SENSITIVE AND SPECIFIC BIFUNCTIONAL ASSAY FOR BOTULINUM NEUROTOXIN TYPE A

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ABSTRACT

Background: The purpose of this study is to develop a highly specific and sensitive method to assess two of the key steps important for Botulinum Neurotoxin type A (BoNT/A) toxicity. This bifunctional assay measures both the integrity of the protein receptor site as well as the enzymatic function. To demonstrate specific binding of the toxin via its protein receptor site, BoNT/A is captured using magnetic beads coated with the luminal domain of SV2c. This binding is then quantitated through measurement of the endopeptidase activity using a specific substrate, SNAPtide®, which is based on the native SNAP-25 protein.

Methods: Recombinant GST-SV2c (List Prod #690) and recombinant GST Synaptobrevin-2 (List Prod #510A), a control protein, were attached to glutathione coated magnetic beads. Each set of coated beads was exposed to varying amounts of BoNT/A in buffer, washed, and incubated with SNAPtide® (List Prod #520), a quenched fluorogenic (FRET) substrate. Detection of cleaved peptide was monitored using reverse phase HPLC with fluorescence detection.

Results: Various conditions were tested to optimize the binding and to limit the non specific binding of BoNT/A to the beads. Conditions were established allowing the detection of as low as 1.25 pg of SV2c bound BoNT/A in an overnight digestion at room temperature.

Conclusions: This highly specific and sensitive assay captured, on the native receptor, SV2c, and detected enzymatic activity using as little as 1.25 pg of BoNT/A, significantly less than one mouse LD_{50} (5 pg). This bifunctional assay of BoNT/A toxicity can be used to assess the integrity of the binding and catalytic domains of BoNT/A.

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. 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.

$$L^{165}\text{-}D\text{-}M\text{-}G\text{-}N\text{-}E^{170}\text{-}I\text{-}D\text{-}T\text{-}Q\text{-}N^{175}\text{-}R\text{-}Q\text{-}I\text{-}D\text{-}R^{180}\text{-}I\text{-}M\text{-}E\text{-}K\text{-}A^{185}\text{-}D\text{-}S\text{-}N\text{-}K\text{-}T^{190}\text{-}R\text{-}I\text{-}D\text{-}E\text{-}A^{195}\text{-}N\text{-}Q\text{-}R\text{-}A\text{-}T^{200}\text{-}K\text{-}M\text{-}L\text{-}G\text{-}S^{205}\text{-}G$$

SNAPtide® is used as the substrate in these studies. It is based on the 13-amino acid sequence shown above (**red**) 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.

SV2c contains a region known as the luminal domain loop between transmembrane domains 7 and 8, and has been shown to be the location of botulinum neurotoxin type A (BoNT/A) binding. Previously we have demonstrated the functionality of the GST-SV2c fusion protein as a receptor for BoNT/A[1,2,3].

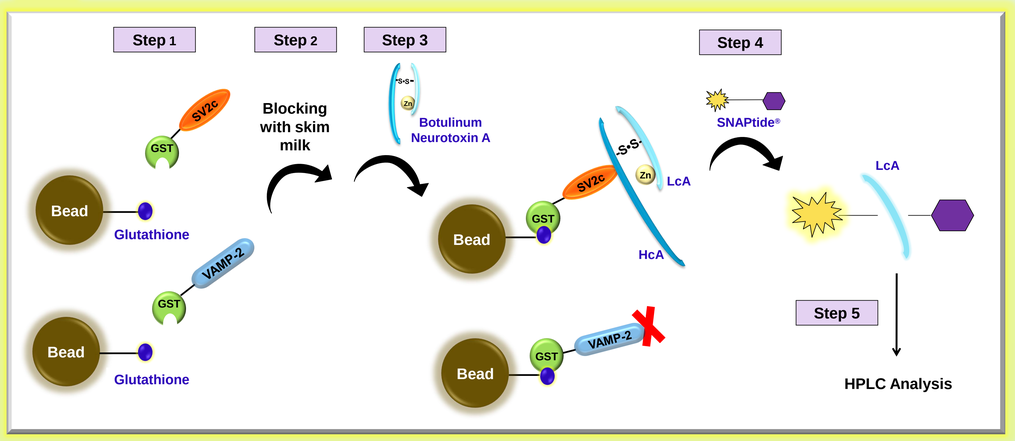
In this assay, the GST fusion protein containing the luminal loop of SV2c is used to specifically capture BoNT/A and the GST fusion protein containing Synaptobrevin-2 is used as the control. Synaptobrevin-2 (VAMP2) is the target protein cleaved by BoNT/B, D, F and G.

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 SV2c is analyzed.

MATERIALS and METHODS

SNAPtide® peptide substrate (Product #520), Unquenched calibration peptide for SNAPtide® 520 (Product #529), Botulinum neurotoxin type A (Product #130A), GST-SV2c luminal domain (Product #690A), GST-Synaptobrevin 2 Protein Substrate (Product #510A) are products of List Biological Laboratories, Inc. MagneGST™ beads are from Promega.

Figure 1



Assay Experimental Design:

A schematic representation of the assay setup is shown above (**Figure 1**). The assay consists of five steps.

Step 1: Coating magnetic beads with protein.
GST-SV2c and GST VAMP-2 protein (62.5 µg) were mixed with 100 µl of immobilized glutathione paramagnetic particles (MagneGST™) in 20 mM HEPES, pH 8.0, 5 mM dithiothreitol (DTT) and incubated for 30 minutes at room temperature with mixing.

Step 2: Blocking the GST-protein coated magnetic beads from any non-specific interaction.
Coated beads were incubated in 1 ml of 10% skim milk for 2 hours at room temperature. The beads were washed and divided into 10 tubes.

Step 3: Binding of BoNT/A to GST-protein coated magnetic beads.
The **BoNT/A** was prepared as a 50 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, 0.1% TWEEN-20. GST-protein coated magnetic beads were exposed to a series of BoNT/A concentrations in buffer and incubated for 1 hour at room temperature.

Step 4: Cleavage of SNAPtide® by BoNT/A.
Stock solutions (5 mM) of the **SNAPtide®** (Product #520) FRET peptide were made in dimethyl sulfoxide (DMSO). The SNAPtide® was diluted in 20 mM HEPES, pH 8.0 containing 1.0 mM $ZnCl_2$, 5 mM (DTT), and 0.1% Tween-20 (reaction buffer). Twenty micromolar SNAPtide® in reaction buffer was added to the BoNT/A bound coated magnetic beads. BoNT/A cleaves SNAPtide® between the fluorophore and the quencher, thereby releasing the fluorophore and restoring full fluorescence. After overnight digestion, the reaction mixture was separated from the beads, 0.2% TFA was added to stop the reaction, and samples were analyzed by HPLC.

Step 5: HPLC analysis of cleaved SNAPtide®.
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: 15% B for 3 min, 15-100% B in 3 min, 100% B for 3 min, and 6 min equilibration with 15% 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 20 µl.

RESULTS

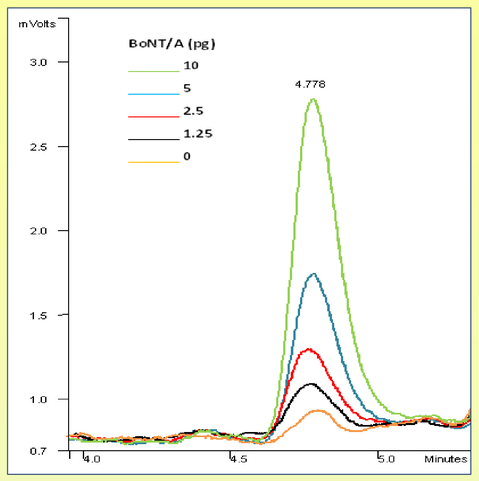


Figure 2. HPLC chromatograms of SNAPtide® 520 cleaved by SV2c captured BoNT/A. After digestion, the o-Abz-containing N-terminal fragment was visible at 4.7 min. As low as 1.25 pg BoNT/A was easily detected after overnight digestion.

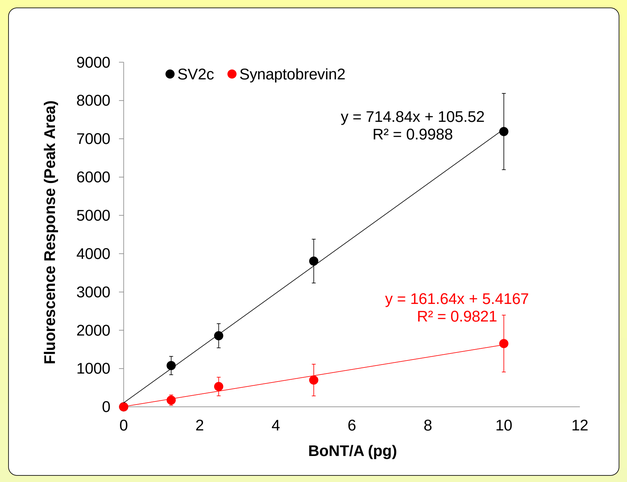


Figure 3. Plot of the peak area corresponding to the amount of cleaved SNAPtide® 520 substrate as a function of SV2c-captured BoNT/A (pg). The fluorescence response of cleaved SNAPtide® generated from SV2c captured BoNT/A (**black**). is linearly poportional to the toxin. Cleaved SNAPtide® generated from the Synaptobrevin-2 captured BoNT/A control is shown in **red**. The fluorescence response to BoNT/A is minimal, as expected. The data is the result of six replicates. These data demonstrate sensitive and specific detection of 1.25 pg SV2c-captured BoNT/A.

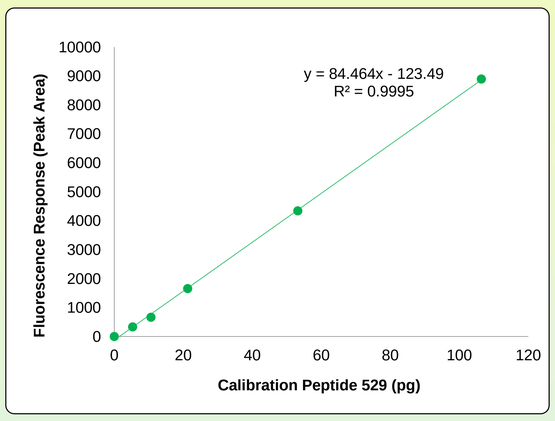


Figure 4. Calibration curve for SNAPtide® 520. Plot of the fluorescence response (peak area) versus amount of calibration peptide 529 (pg). Calibration peptide 529 contains the N-terminally-linked fluorophore, o-aminobenzoic acid (o-Abz). The peptide is used to generate a calibration curve to convert fluorescence response (peak area) of cleaved SNAPtide® 520 to pmoles and pg (Table.1).

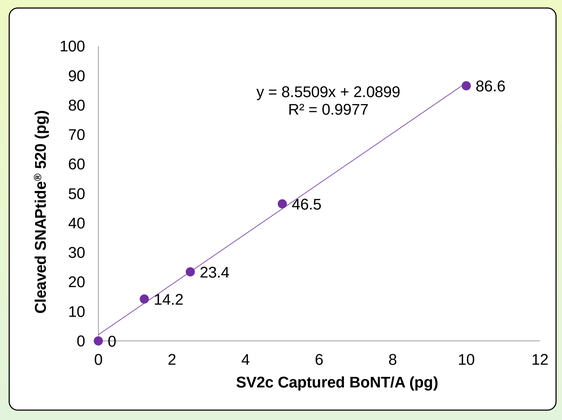


Figure 5. Plot of cleaved SNAPtide® 520 (pg) quantitated from the calibration curve shown in Figure 4 versus the amount of BoNT/A (pg) added in the reaction.

Table 1. Amount of cleaved SNAPtide® 520 detected in pmoles and pg after overnight exposure to various amount of SV2c-captured BoNT/A (pg). The calibration curve shown in Figure 4 was used to convert the data which was obtained from six replicate assays .

SV2c-captured BoNT/A (pg)	Cleaved SNAPtide® 520			
	Response (Peak Area)	% CV	(pmoles)	(pg)
0	0	0	0	0
1.25	1053	23	0.16	14.2
2.5	1804	18	0.27	23.4
5	3670	16	0.53	46.5
10	7055	14	1.00	86.6

CONCLUSIONS

- This assay has been optimized to allow highly sensitive detection of BoNT/A captured, on the native receptor, SV2c, and detected by BoNT/A specific enzymatic activity using as little as 1.25 pg of BoNT/A. This is significantly less than one mouse LD_{50} (5 pg).
- This bifunctional assay of BoNT/A toxicity can be used to evaluate both the integrity of the heavy chain SV2c protein receptor site as well as the enzymatic activity associated with the BoNT/A light chain.
- The data in **Figure 3** demonstrate that GST-synaptobrevin is ideally suited to measure residual non-specific binding of BoNT/A. The results indicate that, following the assay protocol, the contribution from non-specific binding is minimal.

REFERENCES

1. T. Christian and N. Shine. Capture Assay for Botulinum Neurotoxin Type A Utilizing the Neuronal Receptor Protein SV2c. The 6th International Conference on Basic and Therapeutic Aspects of Botulinum and Tetanus Toxins. June 2008 in Baveno, Italy.
2. T. Christian, A. Rummel, and N. Shine. Functional Assay for Botulinum Neurotoxin Type A Utilizing the Neuronal Receptor Protein SV2c. The 45th Annual Interagency Botulinum Research Coordinating Committee Meeting. September 2008 in Philadelphia, PA.
3. T. Christian and N. Shine. Sensitive, *In Vitro*, Bifunctional, Potency Assay For Botulinum Neurotoxin Type A. The 46th Annual Interagency Botulinum Research Coordinating Committee Meeting. October 2009 in Alexandria, VA.