USAMRICD-TR-20-07

In Vitro Stability of Botulinum Neurotoxin Serotypes A and B Recombinant Light Chains in Human Serum, Buffered Solutions, Water and Milk

Heidi Hoard-Fruchey
Michael Adler

July 2020

US Army Medical Research Institute of Chemical Defense
8350 Ricketts Point Road
Aberdeen Proving Ground, MD 21010-5400

an element of the

US Army Medical Research and Development Command

DISPOSITION INSTRUCTIONS:

Destroy this report when no longer needed. Do not return to the originator.

DISCLAIMERS:

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of Army, the Department of Defense, or the National Biodefense Analysis and Countermeasures Center.

The use of trade names does not constitute an official endorsement or approval of the use of such commercial hardware or software. This document may not be cited for purposes of advertisement.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
31-07-2020	Technical Report	July 5, 2004 – April 30, 2006

4. TITLE AND SUBTITLE

In Vitro Stability of Botulinum Neurotoxin Serotypes A and B Recombinant Light Chains in Human Serum, Buffered Solutions, Water and Milk

5a. CONTRACT NUMBER

5b. GRANT NUMBER
BCN04-N832

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Hoard-Fruchey, H., Adler, M.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

US Army Medical Research Institute of Chemical Defense
8350 Ricketts Point Road
Aberdeen Proving Ground, MD 21010-5400

8. PERFORMING ORGANIZATION REPORT NUMBER

USAMRICD-TR-20-07

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Department of Homeland Security
National Biodefense Analysis and Countermeasures Center
National Interagency Biodefense
Fort Detrick, MD 21702

10. SPONSOR/MONITOR'S ACRONYM(S)
DHS/NBACC

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES Funding for this project was provided by the Department of Homeland Security (DHS) National Biodefense Analysis and Countermeasures Center (NBACC), grant BCN04-N832**.** During the period in which the research was conducted H. Hoard-Fruchey was a National Research Council (NRC) fellow.

14. ABSTRACT

Symptoms of botulinum neurotoxin (BoNT) intoxication can persist for many months presumably due to the intracellular stability of the BoNT light chain (LC). LC catalytic activity is highly stable *in vivo* (>11 weeks for serotype A, 6-8 weeks for serotype B) (1-3). In the present study, we determined stability of recombinant BoNT LC (rLC) serotypes A and B in HEPES buffer, intracellular buffer (ICB), bottled water, human serum and milk. BoNT/A and BoNT/B rLCs were incubated in test solutions at 4 °C, 22 °C or 37 °C for up to 6 months. Stability was assessed by monitoring catalytic activity using a fluorescent microplate assay (4). BoNT rLCs were most stable in ICB and least stable in human serum. Regardless of solution, a graded decrease in stability occurred between 4 °C and 22 °C, whereas a striking reduction was observed from 22 °C to 37 °C. The relative instability of the rLC at 37 °C in the test solutions is in marked contrast to the unusually high *in vivo* stability of the LCs. Stability in milk increased with increasing milkfat for the BoNT/A rLC, but not for the BoNT/B rLC, suggesting a greater role of lipids in the stability of the former. The results indicate that the factors regulating the stability of BoNT LCs may be serotype specific and factors relating to localization or trafficking of the LCs may contribute to their stability.

15. SUBJECT TERMS

Botulinum neurotoxin, stability, light chain, *in vitro*

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Michael Adler
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UNLIMITED	17	**19b. TELEPHONE NUMBER** *(include area code)* 410-436-1913

SUMMARY

Symptoms of botulinum neurotoxin (BoNT) intoxication can persist for many months presumably due to the intracellular stability of the BoNT light chain (LC). LC catalytic activity is highly stable *in vivo* (>11 weeks for serotype A, 6-8 weeks for serotype B) (1-3). In the present study, we determined stability of recombinant BoNT LC (rLC) serotypes A and B in HEPES buffer, intracellular buffer (ICB), bottled water, human serum and milk. BoNT/A and BoNT/B rLCs were incubated in test solutions at 4 °C, 22 °C or 37 °C for up to 6 months. Stability was assessed by monitoring catalytic activity using a fluorescent microplate assay (4). BoNT rLCs were most stable in ICB and least stable in human serum. Regardless of solution, a graded decrease in stability occurred between 4 °C and 22 °C, whereas a striking reduction was observed from 22 °C to 37 °C. The relative instability of the rLC at 37 °C in the test solutions is in marked contrast to the unusually high *in vivo* stability of the LCs. Stability in milk increased with increasing milkfat for the BoNT/A rLC, but not for the BoNT/B rLC, suggesting a greater role of lipids in the stability of the former. The results indicate that the factors regulating the stability of BoNT LCs may be serotype specific and factors relating to localization or trafficking of the LCs may contribute to their stability.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

1. INTRODUCTION

The botulinum neurotoxins (BoNTs) are a group of seven immunologically distinct bacterial protein toxins (A-G) produced by the anaerobic bacterium *Clostridium botulinum* (5). BoNTs are potent inhibitors of synaptic transmission in skeletal muscle, acting by inhibiting the release of acetylcholine from motor nerve endings, culminating in muscle paralysis and death (6). As one of the most lethal substances known to mankind (7, 8), BoNTs are considered category A threat agents by the US Centers for Disease Control and Prevention (CDC).

Each of the seven serotypes of BoNT consists of a light chain (LC) of ~50 kDa and a heavy chain (HC) of ~100 kDa linked by a single disulfide bond and non-covalent interactions. The LC has Zn^{2+} metalloprotease activity and cleaves one of three proteins associated with transmitter release: synaptobrevin, SNAP-25 or syntaxin (9, 10). The HC is responsible for the binding of the toxin to the nerve terminal and for internalization of the LC into the cytosol (5, 11).

Human intoxication is caused primarily by serotypes A, B, and E. Symptoms of BoNT intoxication can persist for many months presumably due to the intracellular stability of BoNT LC. LC catalytic activity is highly stable *in vivo*, lasting over 11 weeks for serotype A, 6-8 weeks for serotype B, and less than 3 weeks for serotype E (1-3). The observed differences in stabilities of the serotypes are not easily explained. In addition, limited information is available about the stability of the various serotypes *in vitro*. To begin to address both issues, BoNT/A and /B recombinant LCs (rLCs) were incubated in physiological solutions, milk, and water over a 6-month period to determine stability as assessed by a high-throughput fluorescent microplate activity assay.

2. MATERIALS AND METHODS

2.1 BoNT/A and BoNT/B Fluorescent Microplate Assay

Catalytic activities of BoNT/A and BoNT/B rLCs were determined using a fluorescent microplate assay (4). The plates contained fluorescein-tagged immobilized substrates for BoNT/A (SNAP-25 peptide: (5CF) GGG SNRTRIDEAN**QR**ATRMLG GGC) and for BoNT/B (synaptobrevin peptide: (5CF) GGG LSELDDRADALQAGAS**QF**ETSAAKLK RKYWWKNLKGGC). Cleavage sites are denoted in bold. Buffer was added to the microplate, and an initial fluorescence was measured with a SpectraMax GeminiXS multiwell fluorometer (Molecular Devices, Sunnyvale, CA) using an excitation wavelength of 485 nm and an emission wavelength of 535 nm. All assay conditions included 40 mM HEPES pH 7.3 and 50 μM $ZnSO_4$. Concentrations of rLCs were 60 and 180 nM for type A and type B toxins, respectively. rLCs were incubated in the microplate for 1 hr at 37 °C in the dark without agitation, and fluorescence was measured in the same microplate. At the end of the incubation, 40 μg of trypsin was added to each well, and the plate was incubated at 37 °C for 30 min in the dark to determine the total fluorescence released. Initial fluorescence was subtracted from the BoNT rLC activity, and this was normalized to total fluorescence. Normalized fluorescence readings from four replicate wells were averaged for each time point. GraphPad Prism version 3.02

(GraphPad Software, Inc., San Diego, CA) was used to calculate half-times using a one-phase exponential decay for rLC-mediated substrate cleavage.

2.2 Solutions for Stability Determination

BoNT rLCs were incubated in seven solutions for up to 6 months. BoNT rLCs in fat free, 2% milkfat and whole milk were incubated only at 4 °C, since this temperature corresponds to normal storage conditions for milk. Bottled water samples were incubated at 4 °C and 22 °C. BoNT rLCs in human serum, ICB and HEPES solution were incubated at 4 °C, 22 °C and 37 °C. The composition of ICB was 47 mM potassium phosphate (pH 7.0), 12 mM $NaHCO_3$, 0.1 μM $CaCl_2$, 1 mM $MgSO_4$, and 4 mM KCl. The HEPES solution contained 40 mM HEPES titrated with NaOH to pH 7.3. Stock concentrations of rLC were 600 nM and 1.8 μM for serotypes A and B, respectively. Samples were assayed at 0, 0.25, 1, 2, 3, 7, 14, 21 and 28 days and monthly thereafter for up to 6 months using the fluorescent microplate assay described above.

2.3 Materials

BoNT/A and BoNT/B rLCs were a gift from Dr. Leonard A. Smith (USAMRIID, Ft. Detrick, MD) and were purified as previously described (12). Fluorescent microplates for BoNT/A and BoNT/B assays were obtained from Battelle (Columbus, OH). Fat free, 2% milkfat, and whole milk was purchased from a local source (Giant Brands, Inc., Landover, MD). Bottled water was from Wissahickon Spring Water (Philadelphia, PA). Human serum was obtained from Bioreclamation, Inc. (Hicksville, NY) and consisted of pooled sera from 100 donors. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

3. RESULTS AND DISCUSSION

3.1 BoNT Stability in Fat Free, 2% and Whole Milk

BoNT rLC was incubated in milk containing three levels of milkfat at 4 °C to assess effect on stability. After 5 months of incubation at 4 °C, BoNT/B rLC retained greater than 55% activity in all three levels of milkfat (table 1). BoNT/A rLC stability was dependent upon milkfat content (figure 1). The half-times for loss of activity of BoNT/A rLC in fat free (0.02%) and 2% milk were similar (19.5 and 21 days respectively). However, whole milk (3.6%) greatly enhanced the stability of BoNT/A rLC, which had a half-time of 51 days. Unlike serotype A, increasing milkfat content did not correlate with BoNT/B rLC stability. These data suggest that lipids may play a role in stabilizing BoNT/A rLC activity and that factors regulating BoNT stability may be serotype specific. This is in agreement with reports that BoNT/A rLC associates with the intracellular plasma membrane, whereas BoNT/B and BoNT/E LCs are not membrane associated (13, 14).

3.2 BoNT rLC Stability in Human Serum

To assess BoNT rLC stability in a biological fluid, BoNT/A and BoNT/B rLCs were incubated in human serum at 4, 22, and 37 °C. Due to problems with microbial growth, data for BoNT/B rLC in human serum were not interpretable. In all the fluids examined, BoNT/A rLC was least stable in human serum. Instability of BoNT/A rLC in human serum increased with increasing temperature, resulting in half-times of 6.3, 0.62, and 0.063 days at 4, 22, and 37 °C, respectively (figure 2). These data suggest that in the absence of the HC, the LC is highly unstable outside of the nerve terminal cytoplasm (2).

3.3 BoNT rLC Stability at 37 °C

BoNT rLC stability at 37 °C was also assessed in ICB and 40 mM HEPES. BoNT/A rLC was unstable at 37 °C in all solutions, losing activity within 1 day (figures 2 and 3). However, BoNT/B rLC was more stable than BoNT/A rLC at 37 °C in ICB and in 40 mM HEPES. Half-times for BoNT/B rLC in ICB and 40 mM HEPES were 5.2 and 5.5 days, respectively (figure 4). These data are in marked contrast to stability observed *in vivo* (1-3). The differences between stability *in vivo* and that in the test solution may be due to the following. First, intracellular stabilizing factors may exist that are not present in the solutions tested. For example, interacting proteins or compartmentalization may provide stabilization to the LC *in vivo*. Serotypes A and B display distinct localization patterns that may contribute to the varied persistence *in vivo*. BoNT/A LC localizes to the plasma membrane, whereas BoNT/B LC is found throughout the cell (13, 14). Second, in the absence of HC, rLC may not fold like native LC. This alternate folding of the LC may affect the heat stability and cause the protein to more easily unfold at higher temperatures. Intracellular trafficking of the toxin during intoxication may confer post-translational modifications to stabilize the LC, which would not be present in the rLC. Regardless of the explanation, BoNT/A and BoNT/B rLCs are less stable at 37 °C than expected from the prolonged action of these toxins following local and systemic exposure (1-3).

3.4 BoNT Stability in ICB, 40 mM HEPES and Bottled Water at 4 and 22 °C

For both BoNT/A and BoNT/B rLCs, stability was greatest in ICB, followed by 40 mM HEPES (tables 1 and 2). A reduction in stability of BoNT rLCs in ICB and 40 mM HEPES was observed at 22 °C, but it was not as severe as that observed at 37 °C. The stability observed in ICB suggests that physiological pH and ionic strength favor BoNT LC long-term stability. Stability of BoNT rLCs in bottled water was much greater than expected (tables 1 and 2). BoNT/A rLC in water displayed half-lives of 32.8 and 7.7 days at 4 °C and 22 °C, respectively. BoNT/B rLC was more stable than BoNT/A rLC in water, with 62.3% activity remaining at 4 °C after 5 months and a half-life of 37.2 days at 22 °C. These data suggest that even in the absence of physiological salts and stabilizing factors, BoNT LC structure may confer some inherent stability.

Figure 1

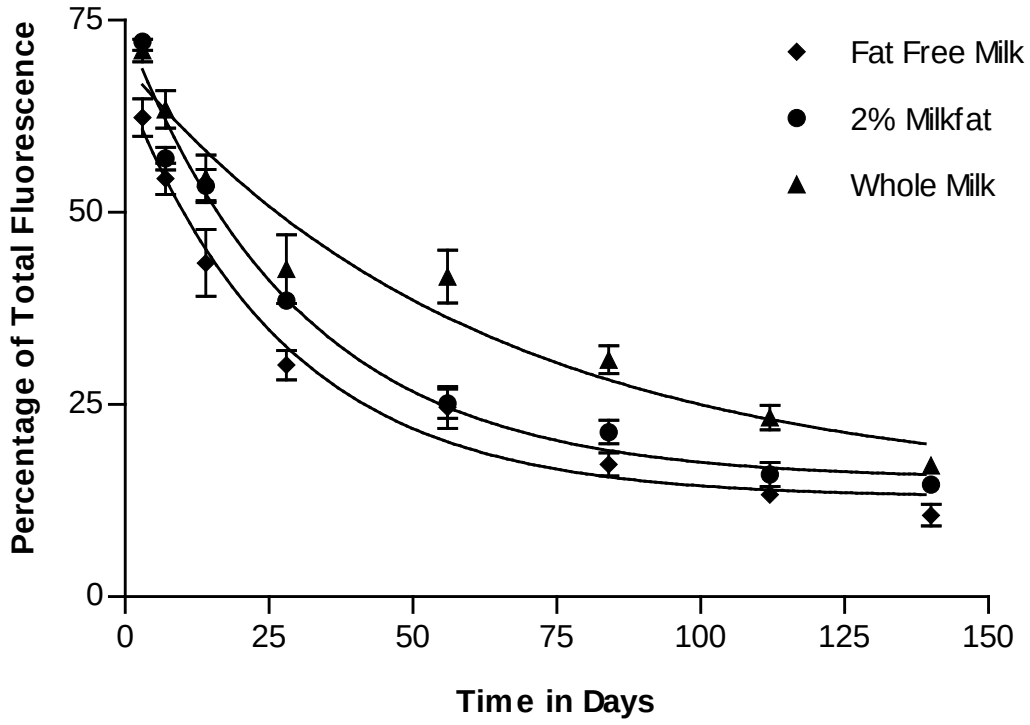

Figure 1: BoNT/A rLC stability increases with increasing milkfat content. Each symbol represents the mean ± SD of 4 replicates. The curves represent the non-linear regression of the data fit for a one-phase exponential decay. Half-times were 19.5, 21, and 51 days for fat free, 2% and whole milk, respectively at 4 ^{o}C.

Figure 2

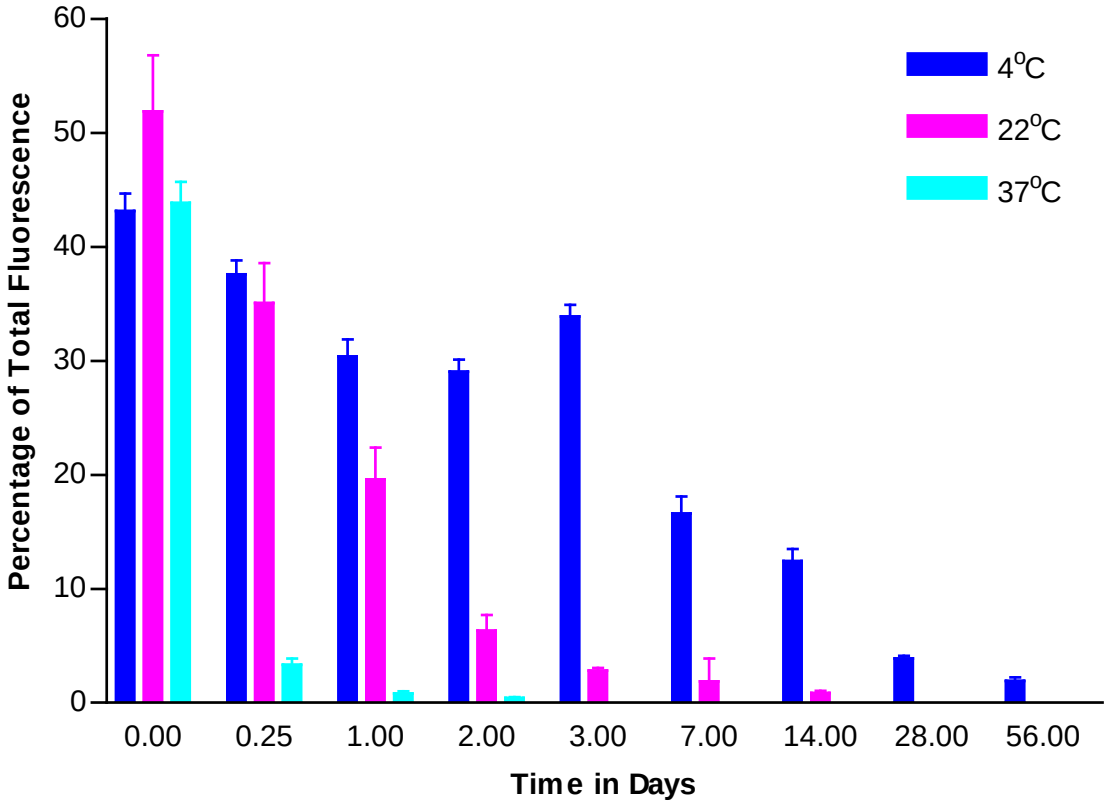

Figure 2: BoNT/A rLC is unstable in human serum. Each bar represents the mean ± SD of 4 replicates. Half-times were 6.3, 0.62, and 0.063 days for BoNT/A rLC in human serum at 4 °C, 22 °C, and 37 °C, respectively.

Figure 3

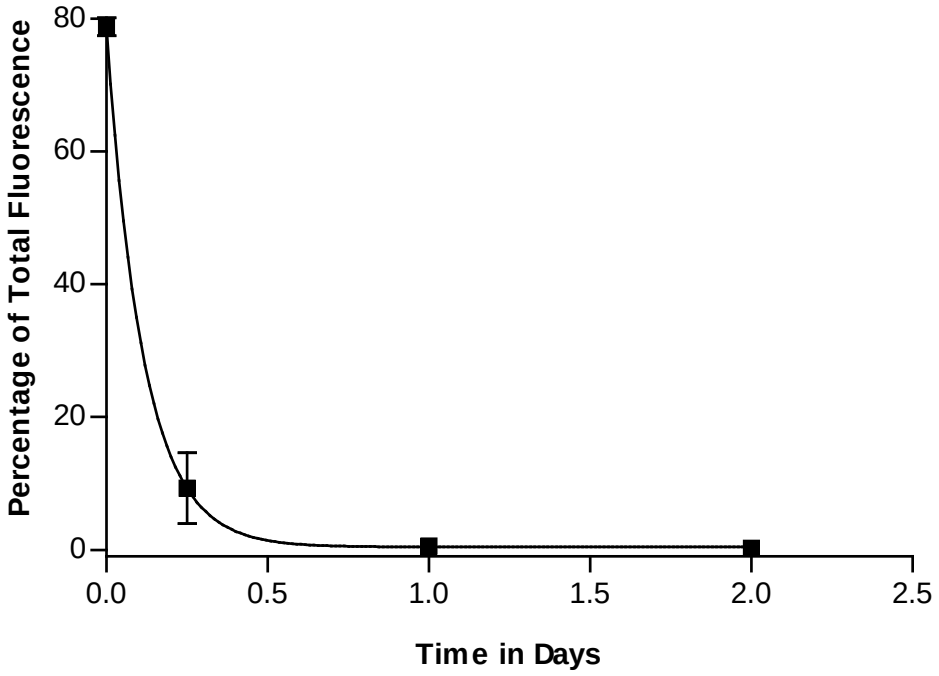

Figure 3: BoNT/A rLC is unstable at 37 °C under all conditions examined. BoNT/A rLC in ICB (shown), 40 mM HEPES or human serum loses all activity within 1 day at 37 °C. Each symbol represents the mean ± SD of 4 replicates. The curve represents non-linear regression of the data fit with a one-phase exponential decay.

Figure 4

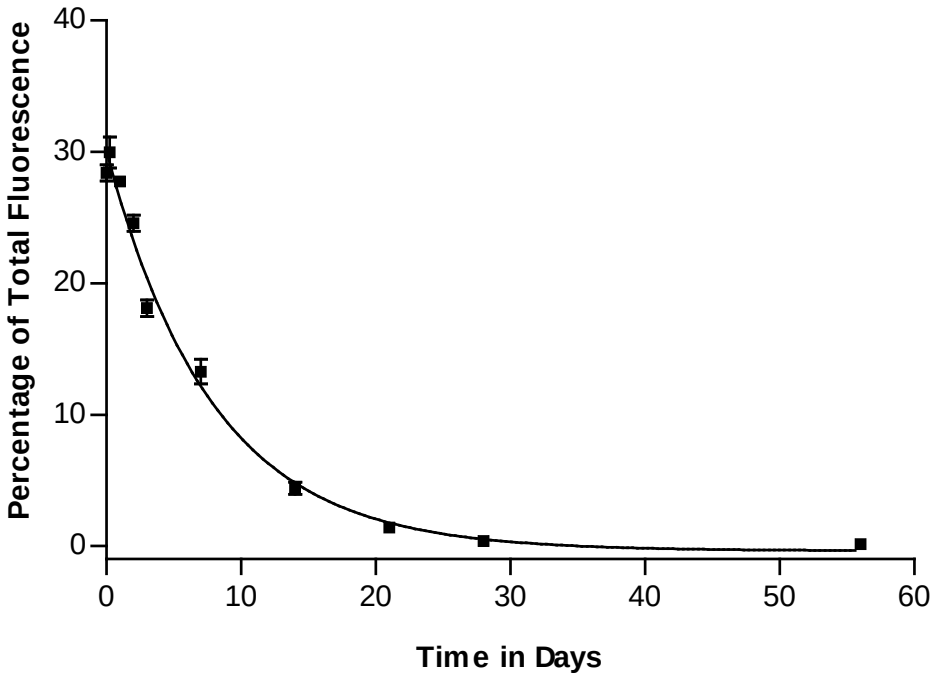

Figure 4: BoNT/B rLC was more stable than BoNT/A rLC at 37 °C in 40 mM HEPES and ICB. BoNT/B rLC in 40 mM HEPES (shown) and ICB displayed half-times of 5.5 and 5.2 days respectively at 37 °C. Each symbol represents the mean ± SD of 4 replicates. The curve represents non-linear regression of the data fit with a one-phase exponential decay.

Table 1: BoNT/B rLC activity remaining after 5 months of incubation

Solution	Temperature	% Initial Activity
Fat Free Milk	4 °C	64.1%
2% Milkfat	4 °C	59%
Whole Milk	4 °C	55.6%
ICB	4 °C	84.2%
	22 °C	43.2%
Bottled Water	4 °C	62.3%
	22 °C	0% (*half-time = 37.2 days)
HEPES Buffer	4 °C	77%
	22 °C	64%

*Half-time values were determined using a one-phase exponential decay equation (GraphPad Prism, San Diego, CA).

Table 2: Half-time values for BoNT/A rLC in ICB, bottled water and 40 mM HEPES after 6 months of incubation

Solution	Temperature	Half-Time*
ICB	4 °C	N/A (64.4% activity remaining)
	22 °C	100.4 days
Bottled Water	4 °C	32.8 days
	22 °C	7.7 days
HEPES Buffer	4 °C	116.7 days
	22 °C	84.4 days

*Half-time values were determined using a one-phase exponential decay equation (GraphPad Prism, San Diego, CA).

4. CONCLUSIONS

In this study, the stability of BoNT/A and /B rLC was assessed in various solutions by monitoring enzymatic activity. BoNT/A rLC stability in milk increased with increasing milkfat content, suggesting that lipids may stabilize BoNT/A rLC activity. However, stability of BoNT/B rLC did not increase with increasing milkfat, suggesting that factors contributing to stability may be serotype specific. These findings are in agreement with previous *in vivo* localization studies (13, 14). In the absence of the heavy chain, BoNT/A rLC was highly unstable in human serum *in vitro* and was temperature-sensitive under all conditions examined, losing all activity within 1 day at 37 °C. For both BoNT/A and BoNT/B rLCs, stability was greatest in ICB, followed by 40 mM HEPES. Stability in bottled water at 4 °C and 22 °C was greater than expected with half-times of >1 week for both serotypes. Overall, BoNT/B rLC was more stable than BoNT/A rLC. This finding is in contrast with previously reported *in vivo* stability studies that indicated BoNT/A activity was more stable than BoNT/B (1-3). This illustrates the need to assess *in vitro* data with caution and carefully compare these with available *in vivo* data prior to drawing conclusions. Continuation of these studies with BoNT purified LC, holotoxin and toxin complex will provide further insight into factors responsible for BoNT stabilization *in vivo*.

REFERENCES

1. Brin, M. F. (1997) Botulinum toxin: chemistry, pharmacology, toxicity, and immunology. Muscle Nerve 20**:**146-168.
2. Keller, J. E., E. A. Neale, G. Oyler, and M. Adler. (1999) Persistence of botulinum neurotoxin action in cultured spinal cord cells. FEBS Letters 456**:**137-142.
3. Tsui, J. K., M. Hayward, E. K. Mak, and M. Schulzer. (1995) Botulinum toxin type B in the treatment of cervical dysonia: a pilot study. Neurology 45**:**2109-2110.
4. Schmidt, J. J., R. G. Stafford, and C. B. Millard. (2001) High-throughput assays for botulinum neurotoxin proteolytic activity: serotypes A, B, D, and F. Analytical Biochemistry 296**:**130-137.
5. Simpson, L. L. (1989) Peripheral actions of the botulinum toxins. In: Simpson, L. L., ed. Botulinum Neurotoxin and Tetanus Toxin. New York: Academic Press, 153-178.
6. Adler, M., G. A. Oyler, J. E. Keller, and F. J. Lebeda. (2001) Pharmacological countermeasures for botulinum intoxication. In: Somani, S. M., Romano, J. A., eds., Chemical Warfare Agents: Toxicity at Low Levels. Boca Raton: CRC Press, 373-391.
7. Gill, M. D. (1982) Bacterial toxins: a table of lethal amounts. Microbiology Review 46**:**86-94.
8. Lamanna, C. (1959) The most poisonous poison. Science 130**:**763-772.
9. Binz, T., J. Blasi, S. Yamasaki, A. Baumeister, E. Link, T. C. Sudhof, R. Jahn, and H. Niemann. (1994) Proteolysis of SNAP-25 by types E and A botulinal neurotoxins. Journal of Biological Chemistry 269**:**1617-1620.
10. Schiavo, G., O. Rossetto, S. Catsicas, P. Polverino de Laureto, B. R. DasGupta, F. Benfenati, and C. Montecucco. (1993) Identification of the nerve terminal targets of botulinum neurotoxin serotypes A, D, and E. Journal of Biological Chemistry 268**:**23784-23787.
11. Montecucco, C., and G. Schiavo. (1993) Tetanus and botulism neurotoxins: a new group of zinc proteases. Trends in Biochemical Sciences 18**:**324-327.
12. Ahmed, S. A., and L. A. Smith. (2000) Light chain of botulinum A neurotoxin expressed as an inclusion body from a synthetic gene is catalytically and functionally active. Journal of Protein Chemistry 19**:**475-487.
13. Fernandez-Salas, E., H. Ho, P. Garay, L. E. Steward, and K. R. Aoki. (2004) Is the light chain subcellular localization an important factor in botulinum toxin duration of action? Movement Disorders Suppl 8**:**S23-34.
14. Fernandez-Salas, E., L. E. Steward, H. Ho, P. E. Garay, S. W. Sun, M. A. Gilmore, J. V. Ordas, J. Wang, J. Francis, and K. R. Aoki. (2004) Plasma membrane localization signals in the light chain of botulinum neurotoxin. Proceedings of the National Academy of Sciences of the United States of America 101**:**3208-3213.

LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

BoNT	Botulinum Neurotoxin
BoNT/A	Botulinum Neurotoxin Serotype A
BoNT/B	Botulinum Neurotoxin Serotype B
BoNT/E	Botulinum Neurotoxin Serotype E
HC	Heavy Chain
ICB	Intracellular Buffer
LC	Light Chain
rLC	Recombinant Light Chain