(54) Title: COMPOSITIONS OF ACTIVATED BOTULINUM TOXIN TYPE B

(57) Abstract: The present invention relates to pharmaceutical compositions of activated botulinum toxin type B. In particular, the present invention relates to botulinum toxin type B pharmaceutical compositions wherein at least 75% of said botulinum toxin type B is activated - i.e., "nicked". The invention also relates to a process of activating botulinum toxin type B wherein at least 75% of said botulinum toxin type B is nicked. The invention further relates to methods for the treatment of a variety of neuromuscular diseases, pain, inflammatory and cutaneous disorders comprising administering a pharmaceutical composition of activated botulinum toxin type B wherein at least 75% of said botulinum toxin type B is nicked.

![Diagram of process flow]
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TITLE OF THE INVENTION
COMPOSITIONS OF ACTIVATED BOTULINUM TOXIN TYPE B

FIELD OF THE INVENTION

[0001] The present invention relates to pharmaceutical compositions of activated botulinum toxin type B. In particular, the present invention relates to botulinum toxin type B pharmaceutical compositions wherein at least 75% of said botulinum toxin type B is activated — i.e., “nicked”. The invention also relates to a process of activating botulinum toxin type B wherein at least 75% of said botulinum toxin type B is nicked. The invention further relates to methods for the treatment of a variety of neuromuscular diseases, pain, inflammatory and cutaneous disorders comprising administering a pharmaceutical composition of activated botulinum toxin type B wherein at least 75% of said botulinum toxin type B is nicked.

BACKGROUND OF THE INVENTION

[0002] The anaerobic, gram positive bacterium Clostridium botulinum produces a potent polypeptide neurotoxin, botulinum toxin, which causes a neuroparalytic illness in humans and animals by attacking peripheral cholinergic motor neurons. Botulinum toxin apparently binds with high affinity to these motor neurons, is translocated into the neuron and blocks the release of acetylcholine.

[0003] Seven immunologically distinct botulinum neurotoxins have been characterized — these being, respectively, botulinum neurotoxin serotypes A, B, C1, D, E, F and G — each of which is distinguished by neutralization with type-specific antibodies. Although all the botulinum toxin serotypes apparently inhibit release of the neurotransmitter acetylcholine at the neuromuscular junction, they do so by affecting different neurosecretory proteins and/or cleaving these proteins at different sites. Consequently, the different serotypes of botulinum toxin vary in the animal species that they affect and in the severity and duration of the paralysis they evoke.

[0004] Botulinum toxin type A is the most lethal natural biological agent known to man. It is also the most widely used serotype in pharmaceutical compositions due to its efficacy and potency. A deadly toxin at high concentrations and quantities, botulinum toxin type A has been used as a valuable therapeutic for the treatment of many neuromuscular diseases (e.g.,
dystonia, hemifacial spasm, bruxism, spasticity, cerebral palsy, torticollis), as well as sensory disorders and cutaneous disorders (myofascial pain, migraine, tension headaches, neuropathy, hyperhidrosis), and in the treatment of disorders involving inflammation.

[0005] One reason for the efficacy and potency of naturally occurring botulinum toxin serotype A is that while all the botulinum toxin serotypes are initially synthesized as inactive single chain proteins which must be cleaved or "nicked" by proteases to become neuroactive, the bacterial strains that make type A possess endogenous proteases. Therefore, the serotype A toxin can be recovered from bacterial cultures in predominantly its active form: approximately 90-95 percent of type A toxin is nicked. In contrast, botulinum toxin serotypes C₁, D and E are synthesized by non-proteolytic strains and are therefore typically unactivated when recovered from culture. Serotypes B and F are produced by both proteolytic and non-proteolytic strains and therefore can be recovered in either the active or inactive form. However, even the proteolytic strains that produce, for example, the botulinum toxin type B serotype only cleave a portion of the toxin produced. The exact proportion of nicked to unnicked molecules depends on the length of incubation and the temperature of the culture. Therefore, a certain percentage of any preparation of, for example, the botulinum toxin type B toxin is likely to be inactive, possibly accounting for the known significantly lower potency of botulinum toxin type B as compared to botulinum toxin type A. Furthermore, the presence of inactive botulinum toxin molecules in a clinical preparation will contribute to the overall protein load of the preparation, which has been linked to increased antigenicity, without contributing to its clinical efficacy.

[0006] The weakness of pharmaceutical compositions comprising botulinum toxin type A, on the other hand, is that although its action on nerve terminals is irreversible, axon sprouting reverses the denervating effects of the toxin within two to six months and, consequently, repeated administration of the neurotoxin is required in a variety of conditions and disorders. However, immunity and resistance to the neurotoxin due to the production of neutralizing antibodies is an important clinical consequence and problem resulting from repeated administrations. The antigenicity of botulinum toxin type A stimulates antibody formation that reduces and most often completely obliterates the therapeutic effectiveness of botulinum toxin type-A-based pharmaceuticals.
SUMMARY OF THE INVENTION

[0007] In some embodiments, a pharmaceutical composition includes botulinum toxin type B and at least one excipient, wherein at least 75% of the botulinum toxin type B is nicked.

[0008] In some embodiments, a process of activating botulinum toxin type B includes the stages of: cell growth, activation, purification, and dilution; wherein at least one exogenous protease is administered to a volume of said botulinum toxin type B, and wherein the level of nicked botulinum toxin type B is increased to at least 75%.

[0009] In some embodiments, a method of treating a variety of disorders includes administering to a patient in need thereof, a pharmaceutical composition including activated botulinum toxin type B and at least one excipient, wherein at least 75% of said botulinum toxin type B is nicked.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] The accompanying drawings, which are included to provide a further understanding of the invention and are incorporated in and constitute a part of this specification, illustrate embodiments of the invention and together with the description serve to explain the principles of the invention. In the drawings:

[0011] FIG. 1 shows an overall manufacturing process flow chart for activated botulinum toxin type B;

[0012] FIG. 2 shows a detailed flow chart for the fermentation stage of the manufacturing process;

[0013] FIG. 3 shows a detailed flow chart for the recovery stage of the manufacturing process;

[0014] FIG. 4 shows a detailed flow chart for the purification stage of the manufacturing process;

[0015] FIG. 5 shows a detailed flow chart for the production and handling of a dilute bulk solution of activated botulinum toxin type B.
DETAILED DESCRIPTION OF THE INVENTION

[0016] Toxins of the different Clostridium botulinum serotypes are produced in culture as aggregates of neurotoxin and non-toxic proteins non-covalently associated into polypeptide complexes of varying molecular weight. As used herein, "botulinum toxin type B" means an approximately 150 kD protein neurotoxin isolated from the Type B (i.e., Bean strain) of Clostridium botulinum, including mixtures of its approximately 300-500 kD protein complexes, toxoid, and/or other clostridial proteins, and may refer to either its single-chain or di-chain ("nicked") neurotoxin form.

[0017] As used herein, "activated botulinum toxin type B" means the single-chain 150 kD protein type B neurotoxin has undergone limited posttranslational proteolysis ("nicking") typically between residues Lys 440 and Ala 441 to form a di-chain protein consisting of an approximately 50 kD light chain linked to an approximately 100 kD heavy chain by a disulfide bridge. This nicked form is essential for the neurotoxin's binding to and translocation across epithelial cells at the neuromuscular junction to produce acetylcholine blockage.

[0018] According to some embodiments, the present invention describes a pharmaceutical composition of activated botulinum toxin type B. In some embodiments, the present invention describes a process of activating botulinum toxin type B. And in some embodiments, the present invention describes a method of treating a variety of ophthalmologic disorders, neuromuscular diseases, otorhinolaryngological disorders, urogenital disorders, dermatological disorders, pain disorders, inflammatory disorders, secretory disorders, and cutaneous disorders or cosmetic treatment by administering an effective amount of a pharmaceutical composition of the present invention to a patient in need thereof.

[0019] I. Compositions of Activated Botulinum Toxin Type B

[0020] A. Activated Botulinum Toxin Type B

[0021] The proteolytic strains that produce the botulinum toxin type B serotype only cleave a portion of the toxin produced: approximately 65% of naturally produced botulinum toxin type B is activated. The present invention discloses a pharmaceutical composition wherein at least 75% of the botulinum toxin type B is activated – i.e., "nicked". In some embodiments, the present invention is directed to pharmaceutical compositions of activated botulinum toxin
type B. In some embodiments, at least 75 percent of the botulinum toxin type B in a pharmaceutical composition is nicked. In some embodiments, greater than 75 percent of the botulinum toxin type B in a pharmaceutical composition is nicked. In some embodiments, about 75 percent to about 85 percent of the botulinum toxin type B in a pharmaceutical composition is nicked. In some embodiments, greater than 80 percent of the botulinum toxin type B in a pharmaceutical composition is nicked. In some embodiments, greater than 85 percent of the botulinum toxin type B in a pharmaceutical composition is nicked. In some embodiments, about 85 percent to about 95 percent of the botulinum toxin type B in a pharmaceutical composition is nicked. In some embodiments, greater than 90 percent of the botulinum toxin type B in a pharmaceutical composition is nicked. In some embodiments, about 95 percent to about 100 percent of the botulinum toxin type B in a pharmaceutical composition is nicked. In some embodiments, greater than 95 percent of the botulinum toxin type B in a pharmaceutical composition is nicked. In some embodiments, greater than 99 percent of the botulinum toxin type B in a pharmaceutical composition is nicked.

[0022] The increased activation of botulinum toxin type B in the present invention results in pharmaceutical compositions with comparable efficacy, potency and specific activity to compositions of botulinum type A while limiting the adverse effects of inactive botulinum toxin molecules. Relative to existing pharmaceutical compositions of botulinum toxin type B, the present invention has a decreased overall protein load which results in decreased antigenicity without diminishing clinical efficacy.

[0023] B. Excipients

[0024] In some embodiments, the pharmaceutical compositions include activated botulinum toxin type B, and at least one excipient. As used herein, the term “excipient” means a pharmaceutically acceptable chemical composition, compound, or solvent with which the activated botulinum toxin type B may be combined, may stabilize the botulinum toxin and does not alter its physical or therapeutic properties. Excipients suitable for use in the present invention may be selected from the group consisting of, but not limited to: carriers, sequestration agents, surfactants, crystalline agents, buffers, polyaccharides, metals, non-oxidizing amino acid derivatives, sodium chloride, surface active agents, dispersing agents, inert diluents, granulating and disintegrating agents, binding agents, lubricating agents, preservatives, physiologically degradable compositions such as gelatin, aqueous vehicles and solvents, oily vehicles and solvents, suspending agents, dispersing or wetting
agents, emulsifying agents, demulcants, salts, thickening agents, fillers, antioxidants, stabilizing agents, and any pharmaceutically acceptable polymeric or hydrophobic materials and other ingredients as known to one of ordinary skill in the art. Examples of excipients that are potentially suitable are disclosed in U.S. Patent No. 7,211,261 which is incorporated herein by reference in its entirety.

1. Sequestration agents

In some embodiments, a pharmaceutical composition of the present invention includes activated botulinum toxin type B, and at least one excipient such as a sequestration agent. As used herein, "sequestration agent" means an agent that enhances localization and/or retention of the botulinum toxin to the site of administration. Examples of proteins, polysaccharides, lipids, polymers, gels and hydrogels that are potentially suitable as sequestration agents are disclosed in U.S. Patent No. 4,861,627, which is incorporated herein by reference in its entirety. Methods of using and making protein microspheres as sequestration agents, including albumin microspheres, are disclosed in U.S. Patent Nos. 6,620,617; 6,210,707; 6,100,306; and 5,069,936 which are each incorporated herein by reference in their entirety.

In some embodiments, the sequestration agent is albumin. Human serum albumin may bind with many pharmaceutical agents, including peptides and proteins such as botulinum toxin, which can influence potency, complication rate, clearance, and other pharmacodynamic properties of these agents. Albumin in botulinum toxin pharmaceutical compositions may maintain biologic activity by promoting nerve and other receptor contact and preventing wash out from free neurotoxin release at injection points. Additionally, albumin can non-covalently bind cations that serve as cofactors for enzymatic reactivity of portions of the botulinum toxin polypeptide complex. Specifically, zinc is a cofactor for the endopeptidase activity of the botulinum toxin light chain which enters the target cells after heavy chain binding to the cell surface protein receptors. Higher quantities of zinc bound to albumin enhance endopeptidase activity and thus enhances the denervating effect of botulinum toxin type B. Finally, although other proteins (e.g., gelatin, lactalbumin, lysozyme), lipids and carbohydrates may serve as effective sequestration agents, albumin, including encapsulated albumin and solid microspheres is the preferred protein sequestration agent, in part, because of its low immunogenicity.
2. Buffers

In some embodiments, the excipient is a buffer. In some embodiments, the buffer is succinate. The buffer may be any buffer able to maintain the adequate pH. In some embodiments, the excipient is a buffer to maintain pH from about 5.0 to about 6.0, more preferably from about 5.2 to about 5.8, and most preferably about 5.6.

II. Process of Activating Botulinum Toxin Type B

In some embodiments, the present invention describes a process of activating botulinum toxin type B. Referring to FIG. 1, which shows an overall manufacturing process flow chart for activated botulinum toxin type B, in some embodiments, a process of activating botulinum toxin type B according to the present invention may generally be divided into four stages: Fermentation (FIG. 2), Recovery (FIG. 3), Purification (FIG. 4), and Dilute Bulk Solution Preparation (FIG. 5).

A. Fermentation (Cell Growth) Stage

FIG. 2 shows a detailed flow chart for the fermentation or cell growth stage 100 of FIG. 1 of the manufacturing process for activated botulinum toxin type B. In some embodiments, a process of activating botulinum toxin type B requires at least one fermentation or cell growth stage 100.

In some embodiments, the fermentation stage 100 includes a media/buffer preparation step 110. In some embodiments, the media buffer preparation step 110 includes autoclaving thioglycollate and Type B mediums for cell growth.

In some embodiments, the fermentation stage 100 includes a working cell bank (WCB) step 120. In some embodiments, the WCB step 120 includes utilizing a frozen culture of Clostridium botulinum, Type B and thawing the frozen culture in BSC. In some embodiments, the WCB step 120 includes taking a sample of the frozen culture for quality control.

In some embodiments, the fermentation stage 100 includes an S1 fermentation step 130 wherein the autoclaved thioglycollate medium of step 110 is inoculated with the thawed frozen culture of the WCB step 120 and incubated. In some embodiments, the S1
fermentation step 130 includes taking a sample of the resulting S1 cell culture for quality control.

[0037] In some embodiments, the fermentation stage 100 includes an S2 fermentation step 140. In some embodiments, the S2 fermentation step 140 includes a three sub-stage progression 141, 142, 143. In some embodiments, the S2 fermentation step 140 includes a first sub-stage 141 wherein the autoclaved Type B medium of step 110 is inoculated with the S1 cell culture of step 130 and incubated. In some embodiments, the S2 fermentation step 140 includes a second sub-stage 142 wherein the autoclaved Type B medium of step 110 is inoculated with the cell culture of the first sub-stage 141 and incubated. In some embodiments, the S2 fermentation step 140 includes a third sub-stage 143 wherein the autoclaved Type B medium of step 110 is inoculated with the cell culture of the second sub-stage 143 and incubated. In some embodiments, the S2 fermentation step 140 includes taking a sample of the resulting cell culture of the third sub-stage 143 for quality control.

[0038] In some embodiments, the fermentation stage 100 includes an S3 fermentation step 150. In some embodiments, the S3 fermentation step 150 includes an integrity test and exhaust filters. In some embodiments, the S3 fermentation step 150 includes sterilizing Type B medium in a fermenter. In some embodiments, the S3 fermentation step 150 includes adding autoclaved glucose via a sterile addition port to the sterilized Type B medium. In some embodiments, the S3 fermentation step 150 includes inoculating the sterilized fermentation media with the resulting step 143 cell culture via aseptic transfer. In some embodiments, the S3 fermentation step 150 includes incubating the fermentation medium with a nitrogen overlay, agitation, and pH control. In some embodiments, the S3 fermentation step 150 includes taking a sample of the resulting cell culture for quality control.

[0039] In some embodiments, the fermentation stage 100 includes an acid precipitation (AP) step 160. In some embodiments, the AP step 160 includes chilling the S3 cell culture of step 150 to less than 20° C. In some embodiment, the AP step 160 includes adjusting the pH of the step 150 fermentation medium with sulfuric acid. In some embodiments, the AP step 160 includes precipitating the cell culture out of the medium and transferring the cell culture to a 20L carboy with sanitary connection and subsequent transfer to bottles within BSC. In some embodiments, the AP step 160 includes centrifuging the precipitated cell culture and discarding the supernatant.
In some embodiments, the fermentation stage 100 includes an AP water wash step 170. In some embodiments, the AP water wash step 170 includes re-suspending the centrifuged pellet of step 160 in filtered water within BSC. In some embodiments, the AP water wash step 170 includes centrifuging the re-suspended cell culture and discarding the supernatant. In some embodiments, the AP water wash step 170 includes storing the centrifuged pellet at about 2-8°C.

B. Recovery (Activation) Stage

FIG. 3 shows a detailed flow chart for the recovery or activation stage 200 of FIG. 1 of the manufacturing process for activated botulinum toxin type B. In some embodiments, a process of activating botulinum toxin type B requires at least one recovery or activation stage 200.

In some embodiments, the recovery stage 200 includes a buffer preparation step 210. In some embodiments, the buffer preparation step 210 includes preparing and adjusting the pH of phosphate buffers. In some embodiments, the buffer preparation step 210 includes filtering the buffers through a 0.2 μm filter, and storing the filtered buffer at room temperature.

In some embodiments, the recovery stage 200 includes an AP buffer wash step 220. In some embodiments, the AP buffer wash step 220 includes transferring the centrifuged pellet of step 170 from the fermentation suite and re-suspension of the pellet in the phosphate buffer of step 210. In some embodiments, the AP buffer wash step 220 includes centrifugation of the re-suspended pellet and saving the supernatant.

In some embodiments, the recovery stage 200 includes an ammonium chloride precipitation step 230. In some embodiments, the precipitation step 230 includes adding an ammonium chloride solution to the suspension of step 210 to achieve target concentration. In some embodiments, the precipitation step 230 includes stirring the mixture while refrigerated to dissolve salts. In some embodiments, the precipitation step 230 includes centrifuging the mixture and saving the supernatant.

In some embodiments, the recovery stage 200 includes an ammonium sulfate precipitation step 240. In some embodiments, the precipitation step 240 includes adding a solution of ammonium sulfate to the supernatant of step 230 to achieve target concentration.
In some embodiments, the precipitation step 240 includes stirring the mixture while refrigerated. In some embodiments, the precipitation step 240 includes centrifuging the mixture and saving the supernatant. In some embodiments, the precipitation step 240 includes adding a second solution of ammonium sulfate to the precipitate to achieve target concentration. In some embodiments, the precipitation step 240 includes stirring the suspension while refrigerated. In some embodiments, the precipitation step 240 includes a second centrifugation and saving the pellet.

[0047] In some embodiments, the recovery stage 200 includes a buffer re-suspension step 250. In some embodiments, the re-suspension step 250 includes dissolving the pellet of step 240 in a succinate buffer of pH 5.5. In some embodiments, the re-suspension step 250 includes centrifuging the suspension and saving the supernatant.

[0048] In some embodiments, the recovery stage 200 includes an activation step 260. In some embodiments, the activation step 260 includes addition of a protease to the supernatant of step 250. In some embodiments, the protease administered is selected from the group consisting of: trypsin, immobilized TPCK-trypsin, metalloproteases, endogenous proteases, bacterial proteases, and gastric proteases. In some embodiments, the protease is an animal free trypsin. In some embodiments, the animal free trypsin used is Trypzean.

[0049] In some embodiments, the recovery stage 200 includes a concentration and filtration step 270. In some embodiments, the concentration and filtration step 270 includes diafiltration of the solution of step 260 with a succinate buffer of pH 5.5 to a concentration of about 300 mL. In some embodiments, the concentration and filtration step 270 includes filtering the buffer through a 0.45 μm filter. In some embodiments, the concentration and filtration step 270 includes storing the filtered buffer at about 2-8° C.
C. Purification Stage

FIG. 4 shows a detailed flow chart for the purification stage 300 of FIG. 1 of the manufacturing process for activated botulinum toxin type B. In some embodiments, a process of activating botulinum toxin type B includes a purification stage 300.

In some embodiments, the purification stage 300 includes a buffer preparation step 310. In some embodiments, the buffer preparation step 310 includes preparing a succinate buffer, sodium hydroxide, and ethanol. In some embodiments, the buffer preparation step 310 includes filtering the succinate buffer and reagents through a 0.2 μm filter. In some embodiments, the filtered buffer and reagents is stored at room temperature.

In some embodiments, the purification stage 300 includes an anion exchange chromatograph step 320. In some embodiments, the chromatograph step 320 includes packing a chromatograph column with DEAE. In some embodiments, the chromatograph step 320 includes cleaning the column with 0.5 N NaOH and rinsing with filtered water. In some embodiments, the chromatograph step 320 includes sampling the column rinse for bioburden, TOC and LAL. In some embodiments, the chromatograph step 320 includes equilibrating the chromatograph column with the succinate buffer of step 310. In some embodiments, the chromatograph step 320 includes loading UFDF on the column. In some embodiments, the chromatograph step 320 includes collecting and analyzing fractions via SDS-PAGE gels. In some embodiments, the chromatograph step 320 includes pooling acceptable fractions. In some embodiments, the chromatograph step 320 includes filtering the pooled fractions through a 0.2 μm filter and sampling the filtered pooled fractions. In some embodiments, the chromatograph step 320 includes storing the filtered pooled fractions at about 2-8° C.

In some embodiments, the purification stage 300 includes a size exclusion chromatography step 330. In some embodiments, the size exclusion chromatography step 330 includes a column packing sub-step 331, a column use sub-step 332, and a column cleaning and storage sub-step 333. In some embodiments, the column packing sub-step 331 includes packing the column with SEC resin. In some embodiments, sub-step 331 includes testing the column for efficiency and peak asymmetry. In some embodiments, sub-step 331 includes cleaning the column with 0.5 NaOH and rinsing with filtered water. In some
embodiments, sub-step 331 includes sampling the column rinse for bioburden, TOC, and LAL. In some embodiments, sub-step 331 includes storing the column in 20% ethanol.

[0055] In some embodiments, the size exclusion chromatography step 330 includes a column use sub-step 332. In some embodiments, sub-step 332 includes cleaning the column with 0.5 NaOH and rinsing with filtered water. In some embodiments, sub-step 332 includes sampling the column rinse for bioburden, TOC, and LAL. In some embodiments, sub-step 332 includes equilibrating the column with the succinate buffer of step 310. In some embodiments, sub-step 332 includes loading the filtered pooled fractions of step 320 on the column. In some embodiments, sub-step 332 includes collection and analyzing fractions via SDS-PAGE gels. In some embodiments, sub-step 332 includes pooling acceptable fractions.

[0056] In some embodiments, the size exclusion chromatography step 330 includes a column cleaning and storage sub-step 333. In some embodiments, sub-step 333 includes cleaning the column with 0.5 NaOH and rinsing with filtered water. In some embodiments, sub-step 333 includes sampling the column rinse for bioburden, TOC, and LAL. In some embodiments, sub-step 333 includes storing the column in 20% ethanol.

[0057] In some embodiments, the purification process 300 includes a filtration step 340. In some embodiments, the filtration step 340 includes filtering the pooled fractions of step 332 through a 0.2 µm filter into a sterile bottle.

[0058] In some embodiments, the purification process 300 includes a concentrated product (CP) step 350. In some embodiments, the filtered concentrated product of step 340 is stored at about 2-8°C.

[0059] D. Dilute Bulk Solution Preparation Stage

[0060] FIG. 5 shows a detailed flow chart for the production and handling of a dilute bulk solution of activated botulinum toxin type B. In some embodiments, a process for activating botulinum toxin type B includes a dilute bulk solution preparation stage 400.

[0061] In some embodiments, the dilute bulk solution preparation stage 400 includes a component preparation step 410. In some embodiments, the component step 410 includes washing and sterilizing the components at 123.5°C for 30 minutes.
In some embodiments, the dilute bulk solution preparation stage 400 includes a succinate buffer preparation step 420. In some embodiments, the buffer preparation step 420 includes weighing sodium succinate and sodium chloride and dissolving them in filtered water. In some embodiments, the sodium succinate weighed is 2.7 mg/mL. In some embodiments, the sodium chloride weighed is 5.8 mg/mL. In some embodiments, the buffer preparation step 420 includes adding human serum albumin (HAS). In some embodiments, the HAS is 0.5 mg/mL. In some embodiments, the buffer preparation step 420 includes addition of filtered water, stirring, and adjustment of the buffer to a pH of 5.6 using hydrogen chloride.

In some embodiments, the dilute bulk solution preparation stage 400 includes a dilution step 430 of the concentrated product with succinate buffer. In some embodiments, the dilution step 430 includes calculating the amount of the concentrated product (CP) of step 350 required and diluting the CP with the prepared succinate buffer of step 420. In some embodiments, the CP is diluted with about 3L of succinate buffer. In some embodiments, the dilution step 430 includes pumping about the succinate buffer of step 420 into a dilute bulk vessel through a 0.2 μm filter. In some embodiments, the dilution step 430 includes pumping the pre-diluted CP into a dilute bulk vessel through a 0.2 μm filter. In some embodiments, the dilution step 430 includes pumping additional succinate buffer through the 0.2 μm filter, stirring for 20-30 minutes, and storing the diluted bulk solution at about 2-8° C.

Method of Treatment Using Activated Botulinum Toxin Type B

The increased percentage of activated botulinum toxin type B molecules in a pharmaceutical composition of the present invention enhances the clinical effectiveness of the botulinum toxin, allows for the decreased protein load of a preparation, and results in decreased antigenicity.

The pharmaceutical compositions of the present invention may be administered by any means known in the art to deliver the activated botulinum holotoxin type B (150 kD) to the desired therapeutic target. In some embodiments, the pharmaceutical compositions are delivered by transmucosal administration. In some embodiments, the pharmaceutical compositions are delivered by transcutaneous administration. In some embodiments, the pharmaceutical compositions are delivered by intramuscular administrations. In some embodiments, the pharmaceutical compositions are delivered by transdermal administration.
In some embodiments, the pharmaceutical compositions are injection. In some embodiments, the pharmaceutical compositions are delivered topically.

[0067] The pharmaceutical compositions of the present invention may be used in any of the methods of treatment disclosed herein. According to the methods disclosed herein, the pharmaceutical compositions of the present invention may be administered as a single treatment or repeated periodically to provide multiple treatments.

[0068] In some embodiments, the present invention describes a method of treating a variety of ophthalmologic disorders, neuromuscular diseases, otorhinolaryngological disorders, urogenital disorders, dermatological disorders, pain disorders, inflammatory disorders, secretory disorders, and cutaneous disorders or cosmetic treatment by administering an effective amount of a pharmaceutical composition of the present invention to a patient in need thereof. As used herein, an "effective amount" is an amount sufficient to produce a therapeutic response. An effective amount may be determined with dose escalation studies in open-labeled clinical trials or bin studies with blinded trials.

[0069] Pharmaceutical compositions according to the invention may be used for preparing medicaments intended to treat a disease, condition, or syndrome may be chosen from, but not limited to, the following:

[0070] A. Ophthalmologic Disorders

[0071] In some embodiments, a method of treating ophthalmologic disorders includes administering an effective amount of a pharmaceutical composition of the present invention to a patient in need thereof. In some embodiments, the ophthalmologic disorder is selected from the group consisting of, but not limited to: blepharospasm, strabismus (including restrictive or myogenic strabismus), amblyopia, oscillopsia, protective ptosis, therapeutic ptosis for corneal protection, nystagmus, estropia, diplopia, entropion, eyelid retraction, orbital myopathy, heterophoria, concomitant misalignment, nonconcomitant misalignment, primary or secondary esotropia or exotropia, internuclear ophthalmop Pegia, skew deviation, Duane’s syndrome and upper eyelid retraction.
[0072] B. Neuromuscular Diseases

[0073] As used herein, “neuromuscular diseases” refer to any disease adversely affecting both nervous elements (brain, spinal cord, peripheral nerve) or muscle (striated or smooth muscle), including but not limited to: involuntary movement disorders, dystonias, spinal cord injury or disease, multiple sclerosis, and spasticity from cerebral palsy, stroke, or other cause.

[0074] In some embodiments, a method of treating neuromuscular diseases includes administering an effective amount of a pharmaceutical composition of the present invention to a patient in need thereof. In some embodiments, the neuromuscular disease is an involuntary movement disorder selected from the group consisting of, but not limited to: hemifacial spasm, torticollis, spasticity of the child or of the adult (e.g., in cerebral palsy, post-stroke, multiple sclerosis, traumatic brain injury or spinal cord injury patients), idiopathic focal dystonias, muscle stiffness, writer’s cramp, hand dystonia, CN VI nerve palsy, oromandibular dystonia, head tremor, tardive dyskinesia, occupational cramps (including musicians’ cramp), facial nerve palsy, jaw closing spasm, facial spasm, synkinesia, tremor, primary writing tremor, myoclonus, stiff-person-syndrome, foot dystonia, facial paralysis, painful-arm-and-moving-fingers-syndrome, tic disorders, dystonic tics, Tourette’s syndrome, neuromyotonia, trembling chin, lateral rectus palsy, dystonic foot inversion, jaw dystonia, Rabbit syndrome, cerebellar tremor, III nerve palsy, palatal myoclonus, akasthesia, muscle cramps, IV nerve palsy, freezing-of-gait, extensor truncal dystonia, post-facial nerve palsy synkinesia, secondary dystonia, off period dystonia, cephalic tetanus, myokymia and benign cramp-fasciculation syndrome.

[0075] C. Otorhinolaryngological Disorders

[0076] In some embodiments, a method of treating otorhinolaryngological disorders includes administering an effective amount of a pharmaceutical composition of the present invention to a patient in need thereof. In some embodiments, the otorhinolaryngological disorder is selected from the group consisting of, but not limited to: spasmodic dysphonia, hypersalivation, sialorrhea, ear click, tinnitus, vertigo, Meniere’s disease, cochlear nerve dysfunction, stuttering, cricopharyngeal dysphagia, bruxism, closure of larynx in chronic aspiration, vocal fold granuloma, ventricular dystonia, ventricular dysphonia, mutational dysphonia, trismus, snoring, voice tremor, aspiration, tongue protrusion dystonia, palatal tremor and laryngeal dystonia; gastrointestinal disorders selected from the group consisting
of achalasia, anal fissure, constipation, temporomandibular joint dysfunction, sphincter of Oddi dysfunction, sustained sphincter of Oddi hypertension, intestinal muscle disorders, puborectalis syndrome, anismus, pyloric spasm, gall bladder dysfunction, gastrointestinal or oesophageal motility dysfunction, diffuse oesophageal spasm, oesophageal diverticulosis and gastroparesis.

[0077] D. Urogenial Disorders

[0078] In some embodiments, a method of treating urogenital disorders includes administering an effective amount of a pharmaceutical composition of the present invention to a patient in need thereof. In some embodiments, the urogenital disorder is selected from the group consisting of, but not limited to: detrusor sphincter dyssynergia, detrusor hyperreflexia, neurogenic bladder dysfunction in Parkinson's disease, spinal cord injury, stroke or multiple sclerosis patients, bladder spasms, urinary incontinence, urinary retention, hypertrophied bladder neck, voiding dysfunction, interstitial cystitis, vaginismus, endometriosis, pelvic pain, prostate gland enlargement (Benign Prostatic Hyperplasia), prostatodynia, prostate cancer and priapism.

[0079] E. Dermatological Disorders

[0080] In some embodiments, a method of treating dermatological disorders includes administering an effective amount of a pharmaceutical composition of the present invention to a patient in need thereof. In some embodiments, the dermatological disorder is selected from the group consisting of, but not limited to: axillary hyperhidrosis, palmar hyperhidrosis, Frey's syndrome, bromhidrosis, psoriasis, skin wounds and acne.

[0081] F. Pain Disorders

[0082] In some embodiments, a method of treating pain disorders includes administering an effective amount of a pharmaceutical composition of the present invention to a patient in need thereof. In some embodiments, the pain disorder is selected from the group consisting of, but not limited to: upper back pain, lower back pain, myofascial pain, tension headache, fibromyalgia, myalgia, migraine, whiplash, joint pain, post-operative pain and pain associated with smooth muscle disorders.
[0083] G. Inflammatory Disorders

[0084] In some embodiments, a method of treating inflammatory disorders includes administering an effective amount of a pharmaceutical composition of the present invention to a patient in need thereof. In some embodiments, the inflammatory disorder is selected from the group consisting of, but not limited to: pancreatitis, gout, tendonitis, bursitis, dermatomyositis and ankylosing spondylitis.

[0085] H. Secretory Disorders

[0086] In some embodiments, a method of treating secretory disorders includes administering an effective amount of a pharmaceutical composition of the present invention to a patient in need thereof. In some embodiments, the secretory disorder is selected from the group consisting of, but not limited to: excessive gland secretions, mucus hypersecretion and hyperlacrimation and holocrine gland dysfunction.

[0087] I. Cutaneous Disorders or Cosmetic Treatment

[0088] In some embodiments, a method of treating cutaneous disorders or cosmetic treatment includes administering an effective amount of a pharmaceutical composition of the present invention to a patient in need thereof. In some embodiments, the cutaneous disorder or cosmetic treatment is selected from the group consisting of, but not limited to: skin defects; facial asymmetry; wrinkles selected from glabellar frown lines and facial wrinkles; downturned mouth; and hair loss.
EXAMPLES

[0089] The following Examples serve to further illustrate the present invention and are not to be construed as limiting its scope in any way.

Example 1
Preparation of an Activated Botulinum Toxin Type B Composition: Fermentation (Cell Growth) Stage

[0090] The drug substance manufacturing process, which utilizes a frozen culture of *C. botulinum*, Type B Bean strain (working cell bank), proceeds through two successive seed cultures (S1 and S2). The S2 seed culture is used as the inoculum for the production culture (S3). In S3, a fermentor containing liquid medium of casein hydrolysate (trypsin case peptone), yeast extract, cysteine hydrochloride, and glucose is inoculated with an S2 culture. After fermentation, the crude toxin complex is precipitated by acidifying the culture.

Example 2
Preparation of an Activated Botulinum Toxin Type B Composition: Recovery (Activation) Stage

[0091] The precipitated toxin is re-suspended in phosphate buffer and purified by a series of salt precipitations including 2 M ammonium chloride/0.7 mM magnesium chloride precipitation step, a 15% ammonium sulfate precipitation step and 30% ammonium sulfate precipitation step. The pellet is re-suspended in succinate buffer. The dissolved toxin is digested with Trypzean® (animal free proteolytic enzyme) to nick and activate the toxin at temperature range of 20°C - 40°C and pH of 5 - 6, for a period of 30 min to 120 minute. Upon completion of incubation, the toxin solution is diafiltered to remove solutes and the added proteolytic enzyme, and then filtered (0.45 μm). The activation yields toxin with percentage nicking of 95% - 100%

Example 3
Preparation of an Activated Botulinum Toxin Type B Composition: Purification Stage

[0092] Purification is accomplished using anion exchange and size exclusion column chromatography, each followed by 0.2 μm filtration. The concentrated product is produced at the completion of the filtering step from the SEC column.
Example 4
Preparation of an Activated Botulinum Toxin Type B Composition:
Dilute Bulk Solution Stage

[0093] The concentrated product (CP) is diluted to 5000 U/mL with 10 mM succinate buffer (pH 5.6) containing 100 mM sodium chloride and 0.5 mg Human Serum Albumin (HSA) per mL to prepare the bulk drug product, also named dilute bulk solution. The dilute bulk is 0.2μm filtered to reduce bioburden and prepared in a 45 L batch size.

Example 5
Preparation of an Activated Botulinum Toxin Type B Composition:
Final Container Preparation

[0094] The dilute bulk solution is shipped to the contact filler where it is sterile filtered through two 0.2 μm filters in series prior to filling. Three final product presentations 0.5 mL, 1.0 mL, and 2.0 mL are filled into USP Type I glass vials (3.5 mL). The vials are closed with siliconized butyl rubber stoppers and sealed with aluminum seals. The final product is stored refrigerated at 5 ± 3°C.
CLAIMS

WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising:
   (a) activated botulinum toxin type B; and
   (b) at least one excipient;
   wherein at least 75% of said botulinum toxin type B is nicked.

2. The pharmaceutical composition of claim 1, wherein greater than 75 percent of said botulinum toxin B is nicked.

3. The pharmaceutical composition of claim 1, wherein approximately about 75 percent to about 85 percent of said botulinum toxin type B is nicked.

4. The pharmaceutical composition of claim 1, wherein greater than 80 percent of said botulinum toxin B is nicked.

5. The pharmaceutical composition of claim 1, wherein greater than 85 percent of said botulinum toxin B is nicked.

6. The pharmaceutical composition of claim 1, wherein approximately about 85 percent to about 95 percent of said botulinum toxin type B is nicked.

7. The pharmaceutical composition of claim 1, wherein greater than 90 percent of said botulinum toxin B is nicked.

8. The pharmaceutical composition of claim 1, wherein approximately about 95 percent to about 100 percent of said botulinum toxin type B is nicked.

9. The pharmaceutical composition of claim 1, wherein greater than 95 percent of said botulinum toxin B is nicked.

10. The pharmaceutical composition of claim 1, wherein greater than 99 percent of said botulinum toxin B is nicked.

11. The pharmaceutical composition of claim 1, wherein said at least one excipient is selected from the group consisting of: buffers, carriers, stabilizers, preservatives,
diluents, vehicles, bulking agents, albumins, gelatins, collagens, proteins, polysaccharides, metals, non-oxidizing amino acid derivatives, and sodium chloride.

12. The pharmaceutical composition of claim 1, wherein said at least one excipient is albumin.

13. The pharmaceutical composition of claim 1, wherein said at least one excipient is a buffer.

14. The pharmaceutical composition of claim 13, wherein said buffer is a succinate buffer.

15. A process of activating botulinum toxin type B, comprising the stages of: cell growth, activation, purification, and dilution; wherein at least one protease is administered to a volume of said botulinum toxin type B, and wherein the levels of nicked botulinum toxin type B is increased to at least 75%.

16. The process of claim 15, wherein said at least one protease administered is selected from the group consisting of: trypsin, immobilized TPCK-trypsin, metalloproteases, endogenous proteases, bacterial proteases, and gastric proteases.

17. The process of claim 16, wherein said protease administered is trypsin.

18. The process of claim 15, wherein greater than 75 percent of said botulinum toxin B is nicked.

19. The process of claim 15, wherein approximately about 75 percent to about 85 percent of said botulinum toxin type B is nicked.

20. The process of claim 15, wherein greater than 80 percent of said botulinum toxin B is nicked.

21. The process of claim 15, wherein greater than 85 percent of said botulinum toxin B is nicked.

22. The process of claim 15, wherein approximately about 85 percent to about 95 percent of said botulinum toxin type B is nicked.
23. The process of claim 15, wherein greater than 90 percent of said botulinum toxin B is nicked.

24. The process of claim 15, wherein approximately about 95 percent to about 100 percent of said botulinum toxin type B is nicked.

25. The process of claim 15, wherein greater than 95 percent of said botulinum toxin B is nicked.

26. The process of claim 15, wherein greater than 99 percent of said botulinum toxin B is nicked.

27. A method of treating a disorder, comprising administering to a patient in need thereof, a pharmaceutical composition comprising:
(a) activated botulinum toxin type B; and
(b) at least one excipient;
wherein at least 75% of said botulinum toxin type B is nicked.

28. The method of claim 27, wherein approximately about 75 percent to about 85 percent of said botulinum toxin type B in said pharmaceutical composition is nicked.

29. The method of claim 27, wherein approximately about 85 percent to about 95 percent of said botulinum toxin type B in said pharmaceutical composition is nicked.

30. The method of claim 27, wherein approximately about 95 percent to about 100 percent of said botulinum toxin type B in said pharmaceutical composition is nicked.

31. The method of claim 27, wherein said at least one excipient of said pharmaceutical composition is selected from the group consisting of: buffers, carriers, stabilizers, preservatives, diluents, vehicles, bulking agents, albumins, gelatins, collagens, proteins, polysaccharides, metals, non-oxidizing amino acid derivatives, and sodium chloride.

32. The method of claim 31, wherein said at least one excipient of said pharmaceutical composition is albumin.
33. The method of claim 31, wherein said at least one excipient of said pharmaceutical composition is a buffer.

34. The method of claim 33, wherein said buffer is a succinate buffer.

35. The method of claim 27, wherein said disorder is selected from the group consisting of: ophthalmologic disorders, neuromuscular diseases, otorhinolaryngological disorders, urogenital disorders, dermatological disorders, pain disorders, inflammatory disorders, secretory disorders, and cutaneous disorders or cosmetic treatment.
<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>Fermentation</td>
</tr>
<tr>
<td>130</td>
<td>S1 Fermentation (Thioglycollate medium)</td>
</tr>
<tr>
<td>140</td>
<td>S2 Fermentation (Type B medium)</td>
</tr>
<tr>
<td>150</td>
<td>S3 Fermentation (Type B medium)</td>
</tr>
<tr>
<td>160</td>
<td>Acid Precipitation ($H_2SO_4$) and Harvest</td>
</tr>
<tr>
<td>220</td>
<td>AP Buffer Wash ($PO_4$ buffer)</td>
</tr>
<tr>
<td>230</td>
<td>$NH_4Cl$ Precipitation</td>
</tr>
<tr>
<td>240</td>
<td>2-Step ($NH_4)_2SO_4$ Precipitation</td>
</tr>
<tr>
<td>260</td>
<td>Activation of toxin</td>
</tr>
<tr>
<td>270</td>
<td>Concentration and Filtration (100 kD Ultrafiltration/diafiltration, 0.45 μm filtration)</td>
</tr>
<tr>
<td>320</td>
<td>Anion Exchange Chromatography</td>
</tr>
<tr>
<td>330</td>
<td>Size Exclusion Chromatography</td>
</tr>
<tr>
<td>350</td>
<td>Drug Substance (Concentrated Product [CP])</td>
</tr>
<tr>
<td>Step Description</td>
<td>Reagents/Conditions</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------------</td>
</tr>
</tbody>
</table>
| 110 Media/Buffer Prep | • Thioglycollate medium for S1 autoclaved  
• Type B medium for S2 autoclaved | 1 day | Autoclave (AUT-1002) | 14 days |                    |
| 120 Working Cell Bank (WCB) | • WCB thawed in BSC  
• Sample taken for QC | 0.25 days | Freezer (FZ-202)  
BSC (BZH-204) | N/A | Culture purity |
| 130 S1 Fermentation | • Thioglycollate inoculated with WCB  
• Incubation  
• QC sample taken | 1.25 days | BSC (BZH-204)  
Incubator (INC-203) | N/A | Culture purity |
| 140 S2 Fermentation (3-stage progression) | Stage 1 141  
• Type B medium inoculated with S1, incubate  
Stage 2 142  
• Type B medium inoculated with Stage 1, incubate  
Stage 3 143  
• Type B medium inoculated with Stage 2, incubate  
• QC sample taken | 1 day | BSC (BZH-204)  
Incubator (INC-203) | N/A | Culture purity |
| 150 S3 Fermentation | • Integrity test inlet and exhaust filters  
• Type B medium sterilized in fermenter  
• Autoclaved glucose added via sterile addition port  
• Fermentation media Inoculated with Stage 3 S2 culture via aseptic transfer  
• Incubate with nitrogen overlay, agitation, pH control  
• QC sample taken | 4 days | Fermentor (FRX-401) | N/A | Pre-use equipment cleaning  
Culture purity OD500 |
| 160 Acid Precipitation (AP) | • S3 culture chilled to <20°C  
• pH adjusted with sulfuric acid  
• Precipitated culture transferred to 20L carboy with sanitary connections – transferred to bottles within BSC  
• Centrifuge precipitated culture, supernatant discarded | 0.25 days | Fermentor (FRX-401)  
BSC (BZH-502)  
Centrifuge (CEN-501) | N/A | pH Temperature Pressure |
| 170 AP Water Wash | • Pellet re-suspended in WFIr within BSC  
• Centrifuge, discard supernatant  
• Store Pellet at 2-8°C | 2 hours | BSC (BZH-502)  
Centrifuge (CEN-501)  
Refrigerator (RF-302) | 3 Months | Pellet Mass |
<table>
<thead>
<tr>
<th>Step Description</th>
<th>Reagents/Conditions</th>
<th>Time</th>
<th>Equipment</th>
<th>Hold Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>210 Buffer Preparation</td>
<td>• Prepare buffers and adjust pH</td>
<td>1 day</td>
<td>Balance pH meter</td>
<td>14 days</td>
</tr>
<tr>
<td></td>
<td>• Filter buffers through 0.2µm filter, store at room temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>220 AP Buffer Wash</td>
<td>• Pellet transferred from fermentation suite</td>
<td>4 hours</td>
<td>BSC (BZP-604)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>• Re-suspend pellet in phosphate buffer</td>
<td></td>
<td>Centrifuge (CEN-605)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Centrifuge, save supernatant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>230 Ammonium Chloride Precipitation</td>
<td>• Chloride solution added to suspension to achieve target concentration</td>
<td>4 hours</td>
<td>BSC (BZP-604)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>• Mixture stirred to dissolve salts</td>
<td></td>
<td>Centrifuge (CEN-605)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Mixture stirred while refrigerated</td>
<td></td>
<td>Refrigerator (RF-602)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Centrifuge, save supernatant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>240 Ammonium Sulfate Precipitation</td>
<td>• Ammonium sulfate solution added to supernatant to achieve target concentration</td>
<td>0.5 days</td>
<td>BSC (BZP-604)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>• Mixture stirred while refrigerated</td>
<td></td>
<td>Centrifuge (CEN-605)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Centrifuge, save supernatant</td>
<td></td>
<td>Refrigerator (RF-602)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Add ammonium sulfate to achieve target concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Suspension stirred while refrigerated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Centrifuge, save pellet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>250 Buffer Resuspension</td>
<td>• Pellet dissolved in succinate buffer pH 5.5</td>
<td>4 hours</td>
<td>BSC (BZP-604)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>• Centrifuge, save supernatant</td>
<td></td>
<td>Centrifuge (CEN-605)</td>
<td></td>
</tr>
<tr>
<td>260 Activation</td>
<td>• Digest with Trypsae (animal free trypsin)</td>
<td>2 hours</td>
<td>BSC(BZP-604)</td>
<td>N/A</td>
</tr>
<tr>
<td>270 Concentration and Filtration</td>
<td>• Dialfiltration with succinate buffer pH 5.5</td>
<td>4 hours</td>
<td>BSC (BZP-604)</td>
<td>3 months</td>
</tr>
<tr>
<td></td>
<td>• Concentrate to ~300mL</td>
<td></td>
<td>Ultrfilter (ULF-41)</td>
<td>Filter integrity</td>
</tr>
<tr>
<td>Step Description</td>
<td>Reagents/Conditions</td>
<td>Time</td>
<td>Equipment</td>
<td>Hold Time</td>
</tr>
<tr>
<td>------------------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td>---------</td>
<td>----------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>310 Buffer Preparation</td>
<td>- Prepare succinate buffer, sodium hydroxide and ethanol</td>
<td>2 days</td>
<td>Balance pH meter</td>
<td>14 days</td>
</tr>
<tr>
<td></td>
<td>- Filter buffer and reagents through 0.2 μm filter, store at room temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>320 Anion Exchange</td>
<td>- Pack column with DEAE</td>
<td>1 week</td>
<td>BSC (BZH-701)</td>
<td>3 months</td>
</tr>
<tr>
<td>Chromatography</td>
<td>- Clean column with 0.5N NaOH, rinse with WFIR</td>
<td>but</td>
<td>Refrigerator (RF-802)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Column rinse sampled for bioburden, TOC and LAL</td>
<td>process</td>
<td>Gradifrac system (CHR-40)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Equilibrate column in succinate buffer</td>
<td>time is</td>
<td>pH meter</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Load UFDF on column</td>
<td>8 hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Collect fractions and analyze – pool acceptable fractions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Store at 2-8°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>330 Size Exclusion</td>
<td>Column Packing 331</td>
<td>2 weeks</td>
<td>BSC (BZH-701)</td>
<td>18 months</td>
</tr>
<tr>
<td>Chromatography</td>
<td>- Pack column with SEC resin</td>
<td>prep time</td>
<td>Refrigerator (RF-802)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Column tested for efficiency and peak asymmetry</td>
<td></td>
<td>Gradifrac system (CHR-40)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Clean column with 0.5N NaOH, rinse with WFIR</td>
<td></td>
<td>XK50/100 Column pH meter</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Column rinse sampled for bioburden, TOC and LAL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Store column in 20% ethanol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Column Use 332</td>
<td>8 hours</td>
<td>BSC (BZH-701)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Clean column with 0.5N NaOH, rinse with WFIR</td>
<td>process</td>
<td>Refrigerator (RF-802)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Column rinse sampled for bioburden, TOC and LAL</td>
<td>time</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Equilibrate column with succinate buffer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Load DEAE pool on column</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Fractions collected and analyzed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Acceptable fraction are pooled</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Column Cleaning and Storage 333</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Clean column with 0.5N NaOH, rinse with WFIR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Column rinse sampled for bioburden, TOC and LAL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Store column in 20% ethanol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>340 Filtration</td>
<td>- SEC pool 0.2 μm filtered into a sterile PP bottle</td>
<td>4 tests</td>
<td>BSC (BZH-701)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Refrigerator (RF-802)</td>
<td></td>
</tr>
<tr>
<td>350 Concentrated</td>
<td>- Store at 2-8°C</td>
<td></td>
<td></td>
<td>1 year</td>
</tr>
<tr>
<td>Product (CP)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Step Description</td>
<td>Reagents/Conditions</td>
<td>Time</td>
<td>Equipment</td>
<td>Hold Time</td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------------------------------------------------------------------------------</td>
<td>-------</td>
<td>-------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>410 Component Preparation</td>
<td>• Wash components</td>
<td>1 day</td>
<td>Autodave</td>
<td>1-3 days</td>
</tr>
<tr>
<td></td>
<td>• Sterilize @ 123.5°C; 30 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>420 Preparation of</td>
<td>• Weigh sodium succinate (2.7 mg/ml) and sodium chloride (5.8 mg/ml), dissolve in</td>
<td>4 hours</td>
<td>BSC (BZH-801) Balances</td>
<td>14 days</td>
</tr>
<tr>
<td>Succinate Buffer</td>
<td>WFI</td>
<td></td>
<td>pH meter</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Add 0.5 mg/ml HSA</td>
<td></td>
<td>Stir plate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• QS to final weight with WFI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Stir for ≥ 10 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Adjust buffer to pH 5.6 using HCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>430 Dilution of CP with</td>
<td>• Calculate amount of CP required</td>
<td>6 hours</td>
<td>BSC (BZH-801) Peristaltic Pump</td>
<td>12 months</td>
</tr>
<tr>
<td>Succinate Buffer</td>
<td>• Dilute CP with &quot;3L Succinate Buffer in a beaker</td>
<td></td>
<td>Balance</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Pump ~15kg Succinate Buffer into Dilute Bulk vessel through 0.2μm filter</td>
<td></td>
<td>Refrigerator (RF-602)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Pump pre-diluted CP into Dilute Bulk vessel through 0.2μm filter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Pump additional Succinate Buffer through 0.2μm filter, QS to final weight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Stir for 20-30 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Store at 2-8°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>440 Ship to Contract</td>
<td>• Pack 50L Dilute Bulk vessel in sealed drum with foam/bubble wrap and temperature</td>
<td>4 hours</td>
<td>Not applicable</td>
<td>N/A</td>
</tr>
<tr>
<td>Filler</td>
<td>monitor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Ship via truck at 2-8°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Store dilute bulk at contractor at 2-8°C</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
 IPC(8) - A61K 39/06 (2009.01)
 USPC - 424/239.1
 According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC - 424/239.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/247.1 (see search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST -- PGPB, USPT, USOC, EPAB, JPAB; Dialog Classic Files ? 654, 652, 351, 349, 315, 6, 35, 65, 155; USPTO Web Page; Google Patents; Google Scholar; Search terms -- pharmaceutical composition, activated/nicked botulinum toxin type B, excipient, albumin, succinate, buffer, trypsin, cell growth, purification, therapeutic administration

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>US 2008/0118532 A1 (ATASSI) 22 May 2008 (22.05.2008) para [0007], [0017], [0190], [0195], [0252]-[0253], [0241], [0251]-[0252], [0332]</td>
<td>1-14, 27-35</td>
</tr>
</tbody>
</table>

☐ Further documents are listed in the continuation of Box C. ☐

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Date of the actual completion of the international search
18 September 2009 (18.09.2009)

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