Ultra Sensitive HPLC Detection Assay for Botulinum Neurotoxin Type A

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There are three steps in the interruption of synaptic transmission by botulinum toxin.

- The first is binding of the toxin to neuronal cells via a specific receptor protein and ganglioside. For Type A Botulinum toxin, it has been shown that SV2c is the protein receptor (Mahrold et al. FEBS Letters. 2006 April 3, 580(8):2011-4).
- The second is translocation of the enzymatic light chain out of the endosome.
- The final step is cleavage of synaptosomal proteins to inhibit neurotransmitter release. For Type A Botulinum toxin, this target protein is SNAP-25.

With this assay we can monitor two of the three steps of toxin activity, binding and cleavage (**FIGURE 1**). Using the luminal domain loop of SV2c, we have shown through GST pull-down experiments that we can detect specific binding of BoNT/A to SV2c using an immunoassay (**FIGURE 2**).

Upon specific binding of BoNT/A to its receptor protein, we can introduce our FRET peptide SNAPtide®, (Mca/Dnp) which is based on SNAP-25, and detect enzymatic cleavage using HPLC (**FIGURE 3**).

FIGURE 1: Capture Method and Detection.
Schematic showing the use of SV2c in the binding and detection of Botulinum Neurotoxin Type A. MagneGST beads were mixed with GST-SV2c and incubated for 1 hour, washed and reconstituted in 50mM HEPES, pH 8 buffer. Fifty microliters of the beads were used in each reaction. Various concentrations of Botulinum Neurotoxin Type A were added to each tube. Toxin was bound at room temperature for 1-4 hours with mixing. Unbound toxin was washed away and 400µl of SNAPtide® reaction buffer (50 mM HEPES, pH 8, 5 mM DTT, 1 mM $ZnCl_2$, 0.1% BSA) and 20µM SNAPtide®, 7-methoxy-coumarin-4-yl acetyl (Mca) / dinitrophenol (Dnp) was added to each tube. The reaction tubes were mixed overnight at room temperature. Reaction mixtures were filtered using a Microcon spin column with a 10,000 MWCO. The filter was rinsed with 0.1% TFA to inactivate residual toxin. The filtered reaction mixtures were then analyzed using HPLC.

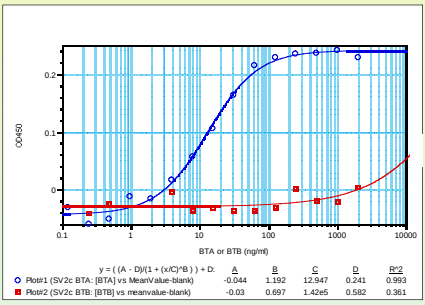

FIGURE 2: "Sandwich" ELISA assay showing specificity of BTA for binding to GST-SV2c.
Microtiter plates (96 wells) were coated with 2 µg of GST human SV2c in 1x carbonate buffer overnight at 4ºC. Plates were blocked with 1% BSA. Botulinum Neurotoxin Type A or B was applied to the wells and incubated for 1 hr at 37ºC. Toxin was detected using one of the following pair of antibodies: horse anti BoNT/A / anti horse HRP or rabbit anti BoNT/B / anti rabbit HRP. The assay was developed using One Step Turbo ELISA TMB from Pierce. These data indicate that the binding to SV2c is specific for Type A neurotoxin. The 50% binding point occurs at approximately 13 ng/ml BoNT/A.

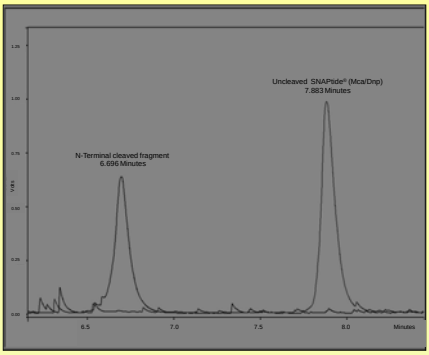

FIGURE 3: HPLC Analysis.
HPLC was performed using a ProSWIFT® RP-1S monolithic reverse phase column, 4.6 x 50 mm (Dionex, Sunnyvale, CA) attached to a Varian HPLC system. Solvent A was 0.1%TFA and solvent B was 100% acetonitrile containing 0.1% TFA. A linear gradient from 0 to 100% B in 10 minutes with a flow of 1 ml/min was used. The column effluent was monitored using a Hitachi model L-2485 Fluorescence detector with excitation at 325 nm and emission at 390 nm.

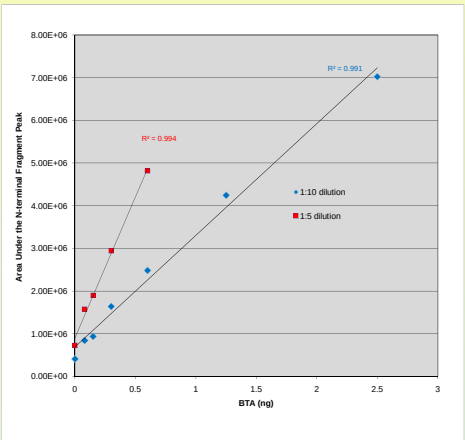

FIGURE 4: Preliminary HPLC Data.
Graph of the fluorescence intensity obtained for cleaved SNAPtide® (Mca/Dnp) as a function of the amount of Botulinum Neurotoxin Type A (BTA). A 1:10 and a 1:5 dilution of the reaction mixture for each BTA concentraton was analyzed via HPLC. Preliminary data indicate that samples containing as low as 80 pg of BTA can be easily detected.

SYNTAXtide™, FRET Substrate for Botulinum Toxin Type C

Botulinum neurotoxin Type C causes botulism in a number of wild animals and birds. The 150 kDa neurotoxin is composed of a 100 kDa heavy chain, and an enzymatically active zinc-dependent light chain (50 kDa).
A new FRET substrate, SYNTAXtide™, has been designed to measure the endoprotease activity of the 50 kDa light chain (LcC) of the type C neurotoxin. The peptide sequence is based on the native substrate, syntaxin, and contains the FRET pair o-Abz/Dnp (**FIGURE 1**). Using this FRET pair, the fluorescence of the o-Abz is initially quenched by the Dnp chromophore. When the SYNTAXtide™ is cleaved, full fluorescence is restored. SYNTAXtide™ was evaluated on the basis of the increase in relative fluorescence units (RFU) observed after addition of LcC. Tests to estimate sensitivity with LcC were also performed (**FIGURE 2**).

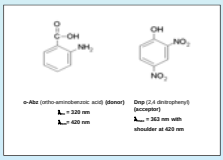

FIGURE 1: The FRET pair used in the Syntaxtide™ peptide.

Fluorimetric assay: Continuous assays were performed on a SPECTRAmax GEMINI XS fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) using Greiner FLUO-TRAC black flat-bottomed, non-binding plates (E&K Scientific, Campbell, CA). Plates containing serial dilutions of LcC were equilibrated for 15 min at 37ºC prior to addition of 10 µM SYNTAXtide™. The time-dependent increase in fluorescence intensity was monitored at 37ºC. The excitation and emission wavelengths were set to 321 nm and 418 nm, respectively.

The hydrolysis of SYNTAXtide™ was evaluated as a function of LcC concentration in order to determine the minimum amount of toxin detectable using this FRET substrate. Preliminary studies suggest that the o-Abz/Dnp FRET substrate can detect ~ 0.078 nM LcC or 4 ng/ml after 3 hrs of hydrolysis at 37ºC and ~ 0.039 nM LcC or 2 ng/ml after 24 hrs of hydrolysis at 37ºC (**FIGURE 2**).

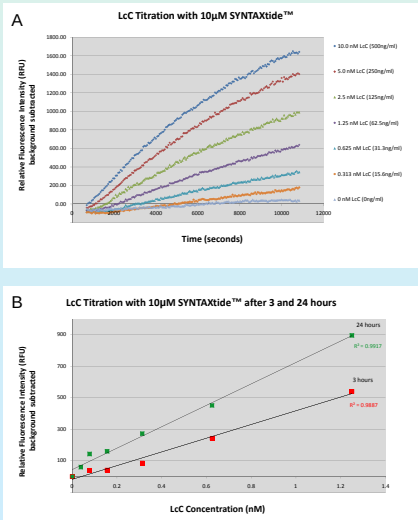

FIGURE 2: LcC Titration with 10 µM SYNTAXtide™.
FRET assay with SYNTAXtide™ was performed in 20mM HEPES pH 6.8, 0.05% TWEEN-20, at 37°C **(FIGURE 2A)**. A plot of 3 hour and 24 hour endpoint fluorescence intensities versus LcC concentrations demonstrates that the SYNTAXtide™ cleavage reaction is linearly proportional to the LcC concentration **(FIGURE 2B).**