Non-Lethal Endpoint in Botulinum Toxin Potency Assay

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How to get your biologic to market and keep it there!

Animal testing was and remains an integral part of the **Lot release system** at the Bureau of Biologics and Radiopharmaceuticals (BBR), Health Canada; now Centre for Biologics Evaluation (CBE) and Centre for Evaluation of Radiopharmaceuticals and Biotherapeutics (CERB).

Botulinum Toxin products are subject to the **Lot release system** .

Lot release is a system of approval given by BBR for release onto the Canadian market of a specific lot of product based upon certification that the lot meets appropriate in process controls and control tests on final product.

The degree to which a product is evaluated by testing at BBR is linked to the product indication, the complexity of the product and the manufacturing process, and the testing history of the product

All protocols at BBR for regulatory testing using animals, must receive prior approval by a departmental **Animal Care Committee** (established in 1973) that operates under the direction of the **Canadian Council on Animal Care (CCAC)**.

The three R's of Russell and Burch , **Reduction, Replacement or Refinement** are encouraged.

Canadian Council on Animal Care

- The CCAC policy statement " **Ethics of Animal Investigation (1989)** states :
  " Animals must not be subjected to unnecessary pain or distress. The experimental design must offer them every practicable safeguard, whether in research, in teaching or in testing procedures:"

Russell and Burch

- **Refinement Alternatives** are described as :

  " methods which alleviate or minimize potential pain, suffering and distress and which **enhance animal well-being**.

CCAC General Guideline

- In experiments involving animals any actual or potential pain, distress or discomfort should be minimized or alleviated by **choosing the earliest end-point that is compatible with the scientific objectives** of the research

CCAC General Guideline

- For purposes of these guidelines , the term **"Endpoint"** is defined as **the point at which an experimental animals pain and/or distress is terminated, minimized or reduced** by taking actions such as killing the animal humanely, terminating a painful procedure or giving treatment to relieve pain and/or distress

Procedure for Selecting an Endpoint

1. There are several considerations in defining an appropriate endpoint in a given experiment. These all depend on an **objective determination of any deviations from an animals normal state**.

Procedure for Selecting an Endpoint

2. Determining which observations are the **most significant indicators of further deterioration in the animals condition** and then **identifying the earliest points at which those signs appear**.
3. Meeting the **scientific demand for an objectively measured and significant endpoint.**

Frequency Of Observations

- CCAC "Guide to the Care and Use of Experimental Animals"(1993), states that **normal healthy experimental animals should be observed at least once a day.**
- Once an animal is in a **potentially critical period with respect to impairment, more frequent observations must be made daily**.

Frequency Of Observations

- Based on previous knowledge during **critical periods** of the experiment and at the onset of adverse reactions, **a minimum of two or three observations** should be made daily.
- The frequency of the observations should increase depending on the potential for increasing pain /distress

ATLA

(Alternatives to Laboratory Animals)

Volume 27, July 1999

Abstracts

Third World Congress on Alternatives and Animal Use in The Life Sciences

29 August – 2 September 1999, Bologna Italy

Enhancing Laboratory Animal Well-Being Through Refinement by Establishing Non-Lethal Endpoints in Regulatory assays of Certain Biologicals

Gerald Calver, Josefina Gavieres , Nazar Shakarchi and Pierre Thibert .
Bureau of Biologics and Radiopharmaceuticals and Animal Resources Division, Health Canada

Abstract from ATLA

- Pain and distress were significantly reduced in test animals involved in the following regulatory tests: pertussis vaccine potency by the intracerebral mouse infection assay, diphtheria and tetanus toxoid vaccine potency by the toxin neutralization assay in guinea–pigs and **botulinum toxin potency by the toxicity assay in mice**.

Abstract from ATLA

- End-point clinical symptoms for euthanasia were chosen in-lieu of death for each assay on the basis that **the limits and criteria of acceptance for the assay remained the same** as previously established for the respective assays when death was used as an end-point

Abstract from ATLA

- Botulinum Reference Toxin lot 003 had comparable potency unitage in tests in which mice were euthanized when presenting with **raised scaphoid in combination with respiratory distress**.

Assay Methodology

1. Contents of 100 unit vial of Botulinum Toxin Standard Lot 003 suspended in 2.8 ml 0.9 % saline.
2. Range of seven dilutions from 1:1.33 to 1:7.49 prepared in 0.9 % saline.
3. Injection of all mice within 90 minutes of reconstitution.

Assay Methodology

4. Swiss Webster (CFW, 17 – 22 g) female, (groups of 10) injected by the intra-peritoneal route with 0.1 ml of diluted toxin.
5. Mortalities or numbers of mice euthanized at an appropriate stage were recorded over 72 hours post injection.
6. LD_{50} determined by Probit analysis or method of Reed and Muench

Test Validity

- The upper and lower mortalities must be ≥ 80 % and ≤ 20 % respectively. There must be a minimum of four distinct non-overlapping values

Presence of botulinum toxin in mice is indicated by the occurrence of a progressive motor paralysis. Flaccid paralysis in the form of scaphoid abdomen " raised rib" occurs . Inhibition of the small muscle of the eye leading to pupillary dilation also takes place.

When large amounts of toxin are present , the first signs may be apparent in 4 to 5 h in the the form of the indrawn scaphoid abdomen with bellows respiration, followed by limb paresis, dragging of the hind legs and increasing weakness leading to paralysis and subsequent death.

In the dilution scheme described, ie 1: 1.33 to 1:7.56 most animals will be symptomatic within 24 h and approximately 50 % of the animals will die within the 72 h observation period.

TOXICITY of *Botulinum toxin* (STD 003) IN MICE ASSESSED BY EUTHANASIA WITH STAGE 3 SYMPTOMS

Dilution Factor	# of Mice	# Euthanized
1: 1.333	10	10
1: 1.78	10	9
1: 2.37	10	7
1: 3.16	10	1
1: 4.22	10	0
1: 5.62	10	0

LD_{50} = 1: 2.819 (Reed and Meunch)

Observations

- Mice exhibited **three successive symptomatic stages** following injection of adequate concentrations of botulinum toxin:
  Stage 1: slightly indrawn scaphoid abdomen (lightly raised rib)

Observations cont'd

- **Stage 2: severe indrawn scaphoid abdomen (highly raised rib)**
- **Stage 3: severe indrawn scaphoid abdomen with respiratory distress in the form of hiccough & pupillatory dilation (eyes wide open, bulging)**

Observations cont'd

- **In a number of different experiments using lethality as the end point it was noted that mice that exhibited Stage 3 symptoms died within the 72 h monitoring period**

Results

- In separate experiments, LD_{50} determinations by Euthanasia at Stage 3 were **similar to those determined by lethal end-point analysis.**

TOXICITY of *Botulinum toxin* IN MICE ASSESSED BY LETHAL END-POINT versus EUTHANASIA WITH STAGE 3 SYMPTOMS

	LD_{50} VALUES (Dilution of Injected Dose)	
	Lethal End-point	**Euthanasia**
Mean	1: 3.06	1: 2.65
Std dev	± 0.04	± 0.11
Number tests	3	3

Time (h) to reach Symptom – Test A

Std 003 Dil.	Stage 1	Stage 2	Stage 3 Euth.	Dead
1:1.33-# - hrs	7 8h (0)	4 10h (4)	10 17h (3)	
1:1.78-# - hrs	8 8h (0)	5 16h (5)	9 21h (5)	
1:2.37-# - hrs	8 19h (3)	5 26h (4)	7 35h (8)	
1:3.16-# - hrs	9 27h (7)	8 39h (9)	1 24h	
1:4.21-# - hrs	7 40h (12)	2 50h (3)	0	
1:5.62-# - hrs	6 48h (7)	1 52h	0	

Time to reach Stage
Std 003 1:1.33 (dil) - Test A

Mouse #	Stage 1 hrs	Stage 2 hrs	Stage 3 hrs
1	8		16
2	8		16
3	8		16
4	8		16
5	8		16
6	8	16	20
7		8	16
8		8	24
9	8		16
10		8	20

Time to reach Stage
Std 003 1:1.78 (dil) - Test A

Mouse #	Stage 1 hrs	Stage 2 hrs	Stage 3 hrs
1	8	16	20
2	8		16
3		8	20
4	8		16
5	8		16
6		20	28
7	8		
8	8	20	24
9	8	16	20
10	8		28

Time to reach Stage
Std 003 1:2.37 (dil) - Test A

Mouse #	Stage 1 hrs	Stage 2 hrs	Stage 3 hrs
1	16	28	
2		28	38
3	16		38
4	20	28	38
5	24		44
6	16	20	38
7			
8	20		24
9	20	28	
10	20		24

Time to reach Stage
Std 003 1:3.16 (dil) - Test A

Mouse #	Stage 1 hrs	Stage 2 hrs	Stage 3 hrs
1	24	44	
2	24	28	
3	24	40	
4	40	52	
5	24	28	
6	24	40	
7			
8	16	20	24
9	38		
10	28	40	

Time (h) to reach Symptom – Test B

Std 003 Dil.	Stage 1	Stage 2	Stage 3 Euth.	Dead
1:1.33-# - hrs	9 14h (6)	3 21h (2)	8 22h (4)	2 28h (5)
1:1.78-# - hrs	7 18h (6)	2 24h (6)	5 27h (4)	2 26h (3)
1:2.37-# - hrs	9 21h (3)	9 26h (3)	7 40h (12)	
1:3.16-# - hrs	7 24h (4)	6 31h (3)	1 64h	
1:4.21-# - hrs	9 28h (7)	6 38h (7)	0	
1:5.62-# - hrs	9 30h (8)	5 44h (5)	0	

Time to reach Stage
Std 003 1:133 (dil) - Test B

Mouse #	Stage 1 hrs	Stage 2 hrs	Stage 3 hrs	Dead
1	4		20	
2	20			24
3	8		20	
4	20		24	
5			20	
6	20	24	28	
7	8	20		32
8	16		20	
9	12	20	28	
10	16		20	

Time to reach Stage
Std 003 1:1.78 (dil) - Test B

Mouse #	Stage 1 hrs	Stage 2 hrs	Stage 3 hrs	Dead
1	12	28	32	
2				
3				
4	12	20		24
5	20		24	
6				
7	20		24	
8	28		32	
9	12			28
10	20		24	

General Observations

1. **The timing of appearance of stage symptoms depended upon the concentration of toxin injected**. Within an experiment, Stage 1 symptoms appear first in the stronger dilutions **1:1.33 and 1:1.76)**

General Observations

2. **Time of appearance of stage 1 symptoms varies however from experiment to experiment**. The majority of mice receiving the stronger dilutions (1:1.33 and 1:1.78 of Std 003) demonstrated **Stage 1 symptoms as early as 8 hours in certain experiments and as late as 24 h post injection in other experiments.**

General Observations

3. In most experiments, the majority of mice receiving the concentrated dilutions of 1:1.33 and 1:1.76 **reach Stage 3 within 24 h (euthanized)**.
4. No definite progression from Stage 1 thru to Stage 2 thru to Stage 3 occurs.
5. In separate experiments 60 % of the mice receiving 1:1.33 dilution go from Stage 1 to Stage 3. Only 10 and 20 % respectively progress from Stage 1 to Stage 2 to Stage 3

General Observations

6. Within experiments, despite variations in time of appearance of Stage 1, **the time between Stage 1 and Stage 3 for 1:1.33 dilution remains at approximately 8 to 10 h.**

Observations cont'd

- **Stage 2 was not appropriate** to use for euthanasia since **significant reversion appeared within the 72 h monitoring period**, particularly in mice injected with more dilute concentrations of toxin
- Indeed within an additional 12 hours of the monitoring period extensive Stage 2 reversion occurred , ie up to 70 %

Stage 2 Non-Progressers or Reversions

Lot # / dil Test A	# mice symptom 2 at end of test	# mice revert symptom 2 to symptom 1
Std 003 / 1:2.37	1	
Std 003 / 1:3.16	3	4
Std 003 / 1:4.21		2
Std 003 / 1:5.62		1
Lot A / 1:1.78	1	
Lot A / 1:2.37	1	
Lot A / 1:3.16	7	
Lot A / 1:4.21		3

Stage 2 Non-Progressers or Reversions

Lot # / dil Test B	# mice symptom 2 at end of test	# mice revert symptom 2 to symptom 1
Std 003 / 1:2.37	1	2
Std 003 / 1:3.16	2	2
Std 003 / 1:4.21	1	5
Std 003 / 1:5.62		8
Lot A / 1:4.21	2	1
Lot A / 1:5.62		8
Lot B / 1:3.16	3	1
Lot B / 1:4.21		6

Future Consideration

- A collaborative study should be undertaken to verify the use of Euthanasia at Stage 3 symptoms as an appropriate non-lethal endpoint for global Botox testing .

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