Abstract:

Title: STABILIZATION OF CLOSTRIDIUM BOTULINUM NEUROTOXIN COMPLEX

23-WEEK INCUBATION OF BOTULINUM NEUROTOXIN TYPE A WITH α,β, AND 7-CYCLODEXTRINS AT ROOM TEMPERATURE

Room Temperature Incubation

<table>
<thead>
<tr>
<th>Area %</th>
<th>Wk. 1</th>
<th>Wk. 2</th>
<th>Wk. 3</th>
<th>Wk. 4</th>
<th>Wk. 12</th>
<th>Wk. 23</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>77.07333333</td>
<td>58.81</td>
<td>55.39333333</td>
<td>52.65666667</td>
<td>56.14</td>
</tr>
<tr>
<td>Alpha</td>
<td>98.78</td>
<td>99.41</td>
<td>94.71333333</td>
<td>83.27</td>
<td>58.65333333</td>
<td>51.673333</td>
</tr>
<tr>
<td>Beta</td>
<td>100</td>
<td>84.06666667</td>
<td>57.75</td>
<td>44.53</td>
<td>40.10666667</td>
<td>35.35</td>
</tr>
<tr>
<td>Gamma</td>
<td>99.80333333</td>
<td>100</td>
<td>87.30333333</td>
<td>74.36</td>
<td>54.36666667</td>
<td>45.123333</td>
</tr>
</tbody>
</table>

(57) Abstract: A stable composition including botulinum neurotoxin and a cyclodextrin and a method of preserving botulinum neurotoxin and for producing a botulinum neurotoxin composition with improved stability properties in an efficient and economically advantageous manner. The invention seeks to alleviate the problems associated with rapid degradation or denaturation of botulinum neurotoxin by providing a novel composition that exhibits improved stability properties. The botulinum neurotoxin is preferably stabilized by forming a cyclodextrin inclusion complex.
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.
STABILIZATION OF CLOSTRIDIUM BOTULINUM NEUROTOXIN COMPLEX

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a pharmaceutical composition comprising Clostridium botulinum neurotoxin and to a method of stabilizing the same. Particularly, the present invention is directed to a stabilized pharmaceutical composition including C. botulinum Type A and a cyclodextrin.

Description of Related Art

Botulinum neurotoxins are produced from anaerobic bacillus Clostridium botulinum. Seven related protein neurotoxins, known as serotypes A through G, are produced by different strains of the bacillus. Each of the seven serotypes of the botulinum neurotoxins is a large protein having a molecular weight of about 150 kDa, and a 100kDa heavy chain of amino acid residues and a 50 kDa light chain of amino acid residues coupled by at least one disulfide linkage.

Clostridium botulinum neurotoxins, which cause the disease of botulism by blocking the release of the neurotransmitter, acetylcholine at the neuromuscular junction, are the most toxic proteins currently known to mankind. Food-borne botulism results from the consumption of improperly stored foods in which anaerobic C. botulinum grows and releases the toxin. In other forms of botulism, C. botulinum also produces the neurotoxin resulting in toxigenesis. For example, wound botulism results when the spores of C. botulinum are introduced into an open skin abrasion. The colonization of the wound is followed by the release of botulinum toxin. Similarly, infant botulism results from the consumption of C. botulinum spores followed by colonization in the intestine and toxigenesis.

Although the botulinum neurotoxins are known to be the most lethal natural toxin known to man, these lethal poisons have become utilized in the medical community as drugs with many indications. In this regard, the botulinum neurotoxins have been used to treat strabismus, and local injections of botulinum neurotoxin are now considered a safe and efficacious treatment for many neurological and non-neurological conditions. Recently, it has been observed that botulinum neurotoxin is useful as a treatment for diseases of the gastrointestinal tract. Botulinum neurotoxin is not only potent in blocking skeletal neuromuscular transmission, but also block cholinergic nerve endings in the autonomic nervous system. The capability to inhibit contraction of smooth muscles of the
gastrointestinal tract was first suggested based on in vitro observations and later demonstrated in vivo, it has also been shown that botulinum neurotoxin does not block non adrenergic non cholinergic responses mediated by nitric oxide. This has further promoted the interest to use botulinum neurotoxin as a treatment for overactive smooth muscles and sphincters, such as the lower esophageal sphincter to treat esophageal achalasia, or the internal anal sphincter to treat anal fissure.

Commercially available pharmaceutical compositions comprising botulinum toxin are marketed under the trademarks including BOTOX® (Allergan, Inc. Irvine CA.), Dysport® (Ipsen Ltd. Berkshire, U.K.) and Myobloc® (Elan Corp. Dublin Ireland). Typically, the pharmaceutical compositions are sold as vacuum-dried form that must be reconstituted with a diluent prior to actual usage. One major drawback to using the commercially available botulinum toxin preparations is the very short shelf life of the composition. In this regard, the actual usage of the pharmaceutical composition should be administered within about four hours after reconstitution because the botulinum toxin is very susceptible to denaturation due to surface denaturation, heat, and alkaline conditions.

The susceptible denaturation of the botulinum neurotoxin reconstitution necessitates a need for methods of preserving the botulinum neurotoxin. It has been found that the Clostridium botulinum bacteria secretes the neurotoxin along with a group of neurotoxin associated proteins ("NAPs"). Research has shown that not only do the NAPs have a critical role in the toxico-infection of the botulinum neurotoxins, but the NAPs have an important role in the toxicity of the botulinum neurotoxin. In particular, it has been demonstrated the oral toxicity of the neurotoxin type A decreases by 43,000-fold upon removal of the NAPs. It has also been shown that the NAPs act to protect the neurotoxin against various environmental conditions including exposure to proteases, acidity, and heat. See, Kitamura, M., Sakaguchi, S., and Sakaguchi, G. (1969) Significance of the 12S toxin of Clostridium botulinum type E. Bacteriol. 98, 1173-1178; Sugii, S., Ohishi, L, and Sakagch, G., (1977) Botulogenic properties of vegetables with special reference to molecular size of the toxin in them, J. Food Safety 1, 53-65. Accordingly, it has been suggested that the NAPs interact with the neurotoxin to protect it from adverse environmental conditions. This protective role has led to the hypothesis that NAPs are important for the preservation of the structural integrity of the neurotoxin, as well as preservation of its activity.

Cyclodextrins are cyclic multiclyclopynose unitecs connected by alpha-(l-4) linkages. The most widely known cyclodextrins are A, B, G-cyclodextrins, and their derivatives. The
cyclic nature of the cyclodextrins, the hydrophobic properties of their cavities and the hydrophilic properties of their outer surfaces enable them to interact with other chemicals and produce inclusion compounds which are characterized by improved solubilities and stabilities. For example, U.S. Patent No. 6,818,662 to Ito et al. discloses that solfobutyl ether B-cyclodextrin increases the solubility and light stability of N-(3-chloro-4-morpholin-4-yl)phenyl-N'-hydroxyimodoforrnadine.

Although cyclodextrins are known to form inclusion complexes with drugs, there has been no disclosure or suggestion that a cyclodextrin can stabilize a botulinum neurotoxin. In fact, it has been thought that cyclic polymers including cyclodextrins can not be used to preserve or stabilize a botulinum neurotoxin. See, U.S. Patent Application Publication 2003/0118598 to Hunt, in which it has been taught that the botulinum neurotoxin can not utilize the cyclodextrin cavity because the cavity is much smaller in size than the neurotoxin. Accordingly, cyclodextrins cannot form inclusion complexes with botulinum neurotoxin.

There remains a need for an efficient and economic method and system for preparing a pharmaceutical composition comprising botulinum neurotoxin and a method for stabilizing and preserving the botulinum neurotoxin. The present invention satisfies these needs.

**SUMMARY OF THE INVENTION**

The purpose and advantages of the present invention will be set forth in and apparent from the description that follows, as well as will be learned by practice of the invention. Additional advantages of the invention will be realized and attained by the methods and compositions particularly pointed out in the written description and claims hereof, as well as from the appended drawings.

The present invention seeks to alleviate the problems associated with rapid degradation or denaturation of botulinum neurotoxin by providing a novel composition that exhibits improved stability properties. In particular, the present invention seeks to provide a method for producing a botulinum neurotoxin composition with improved stability properties in an efficient and economically advantageous manner.

To achieve these and other advantages and in accordance with the purpose of the invention as described herein, the invention includes novel inclusion complexes of botulinum neurotoxin which exhibit improved stability properties. The invention also includes method for stabilizing botulinum neurotoxin. The neurotoxin is preferably stabilized by forming a cyclodextrin inclusion complex.
In accordance with the invention, a composition is provided which comprises a
botulinum neurotoxin and a cyclodextrin or a derivative thereof. The botulinum neurotoxin
can be pure or purified and can be Type A, B, C, D, E, F, or G. Preferably, the botulinum
neurotoxin is Type A. The cyclodextrin can be alpha, beta, or gamma cyclodextrin.

Alternatively, the cyclodextrin can be a cyclodextrin derivative, such as but not limited to
hydroxyalkyl, carboxamide, diethylaminoethyl, carboxymethyl or dihydroxyalkyl derivative
of α-cyclodextrin, β-cyclodextrin, or γ-cyclodextrin.

Advantageously, the cyclodextrin is present in an amount sufficient to form a complex
with the botulinum neurotoxin to provide a stabilized botulinum neurotoxin. Ordinarily, as
known in the art, the botulinum neurotoxin is very susceptible to degradation or denaturation.
Typically, commercially available botulinum neurotoxins, such as BOTOX®, DYSPORT®,
and MYOBLOC®, lose their potency in about 4 hours. It has been surprisingly found that the
formation of the cyclodextrin-botulinum neurotoxin complex preserves the potency of the
botulinum neurotoxin for at least about 23 weeks.

In one embodiment, the pharmaceutical composition comprises botulinum neurotoxin
Type A and alpha-cyclodextrin. Only about 4% of the botulinum neurotoxin degrades over a
23-week period when at 4°C, whereas about 52 to 48% of the neurotoxin degrades over a 23-
week period at 25°C.

In another embodiment, the composition comprises botulinum neurotoxin Type A and
a beta-cyclodextrin. Less than 7% of the botulinum neurotoxin at 4°C degrades over a 23-
week period, while less than 65% of the botulinum neurotoxin at 25°C degrades over the same
time period.

In yet another embodiment, the composition comprises botulinum neurotoxin Type A
and gamma-cyclodextrin. In this embodiment, the degradation of the neurotoxin at 4°C was
less than 2%, and the degradation of the neurotoxin at 25°C was less than 55%, both over a
23-week period. Accordingly, the composition has an increased stability over time.

In one preferred embodiment, the botulinum neurotoxin inclusion complex of the
invention is admixed with a pharmaceutically acceptable diluent, carrier or excipient
(including combinations thereof) to form a pharmaceutical composition. The pharmaceutical
compositions may be for human or animal usage in human and veterinary medicine and will
typically comprise any one or more of a pharmaceutically acceptable diluent, carrier, or
excipient. Acceptable carriers or diluents for therapeutic use are well known in the
pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences,
The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as, or in addition to, the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s). Examples of suitable carriers include lactose, starch, glucose, methyl cellulose, magnesium stearate, mannitol, sorbitol and the like. Examples of suitable diluents include ethanol, glycerol and water. Examples of suitable binders include starch, gelatin, natural sugars such as glucose, anhydrous lactose, free-flow lactose, beta-lactose, corn sweeteners, natural and synthetic gums, such as acacia, tragacanth or sodium alginate, carboxymethyl cellulose and polyethylene glycol. Examples of suitable lubricants include sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride and the like. Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. Examples of preservatives include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid.

Antioxidants and suspending agents may be also used.

The composition in accordance with the invention may be, for example and not limitation, in the form of an injectable solution, a vacuum dried preparation, or a freeze-dried preparation, also include.

In accordance with another aspect of the invention, a method is provided for increasing the stability of botulinum neurotoxin comprising subjecting the botulinum neurotoxin to cyclodextrin to form an inclusion complex. Preferably, the molar ratio of the cyclodextrin to botulinum neurotoxin is at least 25:1 to 50:1, and most preferably 39:1. As used herein, the term "cyclodextrin" refers to a compound having a plurality of cyclopyranose units and include alpha, beta, gamma-cyclodextrins, any derivative or salt thereof and any combination thereof. The method of the invention provides a cyclodextrin-botulinum neurotoxin inclusion complex having markedly improved stability. Consequently, this improved stability serves to provide a botulinum neurotoxin with increased shelf-life. Preferably, the botulinum neurotoxin is subjected to the cyclodextrin in an aqueous system. The aqueous system has a pH of about 6.8 to 7.6 and can contain between 3 and 25 mM cyclodextrin, between 5 and 25nM phosphate buffer. Most preferably, the aqueous system comprises 10mM cyclodextrin, 10mM sodium phosphate buffer, and has a pH 7.4.

In one particularly preferred embodiment of the invention, the aqueous solution of the cyclodextrin inclusion product of botulinum neurotoxin in accordance with the invention can
be used in a variety of forms. For example, the complex may be prepared as an injectable
drug or can be dried to form a reconstitutable powder, as known in the art.

It is to be understood that both the foregoing general description and the following
detailed description are exemplary and are intended to provide further explanation of the
invention claimed.

The accompanying drawing, which is incorporated in and constitutes part of this
specification, is included to illustrate and provide a further understanding of the method and
system of the invention. Together with the description, the drawings serve to explain the
principles of the invention.

**BRIEF DESCRIPTION OF THE DRAWINGS**

FIG. 1 is a bar graph illustrating the results of a 23-week study of the incubation of
neurotoxin Type A complex with alpha, beta, and gamma cyclodextrins at 4°C in accordance
with the invention;

FIGS. 2 and 2a are chromatograms illustrating the spectra from the gamma-
cyclodextrin incubated neurotoxin Type A at 4°C in accordance with the invention;

FIGS. 3, 3a and 3b are chromatograms illustrating the spectra from the alpha-
cyclodextrin incubated neurotoxin Type A at 4°C in accordance with the invention;

FIGS. 4, 4a and 4b are chromatograms illustrating the spectra from the beta-
cyclodextrin incubated neurotoxin Type A at 4°C in accordance with the invention;

FIG. 5 is bar graph illustrating the results of a 23-week study of the incubation of
neurotoxin Type A complex with alpha, beta, and gamma cyclodextrins at room temperature
in accordance with the invention;

FIGS. 6, 6a, 6b and 6c are chromatograms illustrating the spectra from the neurotoxin
Type A control at room temperature in accordance with the invention;

FIGS. 7, 7a, 7b, 7c and 7d are chromatograms illustrating the spectra from the alpha-
cyclodextrin incubated neurotoxin Type A at room temperature in accordance with the
invention;

FIGS. 8, 8a, 8b, and 8c are chromatograms illustrating the spectra from the beta-
cyclodextrin incubated neurotoxin Type A at room temperature in accordance with the
invention;

FIGS. 9, 9a and 9b are chromatograms illustrating the spectra from the gamma-
cyclodextrin incubated neurotoxin Type A at room temperature in accordance with the
invention;
FIG. 10 illustrates SNAP 25 cleavage by Type A Complex at 6 weeks; and FIG. 11 illustrates endopeptidase activity of TANC after storage for 6 weeks.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Reference will now be made in detail to the present preferred embodiments of the invention, an example of which is illustrated in the accompanying drawing. The method and corresponding steps of the invention will be described in conjunction with the detailed description of the system.

The methods and compositions presented herein may be used for alleviating the problems associated with instability of botulinum neurotoxin. The present invention is particularly suited for providing a novel inclusion complex of botulinum neurotoxin which exhibits improved stability, and method of making the inclusion complex. For purpose of explanation and illustration, and not limitation, an exemplary embodiment of the system in accordance with the invention is shown in drawing FIGS. 1 to 11 inclusive.

Specifically, and in accordance with the present invention, composition comprises a botulinum neurotoxin and cyclodextrin. The botulinum neurotoxin and the cyclodextrin form an inclusion complex which exhibits markedly improved stability. The botulinum neurotoxin embodied herein includes Types A, B, C, D, E, F, and G. In the preferred embodiment, the botulinum neurotoxin is Type A. The cyclodextrin embodied herein includes alpha, beta, and gamma-cyclodextrin, and any derivative or salt thereof.

In accordance, with another embodiment of the invention, a method for stabilizing botulinum neurotoxin is provided. The method includes subjecting the botulinum neurotoxin to cyclodextrin to form a stable inclusion complex.

**Comparative Studies**

A series of studies were performed on the stability and preservation of the botulinum neurotoxin Type A and cyclodextrin complex ("TANC/CD"). In the series of studies, the Type A neurotoxin from the bacillus *Clostridium botulinum* ("TANC") was incubated for 23-weeks with a each of the alpha, beta, and gamma cyclodextrins and analyzed by HPLC-Gel filtration ("HPLC-GF") methods, as known in the art.

As depicted in FIG. 1, the results of the 23-week TANC/CD study illustrates the total peak area determined to be non-degraded TANC at 4°C. As used herein the term "total peak area" refers to the amount of protein represented by the elution time peak. A reduction in the total peak area would reflect reduction in the amount of protein at that peak, suggesting
degradation or dissociation into smaller fragments or components of the complex. As shown, the total peak area of the control is 100% throughout the 23-week period. Accordingly, the control has undergone no degradation of the TANC over the 23-week period of time. The control used in this study comprised 10mM cyclodextrin and 10mM sodium phosphate buffer at pH 7.4.

FIG. 1 depicts that each of the alpha, beta, and gamma-cyclodextrin incubated TANC have peaks which indicate at least a small amount of degradation. As illustrated, the gamma-cyclodextrin incubated TANC shows the least degradation of about 2% at 4°C. The beta-cyclodextrin incubated TANC has the most degradation (as compared to the other cyclodextrins) of more than 6%. The small amount of degradation of the gamma-cyclodextrin incubated TANC sample is most likely a result of the denaturation of a small amount of the complex structure.

As shown and depicted in FIG. 5, further control of the stabilization effect of the 10mM sodium phosphate buffer was observed at room temperature (25°C), and which depicts degradation of the complex by about 42%.

The chromatogram extracted at 280 nm as depicted in FIG. 2 indicates that the TANC peak has a relatively stable retention time of about 7.4 minutes. The degradation peak, however, has a retention time of about 18.3 minutes. Thus, smaller protein fragments or components are retained for a longer period of time in a gel filtration column. The longer retention time refers to the proteins of smaller size, which may be produced either by degradation of the complex or dissociation of its components. Further, as depicted in FIG. 2a, a 200nm to 400nm wavelength scan extracted from the gamma-incubated TANC sample chromatogram indicates that the 18.3 minute peak has a λ max value at about 225 and 280 nm thereby indicating the presence of protein. Accordingly, the degradation of the gamma-cyclodextrin incubated TANC is a disruption of the complex structure and not a degradation of the cyclodextrin complex surrounding the TANC.

Each of the alpha-and beta-cyclodextrin incubated TANC samples revealed two degradation peaks. Extracted spectra from the first of the degradation peaks for the alpha and beta cyclodextrin incubated TANC samples displaced results similar to that of the gamma-cyclodextrin peaks, as illustrated in FIGS. 3a and 4a, respectively. Specifically, as depicted in FIG. 3a, a 200nm to 400 nm wavelength scan extracted from the alpha-cyclodextrin incubated TANC sample indicates that the 16.9 minute peak has λ max values at about 280 nm thereby indicating the presence of protein. Additionally, and as depicted in FIG. 4a, a 200 nm to 400
nm wavelength scan extracted from the beta-cyclodextrin incubated TANC sample indicates that the 17.4 minute peak has λ max values at about 280 nm thereby indicating the presence of protein. Further, the spectra depicted in FIG. 4a also indicates a λ max value at about 265 nm, thereby indicating that the beta-cyclodextrin is no longer in complex with the TANC.

The second of the degradation peaks for the alpha-cyclodextrin incubated TANC and the beta-cyclodextrin incubated TANC, as illustrated in FIGS. 3b and 4b, respectively, are characteristic of uncomplexed cyclodextrins. The spectra illustrated in FIGS. 3b and 4b each have λ max values at about 225 nm and at about 265 nm.

As can be seen from FIGS. 3 and 4, the 7.4 minutes peak for the alpha-cyclodextrin TANC complex and the 7.6 minute peak for the beta-cyclodextrin TANC each have a distinctive shoulder at about 9 minutes. An extracted spectra for each of these shoulders illustrated that each shoulder yielded a peak representative of TANC itself. This data indicates that a large portion of the TANC complex (alpha- at about 22%; beta at about 31% by absorbance) which will break away from the TANC/CD complex.

As depicted in FIG. 5 and embodied herein, the results of the 23-week TANC/CD study illustrates the total peak area determined to be non-degraded TANC at room temperature. As shown, each of the cyclodextrin TANC samples including the control indicate greater amounts of degradation than comparable samples incubated at 4°C. As with the 4°C incubation results, the room temperature control sample had the lowest degree of TANC degradation of about 44% degradation over the 23 week period. FIG. 5 also shows that the alpha-cyclodextrin incubated TANC sample, as compared to the beta- and gamma-cyclodextrin incubated TANC complexes, had the least degradation over the 23-week period. The degradation of the alpha-cyclodextrin TANC was about 52 to 48 %, whereas, the beta-cyclodextrin incubated TANC had a 65% degradation, and the gamma-cyclodextrin incubated TANC had a 55% degradation.

As depicted in FIG. 5 the total peak area of the control at room temperature is 100% in week 1. However, the control shows some degradation in week 2 of about 23% and continually degrades over the 23 week period to about 44% degradation in week 23. The other cyclodextrin incubated TANC had greater degrees of TANC degradation. Specifically, the beta-cyclodextrin incubated TANC at room temperature degraded by about 65%, and the gamma-cyclodextrin incubated TANC at room temperature degraded by about 55% over the 23-week period. The alpha-cyclodextrin incubated TANC at room temperature had less degradation (about 52 to 48%) than either the beta and gamma-cyclodextrin incubated TANC.
FIGS. 6, 6a, 6b, and 6c each show chromatograms and extracted spectra of the control cyclodextrin incubated TANC at room temperature. FIGS. 7, 7a, 7b, and 7c each show chromatograms and extracted spectra of the alpha-cyclodextrin incubated TANC at room temperature, FIGS. 8, 8a, 8b, and 8c each illustrate the chromatogram and extracted spectra of the beta-cyclodextrin incubated TANC at room temperature. In each chromatogram, the wavelength scans of the peaks, which represent the degradation products for each of the cyclodextrin incubated TANC complexes are similar to that of the cyclodextrin incubated TANC complexes at 4°C, as illustrated in FIGS. 2 through and including 4b. The wavelength scans represented in FIGS. 6 through 9 indicate either denaturation of the protein complex, cyclodextrin complex, or both. The early degradation peaks in FIGS. 7a, 7b, and 8a, at about 15.3 min., 17.1 min., and 17.3 min., respectively, each depicted max values slightly above 300 nm.

Degradation Product Molecular Mass
An attempt was made to determine the molecular mass of the degradation products using the standard curve (y = -1.667x + 7.409) generated for this column. The elution time for the Blue Dextrin (5.115 min.) served as the void volume (V₀) for the Rᵣ calculations (Vₑ/V₀). Tables 1 and 2, below, each show the values from the calculations.

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C degradation product molecular masses</td>
</tr>
<tr>
<td>Sample</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>α-CD</td>
</tr>
<tr>
<td>β-CD</td>
</tr>
<tr>
<td>γ-CD</td>
</tr>
</tbody>
</table>

* indicates values below the linear range of the GF column.

<table>
<thead>
<tr>
<th>Table 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room temperature degradation product molecular masses</td>
</tr>
<tr>
<td>Sample</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>α-CD</td>
</tr>
<tr>
<td>β-CD</td>
</tr>
<tr>
<td>γ-CD</td>
</tr>
</tbody>
</table>

* indicates values below the linear range of the GF column.
As shown in Tables 1 and 2, above, the values indicate that none of the major TANC proteins have broken away from the complex structure. The small molecular masses of the degraded portions of the ANC complex are significantly below the lower limit of the linear range of the GF column. The calculated molecular masses indicate by virtue of the very small molecular masses that the complex is still intact.

Presence of DNA/RNA

Addition of Rnases to the TANC solution indicated NO presence of RNAs or DNAs. The addition of the nucleases was monitored spectrophotometrically at 260 nm for an increase in absorbance as the nucleotides comprising any RNA/DNA present were released from their tertiary structure. While an increase in absorbance was observed (0.002 increase over 3 hours) it was determined that such a small increase could not account for the presence of RNAs or DNAs in any significant concentration.

Example

Preparation of C. botulinum Type A

The C. botulinum Type A (strain Hall) complex was prepared by the method described in Cai et al. Enhancement of the Endopeptidase Activity of Botulinum Neurotoxin by Its Associated Proteins and Dithiothreitol, Biochemistry, 1999, 38, 6903-6910, the entire contents of which are incorporated herein by reference. The purified Type A complex was subjected to a buffer exchange using a 5 mL Sephadex G-25 column equilibrated with 10 column volumes of 10 mM sodium phosphate, pH 7.4. The Type A complex was determined to have a typical subunit makeup by SDS-PAGE analysis. The Type A complex, at physiological pH of 7.4, was diluted to a 0.75 mg/mL concentration and 1 mL aliquots were placed into eight 1.5 mL microcentrifuge tubes. 1.5 mg mL\(^{-1}\) solutions of the Type A Botulinum neurotoxin complex at both 25°C and 4°C were analyzed by high performance liquid chromatography using a gel filtration (size exclusion) column (HPLC-GF) both in the presence and absence of 0.1 mM α-, β-, or γ-cyclodextrin in the solution. The WATERS HPLC system was equipped with a Waters 996 Photodiode Array (PDA) detector and dual Waters 515 HPLC pumps. The Waters pump system was integrated by a Waters Pump Control Module and controlled by Waters Millennium Software. The sample was introduced to the system via a Rheodyne 77251 Manual Injection System. The column used for separation of the sample was a Waters Protein
Pak 300SW (4.6 x 300 mm). The buffer conditions used for each separation attempt will be listed below.

**Standard Curve Generation**

The gel filtration standards (Sigma Chemical Co.) were used to determine the molecular mass standard curve for the Protein Pak column. The buffer system used for the standard curve determination was a 10 mM sodium phosphate buffer, pH 7.4. The results from the standard curve determination are listed in Table 1 below. The equation of the line determined from a plot of Log Molecular Mass versus V_v/V_ao was calculated to be y = 1.7417x + 7.399, R^2 = 0.9398.

**Neurotoxin A Complex Analysis**

The results from the HPLC-GF analysis indicate that it is possible to stabilize the botulinum neurotoxin and its complexing NAPs at physiological pH. In 50 mM sodium phosphate buffer, pH 6.8, 85% of the protein remained in complex form. A lower % complex was found in a 10 mM sodium phosphate buffer, pH 7.4, (-70%). The lower % complex at a pH of 7.4 is due to the introduction of the complex directly into the new buffer system. Results similar to that of the pH 6.8 buffer system would be the result if the botulinum toxin and NAPs were dialyzed into the pH 7.4 buffer prior to introduction into the HPLC-GF system.

Further, stability studies of the endopeptidase activity of Type A Neurotoxin Complex (TANC) were performed at 5°C and 25°C in the presence and absence of alpha-cyclodextrin. The results of the study suggest that over a six-week period TANC remained enzymatically active, both in the presence and absence of 0.1 mM alpha cyclodextrin at 5° and 25°C. An electrophoresis analysis of the proteolysis (endopeptidase) product of type A botulinum neurotoxin substrate, SNAP-25 (intact SNAP-25 shown with arrow in lane 2) is illustrated in FIG. 10. After treatment with 150 nM TANC (lane 3), the intact SNAP-25 band disappears. Similar results were obtained for TANC stored at 50°C in 10 mM sodium phosphate buffer, pH 7.4, with (lane 6) or without any α-cyclodextrin (lane 5). Endopeptidase activity was also retained at 250°C (lanes 7&8).

FIG. 11 depicts a graphical representation of the endopeptidase activity under different storage conditions. As shown, the endopeptidase activity of TANC in each storage condition remains high. Negative intensity of SNAP-25 intensity in TANC and TANC stored at 50°C in buffer indicates error in the intensity determination of the protein band corresponding to the
SNAP-25. The endopeptidase activity of TANC in each condition tested was therefore identical with the experimental error.

The methods and systems of the present invention, as described above and shown in the drawings, provide for a stabilized *C. Botulinum* neurotoxin complex.

It will be apparent to those skilled in the art that various modifications and variations can be made in the method and system of the present invention without departing from the spirit or scope of the invention. Thus, it is intended that the present invention include modifications and variations that are within the scope of the appended claims and their equivalents.
THE CLAIMS

What is claimed is:

1. A composition comprising:
   a botulinum neurotoxin; and
   a cyclodextrin or a derivative thereof, wherein the cyclodextrin and the botulinum
   neurotoxin form an inclusion complex.

2. The formulation of claim 1, wherein the cyclodextrin and botulinum neurotoxin are
   present in a molar ratio of at least 25:1 to 50:1 to enable the botulinum neurotoxin to exhibit
   improved stability.

3. The composition of claim 1, which has a shelf life of at least four weeks and about
   55% to about 80% of the botulinum neurotoxin remains as the complex for a period of at least
   2 weeks.

4. The composition of claim 1, wherein the botulinum neurotoxin has about 2% to less
   than 65% degradation over a period of time of about 23 weeks at storage temperatures that are
   between 4° and 25°C.

5. The composition of claim 1, wherein the botulinum toxin is selected from the group
   consisting of botulinum toxins types A, B, C, D, E, F, and G.

6. The composition of claim 1, wherein the botulinum toxin is purified prior to forming
   the complex.

7. The composition of claim 1, wherein the cyclodextrin comprises an α-cyclodextrin, β-
   cyclodextrin, γ- cyclodextrin, or a hydroxyalkyl, carboxamide, diethylaminoethyl,
   carboxymethyl or dihydroxyalkyl derivative of α-cyclodextrin, β- cyclodextrin, or γ-
   cyclodextrin.

8. The composition of claim 1, wherein the cyclodextrin is present in a stabilizing
   solution that further comprises a phosphate buffer.
The composition of claim 8, wherein the buffer is sodium phosphate and the pH of the solution is about 6.8 to about 7.6.

The composition of claim 1, in the form of an injectable solution, or dried preparation.

A method for stabilizing botulinum neurotoxin, the method comprising:
providing botulinum neurotoxin; and
subjecting the botulinum neurotoxin to cyclodextrin, wherein the cyclodextrin is provided in an amount sufficient to form an inclusion complex with the botulinum neurotoxin.

The method of claim 11, which further comprises imparting improved stability to the botulinum neurotoxin by providing the cyclodextrin and botulinum neurotoxin in a molar ratio of at least 25:1 to 50:1.

The method of claim 11, which further comprises providing an amount of cyclodextrin to impart a shelf life of at least four weeks to the complex with about 55% to about 80% of the botulinum neurotoxin remaining as the complex for a period of at least 2 weeks.

The method of claim 11, wherein the botulinum neurotoxin has about 2% to less than 65% degradation over a period of time of about 23 weeks at storage temperatures that are between 4° and 25°C.

The method of claim 11, wherein the botulinum toxin is selected from the group consisting of botulinum toxins types A, B, C, D, E, F, and G.

The method of claim 11, wherein the botulinum toxin is purified prior to forming the complex.

The method of claim 11, wherein the cyclodextrin comprises an α-cyclodextrin, β-cyclodextrin, γ-cyclodextrin, or a hydroxyalkyl, carboxamide, diethylaminoethyl, carboxymethyl or dihydroxyalkyl derivative of α-cyclodextrin, β-cyclodextrin, or γ-cyclodextrin.

The method of claim 11, which further comprises providing a stabilizing solution for the cyclodextrin wherein the solution further comprises a phosphate buffer.
19. The method of claim 18, wherein the buffer is sodium phosphate and the pH of the solution is about 6.8 to about 7.6.

20. The method of claim 11, which further comprises providing the complex in the form of an injectable solution or dried preparation.
23-WEEK INCUBATION OF BOTULINUM NEUROTOXIN TYPE A WITH $\alpha$, $\beta$, AND $\gamma$-CYCLODEXTRINS AT 4°C

4-Degree Incubation

![Graph showing the incubation of botulinum neurotoxin type A with different cyclodextrins over 23 weeks at 4°C.](image_url)

<table>
<thead>
<tr>
<th>Incubation Period</th>
<th>Control</th>
<th>Alpha</th>
<th>Beta</th>
<th>Gamma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wk. 1</td>
<td>100</td>
<td>99.47</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Wk. 2</td>
<td>100</td>
<td>99.67</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Wk. 3</td>
<td>100</td>
<td>99.67</td>
<td>100</td>
<td>99.78</td>
</tr>
<tr>
<td>Wk. 4</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Wk. 12</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Wk. 23</td>
<td>100</td>
<td>96.21</td>
<td>93.78</td>
<td>98.03</td>
</tr>
</tbody>
</table>

FIG. 1
CHROMATOGRAM OF α AND β CYCLODEXTRIN INCUBATED BOTULINUM NEUROTOXIN TYPE A AT 4°C

FIG. 2

FIG. 2a
CHROMATOGRAM OF α AND β CYCLODEXTRIN INCUBATED BOTULINUM NEUROTOXIN TYPE A

FIG. 3

FIG. 3a

FIG. 3b
23-WEEK INCUBATION OF BOTULINUM NEUROTOXIN TYPE A WITH α,β, AND γ-CYCLODEXTRINS AT ROOM TEMPERATURE

Room Temperature Incubation

<table>
<thead>
<tr>
<th>Incubation Period</th>
<th>Control</th>
<th>Alpha</th>
<th>Beta</th>
<th>Gamma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wk. 1</td>
<td>100</td>
<td>98.78</td>
<td>100</td>
<td>99.80</td>
</tr>
<tr>
<td>Wk. 2</td>
<td>77.02</td>
<td>99.41</td>
<td>84.09</td>
<td>100</td>
</tr>
<tr>
<td>Wk. 3</td>
<td>68.81</td>
<td>94.71</td>
<td>57.75</td>
<td>87.30</td>
</tr>
<tr>
<td>Wk. 4</td>
<td>55.38</td>
<td>83.27</td>
<td>44.53</td>
<td>74.36</td>
</tr>
<tr>
<td>Wk. 12</td>
<td>52.66</td>
<td>58.65</td>
<td>40.10</td>
<td>54.63</td>
</tr>
<tr>
<td>Wk. 23</td>
<td>56.14</td>
<td>51.67</td>
<td>35.35</td>
<td>45.12</td>
</tr>
</tbody>
</table>

FIG. 5
CHROMATOGRAM OF $\alpha$ AND $\beta$ CYCLODEXTRIN INCUBATED BOTULINUM NEUROTOXIN TYPE A AT ROOM TEMPERATURE

FIG. 7a

FIG. 7b
CHROMATOGRAM OF α AND β CYCLODEXTRIN INCUBATED BOTULINUM NEUROTOXIN TYPE A AT 4°C

FIG. 9
CHROMATOGRAM OF α AND β CYCLODEXTRIN INCUBATED BOTULINUM NEUROTOXIN TYPE A AT 4°C

FIG. 9b

FIG. 9a
Endopeptidase Activity of TANC After Storage for 6-weeks

1, 4, 9 = Molecular Weight Marker
2 = SNAP-25 Control
3 = Fresh Type A complex Cleavage
5 = 5°C Buffer
6 = 5°C α-CD
7 = 25°C Buffer
8 = 25°C α-CD

FIG. 11