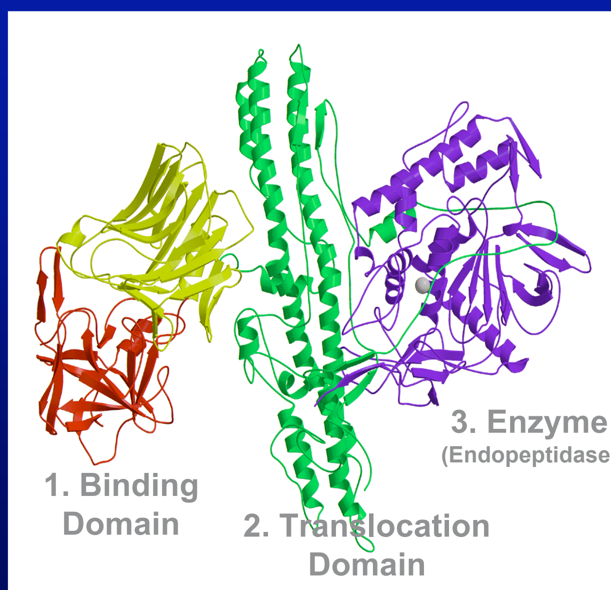
Endopeptidase assays for botulinum toxins

D. Sesardic, NIBSC, UK

Monday 13th November, 2006

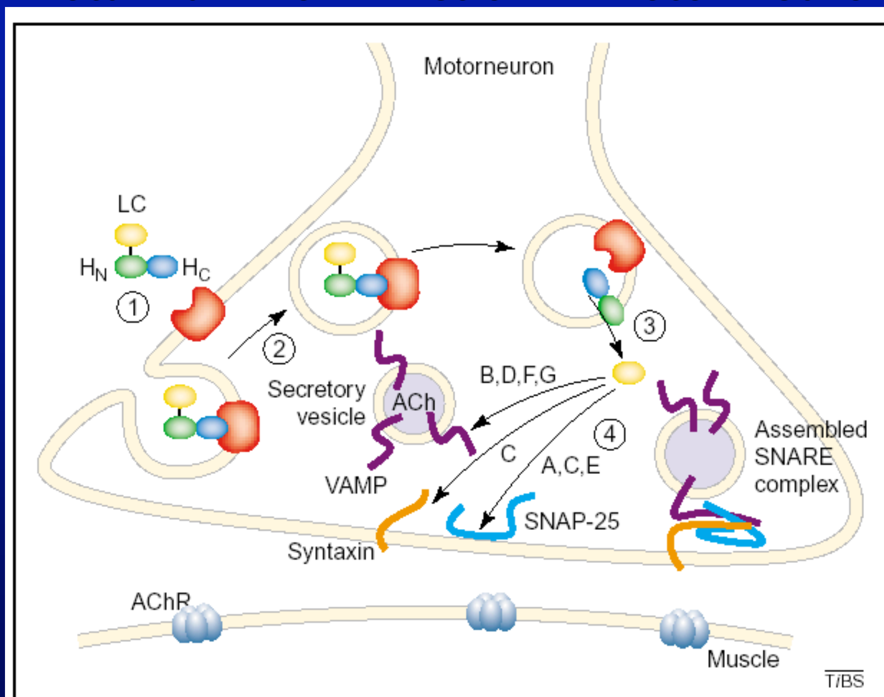
Session 3A: Potential Replacement

Key functions of botulinum toxin



1. Binding domain allows protein to attach to nerve
2. Translocation domain moves enzyme from one compartment to interior of nerve
3. Endopeptidase activity inside the cell – highly specific

Botulinum Toxin Action In Motor Neuron



Kathryn Turton, John A. Chaddock and K. Ravi Acharya
TRENDS in Biochemical Sciences Vol.27 No.11 November 2002: 552

Specificity and location of clostridial neurotoxin cleavage sites

TOXIN	SPECIFICITY
BoNT/A	SNAP-25 Gln $_{197}$ - Arg$_{198}$
BoNT/B	VAMP Gln $_{76}$ - Phe $_{77}$
BoNT/C	SNAP-25 / Syntaxin Lys $_{253}$ Ala $_{254}$
BoNT/D	VAMP Lys $_{59}$ - Leu $_{60}$
BoNT/E	SNAP-25 Arg $_{180}$ -Ile $_{181}$
BoNT/F	VAMP Gln $_{58}$ - Lys $_{59}$
BoNT/G	VAMP Ala $_{81}$ - Ala $_{82}$
TeNT	VAMP Gln $_{76}$ - Phe $_{77}$

NIBSC
Assuring the quality of biological medicines

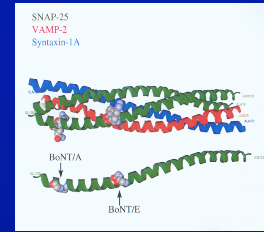
Endopeptidase assays for Botulinum neurotoxins: general principles

- Based on *in vivo* intracellular mode of action
  - Zinc-dependent endopeptidase activity of toxin L-chain
- Require synthetic or recombinant substrate
  - Peptide > 30 aa, structure, binding site
- Require suitable detection system

Examples of detection systems in literature

- Exposed epitope post exposure to botulinum toxin detected with targeted antibody (Hallis et al, 1996, Ekong et al 1997, Witcome et al., 1999)
- Capillary electrophoresis, RP-HPLC (Sesardic et al., & Ekong et al. 1997)
- HPLC with fluorescent substrate (Anne, Cornille et al., 2001)
- Mass Spectrometry detecting substrate size change by MALDI-TOF-MS or HPLC-ESI/MS/MS (Boyer et al, 2005,Barr et al, 2005, Kalb et al, 2006)
- Loss of FRET (Fluorescence Resonance Energy Transfer) fluorescence –based sensors (Schmidt et al, 2003, Dong et al, 2004, Parpura et al,2005)
- Fluorescence polarization (Gilmore et al, 2005)
- Micromechanosensor combined with blotting (Liu et al, 2003)
- Native VAMP (synaptosomes) capture (antibody) via plasmon resonance (Biocore®) (Ferracci et al, 2005)

Why endopeptidase assays ?



- Reflects one of the important modes of toxin action
- Can provide sensitivity comparable to in vivo mouse models
- Highly specific to toxin serotype
- Variety of modifications and detection systems possible

Requirements and challenges

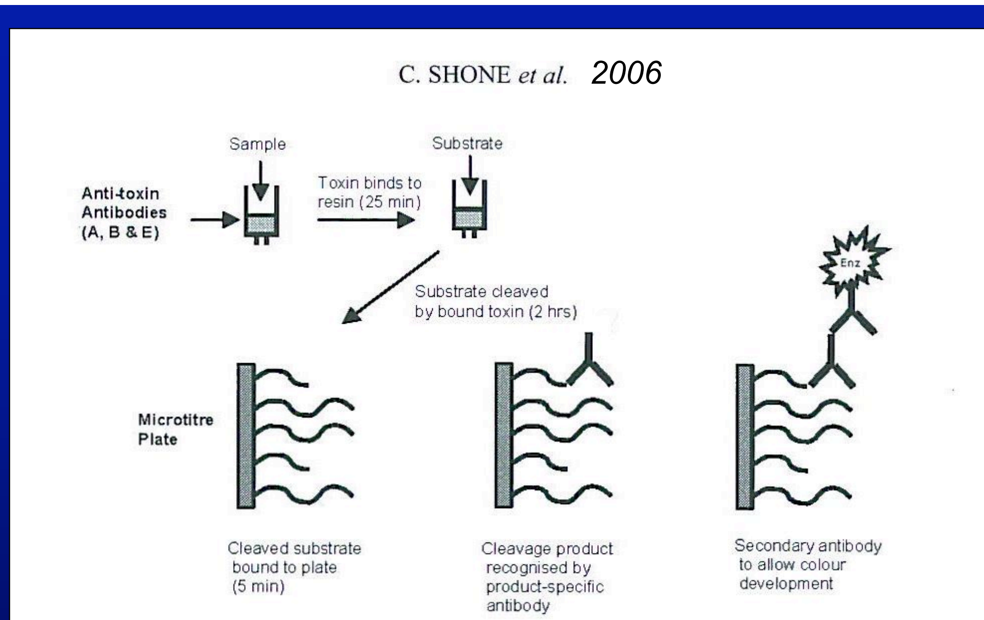
Assay requirements and needs for detection of toxin in environment or biological samples and for potency testing of products are very different

- Detection in environment and biological samples
- Sensitivity
- Specific for serotype and sub-type
- Complex matrix system
- Speed, portability, throughput
- Availability of reagents

- Potency
- Sensitivity
- Relevant for product and production process
- Accurate, precise, reproducible
- Easy and transferable
- Availability of reagents

Assays for toxin detection

- Must be suitable for detection of toxin in complex and difficult samples which interfere in endopeptidase assays
- Purification of toxin with serotype specific antibody prior to endopeptidase assay improves sensitivity
- Most assay development studies with highly purified commercially sourced toxins
- Limited validation studies
- Assay formats designed specifically for toxin detection have not been used in potency testing



Cleavage of SNAP-25 by BoNT/A and BoNT/E in same assay
Sensitivity ~ 0.1 mouse LD50
No loss of sensitivity in presence of human serum

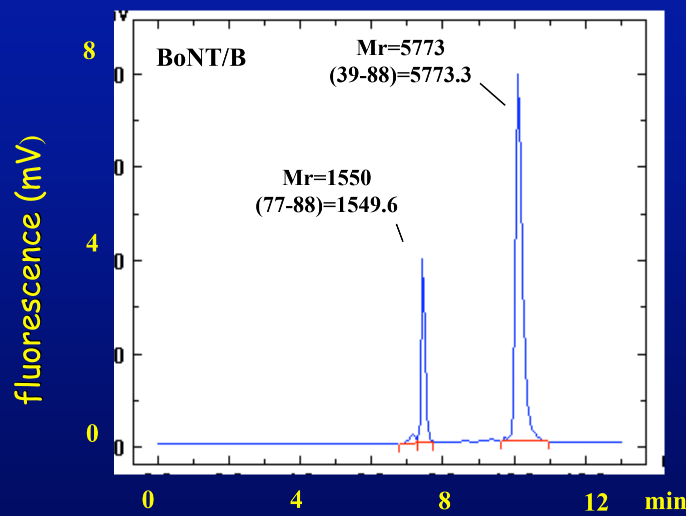
Analysis of BoNT/B digestion of Pya^{88}-VAMP2 $_{39-88}$ by RP-HPLC

fluorescence (mV)

8

4

0



0 4 8 12 min

Substrate from
B. Roques

Gradient : 12-64% AcN in 0.1% TFA over 10 min
100 x 2 mm ODS Hypersil 1 ml/min

Endopeptidase-MS: multiplex format to detect all seven serotypes

BoNTs	BoNT Substrates and Cleaved Peptide Sequences	m/z
	BoNT-A BoNT-C	
1. BoNT-A, -C	Biotin-KGSNRTRIDEANQRATRMLGGK-Biotin	2911.6
A-NTP	Biotin-KGSNRTRIDEANQ	1714.8
A-CTP	RATRMLGGK-Biotin	1215.6
C-NTP	Biotin-KGSNRTRIDEANQR	1871.0
C-CTP	ATRMLGGK-Biotin	1059.6
	BoNT-B BoNT-G	
2. BoNT-B, -G	LSELDDRADALQAGASQFETSAAKLKRKYWWKNLK	4038.2
B-NTP	LSELDDRADALQAGASQ	1759.8
B-CTP	FETSAAKLKRKYWWKNLK	2297.3
G-NTP	LSELDDRADALQAGASQFETSA	2294.6
G-CTP	AKLKRKYWWKNLK	1761.6
	BoNT-F BoNT-D	
3. BoNT-D -F	AQVDEVVDIMRVNVDKVLERDQKLSELDDRADALQAGAS	4311.2
D-NTP	AQVDEVVDIMRVNVDKVLERDQK	2698.4
D-CTP	LSELDDRADALQAGAS	1631.8
F-NTP	AQVDEVVDIMRVNVDKVLERDQ	2570.4
F-CTP	KLSELDDRADALQAGAS	1759.9
	BoNT-E	
4. BoNT-E	IIGNLRHMALDMGNEIDTQNRQIDRIMEKAD	3610.9
E-NTP	IIGNLRHMALDMGNEIDTQNRQIDR	2923.6
E-CTP	IMEKAD	706.3

Barr et al., 2005

Endopeptidase-MS: sensitivity is dependent on sample matrix

Table 2.
LODs for BoNT A, B, E, and F in mouse LD_{50} as determined by mouse bioassay and Endopep–MS in buffer, serum, and stool

Toxin type	Mouse bioassay[a]	Endopep–MS in buffer[b]	Endopep–MS in serum	Endopep–MS in stool
A	1	0.01	10	100
B	1	0.01	0.5	5
E	1	0.08	0.1	0.5
F	1	0.01	0.5	5

[a] Because the mouse bioassay is the standard detection method, the results of the mouse bioassay define the LODs; as a result, all are defined and reported as 1 mouse LD_{50}.
[b] These LODs were determined and reported in [4] and [5].

Kalb et al., 2006

Validation of assays for toxin detection

- Use of endopeptidase assays combined with antibody capture and suitable detection system may replace use of animals for detection of toxins in biological samples
- Validation studies must be designed to fit the purpose and include relevant samples and controls
- Low and high concentration of (specific) reference toxin in defined matrix must be used to determine and monitor sensitivity of methods

Assays for potency

- Must be suitable to accurately quantify small amounts of active toxin in finished product
- Must detect toxin in presence of high concentration of bulking and stabilising material of known quantity
- ELISA plate format with peptide substrate and targeted antibodies to detect exposed epitope selected at NIBSC (Ekong et al., 1997)
- SNAP-25 assay adopted to verify manufacturers potency as a consistency test for clinical samples containing type A toxin (Sesardic et al, 1997, Sesardic et al, 2002)
- Validation undertaken at NIBSC: Gaines Das et al., 1999

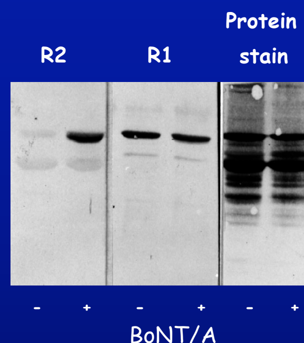
Reagents for BoNT/A and BoNT/E endopeptidase assays

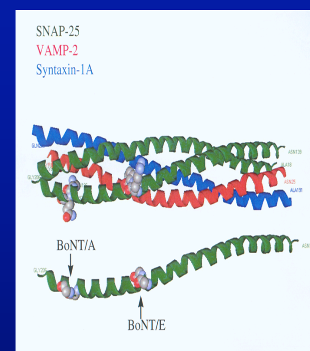
Substrates

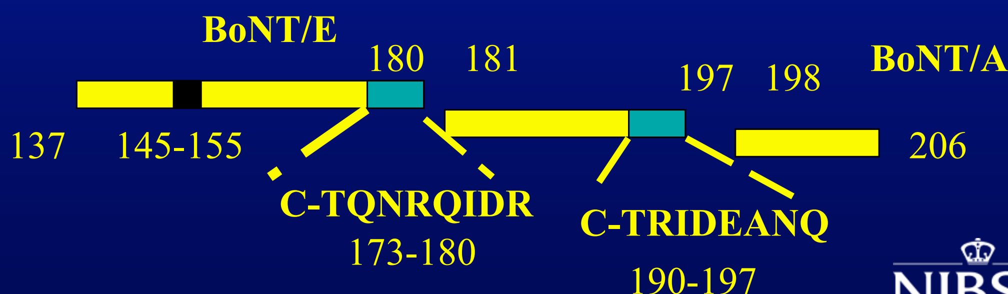
Synthetic SNAP-25 137 - 206

Recombinant SNAP-25 134 - 206

Antibodies







SNAP-25 assay: Dose response curves and correlation with LD50 for type A toxin in clinical samples and reference preparations

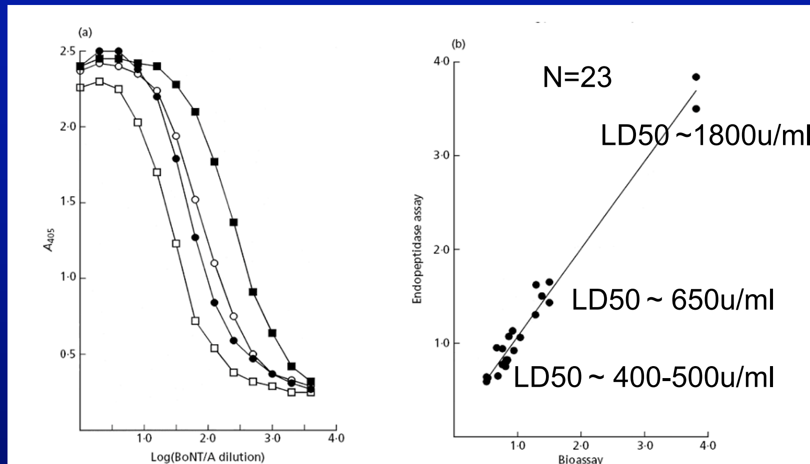
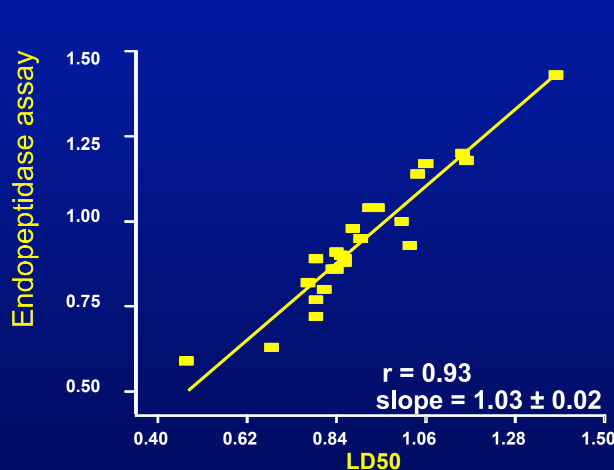
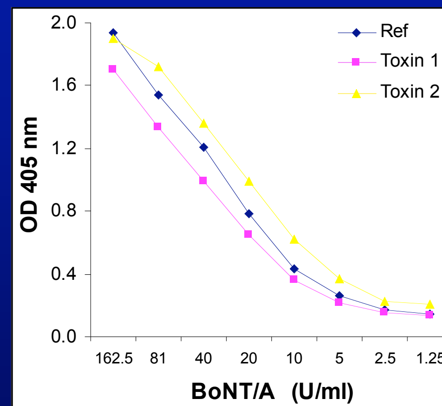


Fig. 4. Endopeptidase activity of therapeutic preparations of BoNT/A. (a) Microtitre plates sensitized with SNAP-25-MBP were treated with serial dilutions (initial dilution = 2 ml per vial) of three different therapeutic preparations of BoNT/A (□, 1x ;○, 2x; ■, 3x; ●, in-house reference standard) which had all previously been reduced as described in the text and incubated for 2 h at 37 °C. Cleaved SNAP-25-MBP was detected using the R2 antibody specific for the cleavage product as described in the Methods. (b) Comparison of the relative potency of 23 different therapeutic preparations of BoNT/A determined using the endopeptidase assay and *in vivo* bioassays. The regression line (r¯0.95) had a slope of 1±03.

Ekong et al., 1997

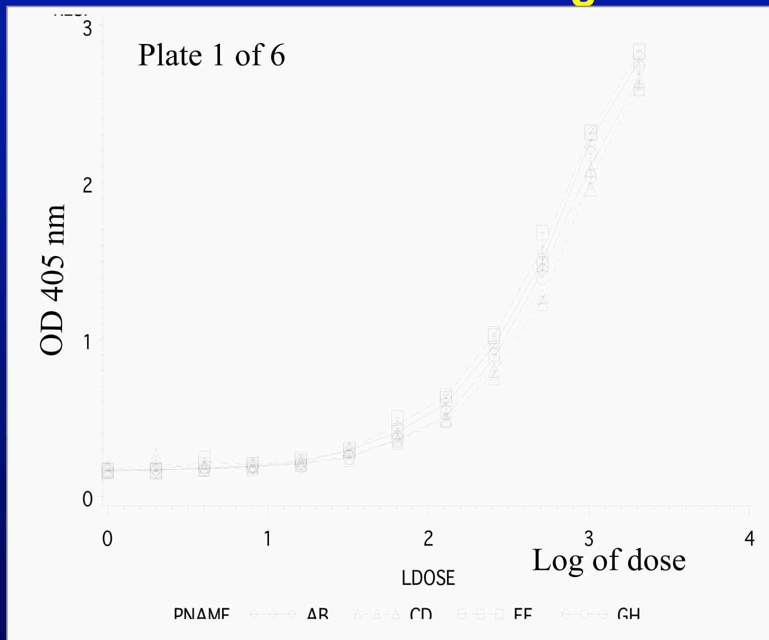
Correlation between LD50 and SNAP-25 endoptidase assay for A toxin in final product





Potency expressed relative to a product specific reference standard

BoNT/A *in vitro* assay design: factors contributing to variability



3 Vials of Ref

3 Vials of Test

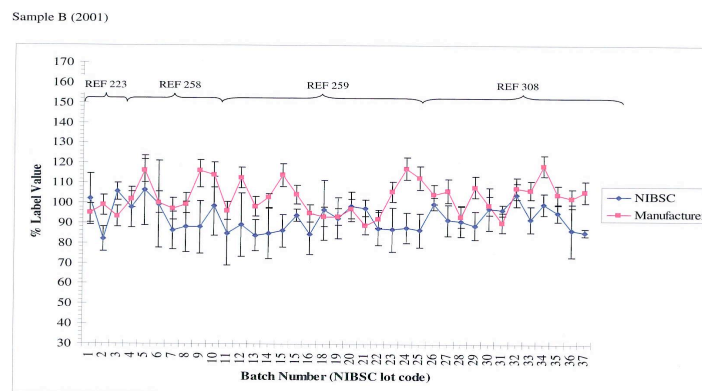
Variability:
between vials
position on plate

Within plates
CV ~ 6-12%
Between plates
CV ~ 11%
Inter-assay
CV < 25%

Gaines-Das et al., 1999

SNAP-25 assay provides an effective method to confirm consistency of finished product

Product B



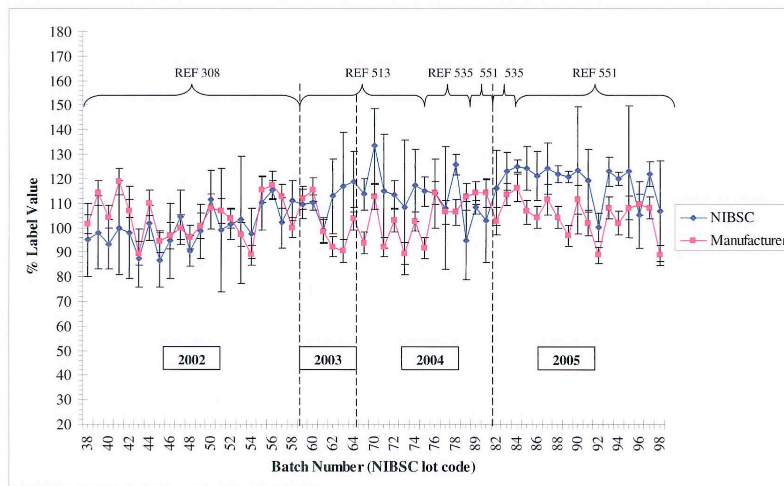
Manufacturer LD50 – 17,760 mice
NIBSC - in vitro

Sesardic et al., 2002

SNAP-25 potency expressed relative to standard, calibrated in LD50 units.

Product B

Sample B (2002-2005)



2001-2005

Manufacturer LD50 – 46,560 mice

NIBSC - in vitro endoptidase assay

Endopeptidase assays at NIBSC: Summary

- SNAP-25 in use since 1999 as a consistency batch release test to confirm manufacturers LD50 data
- Potency generally correlate and are comparable to LD50 values for both products
- Product specific and bulk specific reference used at NIBSC and is essential
- Assay set up for serotypes A, B, C, E and F

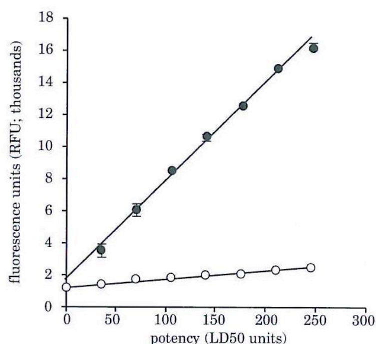
NIBSC
Assuring the quality of biological medicines

Endopeptidase assays for potency: some initiatives by manufacturers

- Immobilised SNAP-25 cleavage
  - Detection by antibody
- In solution cleavage of SNAP-25
  - Fluorescent substrates
- Different readouts / assay times
  - Increase in fluorescence polarization signal

Dose response curve for collision fluorescence activity SNAP-25 endopeptidase assay

Figure 1: Endopeptidase assay calibration curves



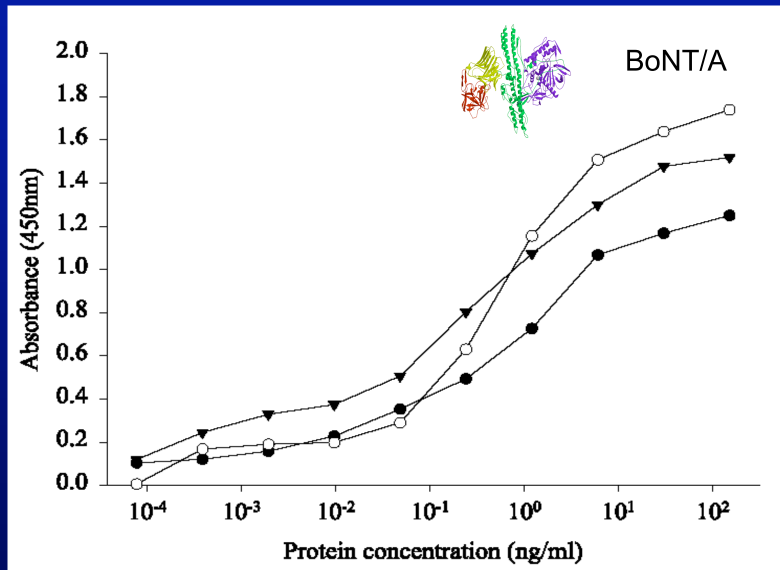
Typical calibration curves for a Dysport® reference preparation, and for an excipient-only control (without toxin), plotting fluorescence units against potency in mouse LD50 units/well.

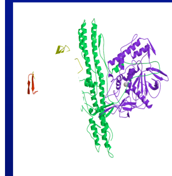
● = Dysport® (R^2 = 0.9954); ○ = excipient-only control (R^2 = 0.983). Each data point is a mean of triplicate measures; error bars represent 1 standard deviation. Data was generated during recent method development work by Ipsen Ltd.

Close agreement to LD50 determined potency
CV <10%

D W Straughan, ALTA 34: 305-313 (2006)

SNAP-25 assay cannot detect changes to the Heavy chain





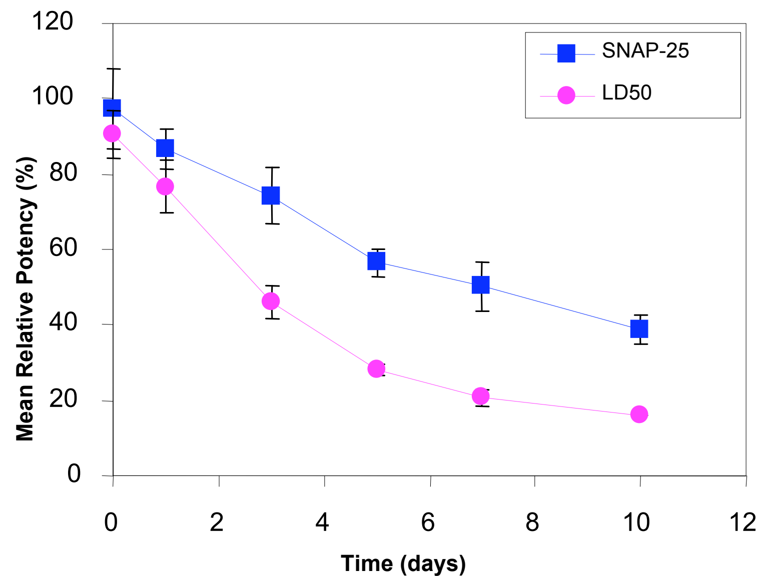
nLHn/A

recLHn/A

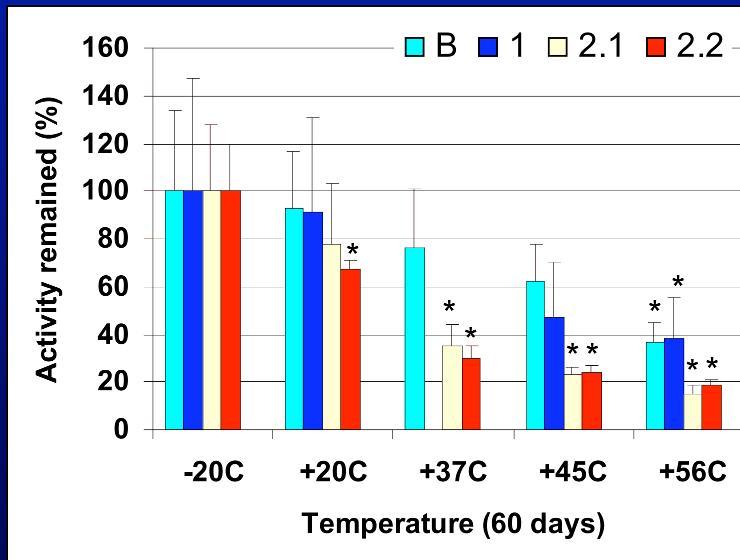
Inactive In Vivo

Chaddock et al. Protein Expression and Purification 25 (2002) 219–228

SNAP-25 and LD50 assay results of finished product exposed to high temperature (75°C) do not agree



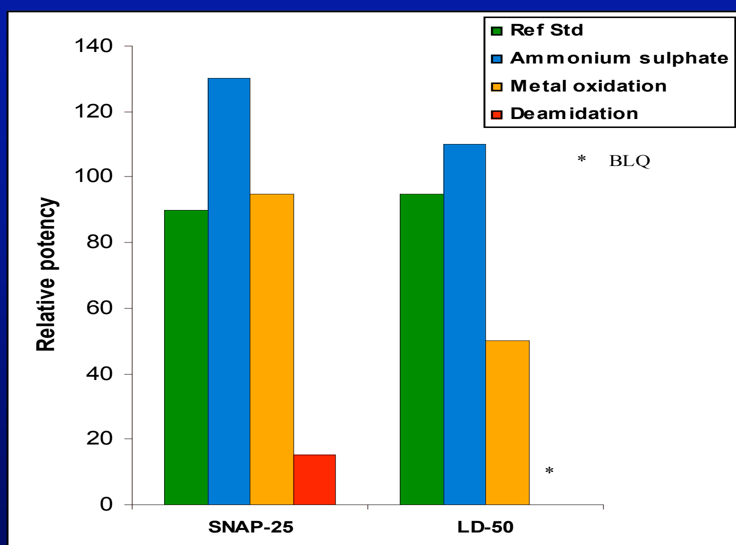
SNAP-25 predicted stability of different formulation



Formulations
B and 1
are more stable
than formulation 2

Order of stability
confirmed *in vivo*

SNAP-25 assay and LD50 may not agree for denatured toxin in product



Enzyme activity is higher than LD50

Endopeptidase as a potency test – Strengths

- Entirely in vitro model
- Objective and measurable response
- Rapid response (minutes to hours)
- Statistically meaningful to calculate potency
- Adequate precision and reproducibility
- Potentially useful for testing of final filling lots
- Excellent model to monitor consistency

Endopeptidase as a potency test – Limitations

- Partially functional assay detecting only L-chain activity
- Changes to Hc domain of toxin are not detected
- Multiple reagents and experimental steps
- Continuing supply of critical reagents
- Equipments and training needed
- May not be useful for stability studies

Validation of endopetidase assays for potency

- Use of endopeptidase assay in the final fill stage of production process is feasible, subject to validation
- Product specific reference calibrated in LD50 is required
- Validation studies must be designed to fit the purpose and include different products of wide range of potency and derived from different bulk toxins
- Validation studies should be based on understanding of critical stages of production process and their effect on toxin activity
- Supported by data from Hc binding studies (antibody or SV2 peptide harbouring receptor for A toxin – Dong et al., 2006)