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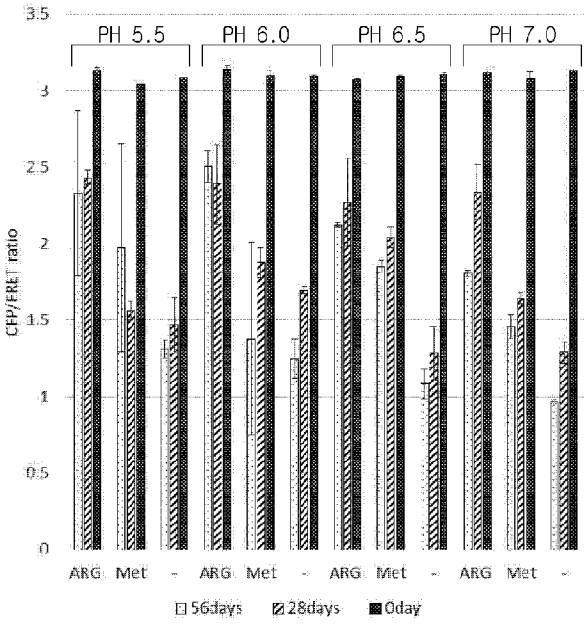
(54) Title: STABILIZED LIQUID FORMULATION OF BOTULINUM TOXIN AND PREPARATION METHOD THEREOF



(57) Abstract: The present invention relates to a stabilized liquid formulation of botulinum toxin and a preparation method thereof. A formulation containing a botulinum toxin and a stabilizer according to the present invention is a liquid formulation which is easily stored and distributed, and it exhibited a remarkable effect on the stabilization of botulinum toxin under suitable conditions according to the temperature and pH of the human body. Thus, it is expected that the formulation of the present invention will greatly contribute to the safe and convenient medical use of botulinum toxin.

WO 2018/038301 A1

Description

Title of Invention: STABILIZED LIQUID FORMULATION OF BOTULINUM TOXIN AND PREPARATION METHOD THEREOF

Technical Field

[1] The present invention relates to a stabilized liquid formulation of botulinum toxin and a preparation method thereof.

[2]

Background Art

[3] A variety of *Clostridium* sp. strains which secrete toxins having neurotoxic effects have been discovered since the 1890s up to the present time, and the characterization of toxins that are secreted from these strains has been made over the past 70 years (Schant, E. J. et al., Microbiol. Rev., 56:80, 1992). Among these toxins, botulinum toxin inhibits the exocytosis of acetylcholine at the cholinergic presynapse of a neuromuscular junction in animals having neurological function to thereby cause asthenia. Thus, efforts have recently been made to use the neurotoxicity of botulinum toxin for cosmetic or therapeutic purposes. Technologies for using botulinum toxin for treatment of optic diseases (US Patent No. 6,265,379), pain (US Patent No. 6,113,915), various autonomic nerve disorders, including sweat gland disorders (US Patent No. 5,766,605), migraine headache pain (US Patent No. 5,714,468), post-operative pain and visceral pain (US Patent No. 6,464,986), psoriasis and dermatitis (US Patent No. 5,670,484), various cancers (US Patent Nos. 6,139,845 and 6,063,768), and neurogenic inflammation (US Patent No. 6,063,768), etc. have been proposed or attempted. However, botulinum toxin, a protein agent, has a problem in that it is not easy to formulate into pharmaceutical compositions and is also not easy to store, distribute and manage. This is attributable to the instability of the protein, and the problem is serious in the case of protein agents such as botulinum toxin, which are formulated into pharmaceutical compositions at a very low concentration. Botulinum toxin protein has the property of adhering to a solid surface, and for this reason, when the protein is injected into a container, a portion of the protein may adhere to the inner wall of the container to cause the loss of the active ingredient, and the protein may be easily oxidized or degraded into small fragments. For this reason, in order to prevent the denaturation of botulinum toxin to the greatest possible extent, botulinum toxin purified in a production process thereof is distributed as freeze-dried powder, which is diluted in a saline immediately before use in clinical applications and administered to patients in the form of liquid. However, in this case, there is also a problem in that medical

accidents are highly likely to occur due to human errors such as a dilution factor error caused by the user or contamination of a dilution saline. Therefore, it is urgently needed to develop stabilizers that can prevent protein denaturation even during the production and distribution of a liquid formulation of botulinum toxin.

[4] In the prior art, albumin was actively used as a stabilizer to maintain the activity of botulinum toxin. However, due to the risk of cross infection and side effects of animal-derived components, the development of non-animal formulations has recently been required. In response to this requirement, US Patent Application Publication No. 2007-0134199 discloses a botulinum toxin composition comprising either glutamine and glutamic acid or asparagine and aspartic acid as amino acids, and Korean Patent No. 1,087,017 discloses a botulinum toxin composition comprising methionine as a stabilizer. However, these patent documents do not suggest remarkable effects under suitable conditions according to the temperature and pH of the human body.

[5] Therefore, the present invention is directed to a stabilized liquid formulation of botulinum toxin and a preparation method thereof. A pharmaceutical composition comprising botulinum toxin according to the present invention is a liquid formulation which is easily stored and distributed through the use of arginine as a stabilizer for botulinum toxin as an active ingredient, and it exhibited a significant effect on the stabilization of botulinum toxin under suitable conditions according to the temperature and pH of the human body. Thus, it is expected that the pharmaceutical composition of the present invention will greatly contribute to the safe and convenient medical use of botulinum toxin.

[6]

Disclosure of Invention

Technical Problem

[7] The present invention has been made in order to solve the above-described problems occurring in the prior art, and it is an object of the present invention to provide a stabilized liquid formulation of botulinum toxin and a preparation method thereof.

[8] However, the technical object to be achieved by the present invention is not limited to the above-mentioned technical object, and other objects that are not mentioned above can be clearly understood by those skilled in the art from the following description.

[9]

Solution to Problem

[10] Hereinafter, various embodiments described herein will be described with reference to figures. In the following description, numerous specific details are set forth, such as specific configurations, compositions, and processes, etc., in order to provide a

thorough understanding of the present invention. However, certain embodiments may be practiced without one or more of these specific details, or in combination with other known methods and configurations. In other instances, known processes and preparation techniques have not been described in particular detail in order to not unnecessarily obscure the present invention. Reference throughout this specification to "one embodiment" or "an embodiment" means that a particular feature, configuration, composition, or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, the appearances of the phrase "in one embodiment" or "an embodiment" in various places throughout this specification are not necessarily referring to the same embodiment of the present invention. Additionally, the particular features, configurations, compositions, or characteristics may be combined in any suitable manner in one or more embodiments.

[11] Unless otherwise specified in the specification, all the scientific and technical terms used in the specification have the same meanings as commonly understood by those skilled in the technical field to which the present invention pertains.

[12] In one embodiment of the present invention, “botulinum toxin” is a neurotoxic protein produced by the bacterium *Clostridium* botulinum. The genus Clostridium has more than 127 species, grouped according to their morphology and functions. The anaerobic, gram-positive bacteria *Clostridium* botulinum produces a potent polypeptide neurotoxin, botulinum toxin, which causes a neuroparalytic illness in humans and animals referred to as botulism. The spores of *Clostridium* botulinum are found in soil and 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 symptoms 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 shows a high affinity for cholinergic motor neurons. Symptoms of botulinum toxin intoxication can progress from difficulty in walking, swallowing, and speaking to paralysis of the respiratory muscles and death.

[13] Botulinum toxin type A is known as the most lethal natural biological agent to man. About 50 picograms of a commercially available botulinum toxin type A (purified neurotoxin complex) is an LD50 (i.e., 1unit). Interestingly, on a molar basis, botulinum toxin type A is about 1.8 billion times more lethal than diphtheria, about 600 million times more lethal than sodium cyanide, about 30 million times more lethal than cobra toxin and about 12 million times more lethal than cholera. One unit (U) of botulinum toxin is defined as the LD50 upon intraperitoneal injection into female Swiss Webster mice weighing 18 to 20 grams each.

[14] Immunologically distinct 7 botulinum neurotoxins have been generally characterized as botulinum neurotoxin serotypes A, B, C1, D, E, F and G, each of which is dis-

tinguished 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 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. Botulinum toxin apparently binds with high affinity to cholinergic motor neurons, is translocated into the neuron and blocks the release of acetylcholine. Additional uptake can take place through low affinity receptors, as well as by phagocytosis and pinocytosis.

[15] Regardless of serotype, the molecular mechanism of toxin intoxication appears to be similar and to involve at least 3 steps. In the first step of the process, the toxin binds to the presynaptic membrane of the target neuron through a specific interaction between the heavy chain (the H chain or HC), and a cell surface receptor. The receptor is thought to be different for each type of botulinum toxin and for tetanus toxin. The carboxyl end segment of the HC appears to be important for targeting of the botulinum toxin to the cell surface.

[16] In the second step, the botulinum toxin crosses the plasma membrane of the target cell. The botulinum toxin is first engulfed by the cell through receptor-mediated endocytosis, and an endosome containing the botulinum toxin is formed. The toxin then escapes the endosome into the cytoplasm of the cell. This step is thought to be mediated by the amino end segment of the heavy chain, the HN, which triggers a conformational change of the toxin in response to a pH of about 5.5 or lower. Endosomes are known to possess a proton pump which decreases intra-endosomal pH. The conformational shift exposes hydrophobic residues in the toxin, which permits the botulinum toxin to embed itself in the endosomal membrane. The botulinum toxin (or at least the light chain of the botulinum toxin) then translocates through the endosomal membrane into the cytoplasm.

[17] The last step of the mechanism of botulinum toxin activity appears to involve reduction of the disulfide bond joining the heavy chain and the light chain. The entire toxic activity of botulinum and tetanus toxins is contained in the light chain of the holotoxin; the light chain is a zinc (Zn^{++}) endopeptidase which selectively cleaves proteins essential for recognition and docking of neurotransmitter-containing vesicles with the cytoplasmic surface of the plasma membrane, and fusion of the vesicles with the plasma membrane. Tetanus neurotoxin, botulinum toxin types B, D, F, and G cause degradation of synaptobrevin (also called vesicle-associated membrane protein (VAMP)), a synaptosomal membrane protein. Most of the VAMP present at the cytoplasmic surface of the synaptic vesicle is removed as a result of any one of these

cleavage events. Serotype A and E cleave SNAP-25. Serotype C1 was originally thought to cleave syntaxin, but was found to cleave syntaxin and SNAP-25. Each of the botulinum toxins specifically cleaves a different bond, except type B (and tetanus toxin) which cleave the same bond. Each of these cleavages blocks the process of vesicle-membrane docking, thereby preventing exocytosis of vesicle content.

[18] Botulinum toxins have been used in clinical settings for the treatment of neuromuscular disorders characterized by hyperactive skeletal muscles (i.e. motor disorders). In 1989, a botulinum toxin type A complex was approved by the U.S. Food and Drug Administration for the treatment of blepharospasm, strabismus and hemifacial spasm. Subsequently, a botulinum toxin type A was also approved by the FDA for the treatment of cervical dystonia and for the treatment of glabellar lines, and a botulinum toxin type B was approved for the treatment of cervical dystonia. Non-type A botulinum toxin serotypes apparently have a lower potency and/or a shorter duration of activity as compared to botulinum toxin type A. Clinical effects of peripheral intramuscular botulinum toxin type A are usually seen within one week of injection. The typical duration of symptomatic relief from a single intramuscular injection of botulinum toxin type A averages about 3 months, although significantly longer periods of therapeutic activity have been reported.

[19] Although all the botulinum toxins serotypes apparently inhibit release of the neurotransmitter acetylcholine at the neuromuscular junction, they do so by affecting different neurosecretory proteins and cleaving these proteins at different sites. For example, botulinum types A and E both cleave the 25 kDa synaptosomal associated protein (SNAP-25), but they target different amino acid sequences within this protein. Botulinum toxin types B, D, F and G act on vesicle-associated membrane protein (VAMP, also called synaptobrevin), with each serotype cleaving the protein at a different site. Finally, botulinum toxin type C1 appears to cleave both syntaxin and SNAP-25. These differences in mechanism of action may affect the relative potency and/or duration of action of the various botulinum toxin serotypes. Particularly, a substrate for a botulinum toxin can be found in a variety of different cell types.

[20] The molecular weight of the botulinum toxin, for all seven of the known botulinum toxin serotypes, is about 150 kDa. Interestingly, the botulinum toxins are released by Clostridial bacterium as complexes comprising the 150 kDa botulinum toxin protein molecule along with associated non-toxin proteins. Thus, the botulinum toxin type A complex can be produced by Clostridial bacterium as 900 kDa, 500 kDa or 300 kDa forms. Botulinum toxin types B and C1 are apparently produced as only a 700 kDa or 500 kDa complex. Botulinum toxin type D is produced as 300 kDa or 500 kDa complexes. Finally, botulinum toxin types E and F are produced as only approximately 300 kDa complexes. The complexes (i.e. molecular weight greater than about 150

kDa) are believed to contain a non-toxin hemagglutinin proteins, a non-toxin, and non-toxic non-hemagglutinin protein. These two non-toxin proteins (which along with the botulinum toxin molecule comprise the relevant neurotoxin complex) may act to provide stability against denaturation to the botulinum toxin molecule and protection against digestive acids when a botulinum toxin is ingested. Additionally, it is possible that the larger (greater than about 150 kDa molecular weight) botulinum toxin complexes result in a slower rate of diffusion of the botulinum toxin away from a site of intramuscular injection of a botulinum toxin complex.

[21] *In* vitro studies have indicated that botulinum toxin inhibits potassium cation-induced release of both acetylcholine and norepinephrine from primary cell cultures of brainstem tissue. In addition, it has been reported that botulinum toxin inhibits the evoked release of both glycine and glutamate in primary cultures of spinal cord neurons and that in brain synaptosome preparations botulinum toxin inhibits the release of each of the neurotransmitters acetylcholine, dopamine, norepinephrine, CGRP, substance P, and glutamate. Thus, when adequate concentrations are used, the stimulus-evoked release of most neurotransmitters can be blocked by botulinum toxin.

[22] Botulinum toxin type A can be obtained by establishing and growing cultures of *Clostridium* botulinum in a fermenter and then harvesting and purifying the fermented mixture in accordance with known procedures. 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 botulinum toxin serotypes A and G possess endogenous proteases and serotypes A and G can, therefore, be recovered from bacterial cultures in predominantly their active form. In contrast, botulinum toxin serotypes C1, D, and E are synthesized by nonproteolytic strains and are therefore typically inactivated when recovered from culture. Serotypes B and F are produced by both proteolytic and nonproteolytic strains, and thus 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. 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. Moreover, it is known that botulinum toxin type B has, upon intramuscular injection, a shorter duration of activity and is also less potent than botulinum toxin type A at the same dose level.

[23] High-quality crystalline botulinum toxin type A can be produced from the Hall A strain of *Clostridium* botulinum with characteristics of $\geq 3\times10^7$U/mg, an A260/A278 of less than 0.60 and a distinct pattern of banding on gel electrophoresis. The known Schantz process can be used to obtain crystalline botulinum toxin type A. Generally, the botulinum toxin type A complex can be isolated and purified from an anaerobic fermentation by cultivating *Clostridium* botulinum type A in a suitable medium. The known process can also be used, upon separation out of the non-toxin proteins, to obtain pure botulinum toxins, such as for example: purified botulinum toxin type A with an approximately 150 kDa molecular weight with a specific potency of $1\text{-}2\times10^8$ LD50 U/mg or greater; purified botulinum toxin type B with an approximately 156 kDa molecular weight with a specific potency of $1\text{-}2\times10^8$ LD50U/mg or greater, and; purified botulinum toxin type F with an approximately 155 kDa molecular weight with a specific potency of $1\text{-}2\times10^7$ LD50U/mg or greater.

[24] Botulinum toxins and/or botulinum toxin complexes are commercially available from compound manufacturers known in the art, and pure botulinum toxin can also be used to prepare a pharmaceutical composition.

[25] As with enzymes generally, the biological activities of the botulinum toxins (which are intracellular peptidases) are dependent, at least in part, upon their three dimensional conformation. Thus, botulinum toxin type A is detoxified by heat, various chemicals surface stretching and surface drying. Additionally, it is known that dilution of a botulinum toxin complex obtained by the known culturing, fermentation and purification to the very low toxin concentrations used for pharmaceutical composition formulation results in rapid detoxification of the toxin unless a suitable stabilizing agent is present. Dilution of the toxin from milligram quantities to a solution containing nanograms per milliliter presents significant difficulties because of the rapid loss of specific toxicity upon such great dilution. Since the botulinum toxin may be used months or years after the toxin containing pharmaceutical composition is formulated, the toxin should be stabilized with a suitable stabilizing agent. Thus, as disclosed in the present invention, the development of optimal stabilizer technology is necessary to control the *in vivo* release of botulinum toxin to a slow release form.

[26] It has been reported that botulinum toxin type A has been used in clinical settings as follows:

[27] The usual duration of an intramuscular injection of botulinum toxin administered *in* vivo is typically about 3 to 4 months. However, in some cases, botulinum toxin subtype A can have an efficacy for up to 12 months (European J. Neurology 6 (Supp 4): S111-S1150:1999), and in some circumstances for as long as 27 months, when used to treat glands, such as in the treatment of hyperhydrosis.

[28] In addition to having pharmacologic actions at the peripheral location, botulinum

toxins may also have inhibitory effects in the central nervous system. Work by Weigand et al, Nauny-Schmiedeberg's Arch. Pharmacol. 1976; 292, 161-165, and Habermann, Nauny-Schmiedeberg's Arch. Pharmacol. 1974; 281, 47-56 showed that botulinum toxin is able to ascend to the spinal area by retrograde transport. As such, a botulinum toxin injected at a peripheral location, for example intramuscularly, may be retrograde transported to the spinal cord.

[29] A botulinum toxin has also been proposed for or has been used to treat skin bone and tendon wounds (US Patent No. 6,447,787); intrathecal pain (US Patent No. 6,113,915); various autonomic nerve disorders, including sweat gland disorders (US Patent No. 5,766,605 and Goldman (2000), Aesthetic Plastic Surgery July-August 24(4):280-282); tension headache (US Patent No. 6,458,365); migraine headache (US Patent No. 5,714,468); post-operative pain and visceral pain (US Patent No. 6,464,986); hair growth and hair retention (US Patent No. 6,299,893); psoriasis and dermatitis (US Patent No. 5,670,484); injured muscles (US Patent No. 6,423,319); various cancers (US Patent Nos. 6,139,845 and 6,063,768), smooth muscle disorders (US Patent No. 5,437,291); nerve entrapment syndromes (US Patent Application 2003-0224019); acne (WO 03/011333); neurogenic inflammation (US Patent No. 6,063,768); optic disorders (US Patent No. 6,265,379); pancreatic disorders (US Patent Nos. 6,143,306 and 6,261,572); prostate disorders, including prostatic hyperplasia, prostate cancer and urinary incontinence (US Patent Nos. 6,365,164 and 6,667,041 and Doggweiler R., et al Botulinum toxin type A causes diffuse and highly selective atrophy of rat prostate, Neurourol Urodyn 1998; 17(4):363); fibromyalgia (US Patent No. 6,623,742), and piriformis muscle syndrome (Childers et al. (2002), American Journal of Physical Medicine & Rehabilitation, 81:751-759).

[30] US Patent No. 5,989,545 discloses that a modified clostridial neurotoxin or fragment thereof, preferably a botulinum toxin, chemically conjugated or recombinantly fused to a particular targeting moiety can be used to treat pain by administration of the agent to the spinal cord. Additionally, it has been disclosed that targeted botulinum toxins (i.e. with a non-native binding moiety) can be used to treat various conditions (WO 96/33273; WO 99/17806; WO 98/07864; WO 00/57897; WO 01/21213; WO 00/10598).

[31] In addition, a botulinum toxin has been injected into the pectoral muscle to control pectoral spasm (Senior M., Botox and the management of pectoral spasm after sub-pectoral implant insertion, Plastic and Recon Surg, July 2000, 224-225). Controlled release toxin implants are known (US Patent Nos. 6,306,423 and 6,312,708) as is transdermal botulinum toxin administration (U.S. Patent Application Serial No. 10/194,805). It is known that a botulinum toxin can be used to: weaken the chewing or biting muscle of the mouth so that self inflicted wounds and resulting ulcers can be

healed (Payne M., et al, Botulinum toxin as a novel treatment for self mutilation in Lesch-Nyhan syndrome, Ann Neurol 2002 Sep.; 52 (3 Supp 1):S157); permit healing of benign cystic lesions or tumors (Blugerman G., et al., Multiple eccrine hidrocystomas: A new therapeutic option with botulinum toxin, Dermatol Surg 2003 May; 29(5):557-9); treat anal fissure (Jost W., Ten years' experience with botulinum toxin in anal fissure, Int J Colorectal Dis 2002 September; 17(5):298-302); and treat certain types of atopic dermatitis (Heckmann M., et al., Botulinum toxin type A injection in the treatment of lichen simplex: An open pilot study, J Am Acad Dermatol 2002 April; 46(4):617-9).

[32] Additionally, a botulinum toxin may have the effect of reducing induced inflammatory pain in a rat formalin model (Aoki K., et al, Mechanisms of the antinociceptive effect of subcutaneous Botox: Inhibition of peripheral and central nociceptive processing, Cephalalgia 2003 September; 23(7):649). Furthermore, it has been reported that botulinum toxin nerve blockage can cause a reduction of epidermal thickness (Li Y, et al., Sensory and motor denervation influences epidermal thickness in rat foot glabrous skin, Exp Neurol 1997; 147:452-462). Finally, it is known to administer a botulinum toxin to the foot to treat excessive foot sweating (Katsambas A., et al., Cutaneous diseases of the foot: Unapproved treatments, Clin Dermatol 2002 November-December; 20(6):689-699; Sevim, S., et al., Botulinum toxin-A therapy for palmar and plantar hyperhidrosis, Acta Neurol Belg 2002 Dec.; 102(4):167-70), spastic toes (Suputtitada, A., Local botulinum toxin type A injections in the treatment of spastic toes, Am J Phys Med Rehabil 2002 October; 81(10):770-5), idiopathic toe walking (Tacks, L., et al., Idiopathic toe walking: Treatment with botulinum toxin A injection, Dev Med Child Neurol 2002; 44(Suppl 91):6), and foot dystonia (Rogers J., et al., Injections of botulinum toxin A in foot dystonia, Neurology 1993 Apr.; 43(4 Suppl 2)).

[33] Tetanus toxin, as wells as derivatives (i.e. with a non-native targeting moiety), fragments, hybrids and chimeras thereof can also have therapeutic utility. The tetanus toxin bears many similarities to the botulinum toxins. Thus, both the tetanus toxin and the botulinum toxins are polypeptides made by closely related species of Clostridium (*Clostridium* tetani and *Clostridium* botulinum, respectively). Additionally, both the tetanus toxin and the botulinum toxins are dichain proteins composed of a light chain (molecular weight: about 50 kDa) covalently bound by a single disulfide bond to a heavy chain (molecular weight: about 100 kDa). Hence, the molecular weight of tetanus toxin and of each of the 7 botulinum toxins (non-complexed) is about 150 kDa. Furthermore, for both the tetanus toxin and the botulinum toxins, the light chain bears the domain which exhibits intracellular biological (protease) activity, while the heavy chain comprises the receptor binding (immunogenic) and cell membrane translo-

cational domains.

[34] Further, both the tetanus toxin and the botulinum toxins exhibit a high, specific affinity for ganglioside receptors on the surface of presynaptic cholinergic neurons. Receptor-mediated endocytosis of tetanus toxin in peripheral cholinergic neurons results in retrograde axonal transport, blocking the release of inhibitory neurotransmitters from central synapses, and causing a spastic paralysis. Contrarily, it has been believed that receptor-mediated endocytosis of botulinum toxin in peripheral cholinergic neurons hardly results in retrograde transport, inhibition of acetylcholine exocytosis from the central synapses, and a flaccid paralysis. However, very recent report has suggested that botulinum toxin also can undergo retrograde transport along axons and possibly inhibit the release of acetylcholine in central synapse (Bomba-Warczak et al., Interneuronal Transfer and Distal Action of Tetanus Toxin and Botulinum Neurotoxins A and D in Central Neurons, Cell Reports, 2016 August; 16, 1974-1987).

[35] Finally, the tetanus toxin and the botulinum toxins resemble each other in both biosynthesis and molecular architecture. Thus, there is an overall 34% identity between the protein sequences of tetanus toxin and botulinum toxin type A, and a sequence identity as high as 62% for some functional domains (Binz T. et al., The Complete Sequence of Botulinum Neurotoxin Type A and Comparison with Other Clostridial Neurotoxins, J Biological Chemistry 265(16); 9153-9158:1990).

[36] In one embodiment of the present invention, "acetylcholine" is an ester of choline and acetic acid, which is the first known neurotransmitter. It is distributed throughout neurons, and has a chemical formula of $C_7H_{16}NO_2$ and a molecular weight of 146.21 kDa.

[37] Typically, only a single type of small molecule neurotransmitter is released by each type of neuron in the mammalian nervous system, although there is evidence which suggests that several neuromodulators can be released by the same neuron. The neurotransmitter acetylcholine is secreted by neurons in many areas of the brain, specifically by the large pyramidal cells of the motor cortex, several different neurons in the basal ganglia, the motor neurons that innervate the skeletal muscles, the preganglionic neurons of the autonomic nervous system (both sympathetic and parasympathetic), the bag 1 fibers of the muscle spindle fiber, the postganglionic neurons of the parasympathetic nervous system, and some of the postganglionic neurons of the sympathetic nervous system. Essentially, only the postganglionic sympathetic nerve fibers to the sweat glands, the piloerector muscles and a few blood vessels are cholinergic as most of the postganglionic neurons of the sympathetic nervous system secret the neurotransmitter norepinephine. In most instances, acetylcholine has an excitatory effect. However, acetylcholine is known to have inhibitory effects at some of the peripheral

parasympathetic nerve endings (for example, inhibition of heart rate by the vagal nerve).

[38] The efferent signals of the autonomic nervous system are transmitted to the body through either the sympathetic nervous system or the parasympathetic nervous system. The preganglionic neurons of the sympathetic nervous system extend from pre-ganglionic sympathetic neuron cell bodies located in the intermediolateral horn of the spinal cord. The preganglionic sympathetic nerve fibers, extending from the cell body, synapse with postganglionic neurons located in either a paravertebral sympathetic ganglion or in a prevertebral ganglion. Since the preganglionic neurons of both the sympathetic and parasympathetic nervous system are cholinergic, application of acetylcholine to the ganglia will excite both sympathetic and parasympathetic post-ganglionic neurons.

[39] Acetylcholine activates two types of receptors, muscarinic and nicotinic receptors. The muscarinic receptors are found in all effector cells stimulated by the post-ganglionic, neurons of the parasympathetic nervous system as well as in those stimulated by the postganglionic cholinergic neurons of the sympathetic nervous system. The nicotinic receptors are found in the adrenal medulla, as well as within the autonomic ganglia, that is on the cell surface of the postganglionic neuron at the synapse between the preganglionic and postganglionic neurons of both the sympathetic and parasympathetic systems. Nicotinic receptors are also found in many nonautonomic nerve endings, for example in the membranes of skeletal muscle fibers at the neuromuscular junction.

[40] Acetylcholine is released from cholinergic neurons when small, clear, intracellular vesicles fuse with the presynaptic neuronal cell membrane. A wide variety of non-neuronal secretory cells, such as adrenal medulla (as well as the PC12 cell line) and pancreatic islet cells release catecholamines and parathyroid hormone, respectively, from large dense-core vesicles. The PC12 cell line is a clone of rat pheochromocytoma cells extensively used as a tissue culture model for studies of sympathoadrenal de-velopment. Botulinum toxin inhibits the release of both types of compounds from both types of cells *in* vitro, when the denervated cells are permeabilized (as by electro-poration) or directly injected with the toxin. Botulinum toxin is also known to block release of the neurotransmitter glutamate from cortical synaptosomes cell cultures.

[41] A neuromuscular junction is formed in skeletal muscle by the proximity of axons to muscle cells. A signal transmitted through the nervous system results in an action potential at the terminal axon, with activation of ion channels and resulting release of the neurotransmitter acetylcholine from intraneuronal synaptic vesicles, for example at the motor endplate of the neuromuscular junction. The acetylcholine crosses the extra-cellular space to bind with acetylcholine receptor proteins on the surface of the muscle

end plate. Once sufficient binding has occurred, an action potential of the muscle cell causes specific membrane ion channel changes, resulting in muscle cell contraction. The acetylcholine is then released from the muscle cells and metabolized by cholinesterases in the extracellular space. The metabolites are recycled back into the terminal axon for reprocessing into further acetylcholine.

[42] In one embodiment of the present invention, the term "basic amino acids" is intended to include arginine, lysine and histidine, and means amino acids that are basic in nature due to an amino ($-NH_2$) content higher than a carboxyl (-COOH) content. Namely, these are amino acids that are dissociated in the neutral pH range so as to have positively charged side chains. Histidine is present in α-helix structures, and lysine is present in helices and β-turns (beta-turns). Arginine tends to adhere to the surface of protein molecules. In many cases, histidine is present in the center of the active site of an enzyme due to the imidazole ring which located on a side chain of the histidine, and it often plays a central role in the enzyme reaction. In many cases, lysine or the carboxyl group of arginine is cleaved by protease in post-translational protein processing.

[43] In one embodiment of the present invention, "arginine" is a kind of basic amino acid, has a molecular formula of $H_2NC(=NH)NH(CH_2)3CH(NH_2)$ COOH and a molecular weight of 174.21, and is water-soluble. This residue is abbreviated as 'Arg' and is denoted by the single letter 'R'. It was first isolated from seedlings of lupin (a kind of bean) by M.J.S. Schulze and E. Steiger. Arginine was named because the nitrate thereof is argent. L-arginine is present as one of amino acids constituting a protein, and is found in the protein protamine present in the sperm of fish. About 70% of constituent amino acids in herrings and salmons are arginines. In plant seeds, arginine is present in a free state. Arginine residues are strongly basic due to their guanidine group. It can be quantified because it shows its peculiar red color when it is reacted with α-naphthol and alkaline hypochlorite. In *in* vivo metabolic pathways, arginine is a component of the ornithine pathway discovered by H. A. Krebs et al., and is cleaved to urea and ornithine by the action of arginase. Arginine is produced from citrulline and asparaginic acid. It is a non-essential amino acid in adults, but is an essential amino acid in infants. It provides protection against the toxicity of ammonia or large amounts of amino acids. Arginase is present in the brain and controls the amount of arginine that is a precursor of γ-guanidinobutyric acid. In invertebrate animals, arginine present in the form of arginine phosphate, plays an important role in muscular contraction with the phosphagen, and is also widely present as a precursor of a special quanidine base (magmatin, octopine).

[44] In one embodiment of the present invention, the term "pharmaceutical composition" refers to a composition that is administered for a specific purpose. For the purpose of

the present invention, the pharmaceutical composition according to the present invention is a botulinum toxin composition comprising arginine as a stabilizer, and may comprise a protein and a pharmaceutically acceptable carrier, excipient or diluent, which are involved in this administration. The term "pharmaceutically acceptable" carrier or excipient means approved by a regulatory agency of a government or listed in the Pharmacopeia or other generally recognized pharmacopeia for use in mammals, and more particularly in humans. For parenteral administration, the pharmaceutical composition of the present invention may be in the form of suspensions, solutions or emulsions in oily or aqueous carriers, and may be prepared in the form of solid or semi-solid. More preferably, it may be a liquid form. In addition, the pharmaceutical composition of the present invention may contain formulating agents such as suspending agents, stabilizers, solubilizing agents and/or dispersing agents, and may be sterilized. The pharmaceutical composition can be stable under the conditions of manufacture and storage, and can be preserved against the contaminating action of microorganisms such as bacteria or fungi. Alternatively, the botulinum toxin composition comprising arginine as a stabilizer according to the present invention may be in the form of sterile powder for reconstitution with suitable carriers before use. The pharmaceutical composition may be present in unit-dose form, microneedle patches, in ampoules, or other unit-dose containers or in multi-dose containers. Alternatively, the pharmaceutical composition can be stored in a freeze-dried (lyophilized) condition requiring only the addition of sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules or tablets.

[45] In some non-limiting embodiments, the botulinum toxin composition containing arginine as a stabilizer according to the present invention may be formulated as liquid, or may be contained in the form of microspheres in liquid. In any non-limiting embodiments, the botulinum toxin composition containing arginine as a stabilizer may contain a botulinum toxin or a pharmaceutically acceptable compound and/or mixture thereof at a concentration of 0.001-100,000 U/kg. In any non-limiting embodiments, excipients suitable for the botulinum toxin composition containing arginine as a stabilizer include preservatives, suspending agents, stabilizers, dyes, buffers, antibacterial agents, antifungal agents, and isotonic agents, for example, sugars or sodium chloride. As used herein, the term "stabilizer" refers to a compound optionally used in the pharmaceutical composition of the present invention in order to increase storage life. In non-limiting embodiments, stabilizers may be sugars, amino acids or polymers. The pharmaceutical composition according to the present invention may contain one or more pharmaceutically acceptable carriers. The carrier can be a solvent or dispersion medium. Non-limiting examples of pharmaceutically acceptable carriers include water,

saline, ethanol, polyol (e.g., glycerol, propylene glycol and liquid polyethylene glycol), oils, and suitable mixtures thereof. Non-limiting examples of sterilization techniques that are applied to the pharmaceutical composition of the present invention include filtration through a bacterial-retaining filter, terminal sterilization, incorporation of sterilizing agents, irradiation, sterile gas irradiation, heating, vacuum drying, and freeze drying.

[46] In one embodiment of the present invention, the term "administration" means introducing the composition of the present invention into a patient by any suitable method. The composition of the present invention may be administered via any general route, as long as it can reach a target tissue. The composition of the present invention can be administered orally, intraperitoneally, intravenously, intramuscularly, subcutaneously, intracutaneously, intranasally, intrapulmonarily, intrarectally, or intrathecally. However, the botulinum toxin composition containing arginine as a stabilizer according to the present invention is most preferably administered by intramuscular injection as a liquid formulation, but is not limited thereto.

[47] A treatment method according to the present invention may comprise administering a pharmaceutically effective amount of the pharmaceutical composition. In the present invention, the effective amount can vary depending on various factors, including the kind of disease, the severity of the disease, the kinds and contents of active ingredient and other ingredients contained in the composition, the kind of formulation, the patient's age, weight, general health state, sex and diet, administration time, the route of administration, the secretion rate of the composition, the period of treatment, and drugs used concurrently.

[48] In one embodiment of the present invention, there is provided a pharmaceutical formulation, containing: a neurotoxin; and a stabilizer. In the pharmaceutical composition, the neurotoxin may be any one or more selected from the group consisting of botulinum toxin, tetanus toxin, cholera toxin, and pertussis toxin. The botulinum toxin may be selected from the group consisting of botulinum toxin type A, B, C, D, E, F and G. The botulinum toxin is preferably botulinum toxin type A. In addition, the botulinum toxin may be either a form containing no complexing protein or a complex form containing a complexing protein. In the pharmaceutical formulation, the stabilizer is any one or more selected from basic amino acid, gluconolactone, and tartaric acid. And the basic amino acid may be any one or more selected from the group consisting of lysine, histidine and arginine. Preferably, the basic amino acid may be arginine. In addition, the basic amino acid may be contained at a concentration of 0.01-1,000 mM per 100 units of the botulinum toxin. The pharmaceutical formulation may have a pH of 5.5-7.0. In addition, the pharmaceutical formulation may further contain polysorbate. The polysorbate is preferably polysorbate 20, and is contained in an

amount of 0.001-1 wt% based on the total weight of the pharmaceutical formulation. In addition, the pharmaceutical formulation may further contain a local anesthetic. In the pharmaceutical formulation, the local anesthetic may be lidocaine, and may be contained in an amount of 0.1-1 wt% based on the total weight of the pharmaceutical formulation. Additionally, the pharmaceutical formulation may be liquid.

[49] In another embodiment of the present invention, there is provided a method for preparing a pharmaceutical formulation, comprising the steps of: purifying a neurotoxin; and adding a stabilizer to the neurotoxin. In the method, the neurotoxin may be botulinum toxin type A. In the method, the stabilizer is any one or more selected from basic amino acid, gluconolactone, and tartaric acid. And the basic amino acid may be arginine. In addition, the pharmaceutical formulation may be liquid.

[50] Hereinafter, each step of the present invention will be described in detail.

[51]

Advantageous Effects of Invention

[52] Botulinum toxin inhibits the exocytosis of acetylcholine at the cholinergic presynapse of a neuromuscular junction in animals having neurological function to thereby cause asthenia. Botulinum toxin has great therapeutic effects on various diseases due to its neurotoxic function, but is lethal even in a very small amount due to its strong toxicity. For this reason, when botulinum toxin is to be used in a living body, it is necessary to minutely control the concentration of botulinum toxin. However, currently available pharmaceutical compositions containing botulinum toxin have problems associated with protein denaturation. Due to such problems, the current pharmaceutical compositions are prepared and distributed in the form of freeze-dried formulations and diluted in liquid saline by the user immediately before use in clinical applications. For this reason, in the case of the current pharmaceutical compositions, there was a problem in that the risk of medical accidents caused by human errors such as dilution factor error or contamination of the dilution saline is high. On the other hand, the present invention is directed to a botulinum toxin composition containing arginine as a stabilizer, and the composition of the present invention exhibited a remarkable effect on the stabilization of botulinum toxin even when it was distributed as a liquid formulation. Thus, it is expected that the composition of the present invention will greatly contribute to the safe and convenient medical use of botulinum toxin.

[53]

Brief Description of Drawings

[54] FIG. 1a shows the results of measuring the potency of botulinum toxin after incubating 0, 5, 10 25, 50 or 100 units of the botulinum toxin for 0 hr, 2 hrs, 4 hrs or overnight.

[55] FIG. 1b shows the results obtained by converting the measured values of FIG. 1a to Delta F values.

[56] FIG. 1c shows the results of plotting a standard curve from the conversed values of FIG. 1b.

[57] FIG. 2 shows the results of measuring the residual potency of botulinum toxin after incubating the botulinum toxin together with arginine or methionine for 28-56 days.

[58] FIG. 3a shows the results of measuring the residual potency of botulinum toxin after incubating a botulinum toxin composition containing polysorbate at a pH of 5.5 for 28-56 days.

[59] FIG. 3b shows the results of measuring the residual potency of botulinum toxin after incubating a botulinum toxin composition containing polysorbate at a pH of 6.0 for 28-56 days.

[60] FIG. 3c shows the results of measuring the residual potency of botulinum toxin after incubating a botulinum toxin composition containing polysorbate at a pH of 6.5 for 28-56 days.

[61] FIG. 3d shows the results of measuring the residual potency of botulinum toxin after incubating a botulinum toxin composition containing polysorbate at a pH of 7.0 for 28-56 days.

[62] FIG. 4 shows the results of measuring the residual potency of botulinum toxin after incubating a botulinum toxin composition containing 0-100 mM of arginine for up to 28 days.

[63] FIG. 5a shows the results of measuring the residual potency of botulinum toxin after incubating a botulinum toxin composition containing arginine or methionine at a pH of 6.0 for 56 days.

[64] FIG. 5b shows the results of measuring the residual potency of botulinum toxin after incubating a botulinum toxin composition containing arginine or methionine at a pH of 6.5 for 56 days.

[65] FIG. 5c shows the results of measuring the residual potency of botulinum toxin after incubating a botulinum toxin composition containing arginine or methionine at a pH of 7.0 for 56 days.

[66] FIG. 6a shows the results of measuring the residual potency of botulinum toxin after incubating a composition containing a stabilizing additive (arginine or methionine), lidocaine (0.3 wt%) and botulinum toxin (50 mM) for 28 days.

[67] FIG. 6b shows the results of measuring the residual potency of botulinum toxin after incubating a composition containing a stabilizing additive (arginine or methionine), lidocaine (0.3 wt%) and botulinum toxin (100 mM) for 28 days.

[68] FIG. 7a shows the results of measuring the residual potency of botulinum toxin after incubating botulinum toxin compositions containing various antioxidants for 28 days.

[69] FIG. 7b shows the results of measuring the residual potency of botulinum toxin after incubating botulinum toxin compositions containing various buffering agents for 28 days.

[70]

Best Mode for Carrying out the Invention

[71] There is provided a method for preparing a pharmaceutical formulation, comprising the steps of: purifying a neurotoxin; and adding an amino acid to the neurotoxin. In the method, the neurotoxin may be botulinum toxin type A, and the amino acid may be arginine. In addition, the pharmaceutical formulation may be liquid.

[72]

Mode for the Invention

[73] Hereinafter, the present invention will be described in further detail with reference to examples. It will be obvious to those skilled in the art that these examples are for illustrative purposes only and are not intended to limit the scope of the present invention.

[74]

[75] **Example** 1: Preparation of Experiments

[76] A botulinum toxin used in the present invention was one produced by Hugel Pharma Co., Ltd. (Korea) and adjusted to a concentration of 0.1 mg/ml and potency of 1,529 units/$\mu\ell$ (based on the BoTest). In all experiments for identification of additives having a stabilizing effect, the botulinum toxin was used after it was diluted to 50 units/$\mu\ell$ with an "optimized protein dilution buffer" having a composition (50 mM $NaPO_4$, pH 7.0, 1 mM DTT, 0.05 wt% polysorbate, and 20 wt% glycerol). For identification of additive candidates having a stabilizing effect, each experimental group (100 $\mu\ell$) was prepared by diluting 200 units of the botulinum toxin and a stabilizer additive candidate in a "stabilizing liquid composition (10 mM $NaPO_4$ (pH 5.5-7.0), 0.01 wt% polysorbate, 130 mM NaCl)". Then, each experimental group was incubated at a temperature of 37°C for 1-11 weeks, and a portion (25%) thereof was used for measurement of the residual potency thereof. The potency of the botulinum toxin was measured using a BoTest® Botulinum Neurotoxin Detection Kit (BioSentinel, USA). For this, 5 mM HEPES-NaOH (pH 7.1), 0.1 wt% polysorbate, 10 uM $ZnCl_2$ (Sigma 229997), 0.2 uM BoTest A/E reporter, and 25$\mu\ell$ stabilizing experimental group were mixed with one another to prepare a final reaction solution (100$\mu\ell$), and the reaction solution was incubated at 37°C for 21 hours. The CFP/FRET ratio of the incubated reaction solution was measured using a Synergy Neo2 Multi-Mode Reader (BioTek, USA) system and applied to a standard curve, thereby determining the residual potency of the botulinum toxin.

[77] The sources of all reagents used in this study are listed in the following (*). In addition, all reagents used in the study on stabilization of the botulinum toxin according to the present invention were dissolved in triple-distilled water and adjusted to a pH of 5.5-7.0 by the addition of hydrochloric acid and sodium hydroxide.

[78] * Gluconolactone (Sigma G2164); L(+)-tartaric acid (Merck 100804); lidocaine hydrochloride monohydrate (Sigma L5647); octanoic acid (Sigma C2875); L-methionine (Merck K45023607 414); L-arginine (Merck K45895542 534); glycine (Bioshop GLN001-1); aspartic acid (Merck K45895542 534); maleic acid (Merck S6858580 534); butylated hydroxyanisole (Sigma SLBM1210V); propyl gallate (Sigma P3130); sodium bisulfite (Sigma MKBR6468V); thioglycolic acid (Sigma T3758); L-cystein hydrochloride (K46446495 513); succinic acid (Merck K46618782 533); sodium phosphate monobasic (Sigma S5011); sodium phosphate dibasic (Sigma S7907); sodium chloride (Merck K47013904 548); polysorbate (Sigma P7949); and DL-dithiothreitol (Sigma D0632).

[79]

[80] **Example** 2: Establishment of Standard Curve for Measurement of Potency of Botulinum Toxin (BoNT/A)

[81] 0, 5, 10 25, 50 or 100 units of botulinum toxin was incubated for 0 hr, 2 hrs, 4 hrs or overnight, and measured using a Synergy Neo2 Multi-Mode Reader (BioTek, USA) system, and the results of the measurement are shown in FIG. 1a. The measured values shown in FIG. 1a were converted to Delta F values based on an equation (Delta F% = (Ratio_sample - Ratio_negative)/Ratio_negative X 100) provided by the manufacturer of the Synergy Neo2 system, and the results of the conversion are shown in FIG. 1b. A standard curve obtained by applying the converted Delta F values to each time point is shown in FIG. 1c. From the standard curve, the equation $Y = 0.0014x2 - 0.0714x + 0.2934$ (R-squared value = 0.86) was obtained. In all stabilizing-effect experiments performed in the present invention, the FRET values measured by the Synergy Neo2 system were converted into Delta F values which were then applied to the above-described equation, thereby obtaining potency values (100% = 50 units) relative to a control group.

[82]

[83] **Example** 3: Comparison of Stabilizing Effects of Arginine or Methionine at Varying pHs

[84] The effects of arginine and methionine as stabilizers on the stabilization of botulinum toxin at a pH ranging from 5.5 to 7.0 were comparatively examined.

[85] For this, botulinum toxin was added to the stabilizing liquid composition described in Example 1, and 50 mM of arginine or methionine was further added to the liquid composition. The resulting composition was incubated at 37°C for 28-56 days, and then the

residual potency of the botulinum toxin was measured. The results of the measurement are shown in Table 1 below and FIG. 2.

[86] [Table 1]

		0 day	28 days	56 days
pH 5.5	50 mM arginine	100	34.2	27.5
	50 mM methionine	100	1.22	9.01
	Negative control	100	0.97	-0.34
pH6.0	50 mM arginine	100	28.2	32.6
	50 mM methionine	100	7.5	-0.66
	Negative control	100	8.38	-0.63
pH6.5	50 mM arginine	100	23.6	16.2
	50 mM methionine	100	13.6	8.78
	Negative control	100	-1.06	-0.8
pH7.0	50 mM arginine	100	19.6	1.72
	50 mM methionine	100	1.63	-0.94
	Negative control	100	-0.83	0.11

[87] As can be seen from the experimental results, in the negative control group containing no stabilizing candidate, the botulinum toxin was unstable at all the pHs, and thus the residual potency of the botulinum toxin was not substantially detected after 28 days. The experimental group containing methionine as a stabilizer a residual potency of about 10% at pH 6.5, whereas the experimental group containing arginine showed a residual potency of up to 30%. In addition, it was measured that the effect of arginine on the stabilization of the botulinum toxin was higher at a pH ranging from 5.5 to 6.0 than at a pH ranging from 6.5 to 7.0.

[88]

[89] **Example** 4: Comparison of Stabilizing Effects of Arginine or Methionine at Varying Concentrations of Polysorbate

[90] Commercially available botulinum toxin composition products (Medy-Tox Inc. and Allergan, etc.) all contain 0.01-0.05 wt% of polysorbate as an additive having a surfactant effect. Thus, for the purpose of optimizing a liquid formulation composition comprising the botulinum toxin, the present inventors examined the effect of the surfactant property of polysorbate on the stabilizing effect of arginine or methionine. For this, the botulinum toxin and 50 mM of arginine or methionine were added to a stabilizing liquid composition containing 0-0.05 wt% of polysorbate, and the resulting

composition was incubated at 37°C for 28-56 days, after which the residual potency of the botulinum toxin was measured. The results of the measurement are shown in Table 2 below and FIG. 3.

[91] [Table 2]

			0 day	28 days	56 days
50 mM arginine	pH5.5	0% polysorbate	100	52	52
		0.01% polysorbate	100	27	59
		0.05% polysorbate	100	47	59
	pH6.0	0% polysorbate	100	78	89
		0.01% polysorbate	100	72	77
		0.05% polysorbate	100	88	104
	pH6.5	0% polysorbate	100	99	38
		0.01% polysorbate	100	110	38
		0.05% polysorbate	100	122	67
	pH7.0	0% polysorbate	100	99	39
		0.01% polysorbate	100	49	17
		0.05% polysorbate	100	75	23
50 mM methionine	pH5.5	0% polysorbate	100	4	7
		0.01% polysorbate	100	12	7
		0.05% polysorbate	100	22	20
	pH6.0	0% polysorbate	100	7	14
		0.01% polysorbate	100	20	23
		0.05% polysorbate	100	9	51
	pH6.5	0% polysorbate	100	36	20
		0.01% polysorbate	100	40	10
		0.05% polysorbate	100	33	18
	pH7.0	0% polysorbate	100	19	0
		0.01% polysorbate	100	3	-3
		0.05% polysorbate	100	12	0

Negative control	pH5.5	0% polysorbate	100	9	-1
		0.01% polysorbate	100	6	0
		0.05% polysorbate	100	0	1
	pH6.0	0% polysorbate	100	9	-1
		0.01% polysorbate	100	8	-2
		0.05% polysorbate	100	10	-1
	pH6.5	0% polysorbate	100	1	-1
		0.01% polysorbate	100	-2	-2
		0.05% polysorbate	100	-2	-2
	pH7.0	0% polysorbate	100	2	0
		0.01% polysorbate	100	-1	3
		0.05% polysorbate	100	-2	2

[92] As can be seen from the experimental results, in the negative control containing no stabilizing candidate substance, the residual level of the botulinum toxin was not measured at a significant level after 4 weeks, and the effect of polysorbate was also not significant. In the case of the stabilizing composition containing methionine, at a pH ranging from 5.5 to 6.0, the stabilizing effect showed a tendency to increase in proportion to the concentration of polysorbate, and at a pH ranging from 6.5 to 7.0, the effect of polysorbate generally did not appear. In the case of the experimental group containing arginine, at a pH ranging from 5.5 to 6.5, the addition of polysorbate showed a tendency to contribute to the stabilizing effect, but the effect of polysorbate was not so significant. Such results suggest that the addition of polysorbate to a liquid botulinum toxin formulation containing arginine as a stabilizer is not essential. It is possible to select the addition of 0.01-0.05 wt% of polysorbate to a liquid botulinum toxin formulation containing arginine as a stabilizer.

[93]

[94] **Example** 5: Determination of Arginine Concentration Optimal For Stabilization of Botulinum Toxin

[95] The standard concentration of arginine used in the present invention is 50 mM, and the concentration of polysorbate is 0.01 wt%. In the case of a commercially available product (e.g., a product manufactured by Medy-Tox Inc.), methionine was used as a stabilizer at a concentration of 0.5-100 mM in order to determine the optimal concentration thereof. Thus, in order to determine an optimal liquid formulation composition of botulinum toxin, the stabilizing effects of varying concentrations of arginine were comparatively examined. For this, arginine was added to an ex-

perimental group at a concentration ranging from 0 to 100 mM, and the experimental group was incubated at 37°C for up to 28 days, and then the residual potency of the botulinum toxin was measured. The results of the measurement are shown in Table 3 below and FIG. 4.

[96] [Table 3]

		0 day	14 days	28 days
pH 5.5	0 mM arginine	100	21.6	12.5
	25 mM arginine	100	110	61.9
	50 mM arginine	100	96	270
	75 mM arginine	100	128	60
	100 mM arginine	100	50.5	16
pH 6.0	0 mM arginine	100	12.2	17.1
	25 mM arginine	100	152	109
	50 mM arginine	100	110	107
	75 mM arginine	100	151	121
	100 mM arginine	100	37	46.2
pH 6.5	0 mM arginine	100	12.3	19.4
	25 mM arginine	100	94.9	112
	50 mM arginine	100	72.1	89.2
	75 mM arginine	100	61.1	56.3
	100 mM arginine	100	58	96.9
pH7.0	0 mM arginine	100	-1.92	-2.15
	25 mM arginine	100	26.3	20.5
	50 mM arginine	100	73.8	81.8
	75 mM arginine	100	52.8	50.3
	100 mM arginine	100	99.6	75.9

[97] As can be seen from the experimental results, the experimental group containing arginine showed botulinum toxin potency higher than that of the control group, but the difference in botulinum toxin potency between the arginine concentrations was not significant. When all the stabilizing experimental groups were compared with one another, it could be seen that the experimental group containing 50 mM arginine had the highest stabilizing effect at pH 6.0 and that the potency of the botulinum toxin was

stably maintained in most of the experimental groups even after 28 days.

[98]

[99] **Example** 6: Comparative Examination of Stabilizing Effects of Arginine or Methionine

[100] The results obtained in the present invention suggest that the effect of arginine on the stabilization of botulinum toxin is similar to or higher than that of methionine. In order to verify such results, a botulinum toxin composition containing arginine (50 mM) or methionine (50 mM) as a stabilizing additive was incubated for 56 days, and the residual potency of the botulinum toxin was measured in three independent experiments. The results of the measurement were statistically processed, thereby comparatively verifying the effects of arginine and methionine. As a control group, a sample containing no additive was used. To determine the significance between the three experimental groups, the one-way ANOVA method was used. When the significance probability (p-value) was 0.05 or less, it was determined that there was a significant difference between the three experimental group, and post-hoc analysis was performed by the LSD (Least Significant Difference) method. The results of the measurement are shown in Table 4 below and FIG. 5.

[101] [Table 4]

	pH	Stabilization at 37°C			
		0 day	14 days	28 days	56 days
50 mM arginine	6.0	100	90.1±5.5	71.6±7.5**	69.1±14**
	6.5	100	71.9±2.1*	70.6±9.6**	38.4±8.2**
	7.0	100	70.1±9**	55.8±8.2***	16.9±6.8*
50 mM methionine	6.0	100	57.6±12	27.2±8.3	22.6±8.5
	6.5	100	55±4.4	36.7±3.3	11.1±3.8
	7.0	100	40.2±2.7	17.1±4.3	0.35±1.4
Negative control	6.0	100	47±14	21.7±5.6	-1.22±0.2
	6.5	100	29.1±7.5	17.3±6.9	-1.27±0.3
	7.0	100	5.16±4.8	3.37±2.7	1.37±0.6

[102] The results obtained in this Example indicate that arginine and methionine all show the high stabilizing effect at a pH of 6.0, and these experimental results are consistent with the above-described experimental results. However, under the same pH condition, arginine showed a higher stabilizing effect compared to methionine in all the experimental groups. For example, in the control group containing no stabilizer, the potency of the botulinum toxin was not measured under all conditions after 56 days of

incubation, but in the experimental group containing methionine, a residual potency of 22.6% was measured at a pH of 6.0, and in the experimental group containing arginine, a potency of 69.1% was measured under the same condition.

[103] The significance of the relative stabilizing effect of arginine and methionine was examined using the one-way ANOVA method, and as a result, it was shown that, at a pH of 6.0, the stabilizing effect values were significant in all the experimental groups except for the experimental group incubated for 14 days. For such results, post-hoc analysis was performed by the LSD (Least Significant Difference) method, and as a result, it was shown that the stabilizing effect of arginine was significantly better than that of methionine at a level of $p<0.05$ to $p<0.001$. For example, the comparison of values measured after 28 days of incubation at a pH of 7.0 indicated that the experimental group containing methionine showed a residual potency of 17.1% and the experimental group containing arginine showed a residual potency of 55.8%, and thus there was a significant difference of $p<0.001$ in the stabilizing effect between the two groups. In addition, the comparison of values measured after 56 days of incubation at a pH of 6.0 indicated that the experimental group containing methionine showed a residual potency of 22.6% and the experimental group containing arginine showed a residual potency of 69.1%, and thus there was a significant difference of $p<0.01$ in the stabilizing effect between the two groups.

[104]

[105] **Example** 7: Identification of Stabilizer for Liquid Botulinum Toxin Formulation Containing Lidocaine

[106] A problem that can occur upon treatment with botulinum toxin is that the patient frequently feels pain. Thus, a botulinum toxin composition containing lidocaine as a local anesthetic was used as a control, various stabilizing candidate substances were added to the composition, after which each of the compositions was incubated for a certain time, and the residual potency of the botulinum toxin was measured. In this manner, an additive that stabilizes a liquid botulinum toxin formulation containing lidocaine was identified.

[107] Thus, the effect of the stabilizing additive arginine, newly identified by the present inventors, on the stabilization of a liquid botulinum toxin composition containing lidocaine, was examined. For this, arginine (50 mM) or methionine (50 mM) was added to a botulinum toxin (200 units) composition containing lidocaine (0.3 wt%), and the composition was incubated for 28 days, after which the residual potency of the botulinum toxin was measured.

[108] The residual potency of the botulinum toxin after 28 days of incubation was measured, and as a result, arginine showed a stabilizing effect, but when the composition contained lidocaine, the stabilizing effect showed a tendency to decrease in

proportion to the concentration of lidocaine. In addition, the experimental groups containing methionine showed a similar tendency, but the residual potency of the botulinum toxin in these compositions was generally measured to be lower than that in the experimental groups containing arginine. The results of the measurement are shown in FIG. 6.

[109]

[110] **Example** 8: Identification of New Stabilizers for Liquid Formulation of Botulinum Toxin

[111] The results of Examples 2 to 7 indicate that arginine has a better effect on the stabilization of a liquid formulation of botulinum toxin compared to methionine. However, detection of new additives showing effects similar to that of arginine enables the development of various products. For this, the present inventors comparatively examined the botulinum toxin-stabilizing effects of various antioxidants and buffers shown in Table 5 below. The results of the examination are shown in Table 5 below and FIG. 7.

[112] [Table 5]

		Stabilizing effect
Buffer for pH adjustment	Arginine	+++
	Gluconolactone	++
	Glycine	-
	Tartaric acid	++
Antioxidant	Sodium bisulfite	-
	Cysteine	-
	Propyl gallate	-
	Sodium hydrosulfite	+
	Thioglycolate	-

[113] FIG. 7a shows the results of measuring the residual potency of botulinum toxin after 28 days of culture of botulinum toxin compositions containing the antioxidants shown in Table 5 above. The results of the measurement indicated that all the antioxidant additives used in the examination showed stabilizing effects lower than that of the control methionine. However, in the case of the buffers, gluconolactone showed a significant stabilizing effect at a pH of 6.5, and tartaric acid showed a stabilizing effect at a pH ranging from 5.5 to 6.5. Such results suggest the possibility of development of new additives having remarkable effects. The results of Examples 2 to 8 indicate that the stabilizing effect of addition of arginine on the stabilization of a liquid formulation

of botulinum toxin is higher than the effect of addition of methionine. Therefore, the present inventors confirmed that arginine is an effective additive for the stabilization of a liquid formulation of botulinum toxin. Also, the present inventor confirmed the possibility of gluconolactone or tartaric acid as the new stabilizer for a botulinum toxin.

[114]

Industrial Applicability

[115] The present invention is directed to a stabilized liquid formulation of botulinum toxin and a preparation method thereof. A pharmaceutical composition comprising botulinum toxin according to the present invention is a liquid formulation which is easily stored and distributed through the use of arginine as a stabilizer for botulinum toxin as an active ingredient, and it exhibited a significant effect on the stabilization of botulinum toxin under suitable conditions according to the temperature and pH of the human body. Thus, it is expected that the pharmaceutical composition of the present invention will greatly contribute to the safe and convenient medical use of botulinum toxin.

[116]

Claims

[Claim 1] A pharmaceutical formulation, containing; a neurotoxin; and a stabilizer.

[Claim 2] The pharmaceutical formulation of claim 1, wherein the neurotoxin is any one or more selected from the group consisting of botulinum toxin, tetanus toxin, cholera toxin, and pertussis toxin.

[Claim 3] The pharmaceutical formulation of claim 2, wherein the botulinum toxin is selected from the group consisting of botulinum toxin type A, B, C, D, E, F and G.

[Claim 4] The pharmaceutical formulation of claim 3, wherein the botulinum toxin is botulinum toxin type A.

[Claim 5] The pharmaceutical formulation of claim 2, wherein the botulinum toxin is either a form containing no complexing protein or a complex form containing a complexing protein.

[Claim 6] The pharmaceutical formulation of claim 1, wherein the stabilizer is any one or more selected from basic amino acid, gluconolactone, and tartaric acid.

[Claim 7] The pharmaceutical formulation of claim 6, wherein the basic amino acid is any one or more selected from the group consisting of lysine, histidine, and arginine.

[Claim 8] The pharmaceutical formulation of claim 7, wherein the basic amino acid is arginine.

[Claim 9] The pharmaceutical formulation of claim 6, wherein the basic amino acid is contained at a concentration of 0.01-1,000 mM per 100 units of the botulinum toxin.

[Claim 10] The pharmaceutical formulation of claim 1, having a pH of 5.5-7.0.

[Claim 11] The pharmaceutical formulation of claim 1, further containing polysorbate.

[Claim 12] The pharmaceutical formulation of claim 11, wherein the polysorbate is polysorbate 20.

[Claim 13] The pharmaceutical formulation of claim 11, wherein the polysorbate is contained in an amount of 0.001-1 wt% based on the total weight of the pharmaceutical formulation.

[Claim 14] The pharmaceutical formulation of claim 1, further containing a local anesthetic.

[Claim 15] The pharmaceutical formulation of claim 14, wherein the local anesthetic is lidocaine.

[Claim 16] The pharmaceutical formulation of claim 14, wherein the local anesthetic is contained in an amount of 0.1-1 wt% based on the total weight of the pharmaceutical formulation.

[Claim 17] The pharmaceutical formulation of claim 1, which is liquid.

[Claim 18] A method for preparing a pharmaceutical formulation, comprising the steps of;
(a) purifying a neurotoxin; and
(b) adding a stabilizer to the neurotoxin.

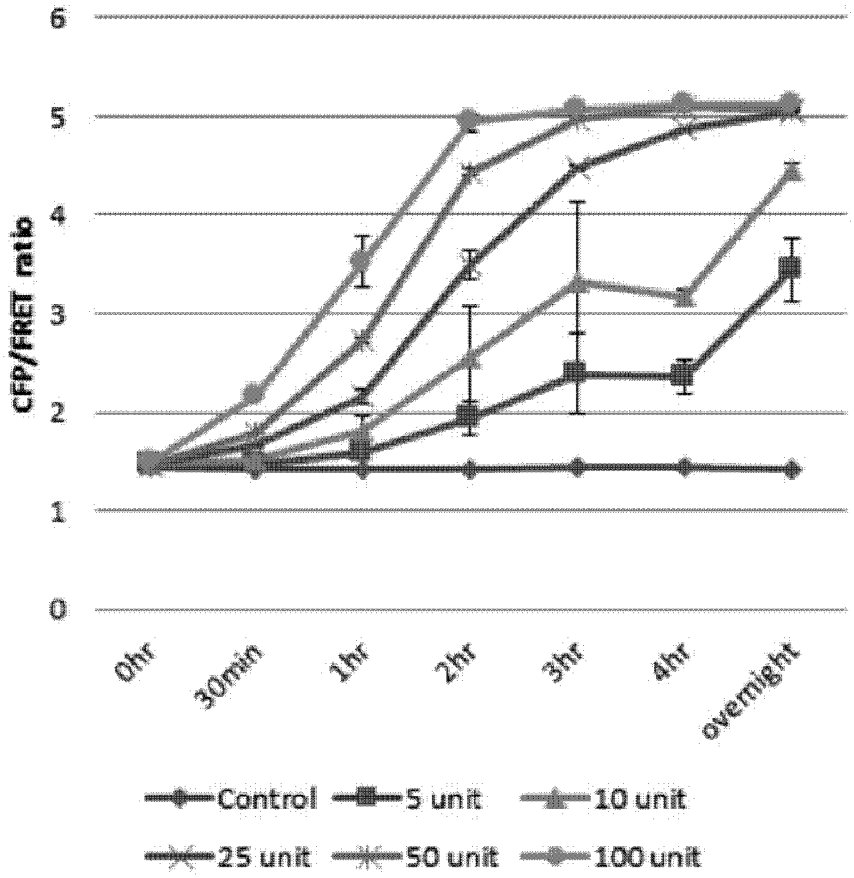
[Claim 19] The method of claim 18, wherein the neurotoxin is botulinum toxin type A.

[Claim 20] The method of claim 18, wherein the stabilizer is any one or more selected from basic amino acid, gluconolactone, and tartaric acid.

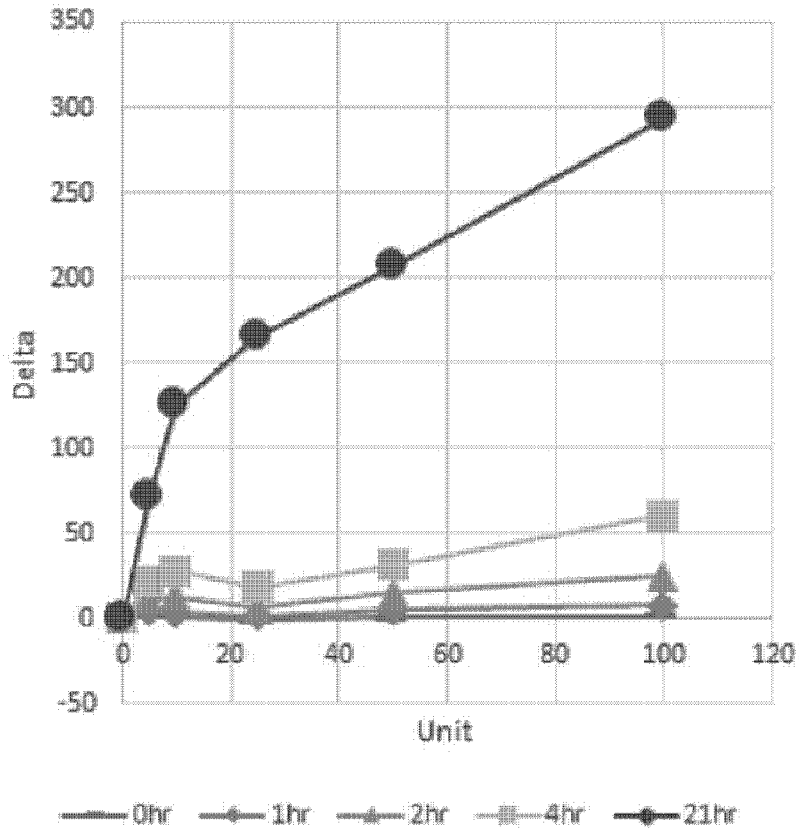
[Claim 21] The method of claim 20, wherein the basic amino acid is arginine.

[Claim 22] The method of claim 18, wherein the pharmaceutical formulation is liquid.

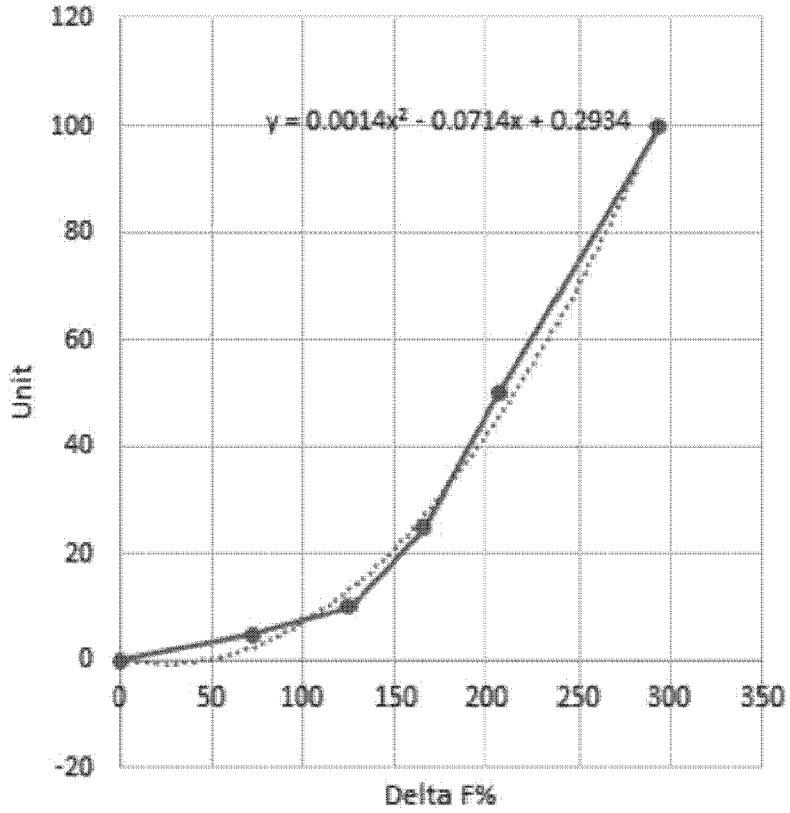
[Fig. 1a]


6
5
4
3
2
1
0
CFP/FRET ratio
0hr
30min
1hr
2hr
3hr
4hr
overnight
Control
5 unit
10 unit
25 unit
50 unit
100 unit

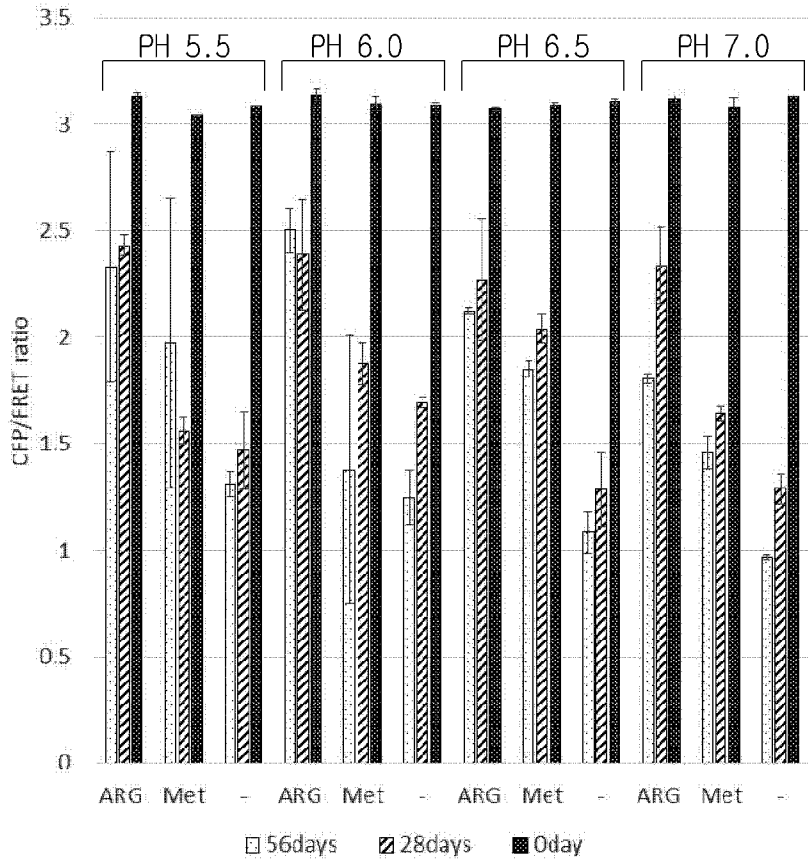
[Fig. 1b]


350
300
250
200
150
100
50
0
-50
Delta
0
20
40
60
80
100
120
Unit
0hr
1hr
2hr
4hr
21hr

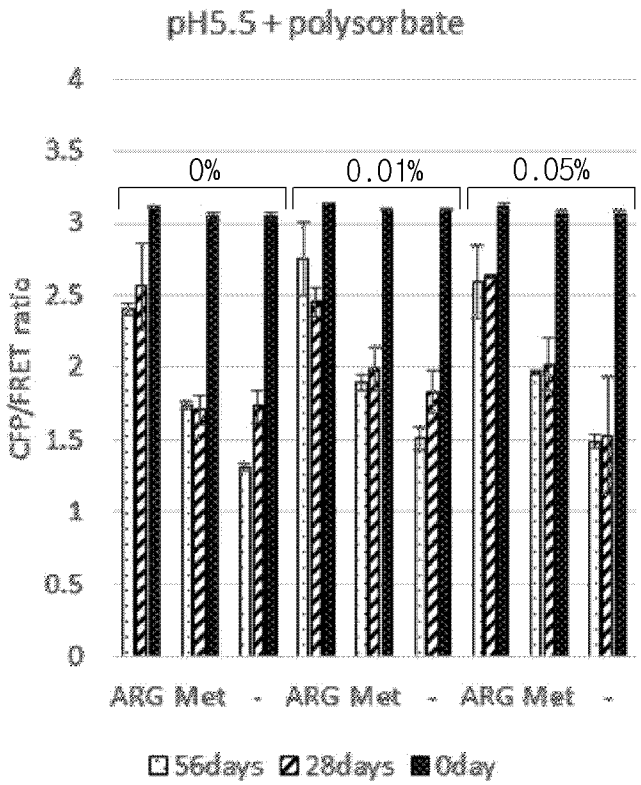
[Fig. 1c]


y = 0.0014x² - 0.0714x + 0.2934
Unit
Delta F%
120
100
80
60
40
20
0
-20
0
50
100
150
200
250
300
350

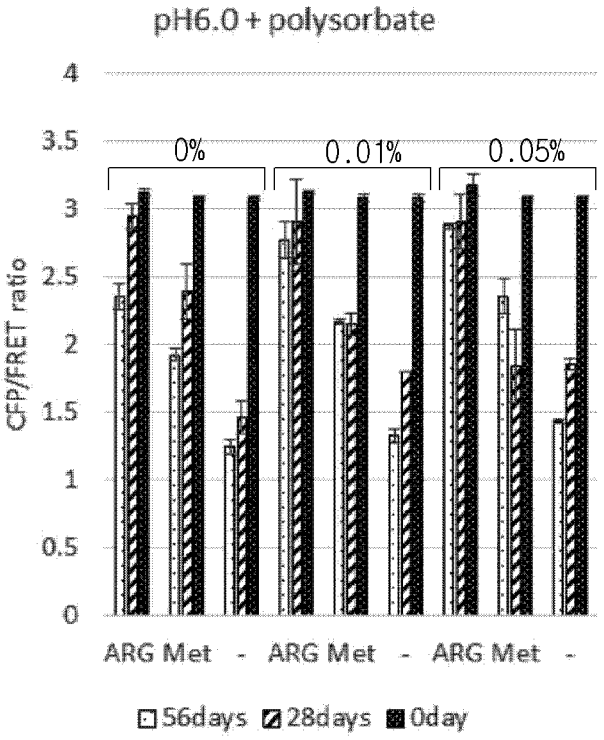
[Fig. 2]


PH 5.5
PH 6.0
PH 6.5
PH 7.0
CFP/FRET ratio
3.5
3
2.5
2
1.5
1
0.5
0
ARG
Met
-
56days
28days
0day

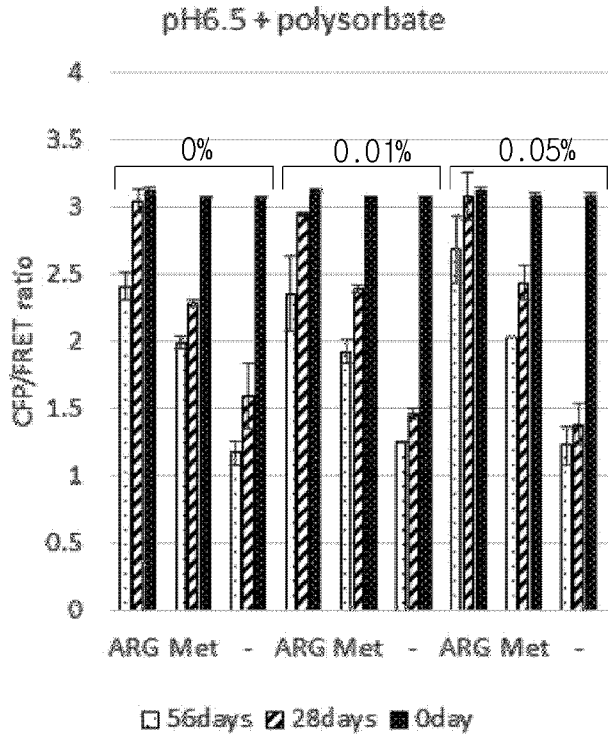
[Fig. 3a]


pH5.5 + polysorbate
4
3.5
3
2.5
2
1.5
1
0.5
0
CFP/FRET ratio
0%
0.01%
0.05%
ARG Met - ARG Met - ARG Met -
56days
28days
0day

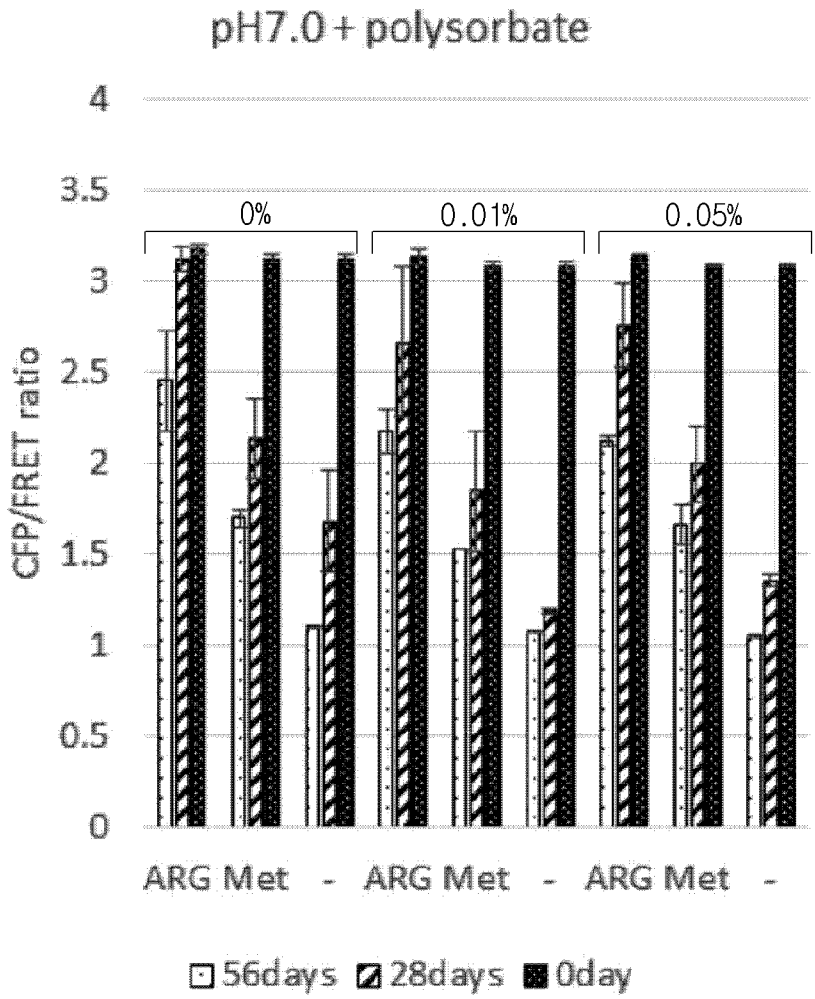
[Fig. 3b]


pH6.0 + polysorbate
4
3.5
3
2.5
2
1.5
1
0.5
0
CFP/FRET ratio
0%
0.01%
0.05%
ARG Met - ARG Met - ARG Met -
56days
28days
0day

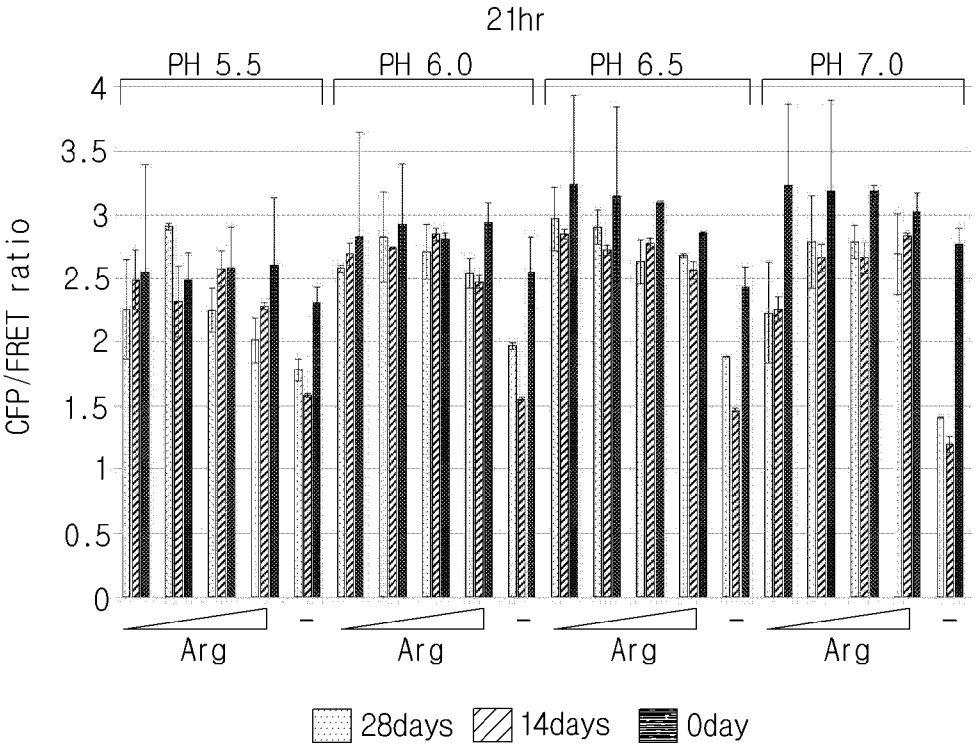
[Fig. 3c]


pH6.5 + polysorbate
4
3.5
3
2.5
2
1.5
1
0.5
0
CFP/FRET ratio
0%
0.01%
0.05%
ARG Met - ARG Met - ARG Met -
56days
28days
0day

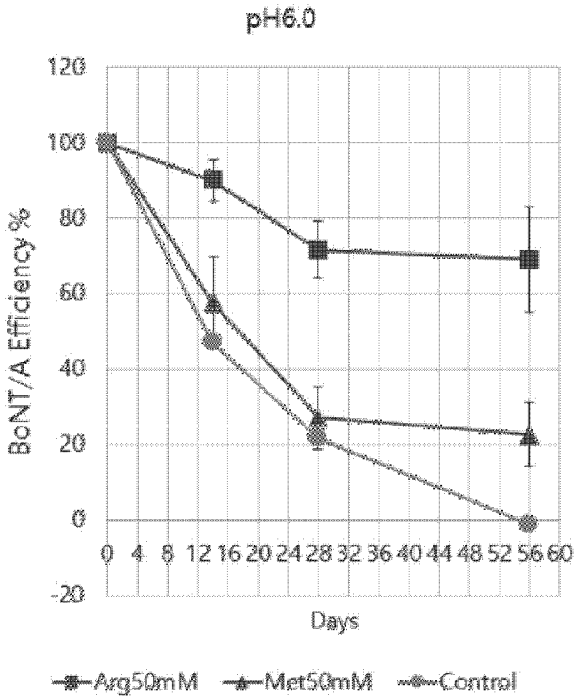
[Fig. 3d]


pH7.0 + polysorbate
4
3.5
3
2.5
2
1.5
1
0.5
0
CFP/FRET ratio
0%
0.01%
0.05%
ARG Met - ARG Met - ARG Met -
56days
28days
0day

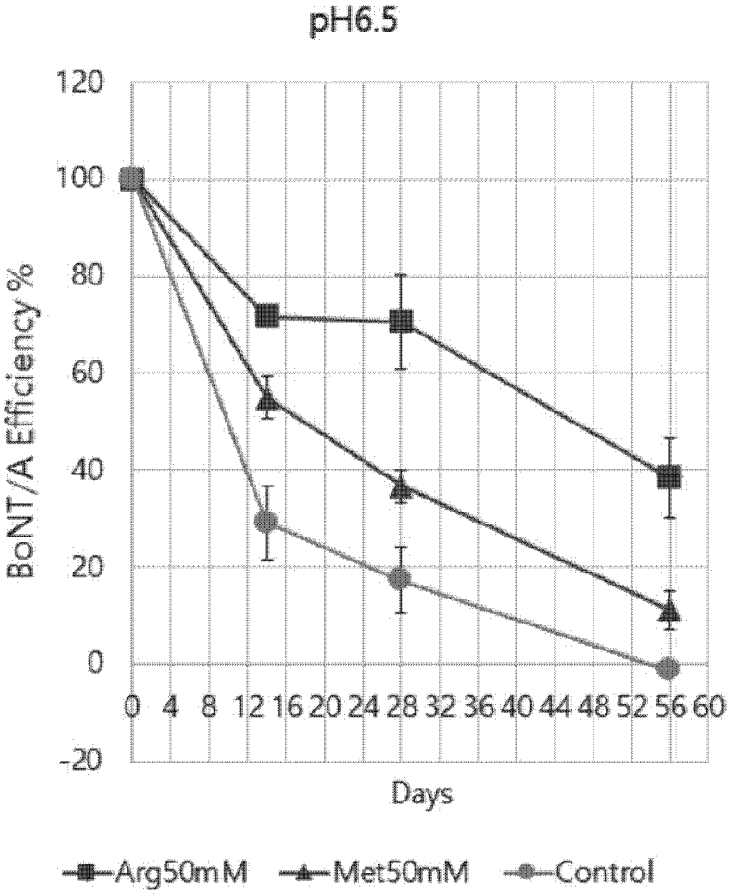
[Fig. 4]


21hr
PH 5.5
PH 6.0
PH 6.5
PH 7.0
CFP/FRET ratio
4
3.5
3
2.5
2
1.5
1
0.5
0
Arg
Arg
Arg
Arg
28days
14days
0day

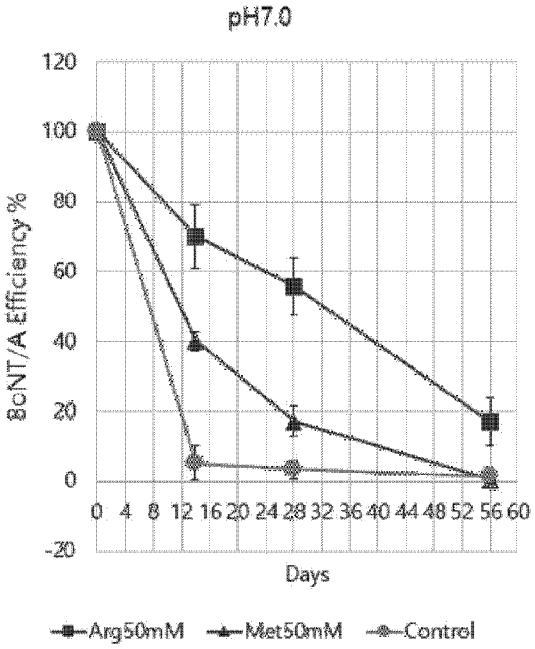
[Fig. 5a]


pH6.0
BoNT/A Efficiency %
120
100
80
60
40
20
0
-20
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60
Days
Arg50mM
Met50mM
Control

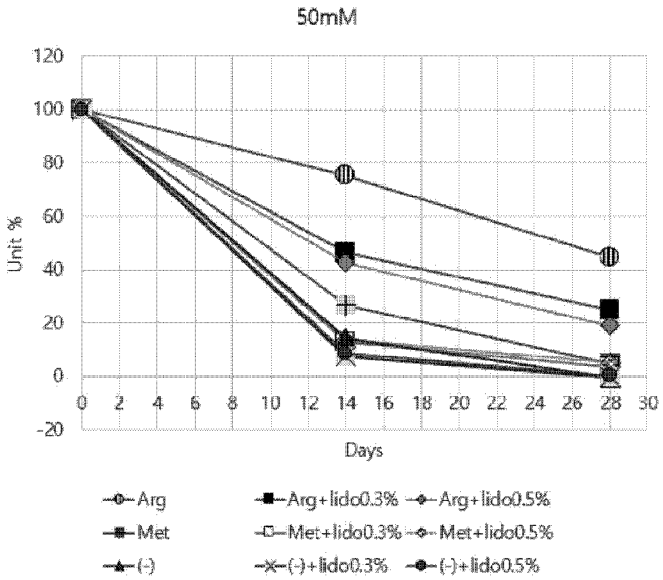
[Fig. 5b]


pH6.5
120
100
80
60
40
20
0
-20
BoNT/A Efficiency %
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60
Days
Arg50mM
Met50mM
Control

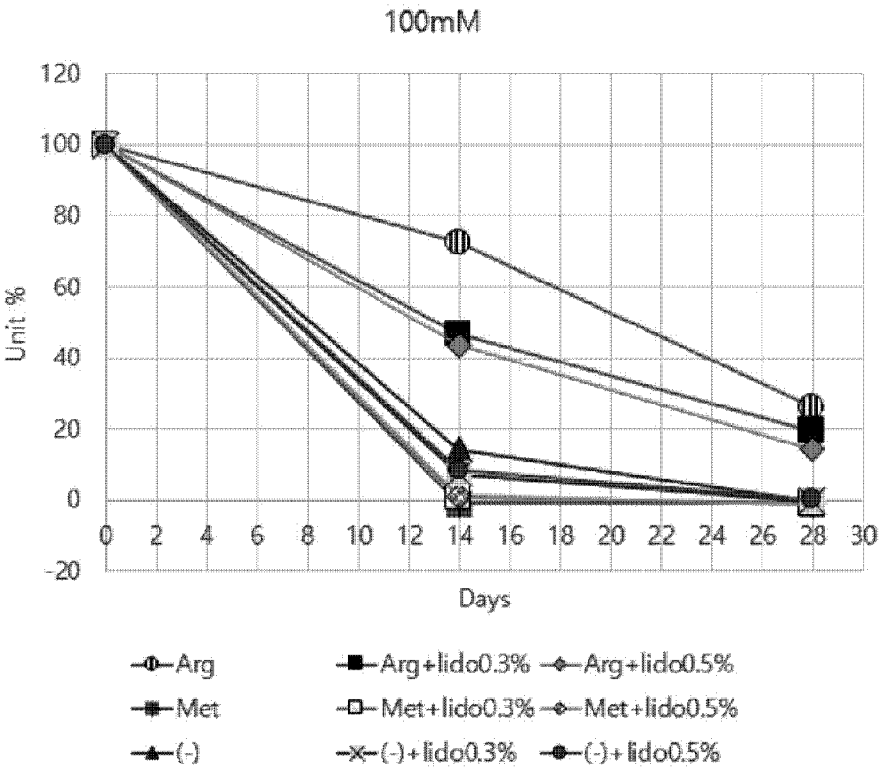
[Fig. 5c]


pH7.0
120
100
80
60
40
20
0
-20
BoNT/A Efficiency %
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60
Days
Arg50mM
Met50mM
Control

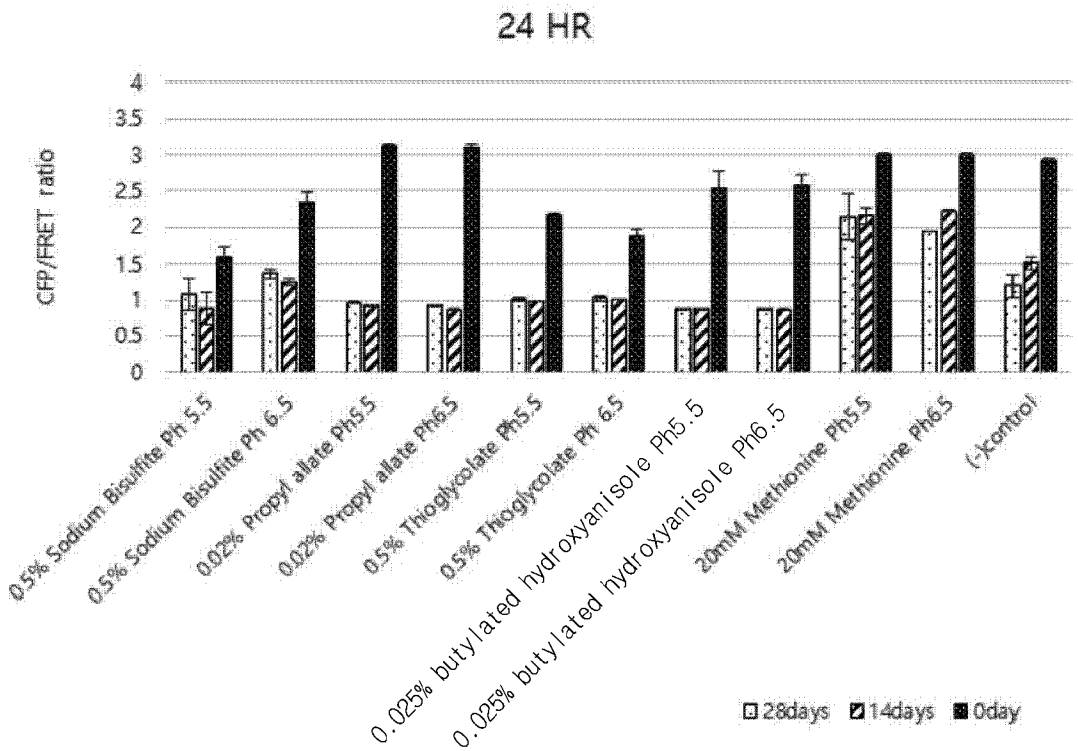
[Fig. 6a]


50mM
120
100
80
60
40
20
0
-20
Unit %
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Days
Arg
Arg+lido0.3%
Arg+lido0.5%
Met
Met+lido0.3%
Met+lido0.5%
(-)
(-)+lido0.3%
(-)+lido0.5%

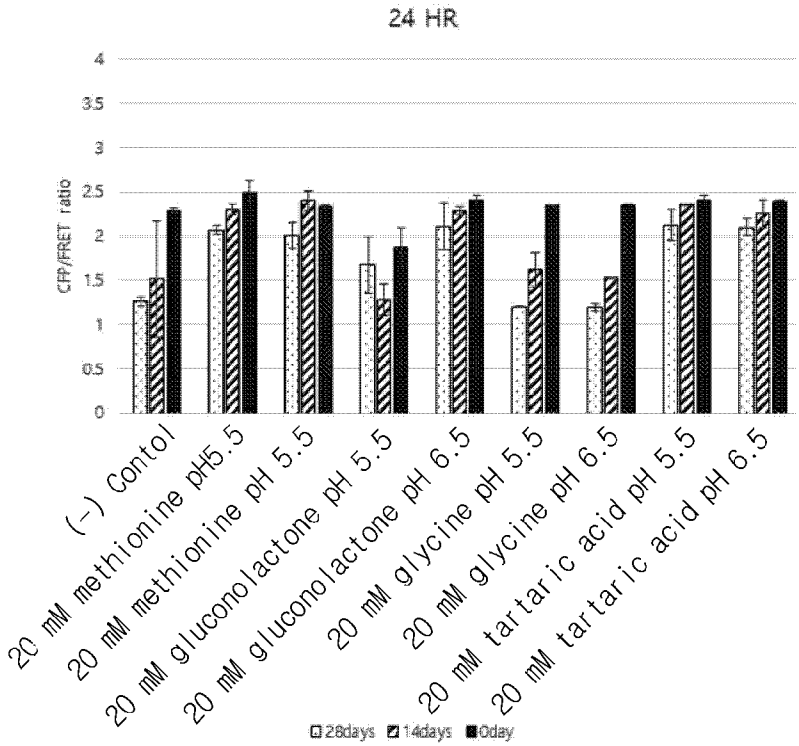
[Fig. 6b]


100mM
120
100
80
60
40
20
0
-20
Unit %
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Days
Arg
Arg+lido0.3%
Arg+lido0.5%
Met
Met+lido0.3%
Met+lido0.5%
(-)
(-)+lido0.3%
(-)+lido0.5%

[Fig. 7a]


24 HR
CFP/FRET ratio
4
3.5
3
2.5
2
1.5
1
0.5
0
0.5% Sodium Bisulfite Ph 5.5
0.5% Sodium Bisulfite Ph 6.5
0.02% Propyl allate Ph5.5
0.02% Propyl allate Ph6.5
0.5% Thioglycolate Ph5.5
0.5% Thioglycolate Ph 6.5
0.025% butylated hydroxyanisole Ph5.5
0.025% butylated hydroxyanisole Ph6.5
20mM Methionine Ph5.5
20mM Methionine Ph6.5
(-)control
28days
14days
0day

[Fig. 7b]


24 HR
CFP/FRET ratio
4
3.5
3
2.5
2
1.5
1
0.5
0
(-) Contol
20 mM methionine pH5.5
20 mM methionine pH 5.5
20 mM gluconolactone pH 5.5
20 mM gluconolactone pH 6.5
20 mM glycine pH 5.5
20 mM glycine pH 6.5
20 mM tartaric acid pH 5.5
20 mM tartaric acid pH 6.5
28days
14days
0day

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2016/009706

A. CLASSIFICATION OF SUBJECT MATTER

A61K 9/08(2006.01)i, A61K 47/18(2006.01)i, A61K 47/08(2006.01)i, A61K 47/12(2006.01)i, A61K 47/26(2006.01)i, A61K 38/48(2006.01)i, A61K 31/167(2006.01)i

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)
A61K 9/08; C07K 14/33; A61K 39/385; A61K 38/36; A61K 39/08; A61K 9/19; A61K 38/16; A61K 38/21; A61K 47/18; A61K 47/08; A61K 47/12; A61K 47/26; A61K 38/48; A61K 31/167

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: neurotoxin, botulinum toxin, stabilizer, base amino acid, arginine, polysorbate 20

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KR 10-2006-0028475 A (ALLERGAN, INC.) 29 March 2006 See abstract; paragraphs [0051], [0060], [0080], [0100], [0115]; and claims 17, 31, 32, 46, 47.	1-10,17-22
A		11-16
X	US 2010-0291136 A1 (JUNG, H. H. et al.) 18 November 2010 See claims 1-12.	1-5,10-13,17-19,22
X	US 2012-0302507 A1 (HAM, J. W.) 29 November 2012 See abstract; paragraph [0022]; and claims 1-4.	1-5,10,14-19,22
X	US 2007-0134199 A1 (FREVERT, J.) 14 June 2007 See paragraph [0044]; and claims 1-14.	1-5,10-13,17-20,22
X	US 9125804 B2 (WEBB, P. et al.) 08 September 2015 See columns 3, 10; and claims 1-4.	1-7,9-13,17-20,22

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

☒ See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 May 2017 (25.05.2017)	**25 May 2017 (25.05.2017)**

Name and mailing address of the ISA/KR
International Application Division
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon, 35208, Republic of Korea
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Authorized officer
PARK, Jung Min
Telephone No. +82-42-481-3516

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2016/009706

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		WO 01-58472 A2	16/08/2001
		WO 01-58472 A3	03/01/2002

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2016/009706

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