SENSITIVE DETECTION OF BOTULINUM NEUROTOXIN TYPE A USING HIGH AFFINITY POLYCLONAL ANTIBODIES

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ABSTRACT

Purpose of Study: The purpose of this study was to determine if a polyclonal antibody directed towards the heavy chain of botulinum toxin type A (BoNT/A) could be used to capture BoNT/A from complex matrices.

Methods Used: Magnetic beads coated with a polyclonal antibody specific for BoNT/A heavy chain, List Prod #730, were used to capture and concentrate the toxin prior to addition of the substrate, FRET peptide SNAPtide®, List Prod #520. Various buffer conditions were tested to optimize the capture of toxin by the antibody. Generation of cleaved peptide was monitored using reverse phase HPLC with a fluorescence detector.

Summary of Results: Initial studies indicated that as low as 1 pg of BoNT/A can be captured and detected using SNAPtide® in an overnight digestion at room temperature. Optimization of this assay is currently in progress.

Conclusions: This highly sensitive assay captures and detects as low as 1 pg of BoNT/A which is significantly lower than one mouse LD_{50} (5 pg) and is in the equivalent range that can be detected by 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.
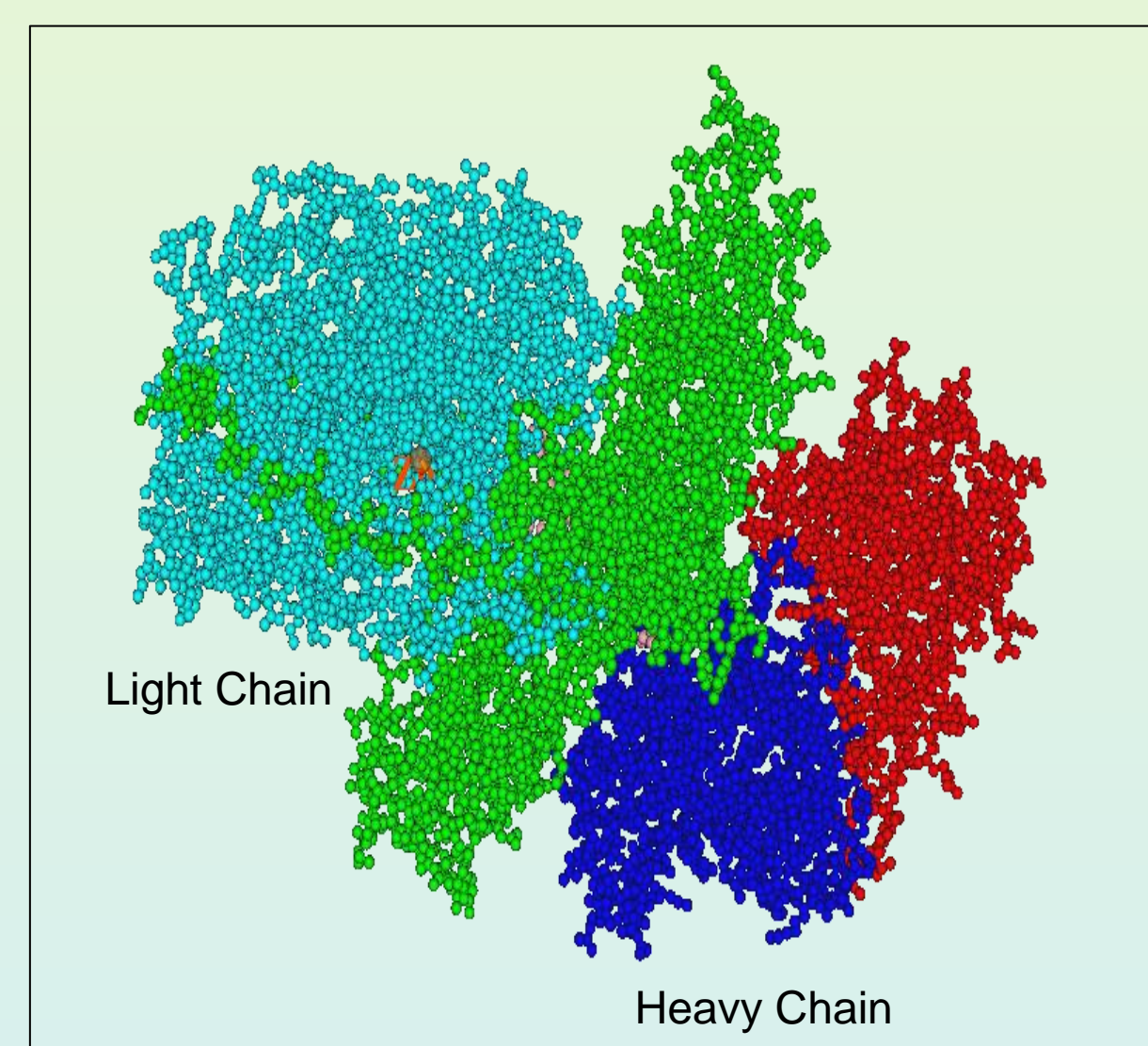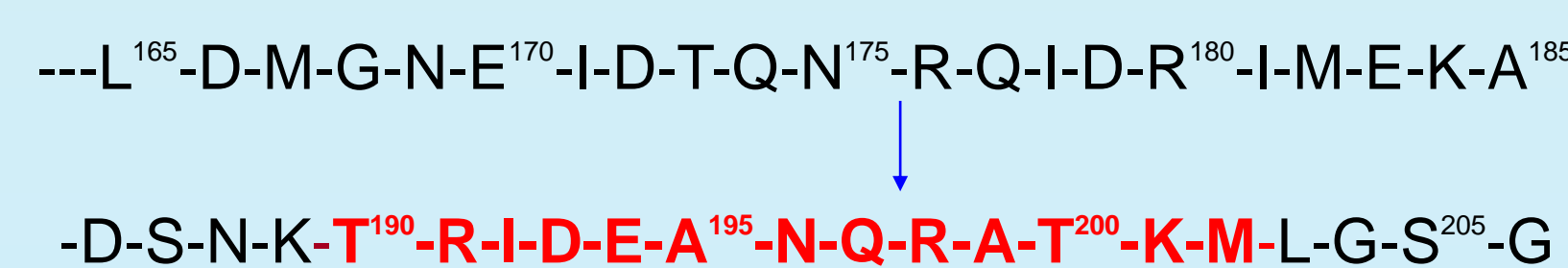

Figure 1: Crystal structure of botulinum neurotoxin type A. Lacy, DB *et al.*, *Nat. Struct. Biol.* 1998, 10:898-902.

The zinc dependent N-terminal light chain is the catalytic subunit which selectively cleaves a SNARE membrane fusion protein. 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. The N-terminally attached fluorophore, o-Abz is quenched by the C-terminally attached chromophore Dnp group. Cleavage of the substrate by BoNT/A releases the fluorophore and full fluorescence is restored.

High affinity **chicken IgY polyclonal anti-BoNT/A antibodies** attached to magnetic beads were used in this analysis to capture and concentrate the BoNT/A. The antibodies were directed toward the heavy chain binding domain of BoNT/A.

The study presented here demonstrates significant increase in the level of detection using HPLC in combination with fluorescence detection. The peak from the fluorescently labeled N-terminal fragment cleaved by the antibody-captured BoNT/A is analyzed. Results obtained in buffer and in complex matrices are presented.

MATERIALS and METHODS

SNAPtide® substrate (Product #520), Botulinum neurotoxin type A (Product #130A), and the chicken IgY polyclonal anti-BoNT/A antibody (Product #730A) are products of List Biological Laboratories, Inc. Skim milk is from Lucerne, pasteurized carrot juice is from Odwalla, and Fetal Bovine Serum (FBS) is from Sigma. The immuno-magnetic beads were made using MyOne™ tosylactivated beads (Invitrogen, Carlsbad, CA). The MyOne™ beads are 1.08 μm in diameter, containing 8 m^2/g of surface area and 1 x 10^{12} beads/ml.

Sample preparation: Stock solutions (2.5 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 50 ng/μl solution in 20 mM HEPES, pH 8.0 containing 0.2% Tween-20. Appropriate dilutions were made in several buffers and in complex matrices. Details are given in **Table 1**.

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 Technologies, Santa Clara, CA) attached to a Varian ProStar HPLC system (Agilent Technologies, Santa Clara, 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 polyclonal Antibody-coated beads.
The immuno-magnetic beads were made by covalently attaching 50 μg of anti-BoNT/A antibodies to 10 μl of magnetic beads according to manufacturer's instructions.

Step 2: Binding of BoNT/A to the anti-BoNT/A antibody-coated beads.
Polyclonal antibody coated beads were exposed to a series of BoNT/A concentrations in buffer and complex matrices, and incubated while rotating gently at room temperature for 2 hours.

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 at room temperature, 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
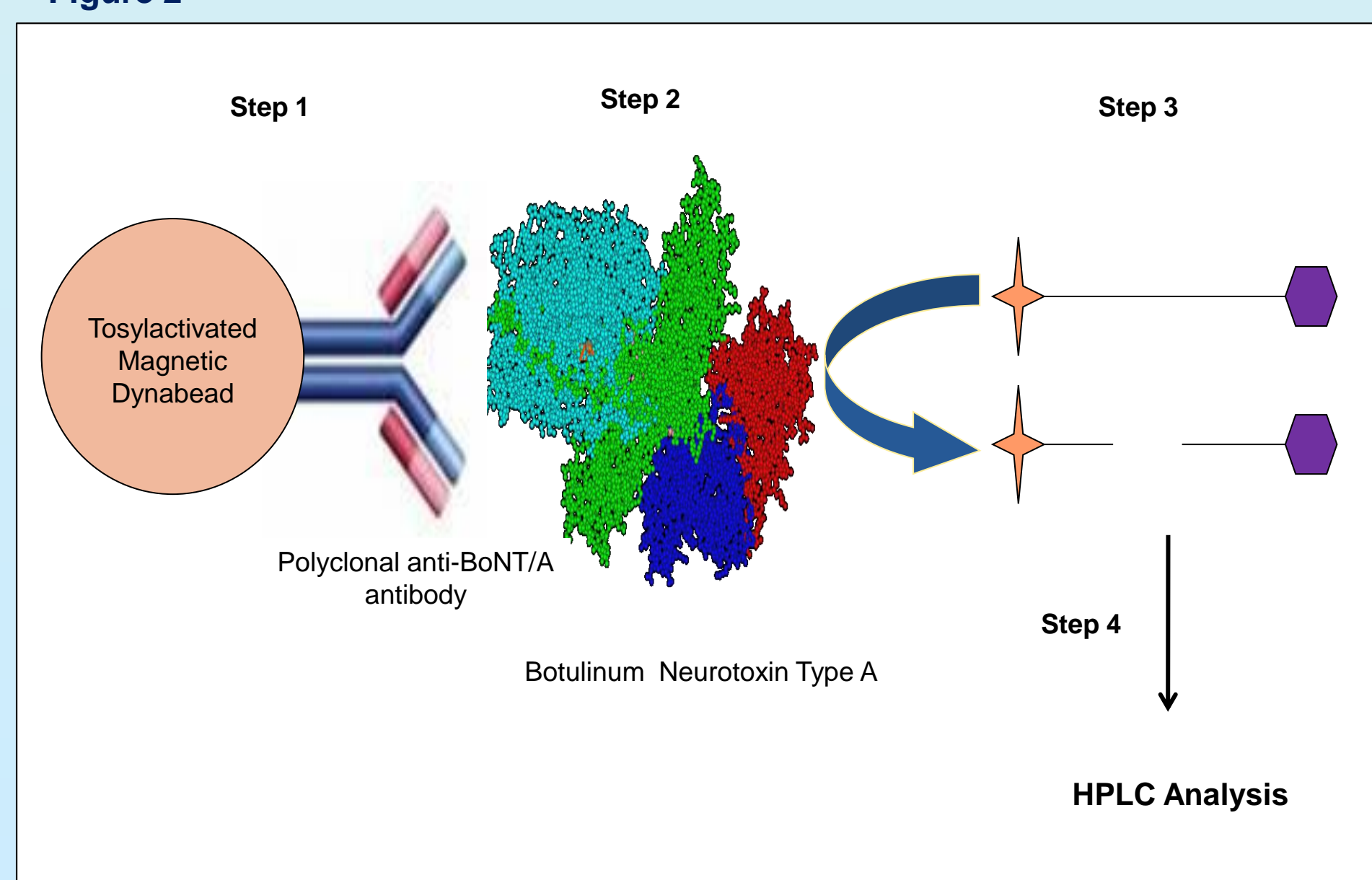
RESULTS

BoNT/A capture by the polyclonal antibody.

In the initial studies, capture of a series of BoNT/A concentrations by anti-BoNT/A antibody-coated beads was tested in 20mM Hepes, pH 8.0 buffer and skim milk. Signals for reaction in skim milk were stronger than for identical concentrations of BoNT/A in 20mM Hepes, pH 8.0 (**Figure 5**). The higher response in skim milk implied a greater capture of BoNT/A by the antibody. A series of buffers were tested to mimic the pH and other characteristics of skim milk. BoNT/A concentration of 5 pg/ml was used to perform the experiments. Of all the buffers tested (**Table 1.A**), the 20mM Hepes, pH 6.8 buffer gives the best signal (**Figure 3.A**), and also a stronger signal compared to skim milk (**Figure 3.A and B**). Components of skim milk, such as lactose, casein, and calcium were tested to optimize the toxin-antibody binding. The signals observed were lower compared to skim milk (data not shown).

Capture of BoNT/A by anti-BoNT/A antibody-coated beads was also tested in complex matrices (**Figure 3.B**). BoNT/A concentration of 5 pg/ml was easily detected in all of these complex matrices, with strongest intensity in 10% FBS, followed by 100%, 50% carrot juice, and skim milk.

Table 1: List of test buffers (A) and complex matrices (B).

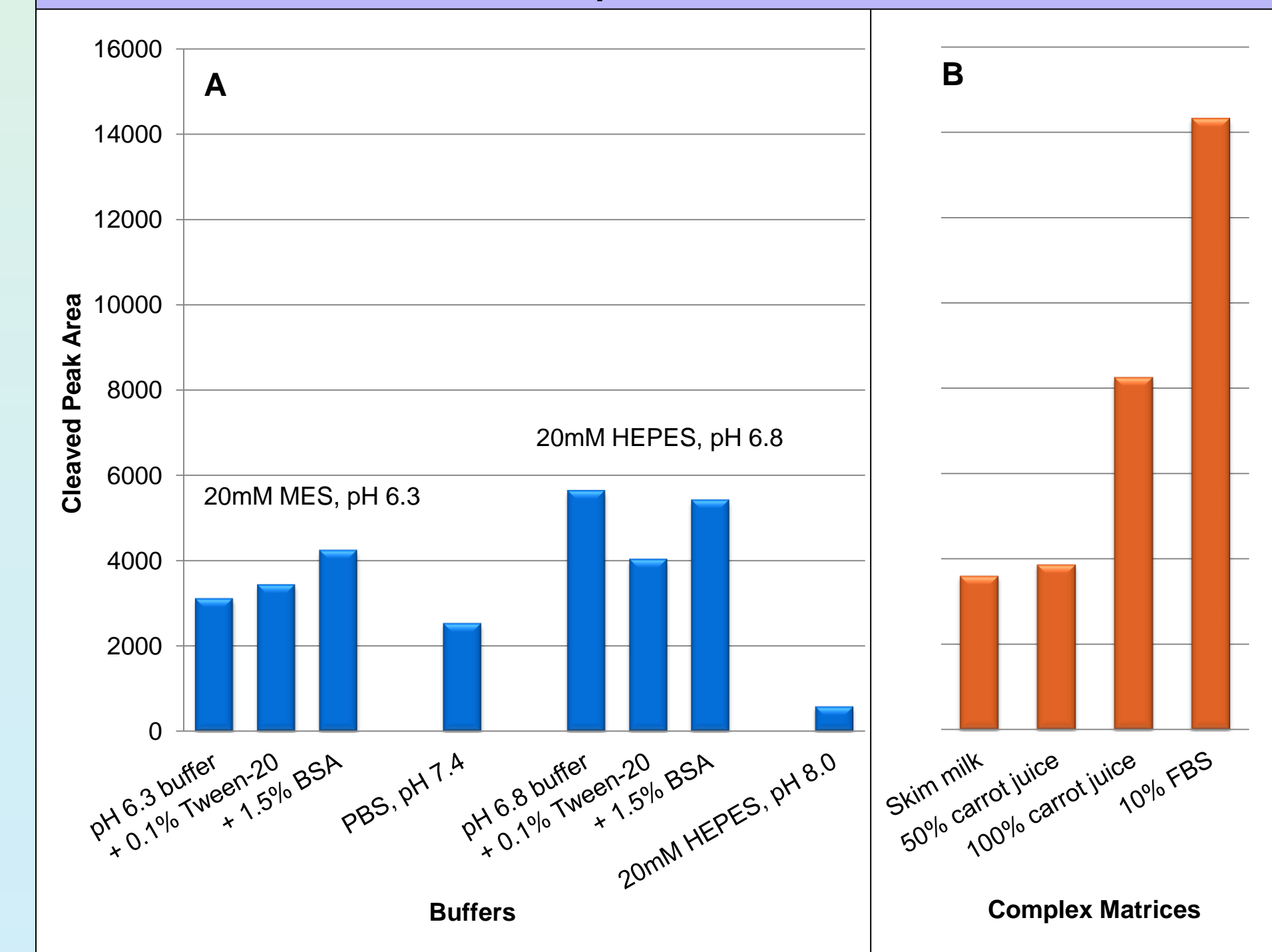| Buffers (A)	
1	20 mM MES, pH 6.3
2	20 mM MES, pH 6.3 + 0.1% Tween-20
3	20 mM MES, pH 6.3 + 1.5% BSA
4	20 mM Hepes, pH 6.8
5	20 mM Hepes, pH 6.8 + 0.1% Tween-20
6	20 mM Hepes, pH 6.8 + 1.5% BSA
7	1 x PBS, pH 7.4
8	20 mM Hepes, pH 8.0
Complex Matrices (B)	
1	Skim milk
2	10% FBS in 20 mM Hepes, pH 6.8
3	50% carrot juice in 20 mM Hepes, pH 6.8
4	100% carrot juice

Figure 3: BoNT/A (5 pg/ml) captured by anti-BoNT/A antibodies in the presence of test buffers (A) and complex matrices (B).

Chromatograms obtained in the absence and presence of a series of BoNT/A concentrations are shown in **Figure 4**. The amount of cleaved SNAPtide® substrate, as reflected in the area of the HPLC peak at 10.5 min, is shown as a function of BoNT/A concentration (pg/ml). The assay allows detection of as little as 0.625 pg/ml and 2.5 pg/ml BoNT/A in 20 mM Hepes, pH 6.8 and skim milk, respectively, see **Figures 4** and **5**.
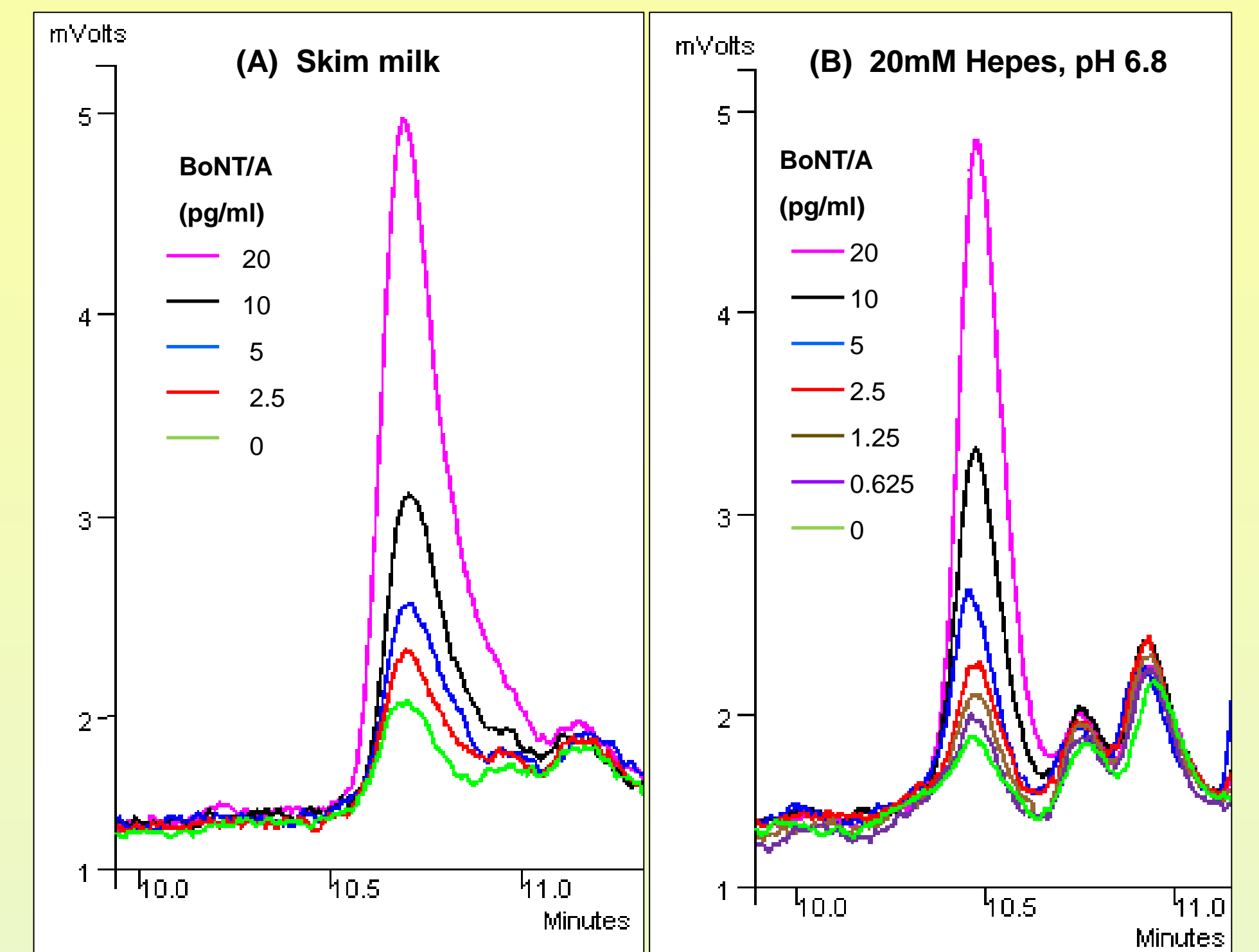

Figure 4: BoNT/A captured by anti-BoNT/A antibodies in the presence of skim milk (A) and 20mM Hepes, pH 6.8 buffer (B). The peak area observed for the cleaved o-Abz containing fragment is plotted as a function of BoNT/A concentration after overnight digestion at room temperature.
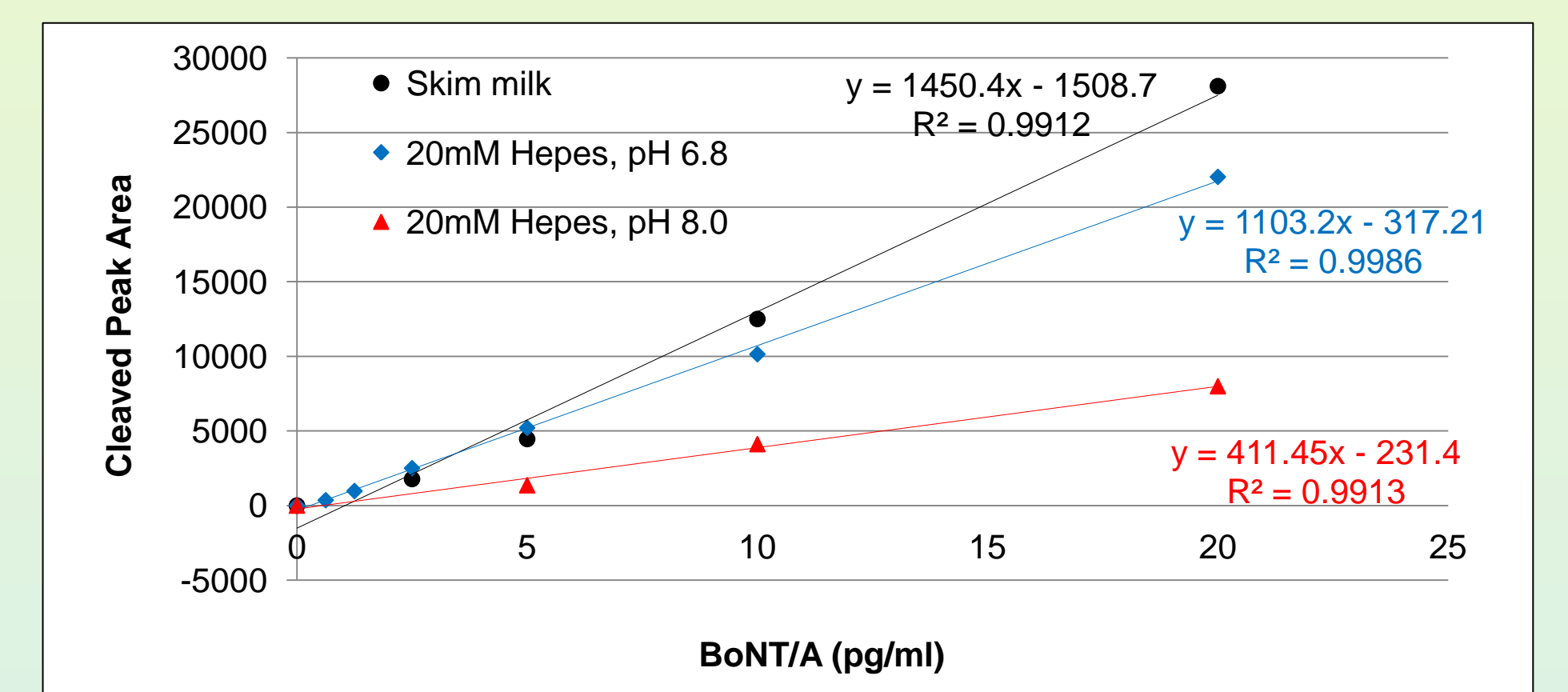

Figure 5. Dose-response curves. The response is proportional to the BoNT/A dose in 20mM Hepes, pH 6.8 and 8.0 buffer, and skim milk.

CONCLUSIONS

A. Sensitivity

Polyclonal antibody directed towards the heavy chain of botulinum toxin type A (BoNT/A) can be used to capture BoNT/A from complex matrices. The amount of BoNT/A captured is measured by monitoring the cleavage of the specific BoNT/A substrate, SNAPtide® #520. Using the anti-BoNT/A antibodies from List Biological Laboratories, Product #730A, as low as 0.625 pg/ml of BoNT/A in 20mM Hepes, pH 6.8 and 2.5 pg/ml BoNT/A in undiluted skim milk is detected. For other complex matrices, such as carrot juice and fetal bovine serum (FBS), 5 pg/ml of BoNT/A is easily detected.

B. Dose dependent response

Using the anti-BoNT/A antibodies to capture the BoNT/A, the dose-response relationship is linear in the dose range studied. The response is proportional to the dose, which starts at 0.625 pg/ml of BoNT/A in 20 mM Hepes, pH 6.8 buffer and 5 pg/ml of BoNT/A in undiluted skim milk.

FUTURE DIRECTIONS

A. 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.

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