Mouse abdominal ptosis or flaccid paralysis: non-lethal mouse model for Botulinum toxin potency testing

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Tuesday 14th November, 2006

Session 4B: Refinement

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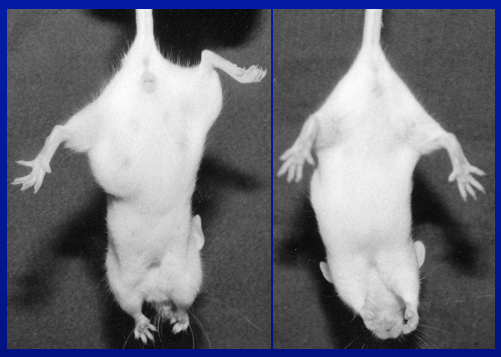
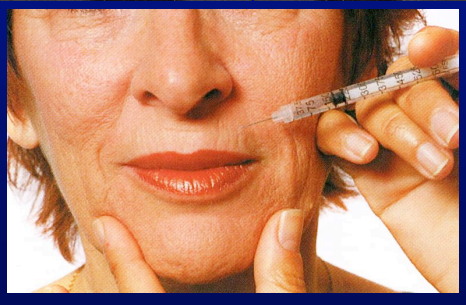
Why mouse flaccid paralysis assay ?





- Humane end point : only sub-lethal toxin dose is used
- Fast : 24-48 hrs compared to 72-96 hrs in lethality test
- Fully functional : all toxin mode of action lead to muscle paralysis
- Easy : no specialised equipment required
- Relevant: dosing similar to clinical use - muscle paralysis rather than systemic toxicity

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Road to replacement of lethality end-point

- Methods, principles and historical development
- Dose response studies with different serotypes
- Validation for use as a potency assay for type A Botulinum toxin products
- Correlation with LD50
- Assay precision, accuracy, reproducibility and transferability
- Use as a batch release potency test
- Steps towards international validation

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Historical developments of methods for botulinum toxins

Assay of Botulinum a Toxin with Goldfish.* (19925)

T. E. Cartwright and Max A. Lauffer.

From the Department of Biophysics, University of Pittsburgh, Pittsburgh, Pa.

The purpose of this report is to describe the successful use of the common goldfish as an assay animal for botulinum toxin. Goldfish have the obvious advantage that they are inexpensive and can be maintained healthy in a very small space.

The goldfish has been used extensively as a laboratory animal in toxicity tests. Pittenger and Vanderkleed(1), Pittenger(2), and Lapenta(3) observed specific reactions in

* Publication of the Department of Biophysics.

Proc. Soc. Exptl. Bio Med., 1952

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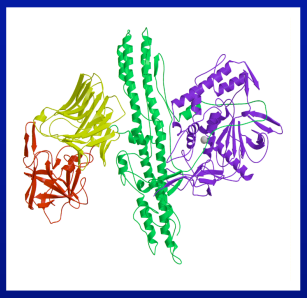
Historical developments of methods for botulinum toxins

- Systemic – mouse LD50
  - IP 1 LD_{50}, Sakaguchi *et al.*, 1964
    Schantz *et al.*, 1964
- Local paralysis models
  - IM 0.1 LD_{50}, Sugiyama *et al.*, 1975
  - SC 0.1 LD_{50}, Takahashi *et al.*, 1990



- Hind limb paralysis, Duchen, 1968, Sugiyama et al., 1975
- Regional chemodenervation, Pearce et al., 1995
- Flaccid paralysis, Sesardic et al., 1996, 2004, Jones et al. 2006
- Digit abduction, Aoki, 2001,2002, Rosales et al., 2006
- Running activity, Keller, 2006

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Mouse local flaccid paralysis/abdominal ptosis: Methods

- Mice injected S.C. with 100µl of varying concentrations of toxin (~ 0.3-3.0 LD50/ml in GPB) in the left inguinocrural region
- Animals scored by two or more independent observers at 24 and 48 hours
- Mice scored according to the size of the local bulge formed from the flaccid paralysis:

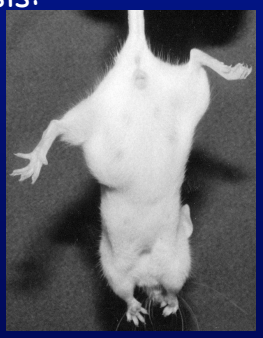
Normal = 0

Limited = 1

Moderate = 2

Substantial = 3

Maximal = 4



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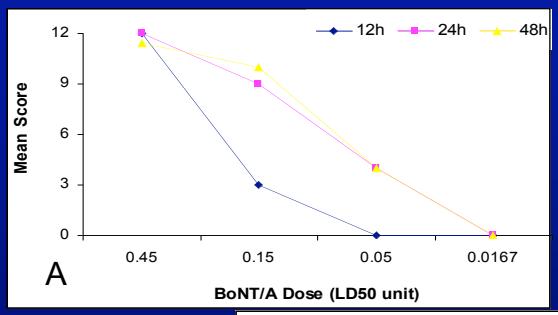
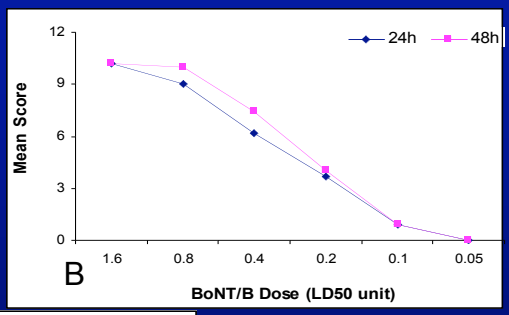
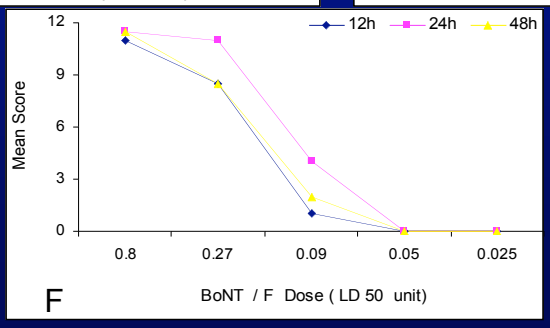
Dose response curves with Botulinum toxins A, B and F







Local muscular paralysis is dose dependent with most Botulinum toxins

Toxin	A	B	C_1	E	F
Paralysis *	0.2	1.0	0.2	1.0	0.2
Linear dose range	0.05-0.2	0.1-1.0	0.05-0.2	0.1-1.0	0.02-0.2

* Highest dose of toxin inducing paralysis without systemic toxicity

Suitable dose response with all serotypes with exception to botulinum type D toxin

Sesardic *et al.*, Movement Disorders, 2004

Validation of paralysis assay for potency testing of products

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Refinement and Validation of an Alternative Bioassay for Potency Testing of Therapeutic Botulinum Type A Toxin

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(Received September 9, 1995; Accepted September 9, 1995)

Abstract: The type A neurotoxin produced by *Clostridium botulinum* is a potent neuromuscular blocking agent which causes paralysis by preventing the release of neurotransmitter from motor neurones. This property has led to the use of the toxin in the treatment of a number of neuromuscular diseases involving muscle spasms. At present, the only recognised assay with the specificity and sensitivity to estimate accurately the potency of botulinum toxin in clinical preparations is bioassay, in which lethality is used as the end point. Refinement of this assay, with respect to the end point, was explored on the basis of the development of flaccid paralysis of muscles following subcutaneous injection of the toxin at the inguinocrural region. Potency estimates, relative to in house reference preparations, for different therapeutic preparations obtained using flaccid paralysis as a scored response gave excellent agreement with estimates obtained in independent assay using the currently required control method. This study demonstrates that an alternative, more humane bioassay for potency testing of clostridia neurotoxins gives valid estimates equivalent to those currently in use.

Pharmacology and Toxicology, 1996

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Dose response curves for same preparation in LD50 and non-lethal assay

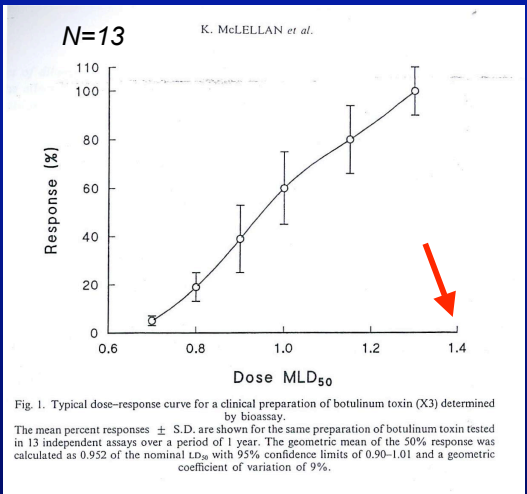

Fig. 1. Typical dose–response curve for a clinical preparation of botulinum toxin (X3) determined by bioassay.
The mean percent responses ± S.D. are shown for the same preparation of botulinum toxin tested in 13 independent assays over a period of 1 year. The geometric mean of the 50% response was calculated as 0.952 of the nominal LD_{50} with 95% confidence limits of 0.90–1.01 and a geometric coefficient of variation of 9%.

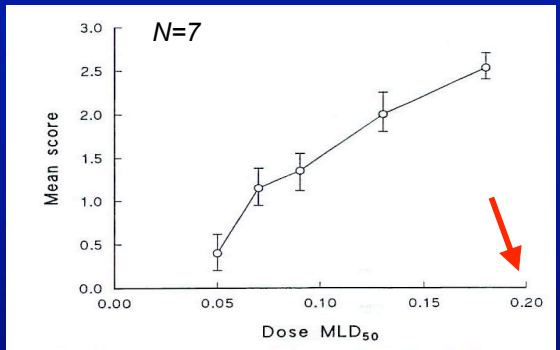

Fig. 1. Dose-response curve for a preparation of botulinum toxin determined by flaccid paralysis assay. The mean score estimates±S.D. for the same preparation of botulinum toxin tested in seven independent assays are shown. The dose of test toxin used was between 0.05–0.2 nominal mouse LD_{50} units. The geometric mean of the 50% response was calculated as 0.096 of the nominal LD_{50} with 95% confidence limits of estimates of 0.084–0.111 and a geometric coefficient of variation of 16%.

Mc Lellan et al 1996

Sesardic et al 1996

Dose response curves suitable for calculation of potency

Maximum dose in paralysis assay is sub-lethal

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Paralysis assay is ten-fold more sensitive than LD50

Table 2.

Comparison of doses giving 50% responses by the conventional (LD_{50}) and the flaccid paralysis (ED_{50}) assay methods. Values for conventional and flaccid paralysis assays are expressed as nominal mouse LD_{50} units/dose where the dose was 0.1 ml. The number of independent estimates of the 50% values is denoted N.

Preparation	N	LD_{50}	N	ED_{50}
A	17	1.15	3	0.092
B	13	0.95	7	0.096
C	8	1.12	3	0.111
D	3	1.90	3	0.206

Mice experience no obvious signs of distress or limitation to move freely and feed. All fully recover from treatment.

Mice in highest toxin dose show weight loss of ~4% relative to body weight of 23 g.

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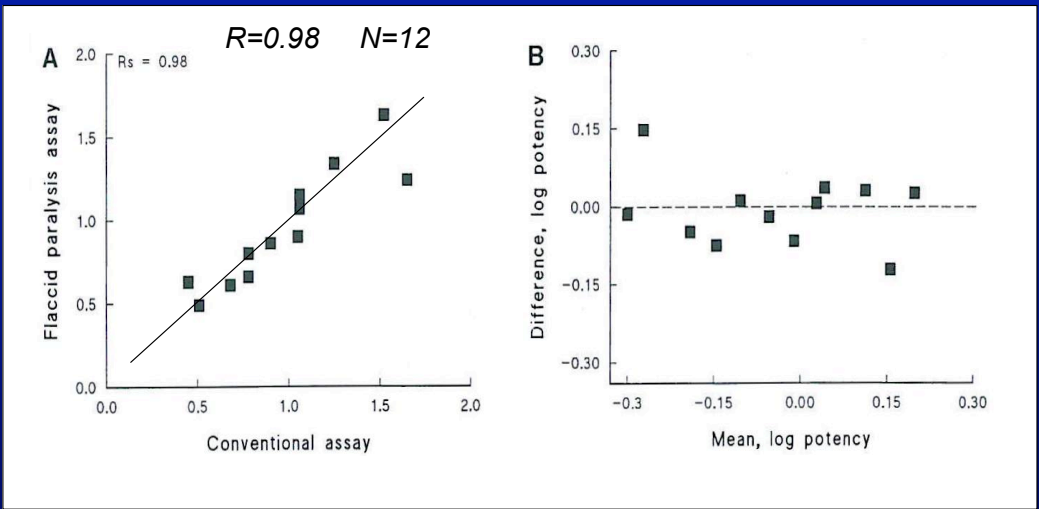
Excellent correlation confirmed between LD50 and flaccid paralysis assays



Agreement between methods for different products including HA-complex and purified toxin

Potency is expressed relative to a reference standard

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Agreement of scores between observers

Table 1.

Comparisons of scores obtained at 48 hr by two independent observers. Scores 0, 1, 2, 3 denote no effect, mild, moderate and severe palsy respectively. Each mouse was individually identified and scored independently by the two observers, who were blind to the treatment. Shown in bold is the total identically scored observations.

Scores	Scores for observer 2				
Obs 1	0	1	2	3	Total
0	**169**	81	21	0	271
1	67	**116**	97	12	292
2	8	56	**192**	53	309
3	0	1	89	**202**	292
Total	244	254	399	267	1164

Identical or 1 score difference in >96% of scores

No extreme score difference recorded

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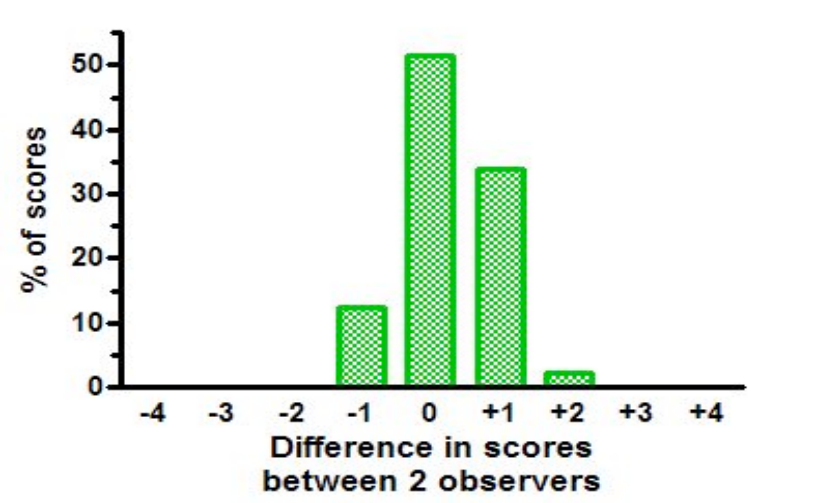
Scoring: accuracy and reproducibility



Further refinement of scoring system introduced (0 to 4) to improve dose response for use in potency comparisons

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Individual scorers may differ but estimates of relative potency are consistent

	Potency	95% Fiducial Limits	
DA 24h	1.11368	(0.94466	1.32257)
DA 48h	1.05579	(0.85097	1.31818)
RC 24h	1.12772	(0.77586	1.71456)
RC 48h	1.06659	(0.79070	1.45996)
RH 24h	0.98505	(0.76575	1.26426)
RH 48h	1.07151	(0.89782	1.28570)
RJ 24h	0.97340	(0.68025	1.38149)
RJ 48h	1.04800	(0.72782	1.53298)

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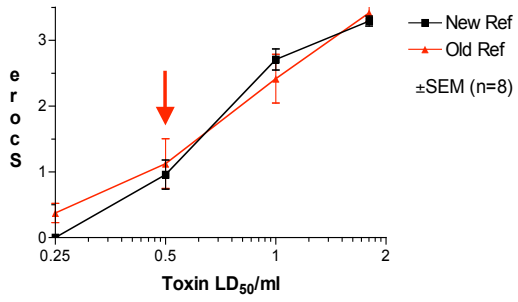
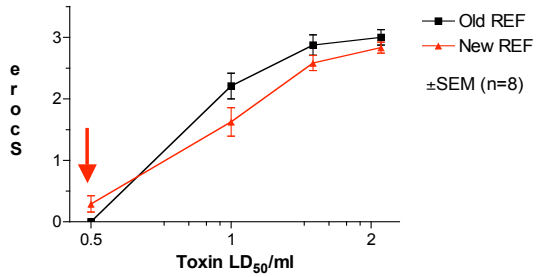
Flaccid paralysis assay is used to verify activity in product specific reference

Comparing Old and new Reference Product A



Comparing Old and New Reference Product B



Product A is more potent per unit as confirmed in LD50 assay and clinical use

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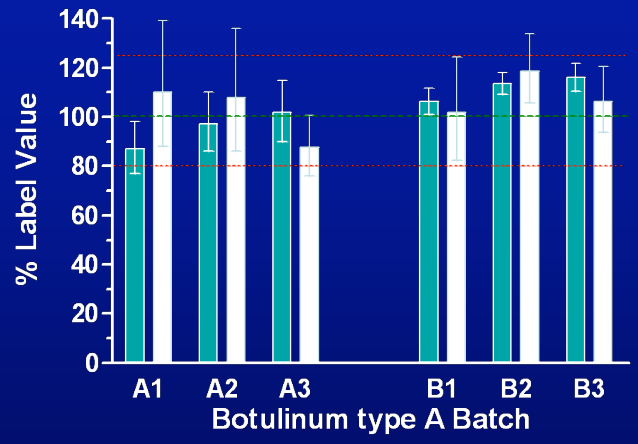
Potency of two products calculated relative to a product specific reference and expressed as % of label value



LD_{50} Manufacturers data (320-480 mice per batch)

Non lethal local flaccid paralysis NIBSC data (68 mice per batch)

±95%CL

Potencies in paralysis and LD50 assays are comparable
Confidence limits decrease with number of animals
NIBSC paralysis data may not meet PL specifications for 95% confidence limits (but <20% of animals used)

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Mouse flaccid paralysis as a batch release test (1)

- Suitable dose response for calculation of potency
- Potency values correlate and are comparable to LD50 values for both products and HA-free purified toxin
- Confidence limits are dependent on number of animals
- Product specific reference, calibrated in LD50, is used at NIBSC to express potency

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Mouse flaccid paralysis as a batch release test (2)

- In routine use at NIBSC since 1995
- From 1999 only for re-calibration of product specific reference standards and to verify in vitro assay data
- Included in new EP monograph for *Botulinum toxin type A for injection (2113)*, effective from 2005
- Transferability exercise with UK testing laboratory initiated in 2006

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Flaccid Paralysis Assay: Summary

- Fully functional assay with dosing more similar to clinical use
- Animals normally exhibit no signs of stress or pain and therefore requires minimal monitoring of animals
- More humane, ethical and economical
- Fast compared to LD50 (48 h vs 72 or 96 h)
- No specialised equipment or reagents required
- Robust and easily transferred to other laboratories
- Potency must be expressed relative to a suitable and stable reference standard of defined mouse LD50 value
- Should be considered as replacement for lethality end point

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