Etanercept Formulations Stabilized with Metal Ions

Field of the Invention

The present invention relates to aqueous pharmaceutical compositions stabilized with metal ions for long-term storage of etanercept, methods of manufacture of the compositions, methods of their administration, and kits containing the same. The invention includes etanercept formulations that do not require arginine for stabilization.

Background of the Invention

Polypeptides must often be stored prior to their use. When stored for extended periods, polypeptides are frequently unstable in solution (Manning et al., 1989, Pharm. Res. 6:903-918). To extend their shelf life, additional processing steps have been developed, such as drying, e.g., lyophilization. However, lyophilized pharmaceutical compositions are less convenient to use.

Typical practices to improve polypeptide stability can be addressed by varying the concentration of elements with the formulation, or by adding excipients to modify the formulation (See, for example, U.S. Pat. Nos. 5,580,856 and 6,171,586). However, the use of additives can still result in inactive polypeptides. In addition, in the case of lyophilization, the rehydration step can result in inactivation of the polypeptide by, for example, aggregation or denaturation (Hora et al., 1992, Pharm. Res., 9:33-36; Liu et al., 1991, Biotechnol. Bioeng., 37:177-184). Aggregation of polypeptides is undesirable, as it may result in immunogenicity (Cleland et al., 1993, Crit. Rev. Therapeutic Drug Carrier Systems, 10:307-377; and Robbins et al., 1987, Diabetes, 36:838-845).

Another way to improve polypeptide stability is to use L-arginine at a specific concentration (U.S. Pat. No. 7,648,702).

One of the polypeptides that is stored for up to two years prior to use is etanercept (Enbrel®, Immunex Corporation), which is a dimeric fusion polypeptide consisting of the extracellular ligand-binding portion of the human 75 kilodalton (p75) tumor necrosis factor receptor (TNFR) linked to the Fc portion of human IgG1. It consists of 934 amino acids and has an apparent molecular weight of approximately

150 kilodaltons (Physicians Desk Reference, 2002, Medical Economics Company Inc.) The Fc component of etanercept contains the constant heavy 2 (CH2) domain, the constant heavy 3 (CH3) domain and hinge region, but not the constant heavy 1 (CH1) domain of human IgG1. An Fc domain can contain one or all of the domains described above. Etanercept is usually produced by recombinant DNA technology in a Chinese hamster ovary (CHO) mammalian cell expression system.

The present invention provides novel stable liquid formulations of etanercept that allow its long-term storage.

Summary of the Invention

The present invention is an aqueous pharmaceutical composition comprising etanercept and a stabilizer to inhibit instability, aggregation, misfolding and/or fragmentation of the etanercept, wherein the stabilizer comprises a stabilizing metal ion.

Various technical terms used in the following discussion are defined below in the section entitled "Definitions" and throughout the remainder of the specification.

The stabilized etanercept formulations of the present invention elicit long term storage stability as characterized by at least one of: (1) SEC analysis at M_3 or T_2 or T_4 of: monomer content greater than about 90%; aggregates content of less than about 3 wt%; and fragment 3 content less than about 5 wt%: and (2) HIC analysis at M_3 or T_2 or T_4 wherein the amount of the composition represented by peak 1 of the HIC chromatogram is less than about 3 wt.%; the amount of the composition represented by peak 2 of the HIC chromatogram is greater than 80 wt.%; and the amount of the composition represented by peak 3 of the HIC chromatogram is less than about 20 wt.%.

In a related aspect the etanercept formulations containing metal ions elicit long term storage stability as characterized by: (1) SEC analysis at M_3 or T_2 or T_4 of greater than about 90 wt.% monomer content; less than about 3 wt.% aggregate(s) content; and less than about 5 wt% fragment 3; and (2) HIC analysis at M_3 or T_2 or T_4 wherein the amount of the composition represented by peak 1 of the HIC chromatogram is less than about 3 wt.%; the amount of the composition represented

by peak 2 of the HIC chromatogram is greater than 80 wt.%; and the amount of the composition represented by peak 3 of the HIC chromatogram is less than about 20 wt.%.

In preferred aspects of the stabilized formulations, the formulations elicit long term storage stability as characterized by: an HIC analysis at M_3 or T_2 or T_4 wherein the amount of the composition represented by peak 2 of the HIC chromatogram is greater than or equal to about 95 wt.%; and wherein, if peak 3 is present on the HIC chromatogram, the amount of the composition represented by peak 3 is less than or equal to about 3 wt.%.

The stabilized etanercept formulation as summarized above, optionally and preferably, contain no arginine, or are essentially free of arginine.

The formulations of the invention have excellent stability as determined by SEC (Size Exclusion Chromatography) and HIC (Hydrophobic Interaction Chromatography) analysis conducted after one, two or three months of storage at 5° C. These formulations are comparable to or better than a commercially available formulation of etanercept, in which arginine is a required component. Accordingly the present invention is further directed to formulations of etanercept, stabilized with metal ions which contain no arginine, or are essentially free of arginine, and wherein the composition, at M_3 or T_2 or T_4, elicits long term storage stability that meets one or both of the following criteria: (A) stability comparable to or better than commercially available etanercept marketed under the trademark Enbrel®, as measured by (i) SEC analysis of the amounts of aggregate(s), monomer and fragment 3 in the composition (as defined in the specification) and (ii) HIC analysis of amounts of material in the composition corresponding to peaks 1, 2 and 3 of the HIC chromatogram (as defined in the specification); and (B) an HIC chromatogram in which (i) peak 3 is absent, or essentially absent and (ii) peak 2 represents greater than about 95 wt% of the composition; an SEC chromatogram containing essentially no peak corresponding to aggregate(s); and an SEC chromatogram in which the monomer content represents at least about 95 wt% of the composition. Formulations of the present invention are able to meet the foregoing criteria without requirement for arginine as a stabilizer.

In one embodiment of the invention in the which the stabilizer is calcium chloride, the calcium chloride is present in an amount not exceeding about 5 mM calcium chloride and the composition further comprises: optionally about 0.5 to 6 wt.% sucrose or trehalose; optionally about 0 to100 mM NaCl; optionally up to about 10 mM xylitol; about 1 to about 30 mM sodium phosphate; wherein the composition has a pH of about 6.0 to 6.6; and wherein, optionally, the composition contains no arginine or is essentially free of arginine.

In a further embodiment the calcium chloride stabilized composition comprises about 50 mg/ml of etanercept; about 2 mM calcium chloride; about 10-30 mM sodium phosphate; less than about 150 mM sodium chloride; and less than about 4 wt.% sucrose; wherein the formulation has a pH of about 6.3 to 6.5.

In a further embodiment the calcium chloride stabilized composition comprises about 50 mg/ml of etanercept; about 2 mM calcium chloride; about 10-30 mM sodium phosphate; less than about 10 wt.% sucrose; wherein the formulation has a pH of about 6.3 to 6.5.

In a further embodiment the calcium chloride stabilized composition comprises about 50 mg/ml of etanercept; about 2 mM calcium chloride; about 10-30 mM sodium phosphate; less than about 100 mM sodium chloride; and less than about 5 wt.% sucrose; wherein the formulation has a pH of about 6.3 to 6.5.

In a further embodiment the calcium chloride stabilized composition comprises about 50 mg/ml of etanercept; about 1 mM calcium chloride; about 10-30 mM sodium phosphate; about 50 mM sodium chloride; and less than about 8 wt.% trehalose; wherein the formulation has a pH of about 6.3 to 6.5.

In a further embodiment in which the stabilizer is magnesium chloride, the stabilized etanercept composition comprises about 1 mM to about 20 mM magnesium chloride; optionally up to about 6 wt.% sucrose; about 25 to 150 mM NaCl; about 1 to about 30 mM sodium phosphate; wherein the composition has a pH of about 6.0 to 6.6; and wherein, optionally, the composition contains no arginine or is essentially free of arginine.

In a further embodiment in which the stabilizer is magnesium chloride, the stabilized etanercept composition comprises about 50 mg/ml of etanercept; about 10 mM magnesium chloride; about 10-30 mM sodium phosphate; less than about 150 mM sodium chloride; and less than about 3 wt.% sucrose; and having a pH of about 6.3 to 6.5.

In a further embodiment in which the stabilizer is magnesium chloride, the stabilized etanercept composition comprises about 50 mg/ml of etanercept; about 5 mM magnesium chloride; about 10-30 mM sodium phosphate; less than about 100 mM sodium chloride; and less than about 4 wt.% sucrose; and having a pH of about 6.3 to 6.5.

In a further embodiment in which the stabilizer is magnesium chloride, the stabilized etanercept composition comprises about 50 mg/ml of etanercept; about 4 mM magnesium chloride; about 10-30 mM sodium phosphate; about 100 mM sodium chloride; and about 2.5 wt.% sucrose; and having a pH of about 6.3 to 6.5.

In a further embodiment in which the stabilizer is magnesium chloride, the stabilized etanercept composition comprises about 50 mg/ml of etanercept; about 10 mM magnesium chloride; about 10-30 mM sodium phosphate; less than about 100 mM sodium chloride; and less than about 5 wt.% sucrose; and having a pH of about 6.3 to 6.5.

The etanercept compositions of the invention further afford the ability to provide formulations which contain acceptable levels of subvisible particles. Accordingly, the invention is further directed to metal ion stabilized etanercept formulations having, at M_3 or T_2 or T_4, no more than, on average, about 10,000 subvisible particles per mL having a size greater than 5μm.

The stabilized etanercept composition of the present invention are further characterized by: (a) an SEC analysis at M_3 or T_2 or T_4 of greater than about 90 wt% monomer content; and less than about 3 wt% aggregate(s) content; and (b) an HIC analysis at M_3 or T_2, or T_4 wherein the amount of the composition represented by peak 1 of the HIC chromatogram is less than about 3 wt%; the amount of the composition represented by peak 2 of the HIC chromatogram is greater than 80 wt%; and the amount of the composition represented by peak 3 of the HIC chromatogram

is less than about 20 wt%, and wherein the formulation contains no arginine or is essentially free of arginine.

The stability of the formulations may be further characterized in that the compositions exhibit an HIC analysis at M_3 or T_2 or T_4 wherein the amount of the composition represented by peak 1 of the HIC chromatogram is less than about 1%; the amount of the composition represented by peak 2 of the HIC chromatogram is greater than about 95 wt%; and the amount of the composition represented by peak 3 of the HIC chromatogram is less than about 3 wt%.

In a further embodiment in which the stabilizer is calcium chloride the etanercept formulation comprises about 50 mg/ml etanercept; 1 to 5 mM calcium chloride; about 1 to 30 mM sodium phosphate; about 0 to 100 mM NaCl; about 0.5 to 5% sucrose or trehalose or combination thereof; and wherein the composition has a pH of about 6.0 to 6.6 and characterized by: an SEC analysis at M_3 or T_2 or T_4 of greater than about 97 wt.% monomer content and less than about 1 wt% aggregate(s) content; an HIC analysis at M_3 or T_2 or T_4 wherein the amount of the composition represented by peak 1 of the HIC chromatogram is less than about 4 wt%; the amount of the composition represented by peak 2 of the HIC chromatogram is greater than about 82 wt%; and the amount of the composition represented by peak 3 of the HIC chromatogram is less than about 15 wt%. Formulations of the present invention utilizing calcium chloride for stabilization are able to meet the foregoing criteria without requirement for arginine as a stabilizer.

In a further embodiment in which the stabilizer is magnesium chloride, the stabilized etanercept composition comprises about 1 mM to about 20 mM magnesium chloride; optionally up to about 6 wt% sucrose; about 25 to 150 mM NaCl; about 1 to about 30 mM sodium phosphate; wherein the composition has a pH of about 6.0 to 6.6; and wherein the composition is characterized by: an SEC analysis at M_3 or T_2 or T_4 of greater than about 97 wt.% monomer content and less than about 1 wt% aggregate(s) content; and an HIC analysis at M_3 or T_2 or T_4 wherein the amount of the composition represented by peak 1 of the HIC chromatogram is less than about 4 wt%; the amount of the composition represented by peak 2 of the HIC chromatogram is greater than about 85 wt%; and the amount of the composition represented by peak 3 of the HIC chromatogram is less than about

14 wt%. Formulations of the present invention utilizing magnesium chloride for stabilization are able to meet the foregoing criteria without requirement for arginine as a stabilizer.

Preferred stabilized compositions of the invention exhibit an HIC analysis at M_3 or T_2 or T_4 wherein the amount of the composition represented by peak 1 of the HIC chromatogram is less than about 2% and preferably less than about 1%; the amount of the composition represented by peak 2 of the HIC chromatogram is greater than about 95 wt.% and preferably greater than about 97%; and the amount of the composition represented by peak 3 of the HIC chromatogram is less than about 3 wt%, and preferably 0 to 1%.

The invention provides a stabilized aqueous pharmaceutical composition comprising etanercept and a stabilizer to inhibit instability, aggregation misfolding and/or fragmentation of the etanercept, wherein said stabilizer comprises a stabilizing metal ion. In a preferred embodiment, the metal ion is selected from the group consisting of calcium, magnesium, zinc, and combinations thereof. In an even more preferred embodiment, calcium, magnesium, zinc are provided as calcium chloride, magnesium chloride and zinc chloride, respectively. Calcium chloride and magnesium chloride are particularly preferred as stabilizers for etanercept.

As differentiated from commercially available etanercept provided in an arginine-containing formulation, we found it surprising in light of US. Pat. No. 7,648,702, that the metal ion stabilized formulations of etanercept described and exemplified herein do not require arginine for long term stabilization, although arginine may still be added if desired. The ability to provide etanercept formulations stabilized without arginine represents a potentially significant benefit to the health care system by providing patients and health care providers with alternative formulations of etanercept that may become available at lower cost compared with present commercial etanercept formulation (i.e., Enbrel®) that require arginine for stabilization.

As used herein the term "instability" or like terms denotes the tendency of the etanercept monomer to undergo a variety of undesired transformations during storage. Such transformations include the formation of oligomers and high molecular weight aggregate(s) (hereinafter termed "aggregates") in which multiple

copies of the essentially intact etanercept monomer become irreversibly associated with one another through a variety of non-covalent attractions (e.g., electrostatic interactions.) Undesired transformations during storage may also include degradation of the etanercept monomer to smaller fragments and/or clipped species. Ideally, a formulaton of etanercept should minimize, to the greatest extent possible, the tendency of the formulation to result, during storage, in the formation of aggregates, misfolded protein, oligomers and/or fragments of the etanercept. An important benefit resulting from the ability to reduce formation of unwanted aggregates or fragments is a reduction in the potential toxicity and/or immunogenicity of the drug.

The metal ion stabilized formulation is optionally and preferably free, or essentially free of arginine. The term "essentially free of arginine" is intended to mean that arginine, even if present, is not contributing to the stabilization of the etanercept monomer in the formulation to such an extent that a person skilled in the art would judge its presence beneficial or necessary from a stabilization standpoint.

These and other aspects will become apparent from the following description of the various embodiments, although variations and modifications therein may be affected without departing from the spirit and scope of the novel concepts of the disclosure.

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

DETAILED DESCRIPTION OF THE INVENTION

Various embodiments of the invention are now described in detail. As used in the description and throughout the claims, the meaning of "a", "an", and "the" includes plural reference unless the context clearly dictates otherwise. Also, as used in the description and throughout the claims, the meaning of "in" includes "in" and "on" unless the context clearly dictates otherwise. Additionally, some terms used in this specification are more specifically defined below.

DEFINITIONS

The terms used in this specification generally have their ordinary meanings in the art, within the context of the invention, and in the specific context where each term is used. Certain terms that are used to describe the invention are discussed below, or elsewhere in the specification, to provide additional guidance to the practitioner regarding the description of the invention. Synonyms for certain terms are provided. A recital of one or more synonyms does not exclude the use of other synonyms. The use of examples anywhere in this specification including examples of any terms discussed herein is illustrative only, and in no way limits the scope and meaning of the invention or of any exemplified term. The invention is not limited to the various embodiments given in this specification.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. In the case of conflict, the present document, including definitions will control.

"Around," "about" or "approximately" shall generally mean within 20 percent, within 10 percent, within 5, 4, 3, 2 or 1 percent of a given value or range. Numerical quantities given are approximate, meaning that the term "around," "about" or "approximately" can be inferred if not expressly stated.

The term "etanercept" or "etanercept monomer" or "monomer" is synonymous with Enbrel®. It refers to a polypeptide which is a dimeric fusion polypeptide consisting of the extracellular ligand-binding portion of the human 75 kilodalton (p75) tumor necrosis factor receptor (TNFR) linked to the Fc portion of human IgG1. It consists of 934 amino acids and has an apparent molecular weight of approximately 150 kilodaltons. For the purposes of the present application, the term "etanercept" also encompasses etanercept with minor modifications in the amino acid structure (including deletions, additions, and/or substitutions of amino acids) which do not significantly affect the function, potency, or avidity of etanercept. The term "etanercept" encompasses all forms and formulations of Enbrel®, including but not limited to concentrated formulations, injectable ready-to-use formulations; formulations reconstituted with water, alcohol, and/or other ingredients, and others.

The term "monomer" as used herein is intended to mean the dimeric etanercept fusion protein referenced above.

The term "sugar" refers to monosaccharides, disaccharides, and polysaccharides. Examples of sugars include, but are not limited to, sucrose, trehalose, dextrose, and others.

The term "metal ion" refers to a metal atom with a net positive or negative electric charge. For the purposes of the present application, the term "metal ion" also includes sources of metal ions, including but not limited to metal salts.

The term "long-term storage" is understood to mean that the pharmaceutical composition can be stored for three months or more, for six months or more, and preferably for one year or more. Long-term storage is also understood to mean that the pharmaceutical composition is stored either as a liquid at 2-8° C, or is frozen, e.g., at -20°C, or colder. It is also contemplated that the composition can be frozen and thawed more than once.

The term "stable" or "stabilized" with respect to long-term storage is understood to mean that etanercept contained in the pharmaceutical compositions does not lose more than 20%, or more preferably 15%, or even more preferably 10%, and most preferably 5% of its activity relative to activity of the composition at the beginning of storage.

The term "mammal" includes, but is not limited to, a human.

The term "pharmaceutically acceptable carrier" refers to a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material, formulation auxiliary, or excipient of any conventional type. A pharmaceutically acceptable carrier is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation.

The term "composition" refers to a mixture that usually contains a carrier, such as a pharmaceutically acceptable carrier or excipient that is conventional in the art and which is suitable for administration into a subject for therapeutic, diagnostic, or prophylactic purposes. It may include a cell culture in which the polypeptide or polynucleotide is present in the cells or in the culture medium. For example, compositions for oral administration can form solutions, suspensions, tablets, pills, capsules, sustained release formulations, oral rinses or powders.

The terms "pharmaceutical composition" and "formulation" are used interchangeably.

The term "treatment" refers to any administration or application of remedies for disease in a mammal and includes inhibiting the disease, arresting its development, relieving the disease, for example, by causing regression, or restoring or repairing a lost, missing, or defective function; or stimulating an inefficient process. The term includes obtaining a desired pharmacologic and/or physiologic effect, covering any treatment of a pathological condition or disorder in a mammal. The effect may be prophylactic in terms of completely or partially preventing a disorder or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disorder and/or adverse affect attributable to the disorder. It includes (1) preventing the disorder from occurring or recurring in a subject who may be predisposed to the disorder but is not yet symptomatic, (2) inhibiting the disorder, such as arresting its development, (3) stopping or terminating the disorder or at least its associated symptoms, so that the host no longer suffers from the disorder or its symptoms, such as causing regression of the disorder or its symptoms, for example, by restoring or repairing a lost, missing or defective function, or stimulating an inefficient process, or (4) relieving, alleviating or ameliorating the disorder, or symptoms associated therewith, where ameliorating is used in a broad sense to refer to at least a reduction in the magnitude of a parameter, such as inflammation, pain and/or tumor size.

The term "disease" refers to any condition, infection, disorder or syndrome that requires medical intervention or for which medical intervention is desirable. Such medical intervention can include treatment, diagnosis and/or prevention.

The term "therapeutically effective amount" refers to an amount which, when administered to a living subject, achieves a desired effect on the living subject. For example, an effective amount of the polypeptide of the invention for administration to the living subject is an amount that prevents and/or treats an integrin αvβ3-mediated disease. The exact amount will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques. As is known in the art, adjustments for systemic versus localized delivery, age, body weight, general health, sex, diet, time of administration, drug interaction and the severity of the

condition may be necessary, and will be ascertainable with routine experimentation by those skilled in the art.

The term "T_1"refers to a point in time at which an etanercept formulation has been stored for about one week at 40° C

The term "T_2"refers to a point in time at which an etanercept formulation has been stored for about two weeks at 40° C

The term "T_4"refers to a point in time at which an etanercept formulation has been stored for about four weeks at 40° C.

The term "M_3" refers, collectively, to three points in time, and in particular to an analytical result being observed for an etanercept formulation after duration of either about one, about two or about three months of storage at a storage temperature of 5° C. For example, reference herein to an analysis being conducted at M_3 should be understand to mean that such analysis is be done at the point in time at which etanercept formulation has been in storage for a duration selected from about one, about two, or about three months. Thus, a requirement herein that an etanercept formulation elicit a certain analytical value or measurement at M_3 is satisfied if the required value is observed at a point in time corresponding to at least one of the following storage durations: at approximately one month, at approximately two months or at approximately three months of storage at 5°C.

The terms "Peak 1," Peak 2" and "Peak 3" when used herein in connection with discussion of HIC chromatography results refers to the same peaks 1, 2 and 3 discussed in US Patent 7,294,481.

Embodiments of the Invention

When pharmaceutical compositions containing etanercept (Enbrel®), including aqueous and lyophilized formulations of etanercept are stored on a long term basis, the activity of etanercept can be lost or decreased due to instability of the etanercept monomer via aggregation and/or chemical degradation including formation of fragments. Thus, the present invention provides several embodiments of aqueous formulations of etanercept that allow stable long-term storage of

etanercept, so that etanercept is stable over the course of storage either in liquid or frozen states. The provided formulations include, but are not limited to formulations that do not contain arginine and do not require any extra steps such as rehydrating.

These embodiments are explained in a greater detail below.

Etanercept

All of the compositions of the present invention comprise etanercept (Enbrel®). As explained in the Background section of this application, etanercept is a dimeric fusion polypeptide consisting of the extracellular ligand-binding portion of the human 75 kilodalton (p75) tumor necrosis factor receptor (TNFR) linked to the Fc portion of human IgG1. Etanercept consists of 934 amino acids. The Fc component of etanercept contains the constant heavy 2 (CH2) domain, the constant heavy 3 (CH3) domain and hinge region of human IgG1. An Fc domain can contain one or all of the domains described above.

Etanercept suitable for storage in the present pharmaceutical composition can be produced by living host cells that express etanercept, such as hybridomas in the case of antibodies, or host cells that that have been genetically engineered to produce the polypeptide in the case of fusion polypeptides or antibodies. Methods of genetically engineering cells to produce polypeptides are well known in the art. See, e.g., Ausubel et al., eds. (1990), Current Protocols in Molecular Biology (Wiley, New York). Such methods include introducing nucleic acids that encode and allow expression of the polypeptide into living host cells. These host cells can be bacterial cells, fungal cells, or, preferably, animal cells grown in culture. Bacterial host cells include, but are not limited to, *Escherichia* coli cells. Examples of suitable *E.* coli strains include: HB101, DH5.alpha, GM2929, JM109, KW251, NM538, NM539, and any E. coli strain that fails to cleave foreign DNA. Fungal host cells that can be used include, but are not limited to, *Saccharomyces* cerevisiae, *Pichia* pastoris and *Aspergillus* cells. A few examples of animal cell lines that can be used are CHO, VERO, BHK, HeLa, Cos, MDCK, 293, 3T3, and W138. New animal cell lines can be established using methods well know by those skilled in the art (e.g., by transformation, viral infection, and/or selection). Optionally, etanercept can be secreted by the host cells into the medium.

Purification of the expressed etanercept can be performed by any standard method. When etanercept is produced intracellularly, the particulate debris is removed, for example, by centrifugation or ultrafiltration. When etanercept is secreted into the medium, supernatants from such expression systems can be first concentrated using standard polypeptide concentration filters. Protease inhibitors can also be added to inhibit proteolysis and antibiotics can be included to prevent the growth of microorganisms.

Etanercept can be purified using, for example, hydroxyapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, and any combination of known or yet to be discovered purification techniques, including but not limited to Protein A chromatography, fractionation on an ion-exchange column, ethanol precipitation, reverse phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSET®, an anion or cation exchange resin chromatography (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation.

Etanercept Stabilized with a Metal Ion

The invention provides a stabilized aqueous pharmaceutical composition comprising etanercept and a stabilizer to inhibit instability, aggregation and/or fragmentation of the etanercept, wherein the stabilizer comprises a stabilizing metal ion.

Without intending to be bound to any particular theory of the invention, it is believed that metal ions such as calcium, magnesium, and zinc act as conformational stabilizers to reduce etanercept's tendency to aggregate. The reduction in aggregation is believed to last for a long period of time, e.g., two years or more. Without wishing to be bound to a particular theory, it is believed that metal ions are able to stabilize aqueous pharmaceutical compositions containing etanercept because the metal can bind to the native state, where the right geometry of ligands occurs. In doing so, there is a net stabilization of the native state. Once the protein unfolds, the binding site is lost, and the denatured state in relatively unaffected in terms of free energy. The result is a net stabilization of the conformation, leading to improved long-term storage. In addition, metal biding may also improve the colloidal stability of the protein, elading to decreased aggregation

and increased solubility. The stabilization effects of metal ion are may not be limited to reduction in aggregates but may also address other aspects of instability of the etanercept monomer in the formulation.

In a preferred embodiment, the metal ion is selected from the group consisting of calcium, magnesium, zinc, and combinations thereof. In an even more preferred embodiment, calcium, magnesium, and zinc are provided as calcium chloride, magnesium chloride and zinc chloride, respectively.

The pharmaceutical compositions of the invention may be prepared by combining, a purified etanercept and a metal ion. Further, a buffer, a tonicity modifier and an additional excipient and other commonly used inactive ingredients can be added as needed. For simplicity, these are discussed more fully later in the specification. A person of ordinary skill in the art will understand that the combining of the various components to be included in the composition can be done in any appropriate order. For example, the buffer can be added first, middle or last, and the tonicity modifier can also be added first, middle or last. A person of ordinary skill in the art will also understand that some of these chemicals can be incompatible in certain combinations, and accordingly, are easily substituted with different chemicals that have similar properties but are compatible in the relevant mixture.

In a preferred embodiment, the concentration of the metal ion in the provided formulations is preferably between about 1 mM to 0.5 M, more preferably about 1 mM to about 100 mM, more preferably about 2 mM to about 20 mM, and yet more preferably about 2 to 10 mM.

Sources of metal ions are available from commercial suppliers.

In an embodiment using calcium chloride for stabilization, an etanercept formulation of the invention comprises about 25 to about 50 mg/ml of etanercept; up to about 5 mM calcium chloride; optionally about 0.5 to 6 wt.% sucrose or trehalose; optionally about 0 to100 mM NaCl; optionally up to about 10 mM xylitol; about 1 to about 30 mM sodium phosphate; wherein the composition has a pH of about 6.0 to about pH 7.0, and more preferably about 6.0 to about 6.6 and most preferably about 6.3 to about 6.5.

In an embodiment using magnesium chloride for stabilization, an etanercept formulation of the invention can comprise about 25 to about 50 mg/ml of etanercept;

about 1 mM to about 20 mM magnesium chloride; optionally up to about 6 wt.% sucrose; about 25 to 150 mM NaCl; about 1 to about 30 mM sodium phosphate; wherein the composition has a pH of about 6.0 to about pH 7.0, and more preferably about 6.0 to about 6.6 and most preferably about 6.3 to about 6.5.

Compositions stabilized with metal ions are preferably characterized as having an SEC analysis at T_2 of: about 80 wt.% to about 95 wt.% monomer content; an SEC analysis at T_2 of aggregate(s) content of less than about 4 wt.%; and an SEC analysis at T_2 of fragment 3 content of less than about 8 wt.%.

In one aspect, etanercept formulations containing a stabilizing metal ion according to the invention elicit stability characterized by:

(a) an SEC analysis at T_4 of greater than about 90, 91, 92, 93, 94, 95, 96, or 97 wt.% monomer content; and less than about 3, 2 or 1 wt.% aggregate(s) content; and

(b) an HIC analysis at T_2 wherein the amount of the composition represented by peak 1 of the HIC chromatogram is less than about 3, 2 or 1 wt.%; the amount of the composition represented by peak 2 of the HIC chromatogram is greater than 80, 81, 82, 83, 84 or 85 wt.%; and the amount of the composition represented by peak 3 of the HIC chromatogram is less than about 20, 19, 18, 17, 16, 15, 14, or 13 wt.%; and

(c) an HIC analysis at T_4 wherein the amount of the composition represented by peak 1 of the HIC chromatogram is less than about 3, 2 or 1 wt.%; the amount of the composition represented by peak 2 of the HIC chromatogram is greater than 80, 81, 82, 83, 84 or 85 wt.%; and the amount of the composition represented by peak 3 of the HIC chromatogram is less than 20, 19, 18, 17, 16, 15, 14 or 13 wt.%.

The etanercept formulations of the present invention containing metal ion for stabilization are more preferably characterized by having an HIC analysis at T_4 or T_2 wherein the amount of the composition represented by peak 1 of the HIC chromatogram is less than about 1%; the amount of the composition represented by peak 2 of the HIC chromatogram is greater than about 95 wt.% and most preferably

greater than about 99 wt.%; and the amount of the composition represented by peak 3 of the HIC chromatogram is less than about 3 wt.%.

The terms "SEC", "T_2" "T_4" "HIC" "monomer content" "aggregate(s)" and "fragment 3" "peak 1," "peak 2," and "peak 3," are defined above and in the examples below.

Preferred etanercept formulations stabilized with calcium chloride comprise: about 50 mg/ml etanercept; 1 to 5 mM calcium chloride; about 1 to 30 mM sodium phosphate; about 0 to 100 mM NaCl; about 0.5 to 5% sucrose or trehalose or combination thereof; and wherein the composition has a pH of about 6.0 to 6.6 and characterized by: an SEC analysis at T_4 of greater than about 97 wt.% monomer content and less than about 1 wt.% aggregate(s) content; an HIC analysis at T_2 wherein the amount of the composition represented by peak 1 of the HIC chromatogram is less than about 4 wt.%; the amount of the composition represented by peak 2 of the HIC chromatogram is greater than about 82 wt.%; and the amount of the composition represented by peak 3 of the HIC chromatogram is less than about 15 wt.%; and an HIC analysis at T_4 wherein the amount of the composition represented by peak 1 of the HIC chromatogram is less than about 2 wt.%; the amount of the composition represented by peak 2 of the HIC chromatogram is greater than about 85 wt.%; and the amount of the composition represented by peak 3 of the HIC chromatogram is less than about 13 wt.%.

Preferred etanercept formulations stabilized with magnesium chloride comprise: about 1 mM to about 20 mM magnesium chloride; optionally up to about 6 wt.% sucrose; about 25 to 150 mM NaCl; about 1 to about 30 mM sodium phosphate; wherein the composition has a pH of about 6.0 to 6.6; and wherein the composition is characterized by: an SEC analysis at T_4 of greater than about 97 wt.% monomer content and less than about 1 wt.% aggregate(s) content; an HIC analysis at T_2 wherein the amount of the composition represented by peak 1 of the HIC chromatogram is less than about 4 wt.%; the amount of the composition represented by peak 2 of the HIC chromatogram is greater than about 85 wt.%; and the amount of the composition represented by peak 3 of the HIC chromatogram is less than about 14 wt.%; and an HIC analysis at T_4 wherein the amount of the composition represented by peak 1 of the HIC chromatogram is less than about 2 wt.%; the amount of the composition represented by peak 2 of the HIC chromatogram is

greater than about 85 wt.%; and the amount of the composition represented by peak 3 of the HIC chromatogram is less than about 14 wt.%.

In particularly preferred embodiments of the invention using calcium chloride for stabilization, a stabilized etanercept formulation having the analytical properties referenced above comprises: about 50 mg/ml of etanercept; about 2 mM calcium chloride; about 15 mM sodium phosphate; about 75 mM sodium chloride; and about 3 wt.% sucrose; wherein the formulation has a pH of about 6.3 to 6.5.

In a further embodiment the metal ion stabilized etanercept composition the stabilizer is calcium chloride or magnesium chloride, the formulation is free or essentially free of arginine, and the formulation elicits long term storage stability as characterized by:

SEC analysis at M_3 of: monomer content greater than about 90%; aggregates content of less than about 3 wt%; and fragment 3 content less than about 5 wt%: and

HIC analysis at M_3 wherein the amount of the composition represented by peak 1 of the HIC chromatogram is less than about 3 wt.%; the amount of the composition represented by peak 2 of the HIC chromatogram is greater than 80 wt.%; and the amount of the composition represented by peak 3 of the HIC chromatogram is less than about 20 wt.%.

In a further embodiment the stabilizer is calcium chloride and/or magnesium chloride, and the stabilized composition elicits long term storage stability as characterized by:

SEC analysis at M_3 or T_4 of greater than about 90 wt.% monomer content; less than about 3 wt.% aggregate(s) content; and less than about 5 wt% fragment 3; and

HIC analysis at M_3 or T_2 or T_4 wherein the amount of the composition represented by peak 1 of the HIC chromatogram is less than about 3 wt.%; the amount of the composition represented by peak 2 of the HIC chromatogram is greater than 80 wt.%; and the amount of the composition

represented by peak 3 of the HIC chromatogram is less than about 20 wt.%.

In a further preferred embodiment of the invention using magnesium chloride for stabilization, a stabilized etanercept formulation having the analytical properties referenced above comprises: about 50 mg/ml of etanercept; about 10 mM magnesium chloride; about 15 mM sodium phosphate; about 75 mM sodium chloride; and about 3 wt.% sucrose; and having a pH of about 6.3 to 6.5.

In another preferred embodiment a calcium chloride or magnesium chloride stabilized etanercept composition according to the invention elicits long term storage stability as characterized by: an HIC analysis at M_3 wherein the amount of the composition represented by peak 2 of the HIC chromatogram is greater than or equal to about 95 wt.%; and wherein, if peak 3 is present on the HIC chromatogram, the amount of the composition represented by peak 3 is less than or equal to about 1 wt.%.

The metal ion stabilized etanercept formulations, containing no arginine, are found to elicit stability over three months storage at 5C that is comparable to or better than the presently available arginine containing formulation of commercially available Enbrel. Accordingly the present invention is further directed to metal ion stabilized formulations of etanercept, which contain no arginine, or are essentially free of arginine, and wherein the composition, after storage at 5° C for one, two or three months, elicits long term storage stability that meets one or both of the following criteria when evaluated at a point in time selected from one or more of the following: at approximately one month, at approximately two months or at approximately three months after initiation of storage of the composition at a storage temperature of 5° C:

(A) stability comparable to or better than commercially available etanercept marketed under the trademark Enbrel®, as measured by (i) SEC analysis of the amounts of aggregate(s), monomer and fragment 3 in the composition (as defined in the specification) and (ii) HIC analysis of amounts of material in the composition corresponding to peaks 1, 2 and 3 of the HIC chromatogram (as defined in the specification); and

(B) an HIC chromatogram in which (i) peak 3 is absent, or essentially absent and (ii) peak 2 represents greater than about 95 wt% of the composition; an SEC chromatogram containing essentially no peak corresponding to aggregate(s); and an SEC chromatogram in which the monomer content represents at least about 95 wt% of the composition.

The terms "SEC", "T_2" "T_4" "HIC" "monomer content" "aggregate(s)" and "fragment 3" "peak 1," "peak 2," and "peak 3," are defined above and in the examples below.

Although the use of stabilizing metal ions according to the invention does not exclude the use of arginine, the etanercept formulations comprising metal ion for stabilization according to the present invention are preferably free or essentially free of arginine.

Additional Components of the Provided Pharmaceutical Compositions

The formulations of the invention may also include buffers, tonicity modifiers, excipients, pharmaceutically acceptable carriers and other commonly used inactive ingredients of the pharmaceutical compositions. For simplicity, these are discussed more fully later in the application.

Buffers maintain pH in a desired range. Suitable buffers include histidine, potassium phosphate, sodium or potassium citrate, maleic acid, ammonium acetate, tris-(hydroxymethyl)-aminomethane (tris), various forms of acetate and diethanolamine. The concentration of the buffer in the formulation is preferably between about 1 mM to about 1M, and more preferably about 10 mM to about 200 mM. Buffers are well known in the art and are manufactured by known methods and available from commercial suppliers.

Examples of suitable buffers are phosphate, histidine, citrate, maleate, tartrate, succinate, acetate, tris-(hydroxymethyl)-aminomethane (tris), bicarbonate.

In a preferred embodiment, the buffer is sodium phosphate.

In a preferred embodiment, the pH of the pharmaceutical composition is at or near physiological levels. Thus, preferably, the pH of the provided compositions is between about 5.8 and about 8.4; and even more preferably, between about 6.2 and about 7.4. A person of ordinary skill in the art will understand that the pH can be

adjusted as necessary to maximize stability and solubility of etanercept in a particular formulation. Thus, etanercept formulations at a pH outside of physiological ranges, yet tolerable to the patient, are also within the scope of the invention.

A tonicity modifier is a molecule that contributes to the osmolality of a solution. The osmolality of a pharmaceutical composition is preferably adjusted to maximize the active ingredient's stability and/or to minimize discomfort to the patient upon administration. It is generally preferred that a pharmaceutical composition be isotonic with serum, i.e., having the same or similar osmolality, which is achieved by addition of a tonicity modifier.

In a preferred embodiment, the osmolality of the provided formulations is from about 180 to about 420 mOsM. However, it is to be understood that the osmolality can be either higher or lower as specific conditions require.

Examples of tonicity modifiers suitable for modifying osmolality include, but are not limited to amino acids (not including arginine) (e.g., cysteine, histidine and glycine), salts (e.g., sodium chloride, potassium chloride and sodium citrate) and/or saccharides (e.g., sucrose, glucose and mannitol).

Preferred tonicity modifiers are glycine, alanine, sodium chloride, potassium chloride, and sodium sulfate.

In a preferred embodiment, the concentration of the tonicity modifier in the formulation is preferably between about 1 mM to about 1 M, more preferably about 10 mM to about 200 mM. Tonicity modifiers are well known in the art and are manufactured by known methods and available from commercial suppliers.

Excipients, also referred to as chemical additives, co-solutes, or co-solvents, that stabilize the polypeptide while in solution (also in dried or frozen forms) can also be added to a pharmaceutical composition. Excipients are well known in the art and are manufactured by known methods and available from commercial suppliers.

Examples of suitable excipients include but are not limited to sugars/polyols such as: sucrose, lactose, glycerol, xylitol, sorbitol, mannitol, maltose, inositol, trehalose, glucose; polymers such as: serum albumin (bovine serum albumin (BSA), human SA or recombinant HA), dextran, poly(viny alcohol) PVA, hydroxypropyl methylcellulose (HPMC), polyethyleneimine, gelatin, polyvinylpyrrolidone (PVP), hydroxyethylcellulose (HEC); non-aqueous solvents such as: polyhydric alcohols,

(e.g., PEG, and glycerol) and dimethylformamide (DMF); amino acids such as: proline, L-serine, sodium glutamic acid, alanine, glycine, lysine hydrochloride, sarcosine and gamma-aminobutyric acid; surfactants such as: Tween®-80 (polysorbate 80), Tween®-20 (polysorbate 20), SDS, polysorbate, poloxamers; and miscellaneous excipients such as: potassium phosphate, sodium acetate, ammonium sulfate, magnesium sulfate, sodium sulfate, trimethylamine N-oxide, betaine, metal ions (e.g., zinc, calcium, and magnesium), CHAPS, monolaurate, 2-O-beta-mannoglycerate or any combination of the above.

Preferred excipients are sucrose, lactose, glycerol, xylitol, sorbitol, mannitol, maltose, inositol, trehalose, glucose, bovine serum albumin (BSA), human serum albumin (HSA), recombinant albumin, dextran, PVA, hydroxypropyl methylcellulose (HPMC), polyethyleneimine, gelatin, polyvinylpyrrolidone (PVP), hydroxyethylcellulose (HEC), polyethylene glycol, ethylene glycol, glycerol, alanine, glycine, lysine hydrochloride, sarcosine, SDS, polysorbate 20, polysorbate 80, poloxamer 188, trimethylamine N-oxide, betaine, zinc ions, calcium ions, magnesium ions, CHAPS, sucrose monolaurate, and 2-O-beta-mannoglycerate.

The concentration of one or more excipients in a formulation of the invention is/are preferably between about 0.001 to 5 weight percent, more preferably about 0.1 to 2 weight percent.

Methods of Treatment

In another embodiment, the invention provides a method of treating a mammal comprising administering a therapeutically effective amount of the pharmaceutical compositions of the invention to a mammal, wherein the mammal has a disease or disorder that can be beneficially treated with etanercept.

In a preferred embodiment, the etanercept is derived from the same species of mammal as is to be treated with the composition.

In a preferred embodiment, the mammal is a human.

Diseases or disorders that can be treated with the provided compositions include but are not limited to rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, Wegener's disease (granulomatosis), Crohn's disease (or inflammatory bowel disease), chronic obstructive pulmonary disease (COPD), Hepatitis C,

endometriosis, asthma, cachexia, psoriasis, and atopic dermatitis. Additional diseases or disorders that can be treated with the compositions of the present invention include those described in WO 00/62790, WO 01/62272, U.S. Patent Application No. 2001/0021380, and US Pat. 7,648,702 B2, the relevant portions of which are incorporated herein by reference.

The provided pharmaceutical compositions may be administered to a subject in need of treatment by injection systemically, such as by intravenous injection; or by injection or application to the relevant site, such as by direct injection, or direct application to the site when the site is exposed in surgery; or by topical application.

In one embodiment, the invention provides a method of treatment and/or prevention of rheumatoid arthritis comprises administering to a mammal in need thereof a therapeutically effective amount of one of the provided etanercept compositions.

The therapeutically effective amount of the etanercept in the provided compositions will depend on the condition to be treated, the severity of the condition, prior therapy, and the patient's clinical history and response to the therapeutic agent. The proper dose can be adjusted according to the judgment of the attending physician such that it can be administered to the patient one time or over a series of administrations.

In one embodiment, the effective etanercept amount per adult dose is from about 1-500 mg/m^2, or from about 1-200 mg/m^2, or from about 1-40 mg/m^2 or about 5-25 mg/m^2.

Alternatively, a flat dose may be administered, whose amount may range from 2-500 mg/dose, 2-100 mg/dose or from about 10-80 mg/dose.

If the dose is to be administered more than one time per week, an exemplary dose range is the same as the foregoing described dose ranges or lower and preferably administered two or more times per week at a per dose range of 25-100 mg/dose.

In another embodiment, an acceptable dose for administration by injection contains 80-100 mg/dose, or alternatively, containing 80 mg per dose.

The dose can be administered weekly, biweekly, or separated by several weeks (for example 2 to 8).

In one embodiment, etanercept is administered at 25 to 75 mg/ml by a single subcutaneous (SC) injection.

In some instances, an improvement in a patient's condition will be obtained by administering a dose of up to about 100 mg of the pharmaceutical composition one to three times per week over a period of at least three weeks. Treatment for longer periods may be necessary to induce the desired degree of improvement. For incurable chronic conditions the regimen may be continued indefinitely. For pediatric patients (ages 4-17), a suitable regimen may involve administering a dose of 0.4 mg/kg to 5 mg/kg of etanercept, one or more times per week.

In another embodiment, the pharmaceutical formulations of the invention may be prepared in a bulk formulation, and as such, the components of the pharmaceutical composition are adjusted to be higher than would be required for administration and diluted appropriately prior to administration.

The pharmaceutical compositions can be administered as a sole therapeutic or in combination with additional therapies as needed. Thus, in one embodiment, the provided methods of treatment and/or prevention are used in combination with administering a therapeutically effective amount of another active agent. The other active agent may be administered before, during, or after administering the pharmaceutical compositions of the present invention. Another active agent may be administered either as a part of the provided compositions, or alternatively, as a separate formulation.

Administration of the provided pharmaceutical compositions can be achieved in various ways, including parenteral, peroral, buccal, sublingual, nasal, rectal, intraperitoneal, intradermal, transdermal, subcutaneous, intravenous, intra-arterial, intracardiac, intraventricular, intracranial, intratracheal, intrathecal administration, intramuscular injection, intravitreal injection, and topical application.

The pharmaceutical compositions of this invention are particularly useful for parenteral administration, i.e., subcutaneously, intramuscularly, intravenously, intraperitoneal, intracerebrospinal, intra-articular, intrasynovial, intravitreal, and/or intrathecal. Parenteral administration can be by bolus injection or continuous

infusion. Pharmaceutical compositions for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. In addition, a number of recent drug delivery approaches have been developed and the pharmaceutical compositions of the present invention are suitable for administration using these new methods, e.g., Inject-ease®, Genject®, injector pens such as GenPen®, and needleless devices such as MediJector® and BioJector®. The present pharmaceutical composition can also be adapted for yet to be discovered administration methods. See also Langer, 1990, Science, 249:1527-1533.

The provided pharmaceutical compositions can also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the formulations may be modified with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The pharmaceutical compositions may, if desired, be presented in a vial, pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. In one embodiment the dispenser device can comprise a syringe having a single dose of the liquid formulation ready for injection. The syringe can be accompanied by instructions for administration.

In another embodiment, the present invention is directed to a kit or container, which contains an aqueous pharmaceutical composition of the invention. The concentration of the polypeptide in the aqueous pharmaceutical composition can vary over a wide range, but is generally within the range of from about 0.05 to about 20,000 micrograms per milliliter (μg/ml) of aqueous formulation. The kit can also be accompanied by instructions for use.

The present invention is more particularly described in the following examples that are intended as illustrative only, since many modifications and variations therein will be apparent to those skilled in the art.

EXAMPLE 1

Etanercept Stabilized with Calcium Chloride

Etanercept formulations may be prepared using the following procedures:

Each solid formulation component is weighed to the amount required for a given volume of formulation buffer. These components are combined into a beaker or vessel capable of carrying and measuring the given volume of formulation buffer. A volume of deionized water equal to approximately ¾ of the target given formulation buffer is added to the beaker, and the components are then solublized. The pH of the buffer is adjusted to the target formulation pH using 1 M sodium hydroxide and/or 1 M hydrogen chloride. The final formulation buffer volume is then raised to the target volume through the addition of deionized water. Etanercept protein solution is placed in dialysis material housing (such as Thermo Scientific Slide-A-Lyzer MINI Dialysis Unit 10,000 MWCO), which is then placed in contact with the desired formulation buffer for 12 hours at 4°C. Formulation buffer volume to protein solution volume ratio should be no less than 1000:1. The dialysis housing and protein solution it contains is then placed in a second, equal volume of formulation buffer for an additional 12 hours at 4°C. Resulting protein solution is removed from the dialysis material housing, and the concentration of protein determined using ultraviolet spectroscopy. Protein concentration is adjusted to the desired level using centrifugation (such as Amicon Ultra 10,000 MWCO Centrifugal Concentrators) and/or dilution with formulation buffer.

The compositions can be tested for long-term stability by size exclusion chromatography (SEC), denatured SEC (dSEC), hydrophobic interaction chromatography (HIC), sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and for binding and bioactivity at various timepoints. The bioactivity can be measured by any number of well-known assays.

For example, the techniques of Size Exclusion Chromatography are described in Hawe et al, Pharm. Res. 2011, 28: 2302 and/or van Marrschalkerweerd et al., Eur. J. Pharm. Biopharm. 2011, 78: 213. Similarly, the techniques of Denatured Size Exclusion Chromatography, Hydrophobic Interaction Chromatography, and Sodium

DodecylSulfate-PolyAcrylamide Gel Electrophoresis are also well known to persons having ordinary skill in the art.

It is believed that the composition will be stable over the term of two years or more.

5

(Formulation P1:1)

Ingredient	concentration
Etanercept (active ingredient)	50 mg/ml
Calcium chloride (inactive ingredient)	2 mM
Sodium phosphate, pH 6.3 (inactive)	25 mM

(Formulation 1:11)

Ingredient	concentration
Etanercept (active ingredient)	50 mg/ml
Calcium chloride (inactive ingredient)	2 mM
Sodium phosphate, pH 6.3 (inactive)	25 mM
NaCl (inactive)	100 mM
Sucrose (inert)	2.5 % (w/v)

10

(Formulation 1:18)

Ingredient	concentration
Etanercept (active ingredient)	50 mg/ml
Calcium chloride (inactive ingredient)	2 mM
Sodium phosphate, pH 6.3 (inactive)	25 mM
Xylitol (inactive)	10 mM

(Formulation 3:6)

Ingredient	concentration
Etanercept (active ingredient)	50 mg/ml
Calcium chloride (inactive ingredient)	2 mM
Sodium phosphate, pH 6.3 (inactive)	15 mM
NaCl (inactive)	75 mM
Sucrose (inactive)	3 % (w/v)

(Formulation 3:9)

Ingredient	concentration
Etanercept (active ingredient)	50 mg/ml
Calcium chloride (inactive ingredient)	1 mM
Sodium phosphate, pH 6.6 (inactive)	10 mM
NaCl (inactive)	50 mM
Trehalose (inactive)	5 % (w/v)

EXAMPLE 2

Etanercept Stabilized with Magnesium Chloride

Etanercept formulations stabilized with magnesium chloride may be prepared and tested using the procedures similar to those described in Example 1. The etanercept formulations exemplified below do not contain arginine.

(Formulation P1:2)

Ingredient	concentration
Etanercept (active ingredient)	50 mg/ml
Magnesium chloride (inactive ingredient)	2 mM
Sodium phosphate, pH 6.3 (inactive)	25 mM

(Formulation 2:15)

Ingredient	concentration
Etanercept (active ingredient)	50 mg/ml
Magnesium chloride (inactive ingredient)	4 mM
Sodium phosphate, pH 6.4 (inactive)	25 mM
NaCl (inactive)	100 mM
Sucrose (inactive)	2.5 % (w/v)

(Formulation 3:7)

Ingredient	concentration
Etanercept (active ingredient)	50 mg/ml
Magnesium chloride (inactive ingredient)	5 mM
Sodium phosphate, pH 6.3 (inactive)	15 mM
NaCl (inactive)	75 mM
Sucrose (inactive)	2.5 % (w/v)

(Formulation 3:14)

Ingredient	concentration
Etanercept (active ingredient)	50 mg/ml
Magnesium chloride (inactive ingredient)	10 mM
Sodium phosphate, pH 6.3 (inactive)	25 mM
NaCl (inactive)	110 mM
Sucrose (inactive)	1 % (w/v)

(Formulation 4:2)

Ingredient	concentration
Etanercept (active ingredient)	50 mg/ml
Magnesium chloride (inactive ingredient)	10 mM
Sodium phosphate, pH 6.5 (inactive)	15 mM
NaCl (inactive)	75 mM
Sucrose (inactive)	3 % (w/v)

EXAMPLE 3

Etanercept Stabilized with Zinc Chloride

Etanercept formulations stabilized with zinc chloride may be prepared and tested using the procedures similar to those described in Example 1.

The etanercept formulation exemplified below does not contain arginine.

(Formulation P1:3)

Ingredient	concentration
Etanercept (active ingredient)	50 mg/ml
Zinc chloride (inactive ingredient)	2 mM
Sodium phosphate, pH 6.3 (inactive)	25 mM

EXAMPLE 4
PREPARATION OF ETANERCEPT

STEP 1. Cell Expansion. In a manner known in the art, cell expansion necessary to generate a sufficient number of cells for inoculation of a production bioreactor is performed using a clone of CHO cells expressing the etanercept fusion protein. The product of this expresson process (a harvested cell culture fluid) results in a mixture of correctly folded etanercept, as well as incorrectly folded and/or aggregated etanercept, along with additional impurities. The harvested cell culture fluid comprising such protein mixture is subjected to detergent viral inactivation.

STEP 2. Affinity Chromatography. Affinity chromatography is performed on the harvested cell culture obtain in Step 1 above using a conventional Protein A affinity column in a well known manner. Product recovery is approximately 85%. The product obtained is a complex protein mixture comprising correctly folded etanercept, incorrectly folded etanercept, and/or aggregates of correctly and/or incorrectly folded etanercept, or protein fragments. The product obtained from this Protein A affinity column purification step is adjusted to pH 3.5 and then subjected to a viral inactivation step. Following viral inactivation the product is adjusted to pH 5.5 and then further clarified in a known manner using a commercially obtained capsule filter.

STEP 3A. Mixed-Mode Cation Exchange Chromatography. A 31.8 L (45 cm diameter X 20 cm bed height) packed bed GE Healthcare Capto MMC chromatography column is used to purify the product obtained in Step 2 above. Prior to use, the column is equilibrated with 2 CV of 25 mM acetate pH 5.5 and sanitized with 2 CV of 0.1 N NaOH, 1 M NaCl and neutralized with 2 CV of 25 mM acetate, 0.7 M NaCl, pH 5.5. The column is then equilibrated with 8-10 CV of 25 mM acetate pH 5.5 until the effluent is pH 5.5 and 3.5 mS/cm. The Protein A pool from step 2 above is diluted to ≤ 6 mS/cm with WFI and applied to a column loading of up to 15 g/L media for each cycle. The column is operated at a linear velocity of 200 cm/h to give a 6 minute residence time. After loading, the column is washed with 2 CV of 25 mM acetate pH 5.5. The product is then eluted with an 8.5 CV, 15% to 85% gradient of 25 mM acetate pH 5.5 to 25 mM acetate, 0.7 M NaCl, pH 5.5. Product collection begins at 0.15 OD (A280, 1.0 cm path length) and collection ends at 50% of peak

maximum. The eluate volume is approximately 5 CV. Residual product and contaminants are stripped from the column with 2 CV of 10 mM Tris, 1 M NaCl, pH 8.0 and discarded. The product obtained from the mixed mode column is filtered using a Millipore Opticap XL10, 0.22 µm Durapore capsule filter, (0.69 m^2). The product obtained from this step represents a recovery of about 70% of the Protein A material obtained in Step 2

STEP 3B. Mixed Mode Anion Exchange Chromatopgraphy. A 27.0 L (45 cm diameter X 17 cm bed height) packed bed GE Healthcare Capto Adhere chromatography column is used to further purify the product obtained in step 3A above. Prior to use, the column is equilibrated with 2 CV of 25 mM Tris, pH 8.0 and sanitized with 2 CV of 0.1 N NaOH, 1M NaCl and neutralized and equilibrated with 2 CV of 25 mM Tris, pH 8.0. Prior to product loading, the column is equilibrated with 3 CV of 10 mM Tris, pH 8.0. The Capto MMC pool from Step 3A above is adjusted to pH 8.1 with ~0.045 kg of 1 M Tris, pH 8.3 per kg of pool. The product from Step 3A above was diluted in-line 1:3.8 with WFI to adjust the conductivity to 12.0 mS/cm and pH 8.0. The resulting material is then applied to a column loading of up to 15 g/L media. The column is operated at a linear velocity of 170 cm/h to give a 6 minute residence time. After loading, the column is washed with 2 CV of 25 mM Tris, pH 8.0. The product is then eluted with a 10 CV gradient (20% to 90%) of 25 mM Tris, pH 8.0 to 10 mM Tris, 1 M NaCl, pH 8.0. Product collection is started at 0.15 OD (A280, 1.0 cm path length) and collection ended at 25% of peak maximum. The eluate volume is 4-6 CV. The eluted product is filtered using a commercially available capsule filter and then subjected in a known manner to viral filtration and tangential flow filtration steps. Overall product recovery from step3B (including the final viral and tangential flow filtration steps) was approximately 68%. Product recovery measured before the filtration steps was about 75%. A schematic representation of HIC data obtained on eluation fractions from this step are representing in Figure 12.

Analysis: The final filtered product obtained in this example is found to have greater than about 90 wt % correctly folded etanercept as determined by HIC; less than 5 wt% incorrectly folded etanercept species as determined by HIC; less than about 3 wt% of clipped material by HIC analysis (believed to be fragments of etanercept in which the TNFR portion thereof has been truncated) and a combined

amount of correctly and incorrectly folded etanercept of greater than 95 wt% as determined by size exclusion chromatography.

Analysis of Etanercept Formulations

A. Thermal Stability Storage

Following dialysis and concentration, samples of the etanercept formulations exemplified above were sterile filtered in a bio safety cabinet. Using sterilized pipettes and autoclaved pipette tips, samples of the etanercept formulations were transferred to pre-labeled and autoclaved 1 mL lyophilization vials. Vials were stoppered with sterile butyl stoppers and crimped with aluminum caps. All vials were then transferred to thermal stability ovens. Samples were subject to two thermal stability regimes: (1) two weeks at 40 °C and (2) four weeks at 25 °C. Throughout this specification, these two temperature regimes are denoted "T_2" and "T_4," respectively.

B. Size Exclusion Chromatography (SEC)

Etanercept formulations disclosed herein were analyzed using the well known technique of Size Exclusion Chromatography (SEC), a high-performance liquid chromatography method in which analytes are separated by size (see Rogner, M. (2000). Size Exclusion Chromatography. Protein Liquid Chromatography. M. Kastner. Amsterdam, Elsevier. **61:** 89-145.). In order to evaluate thermal stability of the Etanercept samples decribed above, the samples were examined by a SEC method based on the literature (van Maarschalkerweerd, A., G. J. Wolbink, et al. (2011). "Comparison of analytical methods to detect instability of etanercept during thermal stress testing." European Journal of Pharmaceutics and Biopharmaceutics **78**(2): 213-221.) The mobile phase buffer was prepared to contain 50 mM sodium phosphate monobasic monohydrate and 150 mM arginine. The pH was adjusted to 6.5 using 1 M HCl. All separations were performed using a Tosoh TSK-Gel SWxl 6 mm x 4 cm guard column (cat. no. 8543) attached linearly to a Tosoh TSK-Gel G4000 SWxl 7.8 mm x 30 cm (cat. no. 8542). To perform a separation, the columns were brought to room temperature (23°C) and equilibrated with mobile phase at a

flow rate of 0.5 mL/min. 5 microliters of 50 mg/mL etanercept formulation were injected onto the column using an autosampler. The separation was accomplished over 30 minutes at a flow rate of 0.5 mL/minute. Column eluent was monitored at a wavelength of 280 nm during this time.

5

C. Integration of Size Exclusion Chromatography Chromatograms

All integration was performed using Chromeleon software (Dionex). Prior to integration, the SEC chromatogram for a buffer containing no etanercept was 10 subtracted from all chromatograms. All integration was performed between retention times of 12 minutes and 26 minutes. Several parameters were used to define a peak. The minimum area for a detected peak was set to 0.05 mAu * min. The two-dimensional sensitivity for peak detection was set to 0.01 mAu and 75 seconds. Peak shoulders were added manually using a manual integration tool. All detected 15 peaks were manually adjusted in two steps. First, peak baselines (the bottom boundary of the peak) were adjusted to horizontal. Secondly, the vertical positions of the peak baselines were adjusted to that of the chromatogram baseline. The chromatogram baseline value was defined as the signal in absence of analyte. The signal in absence of analyte was defined as the absorbance in mAu at 12 minutes 20 retention time.

D. SEC Fractions of Etanercept Formulations

In the SEC analysis of etanercept formulations described above, three SEC chromatography fractions were identified and studied. The fractions that were 25 analyzed were, in the order of elution from the SEC column: (1) a high molecular weight fraction representing aggregates of the intact etanercept TNFR:FC fusion protein likely assembled via non-covalent electrostatic attraction among intact etanercept molecules (hereinafter "aggregate(s)" or aggregate(s) content); (2) monomer content, representing the intact etanercept TNFR:Fc fusion protein 30 (hereinafter referred to as "monomer" of "monomer content"); (3) a fraction likely representing one fragment or a population of fragments of the etanercept molecule in which one portion of the TNFR:molecule fusion protein has become cleaved from the monomer; in the loss of an arm of the Fabportion of the fusion protein at the hinge

region of the molecule. The most common fragment or clipped species, as measured by SEC, is referred to as Fragment 3. In conducting the SEC analysis, it will be observed that aggregates elute first, followed by monomer, followed by fragment 3.

The following tables show the relative amounts of aggregate(s), monomer and fragment 3 determined by SEC analysis as described above.

TABLE 1

SEC ANALYSIS OF MONOMER

Note: Amounts reported Tables I, II and III are percentages by weight

T_0 = formulation maintained at 5 C and analyzed within 24 hours of creation.

T_1 = formulation stored for one week at 40° C

T_2 = formulation stored for two weeks at 40 C

Formulation No.	t_0	t_1	t_2
Commercial Enbrel (comparative) [1:2]	98.81	92.58	87.64
1:11	98.60	92.08	89.71
1:18	98.27	92.89	88.21
2:15	98.18	--	88.22
3:6	98.07	--	90.75
3:7	98.09	--	89.60
3:9	97.90	--	91.44
3:14	98.22	--	90.54
4:2	98.62		90.47

TABLE II

SEC ANALYSIS OF AGGREGATES

Note: Amounts reported Tables I, II and III are percentages by weight

T_0 = formulation maintained at 5 C and analyzed within 24 hours of creation.

T_1 = formulation stored for one week at 40° C

T_2 = formulation stored for two weeks at 40 C

Formulation No.	t_0	t_1	t_2
Commercial Enbrel (comparative)	0.09	0.59	1.02
1:11	0.12	0.50	0.64
1:18	0.24	0.65	1.61
2:15	0.27	--	1.83
3:6	0.23	--	1.27
3:7	0.28	--	0.93
3:9	0.37	--	0.73
3:14	0.25	--	0.91
4:2			1.56

TABLE III

ANALYSIS OF FRAGMENT 3

Note: Amounts reported Tables I, II and III are percentages by weight

T_0 = formulation maintained at 5 C and analyzed within 24 hours of creation.

T_1 = formulation stored for one week at 40° C

T_2 = formulation stored for two weeks at 40 C

Formulation No	t_0	t_1	t_2
Commercial Enbrel (comparative)	0.00	3.30	6.29
1:11	0.00	3.92	**4.71**
1:18	0.00	3.05	**4.65**
2:15	0.00		5.56
3:6	0		4.72
3:7	0		4.37
3:9	0		3.48
3:14	0		3.83
4:2			5.40

TABLE IV

SEC ANALYSIS OF MONOMER CONTENT

(T_4 = 4 weeks/25° C)

Table IV below shows monomer (etanercept) content of etanercept formulations prepared according to the present invention, when stored for four weeks at 25 C°- denoted by the symbol T_4. In the following table T_0 represents SEC measurements conducted within 24 hours of formulation preparation, at sample temperature of 5° C; and T_4 represents etanercept formulation samples subjected to SEC analysis after 4 weeks storage at 25° C.

FORMULATION No.	T_0 Monomer Content	T_4 Monomer Content
Commercial Enbrel (comparative)	98.15	97.86
3:6	98.07	94.84
3:7	98.09	97.75
3:9	97.90	97.44
3:14	98.22	97.79
4:2	98.62	94.70

TABLE V

SEC ANALYSIS OF AGGREGATES CONTENT

(T_4 = 4 weeks/25° C)

Table V below shows aggregate(s) content of etanercept formulations prepared according to the present invention after storage for four weeks at 25 C°. In the following table T_0 represents SEC measurements conducted within 24 hours of formulation preparation, at sample temperature of 5° C; and T_4 represents etanercept formulation samples subjected to SEC analysis after 4 weeks storage at 25° C.

FORMULATION No.	T_0 Aggregate(s) Content	T_4 Aggregate(s) Content
Commercial Enbrel (comparative)	0.28	0.25
3:6	--	0.57
3:7	0.28	0.31
3:9	0.37	0.41
3:14	0.25	0.28
4:2	--	0.57

HIC ANALYSIS OF ETANERCEPT FORMULATIONS

The following tables (Tables VI and VII) show the results of hydrophobic interaction chromatography ("HIC chromatography") conducted on samples 3:5 and 3:8. HIC chromatography was carried out in a manner known in the art and generally described in U.S. Patent 7,294,481, incorporated herein by reference. Samples were evaluated at t_0 (within 24 hours of preparation at 5°C.) and again after either two weeks of storage at 25°C. (t_2) (see Table VI) or after 4 weeks of storage at 25°C. (t_4) (See Table VII) Peak 1 in the HIC chromatogram is believed to be or include "Fragment 3", which is identified and quantified using SEC, as referenced above in the discussion of SEC data; Peak 2 is etanercept monomer as referenced above in the discussion of SEC data; and Peak 3 includes "Aggregate(s)" as referenced above in the discussion of SEC data. It should further be understood that the terms "peak 1", "peak 2" and "peak 3 as used here also constitute a reference to the HIC peak 1, peak 2 and peak 3 referred to and disclosed in Figure 4 of U.S. patent 7,294,481 incorporated herein by reference.

TABLE VI

HIC Data after Two Weeks Storage at 40°C. (T_2)

	PEAK 1		PEAK 2		PEAK 3	
Form. #	T_0	T_2	T_0	T_2	T_0	T_2
Commercial Enbrel (comparative)	0.91	3.23	86.72	83.41	12.33	13.36
3:6	0.72	3.44	85.91	83.26	13.36	13.30
3:7	0.74	3.52	86.11	82.41	13.15	14.07
3:9	0.69	2.39	90.93	85.09	8.38	12.52
3:14	0.71	2.51	87.14	84.54	12.15	12.95

5

TABLE VII

HIC Data after Storage at 25°C for 4 Weeks (T_4)

	PEAK 1		PEAK 2		PEAK 3	
Form. #	T_0	T_4	T_0	T_4	T_0	T_4
Commercial Enbrel (comparative)	0.91	1.09	86.76	86.95	12.33	11.97
3:6	0.55	1.40	85.50	84.07	13.96	14.53
3:7	0.74	1.63	86.11	85.65	13.15	12.72
3:9	0.69	1.05	90.93	86.46	8.38	12.50
3:14	0.71	1.13	87.14	85.58	12.15	13.29
4:2	0.63	1.38	85.16	84.38	14.21	14.25

5

Tables VIII through XVI, below, contain the results of stability testing conducted on formulations 3:6 and 4:2 and containing etanercept material produced in the manner generally described Example 4 (Preparation of Etanercept). Stability of the formulations was evaluated using SEC, HIC and FlowCam analysis for subvisible particles, based on one, two and three months of storage at various temperatures, including 5°C. Following is the methodology used to conduct these stability experiments:

Bulk Etanercept Storage. Non-formulated bulk etanercept was stored at 2-8°C as indicated by the Enbrel® package insert.

UV Spectroscopy. UV spectroscopy was used to determine protein concentration in various stability samples. The absorbance at 280 nm of bulk substance (50 mg/mL Enbrel) was determined to be 0.625 using a 0.1 mm pathlength cell, leading to an extinction coefficient of 1.30 mL/mg*cm. This value was used for all calculations in this project.

Dialysis and Concentration of Etanercept Formulation. All buffers were prepared in two 1 L volumes containing all buffer components. Bulk material was loaded into Slide-A-Lyzer dialysis units (10 kD cutoff, one to three mL volume) following a five-minute rinse of the cassettes in deionized water. Dialysis samples were subjected to a five-hour dialysis at 2-8° C in 1 L of buffer, followed by a second dialysis at 2-8° C overnight in a second 1 L of buffer. When dialyzing the etanercept formulation 3:5, two 4L dialysis events were used as 24 mL of stock protein was needed for the formulation.

All samples were concentrated above their target value using Amicon Ultra 10K cutoff centrifuge filters (2 mL size). UV was used to determine new concentration of samples, which were then diluted to the appropriate level using formulation buffer.

Thermal Stability Sample Incubation. Following dialysis and concentration, thermal stability samples were sterile filtered in a bio safety cabinet. Using sterilized pipettes and autoclaved pipette tips, samples were transferred to pre-labeled and

autoclaved 1 mL lyophilization vials. Vials were stoppered with sterile butyl stoppers and crimped with aluminum caps. All vials were then transferred to thermal stability ovens.

Size Exclusion Chromatography (SEC). Size exclusion chromatography (SEC) was performed using different methods. In one of the SEC methods identified herein as "Method 2" the mobile phase buffer was prepared to contain 50 mM sodium phosphate monobasic monohydrate and 150 mM arginine HCl. The pH was adjusted to 6.5 using 1 M NaOH. Separations were performed using a Phenomonex Yarra 3 micron SEC 3000, 30cm x 4.6 mm. To perform a separation, the columns were brought to room temperature (23°C) and equilibrated with mobile phase at a flow rate of 0.5 mL/min. One microliter of 50 mg/mL etanercept formulation were injected onto the column using an autosampler. The separation was accomplished over 10 minutes at a flow rate of 0.5 mL/minute. Column eluent was monitored at a wavelength of 280 nm during this time.

In an alternate SEC method, hereinafter referred to as "Method 3" NaCl was used as a salt for the mobile phase at a concentration of 100 mM, pH 6.3, in replacement of arginine HCl.

In a further alternate SEC method, referred to herein as "Method 1," the SEC analysis was conducted as follows: The mobile phase buffer was prepared to contain 50 mM sodium phosphate monobasic monohydrate and 150 mM arginine. The pH was adjusted to 6.5 using 1 M HCl. All separation were performed using a Tosoh TSK-Gel SWxl 6 mm x 4 cm guard column (cat. no. 8543) attached linearly to a Tosoh TSK-Gel G4000 SWxl 7.8 mm x 30 cm (cat. no. 8542). To perform a separation, the columns were brought to room temperature (23°C) and equilibrated with mobile phase at a flow rate of 0.5 mL/min. 5 microliters of 50 mg/mL etanercept formulation were injected onto the column using an autosampler. The separation was accomplished over 30 minutes at a flow rate of 0.5 mL/minute. Column eluent was monitored at a wavelength of 280 nm during this time.

Integration of Size Exclusion Chromatography Chromatograms. All integration was performed using Chromeleon software (Dionex). Prior to integration, the SEC chromatogram for a buffer containing no etanercept was subtracted from all chromatograms. All integration was performed between retention times of 2 minutes

and 8 minutes. Several parameters were used to define a peak. The minimum area for a detected peak was set to 0.05 mAu * min. The two-dimensional sensitivity for peak detection was set to 0.01 mAu and 75 seconds. Peak shoulders were added manually using a manual integration tool. All detected peaks were manually adjusted in two steps. First, peak baselines (the bottom boundary of the peak) were adjusted to horizontal. Secondly, the vertical positions of the peak baselines were adjusted to that of the chromatogram baseline. The chromatogram baseline value was defined as the signal in absence of analyte. In this case, the signal in absence of analyte was defined as the absorbance in mAu at 2 minutes retention time.

Hydrophobic Interaction Chromatography (HIC). Formulation samples were loaded into HPLC vials without dilution before injection on chromatography columns. Samples were separated by HIC according to the parameters listed in the table below.

Table 4. HIC method description

Solvent A: 1.8 M ammonium sulfate, 0.1 M sodium acetate pH 6.0 **Solvent B: 0.1 M sodium acetate pH 6.0** **Column Temp: 35° C Column: TSK-Gel Butyl NPR 14947** **Flow Rate: 1.0 mL/min** **Detection: 280 nM** **Injection: 0.5 uL, 50 mg/mL Enbrel**					
Gradient A					
time/minutes	**% B**				
0	0				
40	50				
50	100				
50.1	0				
55	0				

FlowCAM® Method Description for Testing of Formulations 3:6 and 4:2. Method development work was performed on 7/10/12 initially using a Manuel Prime with Non-Sample procedure (liquid to liquid interphase). Pronounced mixing effects were seen in the flow cell so an alternative air gap procedure (Manual Prime with Sample) was selected for sample evaluation.

The baseline (T0) formulations 3:6 and 4:2 samples were received frozen and had been stored at -20°C until thawed at ambient. Once thawed, the formulation was stored at refrigeration temperature (2-8°C). The Tt0 sample method included a matching buffer pre-treatment step to condition the flow cell. Prior to loading the sample, 0.4 mL or more of the matching formulation buffer was flushed thru the system. It was determined that this pre-treatment was not required and was therefore not used for the t3 testing time point.

Samples of formulation 3:6 and 4:2 that had undergone three months of heat stress at 5° C and 25° C were evaluated in the FlowCAM analysis. All samples were thawed on the day they were analyzed. Once thawed, they were stored at refrigeration temperature (2-8° C).

Instrumentation & Accessories

FlowCAM Instrument:	Model VS1, Serial #551 with Sony SX90 camera and C70 pump with a 1 mL syringe (Fluid Imaging Technologies)
FlowCAM Software:	DSP Firmware Version: 54; version 3.0.3
Flow Cell:	Field of View (FOV FC80) with a depth of 80µm and a width of 700µm (Fluid Imaging Technologies)
Objective:	10X

Context Setting (Method & Setting Parameters)

Method:	Manual Prime with Sample (air gap)
Sample Analysis:	0.200mL volume with 0.170 mL analyzed
Flow Rate:	0.100 ml/min
Auto image rate:	22 frames per second
Efficiency:	38.7%
Run time:	1.7 minutes
Distance to Nearest Neighbor:	0 microns
Close Hole:	5 iterations
Images:	Collage image border padding of 5
Particle Segmentation:	Dark Threshold 15.00, Light Threshold 15.00
Acceptable Region:	Left 15, Right 1255, Top 0, Bottom 959
Camera:	Shutter 8
Gain	57
Auto image rate:	22 frames per second
Flash camera delay:	100 microseconds
Flash duration:	18.5 microseconds
Diameter (ESD):	Min 2.00, Max 1000.00 microns

Prior to running samples, the flow cell and objective were installed and the Field of View and Focus optimization were performed. System qualification included running water blanks and particle size standard in multiple replicates. Prior to running samples a cleaning procedure was undertaken to ensure particle counts were at acceptable levels typically below 1000 particles/mL between samples or less than 5% of the sample particles/mL between replicate samples. The routine cleaning process used water (Millipore Direct-Q type 1, 0.22 μm filtered, 18.2 MΩ) between cleaning agents and as a final flush prior to determining count levels. Once the particle count reached an acceptable particle per mL level the sample was carefully pipetted into the sample tip and loaded into the flow prior to initiating the sample analysis.

Run quality was determined during and immediately after each run using a series of diagnostic tools in VisualSpreadSheet including x-y capture plot (to visualize flow pattern dynamics), aspectic ratio to diameter size plot (identify stuck particles), image review during the run and image analysis at completion of run using various particle characteristics (e.g. size, circularity, length, aspect ratio).

Individual particle size was determined with Fluid Imaging Technologies software measurement technique known as Equivalent Spherical Diameter (ESD). ESD is the mean feret measurement of the particle based on 36 sample measurements (conducted every 5°). A feret measurement is the perpendicular distance between parallel tangents touching opposite sides of the particle.

The following data tables describe the behavior of formulation 3:5 over three months of thermal stress at varying temperatures.

(Note: In Tables VIII through XIII, number in parentheses (e.g., 3:6) refers to the formulations being tested. The designation "C" is a control sample in which 50 mg/ml of etanercept prepared according to Example 4 was present in a formulation consisting of 25 mM phosphate buffer, 1% sucrose, 100mM sodium chloride and 25 mM arginine hydrochloride.)

TABLE VIII

ONE, TWO AND THREE MONTH STABILITY SEC DATA MONOMER CONTENT (Formulation 3:6 and 4:2 and Comparator)

Length of Storage	Monomer Content prior to storage and thermal stress	Monomer Content at -80°C Storage Temp	Monomer Content at 5°C Storage Temp	Monomer Content at 25°C Storage Temp	Monomer Content at 40°C Storage Temp
One Month[1]	99.45 (3:6) 99.33 (4:2) 99.45 (C)	99.43 (3:6) 99.16 (4:2) 99.38 (C)	99.20 (3:6) 99.21 (4:2) 99.37 (C)	96.18 (3:6) 95.87 (4:2) 96.54 (C)	86.08 (3:6) 79.32 (4:2) 87.83 (C)
One Month[2]	94.89 (3:6) 93.57 (4:2) 95.52 (C)	--	95.89 (3:6) 95.63 (4:2) 95.01 (C)	93.55 (3:6) 93.55 (4:2) 94.30 (C)	80.83 (3:6) 72.26 (4:2) 84.69 (C)
Two Months[2]	94.89 (3:6) 93.57 (4:2) 95.52 (C)	--	95.05 (3:6) 95.66 (4:2) 95.89 (C)	92.82 (3:6) 91.84 (4:2) 93.60 (C)	72.15 (3:6) 46.23 (4:2) 68.88 (C)
One Month[3]	95.43 (3:6) 93.95 (4:2) 95.08 (C)	--	94.49 (3:6) 94.13 (4:2) 94.04 (C)	93.15 (3:6) 92.23 (4:2) 92.64 (C)	81.46 (3:6) 73.23 (4:2) 83.96 (C)
Two Months[3]	95.43 (3:6) 93.95 (4:2) 95.08 (C)		93.38 (3:6) 93.47 (4:2) 94.34 (C)	91.44 (3:6) 90.90 (4:2) 92.22 (C)	71.92(3:6) 46.27(4:2) 68.34 (C)
Three Months[3]	95.43 (3:6) 93.95 (4:2) 95.08 (C)		93.99 (3:6) 94.07 (4:2) 94.75 (C)	91.16 (3:6) 90.33 (4:2) 91.79 (C)	56.09 (3:6) 9.77 (4:2) 47.46 (C)

[1] Sec Method 1; [2] SEC Method 2. [3] SEC Method 3

TABLE IX

ONE, TWO AND THREE MONTH STABILITY

SEC DATA—"FRAGMENT 3"

(Formulation 3:6 and 4:2 and Comparator)

Length of Storage	Fragment 3 Content prior to storage/thermal stress	Fragment 3 Content at 5°C Storage Temp	Fragment 3 Content at 25°C Storage Temp	Fragment 3 Content at 40°C Storage Temp
One Month[2]	3.89 (3:6) 4.16 (4:2) 3.27 (C)	2.81(3:6) 2.93(4:2) 3.75(C)	4.53 (3:6) 4.20 (4:2) 4.12 (C)	8.28 (3:6) 8.34 (4:2) 7.28 (C)
Two Months[2]	3.89 (3:6) 4.16 (4:2) 3.27 (C)	3.56 (3:6) 2.94 (4:2) 3.00 (C)	4.60 (3:6) 4.94 (4:2) 4.16 (C)	10.48 (3:6) 9.16 (4:2) 10.76 (C)
One Month[3]	3.35 (3:6) 4.36 (4:2) 3.68 (C)	4.00 (3:6) 4.11 (4:2) 4.57 (C)	4.67 (3:6) 5.30 (4:2) 5.82 (C)	---
Two Months[3]	3.35 (3:6) 4.36 (4:2) 3.68 (C)	4.93 (3:6) 4.65 (4:2) 4.22 (C)	5.75 (3:6) 5.84 (4:2) 6.12 (C)	---
Three Months[3]	3.35 (3:6) 4.36 (4:2) 3.68 (C)	4.45 (3:6) 4.21 (4:2) 3.84 (C)	5.82 (3:6) 5.40 (4:2) 5.56 (C)	---

[2] SEC Method 2. [3] SEC Method 3

TABLE X

TWO MONTH STABILITY

SEC DATA—"AGGREGATE(S)"

(Formulation 3:6 and 4:2 and Comparator)

Length of Storage	Aggregates Content at 5°C Storage Temp	Aggregates Content at 25°C Storage Temp	Aggregates Content at 40°C Storage Temp
One Month[2]	0 (3:6) 0 (4:2) 0 (C)	0 (3:6) 0 (4:2) 0 (C)	2.49 (3:6) 10.43 (4:2) 1.28 (C)
Two Months[2]	0 (3:6) 0 (4:2) 0 (C)	0 (3:6) 0 (4:2) 0 (C)	6.14 (3:6) 40.35 (4:2) 8.11 (C)

[2] SEC Method 2.

TABLE XI

HIC PEAK 1

ONE, TWO AND THREE MONTH

STORAGE

(Formulation 3:6 and 4:2 and Comparator)

Length of Storage	PEAK 1 CONTENT prior to storage/thermal stress	PEAK 1 CONTENT at 5°C Storage Temp	PEAK 1 CONTENT at 25°C Storage Temp	PEAK 1 CONTENT at 40°C Storage Temp
One Month	2.16 (3:6) 1.85 (4:2) 1.85 (C)	1.58 (3:6) 2.02 (4:2) 1.71 (C)	2.06 (3:6) 2.28 (4:2) 1.70 (C)	5.62 (3:6) 5.45 (4:2) 5.29 (C)
Two Months	2.16 (3:6) 1.85 (4:2) 1.85 (C)	1.69 (3:6) 1.41 (4:2) 1.78 (C)	2.29 (3:6) 2.70 (4:2) 2.71 (C)	8.32 (3:6) 7.90 (4:2) 8.17 (C)
Three Months	2.16 (3:6) 1.85 (4:2) 1.85 (C)	1.24 (3:6) 1.02 (4:2) 1.42 (C)	3.07 (3:6) 3.10 (4:2) 2.44 (C)	7.78 (3:6) 3.88 (4:2) 8.05 (C)

TABLE XII

HIC PEAK 2

ONE, TWO AND THREE MONTH

STORAGE

(Formulation 3:6 and 4:2 and Comparator)

Length of Storage	PEAK 2 CONTENT prior to storage/thermal stress	PEAK 2 CONTENT at 5°C Storage Temp	PEAK 2 CONTENT at 25°C Storage Temp	PEAK 2 CONTENT at 40°C Storage Temp
One Month	97.84 (3:6) 98.15 (4:2) 98.15 (C)	98.42 (3:6) 97.98 (4:2) 98.29 (C)	97.94 (3:6) 97.72 (4:2) 98.30 (C)	85.35 (3:6) 75.34 (4:2) 88.39 (C)
Two Months	97.84 (3:6) 98.15 (4:2) 98.15 (C)	98.31 (3:6) 98.59 (4:2) 98.22 (C)	97.71 (3:6) 97.30 (4:2) 97.29 (C)	79.03 (3:6) 50.41 (4:2) 73.93 (C)
Three Months	97.84 (3:6) 98.15 (4:2) 98.15 (C)	98.58 (3:6) 98.76 (4:2) 98.58 (C)	96.93 (3:6) 96.90 (4:2) 97.56 (C)	64.57 (3:6) 11.64 (4:2) 54.28 (C)

TABLE XIII

HIC PEAK 3

ONE, TWO AND THREE MONTH

STORAGE

(Formulation 3:6 and 4:2 and comparator)

Length of Storage	PEAK 3 CONTENT prior to storage/thermal stress	PEAK 3 CONTENT at 5°C Storage Temp	PEAK 3 CONTENT at 25°C Storage Temp	PEAK 3 CONTENT at 40°C Storage Temp
One Month	0 (3:6) 0 (4:2) 0 (C)	0 (3:6) 0 (4:2) 0 (C)	0 (3:6) 0 (4:2) 0 (C)	9.03 (3:6) 19.21 (4:2) 6.32 (C)
Two Months	0 (3:6) 0 (4:2) 0 (C)	0 (3:6) 0 (4:2) 0 (C)	0 (3:6) 0 (4:2) 0 (C)	12.66 (3:6) 41.70 (4:2) 17.90 (C)
Three Months	0 (3:6) 0 (4:2) 0 (C)	0 (3:6) 0 (4:2) 0 (C)	0 (3:6) 0 (4:2) 0 (C)	27.64 (3:6) 84.48 (4:2) 37.34 (C)

Formulation 4:2 was evaluated for subvisible particles using the FlowCam flow imaging system. These instruments are designed to measure levels of subvisible particles (SVPs). These were measured initially (Table XIV) and then after three months at 5°C (Table XV) and 25°C (Table XVI). Consistent with the SEC and HIC data shown above indicating low levels of aggregate or misfolded material in formulation 4.2 after three month of thermal stress, formulation 4.2 exhibited low levels of subvisible particles (less than 10000 particles per mL having a size greater than 5μm per mL).

TABLE XIV

Initial Number of Particles/mL of Different Sizes as Measured by FlowCam for Formulation 4.2 (prior to thermal stress)

	Form. 4:2	Comparator
2-5 μm	14000 ± 7200	7400 ± 8000
5-10 μm	3100 ± 1400	1900 ± 1800
10-15 μm	530 ± 100	290 ± 260
15-25 μm	180 ± 90	100 ± 80
25-40 μm	30 ± 40	50 ± 40
40-50 μm	0 ± 10	10 ± 10
> 50 μm	10 ± 10	10 ± 20
> 2 μm	18000 ± 8800	9700 ± 11000
> 5 μm	3900 ± 1600	2300 ± 2200

TABLE XV

Number of particles/mL of Different Sizes as Measured by FlowCam for Formulation 4.2 stored at 5° C for three months

	Formulation 4:2	Comparator
2-5 μm	24000 ± 22000	7900 ± 4200
5-10 μm	5800 ± 4700	1600 ± 700
10-15 μm	760 ± 320	150 ± 30
15-25 μm	120 ± 100	40 ± 30
25-40 μm	60 ± 20	10 ± 10
40-50 μm	0 ± 0	0 ± 0
> 50 μm	0 ± 0	0 ± 0
> 2 μm	30000 ± 27000	9600 ± 4900
> 5 μm	6700 ± 5100	1800 ± 700

TABLE XVI

Number of particles/mL of Different Sizes as Measured by FlowCam for Formulation 4.2 stored at 25° C for three months

	Formulation 4:2	Comparator
2-5 μm	2000 ± 900	15000 ± 10000
5-10 μm	700 ± 280	3200 ± 2000
10-15 μm	100 ± 50	310 ± 190
15-25 μm	20 ± 20	170 ± 140
25-40 μm	10 ± 10	50 ± 40
40-50 μm	0 ± 0	10 ± 10
> 50 μm	0 ± 0	10 ± 10
> 2 μm	2800 ± 1100	19000 ± 13000
> 5 μm	820 ± 280	3800 ± 2300

The data presented in Tables VIII through XVI above demonstrate that a metal ion stabilized formulation according to the present invention is capable of achieving storage stability comparable to or better than that of a comparator formulation comprising arginine as the stabilizer.

Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

WHAT IS CLAIMED IS:

1. An aqueous pharmaceutical composition comprising etanercept and a stabilizer to inhibit instability, aggregation, misfolding and/or fragmentation of the etanercept, wherein the stabilizer comprises a stabilizing metal ion.

2. The composition of claim 1 wherein a source of said stabilizing metal ion is selected from the group consisting of calcium, magnesium and zinc.

3. The composition of claim 2, further comprising one or more additional components selected from: a buffer; a tonicity modifier, and an excipient.

4. The metal ion stabilized etanercept composition of claim 3 where the stabilizer is calcium chloride, the formulation is free or essentially free of arginine, and the formulation elicits long term storage stability as characterized by at least one of:

   SEC analysis at M_3 or T_2 or T_4 of: monomer content greater than about 90 wt.%; aggregates content of less than about 3 wt.%; and fragment 3 content less than about 5 wt%: and

   HIC analysis at M_3 or T_2 or T_4 wherein the amount of the composition represented by peak 1 of the HIC chromatogram is less than about 3 wt.%; the amount of the composition represented by peak 2 of the HIC chromatogram is greater than 80 wt.%; and the amount of the composition represented by peak 3 of the HIC chromatogram is less than about 20 wt.%.

5. The metal ion stabilized etanercept composition of claim 3 wherein the stabilizer is calcium chloride in an amount not exceeding about 5 mM calcium chloride; optionally about 0.5 to 6 wt.% sucrose or trehalose; optionally about 0 to100 mM NaCl; optionally up to about 10 mM xylitol; about 1 to about 30 mM sodium phosphate; wherein the composition has a pH of about 6.0 to 6.6; and wherein, optionally, the composition contains no arginine or is essentially free of arginine.

6. The calcium chloride stabilized composition of claim 5 eliciting long term storage stability as characterized by:

SEC analysis M_3 or T_2 or T_4 of greater than about 90 wt.% monomer content; less than about 3 wt.% aggregate(s) content; and less than about 5 wt% fragment 3; and

HIC analysis at M_3 or T_2 or T_4 wherein the amount of the composition represented by peak 1 of the HIC chromatogram is less than about 3 wt.%; the amount of the composition represented by peak 2 of the HIC chromatogram is greater than 80 wt.%; and the amount of the composition represented by peak 3 of the HIC chromatogram is less than about 20 wt.%.

7. The calcium chloride stabilized composition of claim 6 eliciting long term storage stability as characterized by: an HIC analysis at M_3 wherein the amount of the composition represented by peak 2 of the HIC chromatogram is greater than or equal to about 95 wt.%; and wherein, if peak 3 is present on the HIC chromatogram, the amount of the composition represented by peak 3 is less than or equal to about 3 wt.%.

8. The calcium chloride stabilized composition of claim 6 having, at M_3 or T_2 or T_4 no more than, on average, about 10,000 subvisible particles per mL having a size greater than 5μm.

9. The calcium chloride stabilized composition of claim 6 comprising about 50 mg/ml of etanercept; about 2 mM calcium chloride; less than about 30 mM sodium phosphate; less than about 150 mM sodium chloride; and less than about 4 wt.% sucrose; wherein the formulation has a pH of about 6.3 to 6.5.

10. The calcium chloride stabilized composition of claim 6 comprising about 50 mg/ml of etanercept; about 2 mM calcium chloride; about 10-30 mM sodium phosphate; less than about 10 wt.% sucrose; wherein the formulation has a pH of about 6.3 to 6.5.

11. The calcium chloride stabilized composition of claim 6 comprising about 50 mg/ml of etanercept; about 2 mM calcium chloride; about 10-30 mM sodium phosphate; less than about 100 mM sodium chloride; less than about 5 wt.% sucrose; wherein the formulation has a pH of about 6.3 to 6.5.

12. The calcium chloride stabilized composition of claim 6 comprising about 50 mg/ml of etanercept; about 1 mM calcium chloride; about 10-30 mM sodium phosphate; about 25 to about 75 mM sodium chloride; and less than about 8 wt.% trehalose; wherein the formulation has a pH of about 6.3 to 6.5.

13. The stabilized etanercept composition of claim 3 where the stabilizer is magnesium chloride, the formulation is free or essentially free of arginine, and the formulation elicits long term storage stability as characterized by at least one of:

SEC analysis at M_3 or T_2 or T_4 of: monomer content greater than about 90%; aggregates content of less than about 3 wt%; and fragment 3 content less than about 5 wt%: and

HIC analysis at M_3 or T_2, or T_4 wherein the amount of the composition represented by peak 1 of the HIC chromatogram is less than about 3 wt.%; the amount of the composition represented by peak 2 of the HIC chromatogram is greater than 80 wt.%; and the amount of the composition represented by peak 3 of the HIC chromatogram is less than about 20 wt.%.

14. The stabilized etanercept composition of claim 3 wherein the stabilizer is magnesium chloride and the formulation comprises comprising about 1 mM to about 20 mM magnesium chloride; optionally up to about 6 wt.% sucrose; about 25 to 150 mM NaCl; about 1 to about 30 mM sodium phosphate; wherein the composition has a pH of about 6.0 to 6.6; and wherein, optionally, the composition contains no arginine or is essentially free of arginine.

15. The magnesium chloride stabilized composition of claim 14 eliciting long term storage stability as characterized by:

SEC analysis at M_3 or T_2, or T_4 of greater than about 90 wt.% monomer content; less than about 3 wt.% aggregate(s) content; and less than about 5 wt% fragment 3; and

(b) an HIC analysis at M_3 or T_2 or T_4 wherein the amount of the composition represented by peak 1 of the HIC chromatogram is less than about 3 wt.%; the amount of the composition represented by peak 2 of the HIC chromatogram is greater than 80 wt.%; and the amount of the composition represented by peak 3 of the HIC chromatogram is less than about 20 wt.%; and

16. The magnesium chloride stabilized composition of claim 15 which elicits long term storage stability as characterized by: an HIC analysis at M_3 or T_2 or T_4 wherein the amount of the composition represented by peak 2 of the HIC chromatogram is greater than or equal to about 95 wt.%; and wherein, if peak 3 is present on the HIC chromatogram, the amount of the composition represented by peak 3 is less than or equal to about 3 wt.%.

17. The magnesium chloride stabilized composition of claim 15 having, on average, no more than about 10,000 subvisible particles per mL having a size greater than 5µm.

18. The magnesium chloride stabilized composition of claim 15 comprising about 50 mg/ml of etanercept; about 10 mM magnesium chloride; about 10-30 mM sodium phosphate; less than about 150 mM sodium chloride; and less than about 3 wt.% sucrose; and having a pH of about 6.3 to 6.5.

19. The magnesium chloride stabilized composition of claim 15 comprising about 50 mg/ml of etanercept; about 5 mM magnesium chloride; less than about 30 mM sodium phosphate; less than about 100 mM sodium chloride; and less than about 4 wt.% sucrose; and having a pH of about 6.3 to 6.5.

20. The magnesium chloride stabilized composition of claim 15 comprising about 50 mg/ml of etanercept; about 4 mM magnesium chloride; less than about 30 mM sodium phosphate; about 100 mM sodium chloride; and about 2.5 wt.% sucrose; and having a pH of about 6.3 to 6.5.

21. The magnesium chloride stabilized composition of claim 15 comprising about 50 mg/ml of etanercept; about 10 mM magnesium chloride; less than about 30 mM sodium phosphate; less than about 100 mM sodium chloride; and less than about 5 wt.% sucrose; and having a pH of about 6.3 to 6.5.

22. The stabilized etanercept composition of claim 1, containing no arginine, or essentially free of arginine, wherein the composition, at M_3 or T_2 or T_4 elicits long term storage stability that meets one or both of the following criteria:

(A) stability comparable to or better than commercially available etanercept marketed under the trademark Enbrel®, as measured by (i) SEC analysis of the amounts of aggregate(s), monomer and fragment 3 in the composition (as defined in the specification) and (ii) HIC analysis of amounts of material in the composition corresponding to peaks 1, 2 and 3 of the HIC chromatogram (as defined in the specification); and

(B) an HIC chromatogram in which (i) peak 3 is absent, or essentially absent and (ii) peak 2 represents greater than about 95 wt% of the composition; an SEC chromatogram containing essentially no peak corresponding to aggregate(s); and an SEC chromatogram in which the monomer content represents at least about 95 wt% of the composition.