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(54) **Titre : PREPARATION DE HGF CONVENANT AU TRAITEMENT DE TROUBLES NEUROLOGIQUES**  
(54) **Title: HGF PREPARATION SUITABLE FOR TREATMENT OF NEUROLOGICAL DISORDERS**

(57) **Abrégé/Abstract:**

The present invention provides a hepatocyte growth factor (HGF) preparation in the form of an injection or the like that is highly safe for central nerves and highly stable and can be used for intrathecal or intracerebroventricular administration or for administration into the spinal or cerebral parenchyma for the treatment of central nervous system diseases. The HGF preparation of the present invention contains an HGF protein as an active ingredient and lactose, glycine, sodium chloride, a pH buffering agent and a surfactant as additional ingredients.

## ABSTRACT

The present invention provides a hepatocyte growth factor (HGF) preparation in the form of an injection or the like that  
5 is highly safe for central nerves and highly stable and can be used for intrathecal or intracerebroventricular administration or for administration into the spinal or cerebral parenchyma for the treatment of central nervous system diseases. The HGF preparation of the present invention  
10 contains an HGF protein as an active ingredient and lactose, glycine, sodium chloride, a pH buffering agent and a surfactant as additional ingredients.

## DESCRIPTION

HGF PREPARATION SUITABLE FOR TREATMENT OF NEUROLOGICAL  
DISORDERS

5

## TECHNICAL FIELD

[0001]

The present invention relates to a preparation containing a hepatocyte growth factor (hereinafter may be abbreviated as "HGF") protein. More particularly, the present invention relates to an HGF protein-containing preparation in the form of a lyophilized preparation, an injection or the like. The present invention also relates to an HGF protein-containing preparation in the form of a lyophilized preparation, an injection or the like, the preparation being suitable for the treatment of central nervous system diseases.

## BACKGROUND ART

[0002]

HGF was discovered as a biologically active protein having growth-promoting activity for mature hepatocytes (for example, see Non Patent Literature 1). Subsequent studies have revealed that HGF protein acts on not only hepatocytes but also various epithelial cells, vascular endothelial cells, etc., being involved in repair and regeneration of damaged tissues and organs (see Non Patent Literature 2). HGF protein can be mass-produced as a recombinant protein by bioengineering techniques (for example, see Non Patent Literature 3), and a recombinant HGF protein is expected to be used as a therapeutic

agent not only for hepatitis and liver cirrhosis but also for nephropathy, wounds, etc. (see Non Patent Literature 2).

Furthermore, a large number of recent studies on gene expression analysis and gene functional analysis by  
5 knockout/knockin mouse approaches etc. have revealed that HGF protein also has the effect of promoting neuronal cell survival and neurite outgrowth and is an important neurotrophic factor (see Non Patent Literature 4 and 5).

[0003]

10 HGF protein has neurotrophic activity on neuronal cells such as hippocampal neurons, dopaminergic neurons, cerebellar granule cells, sensory neurons and motor neurons (see Non Patent Literature 6). In particular, HGF protein has a strong effect of promoting the survival of motor neurons (see Non Patent  
15 Literature 7). This effect is comparable to that of glial cell line-derived neurotrophic factor (GDNF), a factor known to most strongly promote the survival of motor neurons.

Based on such neurotrophic activity, HGF protein has been reported to be applicable as a therapeutic agent for various  
20 neurological disorders including amyotrophic lateral sclerosis (ALS) and spinal cord injury (see Patent Literature 1 to 3 and Non Patent Literature 5, 8 and 9).

[0004]

In general, protein pharmaceuticals are injected  
25 intravenously, subcutaneously or intramuscularly. However, proteins administered via such a route can very hardly transfer to central nervous system tissues across the blood-brain barrier between brain tissues and blood vessels, as is commonly known. Therefore, when HGF protein is used for the treatment



of central nervous system diseases, intrathecal or intracerebroventricular administration, which allows direct delivery of HGF protein to central nervous system tissues, is considered to be effective instead of intravenous, subcutaneous  
5 or intramuscular injection, which is a route used for the treatment of common organ diseases (see Non Patent Literature 8 and 9). Intrathecal or intracerebroventricular administration is used also in the anticancer drug treatment of brain tumor. In addition, direct administration of HGF  
10 protein into the cerebral or spinal parenchyma is another possible administration route for the treatment of central nervous system diseases.

[0005]

For the production of HGF protein pharmaceuticals, the  
15 development of stabilized HGF protein preparations is required. Patent Literature 4 discloses an HGF protein preparation which is an aqueous solution containing an HGF protein (also called TCF-II) plus a stabilizer such as albumin, human serum, gelatin, sorbitol, mannitol and xylitol (see Patent Literature 4).  
20 However, this aqueous HGF solution has some disadvantages. One is that the HGF aqueous solution gradually become turbid and gelatinized during storage due to aggregation of HGF protein molecules. Another is that the HGF aqueous solution is poor in physicochemical stability, for example, is prone to  
25 formation of HGF protein-based polymers (formation of HGF polymers), resulting in reduction in biological activity of HGF.

[0006]

In order to provide a solution to prevent such polymer

formation, for example, Patent Literature 5 discloses a lyophilized HGF preparation containing HGF plus a stabilizer such as arginine, lysine, histidine, glutamine, proline, glutamic acid and aspartic acid (see Patent Literature 5).  
5 Patent Literature 6 discloses a lyophilized HGF preparation containing HGF plus a stabilizer such as glycine, alanine, sorbitol, mannitol and dextran sulfate (see Patent Literature 6). Patent Literature 7 discloses a lyophilized HGF preparation containing HGF plus purified sucrose, alanine and  
10 the like (see Patent Literature 7).

[0007]

      Injections prepared from these preparations are supposedly safe to use for intravenous, subcutaneous or intramuscular administration, which is a route used for the treatment of  
15 common organ diseases. However, for example in the case of intrathecal or intracerebroventricular administration, since HGF protein is directly delivered to the central nervous system, all the ingredients of the HGF preparation, including various additives, need to have been fully confirmed safe for the  
20 central nervous system. So far, there is no disclosure of HGF preparations publicly confirmed safe to use for intrathecal or intracerebroventricular administration or for administration into the spinal or cerebral parenchyma.

[0008]

25       There is a need for highly safe HGF preparations that can be used for intrathecal or intracerebroventricular administration or for administration into the spinal or cerebral parenchyma for the treatment of central nervous system diseases.

## CITATION LIST

## Patent Literature

[0009]

- 5 Patent Literature 1: WO 2002/22162 (US Pub. No. 2003/0176347)  
Patent Literature 2: WO 2007/122976 (US Patent No. 8,575,099)  
Patent Literature 3: WO 2008/105507 (US Patent No. 8,518,880)  
Patent Literature 4: WO 90/10651 (EP Patent No. 0462277)  
Patent Literature 5: WO 00/72873 (EP Patent No. 1180368)  
10 Patent Literature 6: JP-A 9-25241 (US Patent No. 7,173,008)  
Patent literature 7: WO 2008/102849 (US Patent No. 8,461,112)

## Non Patent Literature

[0010]

## Non Patent Literature 1:

- 15 T. Nakamura et al., Biochem. Biophys. Res. Commun., vol. 122,  
p. 1450, 1984

## Non Patent Literature 2:

T. Nakamura et al., J. Gastroenterol. Hepatol., vol. 26, Suppl.  
1, pp. 188-202 (2011)

- 20 Non Patent Literature 3:

Jeong Soo Park et al., Protein Expr. Purif., vol. 70, p. 231-235  
(2010)

## Non Patent Literature 4:

Flavio Maina et al., Nat. Neurosci., vol. 2, pp. 213-217 (1999)

- 25 Non Patent Literature 5:

Funakoshi H et al., Current Signal Transduction Therapy vol.  
6, pp. 156-167 (2011)

## Non Patent Literature 6:

Honda, S. et al., Brain Res. Mol. Brain Res. vol. 32, pp. 197-210

(1995)

Non Patent Literature 7:

Allen Ebens et al., Neuron, vol. 17, pp. 1157-1172 (1996)

Non Patent Literature 8:

5 Ishigaki A et al., J Neuropathol Exp Neurol., vol. 66, pp.  
1037-1044 (2007)

Non Patent Literature 9:

Kitamura K et al., PLoS One., vol. 6: e27706 (2011)

## 10 SUMMARY OF INVENTION

### TECHNICAL PROBLEM

[0011]

An object of the present invention is to provide an HGF preparation in the form of an injection or the like that is highly  
15 safe for central nerves and can be used for intrathecal or intracerebroventricular administration or for administration into the spinal or cerebral parenchyma for the treatment of central nervous system diseases. In general, the HGF preparation is also required to be highly stable so that it can  
20 be practically used as a pharmaceutical product. Accordingly, another object of the present invention is to provide a highly stable HGF preparation in the form of an injection, a lyophilized preparation or the like.

## 25 SOLUTION TO PROBLEM

[0012]

The present inventors conducted intensive research to achieve the above-mentioned objects. As a result, the present inventors found that the formation of HGF protein-based



polymers is prevented by addition of lactose, glycine, sodium chloride, a pH buffering agent and a surfactant to an HGF protein. The present inventors also found that an HGF solution containing these ingredients can be used as a stable HGF injection and that  
5 freeze-drying of the HGF solution yields a stable lyophilized HGF preparation. Moreover, it was found that an HGF injection containing the above ingredients is markedly less toxic to the central nervous system and highly safe for the nerve system such as central nerves.

10 Based on these findings, the present inventors conducted further research and completed the present invention. The HGF preparation of the present invention is stable enough to use as a pharmaceutical product. For example, the HGF injection of the present invention can be safely administered  
15 intrathecally or intracerebroventricularly or administered into the spinal or cerebral parenchyma for the treatment of various central nervous system diseases such as ALS and spinal cord injury.

[0013]

20 That is, the present invention provides the following HGF preparation.

(1) A hepatocyte growth factor (HGF) preparation comprising an HGF protein as an active ingredient and lactose, glycine, sodium chloride, a pH buffering agent and a surfactant as additional  
25 ingredients.

(2) The HGF preparation according to the above (1), wherein the HGF preparation is a lyophilized preparation.

(3) The HGF preparation according to the above (2), wherein the HGF preparation is a lyophilized preparation obtained by

freeze-drying of an aqueous solution comprising a hepatocyte growth factor (HGF) protein, lactose, glycine, sodium chloride, a pH buffering agent and a surfactant.

(4) The HGF preparation according to any one of the above (1) to (3), wherein the content of the lactose is in the range of 0.1 to 50 parts by weight relative to 1 part by weight of HGF.

(5) The HGF preparation according to the above (3), wherein the concentration of the lactose in the aqueous solution is in the range of 0.1 to 100 mg/mL.

(6) The HGF preparation according to the above (3), wherein the concentration of the glycine in the aqueous solution is in the range of 0.05 to 50 mg/mL.

(7) The HGF preparation according to the above (3), wherein the concentration of the HGF protein in the aqueous solution is in the range of 0.05 to 40 mg/mL.

(8) The HGF preparation according to the above (1), wherein the pH buffering agent is a combination of citric acid or a hydrate thereof with a salt of citric acid.

(9) The HGF preparation according to the above (1), wherein the surfactant is polysorbate.

(10) The HGF preparation according to the above (1), wherein the HGF preparation is an injection.

(11) The HGF preparation according to the above (10), wherein the injection is an aqueous solution obtained by dissolving the lyophilized preparation according to the above (2) in a pharmaceutically acceptable solvent.

(12) The HGF preparation according to the above (1), wherein the HGF preparation is for use in treatment of a central nervous system disease.

(13) The HGF preparation according to the above (12), wherein the central nervous system disease is amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, Huntington's disease, spinocerebellar ataxia, spinal cord  
5 injury, cerebral infarction, cerebral ischemia or multiple sclerosis.

(14) The HGF preparation according to the above (1), wherein the HGF preparation is administered intrathecally or intracerebroventricularly or administered into spinal or  
10 cerebral parenchyma.

(15) The HGF preparation according to the above (10), wherein the concentration of the lactose in the injection is in the range of 0.1 to 100 mg/mL.

(16) The HGF preparation according to the above (10), wherein  
15 the concentration of the glycine in the injection is in the range of 0.05 to 50 mg/mL.

(17) The HGF preparation according to the above (10), wherein the concentration of the HGF protein in the injection is in the range of 0.05 to 40 mg/mL.

20 (18) The HGF preparation according to the above (1), wherein the HGF protein is a human HGF protein.

(19) The HGF preparation according to the above (18), wherein the HGF protein is a protein consisting of an amino acid sequence represented by SEQ ID NO: 5 or SEQ ID NO: 6.

25 (20) The HGF preparation according to the above (1), wherein the HGF protein is a protein which has 80% or more sequence identity with an amino acid sequence represented by SEQ ID NO: 5 and has a biological activity of HGF.

The present invention also relates to a method for



stabilizing HGF, more particularly preventing formation of HGF protein-based polymers in an aqueous HGF solution or a lyophilized HGF preparation, and the method comprises using lactose, glycine, sodium chloride, a pH buffering agent and a  
5 surfactant.

Furthermore, the present invention relates to a method for treating a central nervous system disease, the method comprising administering the HGF preparation according to the above (1) intrathecally or intracerebroventricularly or  
10 administering the same into the spinal or cerebral parenchyma to a patient with a central nervous system disease.

#### ADVANTAGEOUS EFFECTS OF INVENTION

[0014]

15 The HGF preparation of the present invention is a stable preparation and can be used safely for central nerves. Since the HGF injection of the present invention is highly safe for the central nervous system, for example, it can be administered intrathecally or intracerebroventricularly or administered  
20 into the spinal or cerebral parenchyma for the treatment of various central nervous system diseases such as ALS and spinal cord injury.

#### DESCRIPTION OF EMBODIMENTS

25 [0015]

The HGF preparation of the present invention contains an HGF protein as an active ingredient and lactose, glycine, sodium chloride, a pH buffering agent and a surfactant as additional ingredients.



The HGF preparation of the present invention may further contain one or more additional active ingredients (medicinal ingredients) in addition to the HGF protein, but preferably contain no active ingredient except the HGF protein.

5 [0016]

The dosage form of the HGF preparation of the present invention is not particularly limited, but preferred is, for example, a parenteral dosage form such as a lyophilized preparation and an injection. The lyophilized preparation is  
10 preferably a lyophilized preparation for injection.

[0017]

The injection means a liquid composition which is injectable directly into the living body. In the case where the HGF preparation of the present invention is an injection, it may  
15 be abbreviated simply as an "HGF injection".

The lyophilized preparation means a preparation in which the ingredients are in a freeze-dried solid state. In the case where the HGF preparation of the present invention is a lyophilized preparation, it may be abbreviated simply as a  
20 "lyophilized HGF preparation". Typically, a lyophilized preparation is dissolved in an appropriate solvent (dissolving liquid) before use, and the solution is administered as an injection as it is or if needed after dilution in an appropriate solvent or the like. That is, it can be said that a solution  
25 obtained by dissolving a lyophilized preparation in a solvent is substantially equivalent to an injection.

[0018]

The HGF preparation of the present invention is preferably a lyophilized preparation containing an HGF protein as an active

ingredient and lactose, glycine, sodium chloride, a pH buffering agent and a surfactant as additional ingredients. Another preferable embodiment of the HGF preparation of the present invention is an HGF injection containing an HGF protein  
5 as an active ingredient and lactose, glycine, sodium chloride, a pH buffering agent and a surfactant as additional ingredients.  
[0019]

The HGF preparation of the present invention is highly safe for the central nervous system. The HGF preparation of the  
10 present invention including the HGF injection is so markedly less toxic to the central nervous system that it can be administered, for example, intrathecally or intracerebroventricularly or administered into the spinal or cerebral parenchyma. Therefore, the HGF preparation of the  
15 present invention is suitable for use in the treatment of various central nervous system diseases, etc.  
[0020]

The HGF protein in the present invention may be from any species without particular limitation, and HGF proteins from  
20 various animals (native HGF proteins or recombinant proteins produced by genetic engineering techniques) etc. can preferably be used. In the present invention, it is preferred to use, for example, an HGF protein from an animal for which the HGF preparation of the present invention is intended to be used.  
25 For example, when the HGF preparation of the present invention is intended to be used for humans, an HGF protein from humans (hereinafter may be referred to as a human HGF protein) is suitable as the HGF protein used in the present invention. More preferred is a recombinant human HGF protein. When the HGF

preparation of the present invention is intended to be used for non-human mammals, HGFs from such animals are preferably used, and for example, HGF proteins from monkeys, cattle, horses, pigs, sheep, dogs, cats, rats, mice, rabbits, hamsters, guinea pigs, chimpanzees, etc. are usable. In addition, the HGF protein used  
5 in the present invention may be a 5-amino-acid-deleted-type HGF protein (dHGF).

[0021]

The human HGF protein is preferably a protein encoded by  
10 a DNA consisting of the nucleotide sequence represented by SEQ ID NO: 1 or SEQ ID NO: 2, for example. More specifically, preferred are a protein consisting of the amino acid sequence represented by SEQ ID NO: 3, a protein consisting of the amino acid sequence represented by SEQ ID NO: 4, a protein consisting  
15 of the amino acid sequence represented by SEQ ID NO: 5, a protein consisting of the amino acid sequence represented by SEQ ID NO: 6, etc. In particular, the human HGF protein is preferably a protein having the amino acid sequence represented by SEQ ID NO: 5 or SEQ ID NO: 6, and more preferably a protein consisting  
20 of the amino acid sequence represented by SEQ ID NO: 5 or SEQ ID NO: 6. For example, the HGF protein consisting of the amino acid sequence represented by SEQ ID NO: 6 is a 5-amino-acid-deleted-type HGF protein (dHGF) in which 5 amino acid residues at positions 131 to 135 of the amino acid sequence  
25 represented by SEQ ID NO: 5 are deleted. The protein having the amino acid sequence represented by SEQ ID NO: 5 or SEQ ID NO: 6 is a naturally-occurring HGF protein (native HGF protein) in the human body and has activities of HGF, such as mitogenic activity and motogenic activity.



[0022]

The HGF protein used in the present invention encompasses proteins which have at least about 80% or more, preferably about 90% or more, more preferably about 95% or more sequence identity  
5 with the amino acid sequence of HGF proteins (native HGF proteins) from various animals and have biological activities (mitogenic activity and motogenic activity) of HGF. The term "sequence identity" as used herein in connection with the amino acid sequence means the identity of amino acid residues between  
10 the amino acid sequences (primary structures) of two proteins. A number together with "% or more" represents the degree of the sequence identity.

The mitogenic and motogenic activities of the HGF protein can be confirmed, for example, according to the method described  
15 in J. Biol. Chem. 273, 22913-22920, 1998. Preferably, the HGF protein used for the present invention has mitogenic and motogenic activities as measured according to J. Biol. Chem. 273, 22913-22920, 1998 as high as usually about 50% or more, preferably about 70% or more, more preferably about 80% or more,  
20 still more preferably about 90% or more of those of the native HGF protein.

[0023]

Examples of proteins which have the above-described sequence identity with native HGF proteins include proteins  
25 which have the same amino acid sequence as that represented by SEQ ID NO: 5 or 6 except for substitution, deletion and/or insertion of one to several amino acid residues or modification of one to several amino acid residues and have biological activities of HGF.



The term "several" generally means an integral number of 1 to 8 (1, 2, 3, 4, 5, 6, 7 and 8), and is usually 8, preferably 6, more preferably 5, still more preferably 3, particularly preferably 2. The amino acid to be inserted or substituted for  
5 the original one is preferably a natural amino acid, but may be an unnatural amino acid other than 20 kinds of gene-encoded amino acids. The unnatural amino acid may be any compound that has an amino group and a carboxyl group, and for example is  $\gamma$ -aminobutyric acid or the like.

10 [0024]

The substitution of an amino acid residue means replacement of one amino acid residue with another in a polypeptide, and is preferably conservative substitution. The term "conservative substitution" means replacement of one to several  
15 amino acid residues with another (or other) chemically similar amino acid residue(s) without substantial change in the activity of the polypeptide. Examples of conservative substitution include a case where a hydrophobic amino acid residue is replaced by another hydrophobic amino acid residue,  
20 or a case where a polar amino acid residue is replaced with another polar amino acid residue with the same charge. The functionally similar amino acid(s) for conservative substitution of each amino acid is/are known in the art. Exemplary amino acids with a nonpolar (hydrophobic) side chain  
25 include glycine, alanine, valine, isoleucine, leucine, proline, tryptophan, phenylalanine and methionine. Exemplary neutral amino acids with a polar side chain include serine, threonine, tyrosine, glutamine, asparagine and cysteine. Exemplary positively-charged (basic) amino acids include arginine,

histidine and lysine. Exemplary negatively-charged (acidic) amino acids include aspartic acid and glutamic acid.

[0025]

5 The HGF protein contained in the preparation of the present invention may be of one kind, or a combination of two or more of the above-described ones.

[0026]

10 The HGF protein used in the preparation of the present invention can be prepared by various methods as long as the purity of the prepared HGF protein is suitable for pharmaceutical use. Various preparation methods are known, and for example, the HGF protein can be obtained by extraction and purification from organs such as liver, spleen, lung, bone marrow, brain, kidney and placenta, blood cells such as  
15 platelets and leukocytes, plasma, serum and the like of mammals such as rats, cattle, horses and sheep.

A specific procedure of the extraction and purification of the HGF protein from the above living tissues etc. is, for example, as follows. Carbon tetrachloride is  
20 intraperitoneally injected to rats to induce hepatitis, the liver is isolated and homogenized, and the HGF protein is purified by ordinary protein purification methods such as column chromatography with S-Sepharose®, heparin Sepharose®, etc. and HPLC.

25 [0027]

Alternatively, the HGF protein can be obtained by isolation and purification from the culture (culture supernatant, cultured cells, etc.) of primary cultured cells or established cell lines which produce the HGF protein. Alternatively,

genetic engineering techniques can be used for the preparation of the HGF protein. Specifically, a gene encoding the HGF protein (preferably, a DNA consisting of the nucleotide sequence represented by SEQ ID NO: 1 or 2) is inserted into an appropriate vector, the vector is introduced into an appropriate host for transformation, and a recombinant HGF protein of interest is harvested from the culture of the resulting transformant (for example, see Biochem. Biophys. Res. Commun. 180: 1151-1158, 1991; J. Clin. Invest. 87: 1853-1857, 1991; Protein Expr. Purif. 70: 231-235, 2010; etc.). The host cell is not particularly limited, and various kinds of host cells conventionally used in genetic engineering techniques can be used. For example, *E. coli*, *Bacillus subtilis*, yeasts, filamentous fungi, plant cells, animal cells, etc. can be used. In an example where animal cells are used as the host cell, Chinese hamster ovary (CHO) cells, mouse C127 cells, monkey COS cells or other animal cells are transformed with an expression vector prepared by insertion of a cDNA encoding the amino acid sequence of a human HGF protein, the culture supernatant is separated, and the HGF protein in the supernatant is purified by, for example, column chromatography as exemplified above. [0028]

As long as the thus-obtained HGF protein has biological activities of HGF, it may be different from the native HGF protein in that the amino acid sequence has substitution, deletion and/or insertion of one or more amino acids. In this context, "one or more" is, for example, one to several (the term "several" is as defined above, and is for example 1 to 8, preferably 1 to 6, more preferably 1 to 5, still more preferably



1 to 3, particularly preferably 1 or 2; the same will apply hereinafter). The substitution is preferably conservative substitution. The HGF protein may be modified by substitution, deletion or insertion of a sugar chain(s). The "deletion, substitution and/or insertion of one or more amino acids" as  
5 used herein in connection with the amino acid sequence means deletion, substitution and/or insertion of a certain number of amino acids, which number substantially corresponds to the number of amino acids that can be deleted, substituted and/or  
10 inserted by well-known technical methods such as gene engineering and site-directed mutagenesis or in a naturally-occurring manner (generally one to several amino acids). The "HGF protein modified by substitution, deletion or insertion of a sugar chain(s)" means an HGF protein obtained  
15 by removing a sugar chain(s) from a native HGF protein by treatment with an enzyme or the like; an HGF protein obtained by mutating a glycosylation site(s) in the amino acid sequence of a native HGF protein so as not to allow glycosylation; an HGF protein obtained by mutating the amino acid sequence of a  
20 native HGF protein so as to allow glycosylation of a site(s) other than the natural glycosylation site(s); or the like.  
[0029]

The lactose, glycine, sodium chloride, pH buffering agent and surfactant used in the HGF preparation of the present  
25 invention are preferably the same as described in the pharmacopoeias of various countries (for example, the Japanese Pharmacopoeia, the United States Pharmacopeia, the European Pharmacopoeia, etc.). In the case where those not described in the pharmacopeias are used, pharmaceutically acceptable ones



are preferably used. The term "pharmaceutically acceptable" means being usually safe, less toxic, free from biological and other problems, and useful for preparing pharmaceutical preparations acceptable for animal or human use.

5 [0030]

The lactose used in the HGF preparation of the present invention is preferably the same as described in the pharmacopoeias of various countries (for example, the Japanese Pharmacopoeia, the United States Pharmacopeia, the European  
10 Pharmacopoeia, etc.). The amount of the lactose is preferably about 0.1 to 50 parts by weight, more preferably about 0.5 to 10 parts by weight and still more preferably about 1 to 5 parts by weight (including 1 to 2 parts by weight, 1 to 3 parts by weight, 1 to 4 parts by weight, 1 to 5 parts by weight, 2 to  
15 3 parts by weight, 2 to 4 parts by weight, 2 to 5 parts by weight, 3 to 4 parts by weight, 3 to 5 parts by weight and 4 to 5 parts by weight) relative to 1 part by weight of the HGF protein.  
[0031]

The glycine used in the HGF preparation of the present  
20 invention is preferably the same as described in the pharmacopoeias of various countries (for example, the Japanese Pharmacopoeia, etc.). The amount of the glycine is preferably about 0.01 to 1 part by weight, more preferably about 0.05 to 1 part by weight and still more preferably about 0.1 to 0.5 part  
25 by weight (including 0.1 to 0.2 part by weight, 0.1 to 0.3 part by weight, 0.1 to 0.4 part by weight, 0.1 to 0.5 part by weight, 0.2 to 0.3 part by weight, 0.2 to 0.4 part by weight, 0.2 to 0.5 part by weight, 0.3 to 0.4 part by weight, 0.3 to 0.5 part by weight and 0.4 to 0.5 part by weight) relative to 1 part by

weight of the HGF protein.

[0032]

The pH buffering agent used in the HGF preparation of the present invention means an agent which, once dissolved in a solvent such as water, can serve as a buffer, which has the effect of keeping the pH of the solution within a certain range. A typical example is a combination of a weak acid and a salt thereof. Preferable examples of the pH buffering agent include a combination of phosphoric acid, citric acid or boric acid with the corresponding salt, which can serve as phosphate buffer, citrate buffer or borate buffer once dissolved. More preferred is a combination of citric acid and a salt thereof, which can serve as citrate buffer. These weak acids and their salts may be in the form of a solvate, and the solvate is preferably a hydrate, for example. A solution of the pH buffering agent can serve as a buffer, which has the effects of adjusting the pH of an aqueous HGF solution and maintaining the solubility and stability of the HGF protein. Examples of the aqueous HGF solution in the present invention include an HGF injection; an aqueous solution prepared before a freeze-drying step in the course of the production of the lyophilized preparation described later; and an aqueous solution obtained by redissolving the lyophilized preparation in a solvent. When the HGF preparation is, for example, a lyophilized preparation, the pH buffering agent preferably has the effect of keeping the pH of an aqueous solution obtained by redissolving the HGF preparation within the range of about 4.5 to 8.0. When the HGF preparation is an HGF injection, the pH buffering agent preferably has the effect of keeping the pH of the injection

within the range of about 4.5 to 8.0. Specifically, regardless of the form of the HGF preparations including an HGF injection and a lyophilized HGF preparation, the pH buffering agent in the present invention is preferably a combination of citric acid  
5 or its solvate with a citric acid salt or its solvate; more preferably a combination of citric acid or its hydrate with a citric acid salt; and still more preferably a combination of a citric acid hydrate with sodium citrate (preferably trisodium citrate dihydrate or trisodium citrate (anhydrous)). Citrate  
10 buffer is highly effective for stabilizing the HGF protein in an aqueous HGF solution and can contribute to the stabilization of the HGF protein in the HGF injection; the aqueous HGF solution prepared in the course of the production of the lyophilized HGF preparation; and the aqueous solution obtained by redissolving  
15 the lyophilized HGF preparation in a solvent. In a preferable embodiment, the amount of the pH buffering agent, for example, in the HGF injection, is such an amount as to give a concentration of preferably about 1 to 100 mM, more preferably about 1 to 20 mM in the injection. In a preferable embodiment, the amount  
20 of the pH buffering agent in the lyophilized preparation is such an amount as to give a concentration of preferably about 1 to 100 mM, more preferably about 1 to 20 mM in an aqueous solution before a freeze-drying step in the course of the production of the lyophilized preparation described later.

25 [0033]

Examples of the surfactant used in the HGF preparation of the present invention include polysorbate (for example, polysorbate 20 (polyoxyethylene sorbitan monolaurate), polysorbate 80 (polyoxyethylene sorbitan monooleate), etc.),



Pluronic (registered trademark) F-68 (GIBCO) and polyethylene glycol. Two or more of them may be used in combination. Preferred is polysorbate, and particularly preferred is polysorbate 80. The HGF protein easily adsorbs to the surface  
5 of a container of glass, resin or other materials, but the addition of such a surfactant can prevent the adsorption of the HGF protein to the container in the course of the production of the HGF preparation. The addition of the surfactant can also prevent the adsorption of the HGF protein to a container holding  
10 the HGF injection or the aqueous HGF solution obtained by redissolving the HGF preparation in a solvent. The amount of the surfactant in the HGF injection is, for example, such an amount as to give a concentration of preferably about 0.001 to 2.0% by weight, more preferably about 0.005 to 1.0% by weight  
15 in the injection. The amount of the surfactant in the lyophilized preparation is, for example, such an amount as to give a concentration of preferably about 0.001 to 2.0% by weight, more preferably about 0.005 to 1.0% by weight in an aqueous solution before a freeze-drying step in the course of the  
20 production of the lyophilized preparation described later.  
[0034]

The sodium chloride used in the HGF preparation of the present invention has the effect of maintaining the solubility of the HGF protein. That is, the addition of sodium chloride,  
25 particularly at about 150 mM or higher, increases the solubility of the HGF protein. In addition, the addition of sodium chloride can make the osmotic pressure of an aqueous HGF solution close to the osmotic pressure of the body fluid. The amount of the sodium chloride can be adjusted as appropriate



according to the desired osmotic pressure ratio. Preferably, the amount of the sodium chloride is such an amount as to give an osmotic pressure ratio (relative to physiological saline (osmotic pressure ratio: 1)) of about 1 to 3, which is a range  
5 acceptable for injections for clinical use or animal use. The amount of the sodium chloride, for example, in the HGF injection, is such an amount as to give a concentration of preferably about 150 to 1000 mM, more preferably about 150 to 300 mM in the injection. The amount of the sodium chloride in the lyophilized  
10 preparation is, for example, such an amount as to give a concentration of preferably about 150 to 1000 mM, more preferably about 150 to 300 mM in an aqueous HGF solution prepared in the course of the production of the lyophilized preparation described later.

15 [0035]

The production method of the HGF preparation of the present invention is not particularly limited. For example, the HGF preparation in the form of a lyophilized preparation can be produced by freeze-drying of an aqueous solution containing an  
20 HGF protein, lactose, glycine, sodium chloride, a pH buffering agent and a surfactant. The thus-obtained lyophilized preparation is a preferable embodiment of the HGF preparation of the present invention. For preparation of the aqueous solution used in the production of the lyophilized preparation,  
25 any method may be used without particular limitation as long as the aqueous solution contains an HGF protein, lactose, glycine, sodium chloride, a pH buffering agent and a surfactant. For example, to a solution of a purified HGF protein (typically containing a pH buffer, sodium chloride and a surfactant),

lactose and glycine, and if needed, a pharmaceutically acceptable solvent (for example, sterilized water, distilled water for injection, purified water, buffer, physiological saline, etc.) are added to prepare the aqueous solution. In  
5 a preferable embodiment, the concentration of the HGF protein is adjusted to preferably about 0.05 to 40 mg/mL, more preferably about 0.1 to 40 mg/mL, and still more preferably about 0.1 to 20 mg/mL in the aqueous solution. The lactose is added in such an amount as to give a concentration of preferably  
10 about 0.1 to 100 mg/mL, more preferably about 0.5 to 50 mg/mL, and still more preferably about 1 to 20 mg/mL in the aqueous solution. The glycine is added in such an amount as to give a concentration of preferably about 0.05 to 50 mg/mL, more preferably about 0.05 to 20 mg/mL, and still more preferably  
15 about 0.1 to 10 mg/mL in the aqueous solution. This aqueous HGF solution can further contain one or more additional ingredients such as solubilizers, antioxidants, soothing agents and tonicity agents, if needed. In a preferable embodiment, the aqueous HGF solution is sterilized by  
20 filtration with a filter or the like, distributed into vials or ampules and then freeze-dried. The filter is preferably a sterilizing filter with a pore size of about 0.22  $\mu\text{m}$  or less, for example. Preferable examples of the sterilizing filter include Durapore (registered trademark, manufactured by Merck)  
25 and Sartopore 2 (registered trademark, manufactured by Sartorius).

[0036]

The method for freeze-drying of the aqueous solution is not particularly limited and ordinary freeze-drying methods can be

used. An exemplary freeze-drying method comprises the following three steps: a freezing step in which cooling and freezing is performed under normal pressure; a primary drying step in which solute-free water is sublimed off under vacuum; 5 and a secondary drying step in which water bound to solutes, such as adsorbed water and crystallization water, is removed. The freezing temperature of the freezing step is preferably about  $-60^{\circ}\text{C}$  to  $-40^{\circ}\text{C}$ , the temperature of the primary drying step is preferably about  $-50^{\circ}\text{C}$  to  $0^{\circ}\text{C}$ , and the temperature of the 10 secondary drying step is preferably about  $4^{\circ}\text{C}$  to  $40^{\circ}\text{C}$ . The vacuum pressure is preferably about 0.1 to 1.5 Pa, and particularly preferably about 0.5 to 1.2 Pa. The pressure of the drying chamber is made to recover after the completion of freeze-drying. In a preferable method for pressure recovery, 15 sterile air or inert gas (for example, sterile nitrogen gas, sterile helium gas, etc.) is fed into the chamber to allow the pressure to recover firstly to a level of about 70 to 100 kPa, more preferably about 80 to 95 kPa (primary pressure recovery) and then to atmospheric pressure (secondary pressure recovery). 20 The vials are preferably plugged with stoppers after the primary pressure recovery, and the plugged vials are preferably sealed with caps immediately after the secondary pressure recovery. The ampules are preferably melt-sealed by applying heat to their tips (typically using a gas burner) after the completion of 25 drying.

The lyophilized HGF preparation preferably has a moisture content of about 2% by weight or less.

[0037]

The lyophilized HGF preparation of the present invention



is less prone to formation of HGF protein-based polymers during storage and is highly stable. The term "protein-based polymer" means a substance in which plural protein monomers are bound together for example in a chain or net-like structure. In the present invention, a dimer, a trimer and a tetramer of HGF proteins are included.

[0038]

Typically, the lyophilized HGF preparation of the present invention is dissolved in a pharmaceutically acceptable solvent and used in the form of an aqueous solution. The term "pharmaceutically acceptable" is as defined above. Preferable examples of the pharmaceutically acceptable solvent include distilled water for injection, physiological saline, various kinds of infusion (for example, 5% glucose solution, Ringer's solution, etc.) and artificial spinal fluid. The solvent is more preferably distilled water for injection or physiological saline. In a preferable embodiment, the lyophilized HGF preparation of the present invention is dissolved in a pharmaceutically acceptable solvent, such as distilled water for injection, to prepare a solution containing an HGF protein at a concentration of preferably about 0.05 to 40 mg/mL, more preferably about 0.1 to 40 mg/mL, still more preferably about 0.1 to 20 mg/mL, which solution can preferably be used as an injection.

The lyophilized HGF preparation of the present invention can be packed together with the above-described pharmaceutically acceptable solvent and provided as a kit.

[0039]

The HGF injection of the present invention is preferably



an aqueous solution containing an HGF protein, lactose, glycine, sodium chloride, a pH buffering agent and a surfactant. The lactose, the glycine, the sodium chloride, the pH buffering agent and the surfactant, and their preferable embodiments and  
5 the like are as described above.

[0040]

The production method of the HGF injection of the present invention is not particularly limited. For example, the lyophilized HGF preparation is dissolved in a pharmaceutically  
10 acceptable solvent to prepare the HGF injection. Preferable examples of the pharmaceutically acceptable solvent include distilled water for injection, physiological saline, various kinds of infusion (for example, 5% glucose solution, Ringer's solution, etc.) and artificial spinal fluid. The solvent is  
15 more preferably distilled water for injection or physiological saline. Alternatively, the HGF injection of the present invention can be prepared by adding lactose and glycine, and if needed, a pharmaceutically acceptable solvent (for example, sterilized water, distilled water for injection, purified water,  
20 buffer, physiological saline, etc.) to an about 0.1 to 40 mg/mL aqueous solution of a purified HGF protein (typically containing a pH buffer, sodium chloride and a surfactant). For example, the aqueous solution used in the production of the lyophilized preparation described above, which solution  
25 contains an HGF protein, lactose, glycine, sodium chloride, a pH buffering agent and a surfactant, can be used as the HGF injection.

[0041]

The concentration of the HGF protein in the HGF injection

of the present invention is preferably about 0.05 to 40 mg/mL, more preferably about 0.1 to 40 mg/mL, and still more preferably about 0.1 to 20 mg/mL.

[0042]

5       The concentration of the lactose in the HGF injection of the present invention is preferably about 0.1 to 100 mg/mL, more preferably about 0.5 to 50 mg/mL, and still more preferably about 1 to 20 mg/mL.

[0043]

10       The concentration of the glycine in the HGF injection of the present invention is preferably about 0.05 to 50 mg/mL, more preferably about 0.05 to 20 mg/mL, and still more preferably about 0.1 to 10 mg/mL.

[0044]

15       The pH of the HGF injection of the present invention is preferably about 4.5 to 8.0.

The HGF injection of the present invention can further contain one or more additional ingredients such as solubilizers, antioxidants, soothing agents and tonicity agents, if needed.

20       [0045]

The HGF injection of the present invention is usually a clear solution. The HGF injection of the present invention is less prone to formation of HGF polymers during storage and is highly stable although it is in the state of a solution.

25       [0046]

The intended use of the HGF preparation of the present invention is not particularly limited, but preferably, the HGF preparation is used as a pharmaceutical composition for the treatment or prevention of central nervous system diseases, for

example, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, Huntington's disease, spinocerebellar ataxia, spinal cord injury, cerebral infarction, cerebral ischemia, multiple sclerosis, etc. In particular, the HGF preparation of the present invention is  
5 suitable for use in the treatment of central nervous system diseases.

[0047]

The HGF preparation of the present invention including the  
10 HGF injection and the lyophilized HGF preparation can be administered, for example, intracerebroventricularly or intrathecally. For example, the HGF injection of the present invention is suitable as a preparation for intracerebroventricular or intrathecal administration. The  
15 intrathecal space, into which the HGF injection of the present invention is delivered in the case of intrathecal administration, is a space which is located around the spinal cord and filled with cerebrospinal fluid. This space is surrounded by a double-layer membrane consisting of arachnoid  
20 mater and dura mater. The intrathecal space is a space beneath the arachnoid mater, the inner layer of the double-layer membrane, and therefore, intrathecal administration means administration into the subarachnoid space. The space around the brain and the space around the spinal cord are both filled  
25 with cerebrospinal fluid, and the cerebral ventricles in the brain are also filled with cerebrospinal fluid. The cerebral ventricles, the pericerebral space and the intrathecal space are connected to form one continuous space, in which the cerebrospinal fluid circulates. Therefore,



intracerebroventricular administration and intrathecal administration are both administration of a drug into the cerebrospinal fluid. Usually, intracerebroventricular administration and intrathecal administration are the  
5 substantially same administration route. In addition, the HGF preparation of the present invention including the HGF injection can be administered into the cerebral or spinal parenchyma. For intracerebroventricular or intrathecal administration or administration into the cerebral or spinal  
10 parenchyma, the injection may be administered as a bolus or continuously administered as an infusion using a syringe pump etc.

[0048]

The intended use of the HGF preparation of the present  
15 invention is not limited only to the treatment of central nervous system diseases. Since the HGF preparation of the present invention has a sufficient stability for pharmaceutical use and is highly safe, it can be used for the treatment of diseases other than central nervous system diseases as well.  
20 In this case, an administration route suitable for the treatment of the target disease can be selected, and for example, intravenous injection, subcutaneous injection, intramuscular injection, local administration, etc. can be used.

[0049]

25 The dose of the HGF preparation of the present invention can be determined as appropriate according to the kind of the target disease, the disease condition, etc. For example, in the case where the HGF injection is used for the treatment of central nervous system diseases, the daily dose of the HGF



protein is preferably about 0.01 to 50 mg, and more preferably about 0.1 to 10 mg per adult. In this case, the HGF injection is preferably administered intracerebroventricularly or intrathecally. In addition, the HGF preparation of the present invention including the HGF injection may be diluted as appropriate with an appropriate pharmaceutically acceptable solvent before administration. Examples of the pharmaceutically acceptable solvent include distilled water for injection, physiological saline, various kinds of infusion (for example, 5% glucose solution, Ringer's solution, etc.) and artificial spinal fluid. More preferred are distilled water for injection and physiological saline.

[0050]

The present invention also includes a method for treating a central nervous system disease, comprising administering, to a patient with a central nervous system disease, an HGF preparation containing an HGF protein as an active ingredient and lactose, glycine, sodium chloride, a pH buffering agent and a surfactant as additional ingredients.

The present invention also includes an HGF preparation containing an HGF protein as an active ingredient and lactose, glycine, sodium chloride, a pH buffering agent and a surfactant as additional ingredients for use in the treatment of a central nervous system disease.

The HGF preparation is preferably a lyophilized preparation (lyophilized HGF preparation) or an injection (HGF injection), and is more preferably an injection.

The HGF preparation, the lyophilized HGF preparation and the HGF injection, and their preferable embodiments and the like

are as described above. In the treatment of a central nervous system disease, the HGF preparation is preferably administered intrathecally or intracerebroventricularly or administered into the spinal or cerebral parenchyma.

5 [0051]

The present invention also includes a method for preventing the formation of HGF polymers (HGF protein-based polymers) in an aqueous solution containing an HGF protein, the method comprising adding lactose, glycine, sodium chloride, a pH  
10 buffering agent and a surfactant to the aqueous solution. In this method, the HGF protein, the lactose, the glycine, the sodium chloride, the pH buffering agent and the surfactant, and their preferable amounts and the like added to the aqueous solution are the same as described above for the HGF injection.

15 [0052]

The present invention also includes a method for preventing the formation of HGF polymers (HGF protein-based polymers) in a lyophilized preparation containing an HGF protein, the method comprising adding lactose, glycine, sodium chloride, a pH  
20 buffering agent and a surfactant to the lyophilized preparation. In this method, the HGF protein, the lactose, the glycine, the sodium chloride, the pH buffering agent and the surfactant, and their preferable amounts and the like added to the lyophilized preparation are the same as described above for the lyophilized  
25 HGF preparation.

#### EXAMPLES

[0053]

Hereinafter, the present invention will be illustrated in

more detail by examples, but the present invention is not limited thereto.

[0054]

Various kinds of additives were added to a recombinant human  
5 HGF protein (hereinafter referred to simply as HGF) consisting  
of the amino acid sequence represented by SEQ ID NO: 6, and the  
examination of stability and safety was conducted as below. In  
the following examples, the concentration "%" refers to a  
percent by mass unless otherwise specified. The HGF was  
10 prepared using CHO cells according to the method described in  
Biochem. Biophys. Res. Commun. 180: 1151-1158, 1991.

[0055]

<Example 1>

Lactose and glycine were dissolved at the concentrations  
15 shown in Table 1 below in an HGF solution containing 5 mM citrate  
buffer (pH 6.0), 0.375 M sodium chloride and 0.005% polysorbate  
80.

[0056]

[Table 1]

Ingredient	Concentration
HGF	2.5 mg/mL
Lactose	3.0 mg/mL
Glycine	0.4 mg/mL
Trisodium citrate dihydrate/ citric acid hydrate	5 mM (pH 6.0)
Sodium chloride	0.375 M
Polysorbate 80	0.005%

20 [0057]

The prepared solution was aseptically dispensed into 1-mL  
aliquots in vials (diameter 23 × 43 mm). The vials were  
half-plugged with rubber stoppers and aligned on a tray, and  
the tray was placed into a freeze dryer (Triomaster;



manufactured by Kyowa Vacuum Engineering Co., Ltd).  
Preliminary freezing was performed at -50°C, followed by primary  
drying (-50°C → -20°C/4 hours, -20°C/24 hours or longer, 0.01  
to 0.1 Torr) and secondary drying (-20°C → 20 to 30°C/8 to 10  
5 hours, 20 to 30°C/10 hours or longer, 0.01 to 0.1 Torr) to yield  
a lyophilized preparation. After the completion of  
freeze-drying, sterile nitrogen was fed into the drying chamber  
of Triomaster for pressure recovery (target pressure in the  
chamber: 88.0 kPa; primary pressure recovery). After the  
10 primary pressure recovery, the vials were fully plugged with  
the rubber stoppers, and the pressure in the chamber was  
returned to atmospheric pressure by sterile nitrogen supply  
(secondary pressure recovery). The vials were taken out of the  
chamber and immediately after that, sealed with caps. Thus,  
15 the lyophilized HGF preparation of the present invention was  
obtained.

[0058]

<Example 2>

Another lyophilized HGF preparation was obtained in the same  
20 manner as described in Example 1 except that the concentration  
of the lactose was 7.5 mg/mL.

[0059]

<Example 3>

Another lyophilized HGF preparation was obtained in the same  
25 manner as described in Example 1 except that the concentration  
of the lactose was 10 mg/mL.

[0060]

<Example 4>

Lactose and glycine were dissolved at the concentrations

shown in Table 2 below in an HGF solution containing 2 mM citrate buffer (pH 6.0), 0.15 M sodium chloride and 0.002% polysorbate 80, and thus an HGF injection was obtained. The HGF injection with the composition of Table 2 can also be obtained by another method, i.e., by dissolving the lyophilized HGF preparation obtained in Example 2 in 2.5 mL of distilled water for injection.

[0061]

[Table 2]

Ingredient	Concentration
HGF	1.0 mg/mL
Lactose	3.0 mg/mL
Glycine	0.16 mg/mL
Trisodium citrate dihydrate/ citric acid hydrate	2 mM (pH 6.0)
Sodium chloride	0.15 M
Polysorbate 80	0.002%

[0062]

#### 10 Experimental Example 1

To basic ingredients consisting of 10 mg/mL HGF, 10 mM citrate buffer (pH 6.0), 0.3 M sodium chloride and 0.03% polysorbate 80, the additives shown in Table 3 were added to prepare HGF solutions of formulations 1 to 3 (2 mL each). The HGF solutions were each freeze-dried in vials in the same manner as in Example 1 to give lyophilized preparations. Each lyophilized preparation was stored at 50°C for one week for forced deterioration testing, and the polymer content was measured before and after the storage. The results are shown in Table 4.

[0063]

[Table 3]

Formulation No.	Additive	Basic ingredients
1	-	10 mg/mL HGF

2	10 mg/mL purified sucrose	10 mM citrate buffer (pH 6.0)
3	10 mg/mL purified sucrose 5 mg/mL L-alanine	0.3 M sodium chloride 0.03% polysorbate 80

[0064]

The polymer content of the HGF preparation was determined as follows. Each lyophilized preparation was dissolved in 2 mL of distilled water for injection and the resulting HGF solution was analyzed by high performance liquid chromatography (HPLC) under the conditions shown below. From the HPLC results, the area percentage (%) of the polymer (hereinafter referred to as polymer content (%)) was calculated by the following formula 1.

10 [0065]

[Math. 1]

**Polymer content (%) =  $100 \times A_A / (A_M + A_A)$  Formula 1**

[0066]

In formula 1,  $A_M$  stands for the HGF peak area and  $A_A$  stands for the polymer peak area.

[0067]

#### HPLC conditions

Column: gel-filtration column (trade name: Superdex® 200 10/300, manufactured by GE Healthcare)

20 Mobile phase: 58.44 g of sodium chloride, 2.94 g of trisodium citrate dihydrate and 0.1 g of polysorbate 80 were dissolved in purified water, and then purified water was further added to a total volume of 1 L. This solution was designated as solution A. 58.44 g of sodium chloride, 2.10 g of citric acid monohydrate and 0.1 g of polysorbate 80 were dissolved in purified water, and then purified water was further added to a total volume of 1 L. This solution was designated as solution



B. Solution B was added to solution A and the pH of the mixed solution was adjusted to 6.0. The mixed solution was filtered through a 0.45- $\mu$ m filter (trade name: Millicup-HV, pore size: 0.45  $\mu$ m, manufactured by Merck) and degassed before use. The  
5 solution was stored at room temperature and used within two weeks.

Column temperature: 25°C

Flow rate: 0.5 mL/min

Sample injection volume: 25  $\mu$ L

10 Detection wavelength: 280 nm

[0068]

[Table 4]

Formulation No.	Polymer content (%) (mean, n = 3 each)	
	Initial (before storage)	50°C, 1-week storage
1	0.84	6.12
2	0.58	1.29
3	0.56	0.90

[0069]

For the lyophilized preparation produced from the HGF  
15 solution of formulation 1, which contained only the basic ingredients, the storage under severe conditions resulted in remarkable formation of HGF polymers. On the other hand, for the lyophilized preparations produced from the HGF solutions of formulations 2 and 3, polymer formation was prevented even  
20 under the severe conditions. These results demonstrate that purified sucrose or L-alanine has the effect of maintaining lyophilized HGF preparations in a stable condition, as is known so far.

[0070]

25 Experimental Example 2

To basic ingredients consisting of 2.5 mg/mL HGF, 5 mM

citrate buffer (pH 6.0), 0.375 M sodium chloride and 0.005% polysorbate 80, the additives shown in Table 5 were added to prepare HGF solutions of formulations 4 to 6 (1 mL each). The HGF solutions were each freeze-dried in vials in the same manner as in Example 1 to give lyophilized preparations. Each lyophilized preparation was stored at 50°C for one week and the polymer content was measured before and after the storage in the same manner as in Experimental Example 1. The results are shown in Table 6.

10 [0071]

[Table 5]

Formulation No.	Additive	Basic ingredients
4	0.4 mg/mL glycine	2.5 mg/mL HGF 5 mM citrate buffer (pH 6.0) 0.375 M sodium chloride 0.005% polysorbate 80
5	0.4 mg/mL glycine 7.5 mg/mL lactose	
6	0.4 mg/mL glycine 20.0 mg/mL D-sorbitol	

[0072]

[Table 6]

Formulation No.	Polymer content (%) (mean, n = 3)	
	Initial (before storage)	50°C, 1-week storage
4	0.42	4.66
5	0.38	1.91
6	0.29	3.83

[0073]

15 For the lyophilized HGF preparation of formulation 5, which contained glycine and lactose as additives, polymer formation was prevented, indicating high stability of the preparation.

[0074]

### Experimental Example 3

20 In this experimental example, the stability of lyophilized HGF preparations containing glycine and lactose as additives

was examined. To basic ingredients consisting of 2.5 mg/mL HGF, 5 mM citrate buffer (pH 6.0), 0.375 M sodium chloride and 0.005% polysorbate 80, the additives shown in Table 7 were added to prepare HGF solutions of formulations 4, 5, 7 and 8 (1 mL each).

5 The HGF solutions were each freeze-dried in vials in the same manner as in Example 1 to give lyophilized preparations. Each lyophilized preparation was stored at 25°C for 1 or 2 months or at 50°C for 2 weeks, and the polymer content was measured before and after the storage in the same manner as in

10 Experimental Example 1. The results are shown in Table 8.

[0075]

[Table 7]

Formulation No.	Additive	Basic ingredients
4	0.4 mg/mL glycine	2.5 mg/mL HGF 5 mM citrate buffer (pH 6.0) 0.375 M sodium chloride 0.005% polysorbate 80
5	0.4 mg/mL glycine 7.5 mg/mL lactose	
7	0.4 mg/mL glycine 10 mg/mL lactose	
8	0.4 mg/mL glycine 10 mg/mL purified sucrose	

[0076]

[Table 8]

Formulation No.	Polymer content (%) (mean, n = 3)			
	Initial (before storage)	25°C, 1-month storage	25°C, 2-month storage	50°C, 2-week storage
4	0.91	1.81	1.91	5.63
5	0.45	0.63	0.86	1.37
7	0.50	0.69	0.75	1.29
8	0.49	0.67	0.75	0.97

15 [0077]

For the lyophilized preparations produced from the HGF solutions of formulations 5 and 7, which contained glycine and lactose as additives, increase in the polymer content was only



slight even after 2 months of storage at room temperature (25°C). Moreover, even after the storage under severe conditions, i.e., at 50°C for 2 weeks, the polymer content was as low as only slightly more than 1%, indicating that polymer formation was prevented. The effect of preventing polymer formation in the lyophilized preparations produced from the HGF solutions of formulations 5 and 7 was almost comparable to that in formulation 8, in which the additive other than glycine was purified sucrose, which is known to be effective for the stabilization of lyophilized HGF preparations.

[0078]

#### Experimental Example 4

The lyophilized HGF preparation obtained in Example 2 was dissolved in 2.5 mL of distilled water for injection to give an HGF injection with the composition of Table 2. The injection was stored at 40°C for 2 weeks in an airtight container. The polymer content of the HGF injection was measured before and after the storage in the same manner as in Experimental Example 1. The biological activities of HGF in the HGF injection before and after the storage were evaluated using the growth of the mink lung epithelial cell line Mv1Lu (Riken, BRC ID: RCB0996) as an indicator.

The polymer contents in the HGF injection before and after storage were 1.54% and 2.67%, respectively. That is, the increase in the polymer content during 2 weeks of storage at 40°C was only about 1%. The biological activity of HGF in the HGF injection after 2 weeks of storage at 40°C was 89.4% (relative value calculated on the assumption that the activity before the storage was 100%) of that before the storage and was

maintained at a high level. These results show that the HGF injection of the present invention was kept almost stable during 2 weeks of storage at 40°C.

[0079]

5 Test Example 1

Forty-five microliters of a test sample shown in Table 9, i.e., HGF solution 1 or 2, vehicle (vehicle A or B) or physiological saline, was intrathecally administered as a single bolus to rats for examination of the safety for the  
10 central nervous system. On the ground that the volume of spinal fluid in a rat is only about 200  $\mu$ L, if an excessive volume of a solution is administered thereinto as a single bolus, the bolus administration itself may induce abnormalities in rats. Therefore, the maximum permissible volume per rat for single  
15 bolus intrathecal administration was set at 45  $\mu$ L.

The skin in the area of the neck and the back of each rat was shaved with an electric hair clipper under pentobarbital anesthesia. The shaved area was cleaned and disinfected with ethanol for disinfection and ISODINE solution 10% (trade name,  
20 Meiji, Co., Ltd.: 10% povidone iodine solution). An incision was made through the back skin to expose the vertebral section from the 11th thoracic vertebra to the 2nd lumbar vertebra. The ligament between the 12th and 13th thoracic vertebrae was excised to expose the dura mater. A small incision was made  
25 through the exposed dura mater and the arachnoid mater, and the outflow of spinal fluid was confirmed. Immediately after that, the tip of a polyurethane catheter (a two-piece catheter prepared by connecting MRE025 (OD: 0.25 mm, 10 cm) and MRE010 (OD: 0.65 mm, 2.5 cm); Braintree, USA) filled with physiological

saline (Otsuka Pharmaceutical Factory) was inserted about 2.5 cm from the incision into the intrathecal space (toward the head). The catheter was fixed to peripheral tissues with medical Aron Alpha (trade name: Aron Alpha A "Sankyo", DAIICHI SANKYO Company, Limited). In addition, the open end of the catheter was closed by heat sealing, and the outer end of the catheter was exposed with an appropriate length on the cervical skin surface. The incision was closed with a suture. Each rat was kept warm on a heating pad until emergence from anesthesia, and then returned to a breeding cage. On the day following the catheter placement, 45  $\mu$ L of the test sample shown in Table 9, i.e., HGF solution 1 or 2, vehicle (vehicle A or B) or physiological saline, was administered as a single bolus through the retained catheter to the rats in an awake state. Subsequently, 10  $\mu$ L of physiological saline was injected through the retained catheter (for the purpose that the HGF solution or the vehicle which remained in the catheter was pushed into the intrathecal space). The catheter tip was then closed by heat sealing and placed under the skin, and the conditions of the rats were observed.

[0080]



[Table 9]

Test sample	Composition	Administration volume	Number of animals	Observation of rats
Physiological saline		45 $\mu$ L	3	No rats showed any abnormalities.
Vehicle A	10 mM citrate buffer (pH 6.0) 0.3 M sodium chloride 0.05% polysorbate 80 10 mg/mL <b>purified sucrose</b> 5 mg/mL <b>L-alanine</b>	45 $\mu$ L	3	All rats showed reduced locomotor activity, salivation, convulsions, etc. immediately after administration, but returned to normal 1 hour after administration.
Vehicle B	2 mM citrate buffer (pH 6.0) 0.15 M sodium chloride 0.002% polysorbate 80 0.16 mg/mL <b>glycine</b> 3 mg/mL <b>lactose</b>	45 $\mu$ L	6	No rats showed any abnormalities.
HGF solution 1	1 mg/mL HGF 2 mM citrate buffer (pH 6.0) 0.15 M sodium chloride 0.002% polysorbate 80 0.16 mg/mL <b>glycine</b> 3 mg/mL <b>lactose</b>	45 $\mu$ L	3	No rats showed any abnormalities.
HGF solution 2	1 mg/mL HGF 2 mM citrate buffer (pH 6.0) 0.15 M sodium chloride 0.002% polysorbate 80	45 $\mu$ L	3	All rats showed abnormal phonation, limb rigidity, etc. immediately after administration, but returned to normal 20 minutes after

	0.16 mg/mL <b>glycine</b> 4 mg/mL <b>purified</b> <b>sucrose</b>			administration.
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[0081]

The bolus intrathecal administration of physiological saline to the rats did not cause abnormalities. On the other hand, in the case of the intrathecal administration of vehicle  
5 A, which was prepared by blending additives known to have a stabilizing effect on HGF preparations, neurological abnormalities such as reduced locomotor activity, salivation and convulsions were found in the rats, albeit transiently. In contrast, the intrathecal administration of vehicle B, which  
10 was prepared by blending the additives used in the HGF preparation of the present invention, did not cause abnormalities in the rats as in the case of the administration of physiological saline. Vehicle B was used for the preparation of HGF solution 1 (this solution was exactly the same as the  
15 injection of Example 4 and corresponds to an embodiment of the HGF injection of the present invention), and the intrathecal administration of HGF solution 1 did not cause abnormalities in the rats, either. These results demonstrate that the HGF injection of the present invention does not have adverse effects  
20 on the central nervous system and therefore its composition is very safe. On the other hand, in the case of the intrathecal administration of HGF solution 2, which was prepared with a vehicle having the same composition as that of vehicle B except for containing sucrose instead of lactose, neurologically  
25 abnormal conditions such as abnormal phonation and limb rigidity were observed in the rats for about 20 minutes after the administration.

[0082]

Test Example 2



A rat spinal-cord-injury model was prepared, and from immediately after the onset of the injury, repetitive intrathecal administration of an HGF solution was started (45  $\mu$ L/shot, 3 times/week, for 4 weeks) (HGF administration group; n = 6). The HGF solution used was the same as the HGF solution with the composition of Table 2, and was obtained by redissolving the lyophilized preparation of Example 2 in 2.5 mL of distilled water for injection. For the control group, a vehicle (2 mM citrate buffer (pH 6.0), 0.15 M sodium chloride, 0.002% polysorbate 80, 0.16 mg/mL glycine and 3 mg/mL lactose), which did not contain HGF, was similarly administered to spinal-cord-injury model rats (n = 6).

The spinal-cord-injury model rats were prepared as follows. The skin in the area of the neck down to the waist of each rat was shaved with an electric hair clipper under ketamine and xylazine anesthesia. The shaved area was cleaned with 70% alcohol and ISODINE solution 10% (trade name, Meiji, Co., Ltd.: 10% povidone iodine solution). An incision was made through the back skin to expose the vertebral section from the 6th thoracic vertebra to near the 5th lumbar vertebra. The vertebral arches at the 9th and 10th thoracic vertebrae and the ligament therebetween were excised to expose the dura mater. Immediately after that, a 10-g weight was dropped from a height of 25 mm onto the exposed dura mater at the 10th thoracic level using MASCIS Impactor (Rutgers University, USA) to induce spinal cord injury. Immediately after the onset of spinal cord injury, the ligament between the 1st and 2nd lumbar vertebrae was excised to expose the dura mater, and a small incision was made through the dura mater and the arachnoid mater. The

leakage of spinal fluid was confirmed, and immediately after that, the tip of a polyurethane catheter (a two-piece catheter prepared by connecting MRE025 (OD: 0.25 mm, 10 cm, Braintree, USA) and MRE010 (OD: 0.65 mm, 2.5 cm, Braintree, USA)) was  
5 inserted into the intrathecal space (toward the head) until it reached the neighborhood of the site of spinal cord injury. The catheter was fixed to muscle layers with medical Aron Alpha (trade name: Aron Alpha A "Sankyo", manufactured by DAIICHI SANKYO Company, Limited) and retained. After the first shot  
10 of the test solution (HGF solution or vehicle), the outer end of the catheter was exposed with an appropriate length on the cervical skin surface, and the incision was closed with a suture. Each animal was kept warm on a heating pad until emergence from anesthesia, and then returned to a breeding cage. From then  
15 on, the HGF solution or the vehicle was repeatedly administered through the retained catheter 3 times/week for 4 weeks. At every administration, injection of 45  $\mu$ L of the HGF solution or the vehicle was followed by injection of 10  $\mu$ L of physiological saline (for the purpose that the HGF solution or  
20 the vehicle which remained in the catheter was pushed into the intrathecal space). After every administration, the open end of the catheter was closed by heat sealing.

[0083]

The hindlimb motor function of the rats was evaluated over  
25 time using the BBB scale (highest score of 21: a 21-point rating scale of 0 (complete paralysis) to 21 (normal hindlimb movement)) (Basso DM, Beattie MS, Bresnahan JC: A sensitive and reliable locomotor rating scale for open field testing in rats. J Neurotrauma 12: 1-21, 1995) to examine the therapeutic effect

of HGF on spinal cord injury. The BBB score was zero in all the animals on the day following the onset of spinal cord injury. After 4 weeks from the onset of spinal cord injury, however, the mean of the BBB score in the HGF administration group was  
5 recovered to 10 or more, which was significantly higher than the score (mean: less than 10) in the vehicle administration group (control group). These results show that HGF has a therapeutic effect on spinal cord injury. During the entire 4-week administration period, no abnormal conditions except for  
10 spinal cord injury were observed either in the rats of the vehicle administration group (control group) or in the rats of the HGF administration group. The autopsy of the rats after 4 weeks from the onset of spinal cord injury also showed no abnormalities in the spinal cord except for the site of spinal  
15 cord injury. As shown by these results, the HGF solution prepared by redissolving the lyophilized preparation of Example 2 was safe and effective for the treatment of spinal cord injury.

#### INDUSTRIAL APPLICABILITY

20 [0084]

According to the present invention, an HGF preparation which is excellent in storage stability for pharmaceutical use is provided. The HGF injection of the present invention can be administered intrathecally or intracerebroventricularly or  
25 administered into the spinal or cerebral parenchyma for the treatment of various central nervous system diseases such as ALS and spinal cord injury. Therefore, the present invention is useful in the medical field etc.



### CLAIMS

1. A hepatocyte growth factor (HGF) preparation for use in intrathecal or intracerebroventricular administration or administration into spinal or cerebral parenchyma which is obtained by freeze-drying of an aqueous solution comprising an HGF protein as the active ingredient and lactose, glycine, sodium chloride, a pH buffering agent and a surfactant, wherein the content of the lactose is in the range of 1 to 5 parts by weight relative to 1 part by weight of HGF.
2. The HGF preparation according to claim 1, wherein the concentration of the lactose in the aqueous solution is in the range of 0.1 to 100 mg/mL.
3. The HGF preparation according to claim 1 or 2, wherein the concentration of the glycine in the aqueous solution is in the range of 0.05 to 50 mg/mL.
4. The HGF preparation according to any one of claims 1-3, wherein the concentration of the HGF protein in the aqueous solution is in the range of 0.1 to 20 mg/mL.
5. The HGF preparation according to any one of claims 1-4, wherein the pH buffering agent is a combination of citric acid or a hydrate thereof with a salt of citric acid.
6. The HGF preparation according to any one of claims 1-5, wherein the surfactant is polysorbate.
7. The HGF preparation according to any one of claims 1-6, wherein the HGF preparation is for use in treatment of amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, Huntington's disease, spinocerebellar ataxia, spinal cord injury, cerebral infarction, cerebral ischemia or multiple sclerosis.
8. The HGF preparation according to any one of claims 1-7, wherein the HGF preparation is formulated for intrathecal or intracerebroventricular administration or administration into spinal or cerebral parenchyma, by dissolving in a pharmaceutically acceptable solvent.
9. The HGF preparation according to any one of claims 1-8, wherein the HGF protein is a human HGF protein.
10. The HGF preparation according to claim 9, wherein the HGF protein is a protein consisting of an amino acid sequence represented by SEQ ID NO: 5 or SEQ ID NO: 6.
11. The HGF preparation according to any one of claims 1-8, wherein the HGF protein is a protein which has 80% or more sequence identity over the full-length of an amino acid sequence represented by

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SEQ ID NO: 5 and has a biological activity of HGF.

12. A hepatocyte growth factor (HGF) preparation dissolved in a pharmaceutically acceptable solvent to form an HGF solution for use in intrathecal or intracerebroventricular administration or administration into spinal or cerebral parenchyma, wherein the HGF preparation is obtained by freeze-drying of an aqueous solution comprising an HGF protein as the active ingredient and lactose, glycine, sodium chloride, a pH buffering agent and a surfactant, wherein the content of the lactose is in the range of 1 to 5 parts by weight relative to 1 part by weight of HGF, wherein the concentration of the lactose in the aqueous solution is in the range of 0.1 to 100 mg/ml., wherein the concentration of the glycine in the aqueous solution is in the range of 0.1 to 10 mg/mL, wherein the concentration of the HGF protein in the aqueous solution is in the range of 0.1 to 20 mg/mL, wherein the concentration of the pH buffering agent in the aqueous solution is in the range of 1 to 20mM, wherein the pH buffering agent is a combination of citric acid or a hydrate thereof with a salt of citric acid and having the effect of keeping the pH of the HGF solution within the range of 4.5 to 8.0, wherein the surfactant is polysorbate, wherein the concentration of the polysorbate in the aqueous solution is in the range of 0.001 to 2.0% by weight, and wherein the concentration of the sodium chloride in the aqueous solution is in the range of 150 to 1000 mM.

13. The HGF preparation according to claim 12, wherein the HGF preparation is for use in treatment of amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, Huntington's disease, spinocerebellar ataxia, spinal cord injury, cerebral infarction, cerebral ischemia or multiple sclerosis.

14. The HGF preparation according to any one of claims 12-13, wherein the HGF protein is a human HGF protein.

15. The HGF preparation according to claim 14, wherein the HGF protein is a protein consisting of an amino acid sequence represented by SEQ ID NO: 5 or SEQ ID NO: 6.

16. The HGF preparation according to any one of claims 12-14, wherein the HGF protein is a protein which has 80% or more sequence identity over the full-length of an amino acid sequence represented by SEQ ID NO: 5 and has a biological activity of HGF.