 12% B for 5 min, 12-20% B in 5 min, 20-100% B in 5 min, 100% B for 5 min, and 9 min equilibration with 12% B. The column effluent was monitored using a Hitachi fluorescence detector with excitation set to 320 nm and emission at 418 nm to detect the o-Abz fluorophore on the N-terminal cleaved fragment of SNAPtide® 520. The injection volume was 50 µl. Details of the digestion experiment are described in the figure legends.

**Assay Experimental Design**:

A schematic representation of the assay setup is shown below (**Figure 2**). The assay consists of four steps.

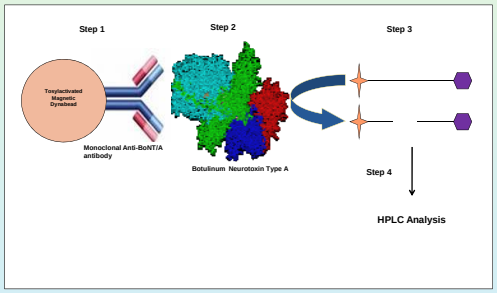
**Step 1:** Preparation of monoclonal Antibody(mAb)-coated beads.
The immuno-magnetic beads were made using the appropriate mAb attached according to manufacturer's instructions.

**Step 2:** Binding of BoNT/A to mAb-coated beads.
Monoclonal antibody coated beads were exposed to a series of BoNT/A concentrations either in buffer or undiluted skim milk and incubated for 2 hours at room temperature.

**Step 3:** Cleavage of SNAPtide® by BoNT/A.
Twenty micromolar SNAPtide® in reaction buffer was added to the BoNT/A bound immuno-magnetic beads. After overnight digestion, samples were separated from the beads, filtered and analyzed by HPLC. Reactions were run at room temperature.

**Step 4:** HPLC analysis of cleaved SNAPtide®.
See description above.

**Figure 2**




## RESULTS

### A. BoNT/A capture using the F1-2 monoclonal antibody.

Capture of BoNT/A was tested in both buffer and undiluted skim milk. The amount of cleaved SNAPtide® substrate, as reflected in the area of the HPLC peak at 10.6 min, is shown as a function of BoNT/A concentration (pg/ml) in **Figure 3**. The assay allows detection of as little as 5 pg/ml in both skim milk and buffer. The dose dependent response also starts at 5 pg/ml.

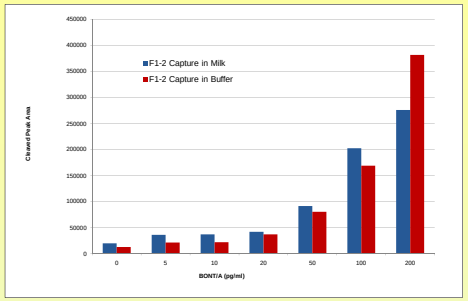



**Figure 3**: BoNT/A captured by mAb F1-2 in the presence of buffer or undiluted skim milk. The peak area observed for the cleaved o-Abz containing fragment is plotted as a function of BoNT/A concentration after overnight digestion at room temperature.

### B. BoNT/A capture using the F1-40 monoclonal antibody.

Chromatograms obtained in the absence and presence of a series of BoNT/A concentrations are shown in **Figure 4**. After digestion, the o-Abz-containing N-terminal fragment was visible at 10.6 min. This peak was easily detected in the 5 pg/ml sample after overnight digestion.

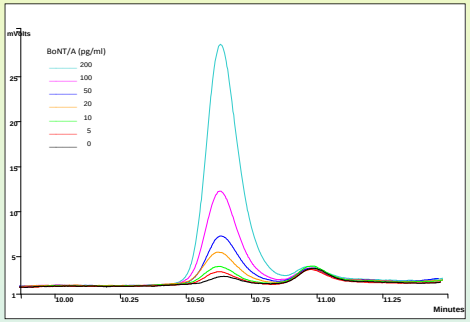



**Figure 4**: HPLC peak for the N-terminal fragment of SNAPtide® cleaved by BoNT/A at room temperature in an overnight digestion. A series of concentrations of BoNT/A (see legend on the left) are captured by mAb F1-40 attached to MyOne™ magnetic beads in reaction buffer.

The sensitivity of the BoNT/A capture assay using the F1-40 monoclonal antibody is demonstrated in **Figure 5**. A series of BoNT/A concentrations were captured using the mAb F1-40 coated magnetic beads in both buffer and undiluted skim milk and incubated with 20 µM SNAPtide® substrate at room temperature. The amount of cleaved SNAPtide® substrate, as reflected in the area of the HPLC peak at 10.6 min, is shown as a function of BoNT/A concentration (pg/ml) after overnight digestion in **Figure 5**. The lowest amount of BoNT/A that can be detected is 5 pg/ml in both buffer and skim milk and the lowest amount that can be quantitated in skim milk is 50 pg/ml and in buffer is 5 pg/ml.

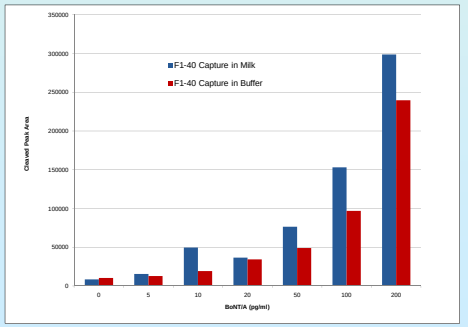



**Figure 5**: BoNT/A captured by mAb F1-40 attached to MyOne™ magnetic beads in both buffer and undiluted skim milk. The peak area observed for the cleaved o-Abz containing fragment as a function of BoNT/A concentration after overnight digestion at room temperature.

## CONCLUSIONS

**A. Comparison of sensitivity**

Two different monoclonal antibodies were evaluated. The amount of BoNT/A captured by each is measured by monitoring the cleavage of the specific BoNT/A substrate, SNAPtide® 520. Using the mAb F1-40 and mAb F1-2 we can detect 5 pg/ml of botulinum toxin type A.

**B. Capture and detection from undiluted skim milk**

For both F1-2 and F1-40 antibodies BoNT/A was captured and detected when the toxin was diluted in skim milk. The sensitivity of the assay remained at 5 pg/ml as it was in buffer.

**C. Dose dependent response:**

Using the F1-2 monoclonal antibody to capture the BoNT/A, the dose dependent response starts at 5 pg/ml in both buffer and undiluted skim milk. The lowest amount that can be quantified with F1-40 is 5 pg/ml in buffer and 50 pg/ml in skim milk.

## FUTURE DIRECTIONS

A. The studies will be extended to include more complex matrices such as serum.

B. A Control Peptide for SNAPtide® #520, which contains all non specific cleavage sites but is not a substrate for BoNT/A will be used to monitor residual non specific hydrolysis of the SNAPtide® #520 in complex matrices.

C. Adaptation of this assay to a 96-well plate format will be evaluated.

## REFERENCES

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