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Toxin inhibition – deconvolution strategies and assay screening of combinatorial peptide libraries

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Abstract

Combinatorial peptide libraries offer an expedient source of structurally diverse molecules that could serve as lead compounds in the development of drug therapies to toxins. The libraries have typical structures of X1 – X2 – hinge – X3 – X4, where X1 through X4 are near-equimolar mixtures of twelve α-L-amino acids and hinge = γ-aminobutyric acid. Screening of the libraries for inhibitory activity in assays for botulinum neurotoxins A and B (BoNT/A, BoNT/B) and saxitoxin uncovered potent library subsets. For effective screening of the peptide libraries, improved methods of analysis were sought. We report on development of a capillary electrophoresis laser-induced fluorescence (CE LIF) method for measuring BoNT/A peptidase activity and for screening peptide libraries for inhibitory effects. A second analytical method for quantitation of BoNT/A assays was employed based on fluorescence resonance energy transfer (FRET). The FRET assay is homogeneous phase, i.e., no separation step is required. Thus assay time was reduced and throughput increased. The research described in this report was supported by the Technology Investment Fund of Defence R&D Canada.

Résumé

Les bibliothèques combinatoires de peptides offrent une source opportune de molécules structurellement diverses qui pourraient servir de premiers composés dans la mise au point de pharmacothérapies contre des toxines. Les bibliothèques ont des structures normales de X1 – X2 – charnière – X3 – X4, où X1 jusqu'à X4 sont des mélanges proches d'équimolaires de douze acides aminés α-L et charnière = acide aminobutyrique – γ. Le triage des bibliothèques à la recherche d'activité inhibitoire des neurotoxines botuliques A et B (BoNT/A, BoNT/B) et saxitoxine dans les biotests ont fait découvrir des sous-ensembles de bibliothèque puissants. On a cherché à améliorer les méthodes d'analyse pour être en mesure de trier les bibliothèques de peptides efficacement. On documente ici la mise au point de la méthode d'électrophorèse capillaire à fluorescence induite par laser (EC-FIL) utilisée pour mesurer l'activité peptidase BoNT/A et pour le triage des bibliothèques peptides pour les effets inhibitoires. On a employé une seconde méthode analytique de quantification de biotests BoNT/A, basée sur un transfert d'énergie de résonance fluorescent. Ce biotest est une phase homogène, l'étape de séparation par exemple, n'est pas requise. On a ainsi réduit la durée du biotest et augmenté sa capacité. La recherche décrite dans ce rapport est financée par le Fonds d'investissement technologique et R & D pour la défense Canada.

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Executive summary

Introduction: Effective medical countermeasures to toxins are deficient in military and civil defence. There are numerous toxins that have potential to be used in bioweapons. Current medical countermeasures such as vaccinations, antiserum or passive vaccines have limited efficacies. There are no drug prophylaxes or therapies available. This technical report describes research to provide new sources and identification methods for compounds that have inhibitory properties against three toxins: botulinum neurotoxin serotype A (BoNT/A), botulinum neurotoxin serotype B and saxitoxin. Other research in the report was directed toward improved analytical methods for screening compounds in BoNT/A bioassays. The research was supported through the Technology Investment Fund (TIF) of Defence R&D Canada. Portions of the work were conducted under PWGSC contracts W7702-00R835/001/EDM (Pepmetics Inc., Victoria, BC) and W7702-01R869/001/EDM (Canada West Biosciences, Camrose, AB).

Results: Analyses identified a number of compounds that showed strong inhibition in each of the bioassays. Significant improvements in analysis methods were achieved.

Significance: The research provided important insights for potentially new prophylaxes and therapies. The focus of the work was toward discovery of compounds that inhibited the damaging action of botulinum neurotoxin. There are at least two other precursor mechanisms where toxin inhibition could be directed that have not been previously investigated. The successful methods reported in this article have pointed the way to developing new broadly applicable drug discovery methods for a wide range of toxins.

Future Work: The work conducted in the TIF project raised and renewed interest in toxin research at DRDC Suffield. TIF provided the initial resources for the creation of a toxin research hub at DRDC Suffield. New toxin research areas have been initiated. A review of botulinum toxin research at DRDC Suffield is given by N. Chan et al., *Botulinum Neurotoxin Research and Development* [DRDC-Suffield TM 2006-233]. Future work will include studies and screening of new classes of compounds. New collaborative partners in university and pharma-industry have been engaged.

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Sommaire

Introduction: Les contremesures médicales efficaces sont déficientes dans les milieux militaires et civils. Il existe de nombreuses toxines qui ont le potentiel d'être utilisées comme armes biologiques. Les contre-mesures médicales actuelles telles que les vaccinations, antisérums et vaccins passifs sont d'une efficacité limitée. Il n'existe pas de thérapies ou de prophylaxies disponibles. Ce rapport technique décrit la recherche dans le but de procurer des nouvelles sources et méthodes d'identification de composés ayant des propriétés inhibitoires contre trois toxines : la neurotoxine botulique de sérotype A (BoNT/A), la neurotoxine botulique de sérotype B et saxitoxine. Un autre aspect de la recherche dans le rapport était dirigé vers les méthodes analytiques concernant le triage des composés dans les bio-essais. La recherche a été financée par le Fonds d'investissement technologique (FIT) et R & D pour la défense Canada. Des portions de ces travaux on été conduits en vertu de contrats avec TPSGC, W7702-00R835/001/EDM (Pepmetics Inc., Victoria, BC) et W7702-01R869/001/EDM (Canada West Biosciences, Camrose, AB).

Résultats: Les analyses ont identifié un certain nombre de composés indiquant une forte inhibition dans chacun des bio-essais. On a accompli des améliorations importantes au niveau des méthodes d'analyse.

Portée des résultats: La recherche procure une meilleure connaissance des nouvelles prophylaxies et thérapies potentielles. Les travaux étaient axés sur la découverte des composants inhibant l'action destructrice de la neurotoxine botulique. Il existe au moins deux autres mécanismes précurseurs qui n'ont pas encore été examinés vers lesquels on pourrait diriger la recherche sur l'inhibition de la toxine. Les méthodes efficaces documentées dans cet article pointent vers la mise au point de nouvelles méthodes de découverte de médicaments facilement applicables à un large éventail de toxines.

Travaux futurs: Les travaux du projet FIT ont soulevé et renouvelé l'intérêt concernant la recherche dans le domaine des toxines à RDDC Suffield. Le FIT a procuré les ressources initiales pour la création d'un centre de discussion concernant la recherche sur les toxines à RDDC Suffield. Des nouveaux domaines de recherche sur les toxines ont été initiés. Une étude concernant la recherche sur la toxine botulique à RDDC Suffield est effectuée par N. Chan et al., *Botulinum Neurotoxin Research and Development* [RDDC-Suffield TM 2006-233]. Les travaux futurs incluront des études et triages de nouvelles classes de composés. On s'est engagé dans de nouveaux partenariats de collaboration avec l'industrie pharmaceutique.

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Introduction

Countermeasures against potential toxic biological weapons are deficiencies for military and civil defence. There are dozens of toxins that are sufficiently toxic to pose a threat should large-scale production be feasible [1]. Until recently, production of militarily significant quantities of toxins was a difficult task. However, advances in protein synthesis and biotechnology make large scale production possible and thus increase the threats posed by toxins. Immunization and antibody-based therapies have limited utility and efficacy for a select few toxins. Conventional drug discovery technologies are expensive and slow. Thus providing protective measures against a wide range of potential toxin weapons is essentially cost-prohibitive. Hence faster, more versatile and capable technologies for drug discovery are required. This report presents new methods for advancing and improving drug discovery to biological toxins.

Synthetic combinatorial libraries offer sources for lead compounds that can be used as potential novel antitoxins or therapies. Traditional chemical libraries can contain upward of 10^8 compounds and require large resources to produce and screen effectively in order to discover useful leads. Peptide libraries can provide a more expedient approach to synthesis, screening and discovery owing to the large chemical content arising from not only molecular diversity but also conformational diversity of the peptide structure [2-4]. The conformational diversity of the peptide libraries can be further augmented by the presence of a centrally located flexible hinge, designed to disrupt the ordered peptide structure. A hinge allows a greater variety of geometries within the construct; it maximizes the potential for the constituent amino acid side chains to interact with the target-receptor sites and to interact with each other to form a cluster recognition motif [5,6]. Hinge peptide libraries have been shown to inhibit in vitro protease assays [7] and have provided a route to the development of lead compounds for breast cancer and multiple sclerosis [8].

Hinge peptide libraries herein consisted of three or four amino acids mounted around a flexible central linker, with capped or uncapped ends. End-capped hinge tetrapeptide libraries in this work have the structure:

$$\text{Acetyl - X1 – X2 – hinge –X3 – X4 –NH}_2$$

where X = one of twelve α-L-amino acid (R, C, Q, E, H, L, K, M, F, S, W, Y) and hinge = a suitable linker that disrupts potential secondary structures in the peptide backbone. The acetyl and NH_2 caps were located at the N- and C-termini, respectively. The hinge linker employed was γ-aminobutyric acid (also known as GABA). GABA is a non-standard amino acid and also functions in nature as neurotransmitter. The amino function on GABA is two carbon-chain units removed from the carbonyl group. Hence there is disruption of the peptide backbone secondary structure. Due to functional group redundancy in mammalian amino acid side chains (e.g. D/E, N/Q, T/S), and the structural rather than functional role of certain amino acids (G, P, A, I, V), it is possible to restrict the amino acid components to twelve without compromising the array of chemico-biological motifs in the library. The restricted library, which is limited to just 20,736 (12^4) hinge peptides, greatly facilitates deconvolution, i.e., the process of identifying the optimal peptide or "best hit". An efficient deconvolution process

termed "positional scanning synthetic combinatorial library" [9] was employed in conjunction with the restricted libraries. The method synthesizes and then screens solution-based peptide mixtures in which sub-libraries of peptides have an amino acid fixed at one designated position while randomized in other positions. In the first part of this study end-capped tetrapeptide libraries were screened in inhibitory assays. In the second part, we dispensed with the capping groups and limited the size of the hinge peptide library even further by reducing the number of variable amino acid positions from four to three:

$$X1 - \text{hinge} - X2 - X3$$

where X and hinge remained as defined previously. The complete library, comprising the 12 functionally essential amino acids outlined above, contained only 1,728 (12^3) individual hinge peptides, again reducing deconvolution. The effectiveness of hinge peptide libraries derives not from the number of compounds in the library, but from the large amount of conformational sampling that occurs for each ligand. Another relevant feature of hinge peptide libraries is that they utilize the very same functional groups, loaded on to a flexible template, that form the actual recognition elements that occur in nature.

Three toxins for which no known drug therapies exist were selected for in vitro studies with hinge peptide libraries as inhibitors. Botulinum neurotoxins (BoNTs), produced by several strains of *Clostridium botulinum*, are zinc proteases that cleave and disable proteins of the neuronal exocytosis apparatus, thereby preventing neurotransmitter release which in turn causes respiratory paralysis and death. BoNT serotype A (BoNT/A) cleaves protein SNAP-25 specifically at Q_{197}-R_{198} [10], and BoNT/B cleaves protein VAMP specifically at Q_{76}-F_{77} [11]. The crystal structures of BoNT/A [10] and BoNT/B have been reported and, in the case of the latter, the substrate-binding site has been characterized [11]. Investigations by others have resulted in a peptide with sub-micromolar affinity for BoNT/A [12], and a high throughput screening assay has been developed for BoNT/B [13]. Each molecule of BoNT/A or B contains a heavy and a light chain held together by a disulfide linkage. The heavy chain is responsible for recognition on the cell surface and insertion through the cell membrane. The light chain (LC), once inserted into the cell and cleaved from the heavy chain by disulfide reduction, then possesses proteolytic activity. The synthetic peptide substrates for in vitro assays of BoNT/A and /B, discussed herein, contained the same amino acid sequences flanking the scissile peptide bonds as their respective protein targets. In our work hinge peptide libraries were added to the LC assays of BoNT/A or /B and screened for modulation of the substrate cleavage activity determined by high performance liquid chromatography (HPLC).

Saxitoxin, the substance responsible for paralytic shellfish poisoning, is produced by "red-tide" marine algae. It is a small nonpeptide molecule (299 Da) that binds to sodium channels in cell membranes preventing sodium ions from entering the cell, thus blocking nerve conduction and causing death by paralyzing muscles of respiration [14]. A sensitive and convenient cell-based in vitro assay for saxitoxin (and inhibitors thereof) invokes the use of the toxin itself plus two pharmacological co-agents. The assay developed for saxitoxin uses veratridine to induce sodium influx and ouabain to prevent simultaneously sodium efflux in mouse neuroblastoma cells. The net effect is cell death due to intracellular hyperosmolarity [15]. However the veratridine/ouabain-induced cell mortality can be rescued by the presence of low doses of saxitoxin that block the sodium influx. The rescue effects of saxitoxin can, in

turn, be reversed by a saxitoxin inhibitor. In this work, saxitoxin inhibition was provided by the hinge peptide libraries. The screening of the libraries was carried out by cell viability measurements on microplates.

The purpose of the work was to determine the effectiveness of hinge peptide libraries as toxin inhibitors. Since combinatorial chemistry can require vast resources to synthesize and to screen we focused our attention on methods that could identify lead compounds expediently. In order to expedite drug discovery, improved methods of analysis are required for in vitro toxin assays. Initially for screening the peptide libraries in proteolytic BoNT LC assays we used HPLC analysis which, although adequate, was less than satisfactory with regards to ease of separation and time required. To fully capitalize on the power of the combinatorial peptide libraries we sought analysis methods that were quicker, and capable of resolving enzyme assays that used substrates in their native states (opposed to substrates modified with spectroscopic labels). Furthermore we required analytical methods that would easily provide kinetic data rather than fixed time endpoint data (as usually the case in HPLC). To this end we investigated two methods for analysis, capillary electrophoresis with laser-induced fluorescence and fluorescence resonance energy transfer.

Capillary electrophoresis (CE) is a powerful separation method for bioanalysis, having high resolution, low sample requirement and short run times and has proven to be an effective technique for analysis of proteins and peptides [16, 17]. The principle of CE separation is the differential electrophoretic mobilities of the analytes in an electric field [18]. The mobilities are dependent on the charge-to-mass ratios of the molecules. The physico-chemical properties of the peptide substrate and products can be determined based on the Offord model for peptide electrophoretic mobility [19, 20] using the **Ex**pert **P**rotein **A**nalysis **Sy**stem (ExPASy) [21]. Detection methods in CE are predominantly UV absorption or fluorescence emission. High sensitivity of fluorescence-labeled biomolecules is achievable in the sub-picomolar range for CE using laser-induced fluorescence (LIF) [22, 23]. For CE LIF analysis of peptide substrates and products, two avenues were available: first, an end-labeled peptide whereby the only species detectable in LIF would be the intact substrate and the peptide fragment that carried the fluorescent label. This approach has been used to develop an assay for cleavable nucleic acid probes using CE LIF [24]. However, preliminary studies for this work suggested that the peptidase activity of the BoNT/A LC was sensitive to end-located substituents such as fluorescein dye. We also considered the possible effect that the label could have on competition between substrate and peptide libraries for the enzymatic site of the activated LC. We preferred substrate and products to be free of fluorescent label during the BoNT/A LC enzyme reaction. Hence we pursued a second approach that employed post-reaction labeling of substrate and products with the primary amine-reactive dye 3-(4-carboxy-benzoyl)-2-quinoline-carboxaldehyde (CBQCA). CBQCA is non-fluorescent until it forms a 7-aza-1-cyano-5,6-benzisoindole covalent adduct (maximum excitation and emission 450 and 550 nm, respectively) with a primary amine in the presence of cyanide ion. CE LIF analysis of amino acids and peptides labeled with CBQCA has been reported [22, 25, 26]. The same labeling reagent has been used in CE LIF for characterization of VAMP-thioredoxin fusion protein, developed as a potential substrate for BoNT/B [27]. A detailed CE-based study of BoNT/B LC activity using UV detection has been reported, although no results were given for quantitation in the presence of potential modulator compounds [28]. Here we describe a technique for measurement of peptidase activity by CE LIF that provides substantial improvements regarding time, resolution and quantitation compared to HPLC. When the

technique was applied to measurement of peptidase activity in the presence of the peptide libraries, modulating properties of the libraries could be observed.

An assay based on fluorescence resonance energy transfer (FRET) [29] was employed to measure the inhibitory properties of peptides and drug compounds towards BoNT/A LC. The assay used SNAPtide™, a synthetic peptide possessing the sequence and native cleavage site for BoNT/A [10, 30, 31]. The fluorophore/donor was located at the N-terminus; the quencher/acceptor was located on the C-terminus side of the cleavage site. The intact substrate does not fluoresce owing to the proximity of the donor and acceptor on the intact substrate. Cleavage of the substrate by BoNT/A releases the fluorophore (donor) from proximity to the quencher and fluorescence occurs. The increase in fluorescence intensity is directly proportional to the extent of cleavage that has occurred and thus allows for accurate determination of BoNT/A LC protease activity in the sample. The main advantage of the FRET method is that the assay is homogeneous phase, i.e., no separation step is required. Thus assay time is reduced and throughput increased. The fluorescence signal from the assay can be monitored continuously from $t = 0$ to the equilibrium state and thus Michaelis–Menten analysis of data and enzyme kinetics can be readily obtained. The SNAPtide assay can be used for highly sensitive and rapid in vitro methods for screening and characterizing toxin inhibitors, that are potential therapeutic agents.

The work presented in this report was initiated under funding from DRDC Technology Investment Fund (TIF) and carried out during 2001-2005. The TIF project was entitled "Drug Design of Peptide Mimetics". The research was conducted in-house at DRDC Suffield and through contract to University of Calgary, Canada West Biosciences Inc. (Camrose AB) and Pepmetics Inc. (Victoria BC). The project was successful on several levels. Original research was conducted on the inhibitory effects of combinatorial peptide libraries on in vitro toxin assays. The findings strongly suggest that hinge peptide libraries are a valuable source of inhibitory "lead compounds" for drug discovery. New methods of assay analysis were developed. Capillary electrophoresis combined with post-reaction labeling of substrate and products provided a significant advantage over previous chromatography methods. The FRET assays has the potential to be powerful high throughput kinetic method of analysis. The work assembled a multi-disciplinary team of protein chemists, biochemists, molecular biologist, computational chemists, analytical chemists and pharmacologists who have continued to collaborate in toxin research. The success of the work created a renewed capability and interest in toxin research at Suffield. A major accomplishment has been the establishment of a toxin research hub at DRDC Suffield.

Materials and Methods

Chemicals

Unless otherwise noted all chemicals and reagents were purchased from Sigma-Aldrich Canada Ltd (Oakville ON). BoNT/A and BoNT/B, recombinant BoNT/A light chain and SNAPtide™ substrates were products of List Biological Laboratories Inc. (distributed by Cedarlane Ltd., Hornby ON). The botulinum neurotoxin substrates and peptide products were obtained from Dr Dennis McMaster at the University of Calgary Peptide Synthesis Laboratory (Calgary AB) and were used in assays with HPLC and CE analysis.

Combinatorial peptide libraries

Materials for peptide synthesis were from Novabiochem (San Diego CA). Peptides and peptide libraries were synthesized by solid phase methods as previously reported [7]. For inhibitory studies, library subsets were freshly solubilized (0.5 mg in 0.2 mL of 5% aqueous DMSO; 5 mM) and the appropriate volume added to assay samples to give a final assay concentration of 0.5 mM. DMSO (0.5%) was found to have no effect on the three assays used in these studies. Captopril (1 – 5 mM) was used as reference inhibitor in BoNT assays [7].

Botulinum neurotoxin A assays for HPLC analysis

Stock solution of intact BoNT/A was prepared in deionized water (NanoPure filtration Barnstead) at a concentration of 50 μg/mL (*ca* 330 nM) and stored at 4 °C for up to two days. Reduction/activation of BoNT/A was accomplished by adding 30 μL of intact toxin stock solution (typically 1 – 2 μg) to 30 μL of 50 mM HEPES pH 8 containing 20 mM fresh dithio-DL-threitol (DTT) and incubating at 37 °C for 30 min. After the first reduction/activation step, 390 μL of 50 mM HEPES buffer pH 6.6 containing 1 μM zinc sulfate was added to the reduced toxin solution, followed by 750 μL of 1 μM zinc sulfate. The mixture (final volume 1200 μL) was incubated at 37 °C for 5 h. The 17-mer peptide, Ac-SNKTRIDQANQ$_{197}$-R$_{198}$ATKML-NH$_2$, which represents SNAP-25 (residues 187-203), was used as the substrate for the enzyme (light chain). Each assay tube (typically 15 tubes total) contained 10 μL of 0.5 mM substrate, 80 μL of activated enzyme solution and 10 μL of deionized water or 10 μL of the combinatorial peptide library solution (5 mM total peptide). The concentration of activated LC enzyme in the assay tubes was nominally 5 nM but some adjustment in concentration was made to yield *ca* 30-70% cleavage of substrate in the positive controls. Inhibitory assays using combinatorial peptide libraries were conducted by adding the library compounds to the enzyme assay immediately prior to substrate. After 30 min incubation at 37 °C the reaction was terminated with 100 μL of 1% TFA. The terminated samples were analyzed in HPLC with UV absorbance detection (data in Tables 1-6) or in capillary electrophoresis as described in this report. The concentrations of the reaction products were determined by HPLC (substrate at 25.0 min, products at 21.4 and 21.5 min) according to Hayden [7] and described below.

Botulinum neurotoxin B assays for HPLC analysis

Stock solution of intact BoNT/B was prepared in deionized water at a concentration of 100 μg/mL with 0.1% bovine serum albumin and stored at -20 °C for up to two months. Assays of BoNT/B were conducted according to methods described by Garcia et al. [32]. Reduction/activation of BoNT/B was accomplished by incubating the whole toxin (typically 0.1 – 1 μg) with 10 mM fresh DTT at 25 °C for 30 min in 50 mM HEPES pH 7.4. After the reduction step, the enzyme was incubated with the 35-mer synthetic peptide substrate Ac–LSELDDRADALQAGASQ$_{76}$–F$_{77}$ETSAAKLKRKYWWKNLK–NH$_2$, which represents VAMP (residues 60-94), for 30 min at 37 °C in 80 mM HEPES buffer containing 4% DMSO and 4% Triton X-100, pH 7.3, in the presence of 11 mM DTT freshly solubilized in H_2O. The reaction was terminated with an equal volume of 2% TFA and the products determined by HPLC with UV detection (substrate at 28 min, products at 22 and 25 min).

HPLC analysis

Separation of products and substrate from one another after cleavage of substrates by BoNTs was achieved by reverse phase HPLC under standard conditions [7]. Separation was carried out on a Bio-Rad Hi-Pore C_{18} RP-304 column (25 cm x 4.6 mm) eluting with a gradient of acetonitrile in 0.1% TFA at 30 °C and a flow rate of 1 mL/min. Eluent A = 0.1% TFA; Eluent B = 0.1% TFA/70% acetonitrile; gradient profile: 100% A (to 2.5 min); linear gradient to 45% B (to 28.5 min); 100% B (to 34 min). Reaction products were detected by UV absorbance at 210 nm. Assays for BoNT/ A and B were conducted such that ~50% cleavage of substrate occurred in controls (no peptide libraries present) as determined by HPLC; inhibitory potencies of libraries were determined from the percentage decrease in cleavage of the substrate in the presence of libraries [7].

Saxitoxin assays

Bioassays of saxitoxin were conducted according to methods described by Manger et al. [15]. Mouse neuroblastoma cells (Neuro-2a, ATCC CCL-131, American Type Culture Collection, Manassas VA) were grown in Gibco RPMI 1640 complete medium (Invitrogen Canada Inc., Burlington ON) supplemented with 10% fetal bovine serum, 25 mM bicarbonate, 50 μg/mL streptomycin and 50 units/mL of penicillin. Cultures were grown at 37 °C in 5% CO_2 and air with a maximum of 10 passages. Cells were passaged using 0.5% trypsin/5.3 mM EDTA (Gibco) to suspend the cells. Assays were initiated using 96 well Costar plates (Fisher Scientific, Edmonton AB) with 200 μL of medium containing 1 X 10^5 cells per milliliter in each well. Outer wells contained 200 μL of phosphate buffered saline. Cells were incubated as above for 18-24 h. Medium was removed and replaced with 100 μL fresh medium for controls, 90 μL medium containing 0.5 mM ouabain and 0.05 mM veratridine to measure cell death, and positive controls which contained ouabain, veratridine and 0.8 ng/well of saxitoxin in medium. Additions to the cells were titrated to give 80% inhibition of the control untreated cells by ouabain and veratridine and approximately 80% reversal of that inhibition with saxitoxin. Inhibition of the saxitoxin rescue of ouabain and veratridine toxicity was determined in the presence of 0.5 mM of inhibitor (10 μL of 5 mM peptide library subset in water). Cells were incubated for 24 h and the cell viability was determined using 5 μL of MTS-tetrazolium compound (Promega, Madison WI) per well followed

by incubation at 37 °C for 4 h. Absorption was determined at 490 nm using a UV-Max kinetic microplate reader (Molecular Devices, Sunnyvale CA) and inhibition values were expressed as a percentage of the positive controls.

Botulinum neurotoxin A assays for capillary electrophoresis analysis

The BoNT/A LC assay samples that were used in CE analysis were performed in a similar manner to those for HPLC. The sequences of the substrate peptide (S) and product peptides (P1; Ac-SNKTRIDQANQ) and (P2; RATKML-NH_2) are shown in Figure 1. The S and P2 peptides were readily dissolved in sterile distilled water at a stock concentration of 10 mg/mL. P1 peptide required the addition of 0.005% Tween-20 to solubilize. Stock peptides were stored at 4 °C for up to five days or longer at -20 °C. For modulation studies of BoNT/A LC assays, stock solutions of the libraries were freshly reconstituted (0.5 mg in 0.2 mL 5% aqueous DMSO) to approximately 5 mM (total for the library) and the appropriate volume of stock solution was added to LC assays to give a final concentration of 0.5 mM (total). No significant effects were observed in the LC assays by the presence of 0.5% DMSO (not shown).

Fluorescent dye labeling for CE LIF

Admixtures of substrate (S) and synthetic products (P1 and P2) in 1% TFA were neutralized by titration with a minimal volume of 2 M NaOH. Aliquots of these admixture samples (10 µL) were subsequently labeled with CBQCA Protein Quantitation kit (Molecular Probes Inc., Eugene OR). Stock solution was prepared by dissolving CBQCA in dry DMSO to yield 40 mM. For CBQCA labeling, each 10-µL sample aliquot was reacted with 5 µL of 5 mM KCN, 1.5 µL of 100 mM *N*-ethylmaleimide and 10 µL of 5 mM CBQCA in HNTE buffer (50 mM HEPES pH 8.4, 20 mM NaCl, 0.005% Tween-20, 1 mM EDTA). Samples were agitated for 30 min at room temperature in the dark on a Nutator shaker, then transferred to microvials for CE LIF analysis. The products of BoNT/A LC assays were labeled in a similar fashion. The labeling conditions provided a theoretic minimum dye-to-amine ratio of 8 in the presence of peptide libraries. All of the labeling reactions were conducted pre-column. With on-column CBQCA-labeling we did not obtain satisfactory fluorescent intensities (data not shown).

CE LIF analysis

CE LIF analyses were performed using a Beckman 5010 CE instrument (Beckman Coulter Inc. Fullerton CA) equipped with an argon ion laser for excitation at 488 nm and detection at 520 nm. Samples were run on a 37 cm x 50 µm i.d. bare fused silica column (PolyMicro Technologies, Phoenix AZ) with a 30-cm separation length and a column temperature of 25 °C. The column was conditioned with 0.1 M NaOH for 10 min and HNTE running buffer for 30 min before use. During sample analysis, the column was rinsed for 1 min with 0.1 M NaOH and 2 min with running buffer before sample injection. The sample was injected at 0.5 psi for 9 s for a sample injection volume of 19.8 nL, determined by the Beckman instrument software. A plug (11 nL calculated volume) of running buffer was injected at 0.5 psi for 5 s

after the sample. The sample was then separated at 18 kV (486 V/cm). Data analysis was performed using P/ACE Version 1.2 software (Beckman Coulter Inc.) on duplicate or triplicate samples. Peak heights and peak areas were calculated and plotted as required.

CE LIF was used for kinetic analysis of the cleavage of the 17-mer peptide substrate by BoNT/A LC. The substrate (at 6 different concentrations) was mixed with LC in assay buffer as described above. Aliquots of 50 µL were removed every 30 seconds over 6 minutes, and stopped with stopping buffer (5 mM sodium borate pH 8, 10 mM EDTA). Samples were labeled with CBQCA then subjected to CE LIF on a 30.2 cm x 50 µm i.d. bare fused silica column in HNTE buffer as described above. The amount of P1 product formed at each time point was calculated from a P1-concentration standard curve and this concentration data was subsequently used to generate P1-concentration versus time curves. V_{max} and K_M were calculated as described below.

FRET Analysis of botulinum neurotoxin A

FRET SNAPtide™ assays were used to screen combinatorial peptide libraries capped with cholic acid (Cho) at the N-terminus and amine at the C-terminus. The substrate, SNAPtide™, is a synthetic peptide containing the native sequence of the cleavage site for BoNT/A. The N-terminally linked fluorophore is o-aminobenzoic acid (oAbz) and the acceptor chromophore is 2,4-dinitrophenol (Dnp) in the SNAPtide (oAbz/Dnp). SNAPtide (FITC/DABCYL) contains fluorescein isothiocyanate and 4-((-4-(dimethylamino)-phenyl)-azo)-benzoic acid, respectively. Each library was dissolved in 25% aqueous DMSO to yield a concentration of 0.5 mg/mL. An aliquot of 10 µL of library solution was added to a standard FRET reaction containing 5 µM SNAPtide and 10 nM recombinant LC in assay buffer (20 mM HEPES pH 8.0/0.3 mM zinc sulfate/0.2% Tween-20/0.5 mM DTT), total volume 100 µL. Samples were incubated for 30 min at 37 °C then stopped in 400 µL of 10 mM TRIS pH 8/100 mM EDTA. Duplicate 200 µL aliquots were loaded into Costar fluorescence microtitre plates and read on a Varian Cary Eclipse spectrofluorometer at 318 nm excitation and 421 nm emission for oAbz/DNP SNAPtide and 490 and 520 nm, respectively, for FITC/DABCYL SNAPtide. The percent LC cleavage was calculated in the presence of each library, corrected for the no-enzyme control, and normalized to the positive control. Each data point represented the mean of four runs.

The SNAPtide assay was used to determine the concentration-dependent inhibition of peptide M1-GABA-C2-C3, and compounds amaranth and ponceau BS. Serial two-fold dilutions of the inhibitor were prepared in assay buffer. Reactions contained inhibitor, 5 µM oAbz/DNP-SNAPtide substrate (with inhibitor M1-GABA-C2-C3) or FITC/DABCYL-SNAPtide (with inhibitors amaranth and ponceau BS), and 10 nM of BoNT/A LC in assay buffer in a total volume of 100 µL. The samples were processed as described above. The percent cleavage was calculated in the presence of the inhibitor and corrected for the no-enzyme control. A sigmoidal dose-response curve was fitted to the data to obtain the IC_{50} (concentration to attain 50% inhibition in cleavage) using the scientific calculation program Prism version 4 (GraphPad Software, San Diego CA). Kinetic studies using the SNAPtide assays were performed in 200-µL micro-cuvettes in the Varian Cary Eclipse spectrofluorometer at 37 °C, taking measurements of product formation every 0.25 seconds for 10 minutes to generate product-versus-time curves at 6 different substrate concentrations. Initial reaction velocities

were then plotted against the substrate concentration and non-linear regression was performed to obtain the Michaelis-Menten constants V_{max} and K_M, using GraphPad Prism v4.

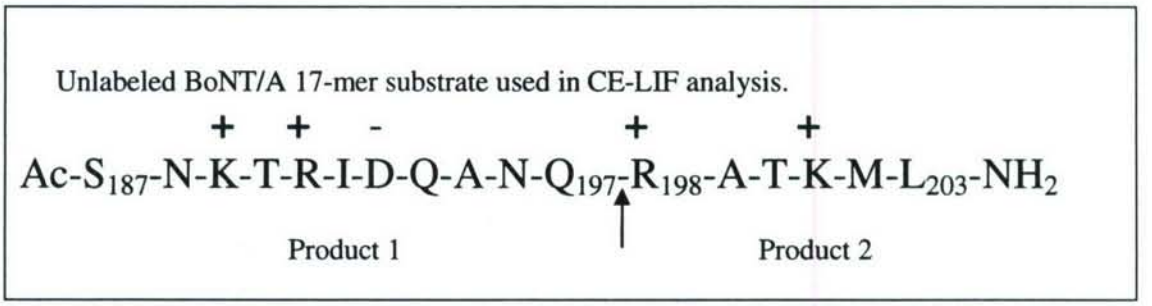

Figure 1. Peptide sequence of substrate and product for CE LIF BoNT/A Assay. Nominal positive and negative charges on the amino acids are indicated by (+) or (-). The arrow indicates the cleavage site by activated BoNT/A light chain. Ac and NH_2 on the ends of the peptide represent acetyl and amide capping of the N-and C-termini, respectively. The 17-mer substrate peptide represents residues 187-203 in SNAP-25.

Results and Discussion

Deconvolution of hinge peptide combinatorial libraries

Capped libraries

A series of primary library subsets of capped hinge peptides was synthesized by fixing, in turn, position 4 of the peptide structure with one of the twelve amino acid residues and then having positions 1, 2 and 3 contain the random residues, i.e.,

$$Ac - X1 - X2 \leftrightarrow X3 - O4 - NH_2$$

where X = approximately equimolar mixture of 12 amino acids (R,C,Q,E,H,K,L,M,F,S,W,Y), O = substitution of one of the 12 amino acids and the double arrow (↔) represents the GABA (γ -aminobutyric acid) hinge. Table 1 shows the percent inhibition and mean inhibition (MI) of the primary peptide libraries with positional scanning at position 4 (O4). For BoNT/A (Table 1 row 1) the subsets were generally inhibitory. At position 4 (O4), the greatest inhibition was shown by C (45%) and R (30%). One of the libraries (S4) displayed slight negative inhibition i.e., the substrate cleavage was increased in the presence of the library. Overall MI was 19%. The same peptide libraries were used in BoNT/B LC cleavage assays of the BoNT/B-specific peptide substrate. Positional scanning of primary libraries in which position 4 was fixed showed no inhibition, but rather pronounced activation effects (Table 1 row 2); MI was –22%. For saxitoxin assays using the same primary library subsets, positional scanning indicated inhibition that was positive or negative (ranging from +27% to -16%). The MI was 11%.

Based on the positional scanning results of the primary libraries subsets in BoNT/A assays (Table 1 row 1), secondary libraries were synthesized containing C or R in position 4. In the secondary libraries either position 2 or 3 was scanned with the twelve amino acids; e.g.,

$$Ac\text{-}X1\text{-}\mathbf{O2} \leftrightarrow X3\text{-}C4\text{-}NH_2 \qquad \text{or} \qquad Ac\text{-}X1\text{-}X2 \leftrightarrow \mathbf{O3}\text{-}C4\text{-}NH_2\,.$$

Thus positional scanning was either on the opposite side or the same side of the hinge with respect to position 4. Table 2 shows the inhibitory potencies of secondary library subsets for BoNT/A activity. When position 4 was R and position 3 was scanned (see, Table 2 row 1, X1X2↔O3R4), the only inhibitors were W (19%) and F (14%). The MI of these library subsets was 3%.

Table 1. Percent inhibition and mean inhibition (MI) of toxin assays by primary capped hinge tetrapeptide libraries: Ac-X1X2↔X3O4-NH_2*

O4	=	R	C	Q	E	H	L	K	M	F	S	W	Y	MI
1	BoTN/A	30	45	21	20	5	5	12	27	22	-5	23	13	19
2	BoTN/B	-20	-40	-25	-24	3	-17	-60	-24	5	-14	-15	-32	-22
3	Saxitoxin	8	3	-13	-7	-14	-16	-7	-8	16	27	-8	-3	11

*Percent inhibition (Tables 1-6) was determined by HPLC from the change in area of the substrate peak in the presence and absence of the inhibitor. The error in the % percent inhibition data was about ± 5 percentage points.

Table 2. Percent inhibition and mean inhibition (MI) of BoNT/A assay by secondary capped hinge tetrapeptide libraries

	O =	R	C	Q	E	H	L	K	M	F	S	W	Y	MI
1	O3 R4	0	0	2	0	0	0	0	4	14	0	19	0	3
2	O2 R4	0	17	0	3	17	14	28	31	25	50	24	22	19
3	O3 C4	4	27	0	0	0	0	0	13	0	4	0	0	4
4	O2 C4	17	37	20	8	26	29	36	75	11	43	4	33	28

Table 3. Percent inhibition and mean inhibition (MI)of BoNT/B assay by secondary capped hinge tetrapeptide libraries

	O =	R	C	Q	E	H	L	K	M	F	S	W	Y	MI
1	O3 R4	-1	-2	-15	-17	-5	-12	-6	-13	-19	-15	-17	-56	-15
2	O2 R4	60	32	22	26	17	40	-5	8	44	22	15	8	24
3	O3 C4	-22	30	4	-22	-10	-37	-80	-38	-39	-38	17	-42	-23
4	O2 C4	54	74	4	10	3	-25	20	4	10	-11	4	38	15

When position 4 was C and position 3 was scanned (Table 2 row 3, Ac-X1X2↔O3C4-NH_2), significant inhibition (>10%) at position 3 occurred for C (27%) and M (13%); MI was 4%. However, when positions 4 was C or R and positional scanning was carried out on position 2, as opposed to position 3, the inhibitory effects of the library subsets showed not only a different order of potency but also significant increases in potencies. Thus when position 4 was R and position 2 was scanned (Table 2 row 2, Ac-X1O2↔X3R4-NH_2), 9 of 12 subsets scored inhibition >10%; the greatest potency was: S2 (50% at 0.5 mM). MI of 19% was substantially higher than observed for the same experiments in which positions 3 was scanned. Similarly the C4 library subsets showed increased potency when position 2 was scanned (Table 2 row 4, Ac-X1O2↔X3C4-NH_2) rather than position 3. The best inhibitory library contained M2 (75% at 0.5 mM); 10 of 12 subsets scored inhibition >10%. MI was 28%.

The same secondary peptide libraries were used in BoNT/B LC cleavage assays of the BoNT/B-specific peptide substrate. When position 4 was R, subsets scanned in position 3 showed only negative inhibition, i.e., activation effects (Table 3 row 1, Ac-X1X2↔O3R4-NH_2); MI was –15%. Secondary library subsets in which position 4 was C and position 3 was scanned (Table 3 row 3, Ac-X1X2↔O3C4-NH_2) did reveal several inhibitory subsets with C (30%) and W (17%) which were masked in the primary subsets (Table 1). However, in general, Ac-X1X2↔O3C4-NH_2 subsets were still more activating than inhibitory; the MI of the subsets was –23%. With position 4 containing R or C and position 2 scanned, a result similar to the BoNT/A above was observed; an increase in potency and a different ordering. Thus R4, which produced no inhibitors when position 3 was scanned, gave several inhibitory subsets when position 2 was scanned (Table 3 row 2, Ac-X1O2↔X3R4-NH_2); the greatest inhibition was R 2 (60%). Scanning the amino acid residues on the opposite sides of the hinge increased MI from –15% to 24%. For C4 the greatest inhibition at position 2 (Table 3 row 4, Ac-X1O2↔X3C4-NH_2) was the C sublibrary (74% at 0.5 mM). MI increased to 15% from –23% when position 2 was scanned instead of position 3.

The overall picture for deconvolution that emerged was that greater potency was observed by scanning on the opposite side of the hinge than on the same side (position 2 versus 3) with respect to fixed position 4. It is noteworthy that the iterative deconvolution step to the secondary libraries (the selection and synthesis) was based on the results of the BoNT/A assay, i.e., selecting R or C in position 4. The primary library subsets that contained R and C in position 4 were in fact activators of the BoNT/B LC assay (Table 1 row 2). Still these libraries contained inhibitory potency toward BoNT/B that was manifested in deconvolution.

Table 4. Percent inhibition and mean inhibition (MI) of saxitoxin bioassay by secondary capped hinge tetrapeptide libraries

	O =	R	C	Q	E	H	L	K	M	F	S	W	Y	MI
1	O3 R4	-7	-3	4	-3	-4	-8	-4	0	-10	-2	-6	-2	-4
2	O2 R4	65	0	0	0	75	0	32	0	4	0	26	4	17
3	O3 C4	-5	-6	-4	-7	-4	-6	-2	-6	-2	-3	-2	-5	-4
4	O2 C4	3	-8	-16	7	-5	4	-17	-7	-8	4	-19	-2	-5

Table 5. Percent inhibition and mean inhibition (MI) of toxin assays by primary uncapped hinge tripeptide libraries: X1↔X2O3

	O_3 =	R	C	Q	E	H	L	K	M	F	S	W	Y	MI
1	BoNT/A	52	28	24	35	21	26	22	14	28	28	46	18	29
2	BoNT/B	-5	-7	-1	15	-16	-14	2	-17	15	3	5	5	-1
3	Saxitoxin	9	2	-13	-6	-13	-14	-7	-8	17	0	-2	-7	-3

Bioassays for saxitoxin used the same hinge peptide libraries as inhibitors. However in contrast to the enzyme-based BoNT LC assays, saxitoxin inhibition was measured by response of neuroblastoma cell cultures as described above. The primary libraries showed some amplification of saxitoxin blockage; i.e., a moderate level of inhibition was observed for S (27%) and F (16%) in position 4 (Table 1 row 3); MI was 11%. In the secondary libraries when position 4 was R or C no significant inhibition was observed for the subsets scanned in position 3 (Table 4 rows 1 and 3, Ac-X1X2↔O3R4-NH_2, Ac-X1X2↔O3C4-NH_2). When position 4 was R and position 2 was scanned, inhibition was observed for H (75%), R (65%), K (32%) and W (26%); MI was 17% (Table 4 row 2, Ac-X1O2↔X3R4-NH_2). With C in position 4, there was no significant inhibition observed when position 2 was scanned (Table 4 row 4, Ac-X1O2↔X3C4-NH_2). Thus the deconvolution picture of saxitonin was similar to BoNT. Where present, inhibition was observed in the secondary libraries by scanning position 2, the opposite side of the hinge.

Uncapped libraries

The modulation effects on toxin assays of uncapped hinge tripeptide libraries were investigated. A series of primary library subsets was synthesized by scanning position 3 of the peptide structure with one of the twelve amino acid residues and then having positions 1 and 2 contain the random residues, i.e.,

$$X1 \leftrightarrow X2 - O3$$

where X, O and ↔ are as defined above. The assays were the same as used with the capped libraries. For the BoNT/A assay, Table 5 row 1 (X1↔X2O3) showed the inhibitory potencies of the primary hinge tripeptide libraries. These results indicate that the best amino acids at position 3 for inhibition were R (52%), W (46%), E (35%). Comparison of the primary tri- and tetrapeptide libraries indicated similar potencies for R, W and C at the C-terminus. None of the primary tripeptide sublibraries displayed negative inhibition with BoNT/A; MI was 29%. This trend was similar to the tetrapeptide libraries with BoNT/A; where only one sublibrary was slightly negative and MI of the tetrapeptides was 19%. For BoNT/B two sublibraries, F and E, gave moderate level of inhibition of 15% (Table 5 row 2). Some individual sublibraries were inhibiting and others activating with respect to the BoNT/B assay

(MI was -1%), whereas the tetrapeptides gave predominantly negative inhibition, i.e., activation. For saxitoxin two tripeptide sublibraries gave inhibition, namely, F (17%) and R (9%, Table 5 row 3). Overall for saxitoxin the MI of the tripeptide libraries was less inhibitory (-3%) than that of the tetrapeptides (11%). Selected secondary uncapped libraries were used in toxin assays and gave inhibitions up to 75-98% at 0.5 mM. Table 6 summarizes library subsets that had the greatest amounts of inhibition.

Table 6. Secondary hinge peptide libraries subsets that gave significant amounts of inhibition

	Percent Inhibition			
Capped tetrapeptide		Uncapped tripeptide		Captopril*
Ac - X1X2↔X3X4 – NH_2		X1↔X2O3		
BoNT/A				
M2C4	**75**	**C1S3**	**75**	75 (at 1 mM)[1]
S2R4	50	S2R3	51	
S2C4	43	M1E3	41	
		W1F3	35	
BoNT/B				
C2C4	**74**	**C1F3**	**80**	20 (at 5 mM)[2]
R2R4	60	K1F3	65	
F2R4	44	M1F3	48	
Saxitoxin				
H2R4	**75**	**E1E3**	**98**	
R2R4	65	E2E3	70	
		C1F3	42	
		C1E3	40	

Note: Best inhibitor subsets in bold; all concentrations 0.5 mM.
*ACE (angiotensin converting enzyme) inhibitor captopril was used as control inhibitor in BoNT/A and /B assays.
[1]Hayden 2003 reference 7, [2]this work.

In this work we observed differences in the effects of library subsets on the activities of BoNT/A and B. Many capped library subsets which were inhibitors of BoNT/A were often activators of BoNT/B (Table 1), reflecting the functional differences in the active sites of the two enzymes. However the capped primary libraries that showed activation of BoNT/B (Table 1) contained inhibitory secondary libraries (Table 3) which were presumably masked by activation effects in the primary library subsets. These findings indicate the importance of examining secondary subsets when the primary subsets have failed to reveal anything useful, even when primary screening has revealed the presence of activation effects. Saxitoxin toxicity derives from its ability to block sodium ion channels in the cell membrane. This

blockage mechanism arises from a target that is totally unrelated to botulinum toxin endopeptidase activity. Thus the structure-activity data for the library subsets in saxitoxin assays bears no relationship to the structure-activity data for either of the two botulinum neurotoxins.

The best secondary library subset 'hit' for each toxin was about 75%. Such hits occurred when position 2 was scanned rather than position 3. Presumably this is because placement on opposite sides of the hinge creates a greater opportunity for interaction of the side chains of these amino acids due to the increased flexibility of the backbone provided by the hinge. In other words the intrinsic dynamic variability resulting from the increase in the degrees of freedom invoked by the hinge moiety leads to increased spatial and conformational sampling of the side chains and a consequential increase in potency. This outcome was in fact predicted previously to be a likely advantage of hinge peptides over conventional peptides, and formed an integral part of the original conceptual basis for the development of hinge peptide libraries [5].

In light of the results with capped tetrapeptide libraries we made changes to the peptide model by decreasing the size to three peptides and removing the end-capping groups. The screening of the primary tripeptide libraries against the three toxins showed features that were similar to the tetrapeptides; that is, overall they were inhibitory toward BoNT/A, less inhibitory toward saxitoxin and slightly activating toward BoNT/B. When selected secondary libraries subsets were assayed, strong inhibition was observed, in fact slightly higher than observed for the tetrapeptides: 75% for BoNT/A, 80% for BoNT/B and 98% for saxitoxin (Table 6).

As with the capped tetrapeptides libraries, greater inhibitory potency was found when the fixed residues were on opposite sides of the hinge. Using uncapped hinge peptide libraries containing as few as three variable amino acid positions, this work has demonstrated the feasibility of gaining rapid access to lead inhibitors with low numbers of syntheses and assays. It was interesting that for both capped and uncapped hinge peptide libraries the inhibitory potencies of the best subsets after two deconvolution steps were similar although limited structural correspondence was observed. The strength of the technology lies in the speed and expediency to discover inhibitory compounds [33]. The only prerequisite is a suitable assay for the toxin. Thus in situations where a toxic threat has been identified, a suitable assay such as those described herein can be quickly put in place. Complete deconvolution of a hinge peptide library requires fewer than 200 assays to be carried out (144 secondary subsets plus a number of tertiary/quaternary subsets) in order to identify the most potent ligand in the library. This is the case whether using capped or uncapped libraries, because there is a requirement to screen all 12 secondary library subsets (144 variants) in each case, since primary library screening (Table 1) does not necessarily provide for the best secondary library candidate. Nevertheless, 200 data points would be attainable in a matter of days rather than weeks or months.

The purpose of capping was to eliminate the electronic charge at each end of the molecule so that only the side chain groups would be 'functional'. In this study we investigated the effects of removing the capping groups, thereby adding two extra terminal charges (positive charge at N-terminus and negative charge at C-terminus). The presence of the charged ends would undoubtedly affect inhibitory activity. Moreover, the ability of uncapped hinge peptides to adopt head-to-tail pseudocyclic conformations because of the attractive nature of the terminal

charges (unpublished NMR observations) would also influence inhibition. Accordingly, uncapped hinge peptide libraries potentially represent a more complex situation than capped libraries.

Analysis of botulinum neurotoxin A assays by CE LIF

Comparison of CE and HPLC for separation of BoNT/A reaction peptides

CE separation of an admixture of the substrate and synthetic products using pre-column CBQCA labeling for LIF detection is shown in Figure 2a. The electrophoretic mobilities and physicochemical properties of substrate and products are given in Table 7. The substrate contained the greatest number of nominal positive charges (Figure 1) and hence displayed the shortest migration time at 2.6 min. The labeled P2 peak was observed at 2.8 min; labeled P1 at 3.2 min. Near baseline separation of all the main labeled peptide product peaks was achieved in CE LIF within 3.5 min separation time. The total time for one CE LIF run, including column rinses and injections, was approximately 8 min. Although labeling shifted the migration times slightly longer (by *ca* 0.1 – 0.2 min) with respect to unlabeled peptides, the order stayed the same (data not shown). The other peaks in Figure 2a were attributable to fluorescence arising from CBQCA-labeled components of the HEPES-based buffer used in the analysis (see Figure 2b). The peaks for the species of interest, i.e., the substrate and product peptides, were sufficiently separated from the buffer peaks that no problems were encountered in making assignments. The presence of the wide buffer peak at 5 min was useful as an internal reference marker for the labeling reaction, electrophoretic mobility and electroosmotic flow. The fluorescence emission of the CBQCA-labeled products was stable at 4 °C up to 14 days (data not shown). Figure 2c (inset) shows the partial separation of a BoNT/A LC assay mixture by HPLC with UV detection at 210 nm. Products (22.8 min) and substrate (24.2 min) were separated, although P1 and P2 peaks were difficult to resolve. The total time in HPLC for separation and column rinse was about 60 min.

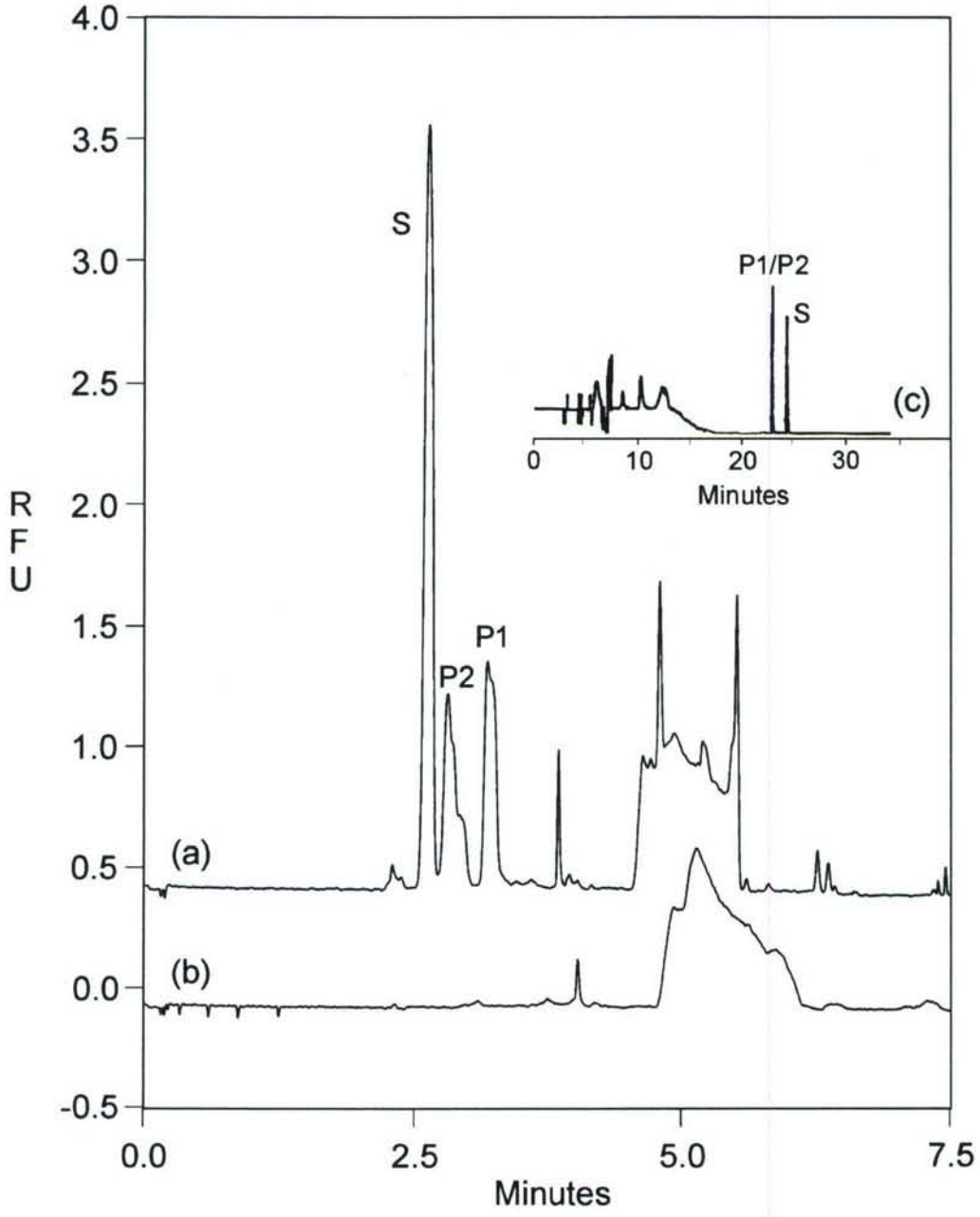

Figure 2. (a) CE separation and LIF detection of an admixture of CBCQA-labeled substrate and synthetic products. S, substrate; P1, product 1; P2, product 2. (b) HEPES-containing running buffer (described in Materials and Methods section as HNTE buffer) reacted with CBQCA reagent. (c) HPLC of BoNT/A LC assay products. Samples were prepared and analyzed as described in the text.

Table 7. Physico-chemical characteristics of peptide substrate and cleavage products.

Compound	Charge[1](at neutral pH)	MW[2] (Da)	pI[2]
substrate	+ 3	1989	11.00
product 1	0 [3]	1288	8.46
product 2	+ 3 [3]	719	11.00

[1] Sum of nominal charges.
[2] Molecular weight (MW) and isoelectric point (pI) were calculated by ExPASy (ref. 12).
[3] Cleavage of the peptide bond introduces a free amino group on P2, which modifies the charge.
Note: CBQCA labeling changes the M/Z ratio peptides by decreasing the net positive charge on lysine residues and adding mass (*ca* 300 Da per adduct).

Quantitation of BoNT/A LC products in CE LIF

A critical issue for the work was whether the labeling process and CE LIF analysis of the BoNT/A assay products could be performed quantitatively. To this end, a set of seven admixtures of substrate and synthetic product fragments were prepared from stock solutions, labeled with CBQCA, and analyzed by CE LIF. The concentrations in the admixtures were adjusted to simulate the concentrations and mole fractions in a BoNT/A LC assay; i.e., the admixtures were prepared at constant total concentration (S + P1) of 100 μM and then diluted 1/1 with running buffer in CE. Thus the concentrations of S, P1 and P2, were given by:

$$[S] + [P1] = 100\,\mu M; \quad [P1] = [P2] \qquad (1)$$

where [ ] denotes concentration of the species in square brackets. The plots of fluorescence intensity obtained from the electropherograms *versus* concentration over the range 0 – 100 μM were shown to be linear for the CBQCA-labeled assay products, i.e., substrate, S, and synthetic peptide fragments, P1 and P2 (Figure 3).

The greater test to whether the methods were quantitative was to compare the fluorescence intensity of a component band in the electropherogram, say P1, to its mole fraction in the sample. Thus in keeping with equation 1,

$$F_S + F_{P1} = 1.0 \qquad (2)$$

where F_S and F_{P1} are the mole fractions of S and P1, respectively. Thus when $F_S = 0.75$, $F_{P1} = (1.0 - 0.75) = 0.25$ etc. A_S and A_{P1} are the fluorescence intensities (by peak area) of the S and P1 bands, respectively, in a particular sample set, e.g., sample in which $F_S = 0.75$, $F_{P1} = 0.25$.

We can express the intensity of P1 as a normalized ratio, R_{P1}, wherein the numerator is the intensity of the P1 band and the denominator is sum of the intensities of S and P1 bands,

$$R_{P1} = A_{P1} / (A_S + A_{P1}) \quad (3)$$

A plot of R_{P1} versus mole fraction P1 (F_{P1}) is shown in the inset to Figure 3. It is to be noted that the ratio is normalized and thus for the sample in which $F_{P1} = 1.0$, eq. 3 gives $R_{P1} = 1.0$. This was the sample where [S] = 0 and [P1] = [P2] = 100 µM. The plot is in fact linear ($R^2 = 0.9935$). In other words in each sample the fractional fluorescence intensity of P1 was proportional to the mole fraction. These results demonstrate that CBQCA labeling and CE LIF analysis provides a quantitative method of measuring the products of BoNT/A LC reactions.

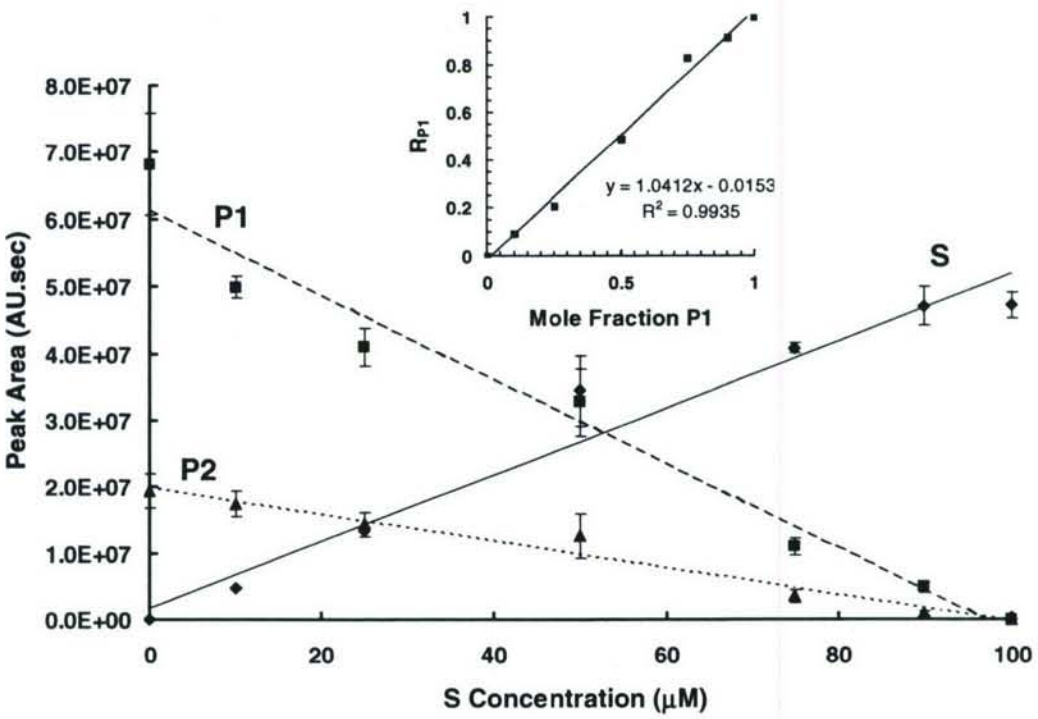

Figure 3. Standard curves of CBQCA-labeled substrate and synthetic products separated and detected in CE LIF. The standard curves were obtained from the peak areas (arbitrary units.sec) of a series of samples in which [S] + [P1] = 100 µM, [P1] = [P2] (see eq 1). The plot ascending from left to right is the linear regression of substrate intensities ($r^2 = 0.9635$). As concentration of substrate increases from 0 to 100 µM, the concentrations of P1 and P2 decrease from 100 to 0 µM. The top plot descending from left to right is P1 ($r^2 = 0.9705$); bottom descending is P2 ($r^2 = 0.9724$). Inset. Quantitation of P1 in the admixtures. The x-axis represents the mole fraction of P1, where [S] + [P1] was constant at 100 µM, i.e., when $F_{P1} = 0.25$, $F_S = 0.75$. The y-axis is the normalized intensity, R_{P1}, calculated according to Eq. 3. The inset plot was derived from data displayed in Figure 3.

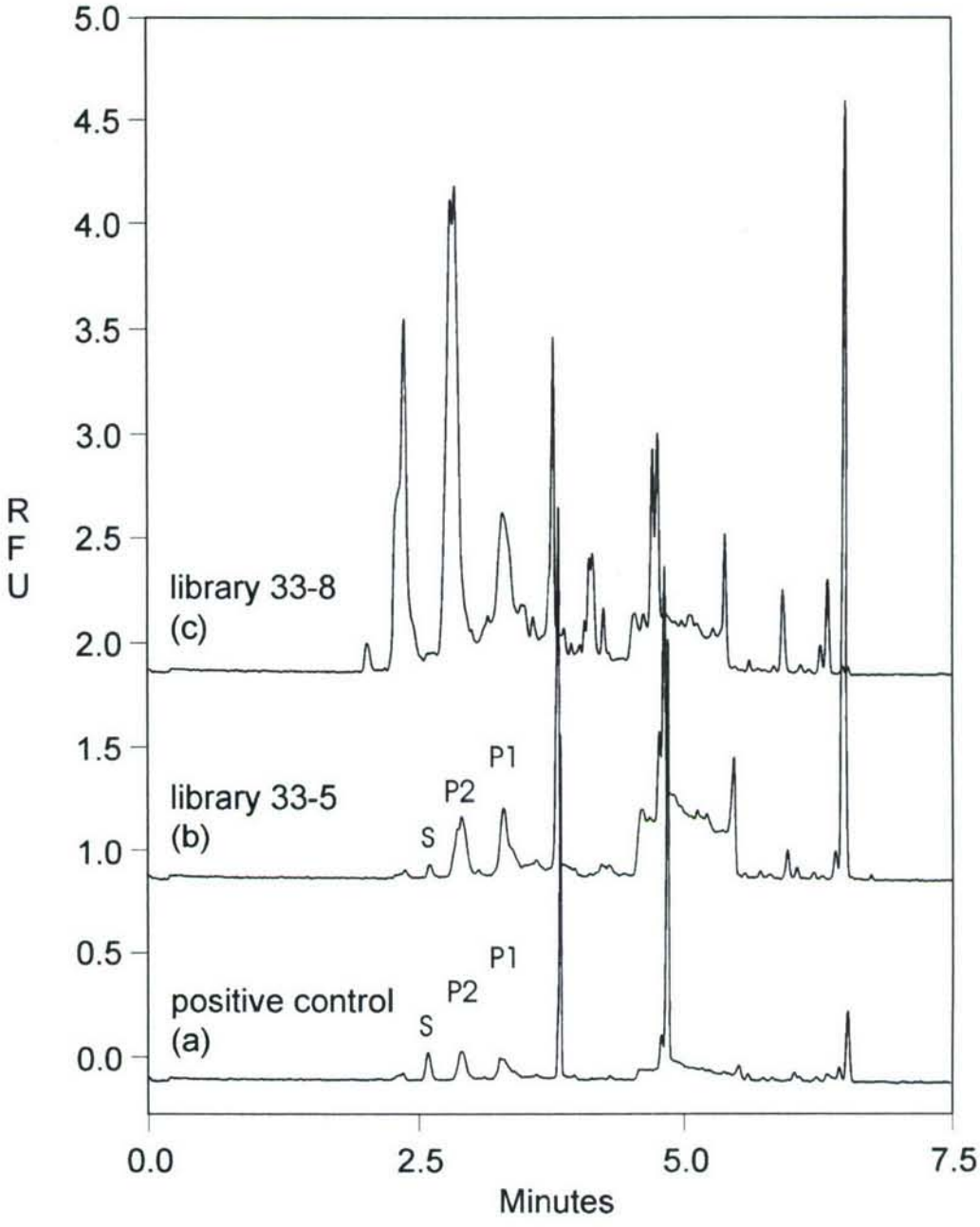

Figure 4. CE LIF separation and detection of products of BoNT/A LC assay in the presence of peptide libraries. Samples were labeled with CBQCA and run as described in the text. (a) Positive control sample (no library): S, P1, P2 peaks were readily assigned. (b) With library 33-5: the products were separated and resolved from the library peptides. (c) With library 33-8: products could not be separated and resolved from the library peptides.

Analysis of BoNT/A LC products in the presence of combinatorial peptide libraries

CE LIF was carried out on samples derived from actual BoNT/A LC reactions that contained substrate, cleaved products and enzyme in the presence of combinatorial peptide libraries. Prior to CE analysis the entire LC reaction mixture was labeled with CBQCA. Thus intact unreacted S, products P1, P2 plus BoNT/A and peptide libraries (if present) were rendered fluorescent. Figure 4a shows the electropherogram of the products of a positive control assay (sample was prepared with only substrate and activated BoNT/A LC, no combinatorial peptide library). The peaks for S, P1, P2 were readily observable. Figures 4b&c show electropherograms of the products of BoNT/A LC assays in the presence of two representative 12-component combinatorial peptide libraries out of a large number of screenings. Most of the libraries screened did not have significant migration overlap with the assay products; the reaction products were readily separated and identified as shown by library 33-5 (Figure 4b). We noted that the BoNT/A LC activity actually increased in the presence of library 33-5 [33]. In a few libraries we observed significant interference of co-migrating library components with the labeled assay products. The greatest amount of interference occurred with the library designated 33-8 in which the labeled constituent peptides co-migrated with S and P2 (Figure 4c). Calculations using ExPASy [21] determined that peptides in this library carried similar mass/charge ratios as the substrate and P2 peptides.

The overlap problem was resolved by reformulating the HNTE separation buffer to contain 50 mM sodium dodecyl sulfate (SDS) and eliminating the Tween-20 detergent. This modified buffer formulation was referred to as HNSE. The addition of SDS detergent in the buffer changed the characteristics of the separation from capillary zone electrophoresis to micellar electrokinetic chromatography (MEKC) [34, 35, 36]. At 50 mM concentration SDS forms micelles; the negatively charged polar sulfate head groups are on the outside of the micelle structure. SDS, being negatively charged and present throughout the column in the separation buffer, migrates counter to the electroosmotic flow. In the presence of SDS micelles, all the peptides in the sample, i.e., substrate, product fragments, and combinatorial peptide library, were partitioned between the micelles and free solution. The most positively charged peptide had the strongest interaction with the negatively charged heads on the SDS micelles. In MEKC the longer the time a molecule is held by the micelles, the greater is the time required to reach the detector. Neutral P1 (see Table 7) migrated faster in MEKC than positively charged S or P2 as shown in Figure 5. The run times were longer than standard CE and the migration order of substrate and products to the detector was reversed. For the admixture (Figure 5b) labeled P1 migration time was 13 min, compared to about 3 min in CE (HNTE buffer). Labeled P2 migrated at 18 min in MEKC; S migrated at 23 min. The migration time of labeled library 33-8 was between 25-30 min (Figure 5c). The MEKC separation of the BoNT/A LC assay products containing library 33-8 is shown in Figure 5a wherein S, P1, P2 were readily resolved from one another and from the components of library 33-8. Overall the resolution and speed of MEKC separation in SDS buffer was still superior to that obtained by HPLC.

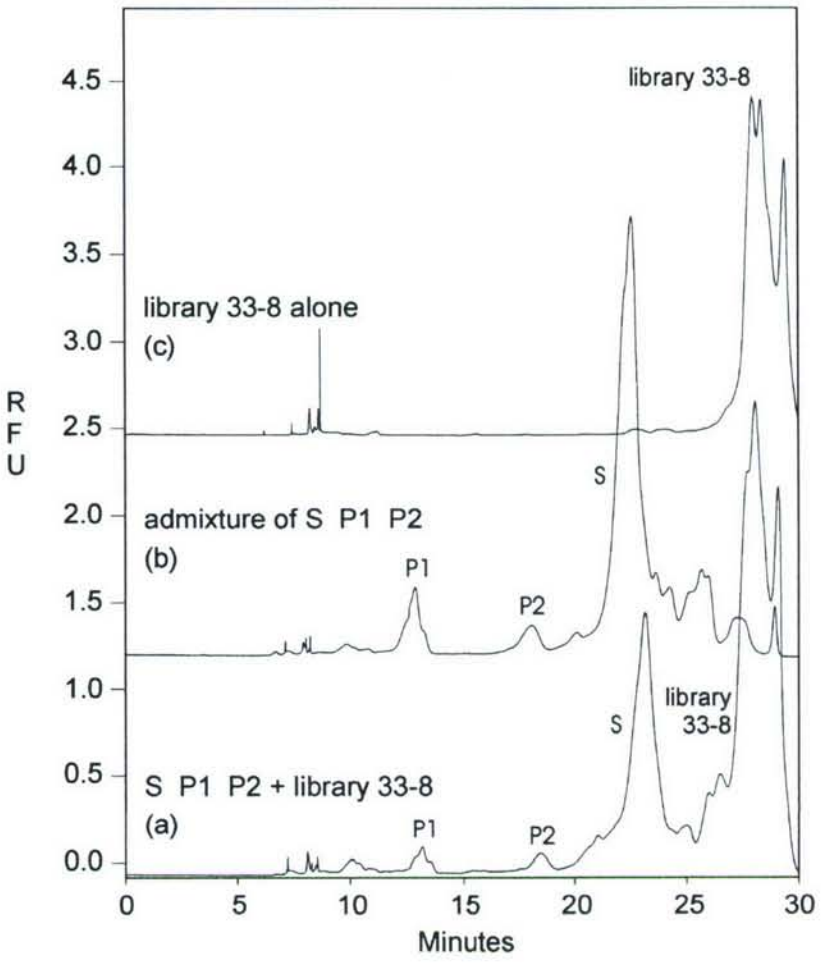

Figure 5. MEKC separation and LIF detection of products of BoNT/A LC assay. All species were labeled with CBQCA. (a) BoNT/A LC assay products containing library 33-8 (same sample of assay products as used in Fig. 5c). With MEKC separation S, P1, P2 are separated and resolved from the peptide library. (b) Admixture of S, P1, P2, no library. (c) Library 33-8 alone, no S, P1, P2.

The analysis of the reaction products of BoNT/A LC by CE LIF presented several advantages over HPLC. All of the reaction products were readily separated by CE LIF within 8 min on-column time. In contrast separation of the reaction products by HPLC and subsequent column washing required approximately 60 min column time, resulted in incomplete resolution products and provide less reliable quantitation of products in the presence of peptide libraries. CBQCA labeling was found to be an effective method for detection of the BoNT/A LC reaction products. An advantage of the method is that the dye itself is non-fluorescent. Only upon reaction with primary amines in the presence of cyanide ion is a fluorescent 7-aza-1-cyano-5,6-benzisoindole covalent adduct formed that is stable for at least two weeks. This limits background interference from unreacted dye and eliminates the need to separate fluorescent adducts from excess labeling reagents. We observed in the electropherograms several large peaks arising from reaction of the dye with components of the buffer (HEPES,

Tween-20, EDTA). We found that other CE buffers (e.g. borate) contained fewer fluorescent adducts when reacted with CBQCA (data not shown). In earlier work significant effort had been made to optimize an assay for BoNT/A LC [6, 8]. In that work the reduction and activation reactions of the intact toxin were carried out in HEPES buffer. As this work was follow-on, it was not feasible to switch to a cleaner buffer system, i.e., one with non-amine-containing compounds and less reactive with CBQCA. Despite the presence of the non-identified peaks, the assignments and quantitation of the critical peaks were readily accomplished. The fact that the extraneous buffer peaks did not cause serious problems actually adds to the utility of the techniques. A caveat with CBQCA labeling is that all species containing primary amines are labeled. Multiple peaks or shoulders may be generated if an analyte contains more than one available primary amine site and labeling of sites is incomplete. For S, P1 and P2, one major peak was identified for each. In CE the bands for P1 and P2 were nonsymmetric; the shoulders suggested that heterogeneous labeling might have occurred.

In capillary zone electrophoresis (CZE) mode, limited migration-overlap of the combinatorial peptide libraries with S, P1, P2 was observed, but it was not usually a significant problem. Since P1 contained a single labeling site, and it was well separated from both S and P2, it was chosen as a convenient reference point for assignments quantitations. We observed a few cases where overlap of the CBQCA-labeled libraries defeated resolution and quantitation by CZE of reaction products. This problem was resolved by MEKC separation [37]. Although it required longer time, MEKC served as a useful back-up method for the difficult libraries. For this work, once the BoNT/A LC assay samples were quenched, labeled and cooled, they were stable for at least two weeks. Thus we routinely ran all samples in normal capillary electrophoresis; the difficult-to-resolve samples were subsequently re-run by MEKC.

CE LIF presents the potential for convenient adaptation to multi-channel and microfluidic formats, which would further improve the ability to screen combinatorial peptide libraries for modulators of BoNT peptidase activity. To date there have been few reports regarding CE analysis of BoNT activity in the literature. The work has demonstrated the utility of CE LIF for quantitation of BoNT/A LC activity.

Analysis of botulinum neurotoxin A assays by FRET

Fluorescence resonance energy transfer (FRET) assays of BoNT/A LC were performed using SNAPtide, a synthetic peptide substrate containing the native cleavage site. Two SNAPtide substrates were used in this work. They contained identical peptide sequences but differed by the pair of FRET donor/acceptor molecules. The SNAPtide assay was very effective for high throughput screening. We used the assay in the positional scanning of a combinatorial peptide library as shown in Table 8. The structure of the libraries is given by

$$\text{Cho} - \text{O1} - \text{GABA} - \text{X2} - \text{E3} - \text{NH}_2.$$

where Cho represents cholic acid capping at the N-terminus, E is the single-letter convention for the amino acid glutamic acid. GABA, O, X, NH_2 are defined in previous sections. The nature of the data (i.e., inhibition) is similar to what could be obtained from CE LIF or HPLC (as in Tables 1-6), however the time and effort required were significantly reduced.

Table 8. Percent inhibition and mean inhibition (MI) of FRET assay by cholic acid-capped tripeptide libraries

O	=	R	C	Q	E	H	L	K	M	F	S	W	Y		MI
		15	-2	-20	-18	-19	62	20	7	-13	15	-8	-6		3.0

The cleavage of substrate was calculated in the presence of each library and corrected for the no-enzyme control, and normalized to the positive control to give percent inhibition. Each inhibition value represents the mean of four runs; standard errors were about ± 8%.

With the SNAPtide assays, the enzyme reactions and data acquisition were completed within about three hours using a microtiter plate format. Comparable effort including sample preparation for 50 samples would be approximately 12 hours for CE LIF and 30 hours for HPLC. Furthermore the microplate format reduces the amount of reagents required for the screening assays. The combinatorial peptides are custom synthesized in relatively small batches, thus expensive. The SNAPtide assay in microplates significantly reduces cost of screening the libraries.

We used the SNAPtide assay in microplate format to measure inhibition of LC activity by individual compounds. The 50% inhibitory concentrations (IC_{50}) were determined for a specific hinge peptide M1-GABA-C2C3 and two select compounds ponceau BS and amaranth. The inhibitory properties of ponceau BS and amaranth had been predicted by in silico screening of chemical data bases (G. J. Moore unpublished work). Figure 6 shows the percent inhibition versus log concentration for the three compounds. The IC_{50} values were 568, 135 and 106 μM for M1-GABA-C2C3, ponceau BS and amaranth, respectively. The SNAPtide reaction provided a homogeneous phase assay method; that is, the cleavage of the FRET-labeled substrate generated the signal. No subsequent separation step was required, as in CE or HPLC. Thus by monitoring the fluorescence emission over the course of the SNAPtide reaction we could observe formation of product as a function of time. Kinetic measurements of SNAPtide cleavage by the BoNT/A LC were carried out to obtain initial velocities versus substrate concentrations. The data were subjected to Michaelis-Menten analysis to determine characteristic K_M and V_{max} values for each SNAPtide (Figure 7, 8). For SNAPtide o-Abz/DNP, K_M = 17.7 μM, V_{max} = 47.2 pmol/min; for SNAPtide FITC/DABCYL, K_M = 32.0 μM, V_{max} = 3.02 pmol/min. In comparison the unlabeled substrate (Figure 1) that was used in capillary electrophoresis yielded K_M = 37.9 μM, V_{max} = 41.2 pmol/min (Figure 9). These results indicated that the o-Abz/DNP SNAPtide yielded Michaelis-Menten parameters more similar to the native sequence substrate than the FITC/DABCYL version.

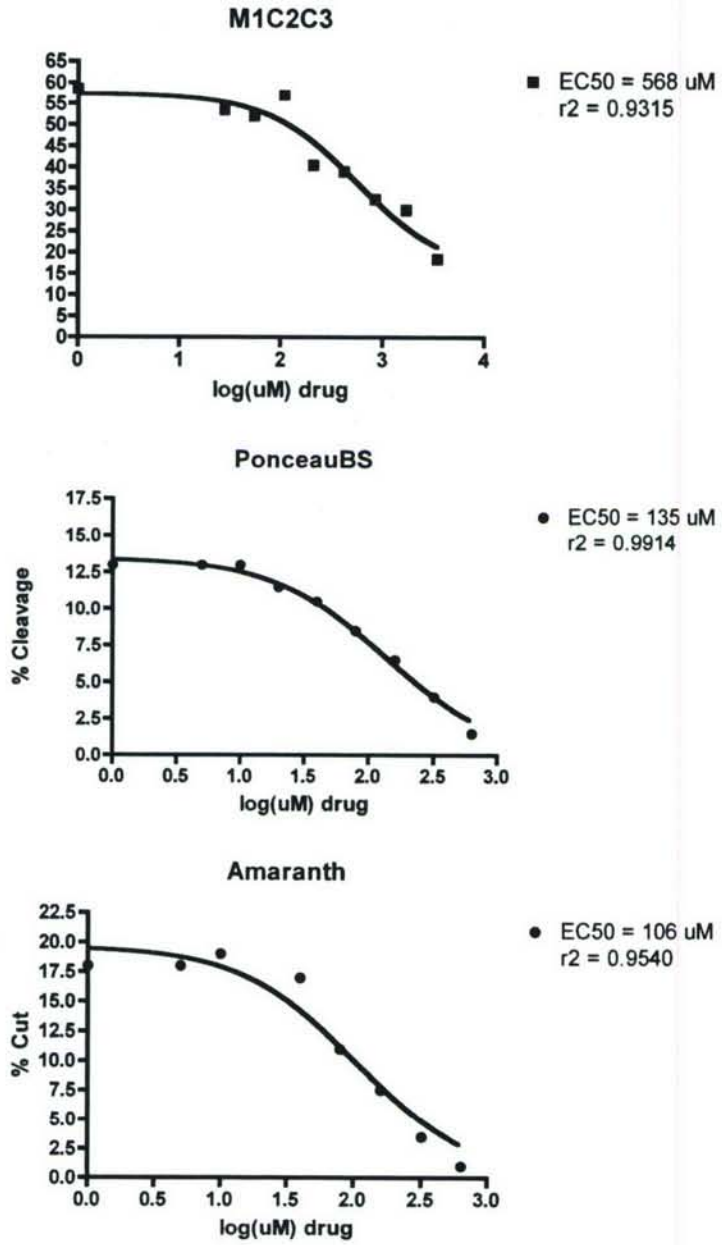

Figure 6. Inhibition (IC_{50}) of BoNT/A LC assay by peptide M1-GABA-C2-C3, ponceau BS and amaranth. Each plot represents mean of two independent assays performed duplicate.

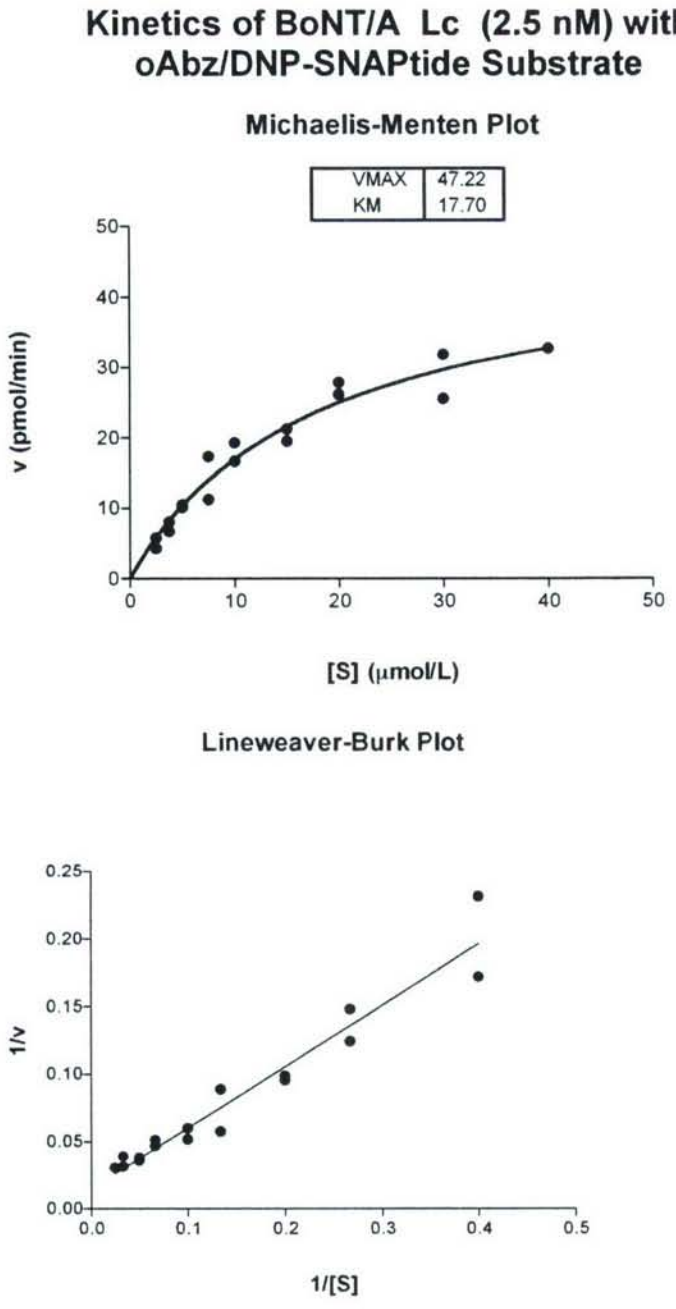

Figure 7. Kinetic study of BoNT/A LC cleavage of oAbz/DNP-SNAPtide substrate. Top: Michaelis-Menten plot, initial velocity versus substrate concentration. Bottom: Lineweaver-Burke reciprocal plot.

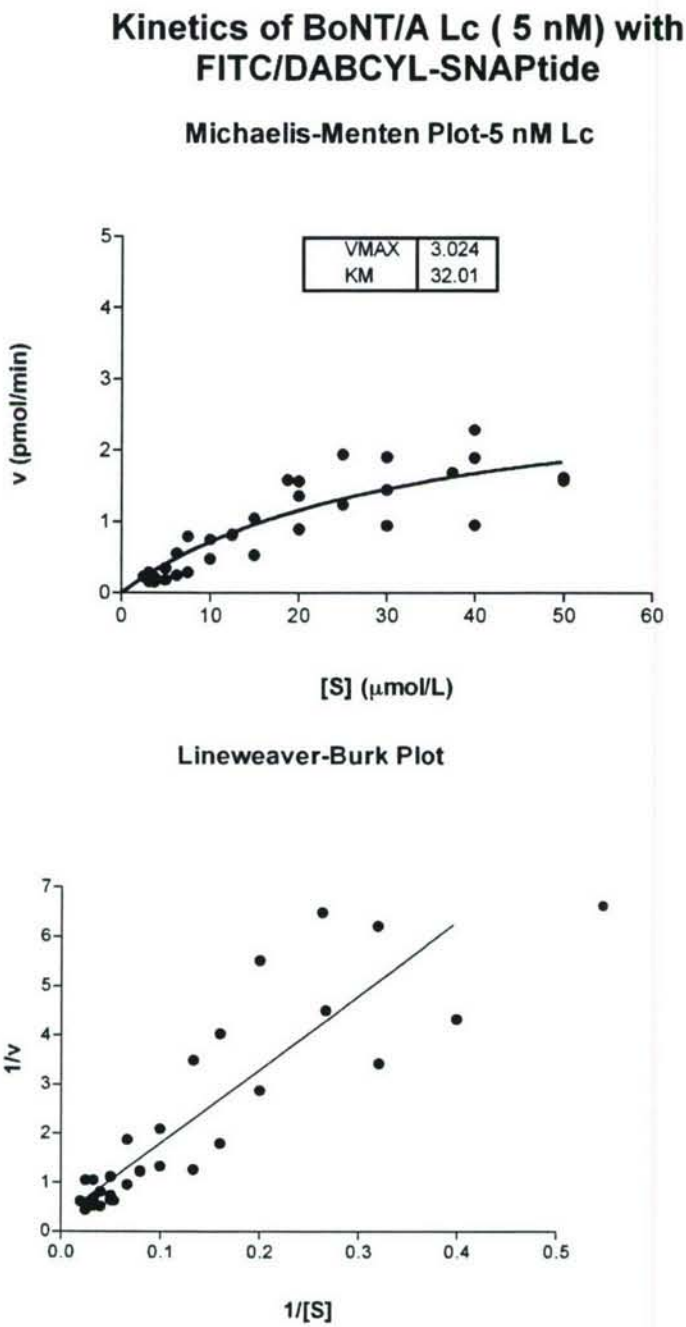

Figure 8. Kinetic study of BoNT/A LC cleavage of FITC/DABCYL-SNAPtide substrate. Top: Michaelis-Menten plot, initial velocity versus substrate concentration. Bottom: Lineweaver-Burke reciprocal plot.

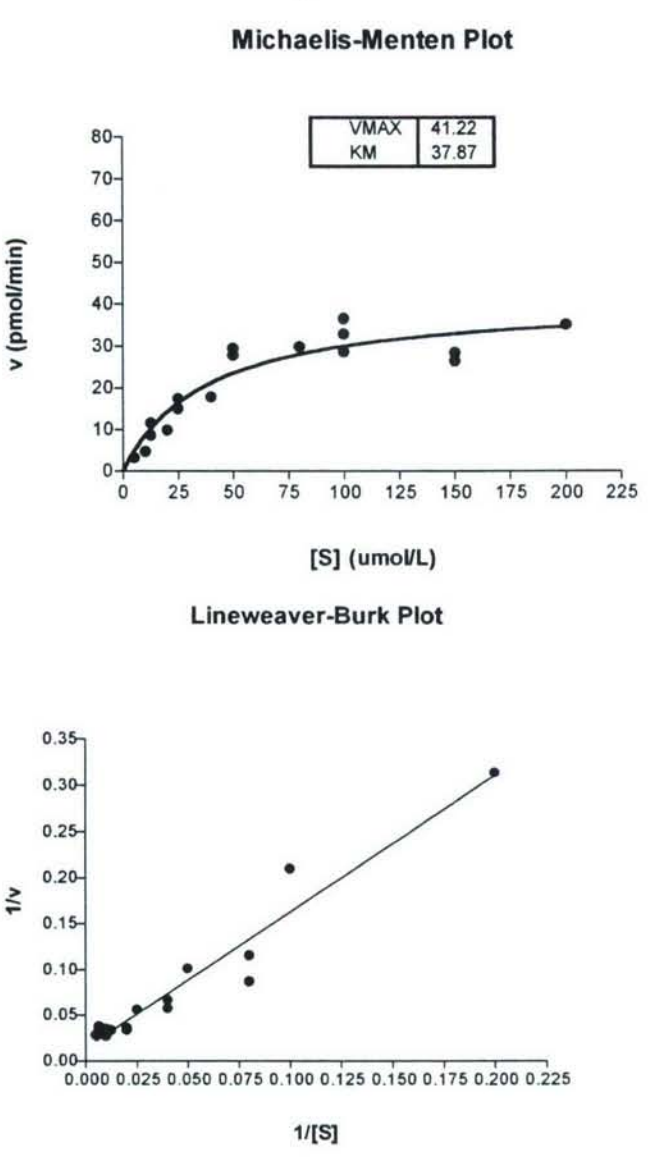

Figure 9. Kinetic study of BoNT/A LC cleavage of unlabeled 17-mer SNAP 25. Top: Michaelis-Menten plot, initial velocity versus substrate concentration. Bottom: Lineweaver-Burke reciprocal plot.

The FRET assays provided an expedient method for in vitro screening potential toxin inhibitors. For screening combinatorial peptide libraries the first step would be positional scanning of library subset as indicated by Table 8. For such initial screening, relatively small amounts of inhibitor are needed since the experiments consist of assays of single concentration of inhibitor (although replicate samples). The process of deconvolution of the combinatorial libraries is the same whether by FRET, CE LIF or HPLC. After individual compounds have been selected for further study through deconvolution, experiments to determine IC_{50} (as in Figure 6) can be performed to provide information with respect to potency and efficacy of the inhibitors. Greater details of the inhibitory mechanism can be gained through kinetic measurements of the SNAPtide assay and Michaelis-Menten analysis to determine modes of inhibition and allerosteric effects. The latter methods are very valuable for the molecular modeling and computation chemistry required for rational drug design.

The two bioanalytical chemistry methods used in the project, CE LIF [37] and FRET (SNAPtide), are complementary approaches to analysis of enzymatic peptidase assays (e.g. BoNT/A LC). CE LIF, although more time and reagent consuming, can accommodate substrates of the natural sequence and form (i.e., unlabeled). FRET assay, while significantly faster and less consuming of reagents, requires labeled substrate. In future work we intend to make selective use of each assay technique to attain high throughput and to minimize the possibility of artifacts in screening process.

Conclusions

The work reported herein has made advances in toxin research toward discovering potential inhibitors and lead compounds. We conclude that combinatorial hinge peptide libraries are a source of potential lead compounds for modulation of bioprocesses. The conformational diversity in the peptides contributes a large amount of chemical function to the libraries. We showed that modulation effects could be uncovered in libraries containing as few as three variable peptide sites. Furthermore through the positional scanning technique we were able to discover active sub-libraries for markedly different bioassays (enzymatic peptidase and cellular) using the same libraries as a start point. These results indicate that such libraries are a rich source of ligands for many different drug discovery applications.

The work developed improved methods of analysis of enzymatic peptidase assays. We conclude that capillary electrophoresis with laser induced fluorescence can be used to quantitate the peptidase activity of the light chain of BoNT/A. In the assay the substrate can be used in its natural state without any labeling and eliminates the potential for assay artifacts associated with the presence of spectroscopic labels. A variation on the capillary electrophoresis technique, micellar electrokinetic chromatography (MEKC), was shown to provide quantitative analysis in such instances where the inhibiting peptide libraries masked the bands of the assay products. The methods are not limited to BoNT/A but are useful for other botulinum serotypes and other classes of toxins such as anthrax or tetanus. More recent research work from the laboratory has employed a homogeneous phase FRET assay for BoNT/A peptidase activity using SNAPtide™. It is amenable to high throughput formats such as 96 or 384-well microplates whereby all wells are analyzed essentially in parallel. Another distinct advantage of the FRET assay is that the enzyme activity data can be obtained as a function of time (rather than endpoint). This kinetic data can be used to perform detailed Michaelis-Menten analysis to determine reaction velocities, the Michaelis-Menten constant (K_M) and the modes of inhibition. These parameters are useful in rational drug design, the process of advancing and developing specific "hits" from screening assays (e.g. hinge peptides) into lead compounds and ultimately into drugs.

This work was conducted through the sponsorship of DRDC Technology Investment Fund. The mandate of the TIF program is to foster forward-looking, potentially high-payoff research projects that lead to important new in-house competencies. In keeping with the mandate, we believe that we were successful. The present report describes the exit point of the project funded under the Technology Investment Fund. Original research from the project was published in scientific journals directed at drug discovery and bioanalytical chemistry. In addition to this original research the TIF project has pointed the way to new directions and future work within DRDC and the contractor network. A new DRDC initiative based on micro affinity chromatography with mass spectrometry detection has been initiated to develop faster and cheaper methods of molecular screening. Also at DRDC, functional assays based on neuronal tissue culture are in development to verify that the rapid affinity methods are biologically valid. To complement the experimental screening methods, we have undertaken in silico screening of chemical data bases, i.e., using computational chemistry and structure function relationships to search for likely inhibitor compounds. A significant accomplishment of the TIF project was the establishment of a toxin research hub at DRDC Suffield. A recent article on botulinum toxin research and development in the Suffield hub has been published by Chan et al. [38].

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List of symbols/abbreviations/acronyms/initialisms

BoNT/A	Botulinum neurotoxin serotype A
BoNT/B	Botulinum neurotoxin serotype B
CBQCA	3-(4-carboxy-benzoyl)-2-quinoline-carboxaldehyde
CE LIF	Capillary electrophoresis laser-induced fluorescence
CHO	Cholic acid
ExPASy	**Ex**pert **P**rotein **A**nalysis **Sy**stem
FRET	Fluorescence resonance energy transfer
GABA	γ-aminobutyric acid
HPLC	High performance liquid chromatography
LC	Light chain of BoNT
MI	Mean inhibition
MEKC	Micellar electrokinetic chromatography
SNAP-25	Synapotsomal associated Protein 25 kDa
S, P1, P2	Substrate and product 1 and product 2 of LC assay
VAMP	Vesicle-associated membrane protein
R, C, Q, E	Arginine, cysteine, glutamine, glutamic acid
L, K, M, F	Leucine, lysine, methionine, phenylalanine
S, W, Y, H	Serine, tryptophan, tyrosine, histidine
G, P, A, I,	Glycine, proline, alanine, isoleucine
V D, N, T	Valine, aspartic acid, asparigine, threonine

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Combinatorial peptide libraries offer an expedient source of structurally diverse molecules that could serve as lead compounds in the development of drug therapies to toxins. The libraries have typical structures of X1 – X2 – hinge – X3 – X4, where X1 through X4 are near-equimolar mixtures of twelve α-L-amino acids and hinge = γ-aminobutyric acid. Screening of the libraries for inhibitory activity in assays for botulinum neurotoxins A and B (BoNT/A, BoNT/B) and saxitoxin uncovered potent library subsets. For effective screening of the peptide libraries, improved methods of analysis were sought. We report on development of a capillary electrophoresis laser-induced fluorescence (CE LIF) method for measuring BoNT/A peptidase activity and for screening peptide libraries for inhibitory effects. A second analytical method for quantitation of BoNT/A assays was employed based on fluorescence resonance energy transfer (FRET). The FRET assay is homogeneous phase, i.e., no separation step is required. Thus assay time was reduced and throughput increased. The research described in this report was supported by the Technology Investment Fund of Defence R&D Canada.

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