LYOPHILIZED FORMULATION OF HGF

TECHNICAL FIELD

[0001]

The present invention relates to a lyophilized formulation comprising hepatic growth factor.

BACKGROUND

[0002]

Hepatic growth factor (HGF) is a bioactive peptide having proliferation activity of hepatic parenchymal cells and has been found in various animal species. In humans, human hepatic growth factor (hHGF) has been found in plasma from patients with fulminant hepatitis (Patent Literature 1), and in recent years, recombinant human hepatic growth factor (rhHGF) can be mass-produced by biotechnology (Non Patent Literature 1). It is expected that these hepatic growth factors may be used as therapeutic and prophylactic agents efficacious for hepatitis and cirrhosis by increasing normal hepatic parenchymal cells and, because of various biological activities thereof, as curative agents for kidney failure and lesions in stomach, duodenum, skin and the like resulting from side effects of anticancer agents.

[0003]

A lyophilized formulation comprising hepatic growth factor has been disclosed in Patent Literature 2, which comprises a hepatic growth factor and a stabilizing agent such as glycine, alanine, sorbitol, mannitol or dextran sulfate.

Patent Literature 3 discloses a lyophilized formulation comprising a hepatic growth factor at a concentration as low as less than 5 mg/mL that is suitable for medicaments, the formulation comprising a stabilizing agent such as arginine, lysine,

histidine, glutamic acid or aspartic acid.

Patent Literature 4 discloses a lyophilized formulation comprising a hepatic growth factor and a stabilizing agent such as purified sucrose.

CITATION LIST

PATENT LITERATURE

[0004]

[PATENT LITERATURE 1] Patent Publication JP-A-S63-22526

[PATENT LITERATURE 2] Patent Publication JP-A-H9-25241

[PATENT LITERATURE 3] WO 2000/072873

[PATENT LITERATURE 4] WO 2008/102849

NON PATENT LITERATURE

[0005]

[NON PATENT LITERATURE 1] Nature, vol.342, p.440-443, 1989

SUMMARY OF THE INVENTION

PROBLEM TO BE SOLVED BY THE INVENTION

[0006]

However, the lyophilized formulations comprising hepatic growth factor disclosed in Patent Publication JP-A-H9-25241, WO 2000/072873 and WO 2008/102849 which aimed to increase stability of hepatic growth factor still fail to provide sufficient storage stability.

In addition, it has not been known how to suppress generation of impurities in conjunction with hepatic growth factor or how to keep the pre-storage isoform ratio in lyophilized formulations comprising hepatic growth factor.

[0007]

Therefore an object of the present invention is to provide a lyophilized formulation having improved storage stability of hepatic growth factor compared to conventional lyophilized formulations.

MEANS FOR SOLVING THE PROBLEM

[0008]

The inventors of the present invention have made intensive studies to achieve the above-described object and, as a result, found that it is possible to achieve the object with a lyophilized formulation comprising (1) a hepatic growth factor, (2) trehalose and (3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof. Thus the inventors have completed the present invention.

[0009]

The present invention provides the following:

[1] A lyophilized formulation comprising (1) a hepatic growth factor, (2) trehalose and (3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof;

[2] A method for producing a lyophilized formulation, comprising the steps of:

(I) prior to addition of (1) a hepatic growth factor, adjusting pH of an aqueous solution comprising at least (3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically

acceptable salts thereof, to pH 4.5 to 6.5, and preparing an aqueous solution comprising (1) the hepatic growth factor, (2) trehalose and (3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof; and

(II) lyophilizing the aqueous solution comprising the hepatic growth factor obtained in the step (I);

[3] A method for stabilizing a hepatic growth factor by comprising (2) trehalose and (3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof in a lyophilized formulation comprising (1) a hepatic growth factor;

[4] A method for stabilizing a hepatic growth factor by comprising (2) trehalose and (3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof in a lyophilized formulation comprising (1) a hepatic growth factor in order to suppress generation of aggregates of the hepatic growth factor, suppress generation of impurities in conjunction with the hepatic growth factor and/or retain a pre-storage isoform ratio;

[5] A method for stabilizing a hepatic growth factor by comprising (2) trehalose and (3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof in a lyophilized formulation comprising (1) a hepatic growth factor in order to suppress

generation of aggregates of the hepatic growth factor;

[6] A method for stabilizing a hepatic growth factor by comprising (2) trehalose and (3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof in a lyophilized formulation comprising (1) a hepatic growth factor in order to suppress generation of impurities in conjunction with the hepatic growth factor;

[7] A method for stabilizing a hepatic growth factor by comprising (2) trehalose and (3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof in a lyophilized formulation comprising (1) a hepatic growth factor in order to retain a pre-storage isoform ratio of the hepatic growth factor; and

[8] The items [1] to [7] as described above, wherein the hepatic growth factor has an amino acid sequence represented by SEQ ID NO: 1.

ADVANTAGEOUS EFFECT

[0010]

The present invention can provide a lyophilized formulation having improved storage stability of hepatic growth factor compared to conventional lyophilized formulations.

BRIEF DESCRIPTION OF DRAWINGS

[0011]

FIG. 1 is a representative chromatogram of impurities in conjunction with hepatic growth factor;

FIG. 2 is a representative chromatogram of isoforms of hepatic growth factor;

FIG. 3 shows the amount of soluble aggregates in lyophilized formulations tested in Test Example 2 initially and after storage at 60°C for 1 month;

FIG. 4 shows the total amount of impurities in lyophilized formulations tested in Test Example 3 initially and after storage at 60°C for 1 month;

FIG. 5 shows the ratio of peak B with respect to isoforms in lyophilized formulations tested in Test Example 4 initially and after storage at 60°C for 1 month;

FIG. 6 shows the correlation between the content of trehalose and the ratio of peak B with respect to isoforms in lyophilized formulations tested in Test Example 4; and

FIG. 7 shows the correlation between the content of arginine and the ratio of peak B with respect to isoforms in lyophilized formulations tested in Test Example 4.

DESCRIPTION OF EMBODIMENTS

[0012]

Embodiments of the present invention are hereinafter specifically described. The present invention is not limited to the embodiments described below and may be variously modified within the scope of the invention.

[0013]

The lyophilized formulation of the present invention comprises (1) a hepatic growth factor, (2) trehalose and (3) one or more compounds (hereinafter also referred to as "the compound (3)") selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof.

A hepatic growth factor for use in the present invention is a protein having the activity of promoting growth of hepatic parenchymal cells and is not particularly limited

as far as it is purified to such extent that it can be used as a medicament. For example, Patent Publication JP-A-H11-56382 may be referred.

[0014]

The hepatic growth factor may be any of those naturally occurring in organ tissue such as liver, spleen and kidney of mammals or in fluidic tissue such as plasma and sera, or those modified having variant amino acid sequences and/or variant carbohydrate chains. Examples of mammals include humans, rats, rabbits, cows, horses, sheep and the like, among which humans are preferred. Examples of the amino acid sequence of human hepatic growth factor include SEQ ID NO: 1.

The hepatic growth factor may be extracted and purified from organ tissue such as liver, spleen and kidney of mammals and from fluidic tissue such as plasma and sera, or isolated from cells producing the hepatic growth factor.

The hepatic growth factor may be obtained by introducing to an appropriate host cell an appropriate vector into which a polynucleotide encoding the hepatic growth factor has been integrated by a genetic engineering technique. The vector is not particularly limited as far as it allows expression of a polynucleotide encoding hepatic growth factor. Examples of the vector include a vector harboring a selection marker that is a gene resistant to antibiotics such as neomycin, a vector harboring a gene encoding dihydrofolate reductase (DHFR) or glutamine synthetase (GS) that can be amplified in animal cells and the like, among which pCI vectors (available from Promega) and pcDNA vectors (available from Invitrogen) are preferred. The host cell is not particularly limited as far as it allows transformation with the vector and expression of a recombinant hepatic growth factor. Examples of the host cell include *Escherichia coli*, *Bacillus subtilis*, yeasts, filamentous fungi, plant cells, insect cells, animal cells and the like, among which animal cells are preferred and Chinese hamster ovary (CHO) cells are particularly preferred.

The hepatic growth factor obtained by a genetic engineering technique may be, for example, a recombinant hepatic growth factor without particular limitation. For example, Patent Publication JP-A-H3-285693 may be referred. The hepatic growth factor used may be, for example, a recombinant human hepatic growth factor (rhHGF) obtained from a host cell which is a CHO cell.

The hepatic growth factor may be a modified hepatic growth factor which is modified by substitution, deletion, addition or insertion or a combination thereof of one or more, preferably 1 to 10 amino acid residues in the amino acid sequence of a naturally occurring hepatic growth factor within the range that the modified amino acid sequence retains the activity of the hepatic growth factor. The modified hepatic growth factor may be the one having, for example, 80%, preferably 85%, more preferably 90% and still more preferably 95% homology to the amino acid sequence of a naturally occurring hepatic growth factor. For example, WO 90/010651, Patent Publication JP-A-H4-030000 and Patent Publication JP-A-H5-111383 may be referred.

[0015]

The concentration of the hepatic growth factor before lyophilization is not particularly limited and may be, for example, 5 mg/mL or less, preferably 3 mg/mL or less and more preferably 1 mg/mL or less.

[0016]

The lyophilized formulation of the present invention is parenterally administered to patients.

When the lyophilized formulation is used for pulmonary administration, the lyophilized formulation of the present invention is dissolved before use and administered to a patient by nebulizer.

When the lyophilized formulation is used for injection, the lyophilized

formulation of the present invention is dissolved before use and intravenously, subcutaneously or intramuscularly administered to a patient.

The concentration of the hepatic growth factor in an aqueous solution obtained by dissolving the lyophilized formulation is not particularly limited and may be, for example, 5 mg/mL or less. Water for dissolving the lyophilized formulation of the present invention is not particularly limited as far as it is water used in medical practice and examples thereof include water for injection, sterilized purified water, saline and the like.

[0017]

Trehalose for use in the present invention is not particularly limited as far as it is purified to a pharmaceutically acceptable extent and examples thereof include trehalose anhydride, trehalose hydrate and the like. Examples of trehalose hydrate include, without particular limitation, trehalose hydrate (trehalose dihydrate) listed in the Japanese Pharmacopoeia 16th edition and the like.

The amount of trehalose added to the lyophilized formulation is such that the mass ratio between the hepatic growth factor and trehalose (hereinafter in this paragraph, expressed as the amount equivalent to trehalose anhydride) is, for example, 1:4 to 1:460, preferably 1:4 to 1:370 and more preferably 1:8 to 1:280. Alternatively, with the total mass of the lyophilized formulation in terms of solid matters being 100.0 parts by mass, trehalose is, for example, 20.0 to 95.0 parts by mass and preferably 30.0 to 94.0 parts by mass. Further, in an aqueous solution comprising 1.0 mg/mL of hepatic growth factor obtained by dissolving the lyophilized formulation, the concentration of trehalose is, for example, 4.0 mg/mL to 460.0 mg/mL and preferably 8.0 mg/mL to 280.0 mg/mL.

[0018]

The compound (3) for use in the present invention is selected from the group

consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof.

The compound (3) is not particularly limited as far as it is purified to a pharmaceutically acceptable extent. One or two or more kinds of the compound (3) may be used.

The compound (3) is preferably, in view of suppression of generation of aggregations or impurities, one or more selected from the group consisting of arginine, histidine, lysine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof.

The compound (3) includes, as a basic substance, arginine, histidine, lysine and meglumine and is preferably one or more selected from arginine, histidine and lysine. The amount of the compound (3) added to the lyophilized formulation can be within the concentration range that allows dissolution of hepatic growth factor without aggregation in an aqueous solution comprising hepatic growth factor used for production of the lyophilized formulation or in an aqueous solution comprising hepatic growth factor obtained by dissolving the lyophilized formulation.

The amount of the compound (3) added to the lyophilized formulation is such that the mass ratio between the hepatic growth factor and the compound (3) (hereinafter in this paragraph, expressed as the amount equivalent to a free form) is, for example, 1:1 to 1:50, preferably 1:1 to 1:40 and more preferably 1:2 to 1:35. Alternatively, with the total mass of the lyophilized formulation in terms of solid matters being 100.0 parts by mass, the amount of the compound (3) is, for example, 1.5 to 60.0 parts by mass and preferably 3.0 to 59.0 parts by mass. Further, in an aqueous solution comprising 1.0 mg/mL of hepatic growth factor obtained by dissolving the lyophilized formulation, the amount of the compound (3) is, for example, 1.0 mg/mL to

50.0 mg/mL, preferably 1.0 mg/mL to 40.0 mg/mL and more preferably 2.0 mg/mL to 35.0 mg/mL.

[0019]

The compound (3) in the present invention may be a pharmaceutically acceptable salt thereof. Examples of the pharmaceutically acceptable salt include a hydrochloride, an acetate salt, a sodium salt, a potassium salt, a magnesium salt and the like.

[0020]

In the present invention, in a lyophilized formulation comprising (1) hepatic growth factor, by further comprising (2) trehalose and (3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof, the storage stability of hepatic growth factor can be improved compared to conventional lyophilized formulations. Storage stability as used herein refers to suppression of generation of aggregates of the hepatic growth factor, suppression of generation of impurities in conjunction with the hepatic growth factor and/or retention of the pre-storage isoform ratio.

The present invention provides a method for stabilizing a hepatic growth factor by comprising (2) trehalose and (3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof in a lyophilized formulation comprising (1) hepatic growth factor.

Namely the present invention provides a method for stabilizing a hepatic growth factor by comprising (2) trehalose and (3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid,

aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof in a lyophilized formulation comprising (1) hepatic growth factor in order to suppress generation of aggregates of the hepatic growth factor.

The present invention also provides a method for stabilizing a hepatic growth factor by comprising (2) trehalose and (3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof in order to suppress generation of impurities in a lyophilized formulation comprising (1) hepatic growth factor in conjunction with the hepatic growth factor.

The present invention further provides a method for stabilizing a hepatic growth factor by comprising (2) trehalose and (3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof in a lyophilized formulation comprising (1) hepatic growth factor in order to retain a pre-storage isoform ratio of the hepatic growth factor.

[0021]

The term "aggregates" as used herein refers to aggregates of hepatic growth factor and examples thereof include soluble aggregates such as dimers and trimers and insoluble aggregates generated by means of interactions.

In the present invention, the amount of aggregates can be evaluated by, for example, measuring the amount of soluble aggregates by size exclusion chromatography.

In the present invention the amount of the soluble aggregates is, when, for example, the amount of aggregates is evaluated for a lyophilized formulation after

storage at 60°C for 1 month by size exclusion chromatography, e.g. less than 1.4% and preferably 1.0% or less. Alternatively, the amount of aggregates measured for a lyophilized formulation after storage at 60°C for 1 month by size exclusion chromatography is, for example, 4.6 times or less, preferably 4 times or less, more preferably 3.5 times or less and still more preferably 3 times or less of the amount of aggregates before storage.

The amount of insoluble aggregates may be evaluated by, for relatively large insoluble aggregates with the size of 1 μm or more, the light obscuration method in the insoluble particulate matter test described in the Japanese Pharmacopoeia 16th edition or the micro flow imaging method and for insoluble aggregates with the size of 1 μm or less, by the light scattering method or the ultracentrifugation method.

[0022]

The term "impurity" or "impurities" as used herein refers to one or more substances corresponding to the one or more peaks observed in reverse phase chromatography other than the peak of the hepatic growth factor.

The amount of impurities is evaluated by reverse phase chromatography. For example, for the lyophilized formulations used in Examples in the present specification, two peaks (hereinafter respectively referred to as "ImpA" and "ImpB") at the retention time of around 10 minutes and other impurities than ImpA and ImpB (hereinafter referred to as "Others imp") at the retention time of around 6 to 7 minutes were observed as shown in FIG. 1.

In the present invention the amount of impurities is, when, for example, the amount of impurities is evaluated for a lyophilized formulation after storage at 60°C for 1 month by reverse phase chromatography, e.g. 1.7% or less, preferably 1.5% or less, more preferably 1.3% or less and still more preferably 1.0% or less. Alternatively, the amount of impurities measured for a lyophilized formulation after storage at 60°C for 1

month by reverse phase chromatography is, for example, less than 9 times, more preferably 7 times or less, more preferably 5 times or less and still more preferably 3 times or less of the amount of impurities before storage.

[0023]

The term "isoform" as used herein refers to a charge variant of hepatic growth factor and refers to a plurality of proteins having different charge state.

The isoform ratio can be evaluated, for example, by ion exchange chromatography. For example, for the lyophilized formulations used in Examples in the present specification, two peaks (hereinafter respectively referred to as "peak A" and "peak B") were observed at 35 to 45 minutes of retention time as shown in FIG. 2. The term "isoform ratio" as used herein refers to the proportion of each isoform with respect to the total amount of isoforms.

[0024]

Biological activity of hepatic growth factor in the present invention can be evaluated according to a cell-based assay which evaluates proliferation of mink lung epithelial cells (Mv.1.Lu cells) or 4BMr5 cells in the presence of transforming growth factor β-1 (TGFβ-1) (see Journal of Immnological Methods. vol. 258, p. 1-11, 2001 and the like) and can be expressed as the 50% effective concentration (EC50). The change in the biological activity of the lyophilized formulation during storage can be expressed as the relative titer (%) obtained by dividing EC50 before storage by EC50 after storage.

[0025]

In order to keep constant pH of an aqueous solution comprising hepatic growth factor used for production of the lyophilized formulation or of an aqueous solution comprising hepatic growth factor obtained by dissolving the lyophilized formulation, the lyophilized formulation may comprise a buffering agent.

The buffering agent for use in the present invention is not particularly limited as far as it is pharmaceutically acceptable and examples thereof include phosphoric acid, sodium dihydrogen phosphate, sodium dihydrogen phosphate monohydrate, citric acid hydrates, sodium citrate hydrates, disodium citrate, acetic acid, glacial acetic acid, sodium acetate hydrates, sodium carbonate hydrates, sodium hydrogen carbonate, glycine, glycylglycine and the like.

The buffering agent may be used in the form of a buffer solution which is generally used such as phosphate buffer solution, citric acid buffer solution and acetic acid buffer solution.

The amount of the buffering agent in the lyophilized formulation is, in terms of the concentration in an aqueous solution comprising hepatic growth factor before lyophilization, e.g. 1 to 100 mM. By adjusting the concentration to this range, constant pH of the aqueous solution comprising hepatic growth factor obtained by dissolving the lyophilized formulation can be achieved.

[0026]

Hepatic growth factor has varied solubility depending on pH, with the solubility being at a minimum at around the isoelectric point. Therefore pH may be selected, while taking the isoelectric point into account, so that hepatic growth factor can be dissolved without aggregation in an aqueous solution comprising hepatic growth factor used for production of the lyophilized formulation or in an aqueous solution comprising hepatic growth factor obtained by dissolving the lyophilized formulation.

In the present invention, the hepatic growth factor used in Examples in the present specification for example has the minimal solubility of 0.1 to 0.2 mg/mL at around neutral pH which is the isoelectric point and has the solubility of 1.6 mg/mL or higher at around pH 5. Therefore an aqueous solution before lyophilization and an aqueous solution obtained by dissolving the lyophilized formulation has pH of, for

example, 4.5 to 6.5 and preferably 5.0 to 6.0.

In the method for producing a lyophilized formulation of hepatic growth factor of the present invention, a pH adjusting agent other than the buffering agent may be used in order to adjust pH of an aqueous solution before lyophilization.

[0027]

The pH adjusting agent for use in the present invention is not particularly limited as far as it is pharmaceutically acceptable and examples thereof include hydrochloric acid, anhydrous citric acid, citric acid hydrates, sodium citrate hydrates, glycine, succinic acid, acetic acid, glacial acetic acid, sodium acetate hydrates, tartaric acid, sodium hydroxide, sodium hydrogen carbonate, sodium carbonate hydrates, dried sodium carbonate, monoethanolamine, triethanolamine, lactic acid, sodium lactate, phosphoric acid, sodium hydrogen phosphate hydrates, anhydrous sodium monohydrogen phosphate, sodium dihydrogen phosphate, anhydrous sodium dihydrogen phosphate, crystalline sodium dihydrogen phosphate, trisodium phosphate, dipotassium phosphate, potassium dihydrogen phosphate and the like.

[0028]

In order to prevent reduction of the content of hepatic growth factor in a drug solution to be administered due to adsorption of the hepatic growth factor to glass or resin constituting a container after reconstitution of the lyophilized formulation, the lyophilized formulation may comprise a surfactant.

The surfactant for use in the present invention is not particularly limited as far as it is pharmaceutically acceptable and examples thereof include nonionic surfactants specifically including polysorbate 80, polysorbate 20, poloxamers, polyethylene glycols, HCO-40, HCO-60 and the like.

The amount of the surfactant in the lyophilized formulation is, in an aqueous solution comprising 1.0 mg/mL of hepatic growth factor obtained by dissolving the

lyophilized formulation, e.g. 0.020 to 1.0 mg/mL, preferably 0.050 to 1.0 mg/mL, more preferably 0.075 to 0.5 mg/mL and still more preferably 0.075 to 0.2 mg/mL.

[0029]

The lyophilized formulation of the present invention comprises, with the total mass of the lyophilized formulation in terms of solid matters being 100.0 parts by mass, 0.1 to 4.5 parts by mass of the hepatic growth factor, 20.0 to 95.0 parts by mass of trehalose (hereinafter in this paragraph, expressed as the amount equivalent to trehalose anhydride) and 1.5 to 60.0 parts by mass of the compound (3) (hereinafter in this paragraph, expressed as the amount equivalent to a free form). In one embodiment, with the total mass of the lyophilized formulation in terms of solid matters being 100.0 parts by mass, the hepatic growth factor accounts for 0.3 to 4.4 parts by mass, trehalose accounts for 30.0 to 94.0 parts by mass and the compound (3) accounts for 3.0 to 59.0 parts by mass. In another embodiment, hepatic growth factor accounts for 0.3 to 4.4 parts by mass, trehalose accounts for 30.0 to 94.0 parts by mass, the compound (3) accounts for 3.0 to 59.0 parts by mass and polysorbate 80 accounts for 0.026 to 0.7 parts by mass.

[0030]

In the present invention, the lyophilized formulation may further comprise, if necessary, an additive such as a vehicle, a tonicity agent and an antioxidant.

[0031]

A method for producing a lyophilized formulation of the present invention comprises the steps of:

(I) prior to addition of (1) a hepatic growth factor, adjusting pH of an aqueous solution comprising at least (3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically

acceptable salts thereof, to pH 4.5 to 6.5, and preparing an aqueous solution comprising (1) the hepatic growth factor, (2) trehalose and (3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof; and

(II) lyophilizing the aqueous solution comprising the hepatic growth factor obtained in the step (I).

The lyophilized formulation of the present invention can be produced by the production method.

[0032]

In the step (I), pH of an aqueous solution comprising at least (3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof is adjusted to pH 4.5 to 6.5 and preferably to pH 5.0 to 6.0.

Prior to adjusting pH, the aqueous solution may comprise (2) trehalose but does not comprise (1) hepatic growth factor. Namely after adjusting pH of the aqueous solution comprising at least the compound (3) to pH 4.5 to 6.5, (1) hepatic growth factor is added thereto or (1) hepatic growth factor and (2) trehalose are simultaneously or sequentially added thereto.

In order to adjust pH, a buffering agent and/or a pH adjusting agent can be used. Reaction temperature during adjusting pH is not particularly limited.

The aqueous solution comprising at least the compound (3) can be prepared by dissolving the compound (3) in a solvent.

The solvent for preparation of the aqueous solution comprising at least the

compound (3) is not particularly limited as far as it is, for example, pharmaceutically acceptable water. Examples thereof include water for injection, sterilized purified water and the like.

[0033]

(1) Hepatic growth factor and (2) trehalose may be added as they are or in the form of aqueous solution. A solvent for preparing an aqueous solution may be the same as or different from the solvent for preparation of the aqueous solution comprising at least the compound (3). Temperature during addition of (1) hepatic growth factor and (2) trehalose is not particularly limited.

[0034]

In the step (I), a surfactant, other additives (a vehicle, a tonicity agent, an antioxidant and the like) and the like may be added, if necessary.

[0035]

In the step (II), the aqueous solution comprising the hepatic growth factor obtained in the step (I) is lyophilized.

The aqueous solution comprising the hepatic growth factor can be lyophilized according to a common method after pouring the aqueous solution comprising the hepatic growth factor into a vial or an ampoule.

It is preferred that the aqueous solution comprising the hepatic growth factor is sterilized by filtration through a filter or the like and lyophilized.

Examples of the lyophilization method include a method comprising three unit operations of a freezing step for cooling and freezing under normal pressure, a primary drying step for sublimation and drying of free water which is not engaged to solutes under reduced pressure and a secondary drying step for removing adsorbed water and crystallization water inherent to the solutes (for reference, Pharm. Tech. Japan, vol.8, no.1, p.75-87, 1992 and the like may be mentioned).

The temperature during the freezing step is, for example, -40°C or less. The temperature during the primary drying step is, for example, -20°C to 10°C. The temperature during the secondary drying step is, for example, 20°C to 30°C. The reduced pressure is, for example, 10 Pa or less.

[0036]

The term "cake" as used herein refers to a porous solid matter in the lyophilized formulation. Characteristics of the lyophilized formulation can be evaluated by visual observation of the porous structure or color of a cake or solubility of the lyophilized formulation in water or visual observation of the thus obtained aqueous solution.

The lyophilized formulation of the present invention is generally an acceptably white cake and is rapidly dissolved in water to give a clear and colorless aqueous solution.

On the other hand, an aqueous solution obtained by dissolving a conventional lyophilized formulation may be turbid potentially due to generation of insoluble aggregates which may be derived from hepatic growth factor and the like. Thus the characteristics of the aqueous solution obtained by dissolving the lyophilized formulation may be indicative of generation of insoluble aggregates. The fact that the aqueous solution is clear after storage of the lyophilized formulation means that generation of insoluble aggregates is suppressed.

EXAMPLES

[0037]

The present invention is hereinafter more specifically described by way of Examples which do not limit the present invention.

[0038]

Hepatic growth factor used in the present Examples was prepared by fully synthesizing a gene on the basis of amino acid sequence information disclosed in Patent Publication JP-A-H11-056382, incorporating the gene in a vector, expressing the protein in CHO cells and isolating and purifying the protein.

[0039]

(Examples 1 to 25 and Comparative Examples 1 to 7)

Lyophilized formulations of Examples and Comparative Examples were prepared by the method described hereinbelow. Tables 1 to 4 indicate the amounts (mg) of components per mL of each aqueous solution comprising hepatic growth factor. The values in Tables are rounded to one decimal place. The aqueous solutions comprising hepatic growth factor were prepared in the volume ranging from 100 mL to 250 mL.

[0040]

Table 1

	Examples				Comparative Examples					
	1	2	3	4	1	2	3	4	5	6
Hepatic growth factor	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.1
Trehalose dihydrate	100.0	100.0	100.0	100.0	-	-	-	-	-	-
Xylitol	-	-	-	-	100.0	-	-	-	-	-
Sucrose	-	-	-	-	-	100.0	-	-	-	10.0
Sorbitol	-	-	-	-	-	-	100.0	-	-	-
Mannitol	-	-	-	-	-	-	-	100.0	-	-
L-arginine	17.4	-	-	-	17.4	17.4	17.4	17.4	17.4	-
L-histidine	-	15.5	-	-	-	-	-	-	-	-
L-lysine	-	-	14.6	-	-	-	-	-	-	-
Meglumine	-	-	-	19.5	-	-	-	-	-	-
L-alanine	-	-	-	-	-	-	-	-	-	5.0
Sodium chloride	-	-	-	-	-	-	-	-	9.0	18.0

Citric acid monohydrate	2.1	
Polysorbate 80	0.1	0.3

[0041]

Table 2

	Examples						
	5	6	7	8	9	10	11
Hepatic growth factor	1.1	1.1	1.1	1.1	1.1	1.1	1.1
Trehalose dihydrate	100.0						
L-arginine	4.2	-	-	-	-	-	-
L-arginine hydrochloride	16.0	-	-	-	-	-	-
Sodium glutamate	-	18.7	-	-	-	-	-
Sodium aspartate	-	-	17.7	-	-	-	-
Proline	-	-	-	11.5	-	-	-
Creatine	-	-	-	-	14.9	-	-
Creatinine	-	-	-	-	-	11.3	-
Tris(hydroxymethyl)aminomethane	-	-	-	-	-	-	12.1
Citric acid monohydrate	2.1						
Polysorbate 80	0.1						

[0042]

Table 3

	Examples						
	12	13	14	15	16	17	18
Hepatic growth factor	0.5	1.0	3.1	5.1	1.1	1.0	1.0
Trehalose dihydrate	100.0	100.0	100.0	100.0	10.0	25.0	50.0
L-arginine	5.5	6.8	12.1	17.4	4.2	4.2	4.2
L-arginine hydrochloride	14.4	12.8	6.4	-	16.0	16.0	16.0
Citric acid monohydrate	2.1						
Polysorbate 80	0.1						

[0043]

Table 4

	Examples							Comparative Example 7
	19	20	21	22	23	24	25	
Hepatic growth factor	1.0	1.0	1.1	1.1	1.1	1.1	1.1	1.1
Trehalose dihydrate	150.0	200.0	250.0	300.0	100.0	100.0	100.0	-
L-arginine	4.2	4.2	4.2	4.2	3.5	8.7	26.1	4.2
L-arginine hydrochloride	16.0	16.0	16.0	16.0	-	-	-	16.0
Citric acid monohydrate	2.1							
Polysorbate 80	0.1							

[0044]

<Preparation of mixing buffer>

According to the volume of each aqueous solution comprising hepatic growth factor and the concentration indicated in Tables 1 to 4, components other than hepatic growth factor or polysorbate 80 were mixed and stirred with an appropriate amount of water for injection (about 80% of the volume of the aqueous solution comprising hepatic growth factor). After visually confirming the dissolution, the pH was adjusted to 5.5 with hydrochloric acid and/or sodium hydroxide and the volume was adjusted with water for injection to give a mixing buffer.

<Preparation of aqueous solution comprising hepatic growth factor>

An aqueous solution comprising hepatic growth factor was prepared by the following procedure.

(a) An aqueous solution comprising hepatic growth factor was adjusted so as to have the concentration of the hepatic growth factor as indicated in Table 1 by ultrafiltration using the above mixing buffer. The concentration of hepatic growth factor was calculated from the following formula (A) after determining the absorbance at 280 nm on an ultraviolet-visible spectrophotometer (using a cell with a light path of 1 cm):

$$\text{Protein concentration (mg/mL)} = (A280/1.73) \times D \qquad (A)$$

wherein:

A280: absorbance at 280 nm;

1.73: theoretical absorbance of a sample solution with 0.1% concentration with the light path of 1 cm and at 280 nm; and

D: dilution factor upon measurement.

(b) Thereafter polysorbate 80 was added so as to obtain the desired concentration as indicated in Table 1 followed by filtration through a sterile filter

(product name: MILLEX GV 0.22 μm filter, available from Millipore) to give the aqueous solution comprising hepatic growth factor.

<Lyophilization step>

The aqueous solution comprising hepatic growth factor was divided at 1 mL into 5-mL glass vials which were then partially closed with rubber caps. After lyophilization according to the program indicated in Table 5, each vial was completely closed with the rubber cap. An aluminum cap was then attached to the glass vial to give a lyophilized formulation.

Comparative Example 5 is a lyophilized formulation produced according to the formulation disclosed in WO 2000/072873. Comparative Example 6 is a lyophilized formulation produced according to the formulation disclosed in WO 2008/102849.

[0045]

Table 5

	Freezing	Hold freezing	Pressure reduction	Heating	Primary drying	Heating	Secondary drying
Temperature (°C)	Room temp. → -40	-40	-40	-40 → -20	-20	-20 → 20	20
Pressure (Pa)	Normal pressure	Normal pressure	Normal pressure → 10	10	10	10	10
Time (h)	1.5	4	1	2	48	8	48

[0046]

(Test Example 1)

The obtained lyophilized formulations of Examples 1 to 11 and Comparative Examples 1 to 6 were examined for characteristics of the lyophilized formulations, solubility of the lyophilized formulations in water for injection and characteristics of the aqueous solutions obtained after dissolution according to the following procedures.

<Method>

Each obtained lyophilized formulation was visually observed (formation of cake and color hue) in white and black backgrounds in an environment with 1000 to 3000 lux. Water for injection (1 mL) was then added to each lyophilized formulation which was then left to stand before visual observation of solubility of the lyophilized formulation and color of the obtained solution.

The term "instant" indicated in Table 6 refers to that the formulation was completely dissolved within 1 minute and the term "slightly soluble" refers to that the formulation was not completely dissolved even after 1 minute.

[0047]

<Results>

The results for the samples of Examples 1 to 4 and Comparative Examples 1 to 6 are shown in Table 6. Examples 1 to 4 formed white cakes and showed preferable solubility. Comparative Example 1 comprising xylitol and Comparative Example 3 comprising sorbitol did not form cakes and were white or colorless molten solids. Comparative Example 5 which did not comprise a saccharide was a white solid without formation of a cake that is generally observed for lyophilized formulations. Examples 5 to 11 also formed white cakes similarly to Examples 1 to 4 and showed preferable solubility.

[0048]

Table 6

	Formation of cake	Color of cake	Solubility	Color of solution
Example 1	Good	White	Instant	Clear
Example 2	Good	White	Instant	Clear
Example 3	Good	White	Instant	Clear
Example 4	Good	White	Instant	Clear
Comparative Example 1	No	Colorless	Slightly soluble	Clear
Comparative Example 2	Good	White	Instant	Clear
Comparative Example 3	No	White	Slightly soluble	Clear
Comparative Example 4	Good	White	Instant	Clear
Comparative Example 5	No	White	Instant	Clear
Comparative Example 6	Good	White	Instant	Clear

[0049]

The obtained lyophilized formulations were stored in a thermostatic chamber at 25°C, 40°C or 60°C for 2 weeks or 1 month followed by the following tests.

[0050]

(Test Example 2)

The amount of soluble aggregates in Examples 1 to 25 and Comparative Examples 1 to 7 was evaluated as follows.

<Method>

Water for injection (1 mL) was added to each lyophilized formulation of Example and Comparative Example for dissolution under ice cooling. The solution was moderately shaken to obtain a uniform solution which was then left to stand for 5 minutes. The obtained solution was distributed into 250-μL vial inserts which were placed in HPLC vials to obtain samples.

Each sample was measured by size exclusion chromatography under the following analytical conditions for the amount of soluble aggregates before and after storage and the amount of soluble aggregates was calculated by the area percentage method.

Detector: UV 280 nm;

Column: guard column (trade name: TSKgel G3000SWXL, available from Tosoh Corporation);

Column temperature: 30°C;

Mobile phase: obtained by dissolving 39 g of sodium dihydrogen phosphate dihydrate, 87.5 g of sodium chloride and 5.0 g of sodium lauryl sulfate in 5000 mL of water and adjusting pH of the solution to 7.5 with a 2 mol/L sodium hydroxide solution;

Sample injection: 50 μL; and

Flow rate: 1 mL/min.

[0051]

<Results>

The results of measurement of the amount of soluble aggregates in samples initially (before storage) and after storage at 25°C, 40°C or 60°C for 2 weeks or 1 month are shown in Tables 7 to 9. The results of measurement of the amount of soluble aggregates in samples of Examples 1 to 4 and Comparative Examples 1 to 6 initially and after storage at 60°C for 1 month are shown in FIG. 3. Further, the correlation between the content of trehalose in lyophilized formulations and the amount of soluble aggregates is shown in Table 10.

[0052]

According to Table 7, the amount of soluble aggregates in Comparative Examples 1 to 6 was increased with an increase in the storage period and storage temperature. The amount of soluble aggregates was significantly increased after storage at 60°C for 1 month.

According to Tables 7 and 8 meanwhile, such a significant change in the amount of soluble aggregates as in Comparative Examples was not observed for Examples 1 to 11 under the storage conditions tested. Thus generation of soluble aggregates was suppressed in Examples 1 to 11 even under the storage condition of 60°C.

[0053]

According to Tables 9 and 10, the amount of soluble aggregates was significantly increased in Comparative Example 7 which did not comprise trehalose.

Meanwhile no significant change in the amount of soluble aggregates was observed for Examples 12 to 15 comprising various amounts of hepatic growth factor and Examples 23 to 25 comprising various amounts of arginine.

[0054]

Table 7

	Amount of aggregates (%)						
	Initial	2-week storage			1-month storage		
		25°C storage	40°C storage	60°C storage	25°C storage	40°C storage	60°C storage
Example 1	0.3	0.2	0.2	0.2	0.2	0.2	0.3
Example 2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Example 3	0.3	0.4	0.4	0.4	0.3	0.3	0.4
Example 4	0.3	0.3	0.3	0.3	0.3	0.3	0.8
Comparative Example 1	0.3	0.6	1.1	26.4	0.8	1.6	44.1
Comparative Example 2	0.3	0.3	0.3	0.6	0.3	0.3	1.4
Comparative Example 3	0.3	0.3	0.7	11.5	0.4	0.9	21.5
Comparative Example 4	0.3	0.3	0.3	1.9	0.3	0.4	5.6
Comparative Example 5	0.3	0.3	0.4	1.9	0.4	0.4	3.1
Comparative Example 6	0.4	0.8	1.1	6.8	1.0	1.4	18.4

[0055]

Table 8

	Amount of aggregates (%)	
	Initial	60°C/2-week storage
Example 5	0.2	0.2
Example 6	0.2	0.3
Example 7	0.3	0.3
Example 8	0.2	0.2
Example 9	0.2	0.3
Example 10	0.2	0.2
Example 11	0.2	0.3

[0056]

Table 9

	Amount of aggregates (%)			
	Initial	1-month storage		
		25°C storage	40°C storage	60°C storage
Example 12	0.3	0.2	0.2	0.2
Example 13	0.2	0.2	0.2	0.2
Example 14	0.2	0.3	0.2	0.2
Example 15	0.2	0.2	0.3	0.3
Example 16	0.2	0.2	0.2	0.6
Example 17	0.2	0.2	0.2	0.4
Example 18	0.2	0.2	0.3	0.2
Example 19	0.2	0.3	0.2	0.3
Example 20	0.2	0.2	0.2	0.2
Example 21	0.2	0.2	0.2	0.2
Example 22	0.2	0.2	0.2	0.2
Example 23	0.2	0.2	0.3	0.3
Example 24	0.2	0.3	0.2	0.3
Example 25	0.3	0.2	0.2	0.2
Comparative Example 7	0.2	0.2	0.2	1.5

[0057]

Table 10

	Trehalose conc. (mg/mL)	Amount of aggregates (%)			
		Initial	1-month storage		
			25°C storage	40°C storage	60°C storage
Comparative Example 7	0	0.2	0.2	0.2	1.5
Example 16	10	0.2	0.2	0.2	0.6
Example 17	25	0.2	0.2	0.2	0.4
Example 18	50	0.2	0.2	0.3	0.2
Example 13	100	0.2	0.2	0.2	0.2
Example 19	150	0.2	0.3	0.2	0.3
Example 20	200	0.2	0.2	0.2	0.2
Example 21	250	0.2	0.2	0.2	0.2
Example 22	300	0.2	0.2	0.2	0.2

[0058]

(Test Example 3)

The amount of impurities in Examples 1 to 25 and Comparative Examples 1 to 7 was evaluated as follows.

<Method>

The samples used were those in HPLC vials prepared for evaluation of the amount of soluble aggregates.

Each sample was measured by reverse phase chromatography under the following analytical conditions for the amount of impurities before and after storage and the amount of impurities was calculated by the area percentage method.

Detector: UV 215 nm;

Column: reverse phase column (trade name: Inertsil WP300-C8, available from GL Sciences Inc.) attached with the GL Cart guard column;

Column temperature: 40°C;

Mobile phase A: water/trifluoroacetic acid (1000:1);

Mobile phase B: acetonitrile/trifluoroacetic acid (1000:0.85);

Sample injection: 25 μL;

Flow rate: 1 mL/min; and

Gradient: the volume ratio of the mobile phase B with respect to the total amount of the mobile phase A and the mobile phase B: 15% (Initial) - 70% (27.5 min) - 70% (30 min) - 15% (30.01 min) - 15% (35 min).

[0059]

<Results>

The results of measurement of the amount of impurities in samples of Examples 1 to 11 and Comparative Examples 1 to 6 initially (before storage) and after storage at 25°C, 40°C or 60°C for 2 weeks or 1 month are shown in Tables 11 and 12.

The results of measurement of the total amount of impurities in samples of Examples 1 to 4 and Comparative Examples 1 to 6 initially and after storage at 60°C for 1 month are shown in FIG. 4.

[0060]

According to Table 11, the total amount of impurities in Examples 1 to 4 was not substantially changed under the storage conditions of 25°C and 40°C. According to Tables 11 and 12, an increase in the total amount of impurities in Examples 1 to 11 was at most 2-fold relative to the initial value even under the storage condition of 60°C, while an increase in the amount in formulations of all Comparative Examples was almost 10-fold or more. Under the storage condition of 60°C, it was found that any of ImpA, ImpB and Others imp was increased in formulations of all Comparative Examples. Thus generation of impurities was suppressed in Examples 1 to 11 even under the storage condition of 60°C.

In Examples 12 to 25, no significant change in the total amount of impurities was observed under any storage conditions.

[0061]

Table 11

	Total amount of impurities (%)						
	Initial	2-week storage			1-month storage		
		25°C storage	40°C storage	60°C storage	25°C storage	40°C storage	60°C storage
Example 1	0.18	0.19	0.19	0.36	0.20	0.20	0.32
Example 2	0.31	0.33	0.34	0.55	0.33	0.35	0.55
Example 3	0.20	0.28	0.21	0.41	0.61	0.23	0.32
Example 4	0.19	0.20	0.21	0.42	0.20	0.20	0.47
Comparative Example 1	0.23	0.51	0.74	2.34	0.38	0.50	4.31
Comparative Example 2	0.20	0.19	0.68	0.95	0.19	0.21	2.25
Comparative Example 3	0.19	0.21	0.31	1.42	0.28	0.48	2.40
Comparative Example 4	0.19	0.20	0.48	0.89	0.29	0.31	1.71
Comparative Example 5	0.22	0.35	0.43	1.02	0.23	0.49	2.69
Comparative Example 6	0.91	1.58	1.75	2.86	1.84	2.11	3.56

[0062]

Table 12

	Total amount of impurities (%)	
	Initial	60°C/2-week storage
Example 5	0.21	0.38
Example 6	0.20	0.41
Example 7	0.21	0.34
Example 8	0.21	0.36
Example 9	0.20	0.38
Example 10	0.21	0.39
Example 11	0.20	0.39

[0063]

(Test Example 4)

The isoform ratio in Examples 1 to 25 and Comparative Examples 1 to 7 was evaluated as follows.

<Method>

The samples used were those in HPLC vials prepared for evaluation of the amount of aggregates.

Each sample was measured by ion exchange chromatography under the following analytical conditions for the amount of isoforms before and after storage and the isoform ratio was calculated by the area percentage method.

Detector: UV 215 nm;

Column: trade name: Agilent Bio SCX NP5 PK, available from Agilent Technologies;

Column temperature: 30°C;

Mobile phase A: 50 mmol/L Tris-HCl buffer (pH 9.0);

Mobile phase B: 50 mmol/L Tris-HCl buffer (pH 9.0), 1 mol/L NaCl

Sample injection: 50 μL;

Flow rate: 0.5 mL/min; and

Gradient: the volume ratio of the mobile phase B with respect to the total amount of the mobile phase A and the mobile phase B: 20% (Initial) - 60% (50 min) - 80% (51 min) - 80% (60 min) - 20% (60.01 min) - 20% (80 min).

[0064]

<Results>

The results of measurement of change in the amount of isoforms expressed as the ratio of peak B with respect to the sum of peak A and peak B in samples initially (before storage) and after storage at 25°C, 40°C or 60°C for 2 weeks or 1 month are shown in Tables 13 to 15. The results of measurement of change in the isoform ratio

expressed as the ratio of peak B in samples of Examples 1 to 4 and Comparative Examples 1 to 6 initially and after storage at 60°C for 1 month are shown in FIG. 5. Further, the correlation between the content of trehalose in lyophilized formulations and the ratio of peak B is shown in FIG. 6 and the correlation between the content of arginine and the ratio of peak B is shown in FIG. 7.

[0065]

According to Tables 13 and 14, little change in the isoform ratio of peak B was observed in Examples 1 to 11 and Comparative Example 5 under the storage conditions tested, while the isoform ratio of peak B was significantly changed in other Comparative Examples under the storage condition of 60°C. Thus change in the isoform ratio was suppressed in Examples 1 to 11 even under the storage condition of 60°C. In Comparative Example 1, a significant change was observed after storage, namely the isoform of peak A was eliminated, so that only the isoform detected was peak B and a peak which was not an isoform was observed.

[0066]

According to FIG. 6, it was found that the isoform ratio of peak B was decreased with an increase in the content of trehalose in formulations. Similarly, a decrease in the isoform ratio of peak B was also observed for the formulation of Comparative Example 7 which did not comprise trehalose.

According to FIG. 7, it was found that the isoform ratio of peak B was decreased with a decrease in the content of arginine in formulations under the storage condition of 60°C for 1 month.

Meanwhile no change in the isoform ratio according to the change in the content of hepatic growth factor in formulations was observed.

[0067]

Table 13

	Ratio of peak B (%)						
	Initial	2-week storage			1-month storage		
		25°C storage	40°C storage	60°C storage	25°C storage	40°C storage	60°C storage
Example 1	90.59	90.10	90.89	89.81	90.87	90.87	90.68
Example 2	90.62	90.60	90.89	90.24	90.96	90.96	90.51
Example 3	91.51	90.22	90.79	91.09	91.04	91.21	90.70
Example 4	90.69	90.84	90.58	90.59	91.08	91.05	90.70
Comparative Example 1	90.28	90.74	89.79	85.37	89.94	90.06	100.00
Comparative Example 2	90.82	90.89	90.62	90.07	90.71	90.78	88.93
Comparative Example 3	90.53	90.99	90.84	88.44	90.75	90.52	84.56
Comparative. Example 4	90.75	91.43	90.57	89.80	90.38	90.89	86.08
Comparative Example 5	91.22	90.72	90.40	90.37	90.27	90.86	90.35
Comparative Example 6	91.17	90.40	90.54	90.24	90.66	91.45	83.05

[0068]

Table 14

	Ratio of peak B (%)	
	Initial	60°C/2-week storage
Example 5	89.95	89.76
Example 6	89.89	89.89
Example 7	89.57	89.82
Example 8	90.00	89.35
Example 9	90.23	89.73
Example 10	89.70	89.96
Example 11	90.04	90.12

[0069]

Table 15

	Ratio of peak B (%)			
	Initial	1-month storage		
		25°C storage	40°C storage	60°C storage
Example 12	90.09	89.94	90.38	89.66
Example 13	90.18	89.97	90.35	89.88
Example 14	89.71	90.20	90.26	89.76
Example 15	89.71	90.00	90.20	89.86
Example 16	90.59	90.44	90.79	90.55
Example 17	90.28	90.38	90.54	90.45
Example 18	90.15	90.19	90.15	90.49
Example 19	89.87	89.97	90.09	90.07
Example 20	89.76	90.02	89.90	89.54
Example 21	89.68	89.63	89.76	89.83
Example 22	89.51	89.72	89.61	89.29
Example 23	90.02	90.26	89.98	88.68
Example 24	90.35	89.95	90.17	89.34
Example 25	90.12	90.30	90.34	89.93
Comparative Example 7	90.05	90.37	90.65	89.77

[0070]

(Test Example 5)

The amount of insoluble aggregates in Examples 1 to 25 and Comparative Examples 1 to 7 was evaluated as follows.

<Method>

Water for injection (1 mL) was added to each lyophilized formulation which was left to stand and then diluted in water for injection or each placebo aqueous solution to give a sample. Each sample was measured on a flow particle imaging analyzer for the number of insoluble microparticles before and after storage and the number of insoluble microparticles was calculated according to the following formula:

Number of microparticles (number/vial) = $P \times 1000 \times V/(n \times v)$

wherein:

P: number of particles detected;

V: volume of the sample solution (mL);

n: number of measurements; and

v: volume of the sample per measurement.

[0071]

<Results>

The results of measurement of the amount of insoluble aggregates in samples of Examples 1 to 11 and Comparative Examples 1 to 6 initially (before storage) and after storage at 60°C for 2 weeks or 1 month are shown in Tables 16 and 17.

[0072]

According to Table 16, the amount of insoluble aggregates in Comparative Examples 1 to 6 was increased after storage at 60°C for 1 month. According to Tables 16 and 17 meanwhile, such a significant change in the amount of insoluble aggregates as in Comparative Examples was not observed for Examples 1 to 11.

Thus generation of insoluble aggregates was suppressed in Examples 1 to 11 even under the storage condition of 60°C for 1 month. No significant change in the amount of insoluble aggregates was observed for Examples 12 to 25.

[0073]

Table 16

	Number of insoluble microparticles (number/vial)					
	< 10 μm		≥ 10 μm		≥ 25 μm	
	Initial	60°C/1-month storage	Initial	60°C/1-month storage	Initial	60°C/1-month storage
Negative control	6786	-	143	-	0	-
Example 1	13929	9286	333	190	0	0
Example 2	7857	3929	143	95	0	48
Example 3	7143	12143	286	333	95	48
Example 4	19643	6429	381	0	95	0
Comparative Example 1	18571	390000	333	87524	190	49857
Comparative Example 2	15357	7857	286	1095	48	333
Comparative Example 3	30000	7392857	381	1476	0	48
Comparative Example 4	28929	334643	238	70286	0	24476
Comparative Example 5	11071	66786	143	143	48	0
Comparative Example 6	3214	5153214	238	197238	48	18524

[0074]

Table 17

	Number of insoluble microparticles (number/vial)					
	< 10 μm		≥ 10 μm		≥ 25 μm	
	Initial	60°C/2-week storage	Initial	60°C/2-week storage	Initial	60°C/2-week storage
Example 5	714	357	0	0	0	0
Example 6	714	357	48	48	0	0
Example 7	2143	0	95	0	48	0
Example 8	1071	357	0	0	0	0
Example 9	714	1786	95	48	95	0
Example 10	1429	357	0	190	0	48
Example 11	357	357	48	0	0	0

[0075]

(Test Example 6)

Biological activity of Example 1, Comparative Example 5 and Comparative Example 6 was evaluated as follows.

<Method>

Mink lung epithelial cells (Mv.1.Lu cells) were cultured, the viability of cells were confirmed, and a cell solution was then prepared at 1×10^5/mL. A solution of transforming growth factor β-1 (TGFβ-1) was added to each well of a 96-well assay plate and a sample solution was added to each well at a hepatic growth factor concentration of 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 or 128 ng/mL. The cell solution of 1×10^5/mL was then added and the plate was incubated in a 5% carbon dioxide incubator at 37°C for 69 hours. The Cell Counting Kit (10 μL) available from Dojindo Molecular Technologies, Inc. was added to the plate which was then incubated for 3 hours and measured for the absorbance at 450 nm.

The obtained absorbance was plotted on the vertical axis relative to the HGF concentration in the samples (horizontal axis) and EC50 of the samples was calculated using an analytical software (SoftMax Pro available from Molecular Devices) employing parallel line analysis and 4 parameter logistic analysis. The relative titer (%) was calculated by dividing EC50 before storage by EC50 after storage.

[0076]

<Results>

The relative titer of EC50 of samples of Example 1 and Comparative Examples 5 and 6 after storage at 60°C for 1 month is shown in Table 18.

[0077]

According to Table 18, a decrease in the relative titer was observed for

Comparative Examples 5 and 6 after storage at 60°C for 1 month.

Meanwhile no decrease in the relative titer was observed for Example 1, indicating that the biological activity was retained even under the storage condition of 60°C for 1 month.

[0078]

Table 18

	Example 1	Comp. Example 5	Comp. Example 6
Relative titer (%)	101	80	45

INDUSTRIAL APPLICABILITY

[0079]

The lyophilized formulation of the present invention has an industrial applicability in that it can provide to medical practice hepatic growth factor formulations having high quality.

SEQUENCE LISTING FREE TEXT

[0080]

SEQ ID NO: 1 represents an amino acid sequence of human hepatic growth factor.

CLAIMS

I (We) claim:

1. A lyophilized formulation comprising:

(1) a hepatic growth factor;

(2) trehalose; and

(3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof.

2. A method for producing a lyophilized formulation, comprising the steps of:

(I) prior to addition of (1) a hepatic growth factor, adjusting pH of an aqueous solution comprising at least (3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof, to pH 4.5 to 6.5, and preparing an aqueous solution comprising (1) the hepatic growth factor, (2) trehalose and (3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof; and

(II) lyophilizing the aqueous solution comprising the hepatic growth factor obtained in the step (I).

3. A method for stabilizing a hepatic growth factor by comprising (2) trehalose and (3) one or more compounds selected from the group consisting of arginine, histidine, lysine, meglumine, glutamic acid, aspartic acid, proline, creatine, creatinine, tris(hydroxymethyl)aminomethane, and pharmaceutically acceptable salts thereof in a lyophilized formulation comprising (1) hepatic growth factor.

4. The method for stabilizing a hepatic growth factor according to claim 3, wherein generation of aggregates of the hepatic growth factor is suppressed.

5. The method for stabilizing a hepatic growth factor according to claim 3, wherein generation of impurities in conjunction with the hepatic growth factor is suppressed.

6. The method for stabilizing a hepatic growth factor according to claim 3, wherein a pre-storage isoform ratio of the hepatic growth factor is retained.