

LIQUID BOTULINUM TOXIN FORMULATION AND USE THEREOF

FIELD OF THE INVENTION

[001] The present invention relates to liquid formulations comprising (i) botulinum toxin, (ii) human serum albumin (HSA), (iii) hyaluronic acid, and (iv) an alditol. The liquid formulations are characterized by an improved stability against light. The invention further relates to a method of making the liquid formulations and to the use of the liquid formulations in the treatment of therapeutic and cosmetic indications.

BACKGROUND OF THE INVENTION

[002] Botulinum neurotoxins (BoNTs; or botulinum toxin (BT)) are a family of bacterial neurotoxins that are widely used to treat a growing variety of neurologic, medical and cosmetic conditions. There are seven widely accepted "classical" BoNT serotypes designated BoNT/A-G). Two serotypes, type A (BoNT/A) and type B (BoNT/B), are currently in clinical use. BoNTs are produced by *Clostridium spp.*, in particular *Clostridium botulinum*, in the form of high molecular weight (up to ~900 kDa) complexes. These toxin complexes are composed of the active 150 kDa neurotoxin and several complexing proteins (non-toxic neurotoxin-associated proteins, NAPs).

[003] The 150 kDa neurotoxin is synthesized as an inactive single chain polypeptide (~150 kDa), which is proteolytically cleaved to give a light chain (LC, ~50 kDa) and a heavy chain (HC, ~100 kDa) connected by an inter-chain disulfide bond. The HC contains a C-terminal domain that mediates binding to receptors and an N-terminal domain that mediates translocation of the LC across endosomal membranes. The LC acts as a protease in neurons and cleaves neuronal SNARE proteins. This blocks the fusion of synaptic vesicles with the plasma membrane, thus inhibiting neurotransmitter release from selected neurons.

[004] The formulation of BoNTs is very challenging, owing to their structural complexity and low product concentrations used. BoNT is highly susceptible to various conditions such as heat and alkaline pH. Thus, since BoNT is only functional if its

structure is intact, the challenge regarding the preparation of a medical dosage form of BoNT is to formulate a composition that protects the BoNT from inactivation or partial loss of biological activity during production, storage or use of the product.

[005] Furthermore, due to the extremely high potency of BoNT, with the human lethal dose being in the range of only about 0.1-1 ng/kg, pharmaceutical formulations of BoNT comprise extremely small quantities of the toxin in the range of only about 1 ng per vial. This aggravates the known problem of loss of toxin activity due to surface denaturation. Therefore, one of the main challenges in the formulation of BoNTs is minimizing activity loss during manufacture and storage.

[006] At the same time, formulations of BoNTs must be suitable for medical use in a wide range of different indications. This means that the BoNT formulation must be compatible with injection into very different types of tissue, such as muscles, different layers of skin (dermis, subcutis), or glands (e.g., salivary gland).

[007] In view of the above, the main BoNT products currently available are provided as lyophilized powders, i.e., in a form that is stable over a long period of time when stored at 2-8 °C or even at room temperature. Lyophilized forms of the BoNT/A complex were first introduced on the market in 1989 (Botox®, Allergan) and 1991 (Dysport®, Ipsen). In 2005, the first stable dosage form of the pure 150 kDa BoNT/A neurotoxin without complexing proteins was approved (Xeomin®; Merz Pharmaceuticals). However, these lyophilized products need to be reconstituted prior to use, a process potentially leading to dosing errors and sterility issues. Therefore, major efforts have been made to develop liquid formulations of BoNTs, which are more convenient to use and can be easily administered.

[008] A commercial liquid formulation of BoNT is available under the trademark Neurobloc® (Eisai). It is a sterile solution of the BoNT/B complex formulated in a buffer containing disodium succinate, sodium chloride, human serum albumin (HSA), sodium caprylate, and sodium N-acetyltryptophanate. Other commercially available liquid formulations are sold under the trademark Innotox® (Medytox), and Alluzience® (Ipsen/Galderma). Both these liquid formulations contain the BoNT/A toxin complex and, in addition to water, sodium chloride, a detergent (Innotox®: polysorbate 20; Alluzience®: polysorbate 80), an amino acid (Innotox®: methionine; Alluzience®:

histidine) and an additional excipient (Innotox®: sodium phosphate as a buffer; Alluzience®: sucrose).

[009] US 8 372 645 discloses a method for stabilizing a botulinum neurotoxin from *Clostridium botulinum* of type A, B, C1, D, E, F, or G or a mixture of two or more botulinum neurotoxins, wherein the neurotoxin or mixture of neurotoxins is free of the complexing proteins which naturally form complexes with botulinum neurotoxins, comprising intermixing the neurotoxin with a non-proteinaceous stabilizing agent in an aqueous solution in an amount effective to retain the biological activity of the neurotoxin, wherein the aqueous solution does not comprise mammalian derived proteins. The non-proteinaceous stabilizing agent is selected from one or more of hyaluronic acid, polyvinylpyrrolidone and polyethylene glycol. The composition may further comprise a polyalcohol selected from one or more of inositol, mannitol, and sorbitol. The aqueous solution comprises a pH buffer. The composition may be freeze-dried.

[0010] US 7 780 967 discloses a pharmaceutical composition consisting essentially of a high molecular weight polysaccharide and a botulinum toxin, wherein the botulinum toxin is stabilized by the high molecular weight polysaccharide and wherein the pharmaceutical composition has a reduced toxicity. The high molecule weight polysaccharide is selected from the group consisting of hydroxymethyl starch, hydroxyethyl starch, hydroxypropyl starch, hydroxybutyl starch, and hydroxypentyl starch.

[0011] US 2012/0141532 discloses a pharmaceutical composition comprising a botulinum neurotoxin and a viscous carrier for the botulinum neurotoxin, wherein the viscous carrier is selected from the group of viscous carriers consisting of hyaluronic acid, carbomer, polyacrylic acid, cellulose polycarbophil, polyvinylpyrrolidone, gelatin, dextrin, polysaccharide, polyacrylamide, polyvinyl alcohol, polyvinyl acetate, chitosans, algenates and derivatives and mixtures thereof.

[0012] WO 2010/090677 discloses an animal-protein free, solid-form Clostridial toxin pharmaceutical composition comprising a Clostridial toxin active ingredient, an effective amount of a sugar excipient and an effective amount of surfactant excipient, wherein the sugar excipient is a monosaccharide, a disaccharide or a trisaccharide,

and wherein the surfactant excipient is a poloxamer, a polysorbate, a polyoxyethylene glycol dodecyl ether, or a polyoxyethylene octyl phenyl ether. The composition is buffered to about pH 5.5 to about pH 6.5 using a citrate buffer, a phosphate buffer, a histidine buffer, or a histidine phosphate buffer. The composition may further comprise an effective amount of sodium chloride. The non-protein polymer excipient is a dextran, a polyethylene glycol, a polyethylene imine, a polyvinyl pyrrolidone, a polyvinyl acetate, an inulin, a starch, or a starch derivative. The composition further comprises an effective amount of a non-protein polymer excipient. The Clostridial toxin active ingredient is stable for at least one-year when stored at either ambient or below freezing temperatures.

[0013] WO2017/148915 finally discloses a botulinum toxin containing composition comprising non-crosslinked hyaluronic acid and human serum albumin.

[0014] Despite these advances in the preparation of liquid botulinum toxin formulations, there is still a need for new options to develop ready-to-use liquid formulations of botulinum toxin, which are stable during transport and storage.

OBJECT OF THE INVENTION

[0015] It is an object of the present invention to provide a stable liquid formulation of botulinum toxin that is ready-to-use for therapeutic and cosmetic treatments.

SUMMARY OF THE INVENTION

[0016] The present invention is based on the unexpected finding that the use of a pre-treated protein stabilizer based on human serum albumin (HSA) in the formulation of a liquid botulinum toxin formulation results in a reduced light sensitivity and, thus, in an improved light stability. Furthermore, it was unexpectedly found that very low amounts of iron ions (Fe^{3+} ions) drastically increase the light sensitivity of liquid botulinum toxin formulations containing human serum albumin (HSA). This is surprising since iron is relatively common and ubiquitous, and moreover, it was found that other metal ions such as copper, cobalt or nickel do not show this destabilizing effect.

[0017] Furthermore, the present invention is based on the surprising finding that tryptophan and N-acetyl-tryptophan decrease the light stability of liquid botulinum toxin

formulations containing human serum albumin (HSA). This finding is highly surprising since commercial HSA products, in addition to water and sodium chloride, usually contain sodium caprylate and N-acetyl-tryptophan to stabilize HSA at high temperatures, which in this way get into the botulinum toxin formulation. It is also surprising because tryptophan is described as stabilizing additive for botulinum toxin (see, e.g., EP 3 679 946).

[0018] It has been further found that the presence of a non-proteinaceous stabilizer comprising a hyaluronic acid and the presence of an alditol contribute to the improved light stability of the liquid formulation.

[0019] Accordingly, in a first aspect, the present invention provides for a liquid formulation comprising water and the following components:

- (i) botulinum toxin,
- (ii) human serum albumin,
- (iii) hyaluronic acid,
- (iv) alditol;

wherein:

- (I) the liquid formulation comprises a chelating agent; or
- (II) the liquid formulation comprises a chelating agent and a metal salt selected from a salt of calcium, magnesium, zinc, and mixtures thereof; or
- (III) said human serum albumin has been subjected to the following process comprising steps (a) and (c) or steps (b) and (c):
 - (a) contacting human serum albumin with a chelating agent to obtain a mixture of human serum albumin and the chelating agent;
 - (b) contacting human serum albumin with a chelating agent and a metal salt selected from calcium, magnesium or zinc, and mixtures thereof to obtain a mixture of human serum albumin, the chelating agent, and the metal salt;
 - (c) removing the chelating agent from the mixture obtained in step (a) or step (b) by dialysis, filtration, crossflow filtration or ultrafiltration, preferably by dialysis; or

(IV) the liquid formulation has been subjected to the following process comprising steps (d) and (f) or steps (e) and (f):

- (d) contacting a liquid formulation comprising components (i) to (iv) with a chelating agent to obtain a mixture of said liquid formulation and the chelating agent;
- (e) contacting a liquid formulation comprising components (i) to (iv) with a chelating agent and a metal salt selected from calcium, magnesium or zinc, or a mixture thereof to obtain a mixture of said liquid formulation, the chelating agent, and the metal salt;
- (f) removing the chelating agent from the mixture obtained in step (d) or step (e) by dialysis, filtration, crossflow filtration or ultrafiltration, preferably by dialysis.

[0020] In a second aspect, the invention provides for a liquid formulation comprising water and the following components:

- (i) botulinum toxin,
- (ii) human serum albumin,
- (iii) hyaluronic acid,
- (iv) alditol, wherein the alditol is sorbitol.

[0021] In one embodiment of the first and second aspect, the liquid formulation further comprises one or both of components (v) and (vi):

- (v) tonicity agent,
- (vi) buffer agent.

[0022] In one embodiment of the first or second aspect, the botulinum toxin is a botulinum neurotoxin complex; or

the botulinum toxin is botulinum neurotoxin free of complexing proteins; or

wherein the botulinum toxin is of serotype A; or

the botulinum toxin is botulinum neurotoxin of serotype A free of complexing proteins;
or

the botulinum toxin is of serotype A and is present at a concentration of 1-1000 U/ml;
or

the botulinum toxin is botulinum neurotoxin of serotype A free of complexing proteins
and is present at a concentration of 1-1000 U/ml.

[0023] In one embodiment of the first or second aspect, the human serum albumin
is present in the liquid formulation at a concentration of 0.001-1.00% w/v.

[0024] In one embodiment of the first aspect and the embodiments referring thereto,
the chelating agent is selected from the group consisting of aminopolycarboxylic acids
having three to six carboxylic acid functional groups, citrate, porphyrins, N,N,N',N'-
tetrakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN), triethylenetetramine (TETA),
and mixtures thereof,

preferably wherein the chelating agent is an aminopolycarboxylic acids of general
formula (I):



wherein R comprises no carboxylic acid group or one or two carboxylic acid groups,
the aminopolycarboxylic acid preferably being selected from the group consisting of
ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(α -aminoethylether)-
N,N,N',N'-tetraacetic acid (EGTA), 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-
tetraacetic acid (BAPTA), diethylenetriaminepentaacetic acid (DTPA),
triethylenetetramine-hexaacetate (TTHA), and mixture thereof; or

wherein the chelating agent is 2,2',2''-nitrilotriacetic acid.

[0025] In one embodiment of the first aspect and the embodiments referring thereto,
the metal salt is present in the liquid formulation in an amount of 0.01-100 mM; or
wherein the metal salt is calcium chloride and is present in the liquid formulation in an
amount of 0.01-100 mM.

[0026] In one embodiment of the first aspect and second aspect or the embodiments
referring thereto, the liquid formulation contains Fe^{3+} ions in a concentration of less
than 1 μM , preferably less than 500 nM, and more preferably less than 250 nM.

[0027] In one embodiment of the first aspect and second aspect or the embodiments referring thereto, the concentration of tryptophan and N-acetyl-tryptophan in the liquid formulation is no more than 50 μM , preferably no more than 20 μM , more preferably no more than 10 μM , still more preferably no more than 1 μM , and most preferably 0 μM , of tryptophan and N-acetyltryptophan (total concentration of both Trp and N-AcTrp).

[0028] In one embodiment of the first and second aspect or the embodiments referring thereto, the hyaluronic acid is not crosslinked; or

hyaluronic acid is present at a concentration of 2 to 10 mg/ml and wherein the hyaluronic acid is not crosslinked.

[0029] In a further embodiment, hyaluronic acid is present at a concentration of 2 to 10 mg/ml and wherein the hyaluronic acid is crosslinked.

[0030] In one embodiment of the first aspect and the embodiments referring thereto, the alditol is selected from glycerol, mannitol, isomalt, lactitol, sorbitol, xylitol, threitol, erythritol and arabitol, preferably from mannitol and sorbitol.

[0031] In one embodiment of the first and second aspect or the embodiments referring thereto, the alditol is present in a concentration of from 20 to 100 mg/ml.

[0032] In one embodiment of the first and second aspect and the embodiments referring thereto,

- (v) the tonicity agent is present in the liquid formulation in an amount of 0.01-2.0 % w/v, or the tonicity agent is sodium chloride, or the tonicity agent is sodium chloride and is present in the liquid formulation in an amount of 0.01-2.0 % w/v;
- (vi) the buffer agent is present in the liquid formulation in a concentration of 1-100 mM, or the buffer agent is citric acid, an amino acid, phosphate, or a mixture thereof, or the buffer agent is histidine, or the buffer agent is histidine, phosphate, or a mixture thereof, and the histidine and phosphate are present in the liquid formulation in a concentration of 1-100 mM.

[0033] In one embodiment of the first and second aspect or the embodiments referring thereto, the pH of the liquid formulation is in the range of from 5.0 to 8, preferably from 5.5 to 6.5.

[0034] In a third aspect, the invention relates to a method of making the liquid formulation as defined in the first aspect or any of the embodiments referring thereto, comprising:

mixing components (i) to (iv) and optionally (v) and/or (vi) in a liquid comprising water; and

- (I) a chelating agent; or
- (II) a chelating agent and a metal salt selected from a salt of calcium, magnesium, zinc, and mixtures thereof; or
- (III) wherein the method comprises steps (a) and (c) or steps (b) and (c):
 - (a) contacting human serum albumin with a chelating agent to obtain a mixture of human serum albumin and the chelating agent;
 - (b) contacting said human serum albumin with a chelating agent and a metal salt selected from calcium, magnesium or zinc, or a mixture thereof, to obtain a mixture of human serum albumin, the chelating agent, and the metal salt; and
 - (c) removing the chelating agent from the mixture obtained in step (a) or step (b) by dialysis, filtration, crossflow filtration or ultrafiltration, preferably by dialysis; or
- (IV) wherein the method comprises steps (d) and (f) or steps (e) and (f):
 - (d) contacting a liquid formulation comprising components (i) to (iv) with a chelating agent to obtain a mixture of said liquid formulation and the chelating agent;
 - (e) contacting a liquid formulation comprising components (i) to (iv) with a chelating agent and a metal salt selected from calcium, magnesium or zinc,

or mixtures thereof to obtain a mixture of said liquid formulation, the chelating agent, and the metal salt;

- (f) removing the chelating agent from the mixture obtained in step (d) or step (e) by dialysis, filtration, crossflow filtration or ultrafiltration, preferably by dialysis;

preferably wherein the contacting in step (a) or step (b) is carried out by mixing the composition comprising human serum albumin with the chelating agent or the chelating agent and the metal salt or wherein the contacting in step (d) and (e) is carried out by mixing a liquid formulation comprising components (i) to (iv) with the chelating agent or the chelating agent and the metal salt such that the concentration of the chelating agent in the liquid formulation is 1 mM – 500 mM;

or the invention relates to a method of making the liquid formulation as defined in the second aspect or any of the embodiments referring thereto, comprising:

mixing components (i) to (iv) and optionally (v) and/or (vi) in a liquid comprising water.

[0035] In a fourth aspect, the invention relates to the liquid formulation as defined in the first and second aspect or any of the embodiments referring thereto for use in therapy, particularly for use in the treatment of neuromuscular diseases, pain, sialorrhea, hyperhidrosis, urological disorders, and neurological disorders,

[0036] In a fifth aspect, the invention relates to the use of the liquid formulation as defined in the first or second aspect or any of the embodiments referring thereto in a cosmetic / non-therapeutic application.

[0037] In a sixth aspect, the invention relates to a method of treating a disease or condition, comprising administering an effective amount of the liquid formulation as defined in the first or second aspect or any of the embodiments referring thereto to a person in need thereof.

DETAILED DESCRIPTION OF THE INVENTION

[0038] Definitions

[0039] As used herein, the term "comprising", like the terms "including" and "containing", and any variations thereof such as "comprises", "includes" and "contains", are intended to refer to a non-exclusive inclusion, such that a process, method, product-by-process, composition or formulation that comprises, includes, or contains an element or list of elements does not include only those elements but can include other elements not expressly listed for such process, method, product-by-process, composition or formulation. In addition, within the framework of the present invention, it is intended that each of the terms "comprise," "comprising", "includes", "including", "contains", "containing", and any variations thereof, may be replaced by the term "consists" or "consisting", or any variation thereof (e.g., "consists essentially of"), which will be understood to refer to an exclusive inclusion of the elements indicated.

[0040] The terms "a" and "an" and "the" and similar reference used in the context of the present invention are to be construed to cover both the singular and the plural and, thus, may also relate to "at least one" or "more than one", unless otherwise indicated herein or clearly contradicted by the context.

[0041] The term "liquid formulation" or "liquid botulinum toxin formulation", as used herein, generally refers to an aqueous formulation and is typically an aqueous solution. Herein, the term "liquid formulation" may be interchangeably used with "liquid composition". Preferably the liquid formulation is a pharmaceutically acceptable liquid formulation or, in other words, a liquid pharmaceutical formulation. The term "pharmaceutically acceptable" as used herein, means that the liquid formulation does not cause unacceptable adverse side effects when administered to a human patient or subject. i.e., it means that the liquid formulation is suitable for human use. The aqueous solution may be a buffered solution, with or without a saline solution, and may be a physiological saline solution such as a buffered (e.g., phosphate and/or histidine buffered) physiological saline solution.

[0042] Botulinum toxin

[0043] As to component (i), the botulinum toxin is not particularly limited and includes botulinum toxin of any serotype (e.g., BoNT/A-G). For example, the botulinum toxin may be of serotype A or B (BoNT/A, BoNT/B). Preferably, the botulinum toxin is of serotype A, more preferably of serotype A1 (BoNT/A1), and most preferably BoNT/A1

produced by *Clostridium botulinum* Hall strain. In addition, the botulinum toxin may be a natural neurotoxin obtainable from the bacteria *Clostridium botulinum* or any other botulinum toxin such as a botulinum toxin obtainable from alternative sources, including recombinant technologies and genetic or chemical modification.

[0044] Furthermore, as used herein, the term "botulinum toxin" ("BT") and the synonymously used term "botulinum neurotoxin" ("BoNT") are intended to refer to the pure botulinum neurotoxin and/or any complex thereof, i.e., any complex of the pure botulinum neurotoxin and complexing proteins (referred to as the "toxin complex").

[0045] The term "pure botulinum neurotoxin", as used herein, means the botulinum neurotoxin free of complexing proteins (sometimes also referred to as the "neurotoxic component"), or more precisely, the botulinum neurotoxin without neurotoxin-associated complexing proteins (NAPs). The pure botulinum neurotoxin is the (active) neurotoxic polypeptide that ultimately inhibits acetylcholine release. It is a di-chain protein comprised of a light chain (LC; about 50 kDa) and a heavy chain (HC; about 100 kDa), held together by a disulfide bond. The active neurotoxic polypeptide may therefore also be referred to herein as the "150 kDa neurotoxin", "*Clostridium botulinum* neurotoxin (150 kD)" or "neurotoxic component".

[0046] The term "toxin complex", as used herein, refers to a high-molecular complex of the neurotoxic component and a set of complexing proteins (NAPs). In particular, the term "toxin complex" includes the 900 kDa, 500 kDa, and 300 kDa *Clostridium botulinum* type A toxin complexes. The complexing proteins are nontoxic non-haemagglutinin (NTNHA) and, in strains of serotypes A-D, different haemagglutinins (HAs). For example, the 900 kDa complex is included in onabotulinumtoxin A (Botox®/Vistabel®, Allergan, Inc., Irvine, CA, USA). Also, a toxin complex as active agent is contained in Dysport® (Azzalure®, Ipsen, Paris, France), Alluzience® (Ipsen/Galderma) and Innotox® (Medytox).

[0047] The concentration of the botulinum toxin in the liquid formulations of the present invention may be in the range of 1-1000 U/ml, preferably in the range of 10-200 U/ml, more preferably in the range of 20-150 U/ml, such as 50 U/ml or 100 U/ml.

[0048] The term "units" or "U", as used herein refers to the biological activity (biological potency) of the toxin and relates to the dose that is lethal to 50% of mice tested (LD₅₀). More specifically, within the context of the present invention, the LD₅₀ is measured using the mouse bioassay (MBA), unless otherwise stated. The MBA determines the mean lethal dose (LD₅₀) of toxin/neurotoxin after intraperitoneal injection in mice, i.e., the dose of toxin/neurotoxin capable of killing 50 % of a group of mice. On this basis, 1 unit (U) of toxin/neurotoxin, as used herein, is defined as one mouse LD₅₀ (1.0 LD₅₀ = 1.0 U). The LD₅₀ mouse bioassay is the gold standard among various biological, chemical or immunological detection methods for botulinum toxin and is known to those skilled in the art (see, e.g., Pearce, L. B.; Borodic, G. E.; First, E. R.; MacCallum, R. D. Measurement of botulinum toxin activity: Evaluation of the lethality assay. *Toxicol. Appl. Pharmacol.* 1994, 128:69-77). A person skilled in the art will be able to determine suitable botulinum toxin concentrations depending on the serotype and the intended use.

[0049] Alternatively, also a cell-based assay can be used to determine botulinum toxin activity, as described in WO 2009/114748, WO 2013/049508 or WO2014/207109. A person skilled in the art will be able to correlate botulinum toxin activity results obtained with a cell-based assay with results obtained in the mouse LD₅₀ assay by calibration using a LD₅₀ reference standard.

[0050] Due to differences in the LD₅₀ tests used by manufacturers of commercial botulinum toxin formulations, the unit potencies indicated by the manufacturers for their commercial botulinum toxin formulations is proprietary and cannot easily be compared. Therefore, within the framework of the present invention, the conversion rates provided below are used to establish the comparative potencies of incobotulinumtoxinA ("INCO"; Xeomin®, Bocouture®; botulinum toxin serotype A, free of complexing proteins; Merz Pharmaceuticals GmbH), onabotulinumtoxinA ("ONA"; Botox®, Vistabel®; botulinum toxin complex of serotype A; Allergan Inc.), abobotulinumtoxinA ("ABO"; Dysport®, Azzalure®; botulinum toxin complex of serotype A; Medicis Pharmaceutical Corp., Galderma Lab.), rimabotulinumtoxinB ("RIM"; Myobloc®, NeuroBloc®; botulinum toxin serotype B; Solstice Neurosciences Inc.), and PurTox® ("TBD"; botulinum toxin serotype A; Mentor Worldwide LLC). For use herein, the conversion rate of ONA and INCO is 1:1. The conversion rate of ONA/INCO:ABO is

1:2.5. The conversion rate of ONA/INCO:RIM is 1:50, and the conversion rate of ONA/INCO:TBD is 1:1.5.

[0051] Human serum albumin (HSA)

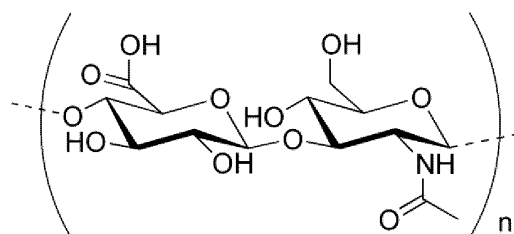
[0052] As to component (ii) of the liquid formulation of the present invention, the human serum albumin (HSA) acts as a stabilizing protein. The term "stabilizing protein" generally refers to a polypeptide that results in an increased stability of the botulinum toxin. The HSA may be present in the liquid formulation in an amount of 0.001-2.0 % w/v, preferably in an amount of 0.001-1.00 % w/v, more preferably in amount of 0.01-0.5 % w/v, still more preferably in an amount of 0.02-0.3 % w/v, and most preferably in an amount of 0.03-0.15 % w/v.

[0053] Within the context of the present invention, the term "human serum albumin" or "HSA" is intended to refer to donor HSA (HSA derived from human blood or, more precisely, from human plasma) and recombinant HSA. In one preferred embodiment, the human serum albumin is donor HSA. In another preferred embodiment, the human serum albumin is recombinant HSA.

[0054] In the liquid formulation according to the first aspect, HSA or a pre-formulation containing HSA is subjected to a chelating agent to bind or remove metal cations, in particular Fe^{3+} ions, which may negatively affect stability, in particular light stability. This is disclosed in more detail hereinunder.

[0055] Hyaluronic acid

[0056] As to component (iii), the term "hyaluronic acid" as used within the context of the present invention, also called HA, hyaluronate, or hyaluronan, is an anionic, non-sulfated glycosaminoglycan which can be represented by the follow structural formula:



[0057] Thus, hyaluronic acid can be regarded as a polymer of disaccharides, wherein said disaccharides are composed of two glucose derivatives, namely D-glucuronic acid and D-N-acetylglucosamine. In the disaccharide, glucuronic acid is glycosidically $\beta(1\rightarrow3)$ -linked to N-acetylglucosamine which in turn is glycosidically $\beta(1\rightarrow4)$ -linked to the next glucuronic acid.

[0058] It is noted that in the present invention the term "hyaluronic acid" comprises all the pharmaceutically acceptable salts, hydrates and/or solvates thereof. Preferably hyaluronic acid is present in form of the sodium salt.

[0059] As used in the context of the present invention, the term "hyaluronic acid" may be understood in the broadest sense as any moiety of hyaluronic acid known in the art.

[0060] Hyaluronic acid can be present in the form of linear chains, which are not crosslinked and in the form of crosslinked hyaluronic acids.

[0061] According to a preferred embodiment of the invention, the hyaluronic acid used in the liquid formulations according to the invention is not crosslinked; or

hyaluronic acid is present at a concentration of 2 to 10 mg/ml and wherein the hyaluronic acid is not crosslinked.

[0062] According to another embodiment, hyaluronic acid is present at a concentration of 2 to 10 mg/ml and wherein the hyaluronic acid is crosslinked.

[0063] In one embodiment, hyaluronic acid used in the liquid formulations according to the invention, may also be subjected to a chelating agent analogously to the purification of HSA in order to remove metal ions that may negatively affect light stability.

[0064] In a preferred embodiment, the hyaluronic acid used in the liquid formulations according to the invention has an average molecular weight of 100 KDa to 8 MDa, preferably from 500 KDa to 7 MDa, more preferably from 1 MDa to 6 MDa, more preferably from 2 MDa to 5 MDa, more preferably from 2 MDa to 4,5 MDa, more

preferably from 2,5 to 4,5 MDa, more preferably from 2,5 MDa to 4.0 MDa, in particular from 3.0 to 3.8 MDa.

[0065] In a further preferred embodiment the hyaluronic acid used in the liquid formulations according to the invention is not crosslinked, and has an average molecular weight of 100 KDa to 8 MDa, preferably from 500 KDa to 7 MDa, more preferably from 1 MDa to 6 MDa, more preferably from 2 MDa to 5 MDa, more preferably from 2 MDa to 4,5 MDa, more preferably from 2,5 to 4,5 MDa, more preferably from 2,5 MDa to 4.0 MDa, in particular from 3.0 to 3.8 MDa.

[0066] Alditol

[0067] As to component (iv), the term “alditol” as used herein is synonymously used with the term “sugar alcohol”. Sugar alcohols have the general formula $\text{HOCH}_2(\text{CHOH})_n\text{CH}_2\text{OH}$. Common sugar alcohols are ethylene glycol, glycerol, erythritol, threitol, arabitol, xylitol, ribitol, mannitol, sorbitol, galactitol, fucitol, iditol, inositol, volemitol, isomalt, maltitol, lactitol, maltotriitol, maltotetraitol, and polyglycitol.

[0068] Storage of the liquid formulation of the invention

[0069] The liquid formulation of the present invention can be stored in any suitable container system. A suitable container system for storing the liquid formulation of the present invention is any device having a partially or fully enclosed space that can be sealed or is sealed and can be used to contain, store, and/or transport liquid formulations. A container system is preferably a closed (or sealed) container made of, or partially or predominantly made of, glass or plastic (e.g., organic polymers) and includes, for example, containers in the form of (i) a syringe, (ii) a vial, (iii) a carpule, or (iv) an ampoule. In a preferred embodiment of the present invention, the liquid formulation is stored in a syringe in the form of a prefilled syringe, as known in the art.

[0070] The liquid formulation of the present invention advantageously provides an improved safety and dosing accuracy compared to lyophilized botulinum toxin preparations in powder form since the formulation does not need to be reconstituted prior to injection but is ready-to-use and can be used, e.g., in the form of a prefilled syringe. Further, the excellent stability of the liquid formulation facilitates and simplifies

the transport and storage as well as the handling by the physician. In particular, the superior light stability of the liquid formulation of the present invention simplifies the manufacturing process, allows the product to be filled and packaged without extensive light protection, and reduces potential loss of activity due to light-exposed storage at the physician's place prior to use. In addition, the liquid formulation of the present invention does not contain substances which results in enhanced injection pain, thereby increasing acceptance of the formulation by physicians and patients, particularly in the aesthetic field.

[0071] The liquid formulation according to the first aspect

[0072] In the first aspect of the invention, the present invention provides for a liquid formulation comprising water and the following components:

- (i) botulinum toxin,
- (ii) human serum albumin (HSA),
- (iii) hyaluronic acid,
- (iv) alditol.

[0073] According to the invention, the liquid formulation contains or is prepared by means of a chelating agent.

[0074] In embodiment (I), the liquid formulation comprises a chelating agent.

[0075] In embodiment (II), the liquid formulation comprises a chelating agent and a metal salt selected from a salt of calcium, magnesium, zinc, and mixtures thereof.

[0076] In embodiment (III), said human serum albumin has been subjected to the following process comprising steps (a) and (c) or steps (b) and (c):

- (a) contacting human serum albumin with a chelating agent to obtain a mixture of human serum albumin and the chelating agent;
- (b) contacting human serum albumin with a chelating agent and a metal salt selected from calcium, magnesium or zinc, and mixtures thereof to obtain a mixture of human serum albumin, the chelating agent, and the metal salt;

- (c) removing the chelating agent from the mixture obtained in step (a) or step (b) by dialysis, filtration, crossflow filtration or ultrafiltration, preferably by dialysis.

[0077] In embodiment (IV), the liquid formulation has been subjected to the following process comprising steps (d) and (f) or steps (e) and (f):

- (d) contacting a liquid formulation comprising components (i) to (iv) with a chelating agent to obtain a mixture of said liquid formulation and the chelating agent;
- (e) contacting a liquid formulation comprising components (i) to (iv) with a chelating agent and a metal salt selected from calcium, magnesium or zinc, or a mixture thereof to obtain a mixture of said liquid formulation, the chelating agent, and the metal salt;
- (f) removing the chelating agent from the mixture obtained in step (d) or step (e) by dialysis, filtration, crossflow filtration or ultrafiltration, preferably by dialysis.

[0078] It is pointed out that the terms "human serum albumin" and "chelating agent" are not intended to imply any restrictions as to their physical form or to exclude the presence of other substances or compounds that are mixed with, or included in, the HSA and the chelating agent. This means that the "human serum albumin" that is contacted in steps (a) and (b) or (d) and (e) with the chelating agent may be present in any form, such as in the form of a solid or a liquid (e.g., an aqueous composition or aqueous solution). Likewise, the "chelating agent" may be present in any form, such as in the form of a solid or a liquid (e.g., an aqueous composition or aqueous solution). Moreover, the expression "contacting human serum albumin with a chelating agent" in embodiments III or IV does not exclude that the human serum albumin is in the form of a composition (e.g., a solid composition or liquid composition, in particular an aqueous composition or aqueous solution) that contains one or more additional components included in the final liquid formulation, such as a tonicity agent or a buffer agent or, and/or the chelating agent is in the form of a composition (e.g., a solid composition or liquid composition, in particular an aqueous composition or aqueous solution) that contains one or more additional components included in the final liquid formulation, such as a tonicity agent or a buffer agent.

[0079] Preferably, the mixture obtained in step (a) or step (d) is an aqueous mixture. This aqueous mixture can be prepared by different ways. For example, the human serum albumin may be in the form of an aqueous composition, e.g., an aqueous solution, which is mixed with the chelating agent that may be present in solid form or in liquid form, e.g., in the form of an aqueous solution of the chelating agent. Furthermore, the human serum albumin may be in the form of a solid, e.g., a lyophilized material, which is mixed with a chelating agent and an aqueous solution or with an aqueous solution of the chelating agent. Preferably, the human serum albumin is in the form of an aqueous composition, more preferably an aqueous solution, and the chelating agent is a solid or an aqueous composition (e.g., aqueous solution).

[0080] In particular, the HSA that is contacted in step (a) or the HSA that is contained in the liquid formulation is contacted in step (d) with a chelating agent may be in the form of an aqueous solution containing at least 5 % w/v HSA, more preferably 10-30 % w/v HSA, and most preferably 20 % w/v HSA. Furthermore, the pH of the mixture obtained in step (a) or step (d) may be adjusted to a pH in the range of 6.0-9.0, preferably 6.5-8.5, more preferably 7.0-8.5, and most preferably 7.0-8.0.

[0081] Moreover, the contacting step (a) or step (d) of the method for preparing the liquid formulation according to the present invention may comprise several sub-steps. For example, in one embodiment, contacting in step (a) comprises, or consists (only) of, the steps of mixing a chelating agent (e.g., EDTA) and human serum albumin, incubating said mixture for a determined time, and optionally subjecting said mixture to dialysis against a buffer containing a chelating agent, the chelating agent being preferably the same as that used in the incubating sub-step. In another embodiment, said mixture is not incubated but (directly) subjected to dialysis against a buffer containing a chelating agent, the chelating agent being preferably the same as that used in the step of mixing a chelating agent (e.g., EDTA) and human serum albumin. In another embodiment, the chelating agent and human serum albumin are not mixed prior to dialysis. This is, contacting step (a) may comprise, or may consist (only) of, the step of subjecting said mixture to dialysis against a buffer containing a chelating agent.

[0082] Preferably, step (a) comprises, or consists (only) of, a step of adding a chelating agent to a composition comprising HSA (e.g., a solution of HSA) or of mixing

a chelating agent with a composition comprising HSA (e.g. a solution of HSA). The resulting mixture is then incubated for some time, e.g., allowed to stand for a given time without stirring or stirred for a given time.

[0083] The time of incubation is not confined to a specific range but is typically at least 0.5 hours, particularly at least 1 hour, and more particularly at least 2 hours. The upper limit of the time of incubation is not critical and may be, e.g., 1 hour, 2 hours, 5 hours or 10 hours. Hence, the incubation time may be, for examples, 0.5 to 5 hours or 1 hour to 10 hours. Likewise, the incubation temperature is not particularly limited and may, for example, be within the range of 0 °C to 60 °C. Preferably, the temperature is from 0 °C to 30 °C. This means that room temperature (20 °C or 25 °C) is a suitable temperature within the present invention. As known to the skilled person, the temperature influences the reaction time. Generally, the incubation conditions (e.g., time and temperature) are selected such that the residual amount of chelating agent (e.g., EDTA) that is contained in the final product is 100 µM or less, preferably 10 µM or less, more preferably 1 µM or less.

[0084] Optionally, the incubation step is followed by further processing such as dialysis of the incubated mixture against a buffer containing a chelating agent, typically the same chelating agent as that used in the incubation step. The chelating agent used in this optional dialyzing step is preferably contained in the dialysis buffer at a concentration of 0.1 mM - 1000 mM, more preferably 1 mM - 200 mM, and most preferably 10 mM - 100 mM. It is also preferred that the buffer used in the dialyzing step (i) has a pH of 7.5 - 8.5, (ii) further comprises a buffer agent, preferably according to the final composition, or (iii) further comprises a tonicity agent, preferably 0.9% sodium chloride w/v, or (i) and (ii), or (i) and (iii), or (ii) and (iii), or (i) and (ii) and (iii).

[0085] The contacting in step (d) may comprise analogous sub-steps.

[0086] Within the present invention, the term "chelating agent", as used herein, is not particularly limited as far as it is capable of binding metal ions. The term "chelating agent", as used herein, may also be referred to "chelator" or "sequestering agent". The chelating agents for use herein are typically metal ion-binding organic compounds. The metal ions generally form multiple coordinate bonds with the organic chelating agents which act as polydentate ligands.

[0087] Suitable chelating agents for use herein include, but are not limited to, aminopolycarboxylic acids (e.g., aminopolycarboxylic acids having three to six, preferably four, carboxylic acid functional groups) and other compounds such as citrate, porphyrins, TPEN (N,N,N',N'-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine), TETA (triethylenetetramine), and mixtures thereof. Exemplary aminopolycarboxylic acids include NTA (nitrilotriacetate, or 2,2',2''-nitrilotriacetic acid), DOTA (1,4,7,10-tetraazacyclododecan-1,4,7,10-tetraacetate), TED (ethylenediaminetriacetate), EDTA (ethylenediaminetetraacetic acid), EGTA (ethylene glycol-bis(β -aminoethylether)-N,N,N',N'-tetraacetic acid), BAPTA (1,2-bis(*o*-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid), DTPA (diethylenetriaminepenta-acetic acid), and TTHA (triethylenetetraminehexaacetate).

[0088] Particularly preferred chelating agents for use herein include compounds of general formula (I):



which are aminopolycarboxylic acid compounds having at least four carboxylic acid functional groups. The R group is not particularly limited and may comprise no carboxylic acid functional group or one or two carboxylic acid functional groups. Preferably, the R group comprises no or one carboxylic acid functional group, most preferably no carboxylic acid functional group.

[0089] Examples of compounds of general formula (I) include, for example, EDTA, EGTA, BAPTA, DTPA, and TTHA. Particularly preferred for use herein are EDTA, EGTA, and DTPA, more preferred are EDTA and DTPA, and most preferred is EDTA. Mixtures of any of the above-mentioned chelating agents may also be used within the present invention, for example as solutions, solids or coupled to matrices.

[0090] In another embodiment, the chelating agent is or comprises 2,2',2''-nitrilotriacetic acid.

[0091] In step (a), the HSA is preferably contacted with an amount of the chelating agent such that the chelating agent is present in the liquid formulation at a concentration of 0.1 mM - 1000 mM or 0.1 mM - 500 mM, more preferably 0.5 mM - 500 mM or 1 mM - 500 mM, and most preferably 10 mM - 100 mM.

[0092] In step (b), the chelating agent may be removed by any appropriate technique such as dialysis (including conventional dialysis using a dialysis bag, counterflow dialysis, etc.), reverse osmosis, filtration, crossflow filtration, ultrafiltration, and chromatographic methods (e.g., ion exchange chromatography or gel filtration chromatography).

[0093] Preferably, the chelating agent is removed by dialysis. The dialysis is typically carried out for 0.5-48 hours, in particular 1-24 hours or 1-12 hours, at a temperature of 0 °C to 30 °C, particularly at a temperature of 2 °C to 30 °C or 4 °C to 25 °C, including room temperature. Further, the dialysis is typically carried out against a volume of dialyzing buffer of 10 to 1000 times the volume of the incubated HSA/chelating agent mixture, and the dialysis buffer is usually changed at least once. The dialysis membrane used may have a molecular weight cut-off of, e.g., 10 kDa.

[0094] Generally, the dialysis conditions (e.g., time, temperature, volume of buffer, number of buffer changes) are selected such that the residual amount of chelating agent (e.g., EDTA) is contained in the final product in a concentration of 100 µM or less, preferably at a concentration of 10 µM or less, more preferably at a concentration of 1 µM or less.

[0095] In accordance with a preferred embodiment of the present invention, a HSA starting material is first pre-treated by contacting same with a chelating agent, followed by removing the chelating agent. The thus obtained pre-treated HSA is then used to prepare the liquid botulinum toxin formulation of the present invention. As used herein, the term "human serum albumin starting material" is intended to refer to donor HSA material (HSA derived from human blood or, more precisely, from human plasma) or recombinant HSA material, as commercially or conventionally obtainable, i.e., without having been subjected to a pre-treatment as described herein. A mixture of the donor HSA material and the recombinant HSA material is also encompassed by the term "human serum albumin starting material", as used herein.

[0096] More specifically, according to this preferred embodiment (III), the liquid formulation comprising (i) botulinum toxin and (ii) human serum albumin is prepared by a method comprising the following steps:

- (a) contacting a human serum albumin (starting material) with a chelating agent to obtain a mixture of the human serum albumin (starting material) and the chelating agent, or
- (b) contacting human serum albumin (starting material) with a chelating agent and a metal salt selected from calcium, magnesium or zinc, and mixtures thereof to obtain a mixture of human serum albumin, the chelating agent, and the metal salt;
- (c) removing the chelating agent from the mixture to obtain a pre-treated human serum albumin material, and
- (g) mixing botulinum toxin with the pre-treated human serum albumin material.

[0097] In the presence of EDTA, the addition of the salts selected from calcium, magnesium or zinc, and mixtures thereof in step (b) will result in the formation of EDTA complexes, i.e., EDTA with the added metal cation complexed as central atom. The nature of the anion of the salt is immaterial, the only requirement being that the salt is soluble in the aqueous formulation of the present invention. For example, the anion may be an anion of pharmaceutically acceptable inorganic and organic acids. Alternatively, the chelating agent itself can form the anion of the metal salt, and components (iii) and (iv) are added to the liquid formulation as, e.g., Na₂CaEDTA.

[0098] Suitable counter anions include, for example, acetate, aspartate, benzenesulfonate, benzoate, besylate, bicarbonate, bitartrate, bromide, camsylate, carbonate, chloride, citrate, decanoate, edetate, esylate, fumarate, gluceptate, gluconate, glutamate, glycolate, glycolylarsanilate, hexanoate, hydrabamine, hydroxynaphthoate, iodide, lactobionate, nitrate, malate, maleate, mandelate, mesylate, methylbromide, methylnitrate, methylsulfate, mucate, napsylate, nitrate, octanoate, oleate, palmoate, pantothenate, phosphate, polygalacturonate, propionate, salicylate, stearate, subacetate, succinate, sulfate, tartrate, and teoclate.

[0099] Preferably, the salts are chloride salts. Particularly preferred salts include calcium chloride (i.e., CaCl₂ and hydrates thereof), magnesium chloride (i.e., MgCl₂ and hydrates thereof) and zinc chloride (i.e., ZnCl₂ and hydrates thereof). In the presence of EDTA, the addition of these salts will result in the formation of EDTA complexes, i.e., EDTA with the added metal cation complexed as central atom.

[00100] Generally, it is envisaged that the concentration of the metal ion of the salt is about equimolar to the concentration of the chelating agent. The salt may be present in the liquid formulation at a concentration of at least 0.01 mM or at least 0.1 mM or at least 1 mM. Preferably, the salt is present in the liquid formulation at a concentration of 0.01-100 mM, preferably 0.1-50 mM, more preferably 0.05-20 mM, and most preferably 1-10 mM.

[00101] Surprisingly, it was found by the inventors of the present invention that the addition of a salt of an alkaline earth metal or a transition metal, in particular a salt of calcium, magnesium or zinc, markedly reduces the injection pain of the liquid formulation of the present invention while at the same time maintaining the storage and light stability. This finding was unexpected because (i) the liquid botulinum toxin formulations as described herein above were found to cause an unpleasant injection pain, which was neither foreseeable nor predictable, (ii) the addition of a salt of an alkaline earth metal such as calcium chloride or a transition metal was surprisingly found to drastically reduce the injection pain and, at the same time, (iii) the storage and light stability of the liquid formulation was maintained despite the belief that a free (non-complexed) chelating agent is required for obtaining the light stabilizing effect, i.e., a chelating agent (e.g., EDTA) containing a central metal atom (e.g., Ca^{2+}) was surprisingly found to be still capable of protecting botulinum toxin from light.

[00102] In accordance with another preferred embodiment of the present invention, a liquid pre-formulation is contacted with a chelating agent, followed by removing the chelating agent to obtain the liquid formulation. The term "liquid pre-formulation", as used herein, refers to a liquid formulation which contains at least components (i) and (ii) (i.e., botulinum toxin and HSA), and preferably contains all the components and substances that are included in the final liquid composition, in particular components (iii) and (iv). In case of the latter, the treatment and removal of the chelating agent results in the final liquid formulation.

[00103] More specifically, according to this preferred embodiment, the liquid formulation comprising (i) botulinum toxin and (ii) human serum albumin is prepared by a method comprising the following steps:

(d) contacting a liquid pre-formulation comprising botulinum toxin and human serum

albumin with the chelating agent to obtain a mixture of the liquid pre-formulation and the chelating agent, or

- (e) contacting a liquid pre-formulation comprising botulinum toxin and human serum albumin with a chelating agent and a metal salt selected from calcium, magnesium or zinc, or a mixture thereof to obtain a mixture of said liquid formulation, the chelating agent, and the metal salt;
- (f) removing the chelating agent from the mixture to obtain a pre-treated human serum albumin material.

[00104] Advantageously, the pretreated HSA provides for a low concentration of Fe^{3+} ions.

[00105] In a preferred embodiment, the liquid formulation contains Fe^{3+} ions in a concentration of less than 1 μM .

[00106] Preferably, the Fe^{3+} concentration in the liquid formulation is less than 1000 nM, less than 750 nM, less than 500 nM, or less than 250 nM, more preferably, the Fe^{3+} concentration in the liquid formulation is less than 200 nM, less than 150 nM, or less than 100 nM, particularly preferable the Fe^{3+} concentration in the liquid formulation is less than 50 nM or less than 10 nM, and most preferably the Fe^{3+} concentration in the liquid formulation is less than 1 nM, less than 100 pM, less than 10 pM, or less than 1 pM.

[00107] Advantageously, the pre-treated HSA contains no or only low concentrations of tryptophan and N-acetyl-tryptophan.

[00108] In a preferred embodiment, the concentration of tryptophan and N-acetyl-tryptophan in the liquid formulation is no more than 50 μM , preferably no more than 20 μM , more preferably no more than 10 μM , still more preferably no more than 1 μM , and most preferably 0 μM , of tryptophan and N-acetyltryptophan (total concentration of both Trp and N-AcTrp).

[00109] The term contains no" tryptophan and N-acetyl-tryptophan, as used herein, means that the liquid formulation is free of, or completely free of, tryptophan and N-acetyltryptophan. Specifically, it means that the formulation contains no added

tryptophan and N-acetyl-tryptophan. Alternatively, it means that the concentration of tryptophan and N-acetyl- tryptophan in the liquid formulation is 0 μM (i.e., according to general rules of rounding $< 0.5 \mu\text{M}$), in particular $\leq 0.1 \mu\text{M}$ or $\leq 0.01 \mu\text{M}$ or $\leq 0.001 \mu\text{M}$, more particularly 0 nM (i.e., according to general rules of rounding $< 0.5 \text{ nM}$). The term "contains no more than" of tryptophan and N-acetyl-tryptophan, as used herein, means that the total amount of tryptophan and N-acetyl-tryptophan may be $\leq 50 \mu\text{M}$, and is preferably $\leq 20 \mu\text{M}$, more preferably $\leq 10 \mu\text{M}$, still more preferably $\leq 1 \mu\text{M}$, yet more preferably $\leq 0.1 \mu\text{M}$, and most preferably $\leq 0.01 \mu\text{M}$ or $\leq 0.001 \mu\text{M}$. Thus, the term "contains no or no more than", as used herein, means that the total amount of tryptophan and N-acetyl-tryptophan in the liquid formulation is between 0 μM and X or between 0 nM and X, with X being 50 μM , 20 μM , 10 μM , 1 μM , 0.1 μM , 0.01 μM and 0.001 μM .

[00110] The concentration of metal ions (e.g., Ca^{2+} , Co^{2+} , Cu^{2+} , Ni^{2+} , or Fe^{3+}) can be determined by measuring methods known to those skilled in the art such as atomic absorption spectroscopy (AAS), inductively coupled plasma mass spectrometry (ICP-MS), and inductively coupled plasma atomic emission spectroscopy (ICP-AES) (also referred to as inductively coupled plasma optical emission spectrometry (ICP-OES)). Preferably, ICP-MS or ICP-OES, in particular ICP-OES, may be used for measuring the concentration of metal ions (see also Second Supplement to USP38-NF 33, Chemical Tests / <233> Elemental Impurities – Procedure 1 (ICP-OES) and Procedure 2 (ICP-MS), 2015).

[00111] The concentration of the amino acids tryptophan and N-acetyl-tryptophan can be determined by various techniques (e.g., DC, HPLC, LC-MS, GC-MS, CE etc.), as known to those skilled in the art. For example, N-acetyl-tryptophane can be determined by separation using liquid chromatography on a reversed-phase column and UV detection at 280 nm as described in Nelis et al. (Nelis et al., J. Chromatogr., 1985, 333(2):381-387) or by a method based on UV spectrophotometry of the acid-soluble fraction remaining after precipitation of the protein as described in Yu and Finlayson (Yu, M. W. and Finlayson, J. S., J. Pharm. Sci., 1984, 73(1):82-86). Tryptophane can, e.g., be quantitatively determined using a method involving liquid chromatography-tandem mass spectrometry as described by Wentao et al. (Wentao et al., Analytical and Bioanalytical Chemistry, 2011, 401:3249- 3261).

[00112] The term "tryptophan", as used herein, refers to L-tryptophan, D-tryptophan or a mixture of L-tryptophan and D-tryptophan (D/L-tryptophan). Likewise, the term "N-acetyltryptophan", as used herein, refers to N-acetyl-L-tryptophan, N-acetyl-D-tryptophan or a mixture of N-acetyl-L-tryptophan and N-acetyl-D-tryptophan (N-acetyl-D/L-tryptophan).

[00113] The human serum albumin used for preparing the liquid formulation is preferably a human serum albumin material containing no more than 50 mM, more preferably no more than 20 mM or 10 mM, still more preferably no more than 1 mM or 0.1 mM, and most preferably no more than 0.01 mM or 0 mM tryptophan and N-acetyl-tryptophan.

[00114] It is pointed out that commercial donor HSA products all contain N-acetyltryptophan in significant amounts (> 10 mM). Thus, such products need to be purified to lower the amount of N-acetyl-tryptophan to the desired level prior to use for formulation of the liquid formulation of the present invention. Suitable methods for preparing the "purified HSA" are described in detail below in connection with the third aspect of the present invention. In brief, human serum albumin with no, or a low content of, tryptophan and N-acetyl-tryptophan (also referred to herein as "purified HSA") can be obtained by subjecting a human serum albumin starting material to dialysis, diafiltration, ultrafiltration, ion exchange chromatography, affinity chromatography, hydrophobic interaction chromatography, field flow fractionation or precipitation (e.g., salt precipitation, ethanol precipitation), resulting in the removal of tryptophan and/or N-acetyl-tryptophan.

[00115] Without being limited by theory, it is believed that the HSA material used for preparing the liquid formulation of the present invention, in particular the donor HSA derived from human blood that contains large amounts of iron ions, contains a significant amount of Fe^{3+} ions which are responsible - at least in part - for the photosensitivity-inducing properties of unpurified (untreated) HSA.

[00116] In addition to the advantages mentioned above, this aspect of the present invention is also advantageous in the development of manufacturing processes and new formulations. For example, equipment (e.g., vessels) and materials (e.g.,

excipients) can be selected in such a way that as little iron as possible is present in the end product.

[00117] Furthermore, the present invention is based on the surprising finding that tryptophan and N-acetyl-tryptophan decrease the light stability of liquid botulinum toxin formulations containing human serum albumin (HSA). This finding is highly surprising since commercial HSA products, in addition to water and sodium chloride, usually contain sodium caprylate and N-acetyl-tryptophan to stabilize HSA at high temperatures, which in this way get into the botulinum toxin formulation. It is also surprising because tryptophan is described as stabilizing additive for botulinum toxin (see, e.g., EP 3 679 946).

[00118] Preferably, the method for preparing the liquid formulation according to the second aspect of the present invention is the same as the method described in connection with the first aspect of the present invention. Furthermore, the liquid formulation according to the first aspect of the present invention may have the same composition as the composition of the liquid formulation according to the second aspect of the present invention. Therefore, all explanations, comments, disclosures, definitions and the like given for the liquid formulation according to the first aspect of the present invention equally apply to the liquid formulation according to the second aspect of the present invention, unless explicitly stated otherwise.

[00119] The liquid formulation according to the second aspect

[00120] In a second aspect, the present invention relates to a liquid formulation, comprising water and the following components:

- (i) botulinum toxin,
- (ii) human serum albumin,
- (iii) hyaluronic acid,
- (iv) alditol, wherein the alditol is sorbitol.

[00121] In a preferred embodiment, the aqueous formulation according to the second aspect contains Fe^{3+} ions in a concentration of less than 1 μM .

[00122] Preferably, the Fe^{3+} concentration in the liquid formulation is less than 1000 nM, less than 750 nM, less than 500 nM, or less than 250 nM, more preferably, the Fe^{3+} concentration in the liquid formulation is less than 200 nM, less than 150 nM, or less than 100 nM, particularly preferable the Fe^{3+} concentration in the liquid formulation is less than 50 nM or less than 10 nM, and most preferably the Fe^{3+} concentration in the liquid formulation is less than 1 nM, less than 100 pM, less than 10 pM, or less than 1 pM.

[00123] In a preferred embodiment, the concentration of tryptophan and N-acetyltryptophan in the liquid formulation is no more than 50 μM , preferably no more than 20 μM , more preferably no more than 10 μM , still more preferably no more than 1 μM , and most preferably 0 μM , of tryptophan and N-acetyltryptophan (total concentration of both Trp and N-AcTrp).

[00124] In a further aspect, the present invention relates to a liquid formulation, comprising water and the following components:

- (iii) botulinum toxin,
- (ii) human serum albumin,
- (iii) hyaluronic acid,
- (iv) alditol.

[00125] In a preferred embodiment, the aqueous formulation according to the second aspect contains Fe^{3+} ions in a concentration of less than 1 μM .

[00126] Preferably, the Fe^{3+} concentration in the liquid formulation is less than 1000 nM, less than 750 nM, less than 500 nM, or less than 250 nM, more preferably, the Fe^{3+} concentration in the liquid formulation is less than 200 nM, less than 150 nM, or less than 100 nM, particularly preferable the Fe^{3+} concentration in the liquid formulation is less than 50 nM or less than 10 nM, and most preferably the Fe^{3+} concentration in the liquid formulation is less than 1 nM, less than 100 pM, less than 10 pM, or less than 1 pM.

[00127] In a preferred embodiment, the concentration of tryptophan and N-acetyltryptophan in the liquid formulation is no more than 50 μM , preferably no more than 20 μM , more preferably no more than 10 μM , still more preferably no more than 1 μM , and

most preferably 0 μ M, of tryptophan and N-acetyltryptophan (total concentration of both Trp and N-AcTrp).

[00128] In accordance with the present invention, the liquid formulation as defined in the first or the second aspect or in the further aspect further comprises one or both components (v) and (vi):

(v) a tonicity agent,

(vi) buffer agent.

[00129] The term "tonicity agent", as used herein, refers to an agent that is added to injectable formulations to render the formulations similar in osmotic characteristics to physiologic fluids. The tonicity agent may also be referred to as "osmotic regulator". The tonicity agent is not particularly limited and may, for example, be selected from the group consisting of sugars, salts, polymers, and mixtures thereof.

[00130] Exemplary tonicity agents include sucrose, glucose, sodium carbonate, amino acids, polyethylene glycol (PEG), dextran, cyclodextrin, and colloids (e.g., colloidal polysaccharides). Typically, the concentration of the tonicity agent is in the range of 0 - 2.0 % w/v, in particular 0.01 - 2.0% w/v or 0.1-1.5 % w/v, more particularly 0.6-1.2 % w/v.

[00131] Preferably, the tonicity agent is sodium chloride (NaCl). The sodium chloride may be present in the liquid formulation of the present invention in an amount of 0.01 - 2.0 % w/v, preferably 0.1 - 1.5 % w/v, more preferably 0.5 - 1.2 % w/v or 0.8 - 1.0 % w/v, and most preferably 0.9 % w/v.

[00132] The term "buffer agent", as used herein, means an agent which maintains the pH of the liquid formulation in an acceptable range, i.e., an agent capable of controlling the pH of the formulation. Suitable buffer agents are those that are not chemically reactive with other ingredients and are present in amounts sufficient to provide the desired degree of pH buffering. Such buffer agents include, for example, amino acids, acetate, malic acid, ascorbate, citrate, tartrate, fumarate, succinate, phosphate, bicarbonate, TRIS, Bis-TRIS, ACES, MES, BES, MOPS, HEPES, TES, PIPES, tricine, and imidazole.

[00133] Preferably, the buffer agent is phosphate (i.e., a phosphate buffer), an amino acid, or a mixture thereof. The term "phosphate", as used herein generally means unprotonated and protonated forms and any salts thereof. The amino acid may be selected from aspartate, glycine, glutamate, histidine, proline, taurine, methionine, serine, tyrosine, tryptophan, and mixtures thereof, and is preferably selected from histidine, proline, taurine, methionine, serine, tyrosine, tryptophan, and mixtures thereof.

[00134] In one embodiment of the present invention component (iv) of the liquid formulation is sorbitol and the buffer agent (vi) is phosphate.

[00135] Most preferably, the amino acid is histidine.

[00136] The most preferred buffer agent for use herein is histidine, phosphate or a mixture thereof.

[00137] The concentration of the buffer agent in the liquid formulation of the present invention is preferably 1-100 mM, preferably 2-50 mM, more preferably 5-20 mM. If the buffer agent is an amino acid (e.g., histidine), it may be present in the liquid formulation at a concentration of 1-100 mM, preferably 2-50 mM, more preferably 5-20 mM, and most preferably 10 mM. If the buffer agent is phosphate, it may be present in the liquid formulation at a concentration of 1-100 mM, preferably 2-50 mM, more preferably 5-20 mM, and most preferably 10 mM.

[00138] The pH of the liquid formulation of the present invention is typically in the range of 5.0-8.0, particularly in the range of 5.5-7.5, and preferably in the range of 5.5-7.0 or 6.0-7.5, more preferably in the range of 6.0-7.0, and most preferably in the range of 6.0-6.5.

[00139] The viscosity of the liquid formulation of the present invention is typically in the range of from 0.1 to 100 Pa•s, preferably 0.3 to 50 Pa•s, more preferably 0.5 to 010 Pa•s, and most preferably in the range of from 0.8 to 05 Pa•s. In a particularly preferred embodiment of the present invention the viscosity of the liquid formulation is in the range of from 1 to 3 Pa•s. The term "viscosity" as used herein means "complex

viscosity" at 1 Hz and is typically determined in a shear rheometer, e.g., by a cone and plate shear rheometer in oscillating mode.

[00140] Moreover, the liquid formulations of the present invention may further comprise one or more additional pharmaceutically acceptable excipients, unless otherwise stated or intended. For example, the liquid formulations may contain one or more of sucrose, lactose, dextran, polyvinylpyrrolidone, lactic acid, citric acid, amino acid(s), benzyl alcohol, lidocaine, gelatine, hydroxyethyl starch (HES), polyethylene oxide, and polysorbate (e.g., polysorbate 20, polysorbate 80). Other suitable pharmaceutically acceptable excipients comprise those well known in the art, see, e.g., Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennsylvania.

[00141] On the other hand, it is also contemplated herein that the liquid formulations of the present invention specifically lack certain components (i.e., compounds, materials or substances), for example chelating agents, detergents, polysaccharides, amino acids, stabilizing peptides, and the like, including any combination thereof.

[00142] As used herein, the term "detergent", is synonymously used with "surfactant" and is intended to include non-ionic and ionic detergents. The term "stabilizing peptide", as used herein, generally means a peptide consisting of 5 to 50 amino acids, such as a peptide of 10 to 40 amino acids or 15 to 30 amino acids. Hence, the term "stabilizing peptide" excludes HSA.

[00143] In one embodiment, the liquid formulation of the present invention does not contain detergents, particularly does not contain polysorbate, more particularly does not contain polysorbate 20 and/or polysorbate 80. In another embodiment, the liquid formulation of the present invention does not contain alginate. In another embodiment, the liquid formulation of the present invention does not contain succinate. In another embodiment, the liquid formulation of the present invention does not contain one or more (e.g., 2, 3, 4 or 5) amino acids selected from the group consisting of: arginine, glutamic acid, methionine, tryptophane, and serine. In another embodiment, the liquid formulation of the present invention does not contain a saccharide, such as a mono-, oligo- or polysaccharide or a mixture thereof. In particular, the liquid formulation of the present invention may not contain one or more (e.g., 2, 3 or 4) of sucrose, lactose,

maltose, and trehalose. In another embodiment, the liquid formulation of the present invention does not contain a chelating agent, in particular those described herein in connection with the present invention. It is also contemplated within the present invention that the liquid formulation lacks more than one, or all of the compounds mentioned above.

[00144] In one embodiment, the liquid formulation of the present invention lacks (i) detergents and mono-, oligo- and polysaccharides, (ii) detergents and any amino acid, or detergents and all amino acids except histidine, (iii) detergents and stabilizing peptides, (iv) mono-, oligo- and polysaccharides and any amino acid, or mono-, oligo- and polysaccharides and all amino acid except histidine, (v) mono-, oligo- and polysaccharides and stabilizing peptides, (vi) any amino acids and stabilizing peptides, or all amino acids except histidine and stabilizing peptides, (vii) detergents, mono-, oligo- and polysaccharides and any amino acid, or detergents, mono-, oligo- and polysaccharides and all amino acids except histidine, (viii) detergents, mono-, oligo- and polysaccharides, and stabilizing peptides, (ix) detergents, any amino acid, and stabilizing peptides, or detergents, all amino acid except histidine, and stabilizing peptides, (x) mono-, oligo- and polysaccharides, any amino acid, and stabilizing peptides, or mono-, oligo- and polysaccharides, all amino acids except histidine, and stabilizing peptides, (xi) detergents, mono-, oligo- and polysaccharides, any amino acid, and stabilizing peptides, or detergents, mono-, oligo- and polysaccharides, all amino acids except histidine, and stabilizing peptides.

[00145] In another embodiment, the liquid formulations of the present invention do not contain any other amino acid than histidine. In another embodiment, the liquid formulation of the present invention does not contain any mono-, di- and trisaccharides. In another embodiment, the liquid formulation of the present invention does not contain any other stabilizing peptide or protein than HSA. In another embodiment, the liquid formulations of the present invention do not contain phosphate such as in the form of a phosphate buffer.

[00146] In yet other embodiments, the liquid formulations of the present invention lack (i) succinate and a detergent (e.g., polysorbate), (ii) succinate and methionine, (iii) succinate and sucrose, (iv) a detergent (e.g., polysorbate) and methionine, (v) a

detergent (e.g., polysorbate) and sucrose, (vi) methionine and sucrose, (vii) succinate, a detergent (e.g., polysorbate) and methionine, (xiii) succinate, a detergent (e.g., polysorbate) and sucrose, (ix) succinate, methionine and sucrose, (x) a detergent (e.g., polysorbate), methionine and sucrose, and (xi) succinate, a detergent (e.g., polysorbate), methionine and sucrose, (xii) a detergent (e.g. polysorbate and histidine), (xiii) a detergent (e.g. polysorbate), histidine and sucrose).

[00147] Furthermore, any of the liquid formulations of the present invention that lacks one or more components (i.e., compounds, materials or substances) may further lack a chelating agent, in particular the chelating agents described herein.

[00148] Further, a preferred liquid formulation of the present invention comprises (i) botulinum toxin at a concentration of 10-200 U/ml, (ii) HSA in an amount of 0.01-1.0 % w/v, (iii) hyaluronic acid in an amount of 2 to 10 mg/ml, (iv) an alditol in an amount of 10 – 100 mg/ml, (v) a tonicity agent, preferably sodium chloride, in an amount of 0.01-2.0% w/v, and (vi) a buffer agent in an amount of 1-100 mM.

[00149] A preferred liquid formulation of the present invention comprises (i) botulinum toxin at a concentration of 10-200 U/ml, (ii) HSA in an amount of 0.01-0.5% w/v, (iii) hyaluronic acid in an amount of 2 to 10 mg/ml, (iv) an alditol in an amount of 10 – 100 mg/ml, (v) a tonicity agent, preferably sodium chloride, in an amount of 0.1-1.5% w/v, and a buffer agent in an amount of 1-100 mM.

[00150] A preferred liquid formulation of the present invention comprises (i) botulinum toxin at a concentration of 10-200 U/ml, (ii) HSA in an amount of 0.05-0.25% w/v, (iii) hyaluronic acid in an amount of 2 to 10 mg/ml, (iv) an alditol in an amount of 10 – 100 mg/ml, (v) a tonicity agent, preferably sodium chloride, in an amount of 0.6-1.2% w/v, and (vi) a buffer agent in an amount of 5-90 mM.

[00151] A further preferred liquid formulation of the present invention comprises (i) botulinum toxin at a concentration of 10-200 U/ml, (ii) HSA in an amount of 0.01-0.5% w/v, (iii) hyaluronic acid in an amount of 2 to 10 mg/ml, (iv) an alditol in an amount of 20 – 90 mg/ml, (v) sodium chloride as a tonicity agent in an amount of 0.9% w/v, and (vi) a buffer agent in an amount of 5-90 mM.

[00152] A further preferred liquid formulation of the present invention comprises (i) botulinum toxin at a concentration of 10-200 U/ml, (ii) HSA in an amount of 0.05-0.25% w/v, (iii) hyaluronic acid in an amount of 2 to 10 mg/ml, (iv) an alditol in an amount of 20 – 90 mg/ml, (v) sodium chloride as a tonicity agent in an amount of 0.9% w/v, and (vi) a buffer in an amount of 5-90 mM.

[00153] It is also contemplated herein that the preferred liquid formulations described in the preceding paragraph as well as in the following paragraphs may be characterized by a concentration of Fe^{3+} ions of less than 1 μM , preferably less than 1000 nM or less than 500 nM, more preferably less than 250 nM or less than 100 nM, and most preferably less than 10 nM or less than 1 nM.

[00154] It is also contemplated herein that the liquid formulations described in the preceding paragraph as well as in the following paragraphs may be characterized in that the concentration of tryptophan and N-acetyl-tryptophan in the liquid formulation is no more than 50 μM , preferably no more than 20 μM , more preferably no more than 10 μM , still more preferably no more than 1 μM , and most preferably 0 μM , of tryptophan and N-acetyltryptophan (total concentration of both Trp and N-AcTrp).

[00155] A further preferred liquid formulation of the present invention comprises (i) botulinum toxin at a concentration of 10-200 U/ml, (ii) HSA in an amount of 0.01-1.0% w/v, (v) a tonicity agent, preferably sodium chloride, in an amount of 0.01-2.0% w/v, and (vi) a buffer agent in a concentration of 1-100 mM. A preferred liquid formulation of the present invention comprises (i) botulinum toxin at a concentration of 10-200 U/ml, (ii) HSA in an amount of 0.01-0.5% w/v, (v) a tonicity agent, preferably sodium chloride, in an amount of 0.01-2.0% w/v, and (vi) a buffer agent in a concentration of 1-100 mM. A preferred liquid formulation of the present invention comprises (i) botulinum toxin at a concentration of 10-200 U/ml, (ii) HSA in an amount of 0.05-0.25% w/v, (v) a tonicity agent, preferably sodium chloride, in an amount of 0.1-2.0% w/v, and (vi) a buffer agent in a concentration of 1-100 mM. A preferred liquid formulation of the present invention comprises (i) botulinum toxin at a concentration of 10-200 U/ml, (ii) HSA in an amount of 0.05-0.25% w/v, (v) a tonicity agent, preferably sodium chloride, in an amount of 0.6-1.3% w/v, and (vi) a buffer agent in a concentration of 2-50 mM. A preferred liquid formulation of the present invention comprises (i) botulinum toxin at a

concentration of 10-200 U/ml, (ii) HSA in an amount of 0.05-0.25% w/v, (v) sodium chloride in an amount of 0.9% w/v, and (vi) a buffer agent in a concentration of 5-20 mM. The concentration of (iii) hyaluronic acid may be in the range of from 2 to 10 mg/ml, and the concentration of (iv) alditol may be in the range of from 10 – 100 mg/ml, respectively.

[00156] It is also contemplated herein that the preferred liquid formulations described above may be characterized by a concentration of Fe^{3+} ions of less than 1 μM , preferably less than 1000 nM or less than 500 nM, more preferably less than 250 nM or less than 100 nM, and most preferably less than 10 nM or less than 1 nM.

[00157] It is also contemplated herein that the liquid formulations described above may be characterized that the concentration of tryptophan and N-acetyl-tryptophan in the liquid formulation is no more than 50 μM , preferably no more than 20 μM , more preferably no more than 10 μM , still more preferably no more than 1 μM , and most preferably 0 μM , of tryptophan and N-acetyltryptophan (total concentration of both Trp and N-AcTrp).

[00158] A preferred liquid formulation of the present invention comprises (i) botulinum toxin, (ii) HSA, (iii) hyaluronic acid, (iv) alditol, (v) sodium chloride as a tonicity agent, and (vi) a buffer agent selected from histidine, phosphate and a mixture thereof as a buffer agent, wherein the buffer agent is preferably histidine.

[00159] A particularly preferred liquid formulation of the present invention comprises (i) botulinum toxin at a concentration of 10-200 U/ml, (ii) HSA in an amount of 0.01-1.0% w/v, (iii) hyaluronic acid in an amount of 2 to 10 mg/ml, (iv) alditol in an amount of 10 – 100 mg/ml, (v) sodium chloride in an amount of 0.01-2.0% w/v, preferably 0.9% w/v, and (vi) a buffer agent selected from histidine, phosphate and a mixture thereof at a concentration of 1-100 mM, wherein the buffer agent is preferably histidine at a concentration of 1-100 mM.

[00160] Another particularly preferred liquid formulation of the present invention comprises (i) botulinum toxin at a concentration of 10-200 U/ml, (ii) HSA in an amount of 0.01-0.5% w/v, (iii) hyaluronic acid in an amount of 2 to 10 mg/ml, (iv) an alditol in an amount of 10-100 mg/ml, (v) sodium chloride in an amount of 0.9% w/v, and (vi) a

buffer agent selected from histidine, phosphate and a mixture thereof at a concentration of 1-100 mM, wherein the buffer agent is preferably histidine at a concentration of 1-100 mM.

[00161] Yet another particularly preferred liquid formulation of the present invention comprises (i) botulinum toxin at a concentration of 10-200 U/ml, (ii) HSA in an amount of 0.05-0.25% w/v, (iii) hyaluronic acid in an amount of 2 to 10 mg/ml, (iv) alditol in an amount of 10 – 100 mg/ml, (v) sodium chloride in an amount of 0.9% w/v, and (vi) a buffer agent selected from histidine, phosphate and a mixture thereof at a concentration of 1-100 mM, preferably at a concentration of 2 mM to 50 mM or, more preferably 5 mM to 20 mM, wherein the buffer agent is preferably histidine at a concentration of 1-100 mM, preferably at a concentration of 2 mM to 50 mM, more preferably 5 mM to 20 mM,

[00162] Furthermore, the preferred and particularly preferred liquid formulations describe above have a pH that is preferably in the range of 6.0-7.5. Moreover, the botulinum toxin is preferably of serotype A and, more preferably, is the neurotoxic component of serotype A.

[00163] A preferred exemplary liquid formulation of the present invention is the following:

[00164] Formulation 1:

BoNT/A (without complexing proteins)	50 U/ml
HSA (pretreated with EDTA and dialyzed)	0.85 mg/ml
Non-crosslinked Hyaluronic acid (3.3 MDa)	5 mg /ml
Sorbitol	35 mg/ml
NaCl	0.9% (9 mg/mL)
Sodium phosphate	10mM
pH	6

[00165] The HSA (EDTA-dialyzed) of exemplary Formulation 1 is prepared by an exemplary process including the following steps:

- addition of 37.2 mg Na₂-EDTA per ml of 20% HSA solution with stirring and adjustment to pH 8.0 with NaOH,
- incubation for 6 hours at room temperature with stirring,
- dialysis against 100-fold volume of EDTA buffer (100 mM Na₂-EDTA; 10 mM histidine; 0.9% NaCl pH 7.0) for 16 h at room temperature with gentle stirring on magnetic stirrer (MWCO of dialysis membrane 10 kDa)
- dialysis against 100-fold volume of 10 mM histidine, 0.9% NaCl pH 6.0 for 24 h at room temperature, followed by replacing dialysis buffer with fresh buffer and dialyzing for another 24 h, and repeating two times more.

[00166] In exemplary Formulation 1, EDTA can optionally be replaced by other chelators such as EGTA, BAPTA or DTPA. As an alternative to adding solid EDTA to the HSA solution, a concentrated EDTA solution can be used (e.g., 200 mM EDTA, pH 8.0). The first dialysis step (against EDTA buffer) can also be omitted. Other methods, such as crossflow dialysis or ultrafiltration, can also be used instead of dialysis.

[00167] Another preferred liquid formulation is Formulation 2:

[00168] Formulation 2:

BoNT/A (without complexing proteins)	50 U/ml
HSA (pretreated with EDTA and dialyzed)	0.85 mg/ml
Non-crosslinked Hyaluronic acid	
(3.3 MDa, pretreated with EDTA and dialyzed)	5 mg/ml
Sorbitol	35 mg/ml
NaCl	0.2% (2 mg/ml)
Sodium phosphate	10mM
pH	6

[00169] Another preferred liquid formulation is Formulation 3:

[00170] Formulation 3:

BoNT/A (without complexing proteins)	50 U/ml
HSA (pretreated with EDTA and dialyzed)	0.85 mg/ml
Non-crosslinked Hyaluronic acid (3.3 MDa)	5 mg /ml
Mannitol	70 mg/ml

NaCl	0.2% (2 mg/ml)
Histidine	10mM
pH	6

[00171] The present invention also relates to a liquid formulation comprising a botulinum toxin, wherein the toxin activity is not reduced by more than 20%, relative to the initial toxin activity, upon storage of the liquid formulation for 4 weeks at an elevated temperature of 40°C. Furthermore, the present invention relates to a liquid formulation comprising a botulinum toxin, wherein the toxin activity is not reduced by more than 20%, relative to the initial toxin activity, upon exposure of the liquid formulation for 7 hours to a light source at 250 W/m². Preferably, for the photostability testing, i.e., for the exposure for 7 hours at 250 W/m², a SUNTEST CPS+ instrument (ATLAS Material Testing Technology LLC) can be used, equipped with a filter set to provide a spectral distribution in the wavelength range of 320-800 nm corresponding to the ID65 (indoor indirect daylight standard) per ISO 10977 with a window glass filter according to ICH Q1B.

[00172] Method of making the liquid formulations as defined in the first and second aspect according to a third aspect

[00173] In a third aspect, the present invention further relates to a method for preparing the liquid formulation as defined in the first aspect or any of the embodiments referring thereto, comprising:

mixing components (i) to (iv) and optionally (v) and/or (vi) in a liquid comprising water; and

- (I) a chelating agent; or
- (II) a chelating agent and a metal salt selected from a salt of calcium, magnesium, zinc, and mixtures thereof; or
- (III) wherein the method comprises steps (a) and (c) or steps (b) and (c):
 - (a) contacting human serum albumin with a chelating agent to obtain a mixture of human serum albumin and the chelating agent;
 - (b) contacting said human serum albumin with a chelating agent and a metal salt selected from calcium, magnesium or zinc, or a mixture thereof, to

obtain a mixture of human serum albumin, the chelating agent, and the metal salt; and

- (c) removing the chelating agent from the mixture obtained in step (a) or step (b) by dialysis, filtration, crossflow filtration or ultrafiltration, preferably by dialysis; or

(IV) wherein the method comprises steps (d) and (f) or steps (e) and (f):

- (d) contacting a liquid formulation comprising components (i) to (iv) with a chelating agent to obtain a mixture of said liquid formulation and the chelating agent;
- (e) contacting a liquid formulation comprising components (i) to (iv) with a chelating agent and a metal salt selected from calcium, magnesium or zinc, or mixtures thereof to obtain a mixture of said liquid formulation, the chelating agent, and the metal salt;
- (f) removing the chelating agent from the mixture obtained in step (d) or step (e) by dialysis, filtration, crossflow filtration or ultrafiltration, preferably by dialysis.

[00174] Preferably, wherein the contacting in step (a) or step (b) is carried out by mixing the composition comprising human serum albumin with the chelating agent or the chelating agent and the metal salt or wherein the contacting in step (d) and (e) is carried out by mixing a liquid formulation comprising components (i) to (iv) with the chelating agent or the chelating agent and the metal salt such that the concentration of the chelating agent in the liquid formulation is 1 mM – 500 mM.

[00175] Additionally, the invention relates to a method of making the liquid formulation as defined in the second aspect or any of the embodiments referring thereto, comprising:

mixing components (i) to (iv) and optionally (v) and/or (vi) in a liquid comprising water.

[00176] The preparation of the liquid formulations of the present invention is not particularly limited and the respective formulation techniques are known to those skilled in the art. As described above, the liquid formulation of botulinum toxin is generally an aqueous solution, preferably a saline solution, more preferably a

physiological saline solution, and most preferably a buffered (e.g., phosphate or histidine buffered) physiological saline solution.

[00177] Preferably, the salts are dissolved first, then HSA is added, the pH is adjusted if necessary, and finally the botulinum toxin is added. This order is not a mandatory requirement but is believed to safely preserve maximum specific activity of the BoNT. Preferably, the method for preparing the liquid botulinum toxin formulation does not comprise the reconstitution of a lyophilized botulinum toxin preparation in powder form.

[00178] Use of the liquid formulations as defined in the first and second aspect according to a fourth aspect

[00179] In a fourth aspect, the present invention relates to a liquid formulation of the present invention for use in therapy.

[00180] In particular, the liquid formulation of the present invention may be used in the treatment of neuromuscular diseases, pain, sialorrhea, hyperhidrosis, urological disorders, and neurological disorders. Exemplary neuromuscular diseases include dystonia, spasm, tremor, hyperkinetic movement disorders, and cerebral palsy. The urological disorders include, among others, conditions characterized by detrusor overactivity, overactive bladder, neurogenic bladder and interstitial cystitis, treatment of vulvodynia and chronic pelvic pain, benign prostate hyperplasia (BPH) and detrusor sphincter dyssynergia (DSD). Exemplary neurological disorders include chronic or episodic migraines, cervical dystonia, peripheral neuropathic pain, (post-stroke) spasticity, and blepharospasm.

[00181] Generally, the liquid formulation of the present invention is administered intramuscularly, subcutaneously, subdermally or intradermally.

[00182] Use of the liquid formulations as defined in the first and second aspect according to a fifth aspect

[00183] In a fifth aspect, the present invention relates to a cosmetic use of the liquid formulation of the present invention for the treatment of a cosmetic condition.

[00184] In an embodiment, the use of the present invention may be a cosmetic use, thus may also be a non-therapeutic use. The use of the present invention may be conducted by cosmetics, cosmetic professionals or health care professionals.

[00185] This aspect of the present invention relates to a purely aesthetic use of the liquid formulation of the present invention. Preferred cosmetic conditions to be treated include skin conditions, in particular the treatment of wrinkles of the skin, in particular of the face.

[00186] The term "wrinkles", as used herein, is to be broadly construed to not only include wrinkles, but also lines, rhytids, creases, furrows, and folds. The words "lines", "wrinkles", "rhytids", "creases", and "folds" share similar definition and are therefore often used interchangeably. Within the present invention, "lines" are generally interchangeable with "wrinkles" but may preferably refer to a cutaneous depression that is less deep than a "wrinkle". A "fold" is interchangeable with wrinkles and lines and is preferably a linear depression. A "crease" is interchangeable with wrinkles, lines and folds. It preferably refers to a mild form of wrinkles and may describe the specific wrinkle in certain locations. A "rhytid", as used herein, has essentially the same meaning of wrinkle. However, a "rhytid" preferably refers to a skin structure that is formed by irregular aggregation of lines. A "furrow" is a deep fold or deep line in the skin.

[00187] Preferably, the wrinkles treated in accordance with the present invention are facial wrinkles including horizontal forehead lines, glabellar lines (e.g. glabellar frown lines), periorbital lines, Crow's feet, bunny lines (i.e., downward radiating lines on the sides of nose), nasolabial folds, perioral lip lines, upper radial lip lines, lower radial lip lines, corner of the mouth lines, marionette lines, perioral lip lines, oral commissures, labiomentale crease and cobblestone chin.

[00188] In order to treat the above-mentioned facial wrinkles, botulinum toxin is usually administered by intramuscular injection to the following muscles: frontalis muscle (horizontal forehead lines), procerus and corrugator muscles (glabellar frown lines), lateral orbicularis oculi muscle (Crow's feet/periorbital lines), nasalis, procerus for transverse nasal muscles (bunny lines), levator labii superioris alaeque nasi (nasolabial folds), orbicularis oris (upper and lower radial lip lines), depressor anguli

oris (corner of the mouth lines, marionette lines, oral commissures, labiomental crease), and mentalis muscles (perioral lip lines, cobblestone chin).

[00189] A further preferred cosmetic use of the liquid formulation of the present invention relates to the use for cosmetic applications comprising rejuvenation and/or improvement of the skin quality of the face and/or body.

[00190] In a further preferred embodiment, the rejuvenation and/or improvement of the skin quality of the face and/or body comprises improving and/or reducing and/or filling and/or preventing wrinkles, in particular of wrinkles resulting from muscular activity such as mimic activity, skin smoothing, improving skin laxity, lifting effect, moisturizing and/or softening the skin, improving and/or reducing and/or filling facial lines, soft-tissue augmentation, improving subdermal support of the brows, malar and buccal fat pads, improving tear troughs, improving nose appearance, resolving facial asymmetries, improving jawlines, or a combination of two or more thereof.

[00191] Generally, the liquid formulation of the present invention is administered intramuscularly, subcutaneously, subdermally or intradermally.

[00192] Use of the liquid formulations as defined in the first and second aspect according to a sixth aspect

[00193] In a sixth aspect, the present invention relates to a method of treating a disease or condition, comprising administering an effective amount of the liquid formulation of the present invention to a person in need thereof.

[00194] The disease or condition may be any one of the diseases and conditions mentioned hereinabove, irrespective of whether it is a therapeutic or cosmetic indication. Thus, in one embodiment, the present invention relates to a method of treating a therapeutic condition, comprising administering an effective amount of the liquid formulation of the present invention to a person in need thereof. In another embodiment, the present invention relates to a (non-therapeutic) method of treating a cosmetic (aesthetic) condition, preferably a skin condition, comprising injecting an effective amount of the liquid formulation of the present invention into a person in need thereof.

[00195] The person to be treated is not particularly limited other than by having a disease or condition that can be treated in accordance with the present invention. Those skilled in the art will be able to determine appropriate administration regimens for the treatment of a given therapeutic or cosmetic indication.

[00196] In particular, the injection may be intradermal, subdermal (subcutaneous), or intramuscular, depending on the disease or condition to be treated.

[00197] Further aspects

[00198] Still further, the present invention relates to a use of a human serum albumin (HSA) for increasing the light and/or the temperature stability of a liquid composition comprising a botulinum toxin, wherein the HSA is prepared by a method comprising the following steps:

- (a) contacting a composition comprising human serum albumin with a chelating agent to obtain a mixture of human serum albumin and the chelating agent; or
 - (b) contacting human serum albumin with a chelating agent and a metal salt selected from calcium, magnesium or zinc, and mixtures thereof to obtain a mixture of human serum albumin, the chelating agent, and the metal salt;
- and
- (c) removing the chelating agent from the mixture.

[00199] Yet further, the present invention relates to a human serum albumin (HSA) which is obtainable by

- (a) contacting a composition comprising human serum albumin with a chelating agent to obtain a mixture of human serum albumin and the chelating agent; or
 - (b) contacting human serum albumin with a chelating agent and a metal salt selected from calcium, magnesium or zinc, and mixtures thereof to obtain a mixture of human serum albumin, the chelating agent, and the metal salt;
- and
- (c) removing the chelating agent from the mixture.

[00200] Further, the present invention relates to a method for stabilizing a liquid botulinum toxin formulation, the method comprising combining botulinum toxin with a

human serum albumin (HSA), wherein the HSA is prepared by a method comprising the following steps:

- (a) contacting a composition comprising human serum albumin with a chelating agent to obtain a mixture of human serum albumin and the chelating agent; or
 - (b) contacting human serum albumin with a chelating agent and a metal salt selected from calcium, magnesium or zinc, and mixtures thereof to obtain a mixture of human serum albumin, the chelating agent, and the metal salt;
- and
- (c) removing the chelating agent from the mixture.

EXAMPLES

[00201] The following examples illustrate the liquid botulinum toxin formulation according to the present invention and its preparation method. Percentages are weight by volume (w/v) unless otherwise indicated.

[00202] It will be further understood that the scope embraces the number values provided as commonly rounded values that embrace the whole rounding limits. For example, the scope of “1 mg” embraces the range of from 0.50 to 1.49 mg.

[00203] The number values of the present invention, however, also disclose the more detailed values of one or more orders of magnitude more in detail. Accordingly, for example, “1 mg” may also include the specific disclosure of “1.0 mg”.

[00204] The biological activity of botulinum toxin was determined using a cell-based potency assay (CBA) as described in WO 2014/207109. In brief, neuronal cells were incubated with the neurotoxin containing sample and a reference standard of known potency. After the incubation period, the cells were lysed and the amount of cleaved SNAP25 protein was determined by an immunoassay. The biological activity of the sample was then calculated by comparing the cleavage rate of the cells treated with the sample with those treated with the reference standard.

EXAMPLE 1

Light and storage stability of liquid botulinum toxin formulations containing HSA pre-treated by conventional dialysis (not according to the present invention)

[00205] In initial experiments, it was found that the biological activity of liquid formulations of the 150 kDa botulinum neurotoxin type A free of complexing proteins (also referred to herein as "150kDa BoNT/A") strongly decreased after exposure to light (daylight or room light) and that the light sensitivity increased with increasing human serum albumin (HSA) concentrations. In further experiments, the present inventors unexpectedly found that the light stability and storage stability at 40°C of liquid botulinum toxin formulations could be significantly increased by adding complexing agents such as EDTA, even if the EDTA was present in the form of its magnesium, calcium or zinc complexes (results not shown).

[00206] In view of these findings, it was assumed that an unknown component in HSA caused the photosensitivity of botulinum toxin and that this component can somehow be inactivated or masked by a chelating agent such as EDTA. However, in further experiments carried out by the present inventors, it was surprisingly found that EDTA resulted in increased injection pain. Therefore, in order to dispense with chelating agents such as EDTA, the present inventors have tried to remove the unknown component in HSA that causes light instability by dialysis.

[00207] For this purpose, a concentrated stock solution of HSA (e.g., 20%) was extensively dialyzed at room temperature against a buffer of 10 mM histidine, 0.9% NaCl, pH 6.0, for 4 x 12 hours (three buffer changes, buffer volume about 100 times the sample volume) to obtain a "dialyzed HSA". The dialyzed HSA and the non-dialyzed HSA were then used to prepare the following liquid formulations of botulinum toxin (150 kDa BoNT/A without complexing proteins):

Formulation 1: 65 U/mL botulinum toxin, 0.1% HSA (non-dialyzed), 10 mM histidine, 0.9% NaCl, pH 6.0

Formulation 2: 65 U/mL botulinum toxin, 0.1% HSA (dialyzed), 10 mM histidine, 0.9% NaCl, pH 6.0

[00208] The light stability of these two formulations was determined by measuring the relative botulinum toxin activity after exposure to light for 7 hours at 250 W/m² relative to a control sample stored in the dark. For the exposure to light for 7 hours at 250 W/m², a SUNTEST CPS+ instrument (ATLAS Material Testing Technology LLC) was used, equipped with a filter set to provide a spectral distribution in the wavelength range of 320-800 nm corresponding to the ID65 (indoor indirect daylight standard) per ISO 10977 with a window glass filter according to ICH Q1B. The results are shown in Table 1.

Table 1. Light stability (7 h, 250 W/m²) of liquid botulinum toxin formulations containing non-dialyzed HSA or dialyzed HSA

BoNT/A formulation	Dialysis	Relative BoNT/A activity (% of control)
1	no	46%
2	yes	56%

[00209] As is evident from Table 1, only a limited influence on the light stability was observed for Formulation 2 containing dialyzed HSA compared to Formulation 1 containing non-dialyzed HSA.

[00210] Furthermore, the storage stability of Formulations 1 and 2 was determined by measuring the relative botulinum toxin activity after storage at 40°C for 2 and 4 weeks relative to a control sample at T₀ (measured immediately after preparation of Formulations 1 and 2). The results are shown in Table 2.

Table 2. Storage stability at 40°C of liquid botulinum toxin formulations containing non-dialyzed HSA or dialyzed HSA

BoNT/A formulation	Dialysis	Relative BoNT/A activity (% of control at T ₀)	
		after storage for 2 weeks at 40°C	after storage for 4 weeks at 40°C
1	no	66%	52%

2	yes	65%	51%
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[00211] As can be seen, dialysis has no impact on the storage stability of the botulinum toxin.

EXAMPLE 2

Light and storage stability of liquid botulinum toxin formulations containing HSA pre-treated with chelating agent and dialysis

[00212] Despite the findings from Example 1 (i.e., EDTA or EDTA complexes required for light stability; dialyzed HSA does not result in a significant improvement of light and storage stability), the present inventors have continued to research for a liquid botulinum toxin formulation that is light and storage stable despite being free of a chelating agent such as EDTA.

[00213] First, EDTA was added to a concentrated stock solution of HSA (e.g., 20%) (100 mM = 37.2 mg Na₂-EDTA per ml HSA solution at pH 8.0) and incubated for 6 hours at room temperature under stirring. Then, the incubated mixture was dialyzed against a buffer with EDTA (100 mM EDTA, 10 mM histidine, 0.9% NaCl, pH 7.0) for 16 hours at room temperature. The dialysis membrane used was a membrane with a MWCO of 10 kDa and the fill volume was about 0.2 ml per cm² dialysis membrane. The volume of the dialysis buffer was about 100 times the volume of the sample volume.

[00214] Thereafter, the EDTA was removed by dialysis for 24 hours at room temperature against a volume of EDTA-free dialysis buffer (10 mM histidine, 0.9% NaCl, pH 6.0) of about 100 times that of the sample volume. The dialysis buffer was then replaced by fresh dialysis buffer and again dialyzed for 24 hours. This was repeated two times to obtain the pretreated HSA ("EDTA-dialyzed HSA").

[00215] This pretreated HSA was then used to prepare liquid Formulation 3 of botulinum toxin (150 kDa BoNT/A without complexing proteins):

Formulation 1: identical to Formulation 1 of Example 2 (65 U/mL botulinum toxin, 0.1% HSA (non-dialyzed), 10 mM histidine, 0.9% NaCl, pH 6.0)

Formulation 3: 65 U/mL botulinum toxin, 0.085% HSA (EDTA-dialyzed), 10 mM histidine, 0.9% NaCl, pH 6.0

[00216] The light stability of Formulations 1 and 3 was determined by measuring the relative botulinum toxin activity after exposure to light for 7 hours at 250 W/m² relative to a control sample stored in the dark. The results are shown in Table 3.

Table 3. Light stability (7 h, 250 W/m²) of liquid botulinum toxin formulations containing non-dialyzed HSA or EDTA-dialyzed HSA

BoNT/A formulation	EDTA/Dialysis	Relative BoNT/A activity (% of control)
1	no	46%
3	yes	84%

[00217] As is evident from Table 3, a marked improvement in light stability was observed for Formulation 3 containing EDTA-dialyzed HSA compared to Formulation 1 containing non-pretreated HSA.

[00218] Furthermore, the storage stability was determined by measuring the relative botulinum toxin activity after storage at 40°C for 2 and 4 weeks relative to a control sample at T0 (measured immediately after preparation of Formulations 1 and 3). The results are shown in Table 4.

Table 4. Storage stability at 40°C of liquid botulinum toxin formulations containing non-dialyzed HSA or EDTA-dialyzed HSA

BoNT/A formulation	EDTA-dialysis	Relative BoNT/A activity (% of control at T0)	
		after storage for 2 weeks at 40°C	after storage for 4 weeks at 40°C
1	no	66%	52%
3	yes	94%	82%

[00219] The results show that the use of EDTA-treated and dialyzed HSA results in a significantly higher biological toxin activity compared to Formulation 1 containing untreated (non-dialyzed) HSA.

[00220] Overall, this example shows that the chelating agent (e.g., EDTA) can be unexpectedly omitted from the liquid formulation if the HSA is pre-treated with a chelating agent followed by removal of the chelating agent by, e.g., dialysis. The resulting formulation is not only light and temperature (storage) stable but also exhibits reduced injection pain when injected compared to liquid formulations containing a chelating agent such as EDTA.

EXAMPLE 3

Impact of iron ions on the light stability of liquid botulinum toxin formulations

[00221] Further experiments to investigate the influence of metal ions on light sensitivity were carried out. To this extent, calcium (1 mM Ca^{2+}), cobalt (1 mM Co^{2+}), copper (1 mM Cu^{2+}), nickel (1 mM Ni^{2+}) or iron (1 mM Fe^{3+}) was added to a liquid formulation containing 50 U/ml of 150 kDa BoNT/A, 0.085% pre-treated HSA prepared in accordance with Example 2 (i.e., EDTA-treated and dialyzed HSA), 10 mM histidine, 0.9% NaCl, pH 6.0. Said liquid formulation was prepared with high purity water, histidine and NaCl, and without using iron or steel equipment. The resulting formulations were exposed to light (7 hours at 250 W/m²) and compared to dark-stored control formulations.

[00222] It was found that the addition of calcium (1 mM Ca^{2+}) as well as the addition of cobalt (1 mM Co^{2+}) have no effect on light stability (74% and 77% residual toxin activity compared to the dark-stored control formulations), when compared to the formulation without added calcium and cobalt (79% residual toxin activity compared to the dark-stored control formulation). Addition of copper (1 mM Cu^{2+}) and nickel (1 mM Ni^{2+}) caused a relatively small reduction of light stability. In contrast, addition of iron (1 mM Fe^{3+}) caused a drastic reduction of light stability, with only 1% residual toxin activity remaining after exposure to light (7 hours at 250 W/m²) compared to the dark-stored control formulation (results not shown).

[00223] In view of these results, the influence of Fe^{3+} ions on the light sensitivity of liquid BoNT/A formulations were investigated in more detail in a concentration range between 10 pM and 100 μM . Analogous to the experiments described above, the same formulation comprising pre-treated HSA (50 U/ml botulinum toxin, 0.085% pre-treated HSA, 10 mM histidine, 0.9% NaCl, pH 6.0) was used and the Fe^{3+} concentration was adjusted to the desired final concentration using stock solutions (50 mM $\text{Fe}(\text{NO}_3)_3$). The biological activity measured after exposure to light for 7 hours at 250 W/m^2 , expressed as percentage of the biological activity measured for the respective sample stored in the dark is shown in Table 5.

Table 5. Light stability (7 h, 250 W/m^2) of liquid botulinum toxin formulations containing pre-treated HSA and varying Fe^{3+} concentrations

BoNT/A formulation	$[\text{Fe}^{3+}]$	Relative BoNT/A activity (% of dark control)
1	100 μM	0.4%
2	1 μM	9%
3	316 nM	66%
4	100 nM	82%
5	32 nM	77%
5	10 nM	75%
4	100 pM	74%
5	10 pM	78%
6	0 pM	78%

[00224] As can be seen from Table 5, at a concentration of 316 nM, the Fe^{3+} ions have a significant impact on the BoNT/A stability, with a stability decrease of > 15%. At

a concentration of 1 μM , the light stability is significantly reduced; however, it is still higher than at concentrations above 1 μM . At a concentration of 1 μM and higher, the added Fe^{3+} ions have an extremely negative effect on the stability of BoNT/A in liquid formulations under the influence of light.

[00225] In another experiment, it was found that stirring of the liquid botulinum toxin formulation containing the pre-treated HSA (i.e., 50 U/ml botulinum toxin, 0.085% pre-treated HSA, 10 mM histidine, 0.9% NaCl, pH 6.0) for 48 hours in a stainless-steel beaker resulted in a relative BoNT/A activity after exposure to light (7 hours at 250 W/m^2) of only about 2% compared to 78% obtained for the same sample stirred for 48 hours in a polypropylene vessel. This is a very surprising finding, likely caused by the leaching of minor amounts of Fe^{3+} ions from the stainless-steel beaker into the liquid botulinum toxin formulation and shows that iron ions are an important factor to consider when preparing liquid botulinum toxin formulations.

EXAMPLE 4

Preparation of stable liquid botulinum toxin formulations

[00226] The preparation of the bulk solution of botulinum toxin formulations was performed with reagents and materials that contain or release minimal amounts of metal contaminants (e.g., purified water, polypropylene vessels, PTFE stirrers etc.)

[00227] 5 g sodium hyaluronate (3.3 MDa non-crosslinked) were mixed with 500 ml of sterile, purified water for 48 h to allow swelling and homogenous dissolution.

[00228] The HSA stock solution was pre-treated with EDTA and dialyzed as described in Example 2.

[00229] 2 g sodium chloride, 1.56 g Monosodium phosphate ($\text{NaH}_2\text{PO}_4 \cdot 2 \text{H}_2\text{O}$) and 35 g sorbitol were dissolved in 450 ml purified water and the pH of this solution was adjusted to pH 6.0 with NaOH. Then HSA (pretreated with EDTA and dialyzed) and BoNT/A stock solutions were added (e.g., 4.25 ml 20% HSA and 50 μl BoNT/A 1 MU/ml). Purified water was added to a final volume of 500 ml and the solution was sterile filtered. Finally, the solution was combined with the hyaluronate solution and mixed for 2 h by gentle stirring.

[00230] The bulk solution was filled into 1 ml syringes and stored at 2-8 °C.

CLAIMS

1. A liquid formulation comprising water and the following components:

- (i) botulinum toxin,
- (ii) human serum albumin,
- (iii) hyaluronic acid,
- (iv) alditol;

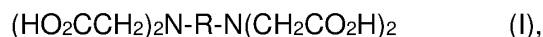
wherein:

- (I) the liquid formulation comprises a chelating agent; or
- (II) the liquid formulation comprises a chelating agent and a metal salt selected from a salt of calcium, magnesium, zinc, and mixtures thereof; or
- (III) said human serum albumin has been subjected to the following process comprising steps (a) and (c) or steps (b) and (c):
 - (a) contacting human serum albumin with a chelating agent to obtain a mixture of human serum albumin and the chelating agent;
 - (b) contacting human serum albumin with a chelating agent and a metal salt selected from calcium, magnesium or zinc, and mixtures thereof to obtain a mixture of human serum albumin, the chelating agent, and the metal salt;
 - (c) removing the chelating agent from the mixture obtained in step (a) or step (b) by dialysis, filtration, crossflow filtration or ultrafiltration, preferably by dialysis; or
- (IV) the liquid formulation has been subjected to the following process comprising steps (d) and (f) or steps (e) and (f):
 - (d) contacting a liquid formulation comprising components (i) to (iv) with a chelating agent to obtain a mixture of said liquid formulation and the chelating agent;
 - (e) contacting a liquid formulation comprising components (i) to (iv) with a chelating agent and a metal salt selected from calcium, magnesium or zinc, or a mixture thereof to obtain a mixture of said liquid formulation, the chelating agent, and the metal salt;

- (f) removing the chelating agent from the mixture obtained in step (d) or step (e) by dialysis, filtration, crossflow filtration or ultrafiltration, preferably by dialysis.
2. A liquid formulation comprising water and the following components:
 - (i) botulinum toxin,
 - (ii) human serum albumin,
 - (iii) hyaluronic acid,
 - (iv) alditol, wherein the alditol is sorbitol.
 3. The liquid formulation of claim 1 or claim 2, further comprising one or both of components (v) and (vi):
 - (v) tonicity agent,
 - (vi) buffer agent.
 4. The liquid formulation of any one of claims 1 to 3, wherein the botulinum toxin is a botulinum neurotoxin complex; or
wherein the botulinum toxin is botulinum neurotoxin free of complexing proteins;
or wherein the botulinum toxin is of serotype A; or
wherein the botulinum toxin is botulinum neurotoxin of serotype A free of complexing proteins; or
wherein the botulinum toxin is of serotype A and is present at a concentration of 1-1000 U/ml; or
wherein the botulinum toxin is botulinum neurotoxin of serotype A free of complexing proteins and is present at a concentration of 1-1000 U/ml.
 5. The liquid formulation of any one of claims 1 to 4, wherein the human serum albumin is present in the liquid formulation at a concentration of 0.001-1.00% w/v.
 6. The liquid formulation of claim 1 or any one of claims 3 to 5, as far as depending on claim 1, wherein the chelating agent is selected from the group consisting of aminopolycarboxylic acids having three to six carboxylic acid functional groups,

citrate, porphyrins, N,N,N',N'-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN), triethylenetetramine (TETA), and mixtures thereof,

preferably wherein the chelating agent is an aminopolycarboxylic acids of general formula (I):



wherein R comprises no carboxylic acid group or one or two carboxylic acid groups, the aminopolycarboxylic acid preferably being selected from the group consisting of ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(β -aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 1,2-bis(α -aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), diethylenetriaminepentaacetic acid (DTPA), triethylenetetraminehexaacetate (TTHA), and mixture thereof; or

wherein the chelating agent is 2,2',2''-nitrilotriacetic acid.

7. The liquid formulation of claim 1 or any one of claims 3 to 6, as far as depending on claim 1, wherein the metal salt is present in the liquid formulation in an amount of 0.01-100 mM; or wherein the metal salt is calcium chloride and is present in the liquid formulation in an amount of 0.01-100 mM.
8. The liquid formulation of any one of claims 1 to 7, wherein the liquid formulation contains Fe^{3+} ions in a concentration of less than 1 μM , preferably less than 500 nM, and more preferably less than 250 nM and/or the concentration of tryptophan and N-acetyl-tryptophan in the liquid formulation is no more than 50 μM , preferably no more than 20 μM , more preferably no more than 10 μM , still more preferably no more than 1 μM , and most preferably 0 μM , of tryptophan and N-acetyltryptophan (total concentration of both Trp and N-AcTrp).
9. The liquid formulation of any one of claims 1 to 8, wherein
 hyaluronic acid is not crosslinked; or
 hyaluronic acid is present at a concentration of 2 to 10 mg/ml and wherein the
 hyaluronic acid is not crosslinked; or

hyaluronic acid is present at a concentration of 2 to 10 mg/ml and wherein hyaluronic acid is crosslinked.

10. The liquid formulation of claim 1 or any one of claims 3 to 9, as far as depending on claim 1, wherein the alditol is selected from glycerol, mannitol, isomalt, lactitol, sorbitol, xylitol, threitol, erythritol and arabitol, preferably from mannitol and sorbitol.
11. The liquid formulation of any one of claims 1 to 10, wherein the alditol is present in a concentration of from 20 to 100 mg/ml.
12. The liquid formulation of any one of claims 3 to 11, wherein
 - (v) the tonicity agent is present in the liquid formulation in an amount of 0.01-2.0% w/v, or wherein the tonicity agent is sodium chloride, or wherein the tonicity agent is sodium chloride and is present in the liquid formulation in an amount of 0.01-2.0% w/v;
 - (vi) the buffer agent is present in the liquid formulation in a concentration of 1-100 mM, or wherein the buffer agent is citric acid, an amino acid, phosphate, or a mixture thereof, or wherein the buffer agent is histidine, or wherein the buffer agent is histidine, phosphate, or a mixture thereof, and the histidine and phosphate are present in the liquid formulation in a concentration of 1-100 mM.
13. The liquid formulation of any one of claims 1 to 12, wherein the pH of the liquid formulation is in the range of from 5.0 to 8, preferably from 5.5 to 6.5.
14. A method of making the liquid formulation as defined in claim 1 or any one of claims 3 to 13, as far as depending on claim 1, comprising:

mixing components (i) to (iv) and optionally (v) and/or (vi) in a liquid comprising water; and

 - (I) a chelating agent; or

- (II) a chelating agent and a metal salt selected from a salt of calcium, magnesium, zinc, and mixtures thereof; or
- (III) wherein the method comprises steps (a) and (c) or steps (b) and (c):
 - (a) contacting human serum albumin with a chelating agent to obtain a mixture of human serum albumin and the chelating agent;
 - (b) contacting said human serum albumin with a chelating agent and a metal salt selected from calcium, magnesium or zinc, or a mixture thereof, to obtain a mixture of human serum albumin, the chelating agent, and the metal salt; and
 - (c) removing the chelating agent from the mixture obtained in step (a) or step (b) by dialysis, filtration, crossflow filtration or ultrafiltration, preferably by dialysis; or
- (IV) wherein the method comprises steps (d) and (f) or steps (e) and (f):
 - (d) contacting a liquid formulation comprising components (i) to (iv) with a chelating agent to obtain a mixture of said liquid formulation and the chelating agent;
 - (e) contacting a liquid formulation comprising components (i) to (iv) with a chelating agent and a metal salt selected from calcium, magnesium or zinc, or mixtures thereof to obtain a mixture of said liquid formulation, the chelating agent, and the metal salt;
 - (f) removing the chelating agent from the mixture obtained in step (d) or step (e) by dialysis, filtration, crossflow filtration or ultrafiltration, preferably by dialysis;

preferably wherein the contacting in step (a) or step (b) is carried out by mixing the composition comprising human serum albumin with the chelating agent or the chelating agent and the metal salt or wherein the contacting in step (d) and (e) is carried out by mixing a liquid formulation comprising components (i) to (iv) with the chelating agent or the chelating agent and the metal salt such that the concentration of the chelating agent in the liquid formulation is 1 mM – 500 mM;

or

a method of making the liquid formulation as defined in claim 2 or any one of claims 3 to 5 and 8 to 13, as far as depending on claim 2, comprising:

mixing components (i) to (iv) and optionally (v) and/or (vi) in a liquid comprising water.

15. The liquid formulation according to any one of claims 1 to 13 for use in therapy, particularly for use in the treatment of neuromuscular diseases, pain, sialorrhea, hyperhidrosis, urological disorders, and neurological disorders; or
the use of the liquid formulation of any one of claims 1 to 13 in a cosmetic application; or
a method of treating a disease or condition, comprising administering an effective amount of the liquid formulation of any one of claims 1 to 13 to a person in need thereof.