



DIRECCIÓN DE NUEVAS CREACIONES
PRESENTACIÓN DE OPOSICIÓN

1. Oposición a solicitud de:

- ☒ Patente de invención
☐ Registro de Diseño Industrial
☐ Patente de Modelo de Utilidad
☐ Esquema de Trazado de Circuitos Integrados

Número de expediente: 12-070-154

Solicitante: REVANCE THERAPEUTICS, INC

Apoderado: LUZ CLEMENCIA DE PAEZ

Título de la Nueva Creación: METODOS Y SISTEMAS PARA PURIFICAR LA NEUROTOXINA BOTULÍNICA LIBRE DE COMPLEJO

Gaceta: 655 de 31 octubre de 2012, No. de publicación 1670

2. Datos del Opositor

☐ Persona Natural

☒ Persona Jurídica

LAFRANCOL S.A.

Nombre o denominación /Razón social completa

Documento de identificación: C.C. ☐ C.E. ☐ NIT ☒ Otro ☐ Cuál _____

Número: 890 301 463-8

Dirección Carrera 1 No. 46-84 Ciudad Cali

Teléfono (092) 449:30:51 Fax: _____ E-mail: _____

☐ Autorizo expresamente a la Superintendencia de Industria y Comercio para que todas las comunicaciones sean enviadas a través de la dirección de correo electrónico

3. Datos del representante o apoderado:

REYES VILLAMIZAR JOSE LUIS

Apellidos y Nombre

79`152.473

Documento de identificación

44655

T.P. No.

Dirección del representante

Cra 17 No. 88-23 Of.205

Dirección electrónica

info@reyes-abogados.com

No. Fax

621.25.42

Número telefónico

621.26.31

En caso de haber presentado poder general para asuntos que se adelanten ante la Delegatura de Propiedad Industrial, sírvase indicar el número de su radicación

6. Fundamentos de la oposición

Causal: Incumplimiento de requisitos de patentabilidad (Título II, Capítulo I, Decisión 486 de 2000)

Justificación: VER ANEXO

Prórroga:

SI

☐

NO

☒

7. Anexos

☒

Comprobante de pago de la tasa para la presentación de la solicitud No.

Fecha:

30-01-2013

☒

Poder, si fuera el caso con el que se acredita la representación.

☒

Pruebas de los fundamentos de la oposición

☒

Copias para traslado

8. Firma

JOSÉ LUIS REYES VILLAMIZAR

Nombre y apellidos

Firma

79.152.473

C.C.

44655

T.P.

Señor

SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

E.

S.

D.

REF: EXPEDIENTE 12-070-154

TÍTULO SOLICITADO: "MÉTODOS Y SISTEMAS PARA PURIFICAR LA
NEUROTOXINA BOTULÍNICA LIBRE DE
COMPLEJO"

PUBLICACIÓN: Gaceta 655 de 31 de octubre de 2012, N° de
publicación 1670.

PRIORIDAD: US 61/253.810 21/10/2009

SOLICITANTE: REVANCE THERAPEUTICS, INC.

APODERADO: LUZ CLEMENCIA DE PAEZ

TRÁMITE: PRESENTACION DE OPOSICIÓN

JOSÉ LUIS REYES VILLAMIZAR, mayor de edad, identificado como aparece al
pie de mi firma, abogado en ejercicio, titular de la tarjeta profesional número 44655
del Consejo Superior de la Judicatura, obrando en calidad de apoderado de la
sociedad **LAFRANCOL S.A.S**, en ejercicio de lo dispuesto por el artículo 42 de la
Decisión 486 de 2000, muy respetuosamente me permito formular la siguiente

I. PETICIÓN

Sírvase negar el beneficio patentario a la solicitud contenida en el expediente **12-070-154**, por no cumplir con lo dispuesto en la Decisión 486 de 2000, con base en los fundamentos que se expondrán a continuación.

II. LEGÍTIMO INTERÉS

Asiste a **LAFRANCOL S.A.S**, legítimo interés para presentar esta oposición, pues la eventual concesión de la solicitud restringiría injustificadamente el empleo de ciertas sustancias, derivados y procesos que a nuestro juicio carecen de nivel inventivo, en detrimento de sus legítimos intereses comerciales. De igual manera mis clientes están interesados, por su misma condición de industrias farmacéuticas, en evitar la concesión de privilegios de patente que incumplan los requisitos materiales de patentabilidad establecidos en las normas vigentes, y que supongan un riesgo para la libre competencia.

III. FUNDAMENTOS DE LA OPOSICIÓN

La solicitud colombiana en trámite 12-070-154, referida a sistemas y métodos para purificar una toxina botulínica libre de complejo que incluye purificar la toxina botulínica libre de complejo en estado crudo para obtener una toxina botulínica libre de complejo purificada, no cumple los requisitos materiales para otorgársele el privilegio de patente de invención, tal como puede determinarse al valorar los siguientes argumentos:

La reivindicación principal en este caso obedece a las siguientes reclamaciones:

1. *Un método para purificar una toxina botulínica libre de complejo, dicho método que comprende:*
 - i. *proporcionar una toxina botulínica libre de complejo en bruto;*
 - ii. *cargar la toxina botulínica libre de complejo en bruto en una columna de intercambio aniónico de manera que permita la captura de dicha toxina por parte de la columna de intercambio aniónico;*
 - iii. *eluir la toxina botulínica libre de complejo de la columna de intercambio aniónico para producir un eluyente que incluya la toxina botulínica libre de complejo;*
 - iv. *cargar una columna de intercambio catiónico con el eluyente de la columna de intercambio aniónico como para permitir la captura de la toxina botulínica libre de complejo por parte de la columna de intercambio catiónico; y*
 - v. *eluir la toxina botulínica libre de complejo purificada de la columna de intercambio catiónico.*

En primer término, lo reclamado por la solicitud en trámite obedece a un método para purificación de una toxina tal como se realizaría en cualquier proteína bajo el

principio de intercambio aniónico; en este caso se emplea un proceso común y conocido de purificación de proteínas siendo en este caso específico la purificación de la toxina botulínica.

En adición a lo expuesto, y aún cuando se estima que la solicitud no cumple siquiera con los requisitos formales para ser valorado de fondo, es necesario observar que existen documentos del estado de la técnica que ya han reclamado la misma materia bajo la fórmula empleada desvirtuando la novedad y el nivel inventivo de la solicitud en trámite, veamos:

- La solicitud americana US 2006/0228780, "*Chromatographic method and system for purifying a botulinum toxin*", publicado por Luo et al, el 12 de octubre de 2006.

Este documento describe un método para purificar una toxina botulínica no acomplexada (ver párrafo 0070, 00415);

[0070] "*Botulinum toxin*" means a neurotoxin produced by *Clostridium botulinum*, as well as modified, recombinant, hybrid and chimeric *botulinum* toxins. A recombinant *botulinum* toxin can have the light chain and/or the heavy chain thereof made recombinantly by a non-Clostridial species. "*Botulinum toxin*," as used herein, encompasses the *botulinum* toxin serotypes A, B, C, D, E, F and G. "*Botulinum toxin*," as used herein, also encompasses both a *botulinum* toxin complex (i.e. the 300, 600 and 900 kDa complexes) as well as pure *botulinum* toxin (i.e. the about 150 kDa neurotoxic molecule), all of which are useful in the practice of

the present invention. "Purified *botulinum toxin*" means a pure *botulinum* toxin or a *botulinum* toxin complex that is isolated, or substantially isolated, from other proteins and impurities which can accompany the *botulinum* toxin as it is obtained from a culture or fermentation process. Thus, a purified *botulinum* toxin can have at least 90%, preferably more than 95%, and most preferably more than 99% of the non-*botulinum* toxin proteins and impurities removed. The *botulinum* C₂ and C₃ cytotoxins, not being neurotoxins, are excluded from the scope of the present invention.

[0415] The FIG. 7 process can also be used to obtain pure (i.e. 150 kDa *botulinum* toxin free of the non-toxin complex proteins) by loading the SP column eluent onto an ion exchange column in a pH 8 buffer to disassociate the non toxin complex proteins from the 150 kDa *botulinum* toxin molecule, thereby providing (in the flow through from the column) a *botulinum* toxin type A (neurotoxic component) with an approximately 150 kD molecular weight, and a specific potency of 1-2x10⁸ LD₅₀ U/mg or greater. This process can also be used to obtain other non toxin components of *botulinum* toxin complex (i.e. non toxin hemagglutinin proteins and/or non toxin non hemagglutinin proteins) by dissociating the complex into its components and next purifying the dissociated components.

Teniendo en cuenta lo anteriormente expuesto y algunos otros apartes de la publicación americana mencionada, es posible comparar el método revelado en la solicitud en trámite y cada etapa que lo conforma, encontrando que dichas etapas se encuentran descritas en el arte previo:

SOLICITUD COLOMBIANA EN TRAMITE	US 2006/0228780
i) proveer una toxina cruda no acomplejada	(párrafo 0070, 0093, 0110)
ii) cargar la toxina botulínica cruda no acomplejada en una columna de intercambio aniónico para permitir la captura de la toxina botulínica no acomplejada por la columna	(párrafo 0476)
iii) eluir la toxina botulínica no acomplejada de la columna de intercambio aniónico para proveer la toxina botulínica no acomplejada	(párrafo 0476)
iv) cargar la columna de intercambio catiónico con el efluente de la columna de intercambio aniónico para permitir la captura de la toxina botulínica no acomplejada por la columna de intercambio catiónico	(párrafo 0270) (párrafo 0293- 0296, 0461-0466)
v) eluir la toxina botulínica no acomplejada purificada de la columna de intercambio catiónico	(párrafo 0296, 0461-0466)

El documento específicamente menciona la carga de la toxina a la columna de cromatografía de intercambio aniónico y su elución en el gradiente de la columna (párrafo 0476).

[0476] We carried out an experiment which determined that anion exchange column chromatography (such as Q Sepharose and DEAE Sepharose resins) should not be used in toxin column chromatography purification because such columns can cause the toxin band pattern to change. Briefly, the experiment was carried out by diluting Phenyl column eluate 10 times with water and loaded onto a Q column (a first Q column), equilibrated with 10 mM Pi, pH 6. After loading, the column was eluted with the step gradient of 100 mM, 150 mM, 200 mM, 300 mM and 500 mM NaCl. The fractions were collected and analyzed by SDS-PAGE. The fractions from the 150 mM -200 mM NaCl elution was diluted with water and reloaded to Q Sepharose column (a second Q column). The column was eluted by using the same step gradients as the first Q column.

Si bien es cierto que los pasos provistos en el documento US 2006/0228780 no se describen en el orden preciso de pasos reclamados en la solicitud en trámite, la forma de llevar a cabo el proceso reclamado obedece a un típico esquema de purificación que cualquier técnico medio versado en la materia podría derivar bajo un ajuste y estandarización de protocolos.

Dichos pasos de purificación por elución en torres de intercambio catiónico y aniónico se basan en el mismo principio y no se traduce en un efecto técnico

diferente el intercambiar el orden en el sistema de purificación, de tal forma que la solicitud no cumple con los requisitos de novedad y mucho menos nivel inventivo.

En adición a lo expuesto, la anterioridad tantas veces mencionada, revela que la toxina botulínica no acomplejada es obtenida a partir de una muestra que comprende toxina botulínica, cargando una columna de intercambio hidrofóbico con la muestra (párrafo 094), lo que permite la captura del complejo de toxina botulínica por la columna, eluyendo dicho complejo y disociando posteriormente el complejo de toxina botulínica obteniendo así una mezcla que comprende toxina botulínica bruta o cruda no acomplejada (párrafo 0415).

De esta forma, el proceso reclamado ya había sido conocido y reclamado en el arte anterior, lo que indiscutiblemente hace que pueda ser reivindicada la misma materia nuevamente y constituye un motivo suficiente para negar de plano la presente solicitud de patente.

Por lo tanto, con los argumentos dados en el presente escrito, un técnico medio en la materia habría fácilmente derivado la materia de la solicitud bajo una selección de las características técnicas sugeridas presentaban los conocimientos y datos necesarios para que el experto medio en la materia llegara a el objeto reclamado.

IV. FUNDAMENTOS DE DERECHO

Son fundamentos de derecho el Capítulo I, (Requisitos de Patentabilidad) y el artículo 42 (Oposiciones) de la Decisión 486 de 2000.

IV PRUEBAS

Ruego que se tenga como tal, sin perjuicio de las que puedan presentarse al vencimiento de la prórroga solicitada, y de aquellas que el Despacho considere oficiosamente, las siguientes:

- ❖ La solicitud americana US 2006/0228780, "*Chromatographic method and system for purifying a botulinum toxin*" publicado por Luo et al, el 12 de octubre de 2006. Portada, página 1, 7, 8, 31 – 34.

VI. ANEXOS

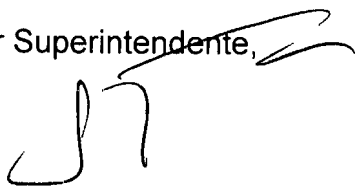
Para efectos del trámite correspondiente, me permito anexar al presente escrito los siguientes anexos:

1. Certificado de existencia y representación legal de Lafranco S.A.
2. Poder para actuar.
3. Constancia de pago de las tasas de tramitación de oposición.
4. Constancia de pago para sustentar la solicitud de prórroga a que se refiere el artículo 42, inciso 2º de la Decisión 486.
5. Copia para correr traslado al solicitante.
6. Las mencionadas en el acápite de pruebas

VII. NOTIFICACIONES

El suscrito recibirá notificaciones en la secretaría de su Despacho o en mi oficina localizada en la Carrera 17 No. 88-23, Oficina 205, Fax (1) 621.25.42 de esta ciudad.

Señor Superintendente,



JOSÉ LUIS REYES VILLAMIZAR

C.C. 79.152.473 de Usaquén
T.P.A. 44.655 de C.S.J.



US 20060228780A1

9

(19) **United States**

(12) **Patent Application Publication** (10) **Pub. No.: US 2006/0228780 A1**
Luo et al. (43) **Pub. Date: Oct. 12, 2006**

(54) **CHROMATOGRAPHIC METHOD AND
SYSTEM FOR PURIFYING A BOTULINUM
TOXIN**

(75) **Inventors: Mingjiang Luo**, Newport Beach, CA
(US); **Hui Xiang**, Irvine, CA (US); **Yao**
Yu, Yorba Linda, CA (US); **Stephen**
Donovan, Capistrano Beach, CA (US)

Correspondence Address:
Stephen Donovan
Allergan, Inc.
2525 Dupont Drive
Irvine, CA 92612 (US)

(73) **Assignee: ALLERGAN, INC.**, Irvine, CA

(21) **Appl. No.: 11/452,570**

(22) **Filed: Jun. 14, 2006**

Related U.S. Application Data

(63) Continuation-in-part of application No. 11/072,673,
filed on Mar. 3, 2005, which is a continuation-in-part
of application No. 11/072,050, filed on Mar. 3, 2005,
which is a continuation-in-part of application No.
10/672,876, filed on Sep. 25, 2003.

Publication Classification

(51) **Int. Cl.**
C12P 21/06 (2006.01)
C07H 21/04 (2006.01)
C12N 15/74 (2006.01)
C07K 14/33 (2006.01)
(52) **U.S. Cl.** **435/69.1; 435/471; 435/252.3;**
530/350; 536/23.7

(57) **ABSTRACT**

Chromatographic processes and systems for purifying a
botulinum toxin from an APF fermentation medium.

CHROMATOGRAPHIC METHOD AND SYSTEM FOR PURIFYING A BOTULINUM TOXIN

CROSS REFERENCE

[0001] This application is a continuation in part of U.S. patent application Ser. No. 11/072,673, filed Mar. 3, 2005, which is a continuation in part of application Ser. No. 11/072,050, filed Mar. 3, 2005, which is a continuation in part of U.S. application Ser. No. 10/672,876, filed Sep. 25, 2003, the entire contents of which applications are incorporated herein by reference.

BACKGROUND

[0002] The present invention relates to systems and processes for purifying a *Clostridium* toxin. In particular, the present invention relates to a chromatographic process for purifying a *botulinum* neurotoxin. A pharmaceutical composition suitable for administration to a human or animal for a therapeutic, diagnostic, research or cosmetic purpose can comprise an active ingredient. The pharmaceutical composition can also include one or more excipients, buffers, carriers, stabilizers, preservatives and/or bulking agents. The active ingredient in a pharmaceutical composition can be a biologic such as a *botulinum* toxin. The *botulinum* toxin active ingredient used to make a *botulinum* toxin pharmaceutical composition can be obtained through a multi step culturing, fermentation and compounding process which makes use of one or more animal derived products (such as meat broth and casein ingredients in one or more of the culture and fermentation media used to obtain a bulk *botulinum* toxin, and a blood fraction or blood derivative excipient in the final compounded *botulinum* toxin pharmaceutical composition). Administration to a patient of a pharmaceutical composition wherein the active ingredient biologic is obtained through a process which makes use of animal derived products can subject the patient to a potential risk of receiving various pathogens or infectious agents. For example, prions may be present in a pharmaceutical composition. A prion is a proteinaceous infectious particle which is hypothesized to arise as an abnormal conformational isoform from the same nucleic acid sequence which makes the normal protein. It has been further hypothesized that infectivity resides in a "recruitment reaction" of the normal isoform protein to the prion protein isoform at a post translational level. Apparently, the normal endogenous cellular protein is induced to misfold into a pathogenic prion conformation.

[0003] Creutzfeldt-Jacob disease is a rare neurodegenerative disorder of human transmissible spongiform encephalopathy where the transmissible agent is apparently an abnormal isoform of a prion protein. An individual with Creutzfeldt-Jacob disease can deteriorate from apparent perfect health to akinetic mutism within six months. Thus, a potential risk may exist of acquiring a prion mediated disease, such as Creutzfeldt-Jacob disease, from the administration of a pharmaceutical composition which contains a biologic, such as a *botulinum* toxin, obtained, purified or compounded using animal derived products.

[0004] Botulinum Toxin

[0005] The genus *Clostridium* has more than one hundred and twenty seven species, grouped by morphology and function. The anaerobic, gram positive bacterium

Clostridium botulinum produces a potent polypeptide neurotoxin, *botulinum* toxin (synonymously "toxin"), which causes a neuroparalytic illness in humans and animals known as botulism. *Clostridium botulinum* and its spores are commonly found in soil and the bacterium can grow in improperly sterilized and sealed food containers of home based canneries, which are the cause of many of the cases of botulism. The effects of botulism typically appear 18 to 36 hours after eating the foodstuffs infected with a *Clostridium botulinum* culture or spores. The *botulinum* toxin can apparently pass unattenuated through the lining of the gut and attack peripheral motor neurons. Symptoms of *botulinum* toxin intoxication can progress from difficulty walking, swallowing, and speaking to paralysis of the respiratory muscles and death.

[0006] *Botulinum* toxin type A is the most lethal natural biological agent known to man. About 50 picograms of *botulinum* toxin (purified neurotoxin complex) type A is a LD₅₀ in mice. On a molar basis, *botulinum* toxin type A is 1.8 billion times more lethal than diphtheria, 600 million times more lethal than sodium cyanide, 30 million times more lethal than cobrotoxin and 12 million times more lethal than cholera. Singh, *Critical Aspects of Bacterial Protein Toxins*, pages 63-84 (chapter 4) of *Natural Toxins II*, edited by B. R. Singh et al., Plenum Press, New York (1976) (where the stated LD₅₀ of *botulinum* toxin type A of 0.3 ng equals 1 U is corrected for the fact that about 0.05 ng of BOTOX® equals 1 unit). BOTOX® is the trademark of a *botulinum* toxin type A purified neurotoxin complex available commercially from Allergan, Inc., of Irvine, Calif. One unit (U) of *botulinum* toxin is defined as the LD₅₀ upon intraperitoneal injection into female Swiss Webster mice weighing about 18-20 grams each. In other words, one unit of *botulinum* toxin is the amount of *botulinum* toxin that kills 50% of a group of female Swiss Webster mice. Seven generally 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. 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. For example, it has been determined that *botulinum* toxin type A is 500 times more potent, as measured by the rate of paralysis produced in the rat, than is *botulinum* toxin type B. Additionally, *botulinum* toxin type B has been determined to be non-toxic in primates at a dose of 480 U/kg which is about 12 times the primate LD₅₀ for *botulinum* toxin type A. The *botulinum* toxins apparently bind with high affinity to cholinergic motor neurons, are translocated into the neuron and block the presynaptic release of acetylcholine.

[0007] *Botulinum* toxins have been used in clinical settings for the treatment of e.g. neuromuscular disorders characterized by hyperactive skeletal muscles. *Botulinum* toxin type A has been approved by the U.S. Food and Drug Administration for the treatment of essential blepharospasm, strabismus and hemifacial spasm in patients over the age of twelve, for the treatment of cervical dystonia and for the treatment of glabellar line (facial) wrinkles. The FDA has also approved a *botulinum* toxin type B for the treatment of cervical dystonia. Clinical effects of peripheral injection (i.e. intramuscular or subcutaneous) *botulinum* toxin type A are usually seen within one week of injection, and often within a few hours after injection. The typical duration of symp-

products in the steps required to obtain a bulk Clostridial toxin. Clearly practice of the present invention subsequent to an APF fermentation process permits an essentially completely APF methodology (fermentation and purification) to be carried out.

[0065] An embodiment of the present invention provides a system and process for obtaining high yield of highly purifying biologically active Clostridial toxin. The present invention accomplishes this through use of a free or substantially animal product free chromatographic system and process to purify a clarified culture obtained from the fermentation processes of a *Clostridium* bacterium, such as a *Clostridium botulinum* bacterium.

DEFINITIONS

[0066] As used herein, the words or terms set forth below have the following definitions.

[0067] "About" means that the item, parameter or term so qualified encompasses a range of plus or minus ten percent above and below the value of the stated item, parameter or term.

[0068] "Administration," or "to administer" means the step of giving (i.e. administering) a pharmaceutical composition to a subject. The pharmaceutical compositions disclosed herein are "locally administered" by e.g. intramuscular (i.m.), intradermal, subcutaneous administration, intrathecal administration, intracranial, intraperitoneal (i.p.) administration, topical (transdermal) and implantation (i.e. of a slow-release device such as polymeric implant or miniosmotic pump) routes of administration.

[0069] "Animal product free" or "substantially animal product free" encompasses, respectively, "animal protein free" or "substantially animal protein free" and means the absence or substantial absence of blood derived, blood pooled and other animal derived products or compounds. "Animal" means a mammal (such as a human), bird, reptile, fish, insect, spider or other animal species. "Animal" excludes microorganisms, such as bacteria. Thus, an animal product free medium or process or a substantially animal product free medium or process within the scope of the present invention can include a *botulinum* toxin or a Clostridial *botulinum* bacterium. For example, an animal product free process or a substantially animal product free process means a process which is either substantially free or essentially free or entirely free of animal derived proteins, such as immunoglobulins, meat digest, meat by products and milk or dairy products or digests. Thus, an example of an animal product free process is a process (such as a bacterial culturing or bacterial fermentation process) which excludes meat and dairy products or meat or dairy by products.

[0070] "*Botulinum* toxin" means a neurotoxin produced by *Clostridium botulinum*, as well as modified, recombinant, hybrid and chimeric *botulinum* toxins. A recombinant *botulinum* toxin can have the light chain and/or the heavy chain thereof made recombinantly by a non-Clostridial species. "*Botulinum* toxin," as used herein, encompasses the *botulinum* toxin serotypes A, B, C, D, E, F and G. "*Botulinum* toxin," as used herein, also encompasses both a *botulinum* toxin complex (i.e. the 300, 600 and 900 kDa complexes) as well as pure *botulinum* toxin (i.e. the about 150 kDa neurotoxic molecule), all of which are useful in the practice of

the present invention. "Purified *botulinum* toxin" means a pure *botulinum* toxin or a *botulinum* toxin complex that is isolated, or substantially isolated, from other proteins and impurities which can accompany the *botulinum* toxin as it is obtained from a culture or fermentation process. Thus, a purified *botulinum* toxin can have at least 90%, preferably more than 95%, and most preferably more than 99% of the non-*botulinum* toxin proteins and impurities removed. The *botulinum* C₂ and C₃ cytotoxins, not being neurotoxins, are excluded from the scope of the present invention.

[0071] "Clostridial neurotoxin" means a neurotoxin produced from, or native to, a Clostridial bacterium, such as *Clostridium botulinum*, *Clostridium butyricum* or *Clostridium beraffi*, as well as a Clostridial neurotoxin made recombinantly by a non-Clostridial species.

[0072] "Entirely free (i.e. "consisting of" terminology) means that within the detection range of the instrument or process being used, the substance cannot be detected or its presence cannot be confirmed.

[0073] "Essentially free" (or "consisting essentially of") means that only trace amounts of the substance can be detected.

[0074] "Modified *botulinum* toxin" means a *botulinum* toxin that has had at least one of its amino acids deleted, modified, or replaced, as compared to a native *botulinum* toxin. Additionally, the modified *botulinum* toxin can be a recombinantly produced neurotoxin, or a derivative or fragment of a recombinantly made neurotoxin. A modified *botulinum* toxin retains at least one biological activity of the native *botulinum* toxin, such as, the ability to bind to a *botulinum* toxin receptor, or the ability to inhibit neurotransmitter release from a neuron. One example of a modified *botulinum* toxin is a *botulinum* toxin that has a light chain from one *botulinum* toxin serotype (such as serotype A), and a heavy chain from a different *botulinum* toxin serotype (such as serotype B). Another example of a modified *botulinum* toxin is a *botulinum* toxin coupled to a neurotransmitter, such as substance P.

[0075] "Patient" means a human or non-human subject receiving medical or veterinary care. Accordingly, as disclosed herein, the compositions may be used in treating any animal, such as mammals.

[0076] "Pharmaceutical composition" means a formulation in which an active ingredient can be a *botulinum* toxin. The word "formulation" means that there is at least one additional ingredient (such as an albumin [such as a human serum albumin or a recombinant human albumin] and/or sodium chloride) in the pharmaceutical composition besides a neurotoxin active ingredient. A pharmaceutical composition is therefore a formulation which is suitable for diagnostic, therapeutic or cosmetic administration (i.e. by intramuscular or subcutaneous injection or by insertion of a depot or implant) to a subject, such as a human patient. The pharmaceutical composition can be: in a lyophilized or vacuum dried condition; a solution formed after reconstitution of the lyophilized or vacuum dried pharmaceutical composition with saline or water, or; as a solution which does not require reconstitution. The active ingredient can be one of the *botulinum* toxin serotypes A, B, C₁, D, E, F or G or a *botulinum* toxin, all of which can be made natively by Clostridial bacteria. As stated, a pharmaceutical composition

can be liquid or solid, for example vacuum-dried. The constituent ingredients of a pharmaceutical composition can be included in a single composition (that is all the constituent ingredients, except for any required reconstitution fluid, are present at the time of initial compounding of the pharmaceutical composition) or as a two-component system, for example a vacuum-dried composition reconstituted with a diluent such as saline which diluent contains an ingredient not present in the initial compounding of the pharmaceutical composition. A two-component system provides the benefit of allowing incorporation of ingredients which are not sufficiently compatible for long-term shelf storage with the first component of the two component system. For example, the reconstitution vehicle or diluent may include a preservative which provides sufficient protection against microbial growth for the use period, for example one-week of refrigerated storage, but is not present during the two-year freezer storage period during which time it might degrade the toxin. Other ingredients, which may not be compatible with a Clostridial toxin or other ingredients for long periods of time, may be incorporated in this manner; that is, added in a second vehicle (i.e. in the reconstitution fluid) at the approximate time of use. Methods for formulating a *botulinum* toxin active ingredient pharmaceutical composition are disclosed in U.S. patent publication 2003 0118598 A1.

[0077] "Substantially free" means present at a level of less than one percent by weight of a culture medium, fermentation medium, pharmaceutical composition or other material in which the weight percent of a substance (such as an animal product, animal protein or animal derived product or protein is assessed).

[0078] "Therapeutic formulation" means a formulation can be used to treat and thereby alleviate a disorder or a disease, such as a disorder or a disease characterized by hyperactivity (i.e. spasticity) of a peripheral muscle.

[0079] The following abbreviations are used herein:

[0080] 3:1:1 culture a *botulinum* toxin culture/fermentation medium containing 3% HySoy, 1% HyYeast, and 1% glucose. HySoy (Quest product no. 5X59022) is a source of peptides made by enzymatic hydrolysis of soy. HyYeast (HyYest, Quest product no. 5Z10102 or 5Z10313 is a baker's yeast extract.

[0081] 5:1:1 culture a *botulinum* toxin culture/fermentation medium containing 5% HySoy, 1% HyYeast, and 1% glucose.

[0082] API active pharmaceutical ingredient

[0083] APF animal product free

[0084] BCA bicinchoninic acid

[0085] CV column volume

[0086] DF diafiltration

[0087] ELISA enzyme linked immunosorbent assay. "Hc" in "Hc-ELISA means a *botulinum* toxin heavy chain.

[0088] MLD50 the amount of a *botulinum* toxin which is a lethal dose to 50% of 18-23 gram Swiss-Weber mice injected intraperitoneally

[0089] SDS-PAGE sodium dodecylsulfate-polyacrylamide gel electrophoresis

[0090] SEC-HPLC size exclusion high performance liquid chromatography

[0091] UF ultrafiltration

[0092] UV ultraviolet

[0093] Our invention includes a process for purifying a *Clostridium* toxin. The process can have the four steps of obtaining a sample of a *botulinum* toxin fermentation culture; contacting a first chromatography column resin with the culture sample so as to permit capture of a *botulinum* toxin by the first column; eluting the *botulinum* toxin from the first column, and; loading a second column chromatography column resin with the eluent from the first chromatography column, thereby obtaining a purified *botulinum* toxin. By "*botulinum* toxin fermentation culture" it is meant a fermentation medium in which a *Clostridium botulinum* bacterium has been fermented so that the bacterium has released *botulinum* toxin into the medium. The sample of a *botulinum* toxin fermentation culture (medium) is preferably a sample of a clarified culture of the fermentation medium.

[0094] The first chromatography column and the second chromatography column can be different columns, and the two different columns can act to purify a *botulinum* toxin through different purification mechanisms. For example, the first chromatography column can be a hydrophobic interaction column and the second chromatography column can be an ion exchange column.

[0095] A process for purifying a *Clostridium* toxin within the scope of our invention can also have the step after the contacting step and before the eluting step, of washing impurities off the first column. Additionally, a process for purifying a *Clostridium* toxin within the scope of our invention can also have the step after the loading step, the step of washing impurities off the second column. Furthermore, a process for purifying a *Clostridium* toxin within the scope of our invention can also have, after the step of washing impurities off the second column, the step of eluting the *botulinum* toxin from the second column.

[0096] Preferably, a process for purifying a *Clostridium* toxin within the scope of our invention is a substantially animal product free (APF) process, such as a substantially animal protein free process, and more preferably it is an essentially APF process for purifying a clostridial toxin, such as a *botulinum* toxin complex. Most preferably, a process for purifying a *Clostridium* toxin within the scope of our invention is an animal product free process.

[0097] The *botulinum* toxin fermentation culture used in a process for purifying a *Clostridium* toxin within the scope of our invention preferably results from an APF process, more preferably results from a substantially APF process, and most preferably results from an essentially APF process.

[0098] Significantly, a process for purifying a *Clostridium* toxin within the scope of our invention can preferably provide a yield of more than about 20 mg of high quality purified *botulinum* toxin complex per batch for each 10 liters of a *botulinum* toxin fermentation culture (APF clarified culture), more preferably more than about 30 mg of high quality purified *botulinum* toxin complex per 10 liters of *botulinum* toxin clarified culture, and most preferably more than about 40 mg of high quality purified *botulinum* toxin complex per 10 liters clarified culture. A particularly pre-

[0263] Among the hydrophobic columns evaluated, the weakly hydrophobic Butyl Sepharose FF gave the best resolution. Therefore, either Butyl Sepharose FF in binding mode or Q Sepharose FF in flowthru mode provided a preferred *botulinum* toxin capturing step.

[0264] Thus, we determined that an efficient capturing step can be carried out using a hydrophobic column, such as the Butyl Sepharose column chromatography. Presumably, the toxin binds to the Butyl column via a hydrophobic interaction. Prior to this experiment it was unknown that a *botulinum* toxin complex could be purified toxin directly from clarified culture using a hydrophobic chromatography column. We found that the Butyl Sepharose Fast Flow column has high binding capacity, allows fast flow rate with low back pressure and is therefore suitable for the capturing step that requires fast removal of impurities.

Example 10

Four Column APF Chromatographic System and Process for Purifying a *Botulinum* Toxin Complex

[0265] Intermediate and Polishing Purification Steps

[0266] Additional (intermediate and polishing) toxin purification steps were carried out using the toxin-containing fractions obtained from the preferred Q and Butyl columns of Example 9.

[0267] Three types of chromatography columns were found effective for such further purification of the *botulinum* toxin complex. A Hydroxyapatite (HA) type I column was a preferred column we used because it showed separation, but some toxin was found in the flow through. Gel filtration with a Superdex 200 column was a more preferred column to use because it permitted purification of the 900 kDa *botulinum* toxin complex from the impurities, but a minor impurity band was still present on SDS-PAGE.

[0268] A most preferred column was a SP Sepharose HP column which we found to separate the *botulinum* toxin from impurities with very good resolution. The *botulinum* toxin was pure after SP Sepharose HP chromatography, based on analysis by SDS-PAGE.

TABLE 4

Summary of Column Chromatography Purification Steps		
Separation technique	Resin	Summary
Mixed mode	Hydroxyapatite type I	Toxin in flow through mode, separated some impurities.
Gel filtration	Superdex 200	Partially purified toxin, difficult to scale-up, low productivity.
Ion exchange	SP Sepharose HP	High resolution separation, pure toxin obtained.

[0269] Based on the results of Examples 8 and 9, and as shown by Table 4, the following four column chromatography purification process was developed:

[0270] 1. use of a Q Sepharose FF column for initial purification of a clarified culture. In this step impurities bound to the column and the toxin flowed through the column;

[0271] 2. the eluent from the Q Sepharose FF column step 1 was then passed through a Butyl Sepharose FF column. The toxin bound to the column and was eluted off with a suitable buffer;

[0272] 3. the eluent from the Butyl Sepharose FF was then passed through a Hydroxyapatite type I column. Impurities bound to the column and the toxin flowed through the column;

[0273] 4. the eluent from the Hydroxyapatite type I was then passed through an SP Sepharose column. The toxin bound to the column and was eluted off with a suitable buffer.

[0274] This four column toxin purification process can be summarized as:

APF clarified culture=>Q(flow through)=>Butyl(binding)=>HA (flow through)=>P (binding)=>purified toxin complex

[0275] This four column bulk *botulinum* toxin complex process allowed direct loading of filtered culture supernatant onto the Q column (step 1). The flow through was supplemented with ammonium sulfate to 0.8M before the second step of loading onto the Butyl column. For the third step, the butyl eluate was loaded onto the HA column directly, while the flow through of the HA was diluted 4 times with deionized water and the pH was adjusted to 4.0 before loading onto the SP column for the fourth column step. This four column process required minimal sample handling at each step, and ensured that the toxin was exposed to mild buffering conditions throughout the four steps of this purification process.

[0276] A scale up of the four column purification process set forth above was used carried out upon 680 ml of filtered culture supernatant obtained from an APF *botulinum* toxin type A fermentation process. The results (see Table 5) show that this four column process resulted in highly in a high yield of highly purified *botulinum* toxin type A complex.

TABLE 5

Results of a Scale Up Purification using the Four Column Purification process.	
Toxin yield	~30 mg per L culture based on UV and Hc-ELISA.
Toxin purity	>98%, monodisperse, 900 kDa complex based on SEC-HPLC and LS. Pure on SDS-PAGE, western blotting conforms to standard.
Toxin potency	3-5 x 10 ⁷ MLD ₅₀ units per mg based on mouse toxicity assays.

Example 11

Additional Multi-Column APF Chromatography Processes for Purifying a *Botulinum* Toxin Complex

[0277] Using the same procedures set forth in Examples 9 and 10 additional column combinations were evaluated. It was determined that each of the following four additional column combinations provided APF methods for obtaining highly purified *botulinum* toxin complex, as determined by SDS-PAGE.

[0278] 1. Q (flow through)=>Butyl=>SP

[0279] 2. Butyl=>Q or HA (flow through)=>SP

[0280] 3. Butyl=>SP=>Q or HA (flow through)

[0281] 4. Butyl=>SP

[0282] The purified toxins were further analyzed by SEC-HPLC with light scattering, capillary electrophoresis, residual DNA assay, Hc-ELISA, and MLD50. No significant differences were found among the toxins from the four different processes set forth above. The results are summarized in Table 6.

TABLE 6

Quality summary of toxin samples purified by different APF processes 1.4. above.	
SEC-HPLC/LS	Purity > 99%, purer than BCC2030, but less homogeneous than BCC2030.
Capillary eletrophoresis	Identical to one another, similar to 19P and 20P Research Grade APF Toxin, but slightly different from BCC2030.
Picogreen DNA assay	2-6 ng/ml, significantly lower than BCC2030.
Mouse toxicity assay, Hc-ELISA	Toxin potency $3.1-4.8 \times 10^7$ MLD ₅₀ units/mg toxin (by UV), or $3.8-12 \times 10^7$ MLD ₅₀ units/mg toxin (by Hc-ELISA).
Silver staining SDS-PAGE	Identical to one another.

Example 12

Two Column APF Chromatography Process and System for Purifying a *Botulinum* Toxin Complex

[0283] Based on the results obtained in Example 11 a two column (Butyl=>SP) column chromatography process was selected for further development.

[0284] Optimization of the First Step: Butyl Sepharose FF Toxin Capture

[0285] Feed: Feed is to the clarified culture loaded on the column. Since ammonium sulfate can affect the buffer pH, the use of NaCl to replace ammonium sulfate in Butyl column was evaluated. We found that addition of NaCl to the feed sufficient to 2M NaCl allowed the *botulinum* toxin complex to bind to the butyl column. Subsequently, we determined that feed at a 4M NaCl increased the binding of *botulinum* toxin complex to the Butyl column, such that the yield of toxin from the Butyl column was increased by 30% to 50%, as determined by Hc-ELISA, as compared to use of feed at 2M NaCl.

[0286] The addition of NaCl to the clarified culture (the feed) caused a small pH shift. However, the acceptable feed pH was established between pH 5 and pH 6 and the final pH of the feed after NaCl addition was within pH 5 and pH 6. Hence the preferred feed to use in this first step of a two column purification process has a 4M NaCl concentration and is at pH 5-6. Solid NaCl was added to the clarified culture directly to obtain the 4M NaCl concentration and this feed was then added to the Butyl column. The bound toxin was eluted from the column using a 1M NaCl elution buffer.

[0287] It was surprising that most of impurity proteins could be washed away from the column and most of toxin bound to the column could be eluted with a 1M NaCl buffer because column purification processes typically consist of 3 or more columns, except for an affinity column process. We determined that this butyl column is unique as it has the ability to remove many of the impurity proteins. Thus, after use of this column the *botulinum* toxin complex purity was approximately 50%.

[0288] A wash step was then carried out to remove impurities from a column. The impurities in the column came from the clarified culture feed (containing 4M NaCl) used. The optimized washing steps were: 1) Wash #1: 5 CV of 50 mM NaPi, 4M NaCl, pH 6.0, and 2) Wash #2: 12 CV of 50 mM NaPi, 2M NaCl, pH 6.0. When 12 CV and 5 CV were compared, it was found that 5 CV is not sufficient in removing the impurities, while the wash is to remove impurities after loading the clarified culture in this case.

[0289] Elution (to remove toxin bound to a column). Toxin elution with 1.2M, 1.0M and 0.8M NaCl were evaluated. It was chosen to elute toxin with 1M NaCl in 50 mM NaPi, pH 6.0, based on toxin recovery and impurity removal. Low salt wash: After elution, the column was further washed with 50 mM NaPi, pH 6.0 to remove residual impurities bound to the column for the characterization of purification process.

[0290] Cleaning: the column was cleaned with 3 CV of 0.1N NaOH to inactivate any residual toxin before the disposal of used resin.

[0291] Running flow rate: The typical flow rate was 100 cm/h. The loading flow rate was between 90 cm/h and 120 cm/h depending on the back pressure. Loading capacity: Typical loading capacity was 12.7 ml culture per ml bed, or at production scale, 10 L culture for 785 ml resin bed (BPG 100 column at 10 cm bed height).

[0292] Bed height: All columns were packed with standard 10 cm bed height.

[0293] Optimization of the Second Step: SP Sepharose HP Purification

[0294] Feed conditioning: The Butyl eluate was diluted 5 times with 20 mM Na citrate buffer, pH 4.0, and the feed pH was adjusted to 4.0. The five times dilution step was carried out to condition the hydrophobic interaction chromatography eluent for use in ion exchange chromatography. We found that the optimal feed pH for best toxin recovery was within the range of pH 4.0 ± 0.2 .

[0295] Wash step: After loading, the column was washed with 1) 5 CV of 20 mM Na citrate, pH 4.0, followed by 2) 3-5 CV of 20 mM Na citrate, 300 mM NaCl, pH 4.0 to remove impurities before the elution of bound toxin.

[0296] Elution step: The toxin was eluted with 20 mM Na citrate, 400 mM NaCl, pH 4.0.

[0297] High salt washing step: After elution, the column was further washed with 20 mM Na citrate, 1M NaCl, pH 4.0 to remove strongly bound impurities.

[0298] Column cleaning: The column was cleaned with ~3 CV of 0.1N NaOH to inactivate residual toxin before the disposal of used resin.

[0299] Flow rate: The typical flow rate was 100 cm/h.

[0300] Load: The entire Butyl eluate was loaded onto the SP column.

[0301] Bed height: All columns were packed with standard 10 cm bed height.

[0302] Detailed operating procedures carried out with regard to this two column *botulinum* toxin complex purification process set forth in this Example 12 are set forth below.

starting volume. This process is then repeated until the required concentration of small molecules (e.g. salts) remaining in the reservoir is reached. Each additional diafiltration volume (DV) reduces the salt concentration further. A diafiltration volume is the volume of sample before the diluting solution is added. Using 5 diafiltration volumes will reduce the ionic strength by ~96% with discontinuous diafiltration. Continuous diafiltration requires less filtrate volume to achieve the same degree of salt reduction as discontinuous diafiltration.

[0398] 3. 0.22 µm Filtration Step

[0399] The low-protein-binding 0.22 µm cellulose acetate (CA) vacuum bottle-top filter was selected for the filtration step.

[0400] 4. Ammonium Sulfate Precipitation Step

[0401] Ammonium sulfate precipitation was then carried out: 3.5M ammonium sulfate was added to the 0.22 µm filtered toxin solution with gentle stirring until the first appearance of opalescence. The purified bulk toxin was then stored at 2-8° C.

[0402] 5. Results from a Typical Post-Column Process

[0403] SP eluate was concentrated from 70.5 ml to 18 ml using Pellicon Biomax-10 (50 cm² surface area, Millipore) on a Labscale TFF system (Millipore) and diafiltered with 8DV of 50 mM NaAc, pH 4.0. The retentate (post-UF/DF fraction) was collected and was filtered with Corning 0.22 µm CA filter (Corning 431154). The UF/DF system was rinsed with acetate buffer. The rinse fraction was collected. Ten ml of the post 0.22 µm filtrate was stored at 2-8° C. for stability studies. Eight ml of the post 0.22 µm filtrate was subjected to ammonium sulfate precipitation. A total of 2.8 ml of 3.5 M ammonium sulfate was added into the filtrate until it became opalescent.

[0404] Toxin recovery was estimated based on UV measurement, which is shown in Table 10. SDS-PAGE results are shown in FIG. 4.

[0405] In FIG. 6 the lanes shown represent:

[0406] Lane 1 is M12, molecular weight standards

[0407] Lane 2 is SP column eluate

[0408] Lane 3 is UF/DF retentate: UF/DF retentate after UF/DF of SP eluate, diluted to the same amount of loaded protein as Lane 2, for comparison

[0409] Lane 4 is UF/DF rinse solution from rinsing UF/DF membrane after completion of UF/DF membrane

[0410] Lane 5 is post 0.2 µm filtration; after UF/DF process and after the sample was filtered with the 0.22 µm filter

[0411] Lane 6 is post column ammonium sulphate suspension; after 0.22 µm filter filtration, the sample was precipitated with ammonium sulphate because the *botulinum* toxin complex is stable in ammonium sulphate

[0412] Lane 7 is UF/DF retentate (same as lane 3), but undiluted, to show the details

[0413] FIG. 6 tells us that the post column purification process steps of UF/DF, 0.22 µm filtration, and ammonium sulphate precipitation do not affect the purity of the *botuli-*

num toxin complex, as determined by SDS-PAGE analysis. Significantly, the MLD₅₀ results showed that the potency of the purified bulk *botulinum* toxin complex was 2.9-3.7×10⁷ MLD₅₀ units/mg.

TABLE 10

Toxin recovery based on UV measurement				
Fraction	Toxin conc. by UV (mg/ml)	Vol. (ml)	Total toxin (mg)	Recovery (%)
SP eluate	0.389	70.5	27.4	100 (defined)
Post UF/DF	1.260	18.0	22.7	82.8
UF/DF rinse	0.220	14.0	3.1	11.3
Post filtration	1.270	18.0	22.8	83.2
Post AS ppt*	N/A (~0.94)	~10.8	N/A	N/A

*from 8 ml post filtration fraction.

[0414] FIG. 7 is a flowchart of a preferred animal protein free, two column chromatographic method for purifying a *botulinum* toxin type A complex. This is a robust, scalable and cGMP compliant process for obtaining purified *Clostridium botulinum* toxin 900 kDa complex. In FIG. 7 it can be noted that the Butyl eluate is conditioned for ion exchange chromatography by a five times dilution with a pH 4 sodium citrate buffer.

[0415] The FIG. 7 process can also be used to obtain pure (i.e. 150 kDa *botulinum* toxin free of the non-toxin complex proteins) by loading the SP column eluent onto an ion exchange column in a pH 8 buffer to disassociate the non toxin complex proteins from the 150 kDa *botulinum* toxin molecule, thereby providing (in the flow through from the column) a *botulinum* toxin type A (neurotoxic component) with an approximately 150 kD molecular weight, and a specific potency of 1-2×10⁸ LD₅₀ U/mg or greater. This process can also be used to obtain other non toxin components of *botulinum* toxin complex (i.e. non toxin hemagglutinin proteins and/or non toxin non hemagglutinin proteins) by dissociating the complex into its components and next purifying the dissociated components.

[0416] The purified toxin complex obtained by our process meets or exceeds the specifications set forth in Table 1. Additionally, the typical yield was approximately 100 mg of 900 kDa toxin complex from a 10 L cell culture, which is higher than the yield obtained from a Schantz (non-APF) process.

Example 16

Three Column APF Chromatographic Method and System for Purifying a *Botulinum* Toxin

[0417] Introduction

[0418] We have developed a highly efficient, high yield system and process for obtaining a *botulinum* toxin with higher purity and higher potency than can be obtained using known process or systems. Our system and process can be used to purify a *botulinum* toxin obtained from a fermentation of an organism which can produce a *botulinum* toxin, such as a *Clostridium* bacterium, or of an organism genetically altered to make a *botulinum* toxin, such as an *E. coli* bacterium or a suitable yeast species inserted with a plasmid which encodes for *botulinum* toxin synthesis.

[0437] A preferred mixed-mode chromatography resin suitable for use in the third chromatography step of our Example 16 process can have one or more of the following characteristics: (1) pH stability at pH 5.5 to 14; (2) can be regenerated in trisodium phosphate; (3) is usable at a flow rate up to a maximum flow velocity of about 5,000 cm/hour; (4) have a dynamic binding capacity (lysozyme) of >25 mg per gram resin; (5) have a nominal particle size of 40 μm ; (6) have a nominal pore size of between about 500 Å and about 1,000 Å.

[0438] A preferred mixed mode chromatography resin suitable for use in the third chromatography step of our Example 16 process can be a ceramic hydroxyapatite type I (40 μm) (available from Bio-Rad) as a CHT™ ceramic hydroxyapatite resin), such as a spherical macroporous form of hydroxyapatite. The binding mechanism of such resins is based on a mixed-mode interaction with Ca^{2+} as the positively-charged functional group and the PO_4^{3-} as the negatively-charged functional group. It has the separation properties of crystalline hydroxyapatite, but can be used reproducibly for several hundred cycles at high flow rates and in large columns. CHT ceramic hydroxyapatite, Type I, differs from the crystalline form in its high protein binding capacity and higher capacity for acidic proteins. Type I has been sintered at different times and lower temperatures than Type II, resulting in differing dynamic capacities; the dynamic capacity of Type I is higher and Type I has a 40 μm particle size.

[0439] Purification Process

[0440] Our Example 16 process and system is an APF chromatographic process for purifying the *botulinum* toxin complex present in the clarified culture obtained from an APF culture and fermentation of a *Clostridium* bacterium. Thus, the starting material used in this Example 16 process was clarified culture. The clarified culture was obtained by filtering the whole culture obtained from an APF *botulinum* toxin culture and fermentation process (such as set forth in the initial parts of Example 6) with depth filter, followed by 0.2 μm filtration.

[0441] I. Day 1: UF/DF and Pellet Wash (Steps 1-6)

[0442] (1) The clarified culture was ultrafiltered (UF) ten to fifteen times with a Millipore 300K Biomax membrane; (2) the ultrafiltered clarified culture was diafiltered (DF) with 0.1M NaPi at pH 6.0 until the permeate absorbance was less than 0.3 at 280 nm; (3) the UF/DF retentate was removed and the filtration membranes rinsed twice with 0.1M NaPi pH 6.0; (4) the UF/DF retentate and the twice rinsed solutions were combined to provide a final UF/DF retentate; (5) the final UF/DF retentate was centrifuged at 3000 rpm for 20 min at room temperature and the supernatant removed (decanted); (6) the resulting pellets were rinsed (to remove residual supernatant and therefore more impurities) with 0.1M Pi, pH 6.0, and again centrifuged followed by removal of supernatant (the supernatant contained most of the many impurities) and storage of the resulting pellet at 2-8° C. for less than 96 hours.

[0443] In step (6) 0.1M Pi pH 6 was the preferred pellet rinse buffer. The effect of rinsing buffer on impurity removal was evaluated by rinsing pellets with 0.1M Pi buffer at pH 4, 5 and 6 and the rinsed solutions were analyzed by SDS-PAGE. The results indicated that 0.1M Pi at pH 6 removed the most impurities in that rinse step.

[0444] The UF/DF membrane used can be a Millipore Biomax or a Regenerated cellulose membrane filters with either 100K, 300K or 500K MWCO. We analyzed the filtrate and retentate by SDS-PAGE and Western Blotting. A Biomax-300 membrane was the preferred membrane for the UF/DF of clarified culture based on the results of contaminant removal, toxin recovery and flow rate. The DF buffer used in the UF/DF process was evaluated by diafiltering the clarified culture with either 1M NaCl in 0.1M Pi pH 6.0, 4M urea in 0.1M Pi pH 6.0, 0.2% octyl glucoside in 0.1M Pi pH 6.0, or 0.1M Pi pH 6.0. The filtrate and retentate were analyzed by SDS-PAGE and Western Blotting. The results indicated that use of 1M NaCl enhanced the removal of impurity proteins in the DF process. The 0.1M Pi pH 6.0 was selected as a preferred DF buffer. To prevent toxin spill during UF/DF, the inlet pressure of UF/DF was maintained at approximately 10 psi by adjusting the pump speed, while outlet valve was open.

[0445] As set forth above, the UF/DF retentate of clarified culture was used in this experiment. To determine retentate relative purities each of 2 ml retentate samples were mixed with either 0, 0.1, 0.3, 0.5, 1, 1.5, or 2 ml of 14% PEG 8000 solution. The mixtures were kept at 4° C. overnight and then clarified by centrifugation. The supernatants and pellets were analyzed by SDS-PAGE. The results (FIG. 10) indicate that toxins were in the pellets and the impurity proteins were in the supernatants for all samples tested, including 0 ml PEG control sample.

[0446] Surprisingly, the results showed that after DF, the toxin in UF/DF retentate was precipitated without any precipitant being added. Lane 6 in FIG. 10 shows a typical result for toxin purified from UF/DF with the unanticipated precipitation of toxin occurring.

[0447] The effect of DF volumes on toxin precipitation was evaluated by collecting retentate samples at various diafiltration volumes (DV). The collected retentates were centrifuged and the pellets were washed with buffer. The supernatant and pellets from different DVs were evaluated by SDS-PAGE. The results indicate that toxin began precipitating from UF/DF retentate when the DVs were higher than three. To ensure the removal of small molecular weight impurities and the precipitation of toxin, more than seven DVs or the permeate absorbance at 280 nm < 0.3 were selected in DF.

[0448] The effect of DF buffer on toxin precipitation were evaluated by dialyzing retentates in either 0.1M Pi pH 6, 0.1M citrate pH 6 or fermentation media overnight at 4° C. After dialysis, the retentates were centrifuged. The toxin in pellets was extracted with 0.1M citrate, pH 4. The supernatant and pellet extract were analyzed by toxin heavy chain enzyme linked immunosorbent assay (Hc ELISA) and SDS-PAGE. The results indicate that the toxin in retentate was precipitated when the dialysis buffer was 0.1M Pi pH 6 and 0.1M citrate pH 6, but not fermentation media. This result indicates that the toxin precipitation from retentate was due to the removal of small molecules during the DF or dialysis, not due to physical shear in UF/DF, since the toxin was precipitated after dialysis.

[0449] In FIG. 10, the following materials were loaded onto the lanes indicated:

[0450] Lane 1: an M12 molecular weight standard

[0451] Lane 2: supernatant from the UF/DF retentate

[0452] Lane 3: a sample of the bulk toxin resulting from the Example 6 process

[0453] Lane 4: Supernatant from the first wash of precipitate

[0454] Lane 5: Supernatant from the second wash of precipitate

[0455] Lane 6: a sample of the toxin purified from the UF/DF and precipitation process of this Example 16

[0456] Lane 7: a sample of the bulk toxin resulting from the Example 6

[0457] Lane 8: a M12 molecular weight standard

[0458] Lane 9: a pre-stained molecular weight marker

[0459] The toxin purified by steps (1) to (6) (UF/DF and precipitation) based on visual inspection of SDS-PAGE gel still contained a small amount of impurities, including protein and nucleic acid, although SDS-PAGE clearly showed that by this stage of the process most impurities had already been removed. To further purify the *botulinum* toxin so as to render it useful for compounding in a pharmaceutical composition the following column chromatography process was used.

[0460] II. Day 2: Extraction and SP Column (Steps 7-13)

[0461] (7) toxin was extracted from the pellets with 0.1M citrate buffer, pH 4.0 with the volume equal to 5% to 10% of the clarified culture volume; (8) the extracted toxin solution was centrifuged at 8000 rpm for 20 minutes at room temperature; (9) the extracted toxin was diluted with an equal volume of water for injection (WFI) and loaded onto an SP column.

[0462] In step (7) 0.1M citrate at pH 4.0 was the preferred toxin extraction buffer. The effect of extraction buffer on toxin extraction was evaluated by comparing the five potential extraction buffers: 0.1M Pi pH 6.0; 0.1M citrate pH 6.0; 0.05M citrate plus 0.1M acetate pH 6.0; 0.2M acetate pH 4, and; 0.1M citrate pH 4.0, in a side by side experiment. The extracted toxins were analyzed by SDS-PAGE. The result indicated that 0.1M citrate pH 4 was most effective in extracting toxin from the pellets.

[0463] (10) the SP column was washed (to remove impurities) with 170 mM NaCl, 50 mM citrate buffer, pH 4.0; (11) the toxin was eluted from the SP column with 400 mM NaCl, 50 mM citrate buffer, pH 4.0; (12) the SP column eluate was mixed with an equal volume of 4M NaCl, 50 mM citrate, pH 6.0; (13) the diluted toxin eluate was filtered with a 0.2 μ m filter and kept at 2-8° C. overnight.

[0464] We evaluated Macroprep High S; SP Sepharose Fast Flow; SP Sepharose High Performance; Source 30S; Source 15S, and Poros HS20 chromatography resins and determined that the Source 15S resins provided good separation between toxin and impurities. The binding capacity of Source 15S resin was approximately 7-10 mg toxin per ml at the flow rate of 100 cm/h.

[0465] A SP Sepharose HP resin was selected because it provided a good separation between the *botulinum* toxin and impurities and because the bead size of this resin is suitable for large scale, low pressure chromatography. We found that

with a SP Sepharose HP at pH>5.0 and conductivity>21 mS/cm, the toxin did not bind to the SP Sepharose HP column resin. We developed preferred conditions for toxin purification with a SP Sepharose HP column which include: use of: 50 mM citrate buffer at pH 4.0; a resin bed height of 10 cm $\pm$ 1 cm; use of a linear flow rate of 100 cm/hour; use of 1:1 dilution of toxin sample with water for loading; elution of impurities from the column using 150-170 mM NaCl, 50 mM citrate pH 4.0, and; elution of bound *botulinum* toxin from the column resin using 400 mM NaCl, 50 mM citrate pH 4.0.

[0466] The toxin eluted from SP column contains a high salt level. Initially, the eluate from the SP Sepharose HP column was mixed at 1:1 ratio with 1.5M ammonium sulfate (AS), 50 mM sodium phosphate, pH 6.0 for loading onto the phenyl column. It was observed that the toxin was not stable in this buffer and tended to form white aggregates. The replacement of phosphate buffer with citrate buffer pH 6.0 prevented toxin aggregation. After loading onto the SP column, the bound toxin was initially eluted with either 0.3 GE Healthcare M AS or OM AS. At 0.3M AS, toxin was eluted in a broad peak with a long tail, while at OM AS, toxin was eluted in a sharp peak. The SP column chromatography conditions were further optimized by using NaCl to replace AS. The eluate from SP Sepharose HP was mixed at 1:1 ratio with 50 mM sodium citrate, 4M NaCl, pH 6.0. The diluted *botulinum* toxin eluate was filtered with a 0.2 μ m filter and kept at 2° C. to 8° C. overnight.

[0467] III. Day 3: Phenyl and HA I Columns (Steps 14-19)

[0468] (14) the filtered and diluted toxin was added to a Phenyl column; (15) the column was washed with 2M NaCl, 50 mM Pi, pH 6.0, followed with 1.0M NaCl, 50 mM Pi, pH 6.0; (16) the toxin was eluted from the Phenyl column with OM NaCl, 50 mM Pi, pH 6.0; (17) the Phenyl eluate was mixed with water and loaded onto a HA I column; (18) the HA I column was washed with the following buffers sequentially:

[0469] (a) 20 mM Pi, pH 6.0

[0470] (b) 500 mM NaCl, 20 mM Pi, pH 6.0

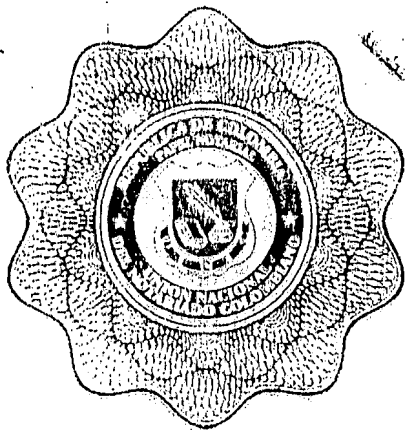
[0471] (c) 20 mM Pi, pH 6.0

[0472] (d) 100 mM Pi, pH 6.0;

[0473] (19) the toxin was eluted from the HA I column with 300 mM Pi, pH 6.0; (20) the eluted toxin was filtered with a 0.2 μ m filter and kept at 2-8° C. overnight.

[0474] After loading onto the Phenyl, the Phenyl column was washed with 50 mM NaPi, 1M NaCl, pH 6.0, and the toxin was eluted from the Phenyl with 50 mM NaPi, pH 6.0. The purified toxin from Phenyl Sepharose HP column had a purity of 98% with monodispersity, based on SEC-HPLC and light scattering analysis.

[0475] The *botulinum* toxin eluate from the Phenyl Sepharose HP column was diluted 10 times with water prior to being and loaded onto the Ceramic Hydroxyapatite Type I (HA I) column (bed height 10 cm), which was equilibrated with 10 mM sodium phosphate, pH 6.0. After loading, the HA I column was washed with 100 mM, 300 mM and 500 mM NaCl in 10 mM sodium phosphate pH 6.0, followed with 100 mM, 300 mM and 500 mM Pi pH 6.0. Based on UV 280 nm, toxin was eluted at 300 mM sodium phosphate in one peak.



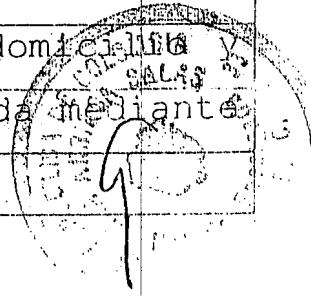
1658 B

AA 8567146

14

REPUBLICA DE COLOMBIA
NOTARIA 33 DE BOGOTA D.C.
ESCRITURA PUBLICA NUMERO: 1 6 5 8
- - MIL SEISCIENTOS CINCUENTA Y OCHO - -
FECHA DE OTORGAMIENTO: CUATRO (4) DE
JULIO - - - - -

DEL AÑO DOS MIL DOS (2002).
ACTO:
1.- PODER ESPECIAL.
OTORGADO POR: LAFRANCOL S.A., con Nit. No.
890.301.463-8
a favor de: JOSE LUIS REYES VILLAMIZAR, con C.C.No.
79.152.473 de Usaquén - Bogotá.
DIANA BEATRIZ LOPEZ
NOTARIA TREINTA Y TRES (33) DE BOGOTA D.C.
EN LA CIUDAD DE BOGOTA, DISTRITO CAPITAL,
DEPARTAMENTO DE CUNDINAMARCA, REPUBLICA DE COLOMBIA,
ANTE MI, DIANA BEATRIZ LOPEZ,
NOTARIA TREINTA Y TRES (33)
DE ESTE CIRCULO SE OTORGA LA ESCRITURA PUBLICA QUE
SE CONSIGNA EN LOS SIGUIENTES TERMINOS:
COMPARECIO: JUAN MARIA RENDON, mayor de edad,
vecino de Bogotá, D.C, identificado con la cédula de
ciudadanía número 17.425.100.000. C. después de la copia por
Expedida en Bogotá - confrontación auténtica única del
Y manifestó: documento de la misma ha firmado y
que se ha remitido a la
PRIMERO: Que obra en su carácter de Representante
Legal de en nombre y representación de LAFRANCOL
S.A, sociedad comercial organizada y existente
conforme a las leyes colombianas, con domicilio y
sede principal en Cali - Valle, constituida mediante
escritura pública número



De la Notaría

De fecha

Reformada en distintas oportunidades, según consta en el certificado expedido por la Cámara de Comercio de Cali - Valle.-----

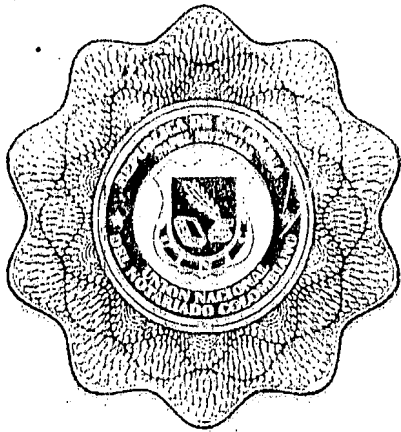
SEGUNDO: Que en tal carácter, por este público instrumento, concede PODER ESPECIAL, PERO AMPLIO Y SUFICIENTE a **JOSE LUIS REYES VILLAMIZAR**, mayor de edad, vecino de Bogotá, D.C, identificado con la cédula de ciudadanía número 79.152.473 de Usaquén, abogado en ejercicio, portador de la T.P.No. 446 55 del C.S. de la J., domiciliado en Bogotá, D.C, República de Colombia, con dirección en la carrera 17 No. 88-23, Oficina 205, de la misma ciudad, como su representante con poder especial, amplio y suficiente, para obtener la protección los derechos de propiedad industrial y de libre competencia de Lafrancol S.A., a cuyo efecto lo autorizo, par que nos represente ante cualesquiera entidad o persona, ejerza o no jurisdicción, especialmente ante las el orden administrativo y judicial, contencioso, administrativo y judicial, concerniente a los siguientes asuntos:-----

a.- Obtención, formulación de observaciones y oposición a patentes de invención, modelos y dibujos industriales y el registro de licencias de uso de esos derechos.-----

b.- Las acciones de oposición, cancelación, nulidad, medidas cautelares, concesión de licencias y en general los procesos civiles, penales, administrativos o contencioso- administrativos relacionados con estos derechos u otros de diferente naturaleza, acciones y procesos que

NOVENO DE
BOGOTÁ, D.C. después de la debida
verificación, se declara esta copia por
la cual se ha tomado y
16 ABR. 2008





16583

AA 8567152

15

promueva Lafrancol S.A., o se
promuevan contra esta y.-----
c.- Para que en todos los asuntos
arriba determinados, tanto en
los términos señalados por los
artículos 42 y complementarios

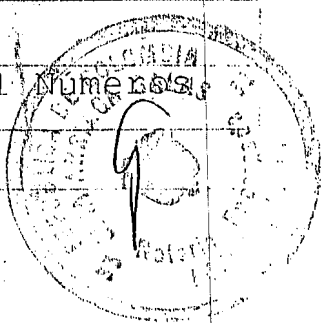
de la decisión 486 de la Comunidad Andina como del
artículo 70 del C.P.C. pueda sustituir este
mandato, transigir, conciliar, admitir los hechos del
proceso, desistir, cancelar, recibir, renunciar y
ratificar los actos de agentes oficiosos y ceder a
título gratuito y/o oneroso, licenciar, inscribir
contratos de cesión y de licencia, inscripción de
prendas, levantamiento de prendas, inscribir
derechos de retención y en general todos los asuntos
que se deban tramitar ante la Superintendencia de
Industria y Comercio.-----

Manifestamos que de la veracidad de las
declaraciones del contenido y autenticidad de los
documentos anexos en éste instrumento público sólo
respondemos los comparecientes como NOTARIO PÚBLICO DE

HASTA AQUI LA MINUTA PRESENTADA BOGOTÁ, D.C. después de la cual
confrontación autentica está copia por
corresponder a la copia autentica del
documento, de la cual se ha tomado y
que se ha tenido a la vista
Bogotá, D.C. 9 FEB. 2009

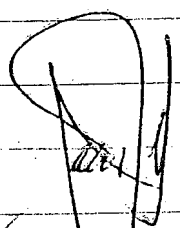
OTORGAMIENTO Y AUTORIZACION: Leído el presente
instrumento Público por los comparecientes
advertidos de la formalidad de su contenido
del término legal, lo hallaron conforme con sus
intenciones y lo aprobaron en todas sus partes y
firmaron junto con la Suscrita Notaria quien da fe y
lo autoriza. -----

Se utilizaron las hojas de papel Notarial Números
AA 8567146, 8567152.



DERECHOS NOTARIALES: \$ 49.600.00

RESOLUCION No. 4188 del 28 de Diciembre del 2001


JUAN MARIA RENDON

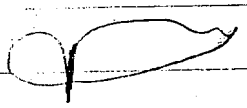
C.C. No. 17.125.100 de Bogotá

LAFRANCOL S.A.

Nit. No. 890.301.463-8

TEL: 622-04-66.

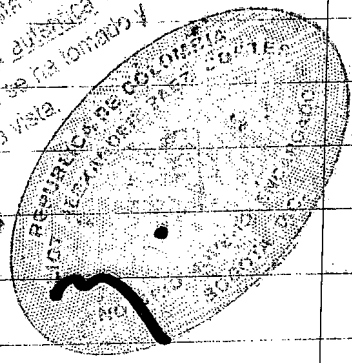
ACEPTO :

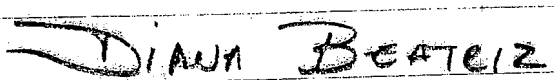

JOSE LUIS REYES VILLAMIZAR

C.C. No. 29.152.473

TEL: 621-2631

Como NOTARIO NOVENO DE
BOGOTÁ, D.C. después de la debida
comparación anterior esta copia por
coincidir con la copia autentica del
documento que se ha tomado y
Bogotá, D.C.
5 ABR. 2002



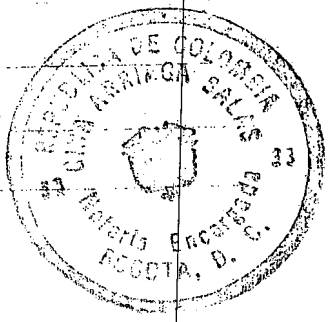

DIANA BEATRIZ LOPEZ

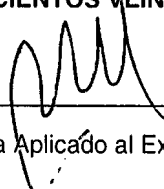
NOTARIA 33 DE BOGOTÁ D.C.

n.c.a.

Es Segunda COPIA (FOTOCOPIA)
tomada de su original que se expide conforme al
Artículo 79 del Decreto 960 de 1970 en 06
hojas útiles con destino a Jose Luis Reyes
Villamizar
Santafé de Bogotá, D.C. 05 JUL. 2002





SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO NIT : 800.176.089-2 -/-					
 Industria y Comercio SUPERINTENDENCIA	RECIBO DE CAJA			No. 13 - 009296	
				Bogotá D.C., Enero 30 de 2013 - 15:27:07	
RECIBIDO DE : REYES Y REYES ABOGADOS				NI 900.048.328	
*** Soporte del Pago ***					
TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VR PAGO
CONSIGNACION	BANCO DE BOGOTA	062754387	383607195	29/01/2013	325.000.00
CONSIGNACION	BANCO DE BOGOTA	062754387	494785760	29/01/2013	975.000.00
CONSIGNACION	BANCO DE BOGOTA	062754387	494785771	29/01/2013	325.000.00
CONSIGNACION	BANCO DE BOGOTA	062754387	494785966	29/01/2013	290.000.00
*** Conceptos Pagados ***					
CANT. RENTISTICO	CONCEPTO			Vr.UNDITARIO	Vr.CONCEPTO
1 50005-01-04 PRESENTACION DE OBSERVACIONES A SOLICITUDES	12 OPOSICION POR CADA CLASE-MARCAS Y LEMAS COMERCIALES			325.000.00	325.000.00
					\$325.000.00
=====					
SON: **TRESCIENTOS VEINTICINCO MIL PESOS MONEDA CORRIENTE***					
Responsable: 					
Recibo de Caja Aplicado al Expediente No. _____					
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO					
					
No. 12-070154- -00004-0000					
Fecha: 2013-01-30 15:49:17 Dep. 2020 DIR.NUEVASCR					
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI					
Act. 400 OPOSIOBSERVTER Folios: 16					
Sede Centro: Carrera 13 No. 27 - 00 Pisos 3,4,5 y 10 Bogotá, D.C.- Colombia					
Web: www.sic.gov.co e-mail: info@sic.gov.co Conmutador: (571) 5870000 Fax: (571) 5870284 Línea: 018000-910165 Call Center: (571) 6513240					
Enero 30 de 2013 - 15:27:08					