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(72) Inventeurs/Inventors:

LEE, JUNG TAE, KR;
KIM, IN HYUK, KR;
YU, JAE KEUN, KR;
YIM, JUNG YIM, KR;
JEONG, MYEONG HYEON, KR;
AHN, YONG HO, KR

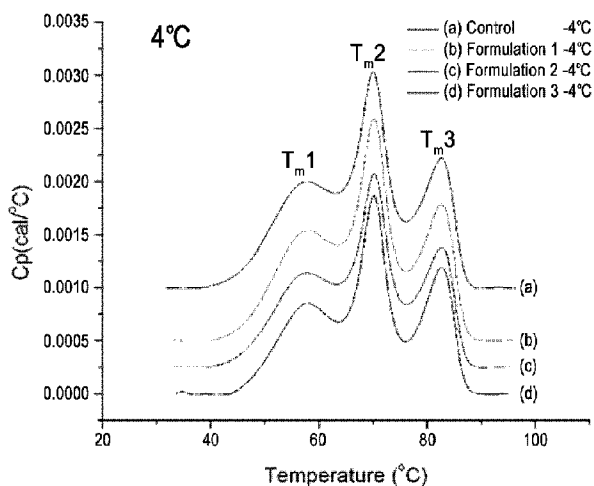
(73) Propriétaire/Owner:

ARES TRADING S.A., CH

(74) Agent: KIRBY EADES GALE BAKER

(54) Titre : FORMULATION LIQUIDE D'UNE PROTEINE DE FUSION COMPRENANT LE TNFR ET UNE REGION FC

(54) Title: A LIQUID FORMULATION OF A FUSION PROTEIN COMPRISING TNFR AND FC REGION



(57) **Abrégé/Abstract:**

Disclosed is a liquid formulation comprising a TNFR-Fc fusion protein and a stabilizer, in which the fusion protein comprises TNFR (tumor necrosis factor receptor) or a fragment thereof and an immunoglobulin Fc region, and the stabilizer comprises one or more amino acids selected from the group consisting of proline and histidine, a buffer solution, and an isotonic agent containing sodium chloride (NaCl) and sucrose, and a preparation method of the liquid formulation. The liquid formulation according to the present invention provides excellent storage stability because long-term storage of TNFR-Fc fusion protein (etanercept) is possible and particular storage conditions are not needed. Since the liquid formulation of the present invention shows excellent storage stability even though the formulation is simple, it is more economical than other stabilizers or lyophilized formulations, and thus the formulation can be effectively applied for uses wherein treatment of TNFR-Fc fusion protein (etanercept) is beneficial.

Abstract

Disclosed is a liquid formulation comprising a TNFR-Fc fusion protein and a stabilizer, in which the fusion protein comprises TNFR (tumor necrosis factor receptor) or a fragment thereof and an immunoglobulin Fc region, and the stabilizer comprises one or more amino acids selected from the group consisting of proline and histidine, a buffer solution, and an isotonic agent containing sodium chloride (NaCl) and sucrose, and a preparation method of the liquid formulation. The liquid formulation according to the present invention provides excellent storage stability because long-term storage of TNFR-Fc fusion protein (etanercept) is possible and particular storage conditions are not needed. Since the liquid formulation of the present invention shows excellent storage stability even though the formulation is simple, it is more economical than other stabilizers or lyophilized formulations, and thus the formulation can be effectively applied for uses wherein treatment of TNFR-Fc fusion protein (etanercept) is beneficial.

A liquid formulation of a fusion protein comprising TNFR and Fc region

Technical Field

The present invention relates to a liquid formulation comprising a TNFR-Fc fusion protein and a stabilizer, in which the fusion protein comprises TNFR (tumor necrosis factor receptor) or a fragment thereof and an immunoglobulin Fc region, and the stabilizer comprises one or more amino acids selected from the group consisting of proline and histidine, a buffer solution, and an isotonic agent containing sodium chloride (NaCl) and sucrose, and a preparation method of the liquid formulation.

Background Art

In general, chemical and physical denaturation of protein drugs can be easily caused by unfavorable temperature, shear stress, vibration, freeze-thawing, UV exposure, excessive pH change, organic solvents, microbial contamination, etc. Chemical denaturation includes dimer dissociation, oxidation, deamidation, isomerization, and polymerization, which are influenced by the amino acids constituting the protein and conditions of the solvent containing the protein (salt, pH and temperature). Physical denaturation includes loss of tertiary structure, covalent/non-covalent aggregation and adhesion of monomers, which are influenced by hydrophobic patches on the protein surface changed by protein-containing surrounding environments such as solvents, complex protein structures such as charge distribution, and thermal stability.

The physical or chemical denaturation of a protein including an antibody causes loss of its physiological activities. Since the denaturation is an irreversible process, proteins, once denatured, may not recover their native properties, leading to a reduction in their therapeutic efficacies. It has been also suggested that phenomenon such as aggregation of monomers causes immune responses. Therefore, many studies have been conducted on formulations containing a physiologically effective amount of protein without aggregates (Ishikawa et al., *Biol. Pharm. Bull.*, 33(8): 1413-1417, 2010).

There are many methods available for preventing protein denaturation in liquid formulations. In some protein drugs, the stability problems are addressed via lyophilization. However, the lyophilization process causes stress related to freezing and drying, such as formation of ice crystals, pH change, and high concentration of solute, and these stresses may cause protein denaturation. In addition, since a large-capacity lyophilizer is needed for the lyophilization process during production, high production costs arise during large scale

production. Dissolving the lyophilized product in sterile aqueous media before use also poses an inconvenience.

As an alternative to solve these limitations, a stabilizer is added to liquid formulations for the improvement of protein stability. Surfactants, serum albumins, polysaccharides, amino acids, polymers, salts or the like are known as stabilizers used in protein drugs (Wang, *Int. J. Pharm.*, 185: 129-188, 1999; Wang et al., *J. Pharm. Sci.*, 96(1): 1-26, 2007).

In order to prepare stable formulations of the drugs, however, appropriate stabilizers should be used considering the physicochemical properties of each active ingredient. When the stabilizers are used in combination, competition therebetween and adverse effects may lead to undesirable effects. In addition, the concentrations of proteins should be within the range suitable for the stabilization, and in particular, to prepare high concentrations of protein drugs, much effort and caution is required to stabilize proteins in solutions (Shire et al., *J. Pharm. Sci.*, 93(6): 1390-1402, 2004).

Meanwhile, etanercept is a biological modulator that functions as a competitive inhibitor of TNF- α , binding to cell surface TNF- α receptor, to inhibit TNF- α mediated immune responses. Etanercept is a macromolecule with a molecular weight of 150 kDa, and is a homodimer of two fusion proteins linked by disulfide bond, each fusion protein consisting of a human soluble p75 TNF (tumor necrosis factor) receptor coupled to the Fc portion of human immunoglobulin G subclass 1 (Goldenberg, *Clinical Therapeutics*, 21(1): 75-87, 1999; Moreland et al., *Ann. Intern. Med.*, 130(6): 478-486, 1999).

This is marketed by Amgen under the trademark of EnbrelTM in 2002. Etanercept is a TNF- α inhibitor used to treat rheumatoid arthritis, psoriasis, and ankylosing spondylitis, and is under clinical trials for the treatment of vasculitis, Alzheimer's disease, and Crohn's disease.

Formulation stabilization technologies for TNFR-Fc fusion proteins such as etanercept aim to develop a liquid formulation for minimizing protein denaturation which may occur during production, storage, and transportation, and maintaining their activity for a long period of time to be identical to that of conventional protein drugs, but it has been difficult to develop satisfactory liquid formulations. Therefore, there is an urgent need to develop a new liquid formulation which is able to stably maintain the activity of TNFR-Fc fusion protein (etanercept) for a long period of time and which is more effective in stabilization of TNFR-Fc fusion protein (etanercept) than the known formulations, including arginine as disclosed in US Patent No. 7,648,702.

Disclosure of Invention**Summary**

Certain exemplary embodiments provide a liquid formulation comprising a TNFR-Fc fusion protein, a mixture of proline and histidine, a buffer solution, and an isotonic agent.

Technical Problem

Accordingly, the present inventors have made many efforts to develop a method for preparing a liquid formulation capable of stably maintaining the activity of etanercept. The present invention provides that a stabilizer comprising one or more amino acids selected from the group consisting of proline and histidine shows remarkable effects on the stabilization of etanercept in a solution, even when used at a lower concentration than the concentrations used for known stabilizers comprising single amino acid or mixed amino acids, thereby completing the present invention.

Solution to Problem

An object of the present invention is to provide a stable liquid formulation comprising a TNFR-Fc fusion protein and a stabilizer, in which the TNFR-Fc fusion protein is a fusion protein comprising TNFR (tumor necrosis factor receptor) or a fragment thereof and an immunoglobulin Fc region, and the stabilizer comprises one or more amino acids selected from the group consisting of proline and histidine, a buffer solution, and an isotonic agent, preferably containing sodium chloride (NaCl) and sucrose.

Another object of the present invention is to provide a preparation method of the liquid formulation.

Advantageous Effects of Invention

A liquid formulation according to the present invention provides excellent storage stability because long-term storage of TNFR-Fc fusion protein (etanercept) is possible and particular storage conditions are not needed. Since the liquid formulation of the present invention shows excellent storage stability even though the formulation is simple, it is more economical than other stabilized or lyophilized formulations, and thus the formulation can be effectively used in treatments with TNFR-Fc fusion protein (etanercept).

Brief Description of Drawings

Figure 1, shows the amino acid sequence (SEQ ID No. 1) for the p75 sTNFR-Fc fusion protein (etanercept).

Figure 2, shows the Differential Scanning Calorimetry (DSC) graphs for the formulations 1, 2, 3, and control as described in Example 3 at 4 °C (figure 2a) and 25 °C

(figure 2b). DSC thermogram at 4°C (2a) and 25°C (2b) of etanercept formulations containing either Arginine and Histidine (formulation 1) or Proline and Histidine (formulations 2 and 3) at the 6 month time point according to storage temperature.

Best Mode for Carrying out the Invention

In one aspect to achieve the above objects, the present invention provides a liquid formulation comprising a TNFR-Fc fusion protein and a stabilizer, in which the fusion protein comprises TNFR (tumor necrosis factor receptor) or a fragment thereof and an immunoglobulin Fc region, and the stabilizer comprises one or more amino acids selected from the group consisting of proline and histidine, a buffer solution, and an isotonic agent, preferably containing sodium chloride (NaCl) and sucrose.

The fusion protein comprising TNFR or a fragment thereof and immunoglobulin Fc region may be etanercept (recombinant p75 sTNFR-Fc fusion protein), in particular, represented by an amino acid sequence of SEQ ID NO. 1. Further, the fusion protein may be a mutated fusion protein which is prepared by amino acid substitution, deletion or insertion in the amino acid sequence of SEQ ID NO. 1, or a peptide analogue showing an activity similar to that of etanercept. The liquid formulation of the present invention is a stabilized liquid formulation or a stable liquid formulation, which comprises one or more amino acids selected from the group consisting of proline and histidine or a pharmaceutically acceptable salt thereof, thereby reducing by-product formation of TNFR-Fc fusion protein (etanercept) and stably maintaining its activity for a long period of time.

As used herein, the term “fusion protein comprising TNFR and immunoglobulin Fc region” refers to a recombinant protein prepared by fusion of tumor necrosis factor receptor (TNFR) or a fragment thereof and an immunoglobulin Fc region, and in particular, the immunoglobulin Fc region may be derived from an immunoglobulin Fc region of IgG1. Preferably, the fusion protein may be etanercept, and represented by the amino acid sequence of SEQ ID NO. 1.

As used herein, the term “etanercept (recombinant p75 sTNFR:Fc fusion protein)” refers to a protein which is a homodimer form of two fusion proteins linked by disulfide bonds, each fusion protein consisting of a human soluble 75 kilodalton (p75) TNF receptor coupled to the Fc portion of human immunoglobulin G (IgG1) subclass 1.

More specifically, etanercept is a homodimer form of two fusion proteins linked by 3 disulfide bonds, each fusion protein consisting of the extracellular ligand-binding portion of the

human soluble p75 TNF receptor linked to the Fc portion of human IgG1. The Fc component of etanercept contains a CH2 domain, a CH3 domain and a hinge region, but not a CH1 domain of IgG1. The etanercept may have a molecular weight of approximately 150 kDa. This etanercept may be currently sold under the trade name ENBREL® (Amgen Inc., Thousand Oaks, CA.), and have CAS number 185243-69-0.

The fusion protein comprising TNFR or a fragment thereof and immunoglobulin Fc region of the present invention, in particular, etanercept may be produced by recombinant DNA technology in a cell expression system, but is not limited thereto.

The etanercept of the present invention is a biological modulator that functions as a competitive inhibitor of TNF- α , binding to cell surface TNF- α receptor, to inhibit TNF- α mediated immune responses, and is used to treat rheumatoid arthritis, psoriasis, and ankylosing spondylitis, and is under clinical trials for the treatment of vasculitis, Alzheimer's disease, and Crohn's disease.

In the liquid formulation of the present invention, the fusion protein comprising TNFR or a fragment thereof and immunoglobulin Fc region of the present invention, in particular, etanercept may be included in a pharmaceutically effective amount, and in an amount of 1 to 100 mg/mL, preferably 20 to 55 mg/mL, and more preferably 30 to 55 mg/mL.

As used herein, the term "stabilized liquid formulation" or "stable liquid formulation" refers to a formulation that retains the physical and chemical identity and integrity of the therapeutically active ingredient, that is, the fusion protein of the present invention, in particular, etanercept, during storage in a solution. An analytical measurement of the stability of the fusion protein of the present invention, in particular, etanercept may be performed by protein stability assay widely known in the art. The stability may be measured at a predetermined temperature for a predetermined time. For rapid assay, the formulation may be stored at a higher or "elevated" temperature, for example, 40°C for 2 weeks to 1 month or longer, and its time-dependent stability is measured at this time.

As used herein, the term "stabilizer" refers to a specific chemical compound or a composition that interacts with a biological molecule and/or a general pharmaceutical excipient in a formulation to improve its stability. The stabilizer generally protects proteins from air/solution interface-induced stress and solution/surface-induced stress which cause protein aggregation. In the present invention, the stabilizer is a component that reduces by-product formation of the fusion protein of the present invention, in particular, etanercept, during storage

in a solution to maintain its activity for a long period of time. Preferably, the stabilizer comprises one or more amino acids selected from the group consisting of proline and histidine.

The amino acid of the present invention may include both L-amino acids and D-amino acids.

As the stabilizer of the present invention, not only the amino acids themselves such as proline and histidine, but also analogues, solvates, hydrates, stereoisomers, and pharmaceutically acceptable salts thereof are within the scope of the present invention as long as they show the substantially identical effect.

As used herein, the term “by-product” refers to an undesirable product that lowers or reduces the ratio of the therapeutically active ingredient, TNFR-Fc fusion protein (etanercept), in the formulation. The typical by-product include “low molecular weight products” resulting from denaturation of the TNFR-Fc fusion protein (etanercept) by deamination or hydrolysis, “high molecular weight products” such as oligomers and aggregates, or mixtures thereof.

As used herein, the term “high molecular weight products” includes TNFR-Fc fusion protein (etanercept) fragments that are subsequently aggregated by denaturation (e.g., produced by polypeptide degradation due to deamination or hydrolysis) or mixtures thereof. Typically, the high molecular weight products are complexes having higher molecular weights than the therapeutic monomer TNFR-Fc fusion protein (etanercept), and may have a molecular weight of more than approximately 150 kDa.

As used herein, the term “low molecular weight products” includes, for example, therapeutic polypeptides produced by deamination or hydrolysis, namely, TNFR-Fc fusion protein (etanercept) fragments. Typically, the low molecular weight products are complexes having lower molecular weights than the therapeutic monomer TNFR-Fc fusion protein (etanercept), and may have a molecular weight of less than approximately 150 kDa.

The liquid formulation of the present invention comprises one or more amino acids, selected from the group consisting of proline and histidine, as stabilizers. Other additional amino acids such as for example glutamic acid, may be added to stabilize the formulation. Preferably, the liquid formulation comprises proline or histidine alone, or both amino acids of proline and histidine. In the combination, the pharmaceutically acceptable salts of proline or histidine may be further included, or modified forms of proline and histidine or their pharmaceutically acceptable salts may be included. Preferably, the liquid formulation comprises a mixture of amino acids of proline and histidine.

In the present invention, the proline concentration may be in the range from 1 to 10 mM, preferably from 1 to 9 mM, more preferably from 3 to 9mM, even more preferably from 3 to 6 mM, and the histidine concentration may be in the range from 0.01 to 5 mM, preferably from 0.01 to 0.1 mM, more preferably from 0.03 to 0.1mM, even more preferably from 0.03 to 0.09mM, even more preferably from 0.06 to 0.09mM, or even more preferably about 0.09mM in the stabilizer. Specifically, in a mixture of the amino acids of proline and histidine included as a stabilizer, the proline concentration may be in the range from 1 to 10 mM, preferably from 1 to 9 mM, more preferably from 3 to 9mM, even more preferably from 3 to 6mM, and the histidine concentration may be in the range from 0.01 to 5 mM, preferably from 0.01 to 0.1 mM, more preferably from 0.03 to 0.1mM, even more preferably from 0.03 to 0.09mM, even more preferably from 0.06 to 0.09mM, or even more preferably about 0.09mM. The addition of additional amino acids, such as glutamic acid, may be in the range from 1 to 20 mM, preferably from 5 to 15 mM, more preferably from 10 to 15 mM. When combined with the mixture of proline and histidine, the concentration of glutamic acid may be in the range from 1 to 20 mM, preferably from 5 to 15 mM, more preferably from 10 to 15 mM.

In one specific embodiment of the present invention, in the preparation of the stabilized liquid formulation for TNFR-Fc fusion protein (etanercept), liquid formulations were prepared by varying the amino acid combination and conditions of the isotonic agent in order to develop a liquid formulation which has advantages over known liquid formulations, including arginine, as the stabilized liquid formulation of etanercept (US Patent No. 7,648,702), and their stabilizing effects were examined.

As a result, the formulations comprising one or more amino acids selected from the group consisting of proline and histidine were found to show remarkable effects of stabilizing etanercept in a solution by preventing its denaturation during storage at a high temperature (Formulation 4 and Formulation 5, Formulation 7 and Formulation 8 of Table 1, Formulation 2 and Formulation 3 of Table 4, Formulation 2 to Formulation 4 and Formulation 6 to Formulation 7 of Table 7). In particular, the formulations comprising a mixture of the amino acids of proline and histidine (Formulation 5 and Formulation 8 of Table 1, Formulation 2 and Formulation 3 of Table 4, Formulation 2 to Formulation 4 and Formulation 6 to Formulation 7 of Table 7) showed more excellent stabilizing effect than the control formulation.

That is, the present invention first demonstrated that a stabilizer comprising one or more amino acids selected from the group consisting of proline and histidine shows remarkable effects on the stabilization of etanercept in a solution, even when used at a lower concentration (6.1

mM) than the concentration (10 mM or more) of the known stabilizers using single amino acid or mixture of amino acids.

The liquid formulation according to the present invention may further comprise any material which is generally contained in liquid formulations of protein drugs or antibody drugs, as long as it does not deteriorates the function of improving stability of TNFR-Fc fusion protein (etanercept) by adding a stabilizer comprising one or more amino acids selected from the group consisting of proline and histidine.

As used herein, the term “buffer solution” refers to the component that improves isotonicity and chemical stability of the formulation, and functions to maintain physiologically suitable pH. The buffer prevents a rapid pH change of the liquid formulation to maintain pH of the solution for the stabilization of TNFR-Fc fusion protein (etanercept). Preferred examples of the buffer solution include an aqueous solution of citrate, phosphate, succinate, tartrate, fumarate, gluconate, oxalate, lactate, acetate, histidine, or Tris, etc., but are not limited thereto. In the specific embodiment, the buffer is an aqueous citrate-phosphate buffer solution. The buffer may be used either alone or in combinations of two or more thereof.

The liquid formulation of the present invention may have pH of approximately 5 to 7.5 or pH of approximately 5.8 to 6.8. In particular, the formulation has pH of approximately 6.0 to 6.6. If necessary, pH may be adjusted by techniques known in the art. The buffer solution may exist at a concentration of 0.1 to 100 mM, preferably 1 to 50 mM, and more preferably, 10 to 35 mM. Preferably, water for injection is used in the aqueous buffer solution of the present invention.

As used herein, the term “isotonic agent” refers to a component that functions to partially maintain isotonicity of the formulation and the protein level, and partially maintain the level, ratio, or proportion of the therapeutically active polypeptide present in the formulation. The isotonic agent maintains the same osmotic pressure as blood plasma, and so can be intravenously injected into a subject without changing the osmotic pressure of the subject's blood plasma. In one embodiment according to the present invention, the osmotic pressure is suitable for intravenous injection of the formulation of the present invention. Often, the isotonic agent serves as a bulking agent as well. As such, the isotonic agent may allow the protein to overcome various stresses such as freezing and shear.

The isotonic agent serves to maintain the proper osmotic pressure in the body, when TNFR-Fc fusion protein (etanercept) in the solution is administered into the body. Examples of the isotonic agent may include the commonly used sodium chloride, potassium chloride, boric acid, sodium borate, mannitol, glycerin, propylene glycol, polyethylene glycol, maltose, sucrose, erythritol, arabitol, xylitol, sorbitol, glucose, etc., but are not limited thereto. These isotonic agents may be used either alone or in combinations of two or more thereof. In the present invention, the isotonic agents may be those comprising sodium chloride and sucrose. The isotonic agent of the present invention allows the solution to maintain an osmotic pressure of 250 to 350 mOsm, and it may comprise preferably 1 to 1000 mM sodium chloride and/or 0.01 to 3% sucrose, more preferably 50 to 250 mM sodium chloride and/or 0.01 to 1.5% sucrose, and even more preferably 105 to 150 mM sodium chloride and/or 0.01 to 1.5% sucrose. When sodium chloride and sucrose are used in the formulation of the present invention, the sodium chloride concentration can be from 1 to 1000 mM, preferably from 50 to 250 mM, more preferably from 100 to 150 mM, even more preferably from 105 to 150 mM, yet even more preferably from 115 to 140 mM, even more preferably from 115 to 130 mM, and the amount of sucrose in the formulation can be from 0.01 to 3% by weight of the total formulation, preferably from 0.01 to 1.5 weight %, more preferably from 0.1 to 1.0 weight %, even more preferably the amount of sucrose in the formulation is selected from 0.1 weight %, 0.5 weight %, and 1.0 weight %.

In one specific embodiment of the present invention, as the isotonic agent in the liquid formulation of the present invention, sodium chloride and sucrose were used, and formulations comprising 115 mM sodium chloride and 1.0% sucrose (Formulation 1 to Formulation 5 of Table 1) or formulations comprising 115 mM sodium chloride and 0.5% sucrose (Formulation 1 to Formulation 4 of Table 7) and formulations comprising 140 mM sodium chloride and 0.1% sucrose (Formulation 6 to Formulation 8 of Table 1) or formulations comprising 130 mM sodium chloride and 0.1% sucrose (Formulation 5 to Formulation 7 of Table 7) were prepared and their stabilizing effects were compared with that of the control formulation (100 mM of sodium chloride, 1.0% sucrose).

As a result, compared to the known etanercept formulation, the formulations of the present invention using the isotonic agents which were prepared by varying concentrations of sucrose and sodium chloride were found to show improvement in the etanercept stability due to the increased sodium chloride concentration and the reduced sucrose concentration in the isotonic agent.

The liquid formulation according to the present invention may further comprise a pharmaceutically acceptable excipient, and examples of the excipient may include sugars and polyols, surfactants, polymers or the like. Examples of the sugars and polyols may include sucrose, trehalose, lactose, maltose, galactose, mannitol, sorbitol, glycerol, etc., examples of the surfactants may include non-ionic surfactants such as polysorbate 20, polysorbate 80, poloxamer, etc., and examples of the polymers may include dextran, polyethylene glycol, carboxyl methylcellulose, hyaluronic acid, cyclodextrin, etc.

The liquid formulation according to the present invention may further comprise a preservative. The preservative refers to a chemical that is added to pharmaceutical formulations as an antimicrobial agent. Examples of the preservative may include benzalkonium chloride, benzethonium, chlorhexidine, phenol, m-cresol, benzyl alcohol, methylparaben, propylparaben, chlorobutanol, o-cresol, p-cresol, chloro-cresol, phenylmercuric nitrate, thimerosal, benzoic acid, etc., but are not limited thereto. These preservatives may be used either alone or in combinations of two or more thereof.

The liquid formulation of the present invention may be a stable liquid formulation comprising TNFR-Fc fusion protein in a pharmaceutically effective amount and the stabilizer, in which the TNFR-Fc fusion protein ("etanercept") is represented by the amino acid sequence of SEQ ID NO. 1, and the stabilizer comprises a citrate-phosphate buffer solution, sodium chloride, sucrose, and a mixture of amino acids of proline and histidine. In particular, the liquid formulation of the present invention may be a liquid formulation comprising 30 to 55 mg/mL of TNFR-Fc fusion protein, and 10 to 35 mM citrate-phosphate buffer solution, 105 to 125 mM NaCl, 0.5 to 1.5% sucrose, 1 to 9 mM proline and 0.01 to 0.1 mM histidine at pH 6.3; or a liquid formulation comprising 30 to 55 mg/mL of TNFR-Fc fusion protein, and 10 to 35 mM citrate-phosphate buffer solution, 120 to 150 mM NaCl, 0.01 to 0.5% sucrose, 1 to 9 mM proline and 0.01 to 0.1 mM histidine at pH 6.3.

In specific embodiments of the present invention, the following liquid formulations are provided.

- Liquid formulation comprising 50 mg/mL of TNFR-Fc fusion protein, 25 mM citrate-phosphate buffer solution, 115 mM NaCl, 0.5% sucrose, 6 mM proline and 0.09 mM histidine at pH 6.3 (Formulation 3 of Table 7).

- Liquid formulation comprising 50 mg/mL of TNFR-Fc fusion protein, 25 mM citrate-phosphate buffer solution, 130 mM NaCl, 0.1% sucrose, 6 mM proline and 0.09 mM histidine at pH 6.3 (Formulation 7 of Table 7).

- Liquid formulation comprising 50 mg/mL of TNFR-Fc fusion protein, 25 mM citrate-phosphate buffer solution, 100 mM NaCl, 1.0% sucrose, 10 mM proline, 5 mM histidine, and 10 mM Glutamic acid at pH 6.3 (Formulation 10 of Table 1).

The formulation of the present invention can be used for treatment of a disease in which etanercept is therapeutically effective. Etanercept is a biological modulator to inhibit TNF- α mediated immune responses, and the formulation of the present invention can be used to treat rheumatoid arthritis, psoriasis, ankylosing spondylitis, vasculitis, Alzheimer's disease, or Crohn's disease, but is not limited thereto.

The formulation according to the present invention may be administered into the body via oral or parenteral route, i.e., subcutaneous, intramuscular, intraperitoneal, intrasternal, transdermal, and intravenous injection, but is not limited thereto.

In another aspect, the present invention provides a preparation method of the liquid formulation.

The liquid formulation of the present invention may be prepared by a method comprising the steps of preparing etanercept; and mixing etanercept prepared in the above step with a stabilizer comprising one or more amino acids selected from the group consisting of proline and histidine, a buffer solution, and an isotonic agent, preferably containing sodium chloride (NaCl) and sucrose. In the above described preparation method the mixing step can comprise exchanging the solution in which etanercept is prepared with a solution comprising the stabilizer.

In still another aspect, the present invention provides a pharmaceutical composition comprising the liquid formulation for prevention or treatment of disorders in which TNF α activity is detrimental.

The liquid formulation is the same as described above.

As used herein, the term "disorders in which TNF α activity is detrimental" include diseases and other disorders in which the presence of TNF α in a subject suffering from the disorder has been shown to be or is suspected of being either responsible for the pathophysiology of the disorder or a factor that contributes to a worsening of the disorder. In the present

invention, examples of the disorders in which TNF α activity is detrimental may include inflammatory diseases such as sepsis, autoimmune diseases, in particular, rheumatoid arthritis, rheumatoid spondylitis, osteoarthritis, gouty arthritis, allergy, multiple sclerosis, autoimmune diabetes, autoimmune uveitis, and nephrotic syndrome, infectious diseases, allograft rejection and graft-versus-host disease (GVHD), malignant tumors, pulmonary disorders, intestinal disorders, cardiac disorders, etc., but are not limited thereto.

The disorders in which TNF α activity is detrimental can be prevented or treated by administering the composition of the present invention into a subject.

As used herein, the term “treatment” refers to any action resulting in improvements in symptoms of the disorders in which TNF α activity is detrimental or the beneficial alteration of the disorders in which TNF α activity is detrimental owing to the administration of the composition of the present invention, and the term “prevention” refers to any action resulting in the suppression or delay of the onset of the disorders in which TNF α activity is detrimental owing to the administration of the composition. The prevention or treatment can be applied to any mammal having the disorders in which TNF α activity is detrimental, for example, humans and primates as well as livestock such as cow, pig, sheep, horse, dog, cat, etc. without limitation, and preferably humans.

As used herein, the term “administration” refers to the introduction of a predetermined amount of substance into a patient by a certain suitable method. The administration route of the composition may be any of the common routes, as long as it is able to reach a desired tissue. The administration route may be intraperitoneal, intravenous, intramuscular, subcutaneous, intracutaneous, oral, topical, intranasal, intrapulmonary, or rectal route, but is not limited thereto. However, since peptides are digested upon oral administration, the active ingredient of a composition for oral administration should be preferably coated or formulated for protection against degradation in the stomach. Preferably, it may be administered in an injectable formulation. In addition, the composition may be administered with the aid of any device that helps transmit of the active ingredient into target cells.

Further, the pharmaceutical composition of the present invention is determined depending on the kind of the active ingredient, together with various related factors such as the disease to be treated, the route of administration, the patient’s age, sex, and body weight, and severity of the disease.

Further, the pharmaceutical composition of the present invention may comprise a pharmaceutically acceptable carrier. As used herein, the term “pharmaceutically acceptable

carrier” refers to a carrier or diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. For oral administration, a binder, a lubricant, a disintegrator, an excipient, a solubilizer, a dispersing agent, a stabilizer, a suspending agent, a coloring agent, a perfume or the like may be used. For injectable administration, a buffering agent, a preserving agent, an analgesic, a solubilizer, an isotonic agent, a stabilizer or the like may be used in combination. For topical administration, a base, an excipient, a lubricant, a preserving agent or the like may be used. The pharmaceutical composition of the present invention may be prepared into various formulations by mixing it with the above described pharmaceutically acceptable carriers. For example, for oral administration, the pharmaceutical composition may be prepared in the form of a tablet, a troche, a capsule, an elixir, a suspension, a syrup, a wafer or the like. The injectable composition may be formulated in unit dosage ampule or multi dosage form. It can be also prepared as a solution, a suspension, a tablet, a pill, a capsule, a sustained release formulation or the like.

The liquid formulation of the present invention comprises a therapeutically effective amount of TNFR-Fc fusion protein (etanercept). For example, a therapeutically effective amount of etanercept(Enbrel) is generally 25 to 100 mg per week.

In still another aspect, the present invention provides a method for preventing or treating disorders in which TNF α activity is detrimental, comprising the step of administering a composition comprising the liquid formulation into a subject having the disorder in which TNF α activity is detrimental.

Since the composition comprising the liquid formulation of the present invention is able to stably maintain high concentration of TNFR-Fc fusion protein (etanercept) for a long period of time, long-term storage is possible and particular storage conditions are not needed. Therefore, it can be widely used for preventing or treating disorders in which TNF α activity is detrimental.

One embodiment of the invention is a liquid formulation comprising a TNFR-Fc fusion protein, one or more amino acids, a buffer solution, and an isotonic agent, wherein the fusion protein comprises TNFR (tumor necrosis factor receptor) or a fragment thereof and an immunoglobulin Fc region, and the one or more amino acids are selected from the group consisting of proline and histidine and glutamic acid.

A second embodiment is the liquid formulation according to first embodiment, wherein the fusion protein is etanercept.

A third embodiment is the liquid formulation according to first embodiment, wherein the fusion protein is has the amino acid sequence of SEQ ID NO. 1.

A fourth embodiment is the liquid formulation according to the first embodiment, wherein the fusion protein is a mutated fusion protein prepared by amino acid substitution, deletion or insertion in the amino acid sequence of SEQ ID NO. 1, or a peptide analogue showing an activity similar to that of etanercept.

A fifth embodiment is the liquid formulation according to any one of the preceding embodiments, wherein the isotonic agent allows the liquid formulation to maintain an osmotic pressure of 280 to 350 mOsm.

A sixth embodiment is the liquid formulation according to any one of the preceding embodiments, wherein the isotonic agent contains sodium chloride (NaCl) and sucrose.

A seventh embodiment is the liquid formulation according to the sixth embodiment, wherein sodium chloride is present at a concentration of 1 to 1000 mM and sucrose is present in an amount of 0.01 to 3% by weight of the total composition.

An eight embodiment is the liquid formulation according to seventh embodiment, wherein sodium chloride is present at a concentration of 105 mM to 150 mM, and sucrose is present in an amount of 0.01 to 1.5% by weight of the total composition.

A ninth embodiment is the liquid formulation according to any one of the preceding embodiment, wherein the concentration of the fusion protein in the liquid formulation is in the range from 20 to 55 mg/mL.

A tenth embodiment is the liquid formulation according to any one of the preceding embodiments, wherein the buffer solution is a citrate-phosphate or phosphate buffer solution.

An eleventh embodiment is the liquid formulation according to the tenth embodiment, wherein the concentration of the buffer solution is in the range from 10 to 35 mM.

A twelfth embodiment is the liquid formulation according to any one of the preceding embodiments, wherein pH of the liquid formulation is in the range from 6.0 to 6.6.

A thirteenth embodiment is the liquid formulation according to any one of the preceding embodiments, wherein the formulation comprises proline at a concentration of less than 10 mM or histidine at a concentration of less than 5 mM.

A fourteenth embodiment is the liquid formulation according to the thirteenth embodiment, wherein the formulation comprises proline at a concentration of less than 9 mM or histidine at a concentration of less than 0.1 mM.

A fifteenth embodiment is the liquid formulation according to any one of first to fourteenth embodiments, wherein the formulation comprises a mixture of proline and histidine.

A sixteenth embodiment is the liquid formulation according to the fifteenth embodiment, wherein proline is present at a concentration from 1 mM to 9 mM and histidine is present at a concentration of less than 0.1 mM.

A seventeenth embodiment is the liquid formulation according to the sixteenth embodiment, wherein proline is present at a concentration of 1 mM to 9 mM and histidine is present at a concentration of 0.01 to 0.09 mM.

An eighteenth embodiment is the liquid formulation according to seventeenth embodiment, wherein proline is present at a concentration of 6 mM and histidine is present at a concentration of 0.09 mM.

A nineteenth embodiment is the liquid formulation according to any one of the first to thirteenth embodiments, further comprising glutamic acid at a concentration of 1 mM to 20 mM.

A twentieth embodiment is the liquid formulation according to nineteenth embodiment, wherein glutamic acid is present at a concentration of 10 to 15 mM.

A twenty first embodiment is a liquid formulation comprising TNFR-Fc fusion protein in a pharmaceutically effective amount, a citrate-phosphate buffer solution, sodium chloride,

sucrose, and a mixture of amino acids comprising proline and histidine, wherein the TNFR-Fc fusion protein is a p75 sTNFR-Fc fusion protein.

A twenty second embodiment is the liquid formulation according to the twenty first embodiment, wherein the liquid formulation comprises 30 to 55 mg/mL of the TNFR-Fc fusion protein, and 10 to 35 mM citrate-phosphate buffer solution, 105 to 120 mM NaCl, 0.5 to 1.5% sucrose, 1 to 9 mM proline and 0.01 to 0.1 mM histidine and has a pH of 6.3.

A twenty third embodiment is the liquid formulation according to the twenty second embodiment, wherein the liquid formulation comprises 50 mg/mL of the TNFR-Fc fusion protein, 25 mM citrate-phosphate buffer, 115 mM NaCl, 1% of sucrose, 6 mM proline, and 0.09 mM histidine and has a pH of 6.3.

A twenty fourth embodiment is the liquid formulation according to the twenty first embodiment, wherein the liquid formulation comprises 30 to 55 mg/mL of TNFR-Fc fusion protein, and 10 to 35 mM citrate-phosphate buffer solution, 120 to 150 mM NaCl, 0.01 to 0.5% sucrose, 1 to 9 mM proline and 0.01 to 0.1 mM histidine and has a pH of 6.3.

A twenty fifth embodiment is the liquid formulation according to the twenty fourth embodiment, wherein the liquid formulation comprises 50 mg/mL of the TNFR-Fc fusion protein, 25 mM citrate-phosphate buffer, 130 mM NaCl, 0.1% of sucrose, 6 mM proline, and 0.09 mM histidine as the stabilizer and has a pH of 6.3.

A twenty sixth embodiment is the liquid formulation according to the twenty first embodiment, further comprising glutamic acid, wherein the liquid formulation comprises 30 to 55 mg/mL of the TNFR-Fc fusion protein, 10 to 35 mM citrate-phosphate buffer, 100-150 mM NaCl, 0.1 to 1.5% of sucrose, 5 to 15 mM glutamate, 1 to 10 mM proline, and 1 to 10 mM histidine and has a pH of 6.3.

A twenty seventh embodiment is the liquid formulation according to the twenty sixth embodiment, wherein the liquid formulation comprises 50 mg/mL of the TNFR-Fc fusion protein, 25 mM citrate-phosphate buffer, 100 mM NaCl, 1% of sucrose, 10 mM glutamate, 10 mM proline, and 5 mM histidine and has a pH of 6.3.

A twenty eight embodiment is a method for preparing a liquid formulation comprising a TNFR-Fc fusion protein, comprising:

- a) preparing the TNFR-Fc fusion protein; and
- b) mixing the TNFR-Fc fusion protein prepared in step a) with a stabilizer comprising one or more amino acids selected from the group consisting of proline and histidine, a buffer solution, and an isotonic agent containing sodium chloride (NaCl) and sucrose.

A twenty ninth embodiment is the method according to the twenty eight embodiment, wherein the mixing in step b) comprises i) preparing a solution comprising the stabilizer, the buffer solution, and the isotonic agent and ii) exchanging the solution in which the TNFR-Fc fusion protein is prepared in step a) with the solution in step i).

Mode for the Invention

Hereinafter, the present invention will be described in more detail with reference to Examples. However, these Examples are for illustrative purposes only, and the invention is not intended to be limited by these Examples.

Example 1: Preparation of etanercept liquid formulation

To stabilize a liquid formulation of the TNFR-Fc fusion protein, etanercept (recombinant p75 sTNFR:Fc fusion protein), an amino acid or mixture of amino acids was/were used as a stabilizer or an isotonic agent was controlled to examine the stabilizing effect. That is, liquid formulations were prepared by using proline or a mixture of proline and histidine as the stabilizer or by controlling the concentrations of NaCl and sucrose as the isotonic agent.

The liquid formulations of the present invention, in detail, Formulation 1 to Formulation 10 were prepared by adding one or more amino acids selected from the group consisting of, arginine, glutamic acid, proline and histidine to a 25 mM citrate-phosphate aqueous buffer solution and then adding sucrose and sodium chloride (NaCl) as an isotonic agent. Thereafter, pH of each formulation was adjusted to 6.3, and the formulations were subjected to dialysis so that the concentration of TNFR-fc fusion protein (etanercept) became 50 mg/mL, thereby preparing Formulation 1 to Formulation 10. Further, a control liquid formulation was prepared including 25 mM arginine, 100 mM sodium chloride and 1.0% sucrose as an isotonic agent in a 25 mM phosphate buffer solution also including 50 mg/mL etanercept, and then adjusting pH to

6.3. Thus, the control formulation was prepared, in which the etanercept concentration was 50 mg/mL.

The compositions of the prepared liquid formulations are as follows.

【Table 1】

Composition of liquid formulation						
Formulation	Composition of formulation					
	Etanercept conc.	Buffer solution	NaCl conc.	Sucrose conc.	pH	Amino acid
Formulation 1	50 mg/ml	25 mM, Citrate-phosphate	115 mM	1.0%	6.3	-
Formulation 2	50 mg/ml	25 mM, Citrate-phosphate	115 mM	1.0%	6.3	arginine 3 mM
Formulation 3	50 mg/ml	25 mM, Citrate-phosphate	115 mM	1.0%	6.3	arginine 3 mM histidine 0.09 mM
Formulation 4	50 mg/ml	25 mM, Citrate-phosphate	115 mM	1.0%	6.3	proline 3 mM
Formulation 5	50 mg/ml	25 mM, Citrate-phosphate	115 mM	1.0%	6.3	proline 3 mM histidine 0.09 mM
Formulation 6	50 mg/ml	25 mM, Citrate-phosphate	140 mM	0.1%	6.3	-
Formulation 7	50 mg/ml	25 mM, Citrate-phosphate	140 mM	0.1%	6.3	proline 3 mM
Formulation 8	50 mg/ml	25 mM, Citrate-phosphate	140 mM	0.1%	6.3	proline 3 mM histidine 0.09 mM
Formulation 9	50 mg/ml	25 mM, Citrate-phosphate	100 mM	1.0%	6.3	glutamic acid 15 mM proline 5 mM histidine 5 mM
Formulation 10	50 mg/ml	25 mM, Citrate-phosphate	100 mM	1.0%	6.3	glutamic acid 10 mM proline 10 mM histidine 5 mM
Control formulation	50 mg/ml	25 mM, phosphate	100 mM	1.0%	6.3	arginine 25 mM

Example 2: Stability test of etanercept liquid formulations

To examine stability of the liquid formulations prepared in Example 1, each formulation was stored under the conditions of 25°C and 40°C for 1, 2, 4 and 8 weeks, and then the

remaining amounts of etanercept were measured by size-exclusion high-performance liquid chromatography (SE-HPLC) and hydrophobic interaction high-performance liquid chromatography (HIC-HPLC).

The measurement results are shown in the following Tables 2 and 3.

【Table 2】

SE-HPLC for measurement of etanercept purity over time											
	Formulation	25°C					40°C				
		0 wk	1 wk	2 wk	4 wk	8 wk	0 wk	1 wk	2 wk	4 wk	8 wk
Reduction in etanercept purity (%)	1	0.0	-0.3	-0.6	-1.4	-2.3	0.0	-4.1	-7.1	-14.8	-25.4
	2	0.0	-0.2	-0.5	-1.3	-2.1	0.0	-4.0	-6.8	-13.6	-24.2
	3	0.0	-0.2	-0.5	-0.8	-1.6	0.0	-2.5	-5.8	-12.4	-21.2
	4	0.0	-0.3	-0.6	-1.4	-2.4	0.0	-4.1	-7.1	-14.4	-24.8
	5	0.0	-0.2	-0.5	-0.9	-1.9	0.0	-3.5	-5.9	-12.0	-23.4
	6	0.0	-0.2	-0.6	-1.4	-2.3	0.0	-4.1	-6.9	-13.5	-22.4
	7	0.0	-0.2	-0.5	-1.4	-2.0	0.0	-3.8	-6.5	-12.3	-21.0
	8	0.0	-0.1	-0.4	-0.8	-1.6	0.0	-3.3	-5.7	-11.4	-20.3
	9	0.0	-0.1	-0.4	-0.6	-1.5	0.0	-1.7	-4.9	-11.1	-24.0
	10	0.0	-0.1	-0.4	-0.7	-1.3	0.0	-1.9	-5.2	-11.2	-24.6
	Control	0.0	-0.2	-0.5	-1.1	-2.2	0.0	-3.8	-6.6	-13.9	-25.7

【Table 3】

HIC-HPLC for measurement of etanercept purity over time											
	Formulation	25°C					40°C				
		0 wk	1 wk	2 wk	4 wk	8 wk	0 wk	1 wk	2 wk	4 wk	8 wk
Reduction in etanercept purity (%)	1	0.0	-0.2	-0.4	-3.3	-4.3	0.0	-4.8	-7.3	-15.9	-26.5
	2	0.0	-0.2	-0.4	-3.2	-4.2	0.0	-4.7	-7.1	-14.7	-25.6
	3	0.0	-0.4	-0.5	-3.1	-3.9	0.0	-4.6	-6.8	-14.6	-23.8
	4	0.0	-0.2	-0.4	-3.2	-4.3	0.0	-4.8	-7.3	-15.4	-25.8
	5	0.0	-0.3	-0.5	-3.2	-4.0	0.0	-4.6	-6.8	-13.6	-25.7
	6	0.0	-0.2	-0.5	-3.4	-4.4	0.0	-4.7	-7.1	-14.6	-23.4
	7	0.0	-0.1	-0.3	-3.1	-4.3	0.0	-4.7	-6.8	-13.6	-22.5
	8	0.0	-0.2	-0.5	-3.1	-4.1	0.0	-4.7	-6.6	-13.4	-22.5
	9	0.0	-0.2	-0.4	N/A	-3.7	0.0	-4.5	-8.4	-14.6	-28.6
	10	0.0	-0.1	-0.4	-3.1	-3.4	0.0	-4.6	-7.3	-15.2	-29.6
	Control	0.0	-0.1	-0.3	-3.4	-4.7	0.0	-5.0	-8.0	-16.4	-29.5

※ N/A : Not Available

As confirmed in Tables 2 and 3 which show the analysis results of SE-HPLC and HIC-HPLC at 25°C and 40°C, formulations using a mixture of the amino acids of proline and histidine (Formulation 5, Formulation 8) showed effects of improving stability of the etanercept solution, compared to the control formulation which was prepared by using arginine alone as a stabilizer in the composition of the conventional etanercept formulation.

These results demonstrated that the etanercept liquid formulation comprising a mixture of the amino acids of proline and histidine as a stabilizer prevents denaturation of etanercept to maintain its activity for a long period of time, and a mixture of the amino acids of proline and histidine is effective as a stabilizer in the stability improvement of the etanercept solution.

Further, etanercept stabilities were examined between the formulations of the present invention which were prepared by varying the composition of sucrose and sodium chloride of the isotonic agent in the composition of the conventional etanercept formulation. The formulation having a higher concentration of sodium chloride and a lower concentration of sucrose showed effects of improving stability of the etanercept solution, compared to the control formulation.

Furthermore, the liquid formulations using a mixture of the amino acids of proline and histidine as a stabilizer (Formulation 5 and Formulation 8) showed effects of improving stability of the etanercept solution, compared to the liquid formulations (Formulation 1 and Formulation 6) without the amino acid stabilizer.

Consequently, these experimental results showed that stability of the etanercept solution can be improved by 1) increasing the NaCl concentration and reducing the sucrose concentration in the isotonic agent and 2) using a mixture of the amino acids of proline and histidine as a stabilizer in the etanercept liquid formulation. That is, through the conditions of 1) or 2), formation of etanercept by-products can be effectively prevented and its stability can be improved so as to stably maintain pharmaceutical efficacy of etanercept for a long period of time, compared to the conventional etanercept liquid formulation.

Example 3: Stability test of etanercept liquid formulations having a composition of a mixture of amino acids during long-term storage

Etanercept liquid formulations comprising one or more amino acids selected from proline and histidine and mixtures thereof, of which stability improvements were confirmed by the experiments of Examples 1 and 2, were subjected to a stability test under long-term storage conditions for 6 months.

In detail, a mixture of amino acids containing 0.09 mM histidine and 3 mM proline or a mixture of 0.09 mM histidine and 3 mM arginine were added to a 25 mM citrate-phosphate buffer solution, and 1.0% or 0.1% sucrose and 115 mM or 130 mM sodium chloride were also added thereto as an isotonic agent. Thereafter, pH of each formulation was adjusted to 6.3, and the formulations were subjected to dialysis so that the concentration of TNFR-fc fusion protein (etanercept) became 50 mg/mL, thereby preparing Formulation 1 to Formulation 3. Further, a control liquid formulation was prepared including 25 mM arginine, 100 mM sodium chloride and 1.0% sucrose as an isotonic agent in a 25 mM phosphate buffer solution also containing

etanercept, and then adjusting pH to 6.3. Thus, the control formulation was prepared, in which the etanercept concentration was 50 mg/mL.

The compositions of the prepared liquid formulations are the same as in the following Table 4.

【Table 4】

Composition of liquid formulation for long-term storage						
Formulation	Composition of formulation					
	Etanercept conc.	Buffer solution	NaCl conc.	Sucrose conc.	pH	Amino acid
Formulation 1	50 mg/ml	25 mM, Citrate-phosphate	115 mM	1.0%	6.3	arginine 3 mM histidine 0.09 mM
Formulation 2	50 mg/ml	25 mM, Citrate-phosphate	115 mM	1.0%	6.3	proline 3 mM histidine 0.09 mM
Formulation 3	50 mg/ml	25 mM, Citrate-phosphate	130 mM	0.1%	6.3	proline 3 mM histidine 0.09 mM
Control formulation	50 mg/ml	25 mM, phosphate	100 mM	1.0%	6.3	arginine 25 mM

To examine stabilities of the liquid formulations prepared as in Table 4, each of the formulations was stored under the conditions of 4°C, 25°C and 40°C for 1, 3 and 6 months (under the condition of 40°C for 3 months), and then the remaining amounts of etanercept were measured by size-exclusion high-performance liquid chromatography (SE-HPLC) and hydrophobic interaction high-performance liquid chromatography (HIC-HPLC).

The measurement results are shown in the following Tables 5 and 6.

【Table 5】

SE-HPLC for measurement of etanercept purity over time in mixed amino acid liquid formulations with improved stability												
Reduction in etanercept purity (%)	Formulation	4°C				25°C				40°C		
		0 M	1 M	3 M	6 M	0 M	1 M	3 M	6 M	0 M	1 M	3 M
	1	0.0	-0.3	-0.6	-1.4	0.0	-2.1	-5.2	-8.9	0.0	-10.4	-25.2
	2	0.0	-0.4	-0.7	-1.4	0.0	-2.2	-6.1	-9.5	0.0	-10.0	-25.3
	3	0.0	-0.3	-0.6	-1.4	0.0	-2.2	-5.4	-9.3	0.0	-10.2	-27.9
	Control	0.0	-0.4	-0.7	-1.4	0.0	-2.2	-5.6	-9.9	0.0	-12.5	-27.4

【Table 6】

HIC-HPLC for measurement of etanercept purity over time in mixed amino acid liquid formulations with improved stability												
Reduction in etanercept purity (%)	Formulation	4°C				25°C				40°C		
		0 M	1 M	3 M	6 M	0 M	1 M	3 M	6 M	0 M	1 M	3 M
	1	0.0	-0.2	-0.2	-0.7	0.0	-1.7	-3.8	-8.8	0.0	-9.5	-26.5
	2	0.0	-0.2	-0.3	-0.8	0.0	-1.8	-4.3	-8.7	0.0	-8.9	-25.9
	3	0.0	-0.3	-0.3	-0.7	0.0	-1.7	-4.3	-9.1	0.0	-9.2	-29.9
	Control	0.0	-0.2	-0.2	-0.8	0.0	-1.7	-4.5	-9.9	0.0	-11.8	-28.6

Also, in order to determine thermal stability of etanercept in the formulations as described in Table 4, and determine the temperature at which the etanercept protein unfolds, the thermograms of etanercept were measured by differential scanning calorimetry (DSC). Figure 2

shows the DSC thermogram of each formulations at 6 month time point at 4°C (figure 2A) and at 25°C (figure 2B).

Three thermal unfolding states with transition temperatures of Tm1, Tm2, and Tm3 were evident, which indicated the presence of two domains originating from the Fc component of etanercept containing the domain of CH2 (Tm2), CH3 (Tm3), and extra-cellular ligand binding protein, tumor necrosis factor receptor (TNFR; Tm1) (N. A. Kim et al., Int. J. Pharm., 460(1-2), 108-118, 2014).

As shown in Figure 2, formulation 1 using a mixture of arginine and histidine at 25°C (figure 2B) showed that the valley between Tm1 and Tm2 peaks disappeared between storage at 4°C (figure 2A) and at 25°C (figure 2B) compared to the control formulation and the other formulations using a mixture proline and histidine. This result showed a decreased stability of CH2 domain in Fc component and extra-cellular ligand binding domain (TNFR) of etanercept in formulation 1.

Further, as confirmed in Tables 5 and 6 which show the analysis results of SE-HPLC and HIC-HPLC at 25°C and 40°C, formulations using a mixture of the amino acids of proline and histidine (Formulations 2 and 3) showed effects of improving stability of the etanercept solution, compared to the control formulation which was prepared by using arginine alone as a stabilizer in the composition of the conventional etanercept formulation.

These results demonstrated that the etanercept liquid formulation comprising a mixture of the amino acids of proline and histidine as a stabilizer prevents denaturation of etanercept to maintain its activity for a long period of time.

Example 4: Stability test of etanercept liquid formulations according to composition ratio of mixed amino acids

To examine stabilities of the etanercept liquid formulations comprising the mixture of proline and histidine according to the composition ratio of the mixture of amino acids, in which these formulations were confirmed to have improved stability compared to the conventional etanercept formulation (single formulation containing arginine alone) in Examples 1 to 3, the stabilizing effect according to the composition ratio of the mixture of amino acids were examined. That is, liquid formulations were prepared by mixing proline and histidine as a stabilizer at various concentrations, and by controlling the concentrations of sucrose and sodium chloride as an isotonic agent.

In detail, to a 25 mM citrate-phosphate buffer solution, a mixture of the amino acids of 0.09 mM histidine and 3 mM to 9 mM proline as a stabilizer were added and 0.5% or 0.1% sucrose and 115 mM or 130 mM sodium chloride were added as an isotonic agent. Thereafter, pH of each formulation was adjusted to 6.3, and the formulations were subjected to dialysis so that the concentration of TNFR-fc fusion protein (etanercept) became 50 mg/mL, thereby preparing Formulation 1 to Formulation 5. Further, a control liquid formulation was prepared including 25 mM arginine, 100 mM sodium chloride and 1.0% sucrose as an isotonic agent in a 25 mM phosphate buffer solution containing 50 mg/mL etanercept, and then adjusting pH to 6.3. Thus, the control formulation was prepared, in which the etanercept concentration was 50 mg/mL.

The compositions of the prepared liquid formulations are the same as in the following Table 7.

【Table 7】

Composition of liquid formulation according to amino acid composition ratio of proline and histidine						
Formulation	Composition of formulation					
	Etanercept conc.	Buffer solution	NaCl conc.	Sucrose conc.	pH	Amino acid
Formulation 1	50 mg/ml	25 mM, Citrate-phosphate	115 mM	0.5%	6.3	
Formulation 2	50 mg/ml	25 mM, Citrate-phosphate	115 mM	0.5%	6.3	proline 3 mM histidine 0.09 mM
Formulation 3	50 mg/ml	25 mM, Citrate-phosphate	115 mM	0.5%	6.3	proline 6 mM histidine 0.09 mM
Formulation 4	50 mg/ml	25 mM, Citrate-phosphate	115 mM	0.5%	6.3	proline 9 mM histidine 0.09 mM
Formulation 5	50 mg/ml	25 mM, Citrate-phosphate	130 mM	0.1%	6.3	
Formulation 6	50 mg/ml	25 mM, Citrate-phosphate	130 mM	0.1%	6.3	proline 3 mM histidine 0.09 mM
Formulation 7	50 mg/ml	25 mM, Citrate-phosphate	130 mM	0.1%	6.3	proline 6 mM histidine 0.09 mM
Control formulation	50 mg/ml	25 mM, phosphate	100 mM	1.0%	6.3	arginine 25 mM

To examine stabilities of the liquid formulations prepared as in Table 7, each of the formulations was stored under the conditions of 25°C and 40°C for 2, 4 and 8 weeks and then the remaining amounts of etanercept were measured by size-exclusion high-performance liquid chromatography (SE-HPLC) and hydrophobic interaction high-performance liquid chromatography (HIC-HPLC).

The measurement results are shown in the following Tables 8 and 9.

【Table 8】

SE-HPLC for measurement of etanercept purity over time in liquid formulation according to change in amino acid composition ratio of proline and histidine									
Reduction in etanercept purity (%)	Formulation	25°C				40°C			
		0 wk	2 wk	4 wk	8 wk	0 wk	2 wk	4 wk	8 wk
	1	0.0	-1.3	-2.3	-3.9	0.0	-6.1	-10.9	-19.0
	2	0.0	-1.1	-2.0	-3.4	0.0	-5.3	-10.0	-18.0
	3	0.0	-1.2	-2.0	-3.4	0.0	-5.2	-10.0	-18.0
	4	0.0	-1.3	-2.2	-3.7	0.0	-5.5	-9.9	-18.1
	5	0.0	-1.3	-2.4	-4.0	0.0	-5.5	-10.2	-18.0
	6	0.0	-1.2	-2.1	-3.6	0.0	-5.5	-10.2	-18.0
	7	0.0	-1.2	-2.1	-3.6	0.0	-5.2	-9.7	-17.7
	Control	0.0	-1.3	-2.4	-4.0	0.0	-6.3	-11.4	-19.8

【Table 9】

HIC-HPLC for measurement of etanercept purity over time in liquid formulation according to change in amino acid composition ratio of proline and histidine									
Reduction in etanercept purity (%)	Formulation	25°C				40°C			
		0 wk	2 wk	4 wk	8 wk	0 wk	2 wk	4 wk	8 wk
	1	0.0	-0.9	-1.6	-3.0	0.0	-5.7	-10.6	-19.0
	2	0.0	-0.9	-1.5	-3.1	0.0	-5.3	-10.2	-19.0
	3	0.0	-0.8	-1.4	-3.0	0.0	-5.1	-10.0	-17.8
	4	0.0	-1.0	-1.6	-2.8	0.0	-5.1	-9.5	-18.2
	5	0.0	-0.9	-1.6	-2.9	0.0	-5.6	-10.4	-18.7
	6	0.0	-0.8	-1.5	-3.0	0.0	-5.3	-10.1	-18.4
	7	0.0	-0.8	-1.5	-3.1	0.0	-5.4	-9.6	-18.0
	Control	0.0	-1.1	-1.8	-3.1	0.0	-6.4	-11.4	-20.3

As confirmed in Tables 8 and 9 which show the analysis results of SE-HPLC and HIC-HPLC at 25°C and 40°C, formulations using a mixture of the amino acids of proline and histidine showed effects of improving stability of the etanercept solution, compared to the control formulation which was prepared by using arginine alone as a stabilizer in the composition of the conventional etanercept formulation.

When the effect of improving stability according to the concentration ratio of the mixed amino acids used as a stabilizer was examined, the formulation (Formulation 3) prepared by mixing 6 mM proline and 0.09 mM histidine showed the most improved stability among the formulations comprising mixed amino acids of proline and histidine (Formulation 2 to Formulation 4) which were prepared by using 115 mM sodium chloride and 0.5% sucrose and varying the proline concentration from 3 mM to 9 mM.

These results demonstrated that the etanercept liquid formulation comprising mixed amino acids of 6 mM proline and 0.09 mM histidine as a stabilizer, as shown in the above composition ratio of proline and histidine, prevents denaturation of etanercept to maintain its activity for a long period of time.

Taken together, the present invention demonstrated that the etanercept liquid formulation comprising mixed amino acids of proline and histidine as a stabilizer prevents denaturation to maintain its activity for a long period of time, and the stability of the etanercept solution can be improved by using an isotonic agent consisting of a high concentration of sodium chloride and a low concentration of sucrose.

Based on the above description, it will be understood by those skilled in the art that the present invention may be implemented in a different specific form without changing the technical or essential characteristics thereof. Therefore, it should be understood that the above embodiment is not limitative, but illustrative in all aspects.

Claims

1. A liquid formulation comprising a TNFR-Fc fusion protein, a mixture of proline and histidine, a buffer solution, and an isotonic agent.
2. The liquid formulation according to claim 1, wherein the fusion protein is etanercept.
3. The liquid formulation according to claim 1, wherein the fusion protein has an amino acid sequence of SEQ ID NO. 1.
4. The liquid formulation according to claim 1, wherein the fusion protein is a p75 sTNFR-Fc fusion protein.
5. The liquid formulation according to any one of claims 1 to 4, wherein the isotonic agent allows the liquid formulation to maintain an osmotic pressure of 280 to 350 mOsm.
6. The liquid formulation according to of any one of claims 1 to 5, wherein the isotonic agent contains sodium chloride (NaCl) and sucrose.
7. The liquid formulation according to claim 6, wherein the sodium chloride is present at a concentration of 1 to 1000 mM and the sucrose is present in an amount of 0.01 to 3% by weight of the total composition.
8. The liquid formulation according to claim 7, wherein the sodium chloride is present at a concentration of 105 mM to 150 mM, and the sucrose is present in an amount of 0.01 to 1.5% by weight of the total composition.
9. The liquid formulation according to any one of claims 1 to 8, wherein the concentration of the fusion protein in the liquid formulation is in the range of from 20 to 55 mg/mL.
10. The liquid formulation according to any one of claims 1 to 9, wherein the buffer solution is a citrate-phosphate or phosphate buffer solution.
11. The liquid formulation according to claim 10, wherein the concentration of the buffer solution is in the range of from 10 to 35 mM.

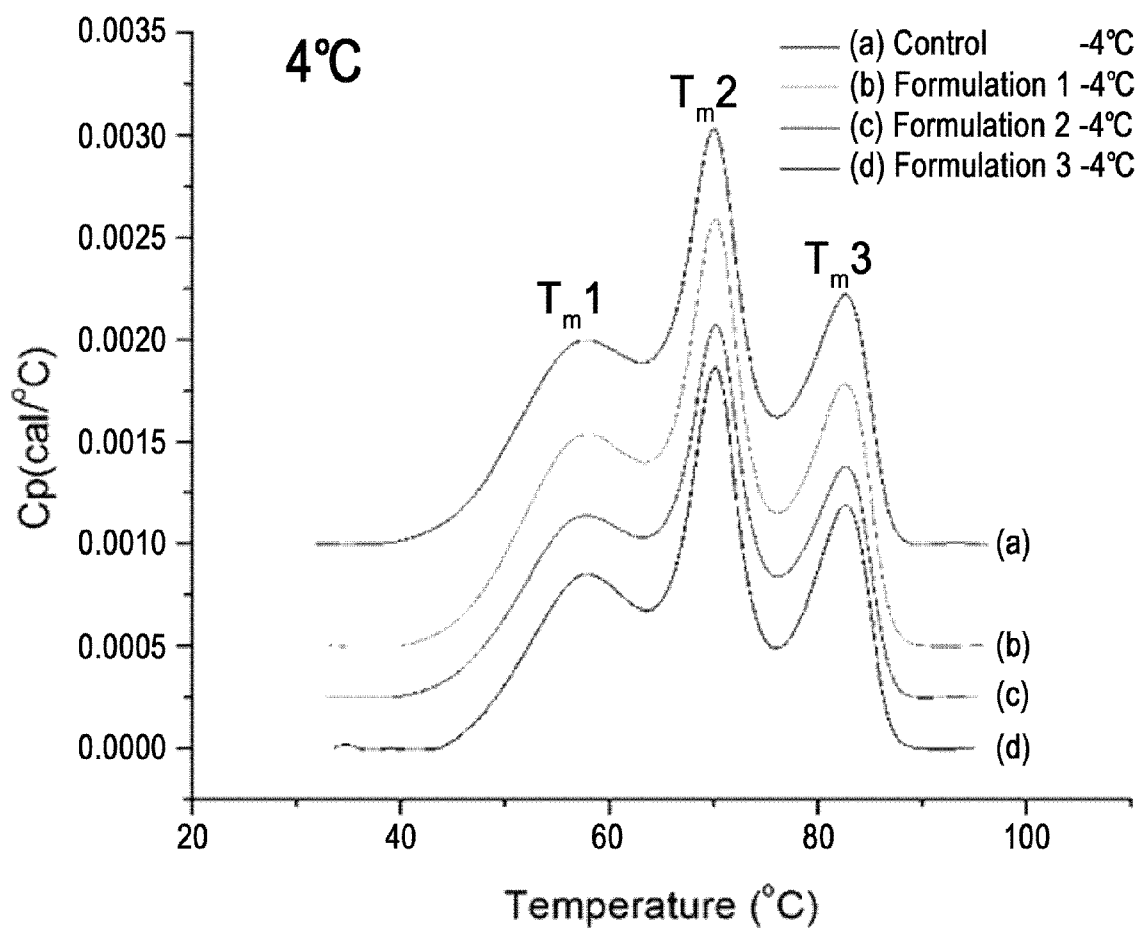
12. The liquid formulation according to any one of claims 1 to 11, wherein the pH of the liquid formulation is in the range from 6.0 to 6.6.
13. The liquid formulation according to any one of claims 1 to 12, wherein the formulation comprises the proline at a concentration of less than 10 mM and the histidine at a concentration of less than 5 mM.
14. The liquid formulation according to claim 13, wherein the formulation comprises the proline at a concentration of less than 9 mM or the histidine at a concentration of less than 0.1 mM.
15. The liquid formulation according to claim 13, wherein the proline is present at a concentration of from 1 mM to 9 mM and the histidine is present at a concentration of less than 0.1 mM.
16. The liquid formulation according to claim 15, wherein the proline is present at a concentration of from 1 mM to 9 mM and the histidine is present at a concentration of from 0.01 to 0.09 mM.
17. The liquid formulation according to claim 16, wherein the proline is present at a concentration of 6 mM and the histidine is present at a concentration of 0.09 mM.
18. The liquid formulation according to any one of claims 1 to 13, further comprising glutamic acid at a concentration of from 1mM to 20mM.
19. The liquid formulation according to claim 18, wherein the glutamic acid is present at a concentration of from 10 to 15 mM.
20. The liquid formulation according to any one of claims 1 to 13, wherein the liquid formulation comprises from 30 to 55 mg/mL of the TNFR-Fc fusion protein, and from 10 to 35 mM citrate-phosphate buffer solution, from 105 to 120 mM NaCl, 0.5 to 1.5% by weight of sucrose, from 1 to 9 mM proline and from 0.01 to 0.1 mM histidine and has a pH of 6.3.

21. The liquid formulation according to claim 20, wherein the liquid formulation comprises 50 mg/mL of the TNFR-Fc fusion protein, 25 mM citrate-phosphate buffer, 115 mM NaCl, 1% by weight of sucrose, 6 mM proline, and 0.09 mM histidine and has a pH of 6.3.
22. The liquid formulation according to any one of claims 1 to 13, wherein the liquid formulation comprises from 30 to 55 mg/mL of TNFR-Fc fusion protein, and from 10 to 35 mM citrate-phosphate buffer solution, from 120 to 150 mM NaCl, from 0.01 to 0.5% by weight of sucrose, from 1 to 9 mM proline and from 0.01 to 0.1 mM histidine and has a pH of 6.3.
23. The liquid formulation according to claim 22, wherein the liquid formulation comprises 50 mg/mL of the TNFR-Fc fusion protein, 25 mM citrate-phosphate buffer, 130 mM NaCl, 0.1% by weight of sucrose, 6 mM proline, and 0.09 mM histidine as a stabilizer and has a pH of 6.3.
24. The liquid formulation according to any one of claims 1 to 13, further comprising glutamic acid, wherein the liquid formulation comprises from 30 to 55 mg/mL of the TNFR-Fc fusion protein, from 10 to 35 mM citrate-phosphate buffer, from 100-150 mM NaCl, from 0.1 to 1.5% by weight of sucrose, from 5 to 15 mM glutamate, from 1 to 10 mM proline, and from 1 to 10 mM histidine and has a pH of 6.3.
25. The liquid formulation according to claim 24, wherein the liquid formulation comprises 50 mg/mL of the TNFR-Fc fusion protein, 25 mM citrate-phosphate buffer, 100 mM NaCl, 1% by weight of sucrose, 10 mM glutamate, 10 mM proline, and 5 mM histidine and has a pH of 6.3.
26. A method for preparing the liquid formulation comprising a TNFR-Fc fusion protein of any one of claims 1 to 25, comprising:
- a) preparing the TNFR-Fc fusion protein in solution; and
 - b) mixing the TNFR-Fc fusion protein prepared in step a) with a stabilizer comprising a mixture of proline and histidine, a buffer solution, and an isotonic agent containing sodium chloride (NaCl) and sucrose.
27. The method according to claim 26, wherein the mixing in step b) comprises i) preparing a solution comprising the stabilizer, the buffer solution, and the isotonic agent and ii) exchanging the solution in which the TNFR-Fc fusion protein is prepared in step a) with the solution in step i).

[Fig. 1]

LPAQVAFTPY	APEPGSTCRL	REYYDQTAQM	CCSKCSPGQH	AKVFCTKTS	D TVCDSCEDST	60
YTQLWNWVPE	CLSCGSRCSS	DQVETQACTR	EQNRICTCRP	GWYCALSKE	GCRLCAPLRK	120
CRPGFGVARP	GTETSDVVCK	PCAPGTFSDT	TSSTDICRPH	QICNVVAIPG	DASMDAVCTS	180
TSPTRSMAPG	AVHLPQPVST	RSQHTQPTPE	PSTAPSTSFL	LPMGPSPPAE	GSTGDEPKSC	240
DKTHTCPPCP	APELLGGPSV	FLFPPKPKDT	LMISRTPEVT	CVVVDVSHED	PEVKFNWYVD	300
GVEVHNAKTK	PREEQYDSTY	RVVSVLTVLH	QDWLNGKEYK	CKVSNKALPA	PIEKTISKAK	360
GQPREPQVYT	LPPSREEMTK	NQVSLTCLVK	GFYPSDIAVE	WESNGQPENN	YKTTTPVLDS	420
DGSFFLYSKL	TVDKSRWQQG	NVFSCSVMHE	ALHNHYTQKS	LSLSPG		

[Fig. 2a]



[Fig. 2b]

